Biosafety: Guidelines for Working with Pathogenic and Infectious Microorganisms

One of the most important emerging technologies used by microbiologists and other life scientists and laboratory workers that handle pathogenic and infectious agents is the technology that manifests in what is collectively referred to as biosafety. Biosafety measures designed to ensure the safety of laboratory workers include the use of various primary and secondary barriers, many of which are due to the advent of new technologies in the fields of material science and engineering. Persons carrying out the protocols in this manual may encounter potentially hazardous materials such as pathogenic and infectious biological agents, as well as toxic chemicals and carcinogenic, mutagenic, or teratogenic reagents (see *UNIT 1A.3*). In the case of biological agents, it has long been recognized that laboratory workers can acquire infections from the agents they manipulate thus making the very nature of their work an occupational hazard. Bacterial agents cause the most commonly acquired laboratory infections but pathogenic agents belonging to all categories of microorganisms can cause infections.

New biosafety technologies and associated evolving guidelines have emerged to significantly improve ways to safely handle microbiological material. In addition, a better understanding of the risks associated with various manipulations of many agents transmissible by different routes has facilitated our ability to apply appropriate biosafety practices to specific laboratory arenas. As this knowledge base grows and new biosafety technologies emerge, evolving safety guidelines will continue to benefit laboratory workers. A combination of engineering controls, management policies, work practices and procedures, as well as medical interventions, collectively defines these safety guidelines.

Several biosafety levels, described in this unit, have been developed for microbiological and biomedical laboratories to provide increasing levels of personnel and environmental protection. *UNIT 1A.2* will provide information related to biosafety practices associated with potential agents of biocrime and biowarfare. *UNIT 1A.3* will provide guidelines for the safe use of hazardous chemicals.

It is important to note that most governments regulate the use of biohazardous materials. Therefore, it is essential that they be used in strict accordance with local and national regulations (see *APPENDIX 1B*). Cautionary notes are included in many instances throughout the manual, and some specific guidelines are provided below (and in references therein). However, we emphasize that users must proceed with the prudence and precautions associated with good laboratory practice, under the supervision of personnel responsible for implementing laboratory safety programs at their institutions. Guidelines for the safe use of radioisotopes are presented in *APPENDIX 1C*.

Precautions described in this unit should be applied to the routine handling of viable pathogenic microorganisms, as well as all human-derived materials, because they may harbor dangerous pathogens such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and a host of bacterial pathogens. In addition to the guidelines provided herein, experimenters can find a wealth of information about handling infectious agents in the appropriate government publications (see Literature Cited and *APPENDIX 1B*).

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GENERAL BIOSAFETY GUIDELINES

Routine Precautions When Working with Biohazards

The following practices are recommended for all laboratories handling potentially dangerous microorganisms, whether pathogenic or not:

1. Decontaminate all work surfaces after each working day using an appropriate disinfectant. Decontaminate all spills of viable material. See discussion under Disinfectants for Biohazards.
2. Decontaminate all liquid or solid wastes that have come in contact with viable material.
3. Do not pipet by mouth.
4. Do not allow eating, drinking, smoking, or application of cosmetics in the work area. Do not store food in refrigerators that contain laboratory supplies.
5. Wash hands with disinfectant soap or detergent after handling viable materials and before leaving the lab. Do not handle telephones, doorknobs, or other common utensils without disinfecting hands.
6. When handling viable materials, minimize creation of aerosols.
7. Wear lab coats (preferably disposable) when in work area, but do not wear them away from the work area.
8. Wear disposable latex gloves when handling viable materials. These should be disposed of as biohazardous waste. Change gloves if they are directly contaminated.
9. Control pest populations. Windows in the lab that can be opened must be equipped with screens to exclude insects.
10. Use furniture that is easy to clean—i.e., with smooth, waterproof surfaces and as few seams as possible.
11. Keep biohazard waste in covered containers free from leaks. Use orange bags or red biohazard bags (or other appropriate color in accordance with local regulations) as required by institutional procedure. Autoclave and dump hazardous waste without undue delay.

See discussion under Disposal of Biohazards (below) for more information.

Disinfectants for Biohazards

Major laboratory suppliers sell disinfectants based on quaternary ammonium compounds that are acceptable for routine biohazard decontamination (see *SUPPLIERS APPENDIX*). These include Roccal (Baxter), Vesphene II (Fisher), and industrial disinfectants such as concentrated Lysol. Additionally, 10% chlorine bleach may also be used for decontamination. An antimicrobial liquid soap (e.g., Vionex; Fisher) should be provided in a dispenser near the sink so that no one need handle the outside of the container to use it.

Disposal of Biohazards

Most institutions have defined procedures for disposal of biohazardous waste, but the following are common to all of these systems:

1. All contaminated material should be placed in autoclavable bags, which should be contained in a plastic trash pail or wire frame. If large numbers of disposable pipets or other pointed instruments are being used, it may be necessary to double-bag the material. All major laboratory supply houses sell autoclavable biohazard bags. In some institutions it is necessary to color code the biohazard waste (e.g., orange bags

for less dangerous waste, red ones for suspected HIV-containing material). All of these bags are marked with the universal biohazard symbol.

2. At time of disposal, the bags are loosely closed (not completely sealed) with temperature-sensitive autoclave tape (also widely available from supply houses), placed in an autoclavable basin, and sterilized at 121°C. When the tape indicates that sterilization temperature has been achieved, it is then possible to dispose of the waste by ordinary means.
3. At many institutions, contaminated hypodermic needles, scalpels, broken glass, and other sharp objects must be disposed of separately. These must be placed in appropriate "sharps" containers (e.g., Baxter), which may be autoclaved when full.

BIOSAFETY LEVELS

For each biosafety level there are specific supervisory qualifications as assurance that laboratory workers are provided appropriate role models and knowledgeable mentors. Various types of specialized equipment are used to provide primary barriers between the microorganism and the laboratory worker. These range from disposable gloves and other personnel protective equipment to complex biosafety cabinets or other containment devices.

The laboratory director is specifically and primarily responsible for the safe operation of the laboratory. His/her knowledge and judgment are critical in assessing risks and appropriately applying these recommendations. The recommended biosafety level represents those conditions under which the agent can ordinarily be safely handled. Special characteristics of the agents used, the training and experience of personnel, and the nature or function of the laboratory may further influence the director in applying these recommendations.

The U.S. Centers for Disease Control and Prevention (CDC; see Internet Resources) defines four levels of biosafety, which are outlined below. Selection of an appropriate biosafety level for work with a particular agent or animal study (see Animal Facilities) depends upon a number of factors. Some of the most important are the virulence, pathogenicity, biological stability, route of spread, and communicability of the agent; the nature or function of the laboratory; the procedures and manipulations involving the agent; the endemicity of the agent; and the availability of effective vaccines or therapeutic measures.

Table 1A.1.1 provides a summary of recommended biosafety levels for infectious agents. For regulations and guidelines applicable outside of the U.S., please refer to *APPENDIX 1B* and Internet Resources.

NOTE: The following information has been adapted from *Biosafety in Microbiological and Biomedical Laboratories*, 4th Ed. (BMBL, 4th Ed.; GPO S/N 017-040-00547-4), which is published jointly by the U.S. Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH), and is available online at *http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm* (see Internet Resources for other online versions). Readers are strongly urged to review this publication prior to initiating any experiment outlined in this manual.

Biosafety Level 1 (BSL-1)

BSL-1 is appropriate for working with microorganisms that are not known to cause disease in healthy human humans. BSL-1 practices, safety equipment, and facility design and construction are appropriate for undergraduate and secondary

Table 1A.1.1 CDC Summary of Recommended Biosafety Levels for Infectious Agents[a,b]

Biosafety level	Agent characteristics	Practices	Safety equipment (primary barriers)[c]	Facilities (secondary barriers)
BSL-1	Not known to consistently cause disease in healthy adults	Standard microbiological practices	None required	Open bench-top sink
BSL-2	Associated with human disease, hazard from percutaneous injury, ingestion, mucous membrane exposure	Standard microbiological practices Limited access Biohazard warning signs "Sharps" precautions Biosafety manual defining any needed waste decontamination or medical surveillance policies	Class I or II biosafety cabinets (BSCs) or other physical containment devices used for all manipulations of agents that cause splashes or aerosols of infectious materials Laboratory coats and gloves Face protection as needed	Open bench-top sink Autoclave
BSL-3	Indigenous or exotic agents with potential for aerosol transmission; disease may have serious or lethal consequences	All BSL-2 practices Controlled access Decontamination of all waste Decontamination of lab clothing before laundering Baseline serum	Class I or II BSCs or other physical containment devices used for all open manipulations of agents Protective lab clothing and gloves Respiratory protection as needed	Open bench-top sink Autoclave Physical separation from access corridors Self-closing, double-door access Exhausted air not recirculated Negative airflow into laboratory
BSL-4	Dangerous/exotic agents which pose high risk of life-threatening disease, aerosol-transmitted lab infections; or related agents with unknown risk of transmission	All BSL-3 practices Clothing change before entering Shower on exit All material decontaminated on exit from facility	All procedures conducted in Class III BSCs, or Class I or II BSCs *in combination with* full-body, air-supplied, positive pressure personnel suit	All BSL-3 facilities plus: Separate building or isolated zone Dedicated supply and exhaust, vacuum, and decontamination systems Other requirements as outlined in the text

[a] Adapted from Biosafety in Microbiological and Biomedical Laboratories, 4th Ed. (GPO S/N 017-040-00547-4), available online at *http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm*. Also see Internet Resources.

[b] The practices, and primary and secondary barriers required for a given biosafety level include those of the all lower levels, as well as the additional required practices, equipment, and/or facilities described for the BSL in question.

[c] See *http://www.cdc.gov/od/ohs/biosfty/bsc/bsc.htm* for more information concerning biological safety cabinets (BSCs).

educational training and teaching laboratories, and for other laboratories in which work is done with defined and characterized strains of viable microorganisms not known to consistently cause disease in healthy adult humans. *Bacillus subtilis*, *Naegleria gruberi*, infectious canine hepatitis virus, *Escherichia coli* K-12 (see BMBL for restrictions), and exempt organisms under the *NIH Recombinant DNA Guidelines* (*http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html*) are representative of microorganisms meeting these criteria. Many agents not ordinarily associated with disease processes in humans are, however, opportunistic pathogens and may cause infection in

the young, the aged, and immunodeficient or immunosuppressed individuals. Vaccine strains that have undergone multiple in vivo passages should not be considered avirulent simply because they are vaccine strains.

BSL-1 represents a basic level of containment that relies on standard microbiological practices with no special primary or secondary barriers recommended, other than a sink for hand washing.

In this manual, when BSL-1 conditions are appropriate to the experiments described, the following note will appear in the unit introduction.

> *CAUTION: <organism name>* is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*).

Biosafety Level 2 (BSL-2)

The facility, containment devices, administrative controls, and practices and procedures that constitute BSL-2 are designed to maximize safe working conditions for laboratorians working with agents of moderate risk to personnel and the environment. BSL-2 practices, equipment, and facility design and construction are applicable to clinical, diagnostic, teaching, and other laboratories in which work is done with the broad spectrum of indigenous moderate-risk agents that are present in the community and associated with human disease of varying severity. With good microbiological techniques, these agents can be used safely in activities conducted on the open bench, provided the potential for producing splashes or aerosols is low. Hepatitis B virus, HIV, *E. coli* O157:H7, the *Salmonella*, and *Toxoplasma* spp. are representative of microorganisms assigned to this containment level.

Biosafety Level 2 is also appropriate when work is done with any human-derived blood, body fluids, tissues, or primary human cell lines where the presence of an infectious agent may be unknown. Laboratory personnel in the United States working with human-derived materials should refer to the U.S. Occupational Safety and Health Administration (OSHA) *Bloodborne Pathogen Standard* (OSHA, 1991), available online at *http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=10051*, for required precautions. For guidelines and regulations appropriate to locations outside the U.S., please refer to *APPENDIX 1B* and Internet Resources.

Primary hazards to personnel working with these agents relate to accidental percutaneous or mucous membrane exposures, or ingestion of infectious materials. Extreme caution should be taken with contaminated needles or sharp instruments. Even though organisms routinely manipulated at Biosafety Level 2 are not known to be transmissible by the aerosol route, procedures with aerosol or high splash potential that may increase the risk of such personnel exposure must be conducted in primary containment equipment, or in devices such as a biological safety cabinet (BSC) or safety centrifuge cups. Other primary barriers should be used as appropriate, such as splash shields, face protection, gowns, and gloves.

Secondary barriers such as hand washing sinks and waste decontamination facilities must be available to reduce potential environmental contamination.

When BSL-2 conditions are appropriate to the organism under investigation, the following note is included in the unit introduction.

CAUTION: <organism name> is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

When BSL-2 conditions are appropriate due to the use of human-derived materials, the following note is included in the introduction.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

Biosafety Level 3 (BSL-3)

BSL-3 is suitable for work with infectious agents, which may cause serious or potentially lethal diseases as a result of exposure by the inhalation route. This may apply to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents with potential for respiratory transmission, and which may cause serious and potentially lethal infection. *Mycobacterium tuberculosis*, St. Louis encephalitis virus, and *Coxiella burnetii* are representative of the microorganisms assigned to this level. Primary hazards to personnel working with these agents relate to autoinoculation, ingestion, and exposure to infectious aerosols.

At BSL-3, more emphasis is placed on primary and secondary barriers to protect personnel in contiguous areas, the community, and the environment from exposure to potentially infectious aerosols (see Table 1A.1.1). For example, all laboratory manipulations should be performed in a BSC or other enclosed equipment, such as a gas-tight aerosol generation chamber. Secondary barriers for this level include controlled access to the laboratory and ventilation requirements that minimize the release of infectious aerosols from the laboratory.

When BSL-3 conditions are appropriate to the organism under investigation, the following note is included in the unit introduction.

CAUTION: <organism name> is a Biosafety Level 3 (BSL-3) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

When BSL-3 conditions are appropriate due to the use of human-derived materials, the following note is included in the introduction.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

Biosafety Level 4 (BSL-4)

BSL-4 practices, safety equipment, and facility design and construction are applicable for work with dangerous and exotic agents that pose a high individual risk of life-threatening disease, which may be transmitted via the aerosol route, and for which there is no available vaccine or therapy. Agents with a close or identical antigenic relationship to Biosafety Level 4 agents also should be handled at this level. When sufficient data are obtained, work with these agents may continue at this or a lower level. Viruses such as Marburg or Congo-Crimean hemorrhagic fever are manipulated at Biosafety Level 4.

The primary hazards to personnel working with Biosafety Level 4 agents are respiratory exposure to infectious aerosols, mucous membrane or broken skin exposure to infectious

droplets, and autoinoculation. All manipulations of potentially infectious diagnostic materials, isolates, and naturally or experimentally infected animals, pose a high risk of exposure and infection to laboratory personnel, the community, and the environment.

The laboratory worker's complete isolation from aerosolized infectious materials is accomplished primarily by working in a Class III BSC or in a full-body, air-supplied, positive-pressure personnel suit. The BSL-4 facility itself is generally a separate building or completely isolated zone with complex, specialized ventilation requirements and waste management systems to prevent release of viable agents to the environment.

As of this printing, there are no experiments described in this manual which specifically require BSL-4 conditions.

ANIMAL FACILITIES

The CDC defines four biosafety levels for activities involving infectious disease work with experimental animals. These combinations of practices, safety equipment, and facilities are designated **Animal Biosafety Levels 1**, **2**, **3**, and **4**, and provide increasing levels of protection to personnel and the environment.

In this manual, when these conditions are necessary, a note is provided in the unit or protocol introduction with the following format, where *x* is the appropriate ABSL.

> *CAUTION:* Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals. This experiment requires Animal Biosafety Level *x* (ABSL-*x*) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

For more information, refer to the Section IV of the BMBL, 4th Ed., available online at *http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s4.htm*.

CLINICAL LABORATORIES

Clinical laboratories, especially those in health care facilities, receive clinical specimens with requests for a variety of diagnostic and clinical support services. Typically, the infectious nature of clinical material is unknown, and specimens are often submitted with a broad request for microbiological examination for multiple agents (e.g., sputa submitted for "routine," acid-fast, and fungal cultures). It is the responsibility of the laboratory director to establish standard procedures in the laboratory, which realistically addresses the issue of the infective hazard of clinical specimens.

Except in extraordinary circumstances (e.g., suspected hemorrhagic fever), the initial processing of clinical specimens and serological identification of isolates can be done safely at Biosafety Level 2 (see above), the recommended level for work with bloodborne pathogens such as hepatitis B virus and HIV. The containment elements described in BSL-2 are consistent with the OSHA standard, *Occupational Exposure to Bloodborne Pathogens* (Richmond, 1994) from the Occupational Safety and Health Administration (OSHA; see Internet Resources). This requires the use of specific precautions with *all* clinical specimens of blood or other potentially infectious material (Universal or Standard Precautions; MMWR, 1988). Additionally, other recommendations specific for clinical laboratories may be obtained from the U.S. National Committee for Clinical Laboratory Standards (NCCLS, 1977).

Biosafety Level 2 recommendations and OSHA requirements focus on the prevention of percutaneous and mucous membrane exposures to clinical material. Primary barriers

such as BSCs (Class I or II; see *http://www.cdc.gov/od/ohs/biosfty/bsc/bsc.htm*) should be used when performing procedures that might cause splashing, spraying, or splattering of droplets. BSCs should also be used for the initial processing of clinical specimens when the nature of the test requested or other information suggests the likely presence of an agent readily transmissible by infectious aerosols (e.g., *M. tuberculosis*), or when the use of a BSC (Class II) is indicated to protect the integrity of the specimen.

The segregation of clinical laboratory functions and limited or restricted access to such areas is the responsibility of the laboratory director. It is also the director's responsibility to establish standard, written procedures that address the potential hazards and the required precautions to be implemented.

Literature Cited

Morbidity and Mortality Weekly Report (MMWR), Centers for Disease Control 1988. Update: Universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus and other bloodborne pathogens in healthcare settings. *MMWR* 37:377-388.

National Committee for Clinical Laboratory Standards (NCCLS) 1997. Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue. Approved Guideline, Dec. 1977, NCCLS Doc. M29-A. NCCLS, Wayne, Penn.

Occupational Safety and Health Administration (OSHA), U.S. Department of Labor 1991. Occupational exposure to bloodborne pathogens, final rule. *In* Federal Register Volume 56, pp. 64175-64182. U.S. Government Printing Office, Washington D.C.

Richmond, J.Y. 1994. HIV Biosafety: Guidelines and Regulations. *In* AIDS Testing, Edition 2 (G. Schochetman and J. R. George, eds.) pp. 346-360. Springer-Verlag, New York.

Key References

U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH) 1999. Biosafety in Microbiological and Biomedical Laboratories (BMBL), 4th Ed. 1999. (J.Y. Richmond and R.W. McKenney, eds.) U.S. Government Printing Office, Washington D.C. (GPO S/N 017-040-00547-4).

Fuscaldo, A.A., Erlick, B.J., and Hindman, B. (eds.) 1980. Laboratory Safety, Theory and Practice. Academic Press, San Diego.

Internet Resources

http://www.OSHA.gov

OSHA web site.

http://www.cdc.gov

The Centers for Disease Control and Prevention website.

http://tis.eh.doe.gov/docs/osh_tr/ch5.html

DOE OSH technical reference chapter on personal protective equipment.

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Online version of the Biosafety in Microbiological and Biomedical Laboratories (BMBL), 4th Ed. See Key References for print version information.

http://bmbl.od.nih.gov

BMBL version with longer than average web pages and minimal graphics for ease of printing.

http://www.cdc.gov/od/ohs/pdffiles/4th%20BMBL.pdf

PDF version of the BMBL. See above for other versions.

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s6.htm

Provides information on recommended biosafety levels for infectious agents and infected animals.

http://www.absa.org/resguides.html

The American Biological Safety Association (ABSA) Biosafety Guidelines website. This page provides links to international biosafety guidelines and websites.

http://www.ebsa.be

The European Biosafety Association homepage. In addition to being a source of information in and of itself, it is also host to the International Biosafety Working Group (IBWG), a compendium of international regulations and guidelines with descriptions and URLs. (Access by clicking International Biosafety from the menu bar on the top of the screen.)

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Biosafety Practices Associated with Potential Agents of Biocrime and Biowarfare

Effective biosafety is built on the concept that some microorganisms present more risk of laboratory-acquired infection or unintended release than others. The risk-assessment process, described in *UNIT 1A.1*, is based on the nature of the microorganism and likelihood of infection, or release, as a result of an unintentional laboratory exposure. Biosecurity, the topic of this unit, is also most effectively applied based on a similar risk assessment. The same features of the microbe, coupled with the likelihood of ill effects from a stolen, misused, or intentionally released organism, are the key factors in a biosecurity risk assessment.

Biosecurity is best applied based on determining (1) what research materials are at risk of being intentionally removed (risk identification); (2) the likelihood of misuse and subsequent damage (risk assessment); and (3) what measures can be applied to limit this risk (risk management).

To begin to consider the security of research materials, a general philosophy must be established. Few researchers would argue that a reasonable level of security is necessary. However, it is less commonly acknowledged that security is, by its nature, inconvenient. There is no "silver bullet" that will render materials secure, while allowing unimpeded access to every person desiring that access. The research environment should include basic laboratory biosecurity as part of good laboratory practice.

It is important to note that, in most cases, an appropriate level of laboratory biosecurity can be achieved without relying on expensive technologies or unusually burdensome procedures. However, some countries require certain pathogens to be strictly secured, regardless of the relative level of risk; thus, the regulations for each locality must be consulted to determine what risk management methods are required by regulation (also see *APPENDIX 1B* and *http://www.absa.org*). Lack of regulations, however, must not be construed to mean that security is unnecessary. Indeed, basic and prudent measures, as described below, are warranted for all laboratories using biological material.

RISK IDENTIFICATION

To most effectively determine appropriate biosecurity measures, an inventory of assets is warranted. In other words, laboratory directors should ask themselves and their research staff, "What needs protection from theft or removal?" The scenarios presented in this unit are most specifically directed towards protection of potentially dangerous microorganisms and toxins, but research activities also involve many other assets such as research equipment (e.g., analytical balances, computer equipment), proprietary materials, chemicals, radioisotopes, research animals, experimental data, and more. Stolen research materials, at the very least, are likely to represent a loss of time and intellectual capital.

RISK ASSESSMENT

While a biosafety risk assessment is based on identifying the potential for the microbe to cause infection and/or illness during routine laboratory use, a biosecurity risk assessment involves evaluating the same organism for its potential for intended removal and malicious use, and the consequences of such use. In addition, an assessment of laboratory activities (e.g., multiple transfers) or an enhanced threat environment (e.g., suspicious activities) are factors in completing a biosecurity risk assessment.

Many dangerous pathogens may present limited biosecurity risk because they are difficult to grow or to disseminate in the form of an active bioweapon. Likewise, other less virulent, easily contained microbes may be of a lower safety concern but may represent a desirable tool for bioterrorism.

A risk assessment for malicious use of biological agents includes consideration of factors that influence both the potential for an organism to be used in a threatening manner (threat potential) and the consequences that might result from the intentional use of the agent to inflict harm (Fig. 1A.2.1). Factors influencing the risk for malicious use have been described by Gaudioso et al. (2005).

Determining the consequences of the release of the agent can include the following:

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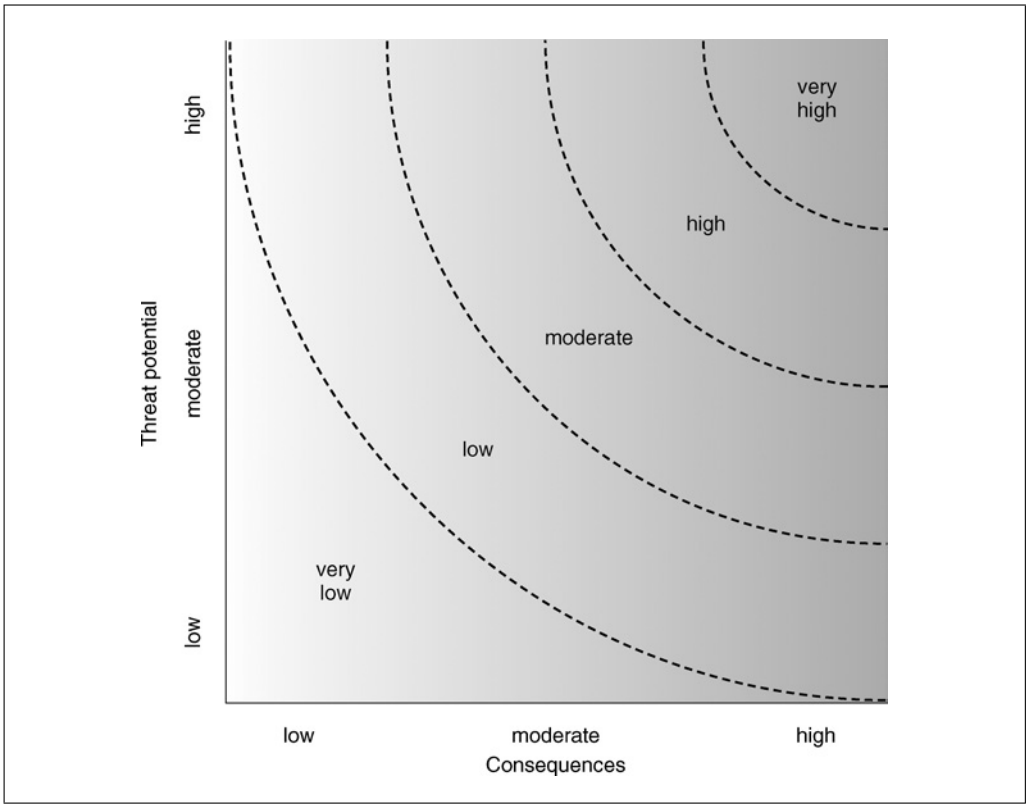

Figure 1A.2.1 Graphic guideline for determining the risk for malicious use of a biological agent. Based on a graphic developed by Sandia Laboratories and used with their kind permission.

(1) level of contagiousness, (2) its ability to cause severe illness or death, (3) its occurrence in the natural local environment (endemic versus exotic agents), and (4) its economic impact and potential for infrastructure disruption.

The threat potential is evaluated by assessing how likely a biological agent could be acquired and manipulated in a manner that favors its release in an infectious state. The evaluation includes: (1) ease of acquisition; (2) ease of growth, processing, and storage; (3) ease of dissemination (e.g., aerosol, ingestion); and (4) environmental stability.

A lesson can be taken from the October 2001 anthrax letters released in the United States. Anthrax is not contagious and, in this case, was associated with a low mortality rate. However, thousands of people were provided preventive treatment and emergency responders worldwide were called on to address false alarms involving "white powder." The potential for weaponization of this microbe contributed to this vast economic and infrastructure disruption.

However, application of these factors in conducting a risk assessment will soon show that few microorganisms present a high risk for malicious use. Therefore, unless otherwise required by regulation, limited risk management techniques will be sufficient to secure the majority of research materials.

RISK MANAGEMENT

Both the World Health Organization (WHO, 2004) and the U.S. Centers for Disease Control and Prevention (CDC/NIH, 1999; MMWR, 2002) have recently included biosecurity discussions as part of commonly used biosafety guidelines. As described by these discussions and elaborated by Sandia National Laboratories (2005), the key components to managing biosecurity risks are (1) physical security measures, (2) personnel security/reliability, (3) materials control and accountability, (4) transfer security, and (5) information security. All of these components are overlapping and interactive in nature (Fig. 1A.2.2). Of particular importance to individuals in microbiology laboratories are physical security, personnel reliability, and materials control and accountability.

Physical Security

Physical security is intended to deter and detect unauthorized access to research areas containing materials of concern. Physical security may be as simple as keeping doors closed at all times and locking the doors when

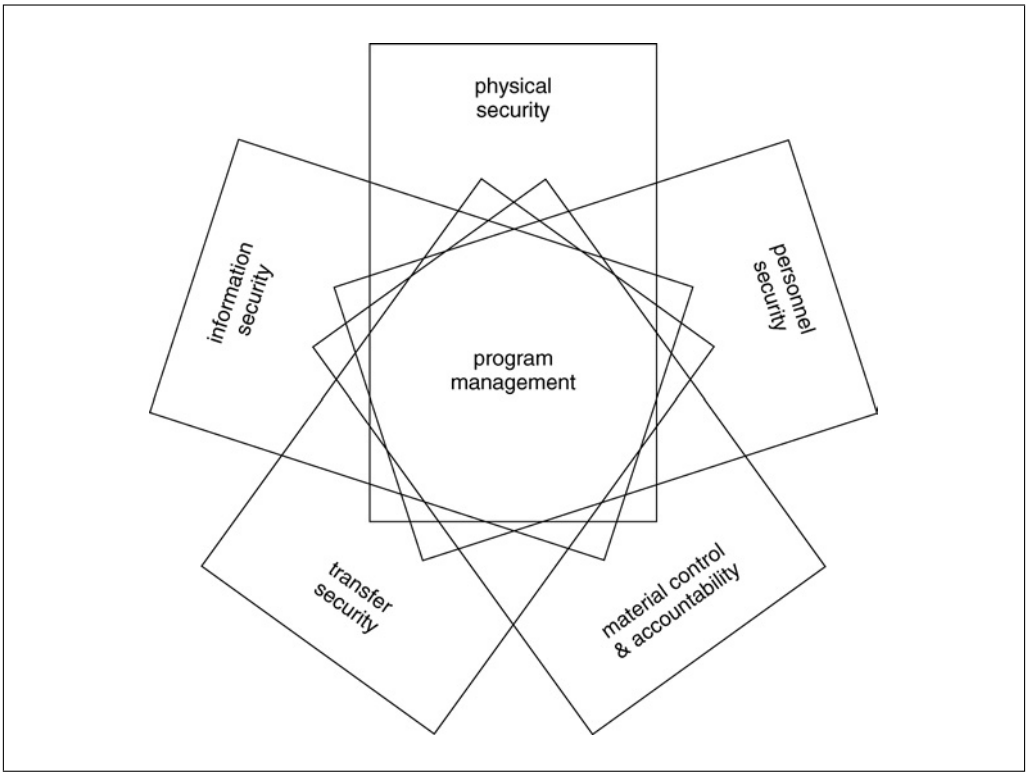

Figure 1A.2.2 Overlapping and interactive components of a comprehensive biosecurity program. Based on a graphic developed by Sandia Laboratories and used with their kind permission.

no one is present. Protecting higher risk assets may require single or multiple keycard or biometric access points or even, in the extreme, checkpoints with security guards. Placing research areas with higher risk assets deeper within a facility may also serve to deter access and intentional removal. In addition, access to these more restricted areas must be monitored, actively or passively, for unauthorized access or attempts at access.

Other considerations to establishing physical security in even benign research settings include: (1) establishing specified access hours; (2) defining what a "suspicious activity" is and how it must be reported; (3) developing a procedure to secure research materials in case of emergencies or to contact security officials if the materials must be left unsecured; and (4) implementing enforcement of security policies—violations must have consequences—e.g., consider revocation of unescorted access into restricted areas.

Personnel Reliability

Physical security may deter an outsider, but will present no barrier to an insider with full access. Personnel reliability is critical in limiting concerns for insider theft. Clearly not all positions within an institution require the same level of scrutiny—the determination of which positions present limited risk or those that carry the most risk is a decision best made by the institution. The type of scrutiny necessary will change depending on the different phases of employment.

Pre-employment screening should include an interviewing process designed to explore reliability issues, and also include a background check.

At time of employment and prior to assignment of access, the following should occur: (1) completion of required employment forms, including appropriate disclosure and conflict of interest declarations, (2) completion of safety and security training, and (3) appropriate medical screening, health evaluations, and immunizations.

During employment, the following options should be considered: (1) assignment of identification badges that clearly indicate access privileges, (2) enforcement of security policies, and (3) availability of employee assistance programs to help resolve personal issues that could contribute to overall stresses.

When an employee is transferred to another area or is terminated, out-processing should include: (1) completion of transfer/termination paperwork; (2) re-inventory of research (and other sensitive) materials and transfer of accountability to another responsible party;

(3) retrieval of security badges, keys, electronic equipment, etc.; and (4) deactivation of computers and electronic access accounts.

Very few, if any, research operations function without requiring access for some sort of visitor. There are several factors to consider with regard to visitors. Determine the different categories of visitors—some visitors are repair or sales persons who provide critical expertise and products for a laboratory, some are observers, and some are working visitors with various lengths of stay. Also, host and escorting procedures and assignments must be documented and followed.

Material Control and Accountability

Physical security and personnel reliability must be coupled with strict attention to material control and accountability. This process works to establish (1) what and where materials are present; (2) how they are stored, used, and transferred securely; and 3) who is accountable for the materials at any given time.

Materials

It is impossible to describe microorganisms in an absolute number because amplification and inactivation are so easily accomplished. A general accounting of the number of containers is more reasonable in this case. A basic inventory is required for the organisms held in repositories, working stocks, master stocks, clinical specimens, genetic material, as well as contaminated materials, such as waste.

Control

Controls are developed to assure that materials stay, or are transported, as intended and that they are used for an authentic, stated purpose by appropriately authorized persons. Physical control may be as simple as a locked freezer. Operational control involves incorporating attention to security issues into standard laboratory procedures. An example of this would be keeping a log to record access to materials of concern. Such a log would be dependent on the nature of the laboratory, the equipment used, and the materials secured. At a minimum, the log should include a brief description of the material, the name of the person accessing the material, the date and time, and when the material was replaced (Fig. 1A.2.3). It is a matter of debate how much information should be kept on a log that could be viewed by persons not involved in the research, but the information recorded must be useful in conducting an investigation if materials are missing. Controls must be developed with regard to both normal and abnormal conditions. For example, the means to secure materials should remain active or be appropriately substituted in the case of a fire alarm where magnetically locked doors are often released.

Other examples of control measures include: (1) confinement to restricted areas, (2) appropriate labeling for accurate identification of materials, (3) clearly documented procedures and inventory for inactivation and disposal, and (4) conducting frequent physical inventories of cataloged research materials.

Accountability

While it is important to inventory and provide control measures for materials of concern, it is imperative that the responsibility for

Logsheet for –80°C freezer
Responsible party: S. Perez

Material description	Removed by	Date	Time	Replaced by	Date	Time
Working stock–*Pseudomonas aeuriginosa* vial #12345	J. Doe	15 March 2005	10:35 a.m.	J. Doe	15 March 2005	2:47 p.m.
Master stock –*Bacillus subtilis* vial #98765	N/A–created master stock			R. Smith	20 April 2005	3:15 p.m.

Figure 1A.2.3 An example of a simple log sheet that may be used to record access to materials of concern in the laboratory.

those materials be clearly assigned to a single, specified person. The person who is the most familiar with the nature of the material, as well as its use and storage, is the best candidate to be held accountable. Other persons who become involved in transfers of materials must also meet appropriate and documented criteria for gaining access to, and accountability for, materials.

An invaluable tool in material control and accountability is the use of a detailed chain of custody document, which records and tracks the materials, control procedures, and the person accountable at each point in the process.

While these processes seem relatively simple, the reality is that material control and accountability are quite complex in microbiological and biomedical research settings. Research materials are routinely transferred between the lab, storage units, and other repositories. Portions of these samples may be divided among different stocks and experiments in progress, involving several different researchers. The daily routine of a laboratory may include transfer of microbes to different persons for the purposes of shipment, destruction, or testing.

DUAL-USE RESEARCH

A parallel concern to laboratory biosecurity of research materials is the concept of "dual-use" research. Dual-use research is that which is conducted for a legitimate purpose but also holds the potential to be misused for bioterrorism. In general, the concern for misuse will not be realized until the research is made public through presentation or publication (National Research Council, 2004). The process of identifying so-called "experiments of concern" is being debated on national and international levels. In the U.S., deliberations of the National Science Advisory Board for Biosecurity (NSABB; see Internet Resources) are focused on permitting critical research to proceed unimpeded, while addressing security issues for research with a potential for misuse. The various groups that are studying the balance between open science and security issues acknowledge that to be truly effective, any decision on how to manage "dual-use" research must be made and implemented internationally.

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Safe Use of Hazardous Chemicals

Persons carrying out the protocols in this manual may encounter toxic chemicals, including carcinogenic, mutagenic, or teratogenic reagents (see Table 1A.3.1). Most governments regulate the use of these materials; it is essential that they be used in strict accordance with local and national regulations. Cautionary notes and some specific guidelines are included in many instances throughout the book; however, users must proceed with the prudence and precautions associated with good laboratory practice, under the supervision of those responsible for implementing laboratory safety programs at their institutions. Guidelines for the safe use of radioisotopes are presented in *APPENDIX 1C*.

It is not possible in the space available to list all the precautions required for handling hazardous chemicals. Many texts have been written about laboratory safety (see Literature Cited and Key References). Obviously, all national and local laws should be obeyed, as well as all institutional regulations. Controlled substances are regulated by the Drug Enforcement Administration (*http://www.usdoj.gov/dea*). By law, Material Safety Data Sheets (MSDSs) must be readily available. All laboratories should have a Chemical Hygiene Plan (29 CFR Part 1910.1450); institutional safety officers should be consulted as to its implementation. Help is (or should be) available from your institutional Safety Office: use it.

Chemicals must be stored properly for safety. Certain chemicals cannot be easily or safely mixed with and should not be stored near certain other chemicals, because their reaction is violently exothermic or yields a toxic product. Some examples of incompatibility are listed in Table 1A.3.2. When in doubt, always consult a current MSDS for information on reactivity, handling, and storage. Chemicals should be separated into general hazard classes and stored appropriately. For example, flammable chemicals such as alcohols, ketones, aliphatic and aromatic hydrocarbons, and other materials labeled flammable should be stored in approved flammable storage cabinets, with those also requiring refrigeration being kept in explosion-proof refrigerators. Strong oxidizers must be segregated. Strong acids (e.g., sulfuric, hydrochloric, nitric, perchloric, and hydrofluoric) should be stored in a separate cabinet well removed from strong bases and from flammable organics. An exception is glacial acetic acid, which is both corrosive and flammable, and which must be stored with the flammables.

Facilities should be appropriate for working with hazardous chemicals. In particular, hazardous chemicals should be handled only in chemical fume hoods, not in laminar flow cabinets. The functioning of the fume hoods should be checked periodically. Laboratories should also be equipped with safety showers and eye-wash facilities. Again, this equipment should be tested periodically to ensure that it functions correctly. Other safety equipment may be required depending on the nature of the materials being handled. In addition, researchers should be trained in the proper procedures for handling hazardous chemicals as well as other laboratory operations—e.g., handling of compressed gases, use of cryogenic liquids, operation of high-voltage power supplies, and operation of lasers of all types.

Before starting work, know the physical and chemical hazards of the reagents used. Wear appropriate protective clothing and have a plan for dealing with spills or accidents; coming up with a good plan on the spur of the moment is very difficult. For example, have the appropriate decontaminating or neutralizing agents prepared and close at hand. Small spills can probably be cleaned by the researcher. In the case of larger spills,

Contributed by George Lunn and Gretchen Lawler
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Table 1A.3.1 Commonly Used Hazardous Chemicals[a,b]

Chemical	Hazards	Remarks
Acetic acid, glacial	Corrosive, flammable liquid	
Acetonitrile	Flammable liquid, teratogenic, toxic	
Acridine orange	Carcinogenic, mutagenic	See Basic Protocol 2
Acrylamide	Carcinogenic, toxic	Use dust mask; polyacrylamide gels contain residual acrylamide monomer and should be handled with gloves; acrylamide may polymerize with violence on melting at 86°C
Alcian blue 8GX		See Basic Protocol 2
Alizarin red S (monohydrate)		
p-Amidinophenylmethanesulfonyl fluoride (APMSF)	Enzyme inhibitor	See Basic Protocol 11
7-Aminoactinomycin D (7-AAD)	Carcinogenic	
4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF)	Mutagenic, enzyme inhibitor	See Basic Protocol 11
Ammonium hydroxide, concentrated	Corrosive, lachrymatory, toxic	
Azure A	Mutagenic	See Basic Protocol 2
Azure B	Mutagenic	See Basic Protocol 2
Benzidine (BDB)	Carcinogenic, toxic	See Basic Protocol 1
Bisacrylamide	Toxic	
Boron dipyrromethane derivatives (BODIPY dyes)	Toxic	
Brilliant blue R	Carcinogenic, mutagenic	See Basic Protocol 2
5-Bromodeoxyuridine (BrdU)	Mutagenic, teratogenic, photosensitizing	
Cetylpyridinium chloride (CPC)	Toxic	
Cetyltrimethylammonium bromide (CTAB)	Corrosive, teratogenic, toxic	
Chloroform	Carcinogenic, teratogenic, toxic	
Chlorotrimethylsilane	Carcinogenic, corrosive, flammable liquid, toxic	Reacts violently with water; see Basic Protocol 3
Chromic/sulfuric acid cleaning solution	Carcinogenic, corrosive, oxidizer, toxic	Replace with suitable commercially available cleanser
Chromomycin A3 (CA3)	Teratogenic, toxic	
Congo red	Mutagenic, teratogenic	See Basic Protocol 2
Coomassie brilliant blue G	Mutagenic	See Basic Protocol 2
Crystal violet		See Basic Protocol 2
Cresyl violet acetate	Mutagenic	See Basic Protocol 2
Cyanides (e.g., KCN, NaCN)	Toxic	Contact with acid will liberate HCN gas; see Basic Protocol 4
Cyanines (e.g., Cy3, Cy5)	Toxic	
Cyanogen bromide (CNBr)	Toxic	See Basic Protocol 4

continued

Table 1A.3.1 Commonly Used Hazardous Chemicals[a,b], *continued*

Chemical	Hazards	Remarks
2′-Deoxycoformycin (dCF, pentostatin)	Teratogenic, toxic	
4′,6-Diamidino-2-phenylindole (DAPI)	Mutagenic	
Diaminobenzidine (DAB)	Carcinogenic	See Basic Protocol 1
1,4-Diazabicyclo[2,2,2]-octane (DABCO)	Toxic	Forms an explosive complex with hydrogen peroxide
Dichloroacetic acid (DCA)	Carcinogenic, corrosive, toxic	
Dichlorodimethylsilane	Corrosive, flammable liquid, toxic	See Basic Protocol 3
Dichloromethane (methylene chloride)	Carcinogenic, mutagenic, teratogenic, toxic	
Diethylamine (DEA)	Corrosive, flammable liquid, toxic	
Diethylpyrocarbonate (DEPC)	Carcinogenic, toxic	
Diethyl sulfate	Carcinogenic, teratogenic, toxic	See Basic Protocol 5
Diisopropyl fluorophosphate (DFP)	Highly toxic, cholinesterase inhibitor, neurotoxin	See Basic Protocol 11
Dimethyl sulfate (DMS)	Carcinogenic, toxic	See Basic Protocol 5
Dimethyl sulfoxide (DMSO)	Flammable liquid, toxic	Enhances absorption through skin
Diphenylamine (DPA)	Teratogenic, toxic	
2,5-Diphenyloxazole (PPO)	Toxic	
Dithiothreitol (DTT)	Toxic	
Eosin B		See Basic Protocol 2
Erythrosin B	Carcinogenic, mutagenic	See Basic Protocol 2
Ether	Flammable liquid, toxic	May form explosive peroxides on standing; do not dry with NaOH or KOH
Ethidium bromide (EB)	Mutagenic, toxic	See Basic Protocol 2 or 6
Ethyl methanesulfonate (EMS)	Carcinogenic, toxic	See Basic Protocol 5
Fluorescein and derivatives	Carcinogenic, toxic	
5-Fluoro-2′-deoxyuridine (FUdR)	Teratogenic, toxic	
Fluoroorotic acid (FOA)	Toxic	
Formaldehyde	Carcinogenic, flammable liquid, teratogenic, toxic	
Formamide	Teratogenic, toxic	
Formic acid	Corrosive, toxic	May explode when heated $>180^{\circ}C$ in a sealed tube
Glutaraldehyde	Corrosive, teratogenic, toxic	
Guanidinium thiocyanate	Toxic	
Hoechst 33258 dye	Mutagenic, toxic	
Hydrochloric acid, concentrated	Corrosive, teratogenic, toxic	

continued

Table 1A.3.1 Commonly Used Hazardous Chemicals[a,b], *continued*

Chemical	Hazards	Remarks
Hydrogen peroxide (30%)	Carcinogenic, corrosive, mutagenic, oxidizer	Avoid bringing into contact with organic materials, which may form explosive peroxides; may decompose violently in contact with metals, salts, or oxidizable materials; see Basic Protocol 7
Hydroxylamine	Corrosive, flammable, mutagenic, toxic	Explodes in air at >70°C
3-β-Indoleacrylic acid (IAA)	Carcinogenic	
Iodine	Corrosive, toxic	See Basic Protocol 8
Iodoacetamide	Carcinogenic, mutagenic, toxic	
Janus green B	Carcinogenic, mutagenic	See Basic Protocol 2
Lead compounds	Carcinogenic, toxic	
2-Mercaptoethanol (2-ME)	Stench, toxic	
Mercury compounds	Teratogenic, toxic	See Basic Protocol 9
Methionine sulfoximine (MSX)	Teratogenic, toxic	
Methotrexate (amethopterin)	Carcinogenic, mutagenic, teratogenic, toxic	
Methylene blue	Mutagenic, toxic	See Basic Protocol 2
Methyl methanesulfonate (MMS)	Carcinogenic, toxic	See Basic Protocol 5
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)	Carcinogenic, mutagenic, teratogenic	Explodes when heated or under impact; contact with base produces toxic, explosive, carcinogenic diazomethane; see Basic Protocol 12
Mycophenolic acid (MPA)	Teratogenic, toxic	
Neutral red	Mutagenic	See Basic Protocol 2
Nigrosin, water soluble		See Basic Protocol 2
Nitric acid, concentrated	Corrosive, oxidizer, teratogenic, toxic	
Nitroblue tetrazolium (NBT)	Toxic	
Orcein, synthetic		See Basic Protocol 2
Oxonols	Toxic	
Paraformaldehyde	Toxic	
Phenol	Carcinogenic, corrosive, teratogenic, toxic	Readily absorbed through the skin
Phenylmethylsulfonyl fluoride (PMSF)	Enzyme inhibitor	See Basic Protocol 11
Phorbol 12-myristate 13-acetate (PMA)	Carcinogenic, toxic	
Phycoerythrins (PE)	Toxic	
Piperidine	Flammable liquid, teratogenic, toxic	
Potassium hydroxide, concentrated	Corrosive, toxic	Produces a highly exothermic reaction when solid is added to water
Propane sultone	Carcinogenic, toxic	See Basic Protocol 5
Propidium iodide (PI)	Mutagenic	See Basic Protocol 2 or 6

continued

Table 1A.3.1 Commonly Used Hazardous Chemicals[a,b], *continued*

Chemical	Hazards	Remarks
Pyridine	Flammable liquid, toxic	
Rhodamine and derivatives	Toxic	
Rose Bengal	Carcinogenic, teratogenic	See Basic Protocol 2
Safranine O	Mutagenic	See Basic Protocol 2
Sodium azide	Carcinogenic, toxic	Adding acid liberates explosive volatile, toxic hydrazoic acid; can form explosive heavy metal azides, e.g., with plumbing fixtures—*do not* discharge down drain; see Basic Protocol 10
Sodium deoxycholate (Na-DOC)	Carcinogenic, teratogenic, toxic	
Sodium dodecyl sulfate (sodium lauryl sulfate, SDS)	Sensitizing, toxic	
Sodium hydroxide, concentrated	Corrosive, toxic	A highly exothermic reaction ensues when the solid is added to water
Sodium nitrite	Carcinogenic	
Sulfuric acid, concentrated	Corrosive, oxidizer, teratogenic, toxic	Reaction with water is very exothermic; always add concentrated sulfuric acid to water, *never* water to acid
SYTO dyes	Toxic	
Tetramethylammonium chloride (TMAC)	Toxic	
N,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (TEMED)	Corrosive, flammable liquid, toxic	
Texas Red (sulforhodamine 101, acid chloride)	Toxic	
Toluene	Flammable liquid, teratogenic, toxic	
Toluidine blue O	Mutagenic, toxic	See Basic Protocol 2
*N*α-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK)	Toxic, enzyme inhibitor	See Basic Protocol 11
N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	Toxic, mutagenic, enzyme inhibitor	See Basic Protocol 11
Trichloroacetic acid (TCA)	Carcinogenic, corrosive, teratogenic, toxic	
Triethanolamine acetate (TEA)	Carcinogenic, toxic	
Trifluoroacetic acid (TFA)	Corrosive, toxic	
Trimethyl phosphate (TMP)	Carcinogenic, mutagenic, teratogenic	May explode on distillation
Trypan blue	Carcinogenic, mutagenic, teratogenic	See Basic Protocol 2
Xylenes	Flammable liquid, teratogenic, toxic	

[a]*CAUTION:* These chemicals should be handled only in a chemical fume hood by knowledgeable workers equipped with eye protection, lab coat, and gloves. The laboratory should be equipped with a safety shower and eye wash. Additional protective equipment may be required.
[b]For extensive information on the hazards of these and other chemicals as well as cautionary details, see Bretherick (1990), Lunn and Sansone (1994a), Bretherick et al. (1999), Lewis (1999), Furr (2000), and O'Neil (2001).

Table 1A.3.2 Examples of Chemical Incompatibility

Chemical	Incompatible with
Acetic acid	Aldehydes, bases, carbonates, chromic acid, ethylene glycol, hydroxides, hydroxyl compounds, metals, nitric acid, oxidizers, perchloric acid, peroxides, phosphates, permanganates, xylene
Acetone	Acids, amines, concentrated nitric and sulfuric acid mixtures, oxidizers, plastics
Acetylene	Copper, halogens, mercury, oxidizers, potassium, silver
Alkali metals, alkaline earth metals	Acids, aldehydes, carbon dioxide, carbon tetrachloride or other chlorinated hydrocarbons, halogens, ketones, plastics, sulfur, water
Ammonia (anhydrous)	Acids, aldehydes, amides, calcium hypochlorite, hydrofluoric acid, halogens, heavy metals, mercury, oxidizers, plastics, sulfur
Ammonium nitrate	Acids, alkalis, chlorates, chloride salts, flammable and combustible materials, metals, organic materials, phosphorus, reducing agents, sulfur, urea
Aniline	Acids, aluminum, dibenzoyl peroxide, oxidizers, plastics
Arsenical materials	Any reducing agent
Azides	Acids, heavy metals, oxidizers
Bromine	Acetaldehyde, alcohols, alkalis, amines, ammonia, combustible materials, ethylene, fluorine, hydrogen, ketones (e.g., acetone, carbonyls), metals, petroleum gases, sodium carbide, sulfur
Calcium oxide	Acids, ethanol, fluorine, organic materials, water
Carbon (activated)	Alkali metals, calcium hypochlorite, halogens, oxidizers
Carbon tetrachloride	Sodium
Chlorates	Acids, ammonium salts, finely divided organic or combustible materials, powdered metals, sulfur
Chlorine	Acetylene or other hydrocarbons, alcohols, ammonia, benzene, butadiene, butane, combustible materials, ethylene, flammable compounds (e.g., hydrazine), hydrogen, hydrogen peroxide, iodine, metals, methane, nitrogen, oxygen, propane (or other petroleum gases), sodium carbide, sodium hydroxide
Chlorine dioxide	Ammonia, hydrogen, hydrogen sulfide, mercury, methane, organic materials, phosphine, phosphorus, potassium hydroxide, sulfur
Chromic acid, chromic oxide	Acetic acid, acetone, alcohols, alkalis, ammonia, bases, benzene, camphor, flammable liquids, glycerin (glycerol), hydrocarbons, metals, naphthalene, organic materials, phosphorus, plastics
Copper	Acetylene, calcium, hydrocarbons, hydrogen peroxide, oxidizers
Cumene hydroperoxide	Acids (organic or inorganic)
Cyanides	Acids, alkaloids, aluminum, iodine, oxidizers, strong bases
Flammable liquids	Ammonium nitrate, chromic acid, halogens, hydrogen peroxide, nitric acid, oxidizing agents in general, oxygen, sodium peroxide

continued

Table 1A.3.2 Examples of Chemical Incompatibility, *continued*

Chemical	Incompatible with
Fluorine	All other chemicals
Hydrocarbons (liquid or gas)	See flammable liquids
Hydrocyanic acid	Alkali, nitric acid
Hydrofluoric acid	Ammonia, metals, organic materials, plastics, silica (glass, including fiberglass), sodium
Hydrogen peroxide	All organics, most metals or their salts, nitric acid, phosphorus, sodium, sulfuric acid
Hydrogen sulfide	Acetylaldehyde, fuming nitric acid, metals, oxidizers, sodium, strong bases
Hydroperoxide	Reducing agents
Hypochlorites	Acids, activated carbon
Iodine	Acetylaldehyde, acetylene, ammonia, hydrogen, metals, sodium
Mercury	Acetylene, aluminum, amines, ammonia, calcium, fulminic acid, lithium, oxidizers, sodium
Nitric acid	Acids, nitrites, metals, most organics, plastics, sodium, sulfur, sulfuric acid
Nitrites	Acids
Nitroparaffins	Amines, inorganic bases
Oxalic acid	Mercury, oxidizers, silver, sodium chlorite
Oxygen	All flammable and combustible materials, ammonia, carbon monoxide, grease, metals, oil, phosphorus, polymers
Perchloric acid	All organics, bismuth and alloys, dehydrating agents, grease, hydrogen halides, iodides, paper, wood
Peroxides, organic	Acids (organic or mineral), avoid friction, store cold
Phosphorus (white)	Air, alkalis, oxygen, reducing agents
Potassium chlorate	Acids, ammonia, combustible materials, fluorine, hydrocarbons, metals, organic materials, reducing agents, sugars
Potassium perchlorate	Alcohols, combustible materials, fluorine, hydrazine, metals, organic matter, reducing agents, sulfuric acid
Potassium permanganate	Benzaldehyde, ethylene glycol, glycerin, sulfuric acid
Selenides and tellurides	Reducing agents
Silver	Acetylene, ammonium compounds, fulminic acid, oxalic acid, ozonides, peroxyformic acid, tartaric acid
Sodium	Acids, carbon dioxide, carbon tetrachloride, hydrazine, metals, oxidizers, water
Sodium nitrate	Acetic anhydride, acids, metals, organic matter, peroxyformic acid, reducing agents
Sodium peroxide	Acetic anhydride, benzaldehyde, benzene, carbon disulfide, ethyl acetate, ethyl or methyl alcohol, ethylene glycol, furfural, glacial acetic acid, glycerin, hydrogen sulfide, metals, methyl acetate, oxidizers, peroxyformic acid, phosphorus, reducing agents, sugars, water
Sulfides	Acids
Sulfuric acid	Alcohols, bases, chlorates, perchlorates, permanganates of potassium, lithium, sodium, magnesium, calcium

the area should be evacuated and help should be sought from those experienced in and equipped for dealing with spills—e.g., the institutional Safety Office.

Protective equipment should include, at a minimum, eye protection, a lab coat, and gloves. In certain circumstances, other items of protective equipment may be necessary (e.g., a face shield). Different types of gloves exhibit different resistance properties (Table 1A.3.3). No gloves resist all chemicals, and no gloves resist any chemicals indefinitely. Disposable gloves labeled "exam" or "examination" are primarily for protection from biological materials (e.g., viruses, bacteria, feces, blood). They are not designed for and usually have not been tested for resistance to chemicals. Disposable gloves generally offer *extremely* marginal protection from chemical hazards in most cases and should be removed immediately upon contamination before the chemical can pass through. If possible, design handling procedures to eliminate or reduce potential for contamination. Never assume that disposable gloves will offer the same protection or even have the same properties as nondisposables. Select gloves carefully and always look for some evidence that they will protect against the materials being used. Inspect all gloves before every use for possible holes, tears, or weak areas. Never reuse disposable gloves. Clean reusable gloves after each use and dry carefully inside and out. Observe all common-sense precautions—e.g., do not pipet by mouth, keep unauthorized persons away from hazardous chemicals, do not eat or drink in the laboratory, wear proper clothing in the laboratory (sandals, open-toed shoes, and shorts are not appropriate).

Order hazardous chemicals only in quantities that are likely to be used in a reasonable time. Buying large quantities at a lower unit cost is no bargain if someone (perhaps you) has to pay to dispose of surplus quantities. Substitute alcohol-filled thermometers for mercury-filled thermometers, which are a hazardous chemical spill waiting to happen.

Although any number of chemicals commonly used in laboratories are toxic if used improperly, the toxic properties of a number of reagents require special mention. Chemicals that exhibit carcinogenic, corrosive, flammable, lachrymatory, mutagenic, oxidizing, teratogenic, toxic, or other hazardous properties are listed in Table 1A.3.1. Chemicals listed as carcinogenic range from those accepted by expert review groups as causing cancer in humans to those for which only minimal evidence of carcinogenicity exists. No effort has been made to differentiate the carcinogenic potential of the compounds in Table 1A.3.1. Oxidizers may react violently with oxidizable material (e.g., hydrocarbons, wood, and cellulose). Before using any of these chemicals, thoroughly investigate all its characteristics. Material Safety Data Sheets are readily available; they list some hazards but vary widely in quality. A number of texts describing hazardous properties are listed at the end of this unit (see Literature Cited). In particular, Sax's *Dangerous Properties of Industrial Materials*, 11th ed. (Lewis, 2004), and the *Handbook of Reactive Chemical Hazards*, 6th ed. (Bretherick et al., 1999), give comprehensive listings of known hazardous properties; however, these texts list only the known properties. Many chemicals, especially fluorochromes, have been tested only partially or not at all. Prudence dictates that, unless there is good reason for believing otherwise, all chemicals should be regarded as volatile, highly toxic, flammable human carcinogens and should be handled with great care.

Waste should be segregated according to institutional requirements, for example, into solid, aqueous, nonchlorinated organic, and chlorinated organic material, and should always be disposed of in accordance with all applicable federal, state, and local regulations. Extensive information and cautionary details along with techniques for the disposal of chemicals in laboratories have been published (Bretherick, 1990; Lunn and Sansone, 1994a; O'Neil, 2001; Furr, 2000). Some commonly used disposal procedures are outlined in Basic Protocols 1 to 12. Incorporation of these procedures into laboratory protocols

Table 1A.3.3 Chemical Resistance of Commonly Used Gloves[a,b]

Chemical	Neoprene gloves	Latex gloves	Butyl gloves	Nitrile gloves
*Acetaldehyde	VG	G	VG	G
Acetic acid	VG	VG	VG	VG
*Acetone	G	VG	VG	P
Ammonium hydroxide	VG	VG	VG	VG
*Amyl acetate	F	P	F	P
Aniline	G	F	F	P
*Benzaldehyde	F	F	G	G
*Benzene	P	P	P	F
Butyl acetate	G	F	F	P
Butyl alcohol	VG	VG	VG	VG
Carbon disulfide	F	F	F	F
*Carbon tetrachloride	F	P	P	G
*Chlorobenzene	F	P	F	P
*Chloroform	G	P	P	E
Chloronaphthalene	F	P	F	F
Chromic acid (50%)	F	P	F	F
Cyclohexanol	G	F	G	VG
*Dibutyl phthalate	G	P	G	G
Diisobutyl ketone	P	F	G	P
Dimethylformamide	F	F	G	G
Dioctyl phthalate	G	P	F	VG
Epoxy resins, dry	VG	VG	VG	VG
*Ethyl acetate	G	F	G	F
Ethyl alcohol	VG	VG	VG	VG
*Ethyl ether	VG	G	VG	G
*Ethylene dichloride	F	P	F	P
Ethylene glycol	VG	VG	VG	VG
Formaldehyde	VG	VG	VG	VG
Formic acid	VG	VG	VG	VG
Freon 11, 12, 21, 22	G	P	F	G
*Furfural	G	G	G	G
Glycerin	VG	VG	VG	VG
Hexane	F	P	P	G
Hydrochloric acid	VG	G	G	G
Hydrofluoric acid (48%)	VG	G	G	G
Hydrogen peroxide (30%)	G	G	G	G
Ketones	G	VG	VG	P
Lactic acid (85%)	VG	VG	VG	VG
Linseed oil	VG	P	F	VG

continued

Table 1A.3.3 Chemical Resistance of Commonly Used Gloves[a,b], *continued*

Chemical	Neoprene gloves	Latex gloves	Butyl gloves	Nitrile gloves
Methyl alcohol	VG	VG	VG	VG
Methylamine	F	F	G	G
Methyl bromide	G	F	G	F
*Methyl ethyl ketone	G	G	VG	P
*Methyl isobutylketone	F	F	VG	P
Methyl methacrylate	G	G	VG	F
Monoethanolamine	VG	G	VG	VG
Morpholine	VG	VG	VG	G
Naphthalene	G	F	F	G
Naphthas, aliphatic	VG	F	F	VG
Naphthas, aromatic	G	P	P	G
*Nitric acid	G	F	F	F
Nitric acid, red and white fuming	P	P	P	P
Nitropropane (95.5%)	F	P	F	F
Oleic acid	VG	F	G	VG
Oxalic acid	VG	VG	VG	VG
Palmitic acid	VG	VG	VG	VG
Perchloric acid (60%)	VG	F	G	G
Perchloroethylene	F	P	P	G
Phenol	VG	F	G	F
Phosphoric acid	VG	G	VG	VG
Potassium hydroxide	VG	VG	VG	VG
Propyl acetate	G	F	G	F
i-Propyl alcohol	VG	VG	VG	VG
n-Propyl alcohol	VG	VG	VG	VG
Sodium hydroxide	VG	VG	VG	VG
Styrene (100%)	P	P	P	F
Sulfuric acid	G	G	G	G
Tetrahydrofuran	P	F	F	F
*Toluene	F	P	P	F
Toluene diisocyanate	F	G	G	F
*Trichloroethylene	F	F	P	G
Triethanolamine	VG	G	G	VG
Tung oil	VG	P	F	VG
Turpentine	G	F	F	VG
*Xylene	P	P	P	F

[a]Performance varies with glove thickness and duration of contact. An asterisk indicates limited use. Abbreviations: VG, very good; G, good; F, fair; P, poor (do not use).

[b]Adapted from the July 8, 1998, version of the DOE OSH Technical Reference Chapter 5 (APPENDIX C at *http://eh.doe.gov/docs/osh_tr/ch5c.html*). For more information, also see Forsberg and Keith (1999) and Forsberg and Mansdort (2003).

Table 1A.3.4 Protocols for Disposal of Some Hazardous Chemicals

Protocol	Method
Basic Protocol 1	Disposal of Benzidine and Diaminobenzidine
Alternate Protocol 1	Decontamination of Spills Involving Benzidine and Diaminobenzidine
Alternate Protocol 2	Decontamination of Aqueous Solutions of Benzidine and Diaminobenzidine
Support Protocol 1	Analytical Procedures to Detect Benzidine and Diaminobenzidine
Basic Protocol 2	Disposal of Biological Stains
Alternate Protocol 3	Continuous-Flow Decontamination of Aqueous Solutions of Biological Stains
Support Protocol 2	Analytical Procedures to Detect Biological Stains
Basic Protocol 3	Disposal of Chlorotrimethylsilane and Dichlorodimethylsilane
Basic Protocol 4	Disposal of Cyanides and Cyanogen Bromide
Support Protocol 3	Analytical Procedure to Detect Cyanide
Basic Protocol 5	Disposal of Dimethyl Sulfate, Diethyl Sulfate, Methyl Methanesulfonate, Ethyl Methanesulfonate, Diepoxybutane, and 1,3-Propane Sultone
Support Protocol 4	Analytical Procedure to Detect the Presence of Dimethyl Sulfate, Diethyl Sulfate, Methyl Methanesulfonate, Ethyl Methanesulfonate, Diepoxybutane, and 1,3-Propane Sultone
Basic Protocol 6	Disposal of Ethidium Bromide and Propidium Iodide
Alternate Protocol 4	Decontamination of Equipment Contaminated with Ethidium Bromide
Alternate Protocol 5	Decontamination of Ethidium Bromide in Isopropanol Saturated with Cesium Chloride
Alternate Protocol 6	Decontamination of Ethidium Bromide in Isoamyl Alcohol and 1-Butanol
Support Protocol 5	Analytical Procedure to Detect Ethidium Bromide or Propidium Iodide
Basic Protocol 7	Disposal of Hydrogen Peroxide
Basic Protocol 8	Disposal of Iodine
Basic Protocol 9	Disposal of Mercury Compounds
Alternate Protocol 7	Decontamination of Waste Water Containing Mercury
Support Protocol 6	Analytical Procedure to Detect Mercury
Basic Protocol 10	Disposal of Sodium Azide
Support Protocol 7	Analytical Procedure to Detect Sodium Azide
Support Protocol 8	Analytical Procedure to Detect Nitrite
Basic Protocol 11	Disposal of Enzyme Inhibitors
Support Protocol 9	Analytical Procedure to Detect Enzyme Inhibitors
Basic Protocol 12	Disposal of *N*-Methyl-*N*′-Nitro-*N*-Nitrosoguanidine (MNNG)
Alternate Protocol 8	Decontamination of Glassware Contaminated with *N*-Methyl-*N*′-Nitro-Nitrosoguanidine (MNNG)
Alternate Protocol 9	Decontamination of Spills of *N*-Methyl-*N*′-Nitro-*N*-Nitrosoguanidine (MNNG)
Support Protocol 10	Analytical Procedure to Detect *N*-Methyl-*N*′-Nitro-*N*-Nitrosoguanidine (MNNG)

can help to minimize waste disposal problems. Alternate Protocols 1 to 9 describe decontamination methods for some of the chemicals. Support Protocols 1 to 10 describe analytical techniques that are used to verify that reagents have been decontaminated; with modification, these assays may also be used to determine the concentration of a particular chemical.

DISPOSAL METHODS

A number of procedures for the disposal of hazardous chemicals are available; protocols for the disposal and decontamination of some hazardous chemicals commonly encountered in microbiology laboratories are listed in Table 1A.3.4. These procedures are necessarily brief; for full details consult the original references or a collection of these procedures (see Lunn and Sansone, 1994a).

CAUTION: These disposal methods should be carried out only in a chemical fume hood by workers equipped with eye protection, a lab coat, and gloves. Additional protective equipment may be necessary.

BASIC PROTOCOL 1

DISPOSAL OF BENZIDINE AND DIAMINOBENZIDINE

Benzidine and diaminobenzidine can be degraded by oxidation with potassium permanganate (Castegnaro et al., 1985; Lunn and Sansone, 1991a). This protocol presents a method for decontamination of benzidine and diaminobenzidine in bulk. It can also be adapted to the decontamination of benzidine and diaminobenzidine spills (see Alternate Protocol 1). Alternatively, these compounds can be removed from solution using horseradish peroxidase in the presence of hydrogen peroxide (see Alternate Protocol 2). Destruction and decontamination are >99%. Support Protocol 1 is used to test for the presence of benzidine and diaminobenzidine.

Materials

Benzidine or diaminobenzidine tetrahydrochloride dihydrate
0.1 M HCl (for benzidine)
0.2 M potassium permanganate: prepare immediately before use
2 M sulfuric acid
Sodium metabisulfite
10 M potassium hydroxide (KOH)
Additional reagents and equipment for testing for the presence of aromatic amines (see Support Protocol 1)

1. For each 9 mg benzidine, add 10 ml of 0.1 M HCl *or* for each 9 mg diaminobenzidine tetrahydrochloride dihydrate, add 10 ml water. Stir the solution until the aromatic amine has completely dissolved.
2. For each 10 ml of solution, add 5 ml freshly prepared 0.2 M potassium permanganate and 5 ml of 2 M sulfuric acid. Allow the mixture to stand for ≥10 hr.
3. Add sodium metabisulfite until the solution is decolorized.
4. Add 10 M KOH to make the solution strongly basic, pH >12.

   CAUTION: *This reaction is exothermic.*
5. Dilute with 5 vol water and pass through filter paper to remove manganese compounds.
6. Test the filtrate for the presence of aromatic amines (i.e., benzidine or diaminobenzidine; see Support Protocol 1).
7. Neutralize the filtrate with acid and discard.

DECONTAMINATION OF SPILLS INVOLVING BENZIDINE AND DIAMINOBENZIDINE

ALTERNATE PROTOCOL 1

Additional Materials *(also see Basic Protocol 1)*

Glacial acetic acid
1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid: prepare immediately before use
Absorbent material (e.g., paper towels, Kimwipes)
High-efficiency particulate air (HEPA) vacuum (Fisher)
Additional reagents and equipment for testing for the presence of aromatic amines (see Support Protocol 1)

CAUTION: This procedure may damage painted surfaces and Formica.

1. Remove as much of the spill as possible using absorbent material and high-efficiency particulate air (HEPA) vacuuming.
2. Wet the surface with glacial acetic acid until all the amines are dissolved, then add an excess of freshly prepared 1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid to the spill area. Allow the mixture to stand $\geq$10 hr.
3. Ventilate the area and decolorize with sodium metabisulfite.
4. Mop up the liquid with paper towels. Squeeze the solution out of the towels and collect in a suitable container. Discard towels as hazardous solid waste.
5. Add 10 M KOH to make the solution strongly basic, pH $\geq$12.

   CAUTION: *This reaction is exothermic*.
6. Dilute with 5 vol water and filter through filter paper to remove manganese compounds.
7. Test the filtrate for the presence of aromatic amines (i.e., benzidine or diaminobenzidine; see Support Protocol 1).
8. Neutralize the filtrate with acid and discard it.
9. Verify complete decontamination by wiping the surface with a paper towel moistened with water and squeezing the liquid out of the towel. Test the liquid for the presence of benzidine or diaminobenzidine (see Support Protocol 1). Repeat steps 1 to 9 as necessary.

DECONTAMINATION OF AQUEOUS SOLUTIONS OF BENZIDINE AND DIAMINOBENZIDINE

ALTERNATE PROTOCOL 2

The enzyme horseradish peroxidase catalyzes the oxidation of the amine to a radical which diffuses into solution and polymerizes. The polymers are insoluble and fall out of solution.

Additional Materials *(also see Basic Protocol 1)*

Aqueous solution of benzidine or diaminobenzidine
1 N HCl or NaOH
3% (v/v) hydrogen peroxide
Horseradish peroxidase (see recipe)
1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid
5% (w/v) ascorbic acid
Porous glass filter *or* Sorvall GLC-1 centrifuge or equivalent

Additional reagents and equipment for testing for the presence of aromatic amines (see Support Protocol 1)

1. Adjust the pH of the aqueous benzidine or diaminobenzidine solution to 5 to 7 with 1 N HCl or NaOH as required, and dilute so the concentration of aromatic amines is $\leq$100 mg/liter.
2. For each liter of solution, add 3 ml of 3% hydrogen peroxide and 300 U horseradish peroxidase. Let the mixture stand 3 hr.
3. Remove the precipitate by filtering the solution through a porous glass filter or by centrifuging 5 min at room temperature in a benchtop centrifuge to pellet the precipitate.

   The precipitate is mutagenic and should be treated as hazardous waste.

4. Immerse the porous glass filter in 1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid. Clean the filter in a conventional fashion and discard potassium permanganate/sulfuric acid solution as described for benzidine and diaminobenzidine (see Basic Protocol 1).
5. For each liter of filtrate, add 100 ml of 5% ascorbic acid.
6. Test the filtrate for the presence of aromatic amines (see Support Protocol 1).
7. Discard the decontaminated filtrate.

SUPPORT PROTOCOL 1

ANALYTICAL PROCEDURES TO DETECT BENZIDINE AND DIAMINOBENZIDINE

Reversed-phase HPLC (Snyder et al., 1997) is used to test for the presence of aromatic amines. The limit of detection is 1 μg/ml for benzidine and 0.25 μg/ml for diaminobenzidine.

Materials

Decontaminated aromatic amine solution
10:30:20 (v/v/v) acetonitrile/methanol/1.5 mM potassium phosphate buffer (1.5 mM K_2HPO_4/1.5 mM KH_2PO_4) (benzidine) *or* 75:25 (v/v) methanol/1.5 mM potassium phosphate buffer (diaminobenzidine)
250-mm × 4.6-mm-i.d. Microsorb C-8 reversed-phase HPLC column (Varian) or equivalent
Additional reagents and equipment for reversed-phase liquid chromatography (Snyder et al., 1997)

Analyze the decontaminated aromatic amine solution by reversed-phase HPLC using a 250-mm × 4.6-mm-i.d. Microsorb C-8 column or equivalent (Snyder et al., 1997). To detect benzidine, elute with 10:30:20 (v/v/v) acetonitrile/methanol/1.5 mM potassium phosphate buffer at a flow rate of 1.5 ml/min and UV detection at 285 nm. To detect diaminobenzidine, elute with 75:25 (v/v) methanol/1.5 mM potassium phosphate buffer at a flow rate of 1 ml/min and UV detection at 300 nm.

BASIC PROTOCOL 2

DISPOSAL OF BIOLOGICAL STAINS

Biological stains (Table 1A.3.5), as well as ethidium bromide and propidium iodide, can be removed from solution using the polymeric resin Amberlite XAD-16. The decontaminated solution may be disposed of as nonhazardous aqueous waste and the resin as hazardous solid waste. The volume of contaminated resin generated is much smaller than the original volume of the solution of biological stain, so the waste disposal problem is greatly reduced. The final concentration of any remaining stain should be less than the

Table 1A.3.5 Decontamination of Biological Stains

Compound	Time required for complete decontamination	Volume of solution (ml) decontaminated per gram resin
Acridine orange	18 hr	20
Alcian blue 8GX	10 min	500
Alizarin red S	18 hr	5
Azure A	10 min	80
Azure B	10 min	80
Brilliant blue R	2 hr	80
Congo red	2 hr	40
Coomassie brilliant blue G	2 hr	80
Cresyl violet acetate	2 hr	40
Crystal violet	30 min	200
Eosin B	30 min	40
Erythrosin B	18 hr	10
Ethidium bromide	4 hr	20
Janus green B	30 min	80
Methylene blue	30 min	80
Neutral red	10 min	500
Nigrosin	2 hr	80
Orcein	2 hr	200
Propidium iodide	2 hr	20
Rose Bengal	3 hr	20
Safranine O	1 hr	20
Toluidine blue O	30 min	80
Trypan blue	2 hr	40

limit of detection (see Support Protocol 2 and Table 1A.3.5). In each case, decontamination should be >99%. This protocol describes a method for batch decontamination in which the resin is stirred in the solution to be decontaminated and removed by filtration at the end of the reaction time. Large volumes of biological stain can be decontaminated using a column (see Alternate Protocol 3). For full details refer to the original literature (Lunn and Sansone, 1991b) or a compilation (Lunn and Sansone, 1994a).

Materials

Amberlite XAD-16 resin (Supelco)
100 μg/ml biological stain in water
Additional reagents and equipment for testing for the presence of biological stain (see Support Protocol 2)

For batch decontamination of 20 ml stain

1a. Add 1 g Amberlite XAD-16 to 20 ml of 100 μg/ml biological stain in water.

For aqueous solutions having stain concentrations other than 100 μg/ml, use proportionately greater or lesser amounts of resin to achieve complete decontamination.

For solutions of erythrosin B, use 2 g Amberlite XAD-16 for 20 ml stain.

2a. Stir the mixture for at least the time indicated in Table 1A.3.5.

For batch decontamination of larger volumes of stain

1b. Add 1 g Amberlite XAD-16 to the volume of 100 μg/ml biological stain in water indicated in Table 1A.3.5.

2b. Stir the mixture for at least 18 hr.

3. Remove the resin by filtration through filter paper.

4. Test the filtrate for the presence of the biological stain (see Support Protocol 2).

5. Discard the resin as hazardous solid waste and the decontaminated filtrate as liquid waste.

ALTERNATE PROTOCOL 3

CONTINUOUS-FLOW DECONTAMINATION OF AQUEOUS SOLUTIONS OF BIOLOGICAL STAINS

For treating large volumes of dilute aqueous solutions of biological stains (Table 1A.3.6), it is possible to put the resin in a column and run the contaminated solution through using a continuous-flow system (Lunn et al., 1994). Limited grinding of the Amberlite XAD-16 resin increases its efficiency.

Additional Materials *(also see Basic Protocol 2)*

25 μg/ml biological stain in water
Methanol (optional)
300-mm × 11-mm-i.d. glass chromatography column fitted with threaded adapters and flow-regulating valves at top and bottom nut and insert connectors, and insertion tool (Ace Glass) *or* 300-mm × 15-mm-i.d. glass chromatography column (Spectrum 124010, Fisher)
Glass wool
1.5-mm-i.d. × 0.3-mm-wall Teflon tubing
Waring blender (optional)
Rubber stopper fitted over a pencil
QG 20 lab pump (Fluid Metering)
Additional reagents and equipment for testing for the presence of biological stain (see Support Protocol 2)

Using a slurry of Amberlite XAD-16

1a. Prepare a 300-mm × 11-mm-i.d. glass chromatography column. To prevent clogging of the column outlet, place a small plug of glass wool at the bottom of the chromatography column. Connect 1.5-mm-i.d. × 0.3-mm wall Teflon tubing to the adapters using nut and insert connectors. Attach the tubing using an insertion tool.

2a. Mix 10 g Amberlite XAD-16 and 25 ml water in a beaker and stir 5 min to wet the resin.

Using a finely ground Amberlite XAD-16 slurry

1b. Prepare a 300-mm × 15-mm-i.d. glass chromatography column. To prevent clogging of the column outlet, place a small plug of glass wool at the bottom of the chromatography column.

2b. Grind 20 g Amberlite XAD-16 with 200 ml water for exactly 10 sec in a Waring blender.

3. Pour the resin slurry into the column through a funnel. As the resin settles, tap the column with a rubber stopper fitted over a pencil to encourage even packing. Attach a QG 20 lab pump.

Table 1A.3.6 Breakthrough Volumes for Continuous-Flow Decontamination of Biological Stains

Compound	Breakthrough volume (ml)		
	Limit of detection	1 ppm	5 ppm
Acridine orange	465	>990	>990
Alizarin red S	120	150	240
Azure A	615	810	>975
Azure B	630	882	>1209
Cresyl violet acetate	706	>1396	>1396
Crystal violet	1020	>1630	>1630
Ethidium bromide	260	312	416
Janus green B	170	650	>870
Methylene blue	420	645	1050
Neutral red	>2480	>2480	>2480
Safranine O	365	438	584
Toluidine blue O	353	494	606

4. Pump the 25-μg/ml biological stain solution through the column at 2 ml/min.

   Alternatively, gravity flow coupled with periodic adjustment of the flow-regulating valve can be used.

5. Check the effluent from the column for the presence of biological stain (see Support Protocol 2). Stop the pump when stain is detected.

   Table 1A.3.6 lists breakthrough volumes at different detection levels for a number of biological stains.

6. Discard the decontaminated effluent and the contaminated resin appropriately.

7. Many biological stains can be washed off the resin with methanol so the resin can be reused. Discard the methanol solution of stain as hazardous organic liquid waste.

ANALYTICAL PROCEDURES TO DETECT BIOLOGICAL STAINS

SUPPORT PROTOCOL 2

Depending on the biological stain, the filtrate or eluate from the decontamination procedure can be analyzed using either UV absorption (A) or fluorescence detection (F).

Materials

Filtrate or eluate from biological stain decontamination (see Basic Protocol 2 or Alternate Protocol 3)
DNA solution: 20 μg/ml calf thymus DNA in TBE electrophoresis buffer, pH 8.1 (*APPENDIX 2A*)
1 M KOH solution
pH 5 buffer (see recipe)

Test the filtrate or eluate from the biological stain decontamination procedure using the appropriate method as indicated in Table 1A.3.7.

Traces of acid or base on the resin may induce color changes in the stain. Accordingly, with cresyl violet acetate or neutral red, mix aliquots of the filtrate with 1 vol pH 5 buffer before analyzing. With alizarin red S and orcein, mix aliquots of the filtrate with 1 vol of 1 M KOH solution before analyzing.

Table 1A.3.7 Methods for Detecting Biological Stains[a]

Compound	Reagent[b]	Procedure	Wavelength(s) (nm)	Limit of detection (ppm)
Acridine orange	DNA solution	F	ex 492, em 528	0.0032
Alcian blue 8GX		A	615	0.9
Alizarin red S	1 M KOH	A	556	0.46
Azure A		A	633	0.15
Azure B		A	648	0.13
Brilliant blue R		A	585	1.0
Congo red		A	497	0.25
Coomassie brilliant blue G		A	610	1.7
Cresyl violet acetate	pH 5 buffer	F	ex 588, em 618	0.021
Crystal violet		A	588	0.1
Eosin B		A	514	0.21
Erythrosin B		F	ex 488, em 556	0.025
Ethidium bromide	DNA solution	F	ex 540, em 590	0.05
Janus green B		A	660	0.6
Methylene blue		A	661	0.13
Neutral red	pH 5 buffer	A	540	0.6
Nigrosin		A	570	0.8
Orcein	1 M KOH	A	579	1.15
Propidium iodide	DNA solution	F	ex 350, em 600	0.1
Rose Bengal		F	ex 520, em 576	0.04
Safranine O		F	ex 460, em 582	0.03
Toluidine blue O		A	626	0.2
Trypan blue		A	607	0.22

[a] Abbreviations: A, absorbance; em, emission; ex, excitation; F, fluorescence.
[b] See Support Protocol 2.

Increase the fluorescence of solutions of acridine orange, ethidium bromide, and propidium iodide by mixing an aliquot of the filtrate with an equal volume of DNA solution. Let the solution stand 15 min before measuring the fluorescence.

BASIC PROTOCOL 3

DISPOSAL OF CHLOROTRIMETHYLSILANE AND DICHLORODIMETHYLSILANE

Silane-containing compounds are hydrolyzed to hydrochloric acid and polymeric silicon-containing material (Patnode and Wilcock, 1946).

1. Hydrolyze silane-containing compounds by cautiously adding 5 ml silane to 100 ml vigorously stirred water in a flask. Allow the resulting suspension to settle.
2. Remove any insoluble material by filtration and discard it with the solid or liquid hazardous waste.
3. Neutralize the aqueous layer with base and discard it.

DISPOSAL OF CYANIDES AND CYANOGEN BROMIDE

BASIC PROTOCOL 4

Inorganic cyanides (e.g., NaCN) and cyanogen bromide (CNBr) are oxidized by sodium hypochlorite (NaOCl; e.g., Clorox) in basic solution to the much less toxic cyanate ion (Lunn and Sansone, 1985a). Destruction is >99.7%.

Materials

Cyanide (e.g., NaCN) or cyanogen bromide (CNBr)
1 M NaOH
5.25% (v/v) sodium hypochlorite (NaOCl; i.e., standard household bleach)
Additional reagents and equipment for testing for the presence of cyanide (see Support Protocol 3)

1. Dissolve cyanide in water to give a concentration ≤25 mg/ml *or* dissolve CNBr in water to give a concentration ≤60 mg/ml.

   If necessary, dilute aqueous solutions so the concentration of NaCN or CNBr does not exceed the limit.

2. Mix 1 vol NaCN or CNBr solution with 1 vol 1 M NaOH and 2 vol fresh 5.25% NaOCl. Stir the mixture 3 hr.

   IMPORTANT NOTE: *With age, bleach may become ineffective; use of fresh bleach is strongly recommended.*

3. Test the reaction mixture for the presence of cyanide (see Support Protocol 3).

4. Neutralize the reaction mixture and discard it.

ANALYTICAL PROCEDURE TO DETECT CYANIDE

SUPPORT PROTOCOL 3

This protocol is used to detect cyanide or cyanogen bromide at ≥3 μg/ml.

Materials

Cyanide or cyanogen bromide decontamination reaction mixture (see Basic Protocol 4)
Phosphate buffer (see recipe)
10 mg/ml sodium ascorbate in water: prepare fresh daily
100 mg/liter NaCN in water: prepare fresh weekly
10 mg/ml chloramine-T in water: prepare fresh daily
Cyanide detection reagent (see recipe)
Sorvall GLC-1 centrifuge or equivalent

1. If necessary to remove suspended solids, centrifuge two 1-ml aliquots of the cyanide or cyanogen bromide decontamination reaction mixture 5 min in a benchtop centrifuge, room temperature. Add each supernatant to 4 ml phosphate buffer in separate tubes.

2. If an orange or yellow color appears, add 10 mg/ml freshly prepared sodium ascorbate dropwise until the mixture is colorless, but do not add more than 2 ml.

3. Add 200 μl of 100 mg/liter NaCN to one reaction mixture (control solution).

4. Add 1 ml freshly prepared 10 mg/ml chloramine-T to each tube. Shake the tubes and let them stand 1 to 2 min.

5. Add 1 ml cyanide detection reagent, shake, and let stand 5 min.

A blue color indicates the presence of cyanide. If destruction has been complete and the analytical procedure has been carried out correctly, the treated reaction mixture should be colorless and the control solution, which contains NaCN, should be blue.

6. If necessary, remove suspended solids by centrifuging tubes 5 min at room temperature. Measure the absorbance at 605 nm with appropriate standards and blanks.

BASIC PROTOCOL 5

DISPOSAL OF DIMETHYL SULFATE, DIETHYL SULFATE, METHYL METHANESULFONATE, ETHYL METHANESULFONATE, DIEPOXYBUTANE, AND 1,3-PROPANE SULTONE

Dimethyl sulfate is hydrolyzed by base to methanol and methyl hydrogen sulfate (Lunn and Sansone, 1985b). Subsequent hydrolysis of methyl hydrogen sulfate to methanol and sulfuric acid is slow. Methyl hydrogen sulfate is nonmutagenic and a very poor alkylating agent. The other compounds can be hydrolyzed with base in a similar fashion (Lunn and Sansone, 1990a). Destruction is >99%. A method to verify that decontamination is complete is also provided (see Support Protocol 4).

NOTE: The reaction times given in the protocol should give good results; however, reaction time may be affected by such factors as the size and shape of the flask and the rate of stirring. The presence of two phases indicates that the reaction is not complete, and stirring should be continued until the reaction mixture is homogeneous.

Materials

Dimethyl sulfate, diethyl sulfate, methyl methanesulfonate, ethyl methanesulfonate, diepoxybutane, or 1,3-propane sultone
5 M NaOH
Acid
Additional reagents and equipment for testing for the presence of dimethyl sulfate, diethyl sulfate, methyl methanesulfonate, ethyl methanesulfonate, diepoxybutane, or 1,3-propane sultone (see Support Protocol 4)

For bulk quantities of dimethyl sulfate

1a. Add 100 ml dimethyl sulfate to 1 liter of 5 M NaOH. Stir the reaction mixture.

2a. Fifteen minutes after all the dimethyl sulfate has gone into solution, neutralize the reaction mixture with acid.

For bulk quantities of diethyl sulfate

1b. Add 100 ml diethyl sulfate to 1 liter of 5 M NaOH. Stir the reaction mixture 24 hr.

2b. Neutralize the reaction mixture with acid.

For bulk quantities of methyl methanesulfonate, ethyl methanesulfonate, diepoxybutane, and 1,3-propane sultone

1c. Add 1 ml methyl methanesulfonate, ethyl methanesulfonate, or diepoxybutane, or 1 g of 1,3-propane sultone to 10 ml of 5 M NaOH. Stir the reaction mixture 1 hr for 1,3-propane sultone, 2 hr for methyl methanesulfonate, 22 hr for diepoxybutane, or 24 hr for ethyl methanesulfonate.

2c. Neutralize the reaction mixture with acid.

3. Test the reaction mixture for the presence of the original compound (see Support Protocol 4).

4. Discard the decontaminated reaction mix.

ANALYTICAL PROCEDURE TO DETECT THE PRESENCE OF DIMETHYL SULFATE, DIETHYL SULFATE, METHYL METHANESULFONATE, ETHYL METHANESULFONATE, DIEPOXYBUTANE, AND 1,3-PROPANE SULTONE

SUPPORT PROTOCOL 4

This protocol is used to verify decontamination of solutions containing dimethyl sulfate, diethyl sulfate, methyl methanesulfonate, ethyl methanesulfonate, diepoxybutane, or 1,3-propane sultone. The detection limit for this assay is 40 μg/ml for dimethyl sulfate, 108 μg/ml for diethyl sulfate, 84 μg/ml for methyl methanesulfonate, 1.1 μg/ml for ethyl methanesulfonate, 360 μg/ml for diepoxybutane, and 264 μg/ml for 1,3-propane sultone.

Materials

Reaction mixture containing dimethyl sulfate, diethyl sulfate, methyl methanesulfonate, ethyl methanesulfonate, diepoxybutane, or 1,3-propane sultone
98:2 (v/v) 2-methoxyethanol/acetic acid
5% (w/v) 4-(4-nitrobenzyl)pyridine in 2-methoxyethanol
Piperidine
2-Methoxyethanol

1. Dilute an aliquot of the reaction mixture with 4 vol water.
2. Add 100 μl diluted reaction mixture to 1 ml of 98:2 (v/v) 2-methoxyethanol/acetic acid. Swirl to mix.
3. Add 1 ml of 5% (w/v) 4-(4-nitrobenzyl)pyridine in 2-methoxyethanol. Heat 10 min at 100°C, then cool 5 min in ice.
4. Add 0.5 ml piperidine and 2 ml of 2-methoxyethanol.
5. Measure the absorbance of the violet reaction mixture at 560 nm against an appropriate blank.

   The absorbance of a decontaminated solution should be 0.000.

DISPOSAL OF ETHIDIUM BROMIDE AND PROPIDIUM IODIDE

BASIC PROTOCOL 6

Ethidium bromide and propidium iodide in water and buffer solutions may be degraded by reaction with sodium nitrite and hypophosphorous acid in aqueous solution (Lunn and Sansone, 1987); destruction is >99.87%. This reaction may also be used to decontaminate equipment contaminated with ethidium bromide (see Alternate Protocol 4; Lunn and Sansone, 1989) and to degrade ethidium bromide in organic solvents (see Alternate Protocol 5 and Alternate Protocol 6; Lunn and Sansone, 1990b). Ethidium bromide and propidium iodide may also be removed from solution by adsorption onto Amberlite XAD-16 resin (see Basic Protocol 2).

Materials

Ethidium bromide– or propidium iodide–containing solution in water, buffer, or 1 g/ml cesium chloride
5% (v/v) hypophosphorous acid: prepare fresh daily by diluting commercial 50% reagent 1/10
0.5 M sodium nitrite: prepare fresh daily
Sodium bicarbonate
Additional reagents and equipment for testing for the presence of ethidium bromide or propidium iodide (see Support Protocol 5)

1. If necessary, dilute the ethidium bromide– or propidium iodide–containing solution so the concentration of ethidium bromide or propidium iodide is ≤0.5 mg/ml.
2. For each 100 ml solution, add 20 ml of 5% hypophosphorous acid solution and 12 ml of 0.5 M sodium nitrite. Stir briefly and let stand 20 hr.
3. Neutralize the reaction mixture by adding sodium bicarbonate until the evolution of gas ceases.
4. Test the reaction mixture for the presence of ethidium bromide or propidium iodide (see Support Protocol 5).
5. Discard the decontaminated reaction mixture.

ALTERNATE PROTOCOL 4

DECONTAMINATION OF EQUIPMENT CONTAMINATED WITH ETHIDIUM BROMIDE

Glass, stainless steel, Formica, floor tile, and the filters of transilluminators have been successfully decontaminated using this protocol. No change in the optical properties of the transilluminator filter could be detected, even after a number of decontamination cycles.

Materials

Equipment contaminated with ethidium bromide
Decontamination solution (see recipe)
Sodium bicarbonate
Additional reagents and equipment for testing for the presence of ethidium bromide (see Support Protocol 5)

1. Wash the equipment contaminated with ethidium bromide once with a paper towel soaked in decontamination solution.

   The pH of decontamination solution is 1.8. If this is too corrosive for the surface being decontaminated, wash with a paper towel soaked in water instead.

2. Wash the surface five times with paper towels soaked in water using a fresh towel each time.
3. Soak all the towels 1 hr in decontamination solution.
4. Neutralize the decontamination solution by adding sodium bicarbonate until the evolution of gas ceases.
5. Test the decontamination solution for the presence of ethidium bromide (see Support Protocol 5).
6. Discard the decontamination solution and the paper towels as nonhazardous liquid and solid wastes.

ALTERNATE PROTOCOL 5

DECONTAMINATION OF ETHIDIUM BROMIDE IN ISOPROPANOL SATURATED WITH CESIUM CHLORIDE

Materials

Ethidium bromide
Isopropanol saturated with cesium chloride
Decontamination solution (see recipe)
Sodium bicarbonate
Additional reagents and equipment for testing for the presence of ethidium bromide (see Support Protocol 5)

1. If necessary, dilute the ethidium bromide in isopropanol saturated with cesium chloride so the concentration of ethidium bromide is ≤1 mg/ml.
2. For each volume of ethidium bromide solution, add 4 vol decontamination solution. Stir the reaction mixture 20 hr.
3. Neutralize the reaction mixture by adding sodium bicarbonate until the evolution of gas ceases.
4. Test the reaction mixture for the presence of ethidium bromide (see Support Protocol 5).
5. Discard the decontaminated solution.

DECONTAMINATION OF ETHIDIUM BROMIDE IN ISOAMYL ALCOHOL AND 1-BUTANOL

ALTERNATE PROTOCOL 6

Materials

Ethidium bromide
Isoamyl alcohol or 1-butanol
Decontamination solution (see recipe)
Activated charcoal
Sodium bicarbonate
Separatory funnel
Additional reagents and equipment for testing for the presence of ethidium bromide (see Support Protocol 5)

1. If necessary, dilute the ethidium bromide in isoamyl alcohol or 1-butanol so the concentration is ≤1 mg/ml final.
2. For each volume of ethidium bromide solution, add 4 vol decontamination solution. Stir the two-phase reaction mixture rapidly for 72 hr.
3. For each 100 ml of reaction mixture, add 2 g activated charcoal. Stir another 30 min.
4. Filter the reaction mixture.
5. Neutralize the filtrate by adding sodium bicarbonate until the evolution of gas ceases. Separate the layers using a separatory funnel.

   More alcohol may tend to separate from the aqueous layer on standing.
6. Test the alcohol and aqueous layers for the presence of ethidium bromide.
7. Discard the alcohol and aqueous layers appropriately. Discard the activated charcoal as solid waste.

   The aqueous layer contains 4.6% 1-butanol or 2.3% isoamyl alcohol.

ANALYTICAL PROCEDURE TO DETECT ETHIDIUM BROMIDE OR PROPIDIUM IODIDE

SUPPORT PROTOCOL 5

This protocol is used to verify that solutions no longer contain ethidium bromide or propidium iodide. The limits of detection are 0.05 parts per million (ppm) for ethidium bromide and 0.1 ppm for propidium iodide.

Materials

Reaction mixture containing ethidium bromide or propidium iodide
TBE buffer, pH 8.1 (*APPENDIX 2A*)
20 μg/ml calf thymus DNA in TBE buffer, pH 8.1

1. Mix 100 µl reaction mixture containing ethidium bromide or propidium iodide with 900 µl TBE buffer, pH 8.1.
2. Add 1 ml of 20 µg/ml calf thymus DNA in TBE, pH 8.1. Prepare a blank solution (100 µl water + 900 µl TBE + 1 ml of 20 µg/ml calf thymus DNA) and control solutions containing known quantities of ethidium bromide or propidium iodide. Let the mixtures stand 15 min.
3. To detect ethidium bromide, measure the fluorescence with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. To detect propidium iodide, measure the fluorescence with an excitation wavelength of 350 nm and an emission wavelength of 600 nm.

   If a spectrophotofluorometer is not available, fluorescence of ethidium bromide can be qualitatively determined using a hand-held UV lamp on the long-wavelength setting (Lunn and Sansone, 1991c).

BASIC PROTOCOL 7

DISPOSAL OF HYDROGEN PEROXIDE

Hydrogen peroxide can be reduced with sodium metabisulfite (Lunn and Sansone, 1994b).

Materials

30% hydrogen peroxide
10% (w/v) sodium metabisulfite
10% (w/v) potassium iodide
1 M HCl
1% (w/v) starch indicator solution

1. Add 5 ml of 30% hydrogen peroxide to 100 ml of 10% sodium metabisulfite. Stir the mixture at room temperature until the temperature starts to drop, indicating that the reaction is over.
2. Test for the presence of hydrogen peroxide by adding a few drops of the reaction mixture to an equal volume of 10% potassium iodide. Add a few drops of 1 M HCl to acidify the reaction mixture, then add a drop of 1% starch indicator solution.

   A deep blue color indicates the presence of excess oxidant. If necessary, add more 10% sodium metabisulfite until the starch test is negative.

3. Discard the decontaminated mixture.

BASIC PROTOCOL 8

DISPOSAL OF IODINE

Iodine is reduced with sodium metabisulfite to iodide (Lunn and Sansone, 1994b).

Materials

Iodine
10% (w/v) sodium metabisulfite
1 M HCl
1% (w/v) starch indicator solution

1. Add 5 g iodine to 100 ml of 10% sodium metabisulfite. Stir the mixture until the iodine has completely dissolved.
2. Acidify a few drops of the reaction mixture by adding a few drops of 1 M HCl. Add 1 drop of 1% starch indicator solution.

   A deep blue color indicates the presence of iodine. If reduction is not complete, add more sodium metabisulfite solution.

3. Dispose of the decontaminated solution.

DISPOSAL OF MERCURY COMPOUNDS

BASIC PROTOCOL 9

Solutions of mercuric acetate can be decontaminated using Dowex 50X8-100, a strongly acidic gel-type ion-exchange resin with a sulfonic acid functionality. Solutions of mercuric chloride can be decontaminated using Amberlite IRA-400(Cl), a strongly basic gel-type ion-exchange resin with a quaternary ammonium functionality. The final concentration of mercury is <3.8 ppm (Lunn and Sansone, 1994a). On a small scale it is most convenient to stir the resin in the solution to be decontaminated, but on a larger scale, or for routine use, it may be more convenient to pass the solution through a column packed with the resin. Although the volume of waste that must be disposed is greatly reduced using this technique, a small amount of waste (i.e., the resin contaminated with mercury) remains and must be discarded appropriately. Resin can be regenerated by washing with acid, but the concentrated metal-containing solution generated by this must also be disposed of appropriately. Mercury may also be removed from laboratory waste water using a column of iron powder (see Alternate Protocol 7). Support Protocol 6 is used to detect the presence of mercury.

Materials

Solution containing ≤1600 ppm mercuric acetate *or* ≤1350 ppm mercuric chloride
Dowex 50X8-100 ion-exchange (mercuric acotate) *or* Amberlite IRA-400(Cl) ion-exchange (mercuric chloride) resin
Additional reagents and equipment to test for the presence of mercury (see Support Protocol 6)

1a. *For mercuric acetate:* For each 200 ml of solution containing ≤1600 ppm mercuric acetate, add 1 g Dowex 50X8-100 ion-exchange resin. Stir the mixture 1 hr, then filter through filter paper.

1b. *For mercuric chloride:* For each 200 ml of solution containing ≤1350 ppm mercuric chloride, add 1 g Amberlite IRA-400(Cl) ion-exchange resin. Stir the mixture 6 hr, then filter through filter paper.

The speed and efficiency of decontamination will depend on factors such as the size and shape of the flask and the rate of stirring.

2. Test the filtrate for the presence of mercury (see Support Protocol 6).

3. Discard the decontaminated filtrate and the mercury-containing resin appropriately.

DECONTAMINATION OF WASTE WATER CONTAINING MERCURY

ALTERNATE PROTOCOL 7

Laboratory waste water that contains mercury is decontaminated by passing it through a column of iron powder. The mercury forms mercury amalgam and stays on the column. Some metallic mercury remains in solution but this can be removed by aeration. The final concentration of mercury is <5 ppb (Shirakashi et al., 1986).

Materials

Iron powder, 60 mesh
Waste water containing ≤2.5 ppm mercury
6-mm-i.d. column

1. Pack a 6-mm-i.d. column with 1 g of 60-mesh iron powder.

Use a fresh column for each treatment.

2. Pass ≤2 liters of water containing ≤2.5 ppm of mercury through the column at a flow rate of 250 ml/hr.

Solutions containing a higher concentration of mercury may also be treated, but this will result in a higher final concentration of mercury (e.g., treating a 100-ppm solution in this fashion yielded 33 ppb final).

Some iron ends up in solution and can be removed by adjusting the pH to 8. The resulting precipitated $Fe(OH)_3$ can then be removed by filtration.

3. Aerate the resulting effluent to remove traces of metallic mercury and continue aeration 30 min after the last of the effluent has emerged from the column. Vent the metallic mercury removed from the solution by aeration into the fume hood or capture it in a mercury trap.

The effluent contains <5 ppb mercury. The presence of iodide or polypeptone may necessitate several treatments to reduce the mercury to an acceptable level.

SUPPORT PROTOCOL 6

ANALYTICAL PROCEDURE TO DETECT MERCURY

Atomic absorption spectroscopy with detection at 253.7 nm, a slit width of 0.7 nm, and a limit of detection of 3.8 ppm can be used to determine the concentration of mercury in solution for experiments involving ion-exchange resins. A Hiranuma mercury meter model HG-1 can be used for experiments involving iron powder.

BASIC PROTOCOL 10

DISPOSAL OF SODIUM AZIDE

Sodium azide can be oxidized by ceric ammonium nitrate (Manufacturing Chemists Association, 1973) to nitrogen (Mason, 1967) or by nitrous acid (National Research Council, 1983) to nitrous oxide (Mason, 1967); destruction is >99.996%. Sodium azide in buffer solution may also be degraded by the addition of sodium nitrite (Lunn and Sansone, 1994a). The reaction proceeds much more readily at low pH, but if sufficient sodium nitrite is added, it will proceed to completion even at high pH. At low pH, it may be possible to completely degrade the azide present in the buffer with less than the amount of sodium nitrite indicated. However, the reaction mixture must be carefully checked to ensure that no azide remains (see Support Protocol 7). At high pH, it is possible for unreacted azide to remain in the presence of excess nitrite. Residual nitrite can be detected using Support Protocol 8.

CAUTION: Some toxic nitrogen dioxide may be produced as a by-product of these reactions, so they should always be carried out in a chemical fume hood.

Materials

Sodium azide or solution containing sodium azide
Ceric ammonium nitrate
10% (w/v) potassium iodide
1 M HCl
1% (w/v) starch indicator solution
Sodium nitrite
4 M sulfuric acid

Additional reagents and equipment to test for the presence of sodium azide (see Support Protocol 7) *or* nitrite (see Support Protocol 8)

Decontamination using ceric ammonium nitrate

1a. For each gram of sodium azide, add 9 g ceric ammonium nitrate to 30 ml water, and stir until it has dissolved.

2a. Dissolve each gram of sodium azide in 5 ml water. Add this solution to the ceric ammonium nitrate solution at the rate of 1 ml each min. Stir 1 hr.

If the reaction is carried out on a larger scale, an ice bath may be required for cooling.

3a. Check that the reaction is still oxidizing by adding a few drops of the reaction mixture to an equal volume of 10% potassium iodide. Acidify the mixture with 1 drop of 1 M HCl and add 1 drop of 1% starch indicator solution.

The deep blue color of the starch-iodine complex indicates that excess oxidant is present. If excess oxidant is not present, add more ceric ammonium nitrate.

4a. Test for the presence of sodium azide (see Support Protocol 8).

5a. Discard the decontaminated reaction mixture.

Decontamination using sodium nitrite

1b. For each 5 g sodium azide, dissolve 7.5 g sodium nitrite in 30 ml water.

2b. Dissolve each 5 g sodium azide in 100 ml water. Add the sodium nitrite solution with stirring. Slowly add 4 M sulfuric acid until the reaction mixture is acidic to litmus. Stir 1 hr.

CAUTION: *It is important to add the sodium nitrite, then the sulfuric acid. Adding these reagents in reverse order will generate explosive, volatile, toxic hydrazoic acid.*

CAUTION: *If the reaction is carried out on a large scale, an ice bath may be required for cooling.*

3b. Check that there is excess nitrous acid in the reaction. Add a few drops of the reaction mixture to an equal volume of 10% potassium iodide. Acidify the mixture with 1 drop 1 M HCl. Add 1 drop starch indicator solution.

The deep blue color of the starch-iodine complex indicates that excess nitrous acid is present. If excess nitrous acid is not present, add more sodium nitrite.

4b. If excess nitrous acid is present, test for the presence of sodium azide (see Support Protocol 7).

5b. Discard the decontaminated reaction mixture.

Decontamination of sodium azide in buffer

1c. If necessary, dilute the buffer solution with water so the concentration of sodium azide is $\leq$1 mg/ml.

2c. For each 50 ml buffer solution add 5 g sodium nitrite. Stir the reaction 18 hr.

3c. Test for the presence of sodium azide (see Support Protocol 7).

4c. Discard the decontaminated reaction solution.

ANALYTICAL PROCEDURES TO DETECT SODIUM AZIDE

SUPPORT PROTOCOL 7

Sodium azide is analyzed by reacting azide ion with 3,5-dinitrobenzoyl chloride to form 3,5-dinitrobenzoyl azide, which can be detected by reversed-phase HPLC. The limit of detection of this assay is 0.2 μg/ml sodium azide. This protocol works only in the absence of nitrite; verify that all of the nitrite has been destroyed by sulfamic acid by using the method detailed later in this unit (see Support Protocol 8).

Materials

Reaction mixture from sodium azide treated with ceric ammonium nitrate or sodium nitrite
1 M KOH
Acetonitrile
Sodium azide indicator solution (see recipe)
0.2 M HCl

20% (w/v) sulfamic acid
3,5-dinitrobenzoyl chloride
50:50 (v/v) acetonitrile/water

Sorvall GLC-1 centrifuge or equivalent
25-cm × 4.6-mm-i.d. Microsorb C-8 reversed-phase HPLC column (Varian) or equivalent

Additional reagents and equipment for reversed-phase liquid chromatography (Snyder et al., 1997)

To analyze for azide in the presence of ceric salts

1a. To a 10-ml aliquot of the reaction mixture from sodium azide treated with ceric ammonium nitrate, add 40 ml water. Add 5 ml of this diluted solution to 3 ml of 1 M KOH and mix by shaking.

If <3 ml of 1 M KOH is used, precipitation of ceric salts will not be complete.

2a. Centrifuge the mixture 5 min, room temperature.

3a. Remove 2 ml supernatant and add to 1 ml acetonitrile. Add 1 drop sodium azide indicator solution, add 0.2 M HCl dropwise until the mixture turns yellow, then add 1 drop more.

To analyze for azide in the presence of nitrite

1b. To 5 ml of the reaction mixture from sodium azide treated with sodium nitrite, add ≥1 ml sulfamic acid to remove excess nitrite. Let stand ≥3 min.

More sulfamic acid solution may be required for strongly basic reaction mixtures or those containing high concentrations of nitrite. Complete removal of nitrite can be checked by using a modified Griess reagent (see Support Protocol 8).

At high pH the reaction between azide and nitrite is quite slow, so the presence of excess nitrite does not mean that all the azide has been degraded.

2b. Add 1 drop sodium azide indicator solution, then basify the mixture by adding 1 M KOH until it turns purple (typically, 3 to 10 ml are required).

3b. Add 2 ml acetonitrile. Add 0.2 M HCl dropwise until the mixture turns yellow, then add 1 drop more.

If >1 ml sulfamic acid is used, add 4 ml acetonitrile.

4. Prepare a 10% (w/v) solution of 3,5-dinitrobenzoyl chloride in acetonitrile.

5. Add 50 µl of 10% dinitrobenzoyl chloride/acetonitrile to the reaction mix (step 3a or 3b). Shake the mixture and let it stand ≥3 min.

Longer standing times have no effect on the HPLC analysis. However, it is crucial to use freshly prepared 3,5-dinitrobenzoyl chloride solution within minutes of its preparation. It is generally most convenient to prepare all the analytical samples with the fresh solution at the beginning of the day and analyze them over the course of the day.

6. Analyze 20 µl of each reaction mixture by reversed-phase HPLC (Snyder et al., 1997) using a 25-cm × 4.6-mm-i.d. Microsorb C-8 reversed-phase HPLC column or equivalent, and a mobile phase of 50:50 (v/v) acetonitrile/water with a flow rate of 1 ml/min and UV detection at 254 nm.

The peak for 3,5-dinitrobenzoyl azide elutes at −9 min.

ANALYTICAL PROCEDURE TO DETECT NITRITE

SUPPORT PROTOCOL 8

This protocol uses a modified Griess reagent to test for the presence of nitrite. The limit of detection of this assay is 0.06 μg/ml nitrite. A similar procedure uses *N*-(1-naphthyl)-ethylenediamine (Cunniff, 1995).

Materials

α-Naphthylamine
15% (v/v) aqueous acetic acid
Sulfanilic acid solution (see recipe)
Reaction mixture treated to remove excess nitrite (see Support Protocol 7, step 1b)

1. Prepare the modified Griess reagent by boiling 0.1 g α-naphthylamine in 20 ml water until it dissolves. While the solution is still hot, pour it into 150 ml of 15% aqueous acetic acid. Add 150 ml sulfanilic acid solution.

   CAUTION: *α-Naphthylamine is a carcinogen.*

   This reagent should be stored at room temperature in a brown bottle.

2. Add 3 ml of the reaction mixture treated to remove excess nitrite to 1 ml modified Griess reagent. Let stand 6 min at room temperature.

3. Measure the absorbance at 520 nm against a suitable blank.

DISPOSAL OF ENZYME INHIBITORS

BASIC PROTOCOL 11

The enzyme inhibitors *p*-amidinophenylmethanesulfonyl fluoride (APMSF), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), phenylmethylsulfonyl fluoride (PMSF; Lunn and Sansone, 1994c), diisopropyl fluorophosphate (DFP; Lunn and Sansone, 1994d), *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK), and *N-p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Lunn and Sansone, 1994c) may be degraded by reaction with 1 M NaOH. Destruction is >99.8% (except TPCK >98.3%). The exact reaction conditions depend on the solvent (see Table 1A.3.8). The solutions that were decontaminated are representative of those described in the literature.

Materials

Solutions of APMSF, AEBSF, PMSF, DFP, TLCK, or TPCK in buffer, DMSO, isopropanol, or water
1 M NaOH
Glacial acetic acid

Additional reagents and equipment for testing for the presence of the enzyme inhibitors (see Support Protocol 9)

1. If necessary, dilute the solutions with the same solvent so that the concentrations given in Table 1A.3.8 are not exceeded.

   Bulk quantities of AEBSF, PMSF, and TPCK may be dissolved in isopropanol and bulk quantities of APMSF and TLCK may be dissolved in water at the concentrations shown in Table 1A.3.8. Bulk quantities of DFP (a liquid) may be mixed directly with 1 M NaOH, taking care to make sure that all the DFP has mixed thoroughly, in the ratio shown in Table 1A.3.8 (e.g., 40 μl DFP with 1 ml of 1 M NaOH).

2. Add 1 M NaOH so that the ratio of solution to 1 M NaOH is that listed in Table 1A.3.8.

3. Shake to ensure complete mixing, check that the solution is strongly basic (pH ≥12), and allow to stand for the time given in Table 1A.3.8.

Table 1A.3.8 Conditions for the Destruction of Enzyme Inhibitors

Compound	Concentration	Solvent	Ratio[a]	Time
AEBSF	1 mM	Buffer (pH 5.0-8.0)	1:0.1	1 hr
AEBSF	20 mM	DMSO	1:10	24 hr
AEBSF	20 mM	Isopropanol	1:10	24 hr
APMSF	2.5 mM	Buffer (pH 5.0-8.0)	1:0.1	1 hr
APMSF	25 mM	DMSO	1:5	24 hr
APMSF	25 mM	50:50 isopropanol:pH 3 buffer	1:5	24 hr
APMSF	100 mM	Water	1:5	24 hr
DFP	10 mM	Buffer (pH 6.4-7.2)	1:0.2	18 hr
DFP	200 mM	DMF	1:2	18 hr
DFP	pure	—	1:25	1 hr
DFP	10 mM	Water	1:0.2	18 hr
PMSF	10 mM	Buffer (pH 5.0-8.0)	1:0.1	1 hr
PMSF	100 mM	DMSO	1:5	24 hr
PMSF	100 mM	Isopropanol	1:5	24 hr
TLCK	1 mM	Buffer (pH 5.0-8.0)	1:0.1	18 hr
TLCK	5 mM	DMSO	1:5	18 hr
TLCK	5 mM	Water	1:0.1	18 hr
TPCK	1 mM	Buffer (pH 5.0-8.0)	1:0.1	18 hr
TPCK	1 mM	DMSO	1:0.1	18 hr
TPCK	1 mM	Isopropanol	1:0.1	18 hr

[a]Ratio refers to the proportion of solution to sodium hydroxide, i.e., solution (compound + solvent):1 M NaOH.

4. Neutralize the reaction mixture with glacial acetic acid and test for the presence of residual enzyme inhibitor (see Support Protocol 9).

5. Discard the decontaminated reaction mixture.

SUPPORT PROTOCOL 9

ANALYTICAL PROCEDURES TO DETECT ENZYME INHIBITORS

DFP can be detected using a complex procedure involving the inhibition of chymotrypsin activity. For more information, refer to Lunn and Sansone (1994d). A gas chromatographic method has also been described by Degenhardt-Langelaan and Kientz (1996). AEBSF, APMSF, PMSF, TLCK, and TPCK may be detected by reversed-phase HPLC (Snyder et al., 1997). The chromatographic conditions and limits of detection are shown in Table 1A.3.9 (Lunn and Sansone, 1994c).

Table 1A.3.9 HPLC Conditions for Enzyme Inhibitors

Compound	Mobile phase	UV detector wavelength	Retention time	Limit of detection
AEBSF	40:60 (v/v) acetonitrile:0.1% trifluoroacetic acid	225 nm	9.5 min	0.1 μg/ml
APMSF	40:60 (v/v) acetonitrile:0.1% trifluoroacetic acid	232 nm	7.7 min	0.5 μg/ml
PMSF	50:50 (v/v) acetonitrile:water	220 nm	8 min	0.9 μg/ml
TLCK	40:60 (v/v) acetonitrile:0.1% trifluoroacetic acid	228 nm	9.5 min	0.37 μg/ml
TPCK	48:52 (v/v) acetonitrile:10 mM pH 7 phosphate buffer	228 nm	10.5 min	2 μg/ml

Materials

Decontaminated enzyme inhibitor solutions
Acetonitrile, HPLC grade
Water, HPLC grade
0.1% (v/v) trifluoroacetic acid in water
10 mM phosphate buffer, pH 7
Compound of interest
250-mm × 4.6 mm-i.d. Microsorb C-8 reversed-phase HPLC column (Varian) or equivalent
Additional reagents and equipment for reversed-phase liquid chromatography (Snyder et al., 1997)

Analyze the decontaminated enzyme inhibitor solutions by reversed-phase HPLC using a 250-mm × 4.6-mm-i.d. Microsorb C-8 reversed-phase column, or equivalent, using the conditions shown in Table 1A.3.9. In each case, the injection volume was 20 μl, the separation occurred at ambient temperature, and the flow rate was 1 ml/min. Check the analytical procedures by spiking an aliquot of the acidified reaction mixture with a small quantity of a dilute solution of the compound of interest.

DISPOSAL OF *N*-METHYL-*N′*-NITRO-*N*-NITROSOGUANIDINE (MNNG)

BASIC PROTOCOL 12

Bulk quantities and solutions in methanol or ethanol of *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) may be degraded by reaction with sulfamic acid in the presence of strong acid (Castegnaro et al., 1983); destruction is >99.5%. This reaction may also be used to decontaminate equipment contaminated with MNNG (see Alternate Protocol 8) and to decontaminate spills (see Alternate Protocol 9; Lunn and Sansone, 1988).

Note that the following procedures may only be used to degrade bulk quantities of MNNG, solutions in methanol (or ethanol), contaminated glassware, or spills. Also the methods are not appropriate for other nitrosamides (e.g., *N*-methyl-*N*-nitrosourea) or the chemically similar nitrosamines (e.g., dimethylnitrosamine). For a full discussion of procedures for the degradation of these compounds see Lunn and Sansone, 1994a.

Materials

N-Methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) as a solid or in solution in methanol or ethanol
6 M hydrochloric acid (*APPENDIX 2A*)
Sulfamic acid
Neutralizing solution (e.g., aqueous NaOH)
Additional reagents and equipment for detecting the presence of MNNG (see Support Protocol 10)

1. Take up bulk quantities of MNNG in methanol so that the concentration does not exceed 30 g/L. If necessary, dilute methanol or ethanol solutions with the same solvent so that the concentration of MNNG does not exceed 30 g/liter.
2. Slowly add an equal volume of 6 M HCl with stirring.
3. For each 1 liter of the resulting solution, add 35 g sulfamic acid. Stir 24 hr.
4. Neutralize the reaction mixture, e.g., with sodium hydroxide solution.
5. Test the reaction mixture for the presence of MNNG (see Support Protocol 10).
6. Discard the decontaminated reaction mixture.

ALTERNATE PROTOCOL 8

DECONTAMINATION OF GLASSWARE CONTAMINATED WITH *N*-METHYL-*N′*-NITRO-*N*-NITROSOGUANIDINE (MNNG)

Materials

Glassware contaminated with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG)
Methanol
70 g/liter sulfamic acid in 2 M HCl
Neutralizing solution (e.g., aqueous NaOH)
Additional reagents and equipment for detecting the presence of MNNG (see Support Protocol 10)

1. Soak the contaminated glassware in a mixture of equal volumes of methanol and 70 g/liter sulfamic acid in 2 M HCl.
2. After 6 hr, drain the glassware and clean it in a conventional fashion.
3. Neutralize the reaction mixture, e.g., with sodium hydroxide solution.
4. Test the reaction mixture for the presence of MNNG (see Support Protocol 10).
5. Discard the decontaminated reaction mixture.

ALTERNATE PROTOCOL 9

DECONTAMINATION OF SPILLS OF *N*-METHYL-*N′*-NITRO-*N*-NITROSOGUANIDINE (MNNG)

Materials

Methanol
70 g/liter sulfamic acid in 2 M hydrochloric acid
Neutralizing solution (e.g., aqueous NaOH)
Additional reagents and equipment for detecting the presence of MNNG (see Support Protocol 10)

1. Soak the area in methanol until the nitrosamide appears to be dissolved, then add an approximately equal volume of 70 g/liter sulfamic acid in 2 M HCl.
2. After 6 hr, remove the reaction mixture and clean the area in a conventional fashion.
3. Check for completeness of destruction by using a wipe soaked in methanol and analyzing it for the presence of the compound (see Support Protocol 10).
4. Neutralize the reaction mixture, e.g., with sodium hydroxide solution.
5. Test the reaction mixture for the presence of MNNG (see Support Protocol 10).
6. Discard the decontaminated reaction mixture.

ANALYTICAL PROCEDURE TO DETECT *N*-METHYL-*N*′-NITRO-*N*-NITROSOGUANIDINE

Reversed-phase HPLC (Snyder et al., 1997) is used to test for the presence of MNNG.

Materials

Neutralized reaction mixture
12:88 methanol/3.5 mM $(NH_4)H_2PO_4$
250-mm × 4.6-mm-i.d. Microsorb C-8 reversed-phase column (Varian) or equivalent
Additional reagents and equipment for reversed-phase liquid chromatography (Snyder et al., 1997)

Analyze the neutralized reaction mixture(s) by reversed-phase HPLC using a UV detector operating at 254 nm and a 250-mm × 4.6-mm-i.d. Microsorb C-8 column (Lunn et al., 1988). Elute with a mobile phase of 12:88 methanol/3.5 mM $(NH_4)H_2PO_4$ flowing at 1 ml/min at ambient temperature. Check the analytical procedure by spiking the neutralized reaction mixture with an aliquot of a dilute solution of MNNG.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cyanide detection reagent

Stir 3.0 g barbituric acid in 10 ml water. Add 15 ml of 4-methylpyridine and 3 ml concentrated HCl while continuing to stir. Cool and dilute to 50 ml with water. Store at room temperature.

CAUTION: *This reaction is exothermic.*

Decontamination solution

Dissolve 4.2 g sodium nitrite (0.2 M final) and 20 ml hypophosphorous acid (3.3% w/v final) in 300 ml water. Prepare fresh.

Horseradish peroxidase

Dissolve hydrogen-peroxide oxidoreductase (EC 1.11.1.7 [Type II]; specific activity 150 to 200 purpurogallin U/mg, Sigma) in 1 g/liter sodium acetate to give 30 U/ml. Prepare fresh daily.

For small-scale reactions, a more dilute solution can be used to avoid working with inconveniently small volumes.

pH 5 buffer

2.04 g potassium hydrogen phthalate (0.05 M final)
38 ml 0.1 M potassium hydroxide (15 mM)
H_2O to 200 ml
Store at room temperature

Phosphate buffer

13.6 g monobasic potassium phosphate (KH_2PO_4; 0.1 M final)
0.28 g dibasic sodium phosphate (Na_2HPO_4; 2 mM final)
3.0 g potassium bromide (KBr; 25 mM final)
1 liter H_2O
Store at room temperature

Potassium bromide is necessary to make the assay for cyanide work correctly.

Sodium azide indicator solution

0.1 g bromocresol purple (0.4% final)
18.5 ml 0.01 M potassium hydroxide (KOH; 7.4 mM final)
H_2O to 25 ml
Store at room temperature

Sulfanilic acid solution

Dissolve 0.5 g sulfanilic acid in 150 ml of 15% (v/v) aqueous acetic acid. Use immediately.

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INTERNET RESOURCES

http://www.ilpi.com/msds/index.html

Where to find MSDSs on the internet. Contains links to general sites, government and nonprofit sites, chemical manufacturers and suppliers, pesticides, and miscellaneous sites.

http://www.OSHA.gov

The OSHA website. Standards can be accessed by clicking on the link in the right sidebar labeled Standards.

http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=10106

Text of OSHA Standard 29 CFR 1910.1450: Occupational Exposure to Hazardous Chemicals in Laboratories.

http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9992

Table Z-1 of OSHA Standard 29 CFR 1910.10000, which provides a list of permissible exposure limits (PELs) for air contaminants.

http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9993

Table Z-2 of OSHA Standard 29 CFR 1910.1000, which provides a list of PELs for toxic and hazardous substances.

http://hazard.com/msds/index.php

Main site for Vermont SIRI. One of the best general sites to start a search. Browse manufacturers alphabetically (for sheets not in the SIRI collection) or do a keyword search in the SIRI MSDS database. Lots of additional safety links and information.

http://siri.uvm.edu/msds

Alternate site for Vermont SIRI.

http://eh.doe.gov/docs/osh_tr/ch5.html

DOE OSH technical reference chapter on personal protective equipment.

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Safe Use of Radioisotopes

The use of radioisotopes to label specific molecules in a defined way has greatly furthered the discovery and dissection of biochemical pathways. The development of methods to synthesize such tagged biological compounds inexpensively on an industrial scale has enabled them to be used routinely in laboratory protocols, including many detailed in this manual. Although most of these protocols involve the use of only microcurie (μCi) amounts of radioactivity, some (particularly those describing the metabolic labeling of proteins or nucleic acids within cells) require amounts on the order of tens of millicuries (mCi). In all cases where radioisotopes are used, depending on the quantity and nature of the isotope, certain precautions must be taken to ensure the safety of laboratory personnel. This unit outlines a few such considerations relevant to the isotopes most frequently used in biological research.

In designing safe protocols for the use of radioactivity, the importance of common sense, based on an understanding of the general principles of isotopic decay, and the importance of continuous monitoring with a hand-held radioactivity monitor (e.g., Geiger counter) cannot be overemphasized. In addition, it is also critical to take into account the rules, regulations, and limitations imposed by the specific institution. These are usually not optional considerations: an institution's license to use radioactivity normally depends on strict adherence to such rules.

Many of the protocols described here have evolved (and are still evolving) over the years at the Department of Molecular and Cell Biology at the Salk Institute. The authors are indebted to those who have trained them in the safe use of radioactivity, in particular to the members of the Salk Institute Radiation Safety Department. Most of the designs for the shields and other safety equipment shown in Figures 1A.4.1A, 1A.4.2, and 1A.4.3 were created at the Salk Institute in collaboration with Dave Clarkin, Mario Tengco, and Steve Berry. Safety equipment of similar design is available from several commercial vendors, including CBS Scientific and Research Products International (see *SUPPLIERS APPENDIX*).

BACKGROUND INFORMATION

The Decay Process

As anyone who has taken a basic chemistry course will remember, each element is characterized by its atomic number (*Z*), defined as the number of protons in the atom's nucleus. Isotopes of a given element exist because some atoms of each element, while by definition having the same number of protons, have a different number of neutrons and therefore a different nuclear weight (*A*), equivalent to the atomic mass number which reflects the total number of nucleons (protons plus neutrons). It should be noted that the number of electrons outside the nucleus remains the same for all isotopes of a given element, and so all isotopes of a given element are equivalent with respect to their chemical reactivity.

Radioactive decay occurs when energy is released from the nucleus of an atom of a particular isotope. This often results in the conversion of an atom of one isotope to an isotope of a different element, a process termed transmutation, as the number of protons in the atom changes after decay. The energy released from naturally occurring radioisotopes can be particulate (α and β particles), or nonparticulate (γ rays).

α particles

An α particle is essentially the nucleus of a helium atom, or two protons plus two neutrons. It is a relatively large, heavy particle that moves slowly and usually only across short distances before it encounters some other atom with which it interacts. These particles are released from isotopes with large nuclei (atomic number >82; e.g., thorium, uranium); such isotopes are not commonly used in biological research except for specific applications such as electron microscopy and X-ray diffraction studies.

β particles

In contrast to α particles, β particles are light, high-speed, charged particles. Negatively charged β particles are essentially electrons of nuclear origin that are released when a neutron is converted to a proton. Release of a β particle thus changes the atomic number and elemental status of the isotope.

Contributed by Jill Meisenhelder and Kentaro Semba

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γ radiation

γ radiation has both particle and wave properties; its wavelength falls within the range of X-ray wavelengths. Presently, γ radiation is defined as that originating from an atomic nucleus, and X-ray radiation as that originating from the electron cloud surrounding the nucleus. Unlike β-particle release, the release of γ radiation by itself produces an isotopic change rather than an elemental one; however, the resultant nuclei are unstable and often decay further, releasing β particles.

Isotopic decay

The energy of all α particles and γ rays (measured in electron volts, eV) is fixed, because they are of specific composition or wavelength. The energy of β particles, however, varies depending on the atom from which they originate (and on the concomitant release of neutrinos or antineutrinos that serve to balance the conservation of energy aspect of the decay equation). Thus, relatively high-energy β particles are released during the decay of ^{32}P and low-energy β particles are released when tritium (^{3}H) decays.

Isotopic decay usually involves a chain or sequence of events rather than a single loss of a particle, because the resultant, equally unstable atoms try to achieve equilibrium. During this course of decay, secondary forms of radiation can be generated that may also pose a hazard to workers. For example, when high-energy β particles released during the decay of ^{32}P encounter the nuclei of atoms with a large atomic number, a strong interaction occurs. The β particle loses some energy in the form of a photon. Such photons are called bremsstrahlung radiation and are detectable using a monitor suitable for the detection of γ or X-rays.

Following their release, α, β, and γ emissions (as well as secondary forms of radiation) travel varying average distances at varying average speeds, depending on their energy and the density of the material through which they are moving. The distance they actually travel before encountering either the electrons or nucleus of another atom is termed their degree of penetrance, or range. This value is expressed as an average for each type of particle. The energy of the particles released (and therefore their potential penetrance) thus dictates what type of shielding, if any, is necessary for protection against the radiation generated by the decay of a given isotope. α, β, and γ emissions all have the potential, upon encountering an atom, to knock out its electrons, thereby creating ions. Thus, these three types of emissions are called ionizing radiation. The formation of such ions may result in the perturbation of biological processes: therein lies the danger associated with radioactivity!

Measuring Radioactivity and Individual Exposure to It

The radioactivity of a given substance or sample is measured in terms of its ionizing activity. A curie, by definition, is the amount of radioactive material that will produce 3.4×10^{10} disintegrations (ions) per second (dps). This, not coincidentally, happens to be the number of disintegrations that occur during the decay of 1 g of radium and its decay products. Exposure to such radiation is measured as the amount of energy absorbed by the recipient, which, of course, is directly related to the potential damage such radiation may cause. One rad is the dose of radiation that will cause 100 ergs of energy to be absorbed per gram of irradiated material. The unit commonly used to measure radiation doses to humans is the rem; this is related to the rad but takes into account a "quality factor" based on the type of ionizing radiation being received. For β particles and γ or X-rays this factor is 1; therefore, rems of β equal rads of β. In contrast, the quality factor associated with α particles is 20, so an exposure of one rad due to α particles would be recorded as 20 rem.

The amount or dose of radiation received by materials (e.g., cells, scientists) near the source depends not only on the specific type and energy (penetrance) of the radiation being produced, but also on the subject's distance from the source, the existence of any intervening layers of attenuating material (shielding), and the length of time spent in the vicinity of the radiation source. To best measure such doses, every person working with or around radioactivity should wear an appropriate type of radiation detection badge (in addition to carrying a portable radiation monitor that can give an immediate, approximate reading). This is normally a requirement (not an option) for compliance with an institution's license to use radioisotopes. Such badges are usually furnished by the safety department and collected at regular intervals for reading by a contracted company. Most institutions currently use either TLDs (thermoluminescent) or OSLDs (optically stimulated luminescent) dosimeters. TLDs take advantage of chemicals such as calcium or lithium fluorides, which, following exposure to ionizing radiation, will luminesce at temperatures below their normal thermal

luminescence threshold. OSLDs are read after stimulating their powdered aluminum trioxide wafer with a laser. Different types of badges are sensitive to different types of radiation: always be sure to wear one that is appropriate for detecting exposure to the isotope being used! Most often, workers will be asked to wear a radiation detection badge on the labcoat lapel in order to measure whole-body radiation. Pregnant women may be asked to wear a dosimeter over or near their stomach area to better monitor their (and the developing fetus's) exposure. When working with >1 mCi ^{32}P or ^{125}I, it is also advisable to wear a ring badge to measure exposure to the unshielded (though gloved!) fingers (extremities). The limit set for "acceptable" exposure to whole-body radiation is several-fold less than the limit set for extremities. Nevertheless, we have found that the exposure recorded on ring badges is often significant with respect to the limit for extremities set by our institution.

What is known about the effects on humans of exposure to low levels of radiation (i.e., levels which would be received when briefly handling mCi or μCi amounts of radioactivity)? Not much, for the obvious reason that direct studies have not been undertaken. Accordingly, guidelines for exposure levels are set using extrapolations—either by extrapolating down from population statistics obtained following accidents or disasters e.g., (the Chernobyl meltdown, atomic bombings) or by extrapolating up from numbers obtained from animal experiments. Each form of extrapolation is subject to caveats, and given that predictions based on such extrapolations cannot be perfect, most health and safety personnel aim for radiation exposure levels said to be ALARA ("as low as reasonably achievable"). An extensive discussion of both the studies and the statistics on which exposure limits are based, updated on a regular basis, may be found in the BEIR series (Biological Effects of Ionizing Radiations; BRER, 2005, available online in an open-book form at *http://www.nap.edu/books/030909156X/html*).

Limiting exposure to radiation can be accomplished by adjusting several parameters: the duration of exposure, distance from the source, and the density of the material (air, water, shielding) between the individual and the source.

Time is of the essence

When designing any experiment using radioactivity, every effort should be made to limit the time spent directly handling the vials or tubes containing the radioactive material. Speed should be encouraged in all manipulations, though not to the point of recklessness! Have everything needed for the experiment ready at hand before radioactivity is introduced into the work area.

Distance helps to determine dose

When possible, experiments involving radioactivity should be performed in an area separate from the rest of the lab. Many institutions require that such work be performed in a designated "hot lab"; however, if many people in the laboratory routinely use radioisotopes, it is less than feasible to move them all into what is usually a smaller space. No matter where an individual is working, it is his or her responsibility to monitor the work area and ensure his or her own safety and the safety of those working nearby by using adequate shielding. Obviously, when handling the radioactive samples, it is necessary to work behind all required shielding. To protect bystanders, remember that the intensity of radiation from a source (moving through air) falls off in proportion to the square of the distance. Thus, if standing 1 foot (~30 cm) away from a source for 5 min would result in an exposure of 45 units, standing 3 feet (~90 cm) away for the same amount of time would result in an exposure $(1/3)^2$ of 45 units, or 5 units. This factor is also relevant when considering the storage of large (mCi) amounts of radioactivity, particularly ^{125}I or ^{32}P, as no amount of shielding can completely eliminate radiation.

Shielding is the key to safety

As mentioned above, the energy of the particle(s) released during the decay of an isotope determines what, if any, type of shielding is appropriate. β particles released during the decay of ^{14}C and ^{35}S possess roughly ten times the energy of those released when ^{3}H decays. All three β particles are of relatively low energy, do not travel very far in air, and cannot penetrate solid surfaces. No barriers are necessary for shielding against this type of β radiation. The major health threat from these isotopes occurs through their accidental ingestion, inhalation, or injection.

β particles released during the decay of ^{32}P have 10-fold higher energy than those released from ^{14}C, and thus pose a more significant threat to workers. (One reported hazard is the potential for induction of cataracts in the unshielded eye.) The fact that these high-energy β particles can potentially generate significant

amounts of bremsstrahlung radiation is the reason that low-density materials are used as the primary layer of shielding for β radiation from ^{32}P. Water, glass, and plastic are suitable low-density materials (as opposed to lead). Obviously water is unsuitable as a shielding layer for work on the bench, although it does a reasonable job when samples are incubating in a water bath. Shields made from a thickness of glass sufficient to stop these particles would be extremely heavy and cumbersome (as well as dangerous if dropped). Fortunately, plastic or acrylic materials—variously called Plexiglas, Perspex, or Lucite—are available for shielding against β radiation from ^{32}P. Shields, as well as storage boxes, constructed of various thicknesses of Plexiglas are necessary equipment in laboratories where ^{32}P is used. When mCi amounts of ^{32}P are used at one time, it is necessary to also block the bremsstrahlung radiation by adding a layer of high-density material (such as 4 to 6 mm of lead) to the outside of the Plexiglas shield (covering the side farthest from the radioactive source).

γ rays released during the decay of ^{125}I have much higher penetrance than the β particles from ^{32}P decay; this radiation must be stopped by very-high-density material, such as lead. Lead foil of varying thicknesses (0.5 to 6 mm) can be purchased in rolls and then cut and molded to cover any container, or taped to a Plexiglas shield (used in this instance for support). Obviously this latter arrangement has the disadvantage that it is impossible to see what one is doing through the shield. For routine shielding of manipulations involving ^{125}I, it is useful to purchase a lead-impregnated Plexiglas shield that is transparent, albeit inevitably very heavy (as well as relatively expensive).

Although it seems logical that the use of more radioactivity necessitates the use of thicker layers of shielding, it is also true that no shielding material is capable of completely stopping all radiation. When deciding how thick is "thick enough," consult the half-value layer measurement for each type of shielding material. This number gives the thickness of a given material necessary to stop half the radiation from a source. In general, 1 to 2 cm of Plexiglas and/or 0.02 mm of lead are sufficient to shield the amounts of radioactivity used in experiments performed in the author's laboratory.

GENERAL PRECAUTIONS

Before going on to a discussion of specific precautions to be taken with individual isotopes, a short list of general precautions to be taken with all isotopes seems pertinent:

1. Know the rules. Be sure that each individual is authorized to use each particular isotope and uses it in an authorized work area.

2. Don the appropriate apparel. Whenever working at the lab bench, it is good safety practice to wear a labcoat for protection; when using radioactivity, wearing a labcoat is imperative almost everywhere. Disposable paper/synthetic coats of various styles are commercially available at relatively low cost. These may be conveniently thrown out if contaminated with radioactivity during an experiment, rather than held for decay as might be preferable with more expensive cloth coats. As an alternative, disposable sleeves can be purchased and worn over those of the usual cloth coat. Other necessary accessories include radiation safety badges, gloves, and protective eyewear. It is convenient to wear two pairs of gloves at once when using radioactivity: when the outer pair becomes contaminated, it is possible to strip it off and continue working without interruption. Contaminated gloves should always be removed over a bench or waste container so that microdroplets of contamination do not fall on the floor and get tracked about!

3. Protect the work area as well as the workers. Laboratory benches and the bases of any shields used should be covered with some sort of disposable, preferably absorbent, paper sheet. Underpads or diapers (the kind normally used in hospitals) are convenient for this purpose.

4. Use appropriate designated equipment. It is very convenient, where use justifies the expense, to have a few adjustable pipettors dedicated for use with each particular isotope. Likewise, it is good practice to use only certain labeled centrifuges and microcentrifuge rotors for radioactive samples, to avoid contamination of all rotors in the laboratory. Although such equipment should be cleaned after each use, complete decontamination is often not possible. A few pipettors or a single microcentrifuge can easily be stored (and used) behind appropriate shielding. Actually, contamination of the insides and tip ends of pipettors can be greatly reduced by using tips supplied with internal aerosol barriers such as those used for PCR reactions. To prevent contamination of the outside of the pipettor's barrel, simply wrap the hand-grip in Parafilm, which can be discarded later.

5. Know where to dispose of radioactive waste, liquid and solid. Most institutions require that radioactive waste be segregated by

isotope. This is done not only so that appropriate shielding can be placed around waste containers, but so that some waste can be allowed to decay prior to disposal in the normal (nonradioactive) trash. With a decreasing number of radioactive waste disposal facilities able or willing to accept radioactive waste for burial (and a concomitant increase in dumping charges from those that still do), this practice of on-site decay can save an institution thousands of dollars a year in disposal charges.

6. Label your label! It is only common courtesy (as well as common sense) to alert coworkers to the existence of anything and everything radioactive that is left where they may come in contact with it! A simple piece of tape affixed to the sample box—with the investigator's name, the amount and type of isotope, and the date—should suffice. Yellow hazard tape printed with the international symbol for radioactivity is commercially available in a variety of widths.

7. Monitor radioactivity early and often. Portable radiation detection monitors are essential equipment for every laboratory using radioactivity. No matter how much or how little radioactivity is being used, the investigator should keep a hand-held monitor nearby—and it should be on! Turn it on before touching any radioactivity to avoid contaminating the monitor's switch. Always check the batteries! Use a monitor with the appropriate detection capacity (β for ^{35}S and ^{32}P; γ for ^{125}I) before, during, and after all procedures. The more frequently fingers and relevant equipment are monitored, the more quickly a spill or glove contamination will be detected. Such timely detection will keep both the potential mess and cleanup time to a minimum. While it is tempting to cover the monitor's detection tube with Parafilm to protect it from contamination, remember that this will preclude the detection of the weaker β of ^{35}S! Because low-energy β emitters such as ^{3}H cannot be detected at all using such monitors, wipe tests of the bench and equipment used, followed by scintillation counting of the wiped samples, are necessary to ensure that contamination of the work area did not occur.

SPECIFIC PRECAUTIONS

The following sections describe precautions to be taken with individual isotopes in specific forms. Although the sections dealing with ^{35}S- or ^{32}P-labeling of proteins in intact cells are presented in terms of mammalian cells, most of the instructions are also pertinent (with minimal and obvious modifications) to the labeling of proteins in other cells (e.g., bacterial, insect, plant).

Working with ^{35}S

As discussed above, the β radiation generated during ^{35}S decay is not strong enough to make barrier forms of shielding necessary. The risk associated with ^{35}S comes primarily through its ingestion and subsequent concentration in various target organs, particularly the testes. As reported several years ago (Meisenhelder and Hunter, 1988), ^{35}S-labeled methionine and cysteine, which are routinely used to label proteins in intact cells and by in vitro translation, break down chemically to generate a volatile radioactive component. The breakdown occurs independently of cellular metabolism. Thus, the radioactive component is generated to the same extent in stock vials as in cell culture dishes. The process seems to be promoted by freezing and thawing ^{35}S-labeled materials. The exact identity of this component is not known, although it is probably SO_2 or CH_3SH. What is known is that it dissolves readily in water and is absorbed by activated charcoal or copper.

The amount of this volatile radioactive component released, despite stabilizers added by the manufacturers, is about 1/8000 of the total radioactivity present. The amount of this radioactivity that a scientist is likely to inhale while using these compounds is presumably even smaller. Nevertheless, such a component can potentially contaminate a wide area because of its volatility, and also tends to concentrate in target organs. Thus, it is advisable to thaw vials of ^{35}S-labeled amino acids in a controlled area such as a hood equipped with a charcoal filter. This charcoal filter will become quite contaminated and should be changed every few months. If such an area is not available, the stock vial should be thawed using a needle attached to a charcoal-packed syringe to vent and trap the volatile compound.

Anyone who has ever added ^{35}S-labeled amino acids to dishes of cells for even short periods knows that the incubators used for such labelings quickly become highly contaminated with ^{35}S. Such contamination is not limited to the dish itself, nor to the shelf on which the dish was placed. Rather, the radioactive component's solubility in water allows it to circulate throughout the moist atmosphere of the incubator and contaminate all of the inside surfaces. For this reason, in laboratories where such metabolic labelings are routine, it is highly convenient to designate one incubator to be used solely for working with ^{35}S-labeled

samples. Such an incubator can be fitted with a large honeycomb-style filter, the size of an incubator shelf, made of pressed, activated charcoal. These filters are available from local air-quality-control companies. Such a filter will quickly become quite contaminated with radioactivity and should therefore be monitored and changed as necessary (usually about every three months if the incubator is used several times a week). The water used to humidify the incubator will also become quite "hot" (contaminated with radioactivity); keeping the water in a shallow glass pan on the bottom of the incubator makes it easy to change it after every use, thus preventing contamination from accumulating. Even with the charcoal filter and water as absorbents, the shelves, fan, and inner glass door of the incubator will become contaminated, as will the tray on which the cells are carried and incubated. Routine wipe tests and cleaning when necessary will help to minimize potential spread of this contamination.

If such labelings are done infrequently or there is no "spare" incubator, dishes of cells can be placed in a box during incubation. This box should be made of plastic, which is generally more easily decontaminated than metal. Along with the dishes of cells, a small sachet made of activated charcoal wrapped loosely in tissue (Kimwipes work well) should be placed in the box. If the box is sealed, it will obviously need to be gassed with the correct mixture of CO_2; otherwise small holes can be incorporated into the box design to allow equilibration with the incubator's atmosphere. In either case, the incubator used for the labeling should be carefully monitored for radioactivity after each experiment.

Working with ^{32}P

Microcurie (μCi) amounts of ^{32}P

The amount of ^{32}P-labeled nucleotide used to label nucleic acid probes for northern or Southern blotting is typically under 250 μCi, and the amount of [γ-^{32}P]ATP used for in vitro phosphorylation of proteins does not usually exceed 50 μCi for a single kinase reaction (or several hundred microcurie per experiment). However, handling even these small amounts, given the time spent on such experiments, can result in an unacceptable level of exposure if proper shielding is not employed. With no intervening shielding, the dose rate 1 cm away from 1 mCi of ^{32}P is 200,000 mrad/hr; the local dose rate to basal cells resulting from a skin contamination of 1 μCi/cm^2 is 9200 mrad/hr (Shleien, 1987). Such skin contamination could easily occur though careless pipetting and the resultant creation of an aerosol of radioactive microdroplets, because the concentration of a typical stock solution of labeled nucleotide may be 10 μCi/μl.

For proper protection during this sort of experiment, besides the usual personal attire (glasses, gloves, coat, and ring and lapel safety badges) it is necessary to use some form of Plexiglas screen between the body and the samples (see Fig. 1A.4.1A). Check the level of radiation coming through the outside of the shield with a portable monitor to be sure the thickness of the Plexiglas is adequate. Consult your Radiation Safety Officer if the monitor reads more than 4000 cpm. Hands can be shielded from some exposure by placing the sample tubes in a solid Plexiglas rack, which is also useful for transporting samples from the bench to a centrifuge or water bath (see Fig. 1A.4.1B).

Experiments of these types often include an incubation step performed at a specific temperature, usually in a water bath. Although the water surrounding the tubes or hybridization bags will effectively stop β radiation, shielding should be added over the top of the tubes (where there is no water)—e.g., a simple flat piece of Plexiglas. If the frequency of usage justifies the expense, an entire lid for the water bath can be constructed from Plexiglas. When hybridization reactions are performed in bags, care should be taken to monitor (and shield) the apparatus used to heat-seal the bags.

The waste generated during the experiments should also be shielded. It is convenient to have a temporary waste container right on the bench. Discard pipet tips and other solid waste into a beaker lined with a plastic bag and placed behind the shield. This bag can then be emptied into the appropriate shielded laboratory waste container when the experiment is done. Liquid waste can be pipetted into a disposable tube set in a stable rack behind the shield (see Fig. 1A.4.1C).

When radiolabeled probes or proteins must be gel-purified, it may be necessary to shield the gel apparatus during electrophoresis if the samples are particularly hot. Be advised that the electrophoresis buffer is likely to become very radioactive if the unincorporated label is allowed to run off the bottom of the gel; check with your Radiation Safety Officer for instructions on how to dispose of such buffer. It is also prudent to check the gel plates with a portable detection monitor after the electrophoresis is completed, because they sometimes become contaminated as well.

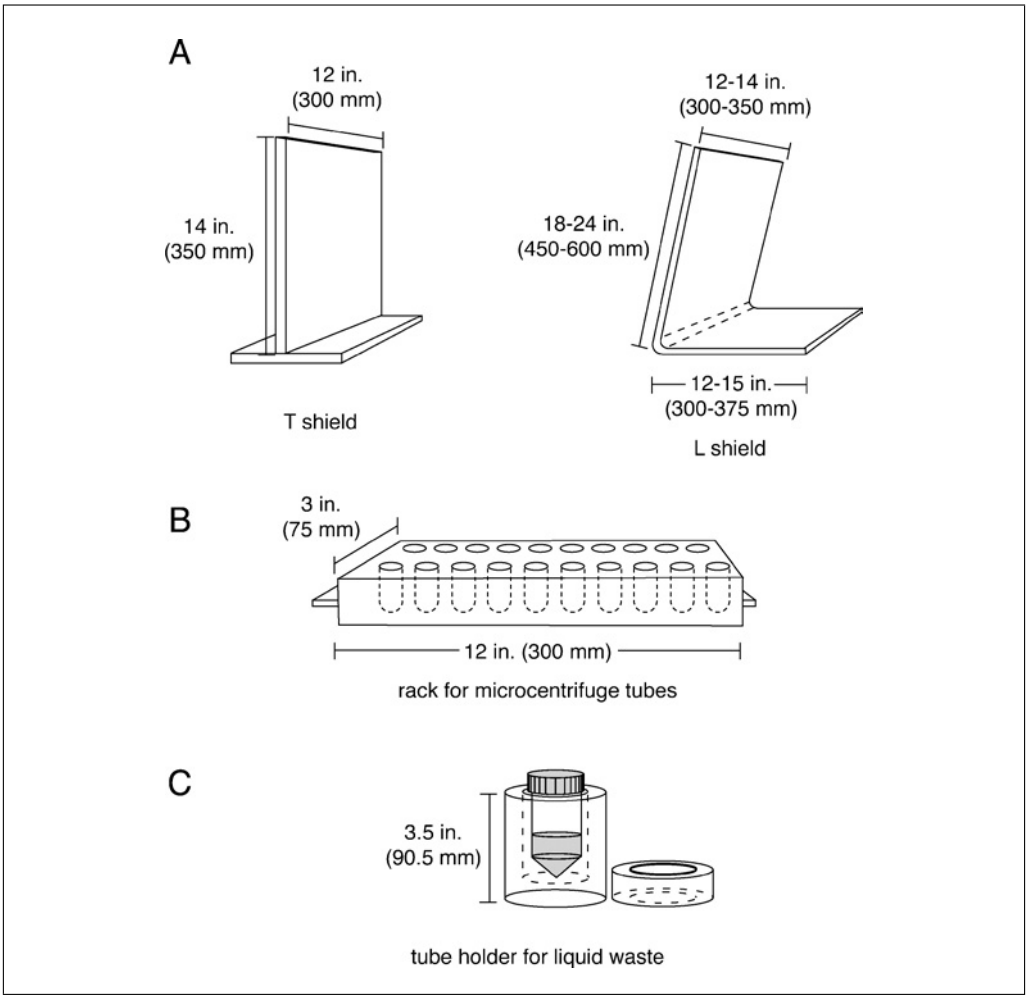

Figure 1A.4.1 Plexiglas shielding for ^{32}P. (**A**) Two portable shields (L and T design) made of 0.5-in. (12.5 mm) Plexiglas. Either can be used to directly shield the scientist from the radioactivity he or she is using. Turned on its side, the L-shaped shield can be used to construct two sides of a cage around a temporary work area, providing shielding for other workers directly across or to the sides of the person working with ^{32}P. (**B**) Tube rack for samples in microcentrifuge tubes. (**C**) Tube holder for liquid waste collection.

Millicurie (mCi) amounts of ^{32}P

In order to study protein phosphorylation in intact mammalian cells, cells in tissue culture dishes are incubated in phosphate-free medium with ^{32}P-labeled orthophosphate for a period of several hours or overnight, to label the proteins. The amount of ^{32}P used in such labelings can be substantial. Cells are normally incubated in 1 to 2 mCi of ^{32}P per ml labeling medium; for each 6-cm dish of cells, 2.5 to 5 mCi ^{32}P may be used. When this figure is multiplied by the number of dishes necessary per sample, and the number of different samples in each experiment, it is clear that the amount of ^{32}P used in one experiment can easily reach 25 mCi or more. Because so much radioactivity is used in the initial labeling phase of such experiments, it is necessary for a researcher to take extra precautions in order to adequately shield him or herself and coworkers.

When adding label to dishes of cells, it is important to work as rapidly as possible. An important contribution to the speed of these manipulations is to have everything that will be needed at hand before even introducing the label into the work area. Prepare the work area, arranging shielding and covering the bench with diapers, in advance. Set out all necessary items, including any pipettors and tips needed, a portable detection monitor, extra gloves, and a cell house (see Fig. 1A.4.2A).

Work involving this much radioactivity should be done behind a Plexiglas shield at least 3/4 in. (~2 cm) thick; the addition of a layer of lead to the outside lower section of this shield to stop bremsstrahlung radiation is also needed. If one shield can be dedicated to this

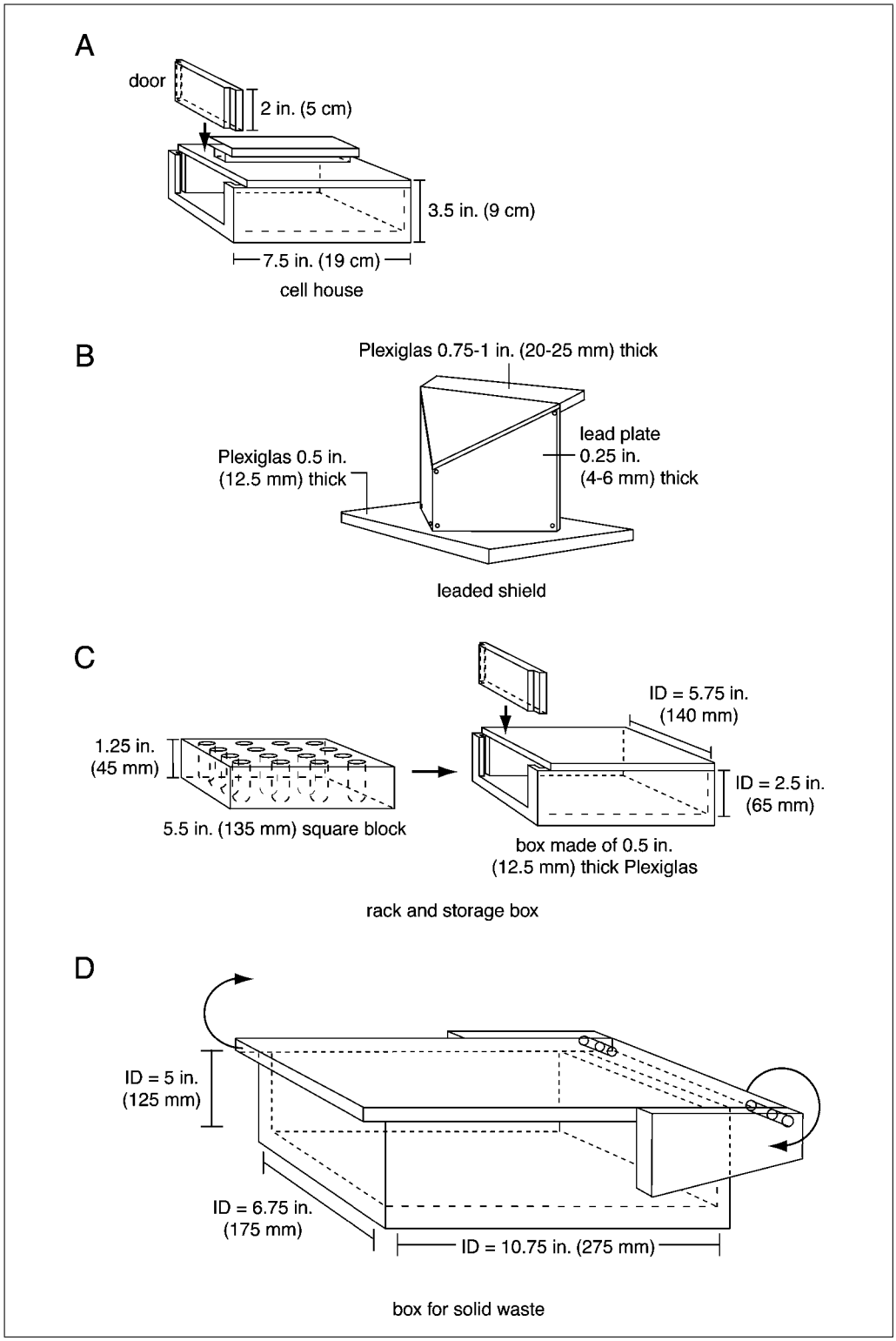

Figure 1A.4.2 (**A**) Box for cell incubation (a “cell house”). (**B**) Box for solid waste collection made of 0.5-in. Plexiglas. (**C**) Sample storage rack and box made of 0.5-in. Plexiglas. (**D**) Box for solid waste collection made of 0.5-in. Plexiglas. Abbreviations: ID, interior dimension.

purpose at a specific location, a sheet of lead several centimeters thick can be permanently screwed to the Plexiglas (as shown in Fig. 1A.4.2B); however, lead makes the shield extremely heavy and therefore less than portable. If space constraints do not permit the existence of such a permanent labeling station, a layer or two of lead foil can be taped temporarily to the outside of the Plexiglas shield.

Again, each worker should take care to shield not only him or herself but also bystanders on all sides. Handling of label should be done away from the central laboratory, if possible, to take maximum advantage of distance as an additional form of shielding. It is also advisable not to perform such experiments in a tissue culture room or any other room that is designed for a purpose vital to the whole laboratory. An accident involving this much ^{32}P would seriously inconvenience future work in the area, if not make it altogether uninhabitable! If care is taken to minimize the amount of time the dish of cells is open when adding the label, use of a controlled air hood to prevent fungal or bacterial contamination of the cells should not be necessary.

Plexiglas dish covers. In the course of doing experiments to determine which hand receives the most exposure during such cell labelings, the authors discovered that extremity exposure can vary as much as seven-fold depending on the finger on which the ring is worn, with the index finger of the left hand receiving the most exposure for a right-handed person (Bursik et al., 1999). As would be expected, the most exposure is received as the worker adds label to the dishes of cells and as the cells are lysed (see below). In order to mitigate this extremity exposure, we designed Plexiglas dish covers to shield each individual dish: the tissue culture dish fits snugly into the bottom Plexiglas piece while the top Plexiglas piece is joined to the top of the tissue culture dish using tape, so that the two lids can be handled together as one unit. Tissue culture dishes of cells are fitted/taped into the Plexiglas dish covers immediately before adding the ^{32}P. As the top and bottom pieces of the dish covers do not form a seal, the medium can equilibrate with the CO_2 of the incubator for proper pH adjustment. Use of such dish covers reduces extremity exposure 8- to 10-fold, despite the stream of radioactivity that passes through the crack between the top and bottom. Figure 1A.4.3 shows a researcher preparing to remove the medium from a dish of cells using the Plexiglas dish covers.

Cell houses. Once the label has been added to the dishes of cells (and whether or not one is using the dish covers discussed above), the dishes will also need to be shielded for transport to and from the incubator and other work

Figure 1A.4.3 Use of Plexiglas dish shields for ^{32}P labels reduces extremity exposure.

areas. Plexiglas boxes that are open at one end (for insertion of the dishes) and that have a handle on top (for safe carrying) make ideal cell houses (see Fig. 1A.4.2A). A Plexiglas door that slides into grooves at the open end is important to prevent dishes from sliding out if the box is tilted (even minutely) during transport. If this door is only two-thirds the height of the house wall, the open slot thus created will allow equilibration of the CO_2 level within the house with that in the incubator. Obviously, this slot will also allow a substantial stream of radiation to pass out of the cell house, so the house should be carried and placed in the incubator with its door facing away from the worker (and others)! Use of the Plexiglas dish covers adds considerable bulk; larger cell houses designed with handles on their sides and a hinged lid are more easily handled (see Fig. 1A.4.3).

Dealing with lysis products. Following incubation with label and any treatments or other experimental manipulations, the cells are usually lysed in some type of detergent buffer. It is during this lysis procedure that a worker's hands will receive their greatest exposure to radiation, because it is necessary to directly handle the dishes over a period of several minutes. It is therefore very important to streamline this procedure and use shielding whenever possible. If the cell lysates must be made at 4°C, as required by most protocols, working on a bench in a cold room is preferable to placing the dishes on a slippery bed of ice. In either case, make the lysate using the same sort of shielding (with lead if necessary) that was used when initially adding the label. Pipet the labeling medium and any solution used to rinse unincorporated radioactivity from the cells into a small tube held in a solid Plexiglas holder (shown in Fig. 1A.4.1C). The contents of this tube can later be poured into the appropriate liquid waste receptacle. If possible, it is a good practice to keep this high-specific-activity ^{32}P liquid waste separate from the lower-activity waste generated in other procedures, so that it can be removed from the laboratory as soon as possible following the experiment. If it is necessary to store it in the laboratory for any time, the shielding for the waste container should also include a layer of lead.

The solid waste generated in the lysis part of these experiments (pipet tips, disposable pipets, cell scrapers, and dishes) is very hot and should be placed immediately into some sort of shielded container to avoid further exposure of the hands. A Plexiglas box similar in design to that in Figure 1A.4.2 is convenient; placed to the side of the shield and lined with a plastic bag, it will safely hold all radioactive waste during the experiment and is light enough to be easily carried to the main laboratory waste container where the plastic bag (and its contents) can be dumped after the experiment is done. If the lid of the box protrudes an inch or so over the front wall, it can be lifted using the back of a hand, thus decreasing the possibility of contaminating it with hot gloves.

When scraping the cell lysates from the dishes, it is good practice to add them to microcentrifuge tubes that are shielded in a solid Plexiglas rack; this will help to further reduce the exposure to which the hands are subjected. At this point, the lysates are usually centrifuged at high speed ($\sim$10,000 $\times$ g) to clear them of unsolubilized cell material. Use screw-cap tubes for this clarification step, as these will contain the labeled lysate more securely than flip-top tubes, which may open during centrifugation. No matter what type of tube is used, the rotor of the centrifuge often becomes contaminated, most probably because of tiny drops of lysate (aerosol) initially present on the rim of the tubes that are spun off during centrifugation. Monitor the rotor and wipe it out after each use.

The amount of ^{32}P taken up by cells during the incubation period varies considerably, depending on the growth state of the culture as well as on the cell type and its sensitivity to radiation. This makes it difficult to predict the percentage of the radioactivity initially added to the cells that is incorporated into the cell lysate; however, this figure probably does not exceed 10%. Thus, the amount of radioactivity being handled decreases dramatically after lysis, making effective shielding much simpler. However, at least ten times more radioactivity than is usual in other sorts of experiments is still involved! It is easy to determine if the shielding is adequate—just use both β and γ portable monitors to measure the radiation coming through it. If the meter reads more than 5000 cpm, additional shielding is needed. Again, be sure to check that people working nearby (including those across the bench) are also adequately shielded. It is sometimes necessary to construct a sort of cage of Plexiglas shields around the ice bucket that contains the lysates.

Storage. At the end of the day or the experiment, it may be necessary to store radioactive samples; in some experiments, it may be desirable to save the cell lysates. These very hot

samples are best stored in tubes placed in solid Plexiglas racks that can then be put into Plexiglas boxes (see Fig. 1A.4.2C). Such boxes may be of similar construction to the cell houses described above; however, they should have a door that completely covers the opening. Be sure to check the γ radiation coming through these layers and add lead outside if necessary.

Working with ^{33}P

Using ^{33}P-labeled nucleotides to label nucleic acid probes or proteins

Several of the major companies that manufacture radiolabeled biological molecules also sell nucleotides labeled with ^{33}P (both α- and γ-labeled forms). ^{33}P offers a clear advantage over ^{32}P with respect to ease of handling, because the energy of the β particles emitted lies between that of ^{35}S and ^{32}P; thus, ^{33}P does not require as many layers of Plexiglas and lead shielding as ^{32}P. In fact, the β radiation emitted can barely penetrate gloves and the surface layer of skin, so the hazard associated with exposure to even millicurie amounts of ^{33}P is thought to be insignificant (as reported in the NEN Life Science product brochure). Gel bands visualized on autoradiograms of ^{33}P-labeled compounds are sharper than bands labeled with ^{32}P because the lower-energy β radiation does not have the scatter associated with ^{32}P. The half-life of ^{33}P is also longer (25 days compared to 14 days for ^{32}P). Despite its higher cost, these features have led many researchers to choose ^{33}P-labeled nucleotides for use in experiments such as band/gel shift assays where discrimination of closely spaced gel bands is important.

The best way to determine what degree of shielding is needed when using ^{33}P is to monitor the source using a portable β monitor and add layers of Plexiglas as necessary, as described previously for ^{32}P.

Working with ^{125}I

Using ^{125}I to detect immune complexes (immunoblots or western blots)

^{125}I that is covalently attached to a molecule such as staphylococcal protein A is not volatile and therefore is much less hazardous than the unbound or free form. Most institutions do not insist that work with bound ^{125}I be performed in a hood, but shielding of the γ radiation is still necessary. Lead is a good high-density material for stopping these γ rays; its drawbacks are its weight and opacity. Commercially available shields for ^{125}I are made of lead-impregnated Plexiglas—though heavy, these are at least see-through. Alternatively, a piece of lead foil may be taped to a structural support, although this arrangement does not provide shielding for the head as a worker peers over the lead!

Incubations of the membrane or blot with the [^{125}I]protein A solution and subsequent washes are usually done on a shaker. For shielding during these steps, a piece of lead foil may simply be wrapped around the container. Solutions of ^{125}I can be conveniently stored for repeated use in a rack placed in a lead box.

Using ^{125}I to label proteins or peptides in vitro

Any experiments that call for the use of free, unbound ^{125}I should be done behind a shield in a hood that contains a charcoal filter to absorb the volatile iodine. Most institutions require that such experiments be done in a special hot lab to which access is limited. Ingested or inhaled iodine is concentrated in the thyroid; a portable γ monitor should therefore be used to scan the neck and throat before beginning and 24 to 36 hr after completing each experiment. Similar thyroid bioassays should routinely be performed on all members of any laboratory in which unbound iodine is used.

DEALING WITH ACCIDENTS

Despite the best intentions and utmost caution, accidents happen. Accidents involving spills of radioactivity are particularly insidious because they can be virtually undetectable yet pose a significant threat to laboratory workers. For this reason, in any laboratory where radioisotopes are routinely used, it is best to foster a community spirit—a sense of cooperativity that extends from shielding each other properly to helping each other clean up when such accidents occur.

The specific measures to be taken following an accident involving radioactivity naturally depend on the type and amount of the isotope involved, the chemical or biological hazards of the material it is associated with, and the physical parameters of the spill (i.e., where and onto what the isotope was "misplaced"). However, following any accident, there are several immediate steps that should be taken:

1. Alert coworkers as well as institutional Radiation Safety personnel to the fact that there has been an accident. This will give them the opportunity to shield themselves if necessary—and to help clean up as well!

2. Restrict access to and away from the site of the accident to ensure that any uncontained radioactive material is not spread around the laboratory. When leaving the site, be sure to monitor the bottoms of the shoes as well as the rest of the body.

3. Take care of all contaminated personnel first, evacuating others if necessary. If anyone's skin is contaminated, first use a portable monitor to identify specific areas of contamination, then wipe these areas with a damp tissue to remove as much surface radioactivity as possible. Try to scrub only small areas at a time to keep the contamination localized. If the contamination is not easily removed with paper tissues, try a sponge or an abrasive pad, but be careful not to break the skin! Sometimes soaking is required: do this only after all easily removed contamination is gone and keep the soaked area to a minimum. Contaminated strands of hair can be washed (or perhaps a new hairstyle may be in order).

4. When attempting to clean any contaminated equipment, floors, benches, etc., begin by soaking up any visible radioactive liquid with an absorbent material. Use a small amount of soap and water to clean the contaminated area, keeping the area wiped each time to a minimum to avoid smearing the contamination over an even greater surface. There are quite a range of foams and sprays available commercially that are made specifically to decontaminate radioactivity. A dilute solution of phosphoric acid works well to pick up ^{32}P. Decontamination of centrifuge rotors can be tricky, as their anodized surfaces are sensitive to many detergents; check with the rotor manufacturer for appropriate cleansing solutions. Many surfaces prove resistant to even Herculean cleaning efforts; in these instances, the best that can be done is to remove all contamination possible and then shield whatever is left until the radioactivity decays sufficiently for safety.

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Growing and Analyzing Static Biofilms

There are a variety of systems available for examining the formation of bacterial biofilms. In this unit, several approaches are described that are useful for studying, in particular, the earlier stages of formation of these communities. Static biofilm systems may be preferable to chemostatic or continuous-flow methods for a number of reasons. First, static assays are particularly useful for examining early events in biofilm formation, in some cases detecting biofilm formation in <60 min. For example, these assays can be used to identify signals that modulate the transition from a planktonic to a biofilm mode of existence. A second major advantage of these systems is the simplicity of the protocols: these assays can be executed primarily using common laboratory equipment. Furthermore, several of the assays outlined below have a relatively high throughput and can easily be adapted to study a variety of biofilm formation conditions, making them excellent tools for performing genetic screens. In addition, simple changes in the configurations of these screens (e.g., media used, medium replacement regimens, incubation time, washing vigor) allow examination of different aspects of these bacterial communities, such as formation or dispersal.

The static nature of these systems does have some drawbacks. Because most of the cultures are neither continuously supplied with fresh medium nor aerated, there may be limitation of nutrients, and an inability to easily generate mature biofilms may be encountered. Continuous-flow and chemostat systems for the production of mature biofilms are described in other units in this chapter.

Presented in this unit are four basic protocols for the growth and analysis of biofilms in static systems. The microtiter plate biofilm assay (see Basic Protocol 1) is a useful method for assessing bacterial attachment by measuring the staining of the adherent biomass. Because it utilizes a 96-well plate format, it is suitable as a tool for screening large numbers of bacterial strains or species. The air-liquid interface (ALI) assay (see Basic Protocol 2) is complementary to the microtiter plate biofilm assay in that it provides a mechanism for direct microscopic viewing of the live attached microbes. A colony-based biofilm system is also described (see Basic Protocol 3), with this system being especially useful for monitoring cell death in biofilms treated with antimicrobial agents. Finally, the Kadouri system (see Basic Protocol 4) is a "low-flow" system that can serve as a bridge between the static assays discussed in this unit and continuous-flow systems.

CAUTION: Follow all appropriate biosafety requirements relevant to the microorganism under investigation. Refer to *UNIT 1A.1* and other pertinent resources (see *APPENDIX 1B*) for instructions on safe handling of microorganisms.

MICROTITER PLATE BIOFILM ASSAY

BASIC PROTOCOL 1

This experimental system, whose most common format is often referred to as the 96-well plate assay, is a simple high-throughput method used to monitor microbial attachment to an abiotic surface. While popularized in the mid-to-late 1990s (Mack et al., 1994; O'Toole et al., 1999), the assay in its typically used form is derived from a protocol published by Christensen et al. (1985). Examples of bacteria studied in this manner are listed in Table 1B.1.1, although in theory, the protocol could be applied to any species amenable to growth in the prescribed format. In brief, cells are grown in microtiter dishes for a desired period of time, and then the wells are washed to remove planktonic bacteria. Cells remaining adhered to the wells are subsequently stained with a dye that allows visualization of the attachment pattern. This surface-associated dye can also be solubilized for semiquantitative assessment of the biofilm formed.

Contributed by Judith H. Merritt, Daniel E. Kadouri, and George A. O'Toole
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Table 1B.1.1 Typical Conditions for Developing and Performing Microtiter Plate Biofilm Assays

Organism	Incubation temperature (°C)	Solvent for solubilization of stained biofilms	Reference
Agrobacterium tumefaciens	28	100% dimethyl sulfoxide (DMSO)	Danhorn et al., 2004
Escherichia coli	25	80% ethanol/20% acetone	O'Toole et al., 1999
Pseudomonas aeruginosa	25-37	95% ethanol	O'Toole et al., 1999
Pseudomonas fluorescens	25-30	95% ethanol	O'Toole et al., 1999
Staphylococcus aureus	37	33% glacial acetic acid	Stepanovic et al., 2001
Streptococcus mutans	37	95% ethanol or 100% DMSO	O'Toole et al., 1999
Vibrio cholerae	25-30	100% DMSO	O'Toole et al., 1999

Materials

Bacterial strains of interest
Appropriate media for bacteria under study (*APPENDIX 2C*)
70% ethanol
0.1% (w/v) crystal violet in water
Solvent (e.g., 95% v/v ethanol; see Table 1B.1.1 for other options) for solubilizing dye and biofilm biomass
96-well microtiter plates, not tissue culture–treated (Becton Dickinson catalog no. 353911) with lids (Becton Dickinson catalog no. 353913)
96-prong inoculating manifold, sterile (DanKar Scientific)
Small trays (e.g., large pipet tip boxes) sufficient in size to hold 96-well microtiter plates
Optically clear flat-bottom 96-well plates, nonsterile
Plate reader or spectrophotometer

For small sample numbers (<20 strains or species):

1a. Inoculate each bacterium of interest in a 3-to-5-ml culture and grow to stationary phase.

2a. Dilute cultures 1:100 in the desired media. Pipet 100 µl of each diluted culture into each of four wells in a fresh microtiter plate which has not been tissue culture treated. Cover plate and incubate at optimal growth temperature for the desired amount of time.

Lids for the microtiter dishes may be reused. Clean lids with 70% (v/v) ethanol and air-dry prior to each experiment.

The time course for attachment varies depending on the organism and must be determined empirically, although when using this system, many organisms commonly studied will form a biofilm within 48 hr.

For large screens (>20 strains or species):

1b. Inoculate cells directly into sterile microtiter plates filled with 100 µl of the appropriate medium per well, using four wells per strain or species. Incubate overnight.

2b. Inoculate biofilm assay plates directly in 100-µl medium per well from the overnight microtiter plate cultures using a sterile 96-prong inoculating manifold. Cover assay plates and incubate at optimal growth temperature for desired amount of time.

3. Set up four small trays in a series and add 1 to 2 inches of tap water to the last three.

The first tray is used to collect waste, while the others are used to wash the assay plates.

4. Remove planktonic bacteria from each microtiter dish (step 2a or 2b) by briskly shaking the dish out over the waste tray. To wash wells, submerge plate in the first water tray and then vigorously shake out the liquid over the waste tray. Replace water when it becomes cloudy.

5. Add 125 µl of 0.1% crystal violet solution to each well. Stain 10 min at room temperature.

6. Shake each microtiter dish out over the waste tray to remove the crystal violet solution. Wash dishes successively in each of the next two water trays (i.e., the two not used in step 4), and shake out as much liquid as possible after each wash.

   This step will remove any crystal violet that is not specifically staining the adherent bacteria. The wash trays can be reused for a number of plates, but the water should be replaced when its color becomes dark or when the efficiency of the washes is observed to decrease.

7. Invert each microtiter dish and vigorously tap on paper towels to remove any excess liquid. Allow plates to air-dry.

   At this stage, the staining is stable and the dried plates may be stored at room temperature for at least several weeks.

8. Add 200 µl of 95% ethanol (or another appropriate solvent) to each stained well. Allow dye to solubilize by covering plates and incubating 10 to 15 min at room temperature.

   A number of reagents may be used to solubilize biofilms. Some suggestions are listed in Table 1B.1.1, but solvents should be tested empirically for each organism.

9. Briefly mix the contents of each well by pipetting, and then transfer 125 µl of the crystal violet/ethanol solution from each well to a separate well in an optically clear flat-bottom 96-well plate. Measure the optical density (OD) of each of these 125-µl samples at a wavelength of 500 to 600 nm.

   The authors suggest four replicate wells for each strain or species used in step 2. The absorbance values from these replicates are sufficient to determine an average and standard deviation for each strain or species and thus provide a measure of the extent of biofilm formation. The number of replicates can be increased as desired.

   The OD wavelengths are presented as a range to allow for variations in the capabilities of plate readers.

DIRECT ENUMERATION OF BACTERIA IN BIOFILMS

ALTERNATE PROTOCOL 1

The biofilm formed in the microtiter dish can also be quantitated by directly enumerating the bacteria adhering to the surface. This method, while more time consuming, avoids one drawback of the crystal violet assay—crystal violet stains not only cells, but essentially any material adhering to the surface of the plate (for example, matrix components), and therefore, crystal violet staining may overestimate the number of adherent bacteria.

Additional Materials *(also see Basic Protocol 1)*

PBS (*APPENDIX 2A*), sterile
Agar plates of appropriate medium
Multichannel pipettor
8-ml plastic tubes with caps
Sonicator (e.g., Sonics and Materials VC-505)
Additional reagents and equipment for counting viable cells (Phelan, 1996)

1. Inoculate biofilm assay plates as described in Basic Protocol 1, steps 1a and 2a or steps 1b and 2b.

2. Wash wells six times, each time using a multichannel pipettor to add 100 µl sterile PBS per well and then vigorously shaking out the liquid over a waste container to remove planktonic cells.

3. Use scissors to cut each individual well from the microtiter plate. Add 100 µl PBS to each well.

4. Add each well (i.e., the actual plastic well plus its contents) to a separate 8-ml tube containing 1.9 ml PBS (for a final liquid volume of 2 ml). Cap before and after addition.

   This step is performed so that each sample can be sonicated (step 5) in an 8-ml tube. Microtiter wells are not suitable for use as sonication vessels, as they are too small.

5. Insert sonicator microtip and sonicate the contents of each tube for 8 sec at 40% power. Perform viable counts (Phelan, 1996) on the resulting suspensions by plating on agar medium to enumerate bacteria that were attached to the microtiter well surface.

   To verify that the sonication procedure does not reduce cell viability, perform viable counts on a separate culture of planktonic cells before and after sonication. If reduced cell viability is seen, adjust the sonication regimen accordingly.

   If desired, use crystal violet staining followed by microscopy to determine the efficacy of sonication in dislodging attached cells from microtiter well walls. Perform crystal violet assay as in Basic Protocol 1. Lack of a crystal violet ring indicates that the bacterial cells have been removed from the wall of the well.

BASIC PROTOCOL 2

AIR-LIQUID INTERFACE ASSAY

Often, a great deal can be learned about the biofilm formation behavior of a particular bacterium simply by observing the early stages of this process, including attachment and early microcolony formation. This point may be particularly relevant for mutants identified in the 96-well plate assay described above.

The air-liquid interface (ALI) assay provides a simple system for microscopic analysis of biofilm formation over a time range of ~4 to 48 hr (Caiazza and O'Toole, 2004). A 24-well plate is placed at an angle of 30° to 50° relative to horizontal, and stationary-phase cultures are diluted and slowly applied to these wells such that the upper edge of each culture aliquot is positioned at the center of a well's bottom. The bacteria of interest are allowed to grow in this manner for an appropriate length of time, and then the wells are rinsed gently and viewed by phase-contrast microscopy. See Background Information for a more thorough discussion of this assay.

Materials

Bacterial strains of interest
Appropriate media for bacteria under study (*APPENDIX 2C*)
Flat-bottom 24-well plate, sterile, with lid
Large paper clips or microcentrifuge tube rack and lab tape
Inverted microscope

1. Adjust a sterile, flat-bottom, 24-well plate so that it sits at a 30° to 50° angle from horizontal.

   The method used will depend on the brand of plate and the available materials. One approach is to affix four large paperclips to one end of the plate. The paperclips stably elevate the end of the plate to which they are attached, while the opposite end remains low (see Fig. 1B.1.1). Alternatively, one end of the plate may be rested on a microcentrifuge tube rack (or anything of the desired height) and stabilized with tape to prevent slipping.

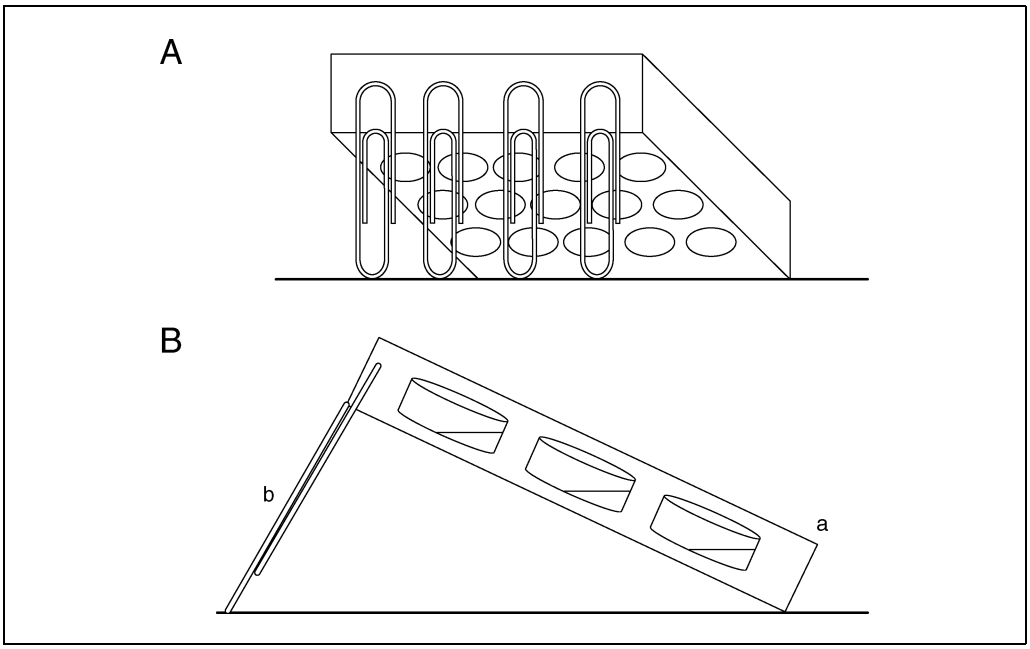

Figure 1B.1.1 (**A**) Schematic of angled plate for air-liquid interface (ALI) assay (back view). (**B**) Side view of ALI assay setup. An angled plate (a) is supported by paper clips (b). The ALI occurs at the center of the bottom of each well, where a meniscus forms.

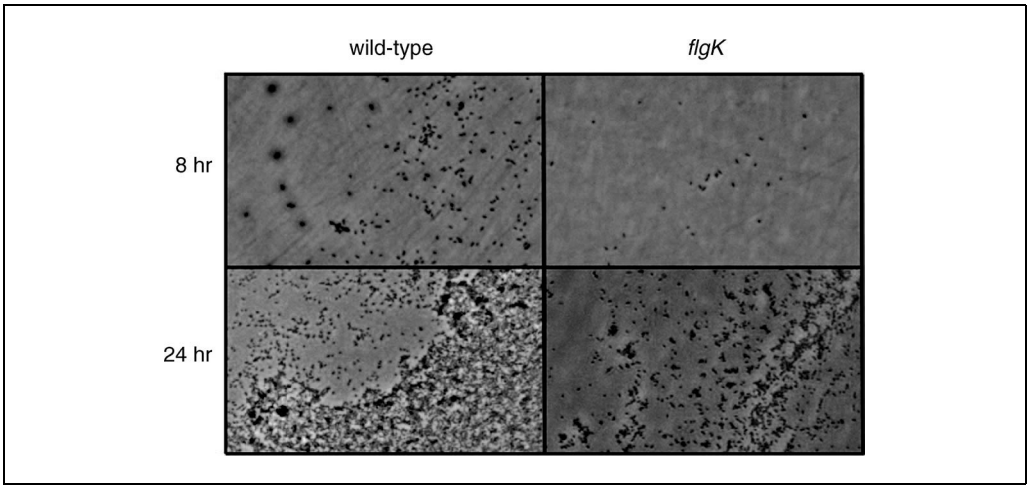

Figure 1B.1.2 Phase-contrast micrographs taken at the air-liquid interfaces of biofilms formed by the wild-type *Pseudomonas aeruginosa* PA14 strain and a nonflagellated mutant (*flgK*), both grown as described in Basic Protocol 2.

If possible, set up the angled plate in the warm room or incubator to be used for sample culturing, and load the wells there (step 4) to avoid potential disruption of the sample meniscuses upon transport.

2. Inoculate each bacterium of interest in a 3- to 5-ml culture and grow to stationary phase (or to some other appropriate stage of growth).

3. Dilute the stationary-phase (or other appropriate) cultures 1:100 in appropriate media.

   Approximately 300 μl diluted culture will be needed for each well that is to be inoculated in the angled 24-well dish.

4. Carefully pipet an aliquot (~300 μl) of each diluted culture into a separate well in the angled 24-well plate such that the upper edge of the aliquot just reaches the center of the bottom of the well. Cover plate with lid and incubate at appropriate temperature for desired period of time.

When inoculating each well, it is important to slowly add the culture down the lower wall to avoid wetting the entire bottom of the well. The meniscus of the liquid should pass through the center of the well, which is often the most optically clear part. This will ensure that the air-liquid interface is appropriately positioned for viewing.

5. Aspirate culture from the wells and gently wash twice, each time by adding 400 µl sterile medium and then aspirating. After completing these two washes, add ~200 µl medium (i.e., enough to cover the bottom of the well) to each well.

   Washing should remove most of the unattached bacteria from the wells. This is important, as planktonic cells can make viewing more difficult and can continue to colonize surfaces, potentially obscuring the air-liquid interface.

6. Lay the plate flat on the stage of an inverted microscope and visualize by phase-contrast or epifluorescent microscopy. Focus just above the center of the bottom of the well to observe bacteria at the air-liquid interface.

   Depending on the bacterial species and conditions, the bacteria may form a well-defined aggregations at the ALI. The ALI will occur wherever the meniscus of the culture was in contact with the plastic, but it will be most visible in the center of the well, where the well tends to be the most optically clear. If desired, the ALI may be circled on the bottom of the plate with a wax pencil prior to microscopic analysis to aid in its identification. Some sample results obtained with Pseudomonas aeruginosa are shown in Figure 1B.1.2.

ALTERNATE PROTOCOL 2

AIR-LIQUID INTERFACE COVERSLIP ASSAY

If an inverted microscope is not available, a plastic or glass coverslip can be placed in a well of a in a 24-well plate such that the air-liquid interface of the culture is in the approximate center of the coverslip. After an appropriate incubation, the coverslip is rinsed, stained with crystal violet, and visualized by conventional microscopy using an upright microscope.

Additional Materials (*also see Basic Protocol 2*)

0.1% (w/v) crystal violet in water
Flat-bottom multiwell (e.g., 12-well) plates, sterile, with lids
Glass or plastic coverslips (e.g., Fisherbrand unbreakable 22-mm^2 coverslips; Fisher Scientific catalog no. 12-547)
Conventional, upright microscope

1. Inoculate each bacterium of interest in a 3- to 5-ml culture and grow to stationary phase (or to some other appropriate stage of growth).

2. Dilute the stationary-phase (or other appropriate) cultures 1:100 in appropriate media.

3. Use each diluted culture to fill a well in a flat-bottom multiwell plate to approximately half of its capacity.

   Almost any size multiwell dish is acceptable for use in this assay, as coverslips are available in a variety of sizes. Plastic coverslips, such as those listed above, can easily be cut to the appropriate size with a pair of scissors.

4. Insert a coverslip into each well so that it is held at a 90° angle relative to the bottom of the well (i.e., perpendicular to the bottom of the well) so that the meniscus of the medium is at the center of the coverslip. Cover plate and incubate at appropriate temperature for desired period of time.

5. Remove each coverslip from its well and rinse off nonadherent cells by dipping in sterile medium.

6. Stain bacteria by submerging coverslips in 0.1% crystal violet for 10 min.

7. Rinse off excess dye by dipping each coverslip in two successive water baths, and then allow coverslips to air-dry.
8. Visualize the bacteria at the air-liquid interface on each coverslip by microscopy.

COLONY BIOFILM ASSAY

BASIC PROTOCOL 3

A colony biofilm is grown on a semipermeable membrane that sits on an agar plate (Fig. 1B.1.3). The bacteria in this type of biofilm can be given a new supply of nutrients simply by relocating the membrane-grown cells to a fresh agar plate. One therefore can easily change the carbon source or type of drug treatment while avoiding the need to wash the cells. The colony biofilm has been especially useful in the study of the antibiotic-resistant properties of cells. The method provided below is an example of the use of a colony biofilm to study the effects of antibiotic treatment.

The other biofilm systems presented in this unit involve attachment of bacteria to a surface while bathed in liquid medium. This is the only protocol where the substratum to which the bacteria attach themselves is also the avenue for meeting nutritional needs and removing waste. For that reason, this assay is more analogous to growing bacteria on plates rather than in broth. In this protocol, however, the colonies can grow much larger, because they are periodically relocated to fresh plates and thus have a fresh supply of nutrients. An advantage of this system is that the biofilms formed are derived from clonal growth of the original population of bacteria deposited on the filter, whereas in liquid media, motile bacteria may be able to "invade" an aggregation of cells. Likewise, in the system described below, bacteria have limited ability to detach and migrate or drift away from the biofilm formed. The stable and spatially restricted nature of this system makes the colony biofilm especially useful for observing the effects of antimicrobial agents, as changes in cell number can be more easily attributed to cell death rather than detachment.

Materials

Bacterial strain of interest
Appropriate liquid medium for bacterial strain under study (*APPENDIX 2C*)
Appropriate agar medium with and without antibiotic (or other) supplementation
PBS (*APPENDIX 2A*), sterile
Forceps, sterilized (e.g., autoclaved or flame-sterilized in 70% v/v ethanol)
Poretics 25-mm-diameter black polycarbonate membranes with pore size of 0.22 μm (GE Osmonics catalog no. K02BP02500)
100-mm-diameter Petri dishes, sterile
UV light source (e.g., UVP 8-W multiple-ray laboratory lamp)
15-ml tubes, sterile, with tightly fitting lids
Vortex mixer

1. On the night before the colony biofilm assay is to be started, inoculate the bacterium of interest in 3- to 5-ml cultures and grow overnight (i.e., to stationary phase).

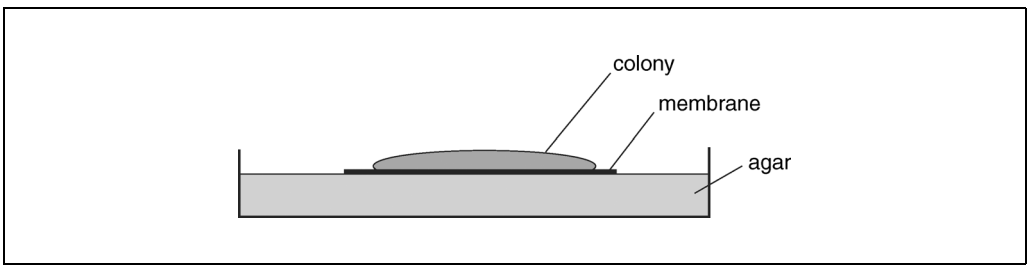

Figure 1B.1.3 Side view of a colony biofilm assay.

2. Using sterilized forceps, place 25-mm black polycarbonate membranes with a pore size of 0.22 μm in a sterile Petri plate. Position plate ~30 cm from a UV light source and treat with UV light in a sterile environment (e.g., a biological control hood) for 10 min per side, using the sterilized forceps to flip the plate.

   Here and in all subsequent steps, the Petri plate should be kept covered whenever possible to maintain the sterility of the membranes.

   The number of membranes needed depends on the desired length of the experiment. Two membranes are required for the initial baseline count of cells (in duplicate), and four more are needed for each time point (for duplicate sampling of treated and untreated cells). Additionally, a few extra membranes may be desirable as backups. For example, starting with sixteen membranes per strain for an experiment with a 72-hr antibiotic treatment period will allow duplicate sampling of treated and untreated cells every 24 hr, leaving two extra backup membranes per strain.

3. Dilute stationary-phase cultures (step 1) in the appropriate medium to an OD_{600} of 0.05 (~1.64×10^8 cfu/ml).

4. Place a membrane (step 2) with its shiny side up on an agar medium plate not containing antibiotic, and inoculate with 5 μl diluted culture. Repeat this procedure for all remaining membranes from step 2, inoculating up to six membranes on the same agar plate. When the liquid on the membranes has dried, incubate plates upright at appropriate temperature for 24 hr.

5. Using sterile forceps, gently lift each membrane off of its agar plate and transfer to a fresh agar plate at up to six membranes per plate. Incubate plates 24 hr at appropriate temperature.

   Flame-sterilized forceps must be cooled before touching the membranes.

   The entire surface of the membrane should be in direct contact with the agar in the fresh plate. Air bubbles may be removed simply by gently lifting the membrane and repositioning it on the plate.

   Following this step, the colony biofilms will have been grown for a total of 48 hr. Membranes are transferred to new plates every 24 hr hereafter to provide a fresh nutrient supply or to expose the colony biofilms to fresh antimicrobial agents.

6. After the incubation in step 5 is complete, sample biofilm growth in the following way.
   a. Aseptically transfer a membrane to each of two separate 15-ml tubes containing 10 ml sterile PBS, such that each tube contains a single membrane.

      The membrane can be gently folded over onto itself before it is lifted off the plate. Doing so will allow the membrane to fit easily into a 15-ml tube.

   b. Cap tubes and vortex samples for two pulses of 60 sec each (or as necessary) to detach all bacteria.
   c. Prepare dilution series of the vortexed samples, and plate each dilution on a separate agar plate. Incubate plates overnight at the appropriate temperature and determine the average number of colony-forming units (cfu) per membrane.

      The counts obtained here will serve as the baseline for future experimentation; this sampling time point is designated $T = 0$ with respect to the start of experimental treatments.

7. Transfer half of the remaining membranes (step 5) to fresh agar plates not containing antibiotic, and transfer the other half to agar plates supplemented with antibiotic at the appropriate concentration. Incubate all plates at appropriate temperature for 24 hr.

8. When incubation is complete, sample biofilm growth (as in step 6) in two untreated membranes and two treated membranes. Transfer the remaining untreated membranes to fresh agar plates not containing antibiotic, and transfer the remaining treated membranes to fresh agar plates supplemented with antibiotic at the appropriate concentration. Incubate plates at appropriate temperature for another 24 hr.
9. Repeat step 8 until all colony biofilms have been sampled.

KADOURI DRIP-FED BIOFILM ASSAY

BASIC PROTOCOL 4

This system overcomes one limitation of the other static systems presented in this unit by constantly refreshing the bacterial growth medium. Bacterial growth can therefore be maintained for a much longer period, allowing formation of mature biofilms. A 6-well plate is inoculated with culture, and growth medium is pumped into each well through a needle that has been inserted through the plate lid; waste and planktonic cells exit through a second needle and are pumped away from the well. Biofilm formation can be monitored as desired by visualizing the bottom of each well using an inverted microscope. See Background Information for a more thorough discussion of this assay.

Materials

Silicone sealant
Sodium hypochlorite
70% (v/v) ethanol
Bacterial strain of interest
Appropriate liquid medium for bacterial strain under study (*APPENDIX 2C*)
20-G needles, sterile (Becton Dickinson catalog no. 305175)
Flat-bottom 6-well plates, sterile, with lids
1/16 × 1/16–in. (~0.16 × 0.16–cm) straight and 90°-elbow, barbed, polypropylene fittings (such as those included in Cole-Parmer catalog no. 6365-90)
0.8- to 1.6-mm-i.d. silicone tubing (e.g., Watson-Marlow catalog no. 913.A008.016 or 913.A016.016)
2-liter flasks
Two-hole rubber stopper (no. 8) equipped with a long glass tube (i.e., one that can reach almost to the bottom of a 2-liter flask) and a short glass tube (for gas exchange)
Peristaltic pump (e.g., Watson-Marlow PumpPro) equipped with Marprene manifold tubing (inner diameter, 0.8 mm; e.g., Watson-Marlowe catalog no. 978.0165.000)
Inverted microscope (*UNIT 2A.1*)

Modify 6-well plate lid

1. Heat a 20-G needle in the flame of a Bunsen burner. Push the heated needle through the lid of a 6-well plate such that it enters directly above one side of a well. Remove the needle and repeat this process to make a hole in the lid directly above the opposite side of the same well.

   The holes should be large enough for the needle to pass through freely.

   These holes will serve as ports for connecting a peristaltic pump that will supply growth medium to and carry waste away from the well. If desired, multiple wells may be prepared on the same plate and/or multiple plates may be run from the same pump simultaneously, depending on the number of channels the pump has for tubing.

2. Obtain two straight 1/16 × 1/16–in. (~0.16 × 0.16–cm) barbed polypropylene fittings (i.e., the type used to connect two pieces of tubing). Using a sharp razor blade, carefully cut off one of the two male ends on each fitting to give a modified fitting with one male end and a flat base (see Fig. 1B.1.4).

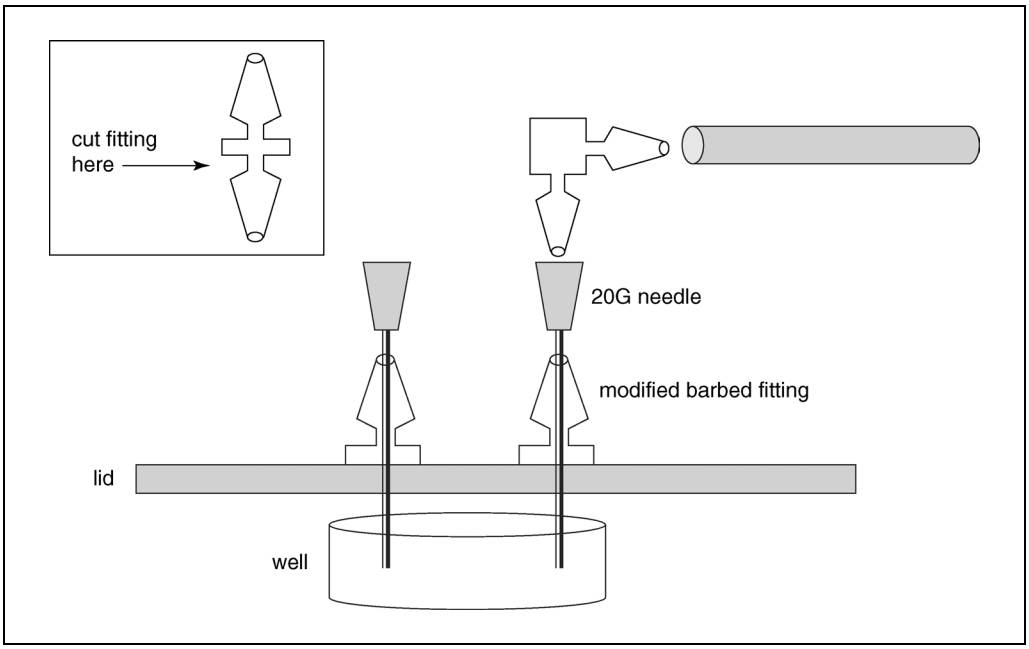

Figure 1B.1.4 Side view of modified 6-well plate lid for Kadouri system.

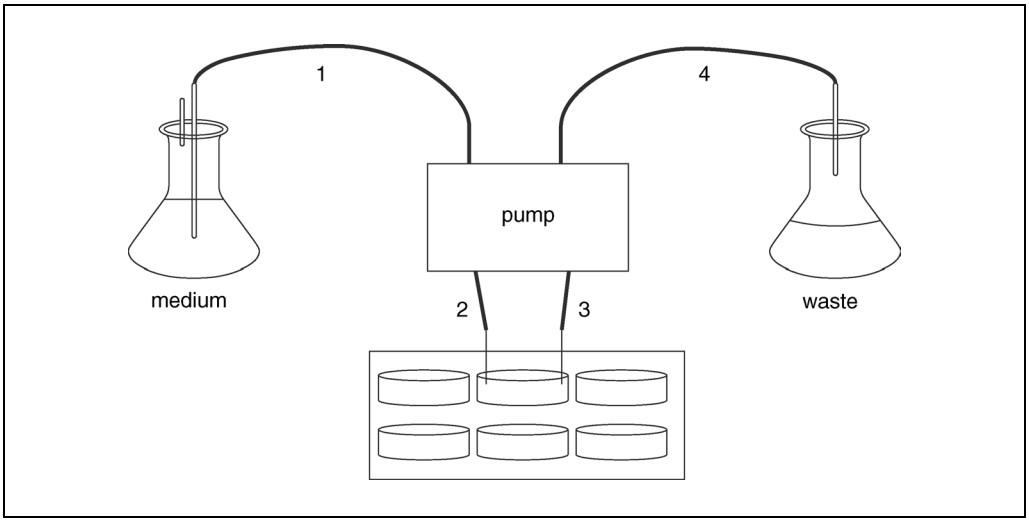

Figure 1B.1.5 Diagram of Kadouri drip-fed biofilm system. Fresh culture medium is pumped onto a biofilm grown in the bottom of a well in a 6-well plate, while planktonic bacteria and spent medium are removed through a needle placed on the opposite side of the well. Numbers are explained in Basic Protocol 4, step 4.

3. Set the flat base of each modified fitting on top of the 6-well plate lid (step 1) such that the hole in the fitting lines up with one of the holes in the lid, and then pass a 20-G needle through both the fitting and the hole in the lid to maintain the alignment. Secure each fitting to the lid of the plate using a silicone sealant, and let the sealant dry completely (see Fig. 1B.1.4).

   Once constructed, this modified lid can be sterilized with ethanol or UV irradiation and reused multiple times with fresh plates in subsequent experiments.

Set up pump apparatus

4. Taking into account the Marprene manifold tubing already installed at the various ports on the peristaltic pump, cut lengths of silicone tubing to sizes appropriate for the following connections (Fig. 1B.1.5): (1) medium reservoir to pump; (2) pump to 6-well plate; (3) 6-well plate back to pump; and (4) pump to waste receptacle. Attach 90°-elbow fittings to the tube ends that are to be connected to the modified plate lid.

Prepare to run the system

5. Fill a 2-liter flask with 500 ml water and fit with a no. 8, two-hole, rubber stopper equipped with one long and one short glass tube. Cover the tube ends with foil. Fill separate 2-liter Erlenmeyer flasks with 1.5 liters water, 500 ml water, and 1.5 liters of medium appropriate for growing the biofilm of interest.

   The 500-ml portion of autoclaved water in the stoppered flask will be used to adjust the liquid flow rate in step 8. The other 500-ml portion of autoclaved water will serve as the solvent for the hypochlorite solution used to sterilize the pumping system in step 10, and the 1.5-liter portion of autoclaved water will be used to rinse the hypochlorite solution out of the system in step 11.

6. Submerge the silicone tubing from step 4, not including the Marprene manifold tubing already installed on the pump, in a beaker containing distilled water and cover with foil. Autoclave along with the flasks prepared in step 5.

7. Connect the plate lid to the medium reservoir line and the waste line as follows (also see Fig. 1B.1.5).
   a. Using polypropylene fittings, connect one of the intake lines on the pump to the silicone tubing that will eventually lead to the medium reservoir. Connect the corresponding outflow line to the end opposite the 90° elbow on one of the silicone tubes that will eventually connect to the modified plate lid.
   b. Connect another outflow line on the pump to the silicone tubing that will eventually lead to the waste receptacle. Connect the corresponding intake line to the end opposite the 90° elbow on the other silicone tube that will eventually connect to the modified plate lid (Fig. 1B.1.5).
   c. Attach a sterile 20-G needle to the elbow fitting on each of the two silicone tubes that are to be connected to the modified plate lid.

8. Adjust the growth medium influx and efflux rates in the following way.
   a. Place a sterile, capped microcentrifuge tube in a rack and make two small, diametrically opposed holes in the lid using a 20-G needle heated over a flame.
   b. Pipet ~1 ml of flow-adjustment water (step 5) into the open tube, close the lid, and then insert the 20-G needles from step 7 through the holes in the lid.
   c. Return the two-hole rubber stopper to the flask containing the flow-adjustment water. Connect the silicone tubing that will eventually serve as the growth medium intake line to the long glass tube on the stopper, and place the open end of the appropriate silicone tube in the waste receptacle.
   d. Start the peristaltic pump at a flow rate of 12 ml/hr (2.0 rpm on a Watson-Marlow PumpPro). Adjust the tension on the manifold tubing such that the water level in the microcentrifuge tube is steady or slightly decreasing.

      The purpose of this step is to adjust the liquid flow such that the well will neither dry out nor overflow during the actual experiment. Increasing the flow rate and observing for a period of time will make subsequent recognition of steady or slightly decreasing water levels easier.

9. Stop the pump, remove the 20-G needles from the microcentrifuge tube, and insert them into the modified fittings on the 6-well plate lid.

10. To 500 ml sterile H_2O (step 5), add sodium hypochlorite to a final concentration of 0.05% (v/v). Remove the two-hole rubber stopper (still connected to the eventual growth medium intake line) from the flask containing the flow-adjustment water, squirt 70% ethanol around its sides, and transfer it to the flask containing the sodium hypochlorite solution. Restart the pump and run the sodium hypochlorite solution through the system for at least 3 hr at a flow rate of 12 ml/hr.

Rinsing with 70% ethanol solution will help ensure sterility each time the rubber stopper is transferred to a new flask. In addition, flushing with sodium hypochlorite sterilizes the pump's Marprene tubing, which cannot be autoclaved.

11. Turn off the pump and aseptically transfer the two-hole rubber stopper (still connected to the eventual growth medium intake line) to the flask containing 1.5 liters of sterile water for rinsing. Restart the pump and flush the system with this water for 3 hr or overnight, adjusting the flow rate as necessary to equalize the inflow and ouflow of liquid.

    This rinsing step removes the sodium hypochlorite from the system and helps ensure that the input and output flow are balanced.

Grow and monitor biofilm

12. Inoculate the bacterium of interest in a 3- to 5-ml culture and incubate overnight at appropriate temperature.

13. Dilute the stationary-phase culture 1:100 in appropriate medium, and inoculate ~3 ml of this diluted culture into the appropriate well of a new 6-well plate (i.e., a well whose position on the plate will allow the bacteria to receive growth medium through the modified plate lid). Allow bacteria to adhere to the well for 3 hr or as appropriate for the organism of interest.

14. At the end of the adhering period, pipet excess medium from the well and replace with ~3 ml fresh medium.

15. Turn off the pump and transfer the two-hole rubber stopper (still connected to the growth medium intake line) to the flask containing 1.5 liters sterilized medium.

16. Transfer the modified plate lid (still connected to the peristaltic pump through two 20-G needles) to the 6-well plate from step 14. Pass medium into the well of interest by restarting the pump at a rate of 10 ml/hr, and maintain this flow throughout biofilm growth.

17. Establish biofilm by incubating the system at appropriate temperature and replenishing the growth medium reservoir flask as necessary.

18. Monitor biofilm growth at desired intervals in the following way.
    a. Pause the pump, transfer the modified lid to another sterile 6-well plate, and place a new sterile lid over the biofilm-containing plate for transport.
    b. Gently remove planktonic cells from the biofilm-containing well by pipetting off the liquid medium, and then wash the well twice, each time by adding 1.0 ml sterile medium and aspirating.
    c. Cover the bottom of the well with a small amount of fresh medium to keep the biomass hydrated, and then view through an inverted microscope.
    d. When viewing is complete, reconnect the plate to the pump system (through the modified plate lid) and resume the flow of growth medium.

Dismantle system and prepare for reuse

19. Upon completion of experiment, detach the modified 6-well plate lid and 20-G needles from the elbow fittings on the silicone tubing, and then use a small (~15-cm) piece of silicone tubing to bridge the two elbow fittings.

20. Sterilize the system by flushing with 0.05% sodium hypochlorite as in step 10, and then rinse by flushing with sterile water as in step 11.

21. Dismantle all tubing and coil it gently into the bottom of a 1-liter beaker. Cover with distilled water, cover beaker with foil, and autoclave to sterilize.

After autoclaving, the tubing may be reused for future experiments; however, before setting up a new system, be sure to examine the entire length of each piece of tubing for clogs or holes that could cause pressure build-ups or leaks.

COMMENTARY

Background Information

Microtiter plate assay

Permutations of the microtiter dish biofilm assay (see Basic Protocol 1) have been in use for a number of years, due to their ease of use and the adaptability of the general protocol to a variety of applications. This assay is equally well suited to the analysis of tens or hundreds of strains or species and is particularly useful as the basis of a genetic screen for mutants defective in surface attachment capabilities. Once conditions have been determined in which the wild-type strain forms a biofilm, a library of mutants can be screened to identify those that differ in terms of the extent or kinetics of attachment. Additionally, slight modifications to this system would allow one to look at the influence of other variables that may affect biofilm formation, such as nutritional provision (e.g., the carbon or nitrogen source used or the availability of phosphate or iron) or the presence of antibiotics or detergents. It is important to keep in mind that due to the indirect nature of biofilm assessment in this system, it is desirable to pair this assay with another method to further examine biofilm formation. For example, using direct microscopic observation of the biofilm to confirm the crystal violet staining pattern is suggested.

Air-liquid interface assay

This technique (see Basic Protocol 2) emerged from a simple adjustment to the typical practice of growing biofilms in multiwell plates. Many bacteria are limited to or prefer aerobic growth and therefore are most prone to forming biofilms at the interface between the medium and air. By tilting the 24-well plate such that the air-liquid interface (ALI) falls on an optically clear portion of the plate, the potential for microscopic analysis is improved. This system makes an excellent companion to the microtiter dish assay (see Basic Protocol 1), as both are useful at many of the same time points and stages of biofilm formation. This assay also allows the visualization of live bacteria without the need for crystal violet staining; therefore, real-time interactions of the bacteria with the surface can be monitored (Caiazza and O'Toole, 2004). The ALI assay can be extended to later time points by replacing the growth medium periodically, but for an accurate study of a mature biofilm, a system in which the medium is continually replaced, such as the Kadouri system (see Basic Protocol 4) or a flow cell–based system (*UNIT 1B.2*), is recommended.

Colony biofilms

Colony biofilms (see Basic Protocol 3) have typically been used for the purpose of determining antibiotic resistance (Anderl et al., 2000; Walters et al., 2003). Such systems are well suited to studies of the viability of cells within a biofilm in response to various assaults, such as exposure to antimicrobial agents or UV light.

Kadouri system

The Kadouri drip-fed biofilm system (named for the postdoctoral researcher who developed it) is a close relative of the continuous-flow biofilm systems presented in other units in this chapter. Although the medium is constantly being exchanged in this system (see Basic Protocol 4), the shear forces on the bacteria are minimal; therefore, this system may apply less mechanical stress to the biofilms. Planktonic cells in this system are not swiftly swept away but may linger in the well for longer periods. Hence, the system may be more closely representative of an equilibrium state between the attached and planktonic lifestyles. This system is typically considered a "low-flow" scheme and serves as an intermediate regimen between static assays and standard flow cell systems; however, because the system involves continuous feeding with growth medium, it allows observation of the entire process of biofilm formation, from reversible to irreversible attachment and on to microcolony formation, macrocolony formation, and detachment. One advantage this system provides is the potential to generate and easily access a relatively large amount of biomass that can be scraped from the bottom of the well and used for applications such as DNA microarrays and proteomics. This system is also useful for many of the microscopy assays typically done by flow cell–based methods. The 6-well plate format also has the advantage of being

easily adaptable to the testing of multiple growth conditions or bacterial species.

Critical Parameters and Troubleshooting

Microtiter plate assay

When using the microtiter plate assay, it is important to have a strong positive control (i.e., a wild-type strain) and, if possible, a negative control (e.g., uninoculated medium) on each test plate. Enough variability can emerge in terms of incubation conditions or washing so that intraplate comparisons are preferable, especially when one strives to observe a subtle difference between bacterial species or strains. Additionally, it is important to note that there are factors that can affect the results of this assay other than an organism's ability to adhere to the surface. For example, a growth defect could easily be mistaken for a lack of attachment capability.

The microtiter plate suggested for the studies described in this protocol is U-shaped, nonsterile, and relatively inexpensive. Nonsterile plates work well with fast-growing organisms such as *E. coli* or pseudomonads, which easily outcompete contaminants in the 8 to 10 hr it takes to complete the biofilm assay. For slow-growing or fastidious organisms, however, it is recommended that sterile plates be used. For nonmotile organisms, such as staphylococci, sterile, flat-bottom plates are recommended.

ALI assay

Although the ALI assay involves a relatively simple system, there are a few important points necessary for successful execution. The ALI should be a well-defined boundary, so care must be taken to avoid wetting the entire bottom of the well during inoculation. Setting up the angled plate in an incubator or warm room and inoculating it in place is a simple way to prevent disturbances to the system. Placement of the air-liquid interface at the center of the bottom of the well will provide the best opportunity for obtaining sharp images by phase-contrast microscopy. In addition, the wells should never be allowed to dry out. If this is a problem, consider adding more medium in the initial setup, decreasing the incubation time, or placing the entire system inside a humidified chamber, which can be improvised by placing some dampened paper towels in the bottom of a plastic container with a lid.

Occasionally, there may be difficulty in locating the air-liquid interface for microscopic examination. There are a number of situations that could lead to this problem. If the initial inoculum has a low cell count and the incubation time is short, there may be an insufficient number of bacteria adhered to the surface at the time of viewing, and this may make identification of the ALI more difficult. If this is the case, consider starting with a less dilute inoculum or increasing the incubation time. Alternatively, circling the ALI with a wax pencil may make it easier to locate under the microscope. Stringent washing of the wells is useful for removing any unattached bacteria; this wash step will help prevent colonization of the formerly dry portion of the well after the whole well has been bathed in medium. Even under the best washing conditions, bacteria can detach from surfaces and colonize other portions of the well. For this reason, it is important to work quickly and analyze plates on a microscope immediately after they have been prepared for observation. If there are many strains or species to analyze, it may be advisable to distribute them over a number of plates so that only a few need be prepared for viewing at one time.

Colony biofilms

One important aspect of this assay is the predetermination of appropriate experimental conditions (e.g., antibiotic concentration). The resistance of a bacterium to antimicrobial agents in a colony biofilm may differ significantly from the resistance of the same bacterium in a different biofilm system, such as the microtiter plate biofilm assay system described earlier in this unit (see Basic Protocol 1). It is recommended that the organism be tested on various concentrations of antibiotic to find appropriate experimental conditions.

The initial inoculation of the colony biofilm is an important step that can affect the success of the experiment. It is important to ensure that each membrane receives an equal amount of cells and that there is only a single point of inoculation in the center of the membrane. To prevent any of the culture from spraying elsewhere on the membrane, touch the drop of liquid at the end of the pipet tip gently to the membrane before depressing the pipet plunger to its final stop. A single, carefully centered spot will ensure the greatest experimental reproducibility, as the counting is no longer accurate if the bacteria have grown off the edges of the membrane. In addition, complete and uniform removal of the bacteria from the membrane is important for accuracy. Empirically testing different conditions to maximize removal of bacteria from the membrane during the vortexing step while preserving viability is recommended.

Kadouri system

Two common problems that are experienced in this system are overflow and drying out of the wells. Both can be addressed by very carefully calibrating the influx and efflux rates (see Basic Protocol 4, step 8). If the medium is evaporating too quickly from the system, consider increasing the influx rate to compensate. Likewise, ensuring that there is sufficient medium in the reservoir is especially crucial when the system will be left unattended for long periods. Due to the nature of biofilm-forming bacteria, it is also important to carefully monitor the tubing prior to each experiment. The waste tubes are especially prone to being colonized, and the colonizing bacteria can completely block the flow, causing pressure to build up and rupture the junctions between pieces of tubing. If blockage is found, the piece of tubing must be trimmed to remove the clogged portion, or else the entire section must be replaced.

This system is designed for observation of biofilms forming on the bottoms of the wells, but depending on the conditions and the strain or species used, the cells may in addition (or instead) form a biofilm, known as a pellicle, at the air-liquid interface. To prevent this, the environment at the bottom of the plate should be made as hospitable as possible for the bacteria. A common problem is the lack of oxygen at the bottom of the well. This may be overcome by increasing the flow rate in the system to deliver more oxygenated medium to the cells while more rapidly removing unattached bacteria. In some cases, adjusting the composition of the medium (e.g., by providing an alternative electron acceptor) will decrease the requirement for oxygen. As with all of the systems presented in this unit, optimal conditions must be determined empirically for each organism.

After the setup is complete, this system requires little daily attention as the cells grow. It should be monitored periodically (twice a day) for overflow or evaporation of the media in the wells and for potential rupture of tubing, and also to ensure that there is an ample supply of medium in the reservoir. However, biofilms may take over a week to develop to the desired stage (as determined by microscopic observation), and this should be taken into consideration when planning experiments.

Anticipated Results

Microtiter plate assay

Using the microtiter plate assay is an effective way to monitor bacterial attachment to an abiotic surface over time. In general, if this assay were performed over several time points, one would expect to see a progressive increase, possibly followed by a decrease, in attached biomass. The eventual decrease in crystal violet staining is presumed to occur because the lack of nutrients may stimulate the bacteria to detach from the surface (Sawyer and Hermanowicz, 1998; Hunt et al., 2004). The time course of biofilm growth must be determined empirically for each organism and set of conditions used. The staining pattern also varies from one organism to another. For example, when *P. aeruginosa* is grown with only oxygen as an electron acceptor, a ring of staining forms at the air-medium interface. In contrast, when this species is grown on arginine, a carbon source used aerobically and anaerobically by *P. aeruginosa*, the entire well shows positive crystal violet staining. Examples of typical results with this assay for *P. aeruginosa* are shown in Figure 1B.1.6.

Direct enumeration of bacteria from microtiter plate assay

When bacteria are grown in the microtiter plate assay, one generally expects to see increased adherent biomass over a period of time followed by a decrease as the bacteria detach or die. The number of cfu eluted from each well is expected to correlate with the amount of crystal violet staining obtained at the corresponding time point.

ALI assay

For bacterial species that form a ring around the well in the microtiter plate assay, the air-liquid interface should be heavily colonized with attached bacteria in this system. For nonmotile or facultative anaerobic bacteria, the boundary may not be as well defined. Some typical results obtained with the bacterium *P. aeruginosa* are displayed in Figure 1B.1.2.

ALI coverslip assay

The results for this variation of the ALI assay should be very similar to those noted above, with the exception that the crystal violet staining kills the bacteria. Because of this, the stained coverslips are relatively stable and may be stored upwards of a week prior to microscopy.

Colony biofilms

The colony biofilm protocol allows assessment of the effects of antibiotics (or other experimental treatments) on a colony of bacteria grown on a solid substratum. Typically, it is expected that bacteria within a biofilm will

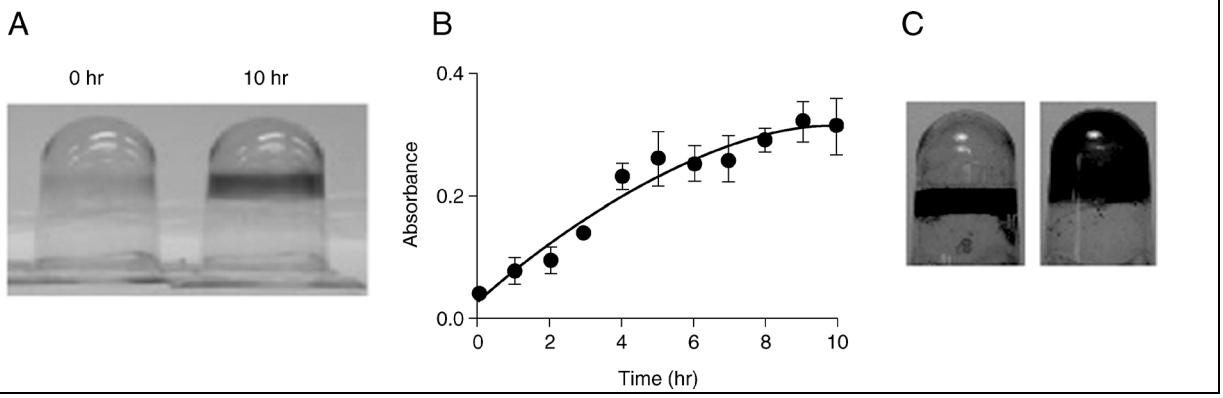

Figure 1B.1.6 Microtiter plate biofilm assay of *Pseudomonas aeruginosa.* (**A**) Crystal violet–stained wells from a microtiter dish at 0 and 10 hr postinoculation. The bacteria were grown on glucose supplemented with casamino acids. Wells were inverted to facilitate photography. (**B**) Quantification of staining at various time points over 10 hr, based on absorbance readings made at 600 nm. (**C**) The wells show formation of a biofilm when *P. aeruginosa* is grown on LB (left; requires aerobic growth) or on arginine (right; can be utilized anaerobically).

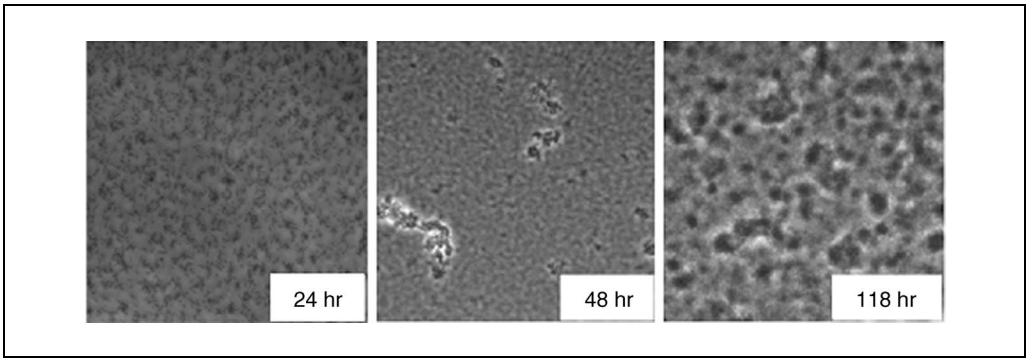

Figure 1B.1.7 Using the Kadouri system, a biofilm of *P. aeruginosa* was grown in minimal medium supplemented with arginine at room temperature and photographed by phase-contrast microscopy at 24, 48, and 118 hr. Microcolonies can be observed as early as 24 hr, with large macrocolonies formed by 118 hr.

display a greater resistance to bacteriocidal agents than will planktonic bacteria.

Kadouri system

Various stages of biofilm formation can be observed with this system. An example of the formation of a *P. aeruginosa* biofilm over 48 hr is shown in Figure 1B.1.7.

Time Considerations

Microtiter plate assay

After the biofilm plates have been inoculated from stationary-phase cultures, results may be obtained in 2 to 30 hr. This, of course, depends on the desired time course and the size of the experiment. Only a small percentage of this time involves hands-on work, however, including the initial inoculation, staining of plates, and dye solubilization. If only a few bacterial strains or species are being tested, these steps may take as little as an hour in total. Alternate Protocol 1, in which viable counts are performed to enumerate the attached bacteria, will take longer, as more handling is involved and the plated bacteria will need to be incubated for another 12 to 24 hr for the colonies to grow large enough to be counted.

ALI assay

The ALI assay follows a time course similar to that of the microtiter plate assay. The length of the assay will again depend on the desired time frame to be studied. Washing of plates followed by microscopic analysis takes ~15 min per well. Alternate Protocol 2 is similar to the ALI assay in terms of its time requirements.

Colony biofilms

During the growth of colony biofilms, the bacteria require little attention, except that the membranes must be transferred to fresh plates every 24 hr. Over the course of an experiment, the separation of bacteria from the membrane and subsequent enumeration are the

most labor-intensive steps. An important consideration regarding the experimental timing in this protocol is the requirement for pregrowth of the colony biofilm (i.e., growth prior to the experimental treatment). This requires membranes to be inoculated 48 hr in advance of antibiotic testing.

Kadouri system

The amount of time required to grow a biofilm in this system depends on the desired maturity, the growth conditions (e.g., medium, temperature), and the organism used. In general, it can be expected that a biofilm will be able to reach maturity within a week of inoculation. Setup of the apparatus should be done 24 hr before inoculation to allow sufficient time for bleaching and rinsing of the system prior to use.

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Growing and Analyzing Biofilms in Flow Cells

This unit describes methods for growing and analyzing biofilms under hydrodynamic conditions in flow cells. The flow in these cells is essentially laminar, which means that the biofilm can be grown under highly controlled conditions and that perturbations such as addition of antibiotics or change of the growth medium can be done efficiently at a defined time point. The Basic Protocol includes inoculation of the flow cells, running of the system, microscopic inspection and imaging of flow cell–grown biofilms, image capture and analysis, and disassembly and cleaning of the flow cells. Support Protocols 1, 2, and 3 describe, respectively, construction of a flow cell system, construction of a bubble trap, and procedures related to fluorescent in situ hybridization (FISH) of species-identifying probes for a biofilm that has been grown in a flow cell.

Confocal laser scanning microscopy (CLSM) is used for monitoring and imaging of structure development in the flow cell–grown biofilms. For a detailed description of CLSM, see *UNIT 2C.1*. The COMSTAT image-analysis program is used for calculation of objective parameters such as surface coverage, biofilm thickness, and biovolume from the acquired biofilm images.

CAUTION: Follow all biosafety requirements relevant to the microorganism under investigation. Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

USE OF FLOW CELLS FOR GROWTH AND ANALYSIS OF BIOFILMS

BASIC PROTOCOL

The flow cells are inoculated by preparing a solution of the microorganisms in a syringe, stopping the medium flow, inserting the syringe needle in the tubing close to the flow cell inlet, carefully injecting the microorganisms, and resuming the medium flow. The flow cell is mounted directly on a microscope for microscopic investigation and imaging of the biofilm.

Materials

Inoculum, e.g., fresh overnight culture of desired microorganisms
70% and 96% (v/v) ethanol
Medium (e.g., see *APPENDIX 2C*)
Silicone glue (3M Super Silicone Sealant Clear)
Flow cell system (BioCentrum-DTU, Technical University of Denmark, or see Support Protocol 1)
Syringes with needles (e.g., Terumo LU-100, 27-G (0.4 × 12 mm), 0.5 ml)
Clamps
Microscope (Zeiss LSM5 META; Chapter 2)
Scalpels
Computer software:
  Imaris (Bitplane; *http://www.bitplane.com*)
  COMSTAT (BioCentrum-DTU, Technical University of Denmark)
  Matlab (required for COMSTAT; MathWorks; *http://www.mathworks.com*)

Contributed by Claus Sternberg and Tim Tolker-Nielsen
Current Protocols in Microbiology (2005) 1B.2.1-1B.2.15

Inoculate the flow cell

1. Load a 0.5-ml sterile syringe with inoculum, typically a dilution of a fresh overnight culture. Stop the medium flow in the flow system. Wipe the flow cell inlet silicone tube with 70% ethanol and penetrate the silicone tubing with the 27-G syringe needle as near to the flow cell as possible.

   Dilution depends on strain and experiment, e.g., for Escherichia coli an OD_{450} of 0.01 is suitable for most experiments.

2. Inject the inoculum carefully into the flow chamber. Wipe the tubing with 70% ethanol after removing the needle. Reseal the tubing by applying a thin layer of silicone glue at the needle hole.

3. After inoculation of all flow channels, clamp the effluent tubes immediately after the flow cell and place the entire flow cell on a flat surface with the substratum downward to allow cell adhesion to the substratum.

4. After 1 hr, remove the clamps, turn the flow cell over, and resume the medium flow.

   The substratum is now on top of the flow cell.

Run the system

5. During the first hours of running, inspect the system periodically for possible leaks, which must be dealt with quickly (see Critical Parameters and Troubleshooting). Ensure uninterrupted supply of medium and adequate disposal of effluent waste during the entire run.

   The system typically runs for several days or weeks.

Perform microscopic inspection and imaging of flow cell–grown biofilms

Microscopy techniques are described in detail in Chapter 2. Only a few comments relevant to microscopy of a biofilm grown in a flow cell with transparent substratum are given here.

6. Mount the flow cell directly on the microscope stage. If the microscope has automatic resetting of the stage, adjust the zero-level by focusing on the substratum.

   CAUTION: *This adjustment is necessary since the flow cell is much thicker than a normal microscope slide and must be accommodated to prevent the microscope focus motor from moving the objective into the flow cell and destroying the sample.*

7. Once the flow cell is on the microscope stage, manipulate the system on site, e.g., by injecting reagents into the flow channels, stopping the flow, changing the flow rate, or switching the medium.

   Take care to avoid air bubbles at this point, since most changes must be performed downstream of the bubble traps.

8. Record images at desired time points or upon manipulation of the system.

   When recording time series or videos, ensure that temperature control is applied both to the environment and to the microscope stage and objective, or focus drift may occur.

Disassemble and clean flow cell

9. At the conclusion of the flow cell experiment, fill the system with air and detach all tubing. Massage and flush with water any tubing with notable cell growth inside (typically effluent tubing).

10. Remove the substratum from the flow cell using a scalpel.

    If the substratum is made of glass, it will inevitably break in the process.

11. Remove any remaining silicone glue using 96% ethanol.

Perform image analysis (optional)

12. Prepare images for presentation using a suitable software package, e.g., Imaris.

    The Imaris software can process CLSM image stacks to create three-dimensional images, or different sections. Furthermore, the software can make virtual fly-throughs and fly-bys where the observer experiences the sample displayed from several angles in an animation. Imaris has also the capability to allow 3-D measurements of the samples.

13. Calculate objective parameters from the acquired images by using suitable software such as the COMSTAT image-analysis program. Parameters include:

    Biovolume
    Area occupied by biomass in each layer
    Thickness distribution and average thickness
    Area distribution of microcolonies at substratum level
    Volumes of microcolonies identified at the substratum level
    Fractal dimension of each microcolony identified at substratum level (Minkovski method)
    Fractal dimension (cross-correlation function method)
    Roughness coefficient
    Distribution of diffusion distances and average diffusion distance
    Surface area (substratum coverage) and surface to volume ratio
    Maximum thickness of the biofilm.

    The COMSTAT program and user instructions are available from http://www.im.dtu.dk/comstat. The parameters obtained from COMSTAT analysis can be paired and plotted in a diagram for ease of interpretation. By inspecting the diagrams, it is possible to compare several samples and determine if a sample is more similar to one group of samples than to another group, e.g., Figure 1B.2.1. For a detailed description of parameters see Heydorn et al. (2000b).

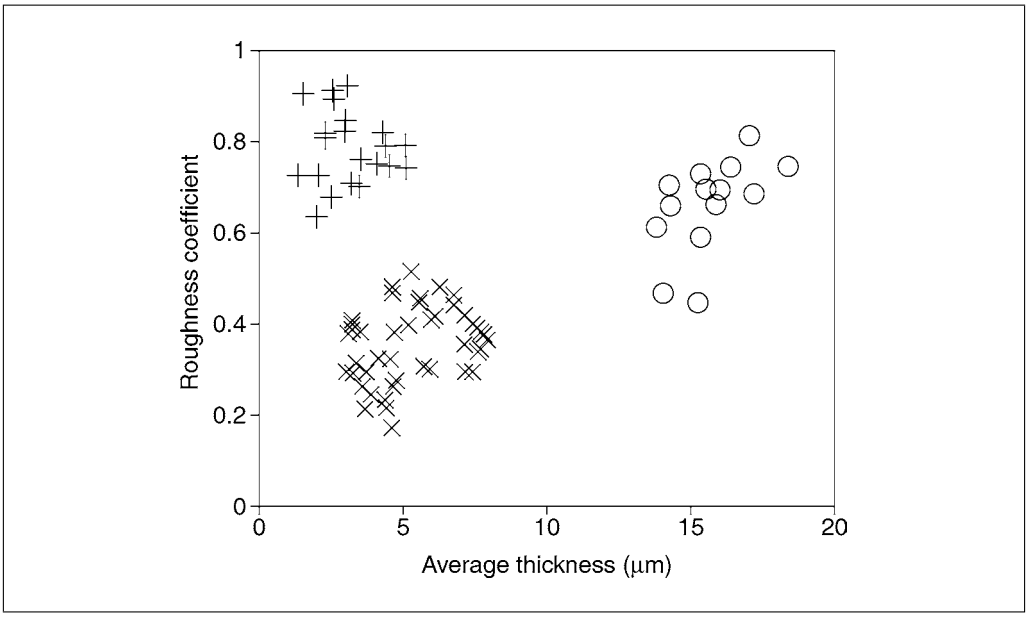

Figure 1B.2.1 COMSTAT analysis of biofilms formed by three different bacterial species. Each point represents roughness and average thickness calculated from one biofilm CLSM image. The symbols ×, +, and ○ represent each of the three species. The two-dimensional analysis of the correlation between the roughness coefficient and average thickness of the biofilms reveals that it is possible to categorize biofilms produced by the three species into three separate groups. Analysis based on either one of the two parameters alone would not indicate a characteristic specific to each of the three species. Combining several of the COMSTAT parameters can refine the analysis further.

SUPPORT PROTOCOL 1

CONSTRUCTION OF A FLOW CELL SYSTEM

The flow cell system consists of a medium bottle, a peristaltic pump, bubble traps, the flow cell, a waste container, tubing, and various connectors. The flow cell described here consists of a plastic base with holes for inlet and outlet tubing and with channels on which coverslips are glued. The bubble trap consists of a syringe mounted on top of a plastic base with an inlet and an outlet (see Support Protocol 2). The system is assembled with tubing and connectors so that the medium can flow from the medium bottle through the peristaltic pump, to the bubble traps, through the flow cell, and to the waste container. The flow system is sterilized with 0.5% sodium hypochlorite, then washed with water, after which the flow of medium can be started. Several suppliers (e.g., BioCentrum-DTU, Technical University of Denmark) offer single- or multiple-use flow cell systems, but many researchers use homemade devices. One particular design modified from a flow cell originally developed by Wolfaardt et al. (1994) is described here.

Materials

Silicone glue (3M Super Silicone Sealant Clear)
Medium appropriate for organisms and type of biofilm being grown (see *APPENDIX 2C*)
70% and 96% (v/v) ethanol
0.5% (w/v) sodium hypochlorite
H_2O, sterile
1% hydrogen peroxide

Polycarbonate sheet plastic, 6 and 35 mm thick
CNC tooling machine, or a drilling machine mounted on an upright stand and equipped with a milling drill-tool (3 mm)
Substratum: 50 × 24–mm glass coverslips or other appropriate material
2-ml syringe (Terumo)
Tubing:
  Silicone, 3 mm outer diameter, 1 mm inner diameter
  Silicone, 4 mm outer diameter, 2 mm inner diameter
  Silicone, 7 mm outer diameter, 5 mm inner diameter
  Marprene, 3 mm outer diameter, 1 mm inner diameter (Watson-Marlow)

Peristaltic Pump (Watson-Marlow, 205S)
Medium bottles (Schott or BIO 101)
Rubber stoppers (to fit medium bottles) with holes for glass tubes
Glass tubes, 6 mm outer diameter, 3 mm inner diameter
Air filter, autoclavable (optional)
Clear polypropylene plastic connectors and T-connectors (Cole Parmer, E-06365-*xx*), 1/8 in. (3.175 mm) and 1/16 in. (1.588 mm)
Bubble traps (BioCentrum-DTU, Technical University of Denmark, or see Support Protocol 2)
Microscope (*UNIT 2A.1*)
Rolling cart for flow systems and pumps (optional)
Waste container

Construct the flow cell

1. Construct the flow cell from a 6-mm thick sheet of polycarbonate according to the working drawing shown in Figure 1B.2.2 using a CNC tooling machine, or a drilling machine mounted on an upright stand and equipped with a milling drill-tool (3 mm).

2. Cut a 6 × 76 × 26–mm flow cell base from a sheet of polycarbonate. Mill 8 mm into the plastic base from each end to remove the top 5 mm.

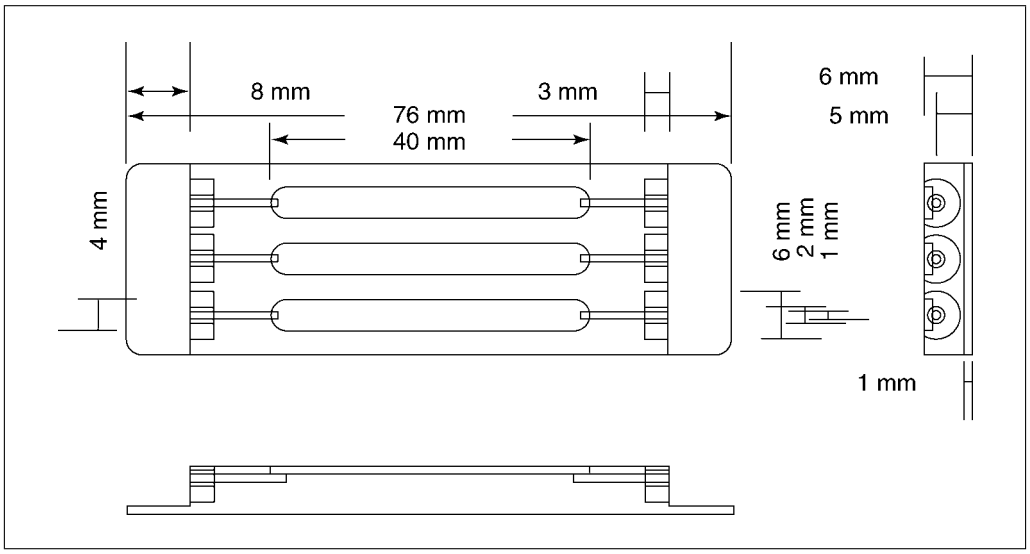

Figure 1B.2.2 Working drawing of the flow cell base (design, copyright Biocentrum-DTU, 2005).

This leaves a central base portion (60 × 26 mm, 6 mm thick) with two 8 × 26–mm ends, each 1 mm thick. The ends facilitate the mounting of the flow cell on a standard microscope specimen holder, like a normal microscope slide.

3. Mill three channels, 1 mm deep and 40 mm long, in the central base portion of the plastic base. If advanced tooling equipment is available, carve connectors to the tubing from the plastic (see Fig. 1B.2.2). Otherwise, still using the milling tool, vertically drill 3-mm wells into the base at each end of the channels and horizontally drill pipes (3-mm drill) from the ends of the central portion of the flow cell, until the pipes connect to the wells.

4. Glue substratum of suitable material and size (e.g., a 50 × 24–mm glass coverslip) to the flow cell base using silicone glue. Apply the silicone glue as very thin threads using a 2-ml syringe. Place the threads between each channel and along the perimeter of the top of the base. Avoid holes in the glue threads to prevent liquid leakage from the flow cell.

5. Place the substratum on top of the glue and press the substratum gently onto the flow cell base. Inspect the glue through the substratum (if transparent) to ensure that the glue is effectively sealing the substratum to the flow cell.

   The handle of the syringe piston is an excellent tool for pressing the substratum onto the flow cell base.

6. If areas with insufficient gluing at the edges are observed, reseal by applying extra glue outside the flow cell adjacent to the potential leak. If the gluing is insufficient between the channels, remove the substratum, clean the base with 96% ethanol, and repeat the mounting procedure (steps 4 and 5).

7. If the flow cell has connectors, attach silicone tubing to them; otherwise, place tube ends inside the drilled holes and attach with silicone glue.

Assemble the flow cell system

The flow system is assembled with autoclaved tubing, bubble traps, and flow cells as shown in Figure 1B.2.3. Use silicone tubing except for the part that goes through the peristaltic pump, where Marprene tubing should be used. Assemble all tubing, except for the connection between the medium supply bottle and the down-stream parts.

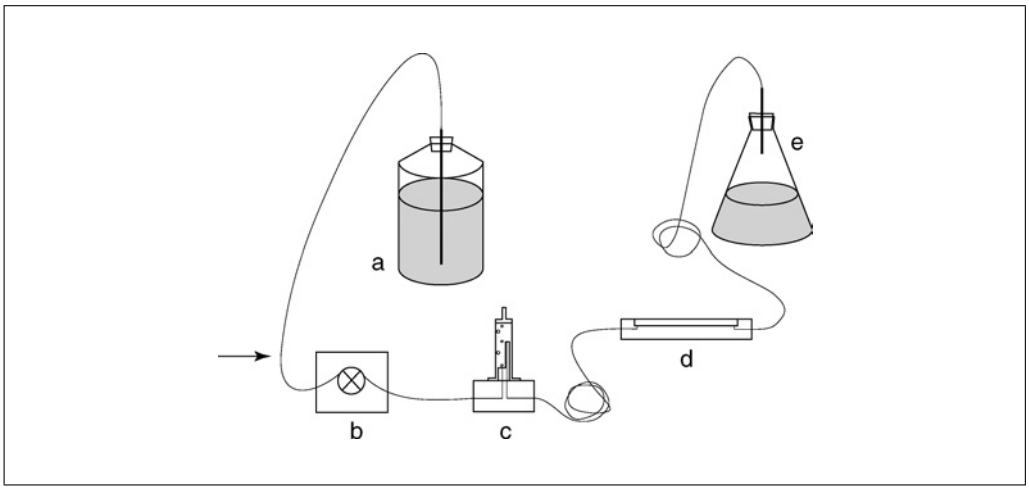

Figure 1B.2.3 Schematic of the flow cell system: a, medium bottle; b, peristaltic pump; c, bubble trap; d, flow cell; e, waste container. Silicone tubing with inner diameter (i.d.) of 2 mm and outer diameter (o.d.) of 4 mm goes from the medium bottle to the point (arrow) where the tube is fanned out. All other tubing has an i.d. of 1 mm and o.d. of 3 mm and is made of silicone, except for the segment that goes through the pump, which consists of Marprene tubing. The connectors used for assembly of the system are listed under Materials in Support Protocol 1.

8. Equip the medium bottle with a rubber stopper of a suitable size with two holes. Place one glass tube, long enough to almost reach the bottom of the medium bottle, into one of the holes (to be used as a siphon tube for medium). Place another glass tube, short enough to not reach into the liquid, into the other hole (to be used as an air vent). Cover the short glass tube with metal foil or mount an air filter via a short connecting plastic tube.

9. Connect the siphon tube to the feed silicone tubing (3 mm i.d., ~1 m long). Mount a straight connector in the end of the feed line to enable easy attachment to the downstream part of the system after autoclaving. Cover this connector with metal foil. Assemble and autoclave the remaining parts of the system together.

   Connect all tubing either directly to the flow cells or bubble traps, or use straight or T-shape connectors between the individual pieces of tubing.

   Do not attach the medium bottle until the system is sterilized.

10. Split a common feed tube into the required number of channels in the experiment by using T-connectors to fan out the feed supply line and reduce the tube dimension from 3- to 1-mm i.d.

11. For each individual channel in the experiment perform the following steps:

a. Connect Marprene tubing to the feed tubing. Connect silicone tubing from the Marprene tubing to the bubble traps.

b. Connect the bubble traps to the flow cell inlet tubing. Connect outlet tubing from the flow cell so that it can reach a waste container.

   Make sure that the inlet and outlet tubes are sufficiently long to allow the flow cell to be moved to the microscope stage.

Sterilize the flow system

12. Cover the free ends of the assembled components with foil and autoclave. Assemble the autoclaved flow cell system as in Figure 1B.2.3 and place the assembled flow cell system near the microscope or, preferably, on a rolling cart. Fill the system with sterile distilled water, and observe for possible leaks. If leaks are present, seal them before using the system.

13. Remove the stoppers from the top of the bubble traps and place them in 70% ethanol. Fill the system with 0.5% sodium hypochlorite solution by pumping at a high flow rate for 5 to 10 min.

    The liquid will pour from the top of the bubble traps; there will be minimal flow downstream from the traps.

14. Place the stoppers back onto the bubble traps and remove any air bubbles from the system components by tapping onto a hard surface while the pump is still running. Continue pumping the disinfectant through the system for 5 to 10 min.

    After replacing the stoppers onto the bubble traps, the remainder of the system will quickly fill with disinfectant.

15. Stop the flow and continue sterilization by allowing the sodium hypochlorite solution to stand in the system from 2 hr to overnight.

16. Remove all traces of sodium hypochlorite by emptying the disinfectant from the system and filling and emptying it with sterile distilled water two to three times. Ensure all disinfectant is removed by purging the system with air between each pass of water. Conclude by passing 1 to 2 liters sterile distilled water through the system at lower flow rate overnight (e.g., 0.2 mm/sec, ~3 ml/hr).

    Emptying and purging the system with air is accomplished by running the pump with the feed tubing taken out of the liquid.

17. Additionally, pass a solution of 1% hydrogen peroxide through the system, as this will eliminate biomass not cleaned by the sodium hypochlorite solution.

    This is mainly for systems that are not autoclaved.

18. Purge the system with air and fill with medium just prior to starting the experiment. Set the laminar flow in the channels to ~0.2 mm/sec (corresponding to 3 ml/hr/channel in the flow cells described here).

CONSTRUCTION OF THE BUBBLE TRAP

SUPPORT PROTOCOL 2

A bubble trap is a simple device basically consisting of a liquid-filled vertical cylinder mounted on top of the medium line. Any air bubble that passes the bubble trap will float to the top of the cylinder and be prevented from passing to the downstream segment. Two types of bubble traps are described here, one that can be made using very simple tools, and an advanced model that requires a CNC tooling machine. Inspect the working drawing shown in Figure 1B.2.4 before constructing the bubble trap.

Materials

10-mm-thick polycarbonate sheet plastic (simple trap) or 35 × 80 × 45–mm polycarbonate block (advanced trap)

Drilling machine mounted on an upright stand and equipped with a 3-mm milling drill-tool and an 8-mm pointed drill (simple trap) or CNC tooling machine (advanced trap)

2-ml syringes with inner diameter of 8.5 mm (simple trap) or 5-ml syringes with inner diameter of 12.5 mm (advanced trap)

9 × 2–mm rubber gaskets (advanced trap; M-seals, 221355; *http://www.m-seals.dk/cms.ashx*)

Silicone glue (3M Super Silicone Sealant Clear)

Silicone tubing, 3 mm outer diameter (simple trap)

Stoppers (e.g., EFD; 5113-B; *http://www.efd-inc.com/catalogs/EFD-Barrel-Reservoirs.pdf*)

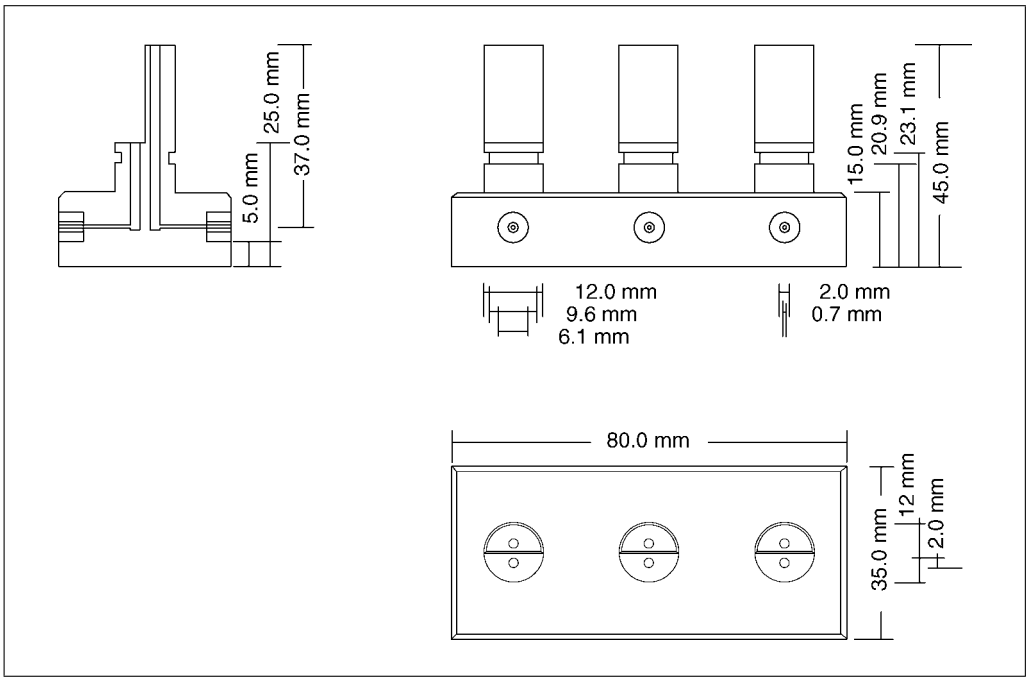

Figure 1B.2.4 Working drawing of the advanced bubble trap base (design copyright Biocentrum-DTU, 2005).

For simple bubble trap

1a. For construction of the simple bubble trap, cut a piece of 10-mm-thick polycarbonate or Plexiglas to 30 × 110 × 10 mm. Drill holes 8 mm into the base using an 8-mm drill. Use a 3-mm drill to create the connecting holes from each side, the center of the drill placed 4 mm from the top of the base.

2a. Glue 2-ml syringes (without the piston) on top of each 8-mm hole. Insert 3-mm-o.d. silicone tubing covered with silicone glue near the ends, 10 mm into the side holes.

3a. Allow the bubble trap to dry at least 24 hr.

Note that this device is somewhat sensitive to physical handling and the syringes can easily break off.

For advanced bubble trap

1b. Cut the plastic base from a single block of polycarbonate using a CNC tooling machine, according to Figure 1B.2.4. Drill holes into the sides and carve connectors directly into the base of the bubble trap in the same way as done for the flow cells (see Support Protocol 1, step 3), eliminating the need for glue when connecting silicone tubing to the base.

2b. Place rubber gaskets on the ring near the top of the three vertical columns.

3b. Press 5-ml syringes (i.d., 12.5 mm; without the piston) onto the vertical columns.

The rubber gaskets seal the trap efficiently and no glue is required.

4. Put a stopper on top of the syringe.

EMBEDDING AND FLUORESCENT IN SITU HYBRIDIZATION OF A FLOW CELL–GROWN BIOFILM

Embedding of a biofilm in acrylamide preserves the three-dimensional structure and allows subsequent FISH for in situ species identification. The embedding procedure presented here describes volumes of reagents specific to flow cells of the dimensions described in Support Protocol 1. Modify the protocol if the dimensions of the flow channels at hand do not match that description.

Materials

Biofilms adhered to the substratum of flow cell system (see Basic Protocol)
Paraformaldehyde fixative (see recipe)
1× phosphate buffered saline (PBS; see recipe)
20% (w/v) acrylamide monomer (200:1 acrylamide:bisacrylamide)
N,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)
1% (w/v) ammonium persulfate (APS) activator, freshly prepared
Fluorescently labeled oligonucleotide probes (e.g., FITC, Cy3, Cy5; e.g., Eurogentec)
FISH prehybridization buffer (see recipe)
FISH hybridization buffer (prehybridization buffer with an appropriate concentration of formamide; see Stahl and Amann, 1991)
FISH washing buffer (see recipe)
Antifade agent (e.g., SlowFade, Molecular Probes)

Clamps
50-ml beaker
2-ml microcentrifuge tubes
Humidified storage container (e.g., Petri dish with wetted paper or cotton)
Microscope slides with 8- to 10-mm diameter wells for FISH (Novakemi AB)
Incubation chamber (50-ml polypropylene centrifuge tube with FISH hybridization buffer–saturated paper toweling)
Microscope slides and coverslips
Rubber gaskets, 0.7 mm thick
Additional reagents and equipment for calibrating oligonucleotide probes (e.g., Stahl and Amann, 1991)

Fix the biofilm

1. Stop the feed pump and clamp off the effluent tubes from the flow chambers. Cut off the inlet tubes so that 3 to 4 cm of inlet tube is left on the flow chamber (i.e., enough to reach the bottom of a 2-ml microcentrifuge tube).

2. Place the pump on the effluent side of the flow cell, and, after mounting the tubes to the pump, gently remove the clamps. Using reverse flow, slowly activate the pump so that a drop of medium appears on the end of the inlet tubes.

3. Put paraformaldehyde fixative in a 50-ml beaker and submerge the inlet tube into the fixative. Draw the fixative into the channels using a flow rate of ~0.2 mm/sec for 15 min. Stop the flow and allow the fixative to work for 1 hr.

   CAUTION: *Paraformaldehyde is carcinogenic. Handle in a ventilated fume hood, wearing suitable gloves (see UNIT 1A.3).*

Embed the biofilm

4. Wash the fixed biofilm for 20 min by pumping 1× PBS through the flow channel at a rate of 0.2 mm/sec.

5. Mix 1 ml 20% acrylamide monomer and 8 µl *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED) in 2-ml microcentrifuge tubes. Immediately before starting the embedding, add 20 µl freshly prepared 1% ammonium persulfate (APS) activator, and invert the reaction tube only once.

   CAUTION: *Acrylamide is carcinogenic and toxic. Handle in a ventilated fume hood, wearing suitable gloves.*

   Because small differences in concentrations, residual oxygen, and ambient temperature can affect the solidification process, test the solidifying time prior to embedding. An optimal solidifying time is 3 to 5 min. If it is shorter, adjust the amount of APS and test again. Because the solidification occurs relatively quickly, it is important to have a working routine, and it is recommended that the technique be tried on empty flow cells before embedding important samples. The acrylamide solution is sufficient for embedding of two 4 mm × 1 mm × 40–mm flow cells. Use the peristaltic pump as described above.

6. Reverse the flow briefly to let a small drop appear on the end of the inlet tubes. Then insert the tubes into the activated acrylamide solution and draw it into the flow channel at a pump speed of 0.5 mm/sec.

   Note that the pump rate is increased to ensure that the chamber is filled with the acrylamide solution quickly before it starts to solidify. In the described system, this takes exactly 1 min, 50 sec. It is recommended that the time it takes to fill the flow cell chamber be measured using an empty channel.

7. Gently clamp off the effluent tubing and remove the pump. Allow to the acrylamide to solidify for at least 1 hr at room temperature.

Prepare the embedded biofilm for FISH

8. Remove the substratum from the flow cell using a scalpel and transfer the solidified acrylamide slab to a humidified storage container. Cut the slab in suitable pieces, 0.5 cm in length.

   Note the orientation of the slabs, since the biofilm is located at the edge that was adjacent to the substratum.

   The solidified sample can be stored in the sealed container for several months at 4°C.

9. Clean used equipment, taking special care to remove solidified acrylamide from the flow channels and the inlets and outlets of the flow cells. Use a dishwashing brush for the channels and a needle for the inlets and outlets.

Perform FISH on embedded biofilm

10. Place the acrylamide slab in a well on a microscope slide with 8- to 10-mm diameter wells, the biofilm side facing downward.

11. Calibrate the fluorescently labeled oligonucleotide probes before use, in order to determine the optimal concentration of formamide in the FISH hybridization buffer (e.g., Stahl and Amann, 1991).

12. Saturate the embedded biofilm with FISH prehybridization buffer by adding 50 µl to each well containing an acrylamide slab and incubating for 30 min at 37°C in a formamide-saturated incubation chamber.

    Make the incubation chamber from a large centrifuge tube (to accommodate a microscope slide), and place a piece of paper towel in the tube, wetted with FISH prehybridization buffer to keep the air saturated with formamide.

13. Meanwhile, add the probe(s) to 30 µl-aliquots of FISH hybridization buffer for a final concentration of ~2.5 µg/ml.

    More than one probe can be used in each hybridization.

14. Remove the FISH prehybridization buffer from the wells containing the slabs, add the hybridization solution containing the probes, and incubate in the formamide-saturated incubation chamber at 37°C, in the dark for 2 hr or longer.

15. After incubation, remove the hybridization solution and wash the slabs as follows:

    a. Add 50 µl FISH prehybridization buffer, and incubate 30 min.

    b. Remove the FISH prehybridization buffer, add 50 µl FISH washing buffer, and incubate 30 min.

    c. Remove the washing buffer, add distilled water, leave for 15 to 30 sec, and remove the water.

    The incubations in the washing steps are also performed in the formamide-saturated incubation chamber at 37°C, in the dark.

16. Mount the slabs on microscope slides with the biofilm facing up. Place a rubber gasket to protect the embedded biofilm from being squashed by the coverslip.

17. Before sealing the sample, add a drop of antifade agent to the slab. Seal the sample with tape and store in a dark cool place until microscopy is performed.

    The mounted samples can be stored for several months at 4°C if kept moist and in the dark.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

FISH prehybridization buffer

x% formamide
0.9% NaCl
0.1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.1% SDS
Store at 4°C for several weeks

The concentration of formamide is determined by calibrating the probes (see Stahl and Amann, 1991).

FISH washing buffer

0.1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.9 M NaCl
Store indefinitely at room temperature

Paraformaldehyde fixative

Add 2.0 g of paraformaldehyde and 10 µl of 10 M NaOH to 33 ml distilled water. Stir at 60°C until all paraformaldehyde is dissolved (~5 min). Next, add 16.5 ml of 3× PBS (see recipe) and cool on ice. Prepare fresh and hold on ice.

CAUTION: *Paraformaldehyde is carcinogenic. Prepare and store this solution in the fume hood.*

Phosphate-buffered saline (PBS), 1× and 3×

390 mM NaCl
30 mM sodium phosphate buffer, pH 7.2 (*APPENDIX 2A*)
If indicated, dilute to 1× before use
Store indefinitely at room temperature

COMMENTARY

Background Information

Studies of the fouling of industrial tubular systems in the early 1980s required reliable and reproducible microscopic observations of biofilm development and encouraged the design of the first flow cells. One of the first flow cells used for biofilm research was the Robbins device (McCoy et al., 1981). This apparatus is constructed of admiralty brass and does not allow direct microscopic inspection but rather has replaceable plugs with sampling surfaces allowing microscopic inspection of relatively undisturbed biofilm. The emerging realization in the mid-1980s of the importance of biofilms in medical context prompted the development of a modified Robbins device which was constructed from an acrylic block, and contained plugs with medically relevant surfaces such as catheter material (Nickel et al., 1985). A need for direct microscopic observation of developing biofilms led to the development of flow cells with glass surfaces mountable on microscopes. One of the first flow cells of this type consisted of two microscope slides, two cover slips, and silicone tubing, and was assembled with silicone adhesive (Caldwell and Lawrence, 1986). Doug Caldwell's group at the University of Saskatchewan subsequently developed the flow cell constructed of Plexiglas and mounted with a microscope slide similar to the flow cell described in detail in this unit (Wolfaardt et al., 1994). These flow cells are well suited for laminar flow conditions, whereas studies of biofilm development under high-shear turbulent flow conditions may use other systems such as square glass tubing flow cells (Stoodley et al., 1999). Biofilm growth in traditional flow cells results in relatively little biomass, but in cases where a large amount of biomass is needed, e.g., for proteome or transcriptome analysis, the biofilms can be grown in silicone tubing (Sauer et al., 2002). To estimate the extent of biofilm development in the silicone tubing, a glass capillary with a square cross-section can be inserted into the tubing during the setup of the system to provide a window for microscopic investigation (Sauer et al., 2002).

Although conventional light microscopy can be used, confocal laser scanning microscopy (CLSM) is particularly well suited for monitoring of three-dimensional structure formation in flow cell–grown biofilms. As a result of its noninvasiveness and nondestructive character, CLSM allows imaging of the three-dimensional structure of microbial biofilms in their naturally hydrated form (Lawrence et al., 1991). The fluorescence required for CLSM can be emitted from the bacteria if they are hybridized with fluorescently labeled nucleic acid probes or are stained with fluorescent dyes. Staining with fluorescently labeled lectins may visualize extracellular substances that surround and interconnect the bacteria in biofilms (e.g., see Neu et al., 2001). In addition, the microorganisms studied in flow cells are often manipulated so that they harbor genes encoding fluorescent proteins to allow analysis by CLSM or epifluorescence microscopy. A description of these tagging techniques is outside the scope of this unit, but useful information on this can be found in Nielsen et al. (2000), Hentzer et al. (2002), and Klausen et al. (2003).

In order to analyze structural biofilm development objectively, it is often necessary to subject the acquired micrographs to image analysis (e.g., see Heydorn et al., 2000a). Korber et al. (1992) devised procedures for determining statistically representative areas suitable for image analysis of the inherent heterogeneous biofilms. The Image Structure Analyzer (ISA) software package, developed at Montana State University, enables the calculation of a number of textural and areal parameters from two-dimensional biofilm images taken by light microscopy or CLSM (Yang et al., 2000). One of the first programs for objective analysis of three-dimensional CLSM biofilm images allows calculation of surface coverage and biovolume (Kuehn et al., 1998). The COMSTAT program, which is described briefly in this unit, enables the calculation of a variety of parameters from three-dimensional CLSM images (Heydorn et al. 2000b). Although the image-analysis procedures are objective, they contain the subjective operation of thresholding, where the operator attempts to find the value of the image grayscale pixels that best represents the distinction between biomass and void space. An image-analysis program with integrated objective threshold selection, enabling fully automated biofilm morphology quantification from CLSM imaging, was developed by Xavier et al. (2003).

Critical Parameters and Troubleshooting

Leakage

The main problem with flow cell experiments is leakage. Even a small leak will render the entire channel useless if not corrected. It is usually very difficult to seal leaks once the system is filled with liquid, which makes it very important to be careful when assembling the system. The primary component vulnerable to leakage is the actual flow cell. If the bubble traps are home-made, using syringes glued to a plastic base, they too can leak.

If the flow cell leaks while mounted on the microscope, it can become critically dangerous to the equipment: either immediately attempt to reseal the leak or remove the flow cell from the microscope. When recording time series, it is particularly important to make sure that no leaks are present, as leaks occurring during the unattended run of time series can cause problems.

If the system is in preparation for an experiment that has not been started, empty the leaking component and dry it. Cover the area of the leak with a layer of silicone glue and allow it to dry for at least a few hours, preferably overnight, before filling the system again. A better method is to have spare components at hand and replace the failing unit with a new one.

While running an experiment, it is not possible to drain the system or even stop the flow temporarily. Clean and dry the area containing the leak as well as possible, and keep a piece of paper towel at hand to repeatedly soak away leaking fluid. Apply a large excess of silicone glue to the leak and continue to remove leaking liquid until it stops.

Air bubbles

Since air bubbles can destroy or alter the three-dimensional structure of biofilms, care must be taken to prevent them from entering the flow cells. Two important measures are taken. First, the system contains bubble traps (see Support Protocol 2) to catch air in the medium supply. Second, the authors recommend not cooling the medium after autoclaving, but rather placing it immediately at the correct temperature for the experiment. If the medium is colder than the ambient temperature of the experiment, air bubbles tend to emerge throughout the system if it is running as the temperature of the medium rises.

Backgrowth

In the standard flow cell setup, there are no special precautions to prevent fouling of the tubing upstream of the flow cells. In most cases there will be little backgrowth, but, if the cells are highly motile, it may occur. Because biofilm in the upstream tubing will use substrate components and release waste products, it may affect the biofilm in the flow cell and result in less controlled conditions: therefore, it should be removed. For the same reasons, microscopic inspection and bioimaging of flow cell–grown biofilms should be done near the inlet. One way to reduce backgrowth is to quickly disconnect the inlet tubing from the flow cell periodically (e.g., once a day), cut off a few centimeters, and reconnect. It may also be necessary to exchange effluent tubes if they become heavily fouled.

Arrangement of the effluent tubes

The effluent tubes must be arranged carefully, to avoid reverse siphoning from the waste container (by placing the end of the tubes above the surface of the waste reservoir), and to avoid accidental entrance of air in case of leaks (by placing the effluent tubes above the level of the flow cells).

Flow stoppage

The biofilms formed by some microbial species are intolerant to interruption of medium flow, and even short periods of flow stoppage will result in detrimental effects, notably complete dispersal of the biofilm. Hence, it is important to plan ahead of the experiment. For instance, if the growth of the biofilms is carried out in a different room than the one to be used for microscopic analysis, electrical power must be available for the pump along the route from one room to another, e.g., by having a very long power cord. Also, when the supply flasks are exchanged, it must be done very quickly, with the pump running. The small amount of air that enters the medium lines at this point is removed by the bubble traps.

Selection

The researcher should keep in mind that flow cell experiments are selection experiments. Biofilms are dynamic systems, and mutants may efficiently out-compete wild-type organisms. Biofilm experiments should therefore always be done in replicates, and biofilm development properties of organisms from

mature biofilms should be retested to verify that the organisms have not undergone irreversible changes during the experiment.

Anticipated Results

Because many different aspects of biofilm biology can be investigated, many different results can be expected. Some of the more general results include: information about biofilm formation steps including adhesion, microcolony formation, and further structural biofilm development; information about structure-function relationships in complex biofilms, including multispecies biofilms; quantitative data to compare biofilms, e.g., those formed by a mutant and its wild-type.

Time Considerations

Assembly and sterilization of the flow cell system will take 1 to 2 days. After that, the system may run for several weeks, but many biofilms reach a mature state within 5 to 7 days. Embedding and FISH labeling can take up to two days, after which the sample can be examined; however, live systems can be observed instantly. Image analysis can take several days.

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Key References

Wolfaardt et al., 1994. See above.

The first paper reporting the use of the flow cell which is described here and widely used.

Lawrence et al., 1991. See above.

The first paper reporting the use of CLSM in biofilm research.

Heydorn et al., 2000b. See above.

A paper describing development and application of the COMSTAT image-analysis program.

Internet Resources

http://www.im.dtu.dk/comstat

Web interface to the COMSTAT program and instructions.

http://www.itqb.unl.pt:1111/clsmip/

Web interface to CLSM biofilm imaging database and image processing application.

http://www.erc.montana.edu/

Contains useful information for the biofilm researcher.

Contributed by Claus Sternberg and
Tim Tolker-Nielsen
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Growing and Analyzing Biofilms in Fermenters

The study of biofilms has stimulated the development of laboratory-based model systems which can be used to recreate various features of natural biofilm communities. Whereas naturally formed biofilms are sufficiently complex that they are not experimentally tractable, model systems can provide controlled conditions and reproducible sample sets. In a biofilm model, questions may be asked and conditions altered so that specific elements of the biofilm structure, physiology, metabolism, and gene expression can be described. Batch culture and flow-cell models are described in *UNITS 1B.1 & 1B.2*, this unit describes two continuous culture laboratory systems, and *UNIT 1B.4* describes the study of natural biofilms in the field. Most biofilm researchers will find that different model systems should be used, or standard methods optimized, to properly address their questions.

In this unit, two models for the growth of biofilms under continuous culture conditions using spinning discs (see Basic Protocol 1) and silicone tubes (see Basic Protocol 2) are described. The spinning disc model consists of a plastic plate with an integrated stir bar and slots for eighteen small glass or plastic chips. The spinning disc is submerged in a reactor flask containing medium and incubated on a stir plate under continuous flow. The silicone tube model consists of a length of silicone tubing through which a medium is supplied under continuous flow. Both model systems provide multiple samples for viable count analyses, and tube-grown biofilm samples may also be used for protein and transcriptional expression profiling and biochemical analysis.

In comparison to the flow cells and static systems, the spinning disc and tube biofilm models have advantages and disadvantages. For example, the biofilms which grow on the chips of a spinning disc reactor are similar in structure and metabolic profile, providing reproducible and statistically clean results. In contrast, more heterogeneous biofilms are generated in flow cells and tube biofilms as nutrient and waste gradients are formed along the length of the reactor and flow characteristics are altered. In static culture biofilms, steep oxygen and nutrient gradients contribute to the formation of extremely heterogeneous biofilms. Tube-grown biofilms are also advantageous in that they can provide a homogeneous biofilm and large amounts of biomass for expression analyses. Generally, spinning disc and tube-grown biofilms are not easily studied microscopically, although tube biofilms may be grown in such a manner that nondestructive microscopic examination is possible. For such studies, flow cell or static culture biofilms are more appropriate.

CAUTION: Follow all biosafety requirements relevant to the microorganism under investigation. Refer to *UNIT 1A.1* and other pertinent resources (see *APPENDIX 1B*) for instructions on safe handling of microorganisms.

BIOFILM CULTURE ON SPINNING DISCS

BASIC PROTOCOL 1

This protocol describes the use of the spinning disc reactor for the study of *Pseudomonas aeruginosa* biofilms. This method was first described by Pitts et al. (2001) for use in the study of toilet bowl biofilms. The method has since been adapted for use with *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Hentzer et al., 2001; Boles et al., 2004; Lee et al., 2004; Lin et al., 2004). As demonstrated by the diversity of these studies, several elements of the protocol, including the substrate materials, may be adapted to the specific requirements of virtually any microbial biofilm former. Whereas the original

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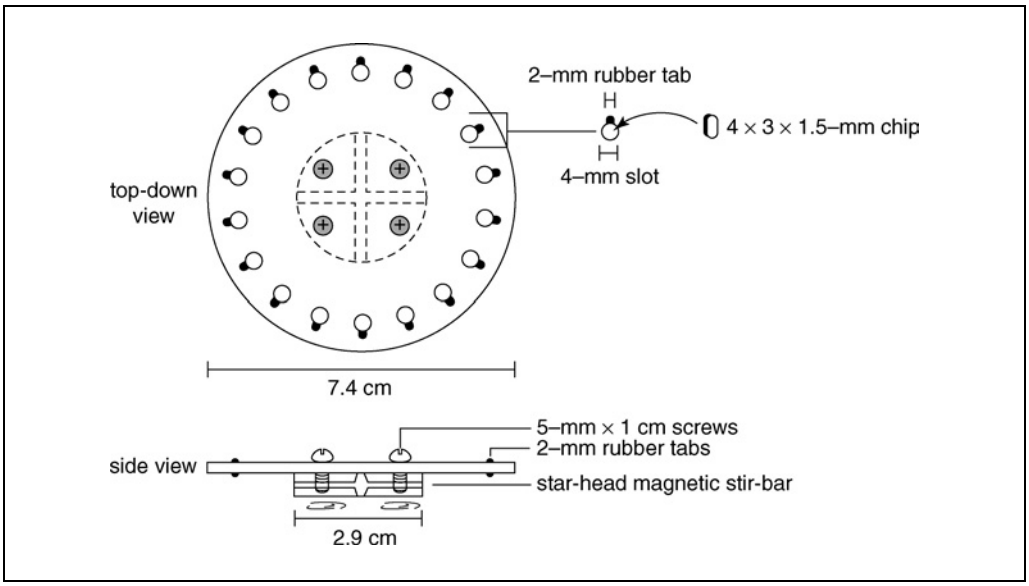

Figure 1B.3.1 The spinning disc is made of polyvinylchloride into which eighteen slots have been drilled, 4-mm wide and 1.5-mm deep. The biofilm substrate (chips) are made of ground glass and placed into the slots and held in place by red silicone rubber tabs. Also visible in the top-down view are the screws that hold the star-head magnetic stir-bar in place to the underside of the disc. The side view shows the placement of the stir bar relative to the chips.

Table 1B.3.1 Timeline for Culturing Biofilms on Spinning Discs

	Action	Steps
Day 1	Assemble and set up medium reservoir and reactor assembly, prepare culture, inoculate biofilm reactor	1–17
Day 2	Culture biofilms (flow phase)	18
Day 3	Test antimicrobial sensitivity	19–27
Day 4	Calculate experimental results	28

protocol utilized porcelain discs, recent adaptations have utilized stainless steel, glass, and plastic discs. The most important piece of equipment in this protocol is the spinning disc. Made of Teflon or polyvinylchloride and silicone rubber, the disc is attached to a magnetic stir bar on the underside, with chips of varying materials on the upper side (see Fig. 1B.3.1). In the protocol described here, the disc is fitted with eighteen glass chips. The biofilm reactor consists of a 1-liter glass beaker with an overflow port. Lastly, an adjustable-speed peristaltic pump is required. Ideally, a multichannel pump is used to allow multiple experiments to be run simultaneously. Because the bacterial culture within the reactor is continually supplied with fresh medium, the culture is maintained in the logarithmic phase, as in a chemostat. The chemostat allows the culture to approach steady-state so that biofilm growth is uncoupled from the planktonic growth phase. Therefore, observed changes in the biofilms will be independent of growth phase phenomena. Chip-grown biofilms are particularly well-suited for antimicrobial susceptibility and viability testing, and will yield highly reproducible results.

Table 1B.3.1 presents a timeline for the procedures involved in this method.

Materials

Silicone glue
Starter culture (mid-log phase bacterial culture)
Rich medium (e.g., LB, TBS, or other; *APPENDIX 2C*)
95% ethanol
Antibiotic solution
PBS or KPBS (*APPENDIX 2A*)
Agar medium plates (for viable counting; see *APPENDIX 2C*)

1-liter glass (e.g., Pyrex) beaker with overflow effluent port (see Fig. 1B.3.2)
Rubber stopper (no. 15; 100-mm top diameter, 81-mm bottom diameter) with four predrilled holes
Glass tubing, 5-mm o.d. or sized to fit holes in stopper, of appropriate length
Flexible silicone tubing, autoclavable: 12-mm o.d./6-mm i.d. and 6-mm o.d./3-mm i.d., and of size appropriate to fit through peristaltic pump (Marprene Manifold tubing from Watson-Marlow, cat. no. 978.0102.000, if Watson-Marlow peristaltic pump is used)
0.22-μm filter cartridges
Small test tube or vial with bottom removed
Medium reservoir and waste bottle: 2-liter, 4-liter, or larger-capacity autoclavable plastic bottles (e.g., Nalgene jugs)
Elbow joint (Cole-Parmer), 0.25-in. (~0.625 cm)
Peristaltic pump (e.g., Watson Marlow; *http://www.watson-marlow.com*)
Incubator with shaker
Aluminum foil
Sandblasted ground-glass biofilm chips
Spinning disc (Fig. 1B.3.1)
Magnetic stir bar
Tube clamps
Stir plate
96-well microtiter plate
Hemostats and forceps, sterile
1.5-ml microcentrifuge tubes
Bath sonicator or tissue homogenizer

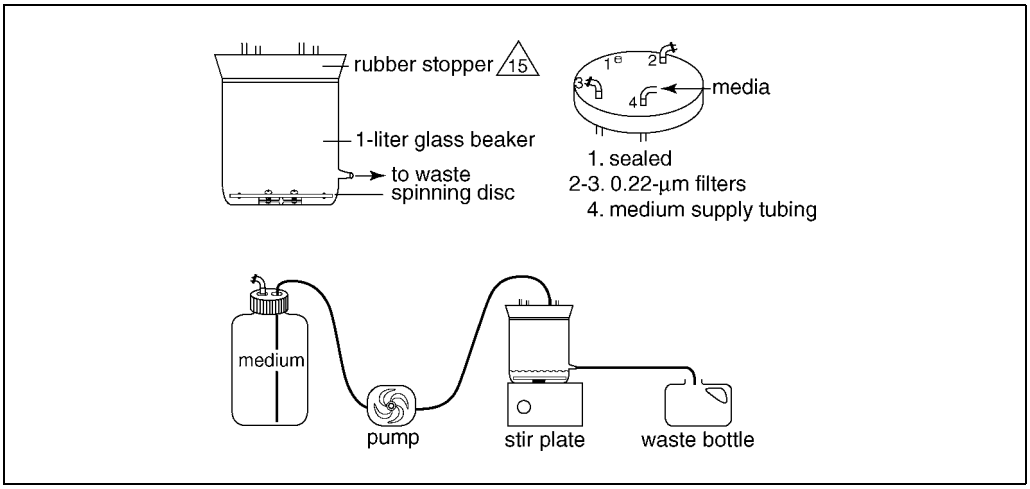

Figure 1B.3.2 The reactor set-up consists of a 1-liter Pyrex jar with an overflow spout and a no. 15 rubber stopper. The ports in the rubber stopper are fitted with (1) a cap, (2-3) filter units, and (4) medium supply tubing, as shown. When fully assembled, medium is pumped from the reservoir (bottom left) through a peristaltic pump and into the reactor. The reactor is placed on a stir plate with the spinning disc inside. Waste medium and culture fluid flows through the overspill spout to the waste bottle.

Assemble reactor

1. Set up the spinning disc biofilm reactor using a 1-liter glass beaker that has an overflow effluent port and a no. 15 rubber stopper with four pre-drilled holes (ports). See Figure 1B.3.2 for a schematic diagram.

   It is best to buy a solid stopper and drill holes in it. Three of the holes should be ∼5 mm in diameter. The fourth port is optional, providing clean access to the planktonic culture during the experiment (e.g., for OD readings); if such access is desired, create an access port sized to fit a small capped vial or test tube.

2. Insert short segments of rigid tubing (e.g., 10-cm lengths of 5-mm i.d. glass tubing) through the small ports in the rubber stopper to provide solid support for ventilation and medium inlets. Using short segments of flexible, autoclavable tubing (silicone, 3-mm i.d.), connect 0.22-μm filter cartridges to two of the inlets to allow for ventilation and prevent contamination. Fit the access port with a small test tube or vial from which the bottom has been removed. Cap the tube/vial.

3. Prepare the medium reservoir as follows.

   a. Create a port for medium delivery by drilling a hole in the lid and fitting it with a segment of 6-mm i.d. silicone tubing that is long enough to reach the bottom of the reservoir and is linked to the exterior of the lid with a 0.25-in. (∼0.625-cm) elbow joint.

   b. Create a port for ventilation in the lid of the medium reservoir by attaching a 0.22-μm filter to the lid using rigid and flexible tubing (as described in step 2 for the stopper of the reactor) *or* create a ventilation port in the shoulder of the medium reservoir by directly inserting the filter, without the use of tubing adapters.

4. To the exterior of the medium outlet, attach silicone tubing long enough to reach from the medium reservoir, through the peristaltic pump, and to the reactor vessel, in three segments connected by barbed mini-tube fittings.

   The first and third segments are of 3-mm i.d. and 6-mm o.d. The middle segment of tubing must be of appropriate size to fit through the peristaltic pump. If a Watson-Marlow pump is used, manifold tubing is available for this purpose.

   The medium reservoir should be sized appropriately with respect to the amount of medium required for the experiment, such as a 2-liter, 4-liter, or larger autoclavable Nalgene jug.

5. Use silicone glue to seal the tubing into the lid of the biofilm reactor and medium reservoir. Cap and seal the fourth port on the stopper.

6. Fit another length of tubing (12-mm o.d./6-mm i.d.) to the outflow port on the side of the 1-liter reactor flask. Use sufficient tubing to reach from the reactor to the waste bottle.

Prepare culture

7. In the morning of day 1, inoculate a 5-ml starter culture from an isolated colony or overnight culture, and incubate with shaking at the appropriate temperature.

   The reactor, growth medium reservoir, and starter culture should be prepared on the same day.

   It is best to use a primary culture, such as an overnight culture or isolated colony grown under antibiotic selection to inoculate the starter culture, as this will ensure culture purity and growth phase when the culture is used later in the day to inoculate the reactor. Using starter cultures of similar age/growth phase also helps ensure consistency between experiments.

Set up medium reservoir and reactor

8. Prepare 4 liters medium, and place it in the medium reservoir (4-liter Nalgene bottle).

   Depending on the planned length of the experiment, it may be necessary to make a much larger volume of medium. Be sure to adjust the autoclave time to accommodate the medium volume used.

9. Replace the lid to the reservoir, with the supply tubing on the interior (tubing must be long enough to rest on the bottom of the reservoir). Use aluminum foil to cover the exterior end of the hard support tubing, as well as the filter units.

10. Wash eighteen biofilm chips one time with 95% ethanol and three times with distilled water, then place the chips into the slots of the spinning disc.

    Neither the discs nor the chips are commercially available to the specifications described in Fig. 1B.3.1. They can be easily constructed or fabricated by a university machine/instrument shop. Alternatively, a similar reactor can be purchased from Biosurface Technologies.

11. Place the spinning disc into the reactor, making sure that the chip side faces up and the stir-bar side is on the bottom.

    The spinning disc may be autoclaved separately, wrapped in aluminum foil. It is best to do this on the dry setting, but if the liquid setting is used, place the foil-wrapped disc in an empty pipet tip box to keep it dry. Use extra caution when assembling the reactor if this alternative method is chosen. An experimental timeline for this alternative technique is described below.

12. Fill the reactor with ~250 ml water and cover with the tube-fitted rubber stopper.

13. Place tube clamps on every tube to avoid backflow and contamination. Cover the ends of the tubing and the filter units with aluminum foil.

14. Autoclave the reactor assembly and medium reservoir on the liquid setting.

Inoculate the biofilm reactor—batch phase

15. Replace the water in the reactor with ~250 ml sterile medium.

16. At the end of day 1, inoculate the reactor with 1 ml of the 5-ml starter culture started that morning (step 7).

17. Stir the reactor at 250 rpm on a stir plate and incubate overnight at room temperature.

Start biofilm culture—flow phase

18. In the morning of day 2, connect the inflow tubing to the supply port of the medium reservoir, fitting it through the peristaltic pump, and remove all of the clamps to allow flow through the entire system. Then, start the pump in a clockwise direction.

    Remember to remove the clamp from the effluent tubing to permit flow through the system.

    Pump settings vary by model. Set the pump so that the flow rate is ~0.5 ml/hr.

    Flow can also be altered to determine the specific growth rate of the test strain.

Test antimicrobial sensitivity

19. In a 96-well microtiter plate, add 100 µl medium to the wells in rows B through H for the first four columns.

    During the test, the cells should not double. Therefore, if a rich medium is used in the reactor, use 1× PBS or KPBS as the diluent in the reactor plate.

20. In row A, add the desired amount of antibiotic solution to the medium for a total volume of 200 µl. Mix the wells in row A and transfer 100 µl from row A to row B. Continue the serial dilution down the column to row G; transfer 100 µl from row G to waste, leaving row H with only 100 µl medium.

    For example, to achieve a final concentration of 1 mM for a given antimicrobial agent, in row A, add 20 µl of 10 mM stock solution to 180 µl medium.

    Modify the concentrations and the dilution schemes for specific experimental needs. Maintain the 200-µl volume for convenience and ease of use in the microtiter plate.

21. Twenty four hours after starting the flow phase of the reactor culture (day 3), stop the reactor and take a 1-ml sample of the planktonic culture and set aside on ice (source of samples for susceptibility testing in parallel with the biofilm samples that will be harvested). Measure the optical density (OD_{600}) of this sample and/or perform a viable count to determine the planktonic culture density.

    It may be necessary to dilute this sample. To ensure comparable results, expose the same number of planktonic and biofilm cells to the antimicrobial stress in the microtiter plate.

22. Remove the disc by reaching into the reactor and pulling it out without touching the biofilm chips. Remove the chips from the disc with sterile forceps and dip each one into fresh medium to remove any loosely attached cells. Place one chip into each well in columns 1 and 2 of the 96-well microtiter plate, and 10 µl planktonic culture into the wells of columns 3 and 4. Mix well.

23. Incubate the plate 5 hr at 37°C.

    Temperature and incubation time may vary depending on the organism used and the experiment at hand.

24. Transfer the chips into 1.5-ml microcentrifuge tubes containing 1 ml of 1× PBS or KPBS. Likewise, transfer 100 µl of the planktonic cultures into 1.5-ml microcentrifuge tubes containing 900 µl of 1× PBS or KPBS.

25. Sonicate the tubes for 10 min, and then briefly vortex.

    Alternatively, a homogenizer can be used to disperse the cells. Place the chips, cells, and PBS or KPBS solution in sterile, capped tubes.

26. Serially dilute the cells from each tube by taking 10 µl cells and mixing with 90 µl PBS or KPBS. Spot 10 µl of each dilution in triplicate onto pre-dried agar plates of appropriate medium.

27. Incubate the plates overnight at 37°C.

Calculate experimental results

28. On the following day (day 4), remove plates from the incubator and count colonies.

29. Clean the entire apparatus with mild soap and water, rinse in distilled water, and allow to dry. Replace the 0.22-µm filters only if they have become soiled or wet. After washing, store the chips in a petri dish until needed; wash in ethanol before reuse.

    There is no need to completely disassemble the tubing. Replace individual segments as needed, should they become dirty or clogged after multiple cleaning/autoclaving cycles.

CONTINUOUS FLOW CULTURE INOCULATION OF SPINNING DISCS

ALTERNATE PROTOCOL 1

The execution of this modified technique is nearly identical to that described in Basic Protocol 1. The critical difference is the residence time of the spinning disc reactor in culture. Whereas Basic Protocol 1 allows for substrate adherence during the batch culture phase, this alternative method begins the biofilm culture during the continuous flow (chemostat) phase, when the planktonic culture has reached near steady-state.

The reservoir and reactor are assembled and sterilized as described in Basic Protocol 1, with one exception; a stir bar is placed in the reactor before autoclaving, and the spinning disc is wrapped in an envelope of aluminum foil and autoclaved separately.

As described in Basic Protocol 1, steps 16 to 18, the reactor is inoculated and batch culture incubation is begun. In this case, however, the culture is simply stirred with a stir bar. On the morning of day 2, start the chemostat phase of the culture by attaching the medium reservoir to the reactor, unclamping the tubing, and starting the pump. Allow the chemostat to run for the day, and in the evening begin the biofilm culture. To do this, aseptically remove the stir bar from the reactor, and replace it with the spinning disc. This must be done very carefully, taking care not to touch the spinning disc as the foil envelope is peeled away. The disc should be gently dropped into the tilted reactor, so that the biofilm chips face upward. Replace the reactor on the stir plate, begin stirring and restart the flow.

IMPORTANT NOTE: Remember to clamp the influent and effluent tubing whenever the pump is stopped; this will prevent backflow in the system. Do not forget to unclamp the tubing when restarting the pump.

Perform biofilm sampling and testing as described in Basic Protocol 1, steps 19 to 21.

BIOFILM CULTURE IN TUBES

BASIC PROTOCOL 2

Tube reactors are a commonly used model system for the study of biofilms, particularly those of clinical relevance. Biofilms may be grown in tubing of varying composition and diameter and under varying flow rates. Medium composition may reflect clinical situations, such as artificial urine or saliva, or may be comprised of standard laboratory media (*APPENDIX 2C*). The effects of shear stress can be tested by varying the flow rate of the supplied medium. The tube biofilm model provides both biofilm and planktonic samples that may be used for viability and antibiotic susceptibility testing, but more complex tests may also be applied. In its most basic form, a tube biofilm model experiment is simple to perform and requires a minimum of special equipment—a little more than tubing, medium, and a peristaltic pump. The biofilm material may be used for antimicrobial susceptibility and viability testing, and can also provide sample material for expression analyses, including both β-galactosidase-based and microarray analyses. The protocol described here offers numerous opportunities for adaptation and specialization.

Materials

30% (v/v) hydrogen peroxide (H_2O_2)
1:8 diluted LB or other rich medium (*APPENDIX 2C*)
Mid-log phase bacterial culture
95% ethanol
PBS or KPBS (*APPENDIX 2A*)
Petri plates containing appropriate solid medium
30% H_2O_2

Medium reservoir (see Basic Protocol 1)
Silicone tubing (2- to 3-mm i.d.)

Mini tube fittings, barbed (Cole-Parmer)
Multichannel peristaltic pump (e.g. Watson-Marlow)
Marprene Manifold tubing from Watson-Marlow, cat. no. 978.0102.000, if Watson-Marlow peristaltic pump is used (or other size silicone tubing compatible with peristaltic pump used)
Bubble traps (*UNIT 1B.2*; optional)
Aluminum foil
Tube clamps
Incubator with shaker
Spectrophotometer
1-ml syringe with a 12-G needle attached
Petri dishes
Razor or scalpel blades
1.5-ml microcentrifuge tubes
50-ml conical tubes (optional)
Bath sonicator (optional)
96-well microtiter plate

Assemble reactor tube

1. Set up a medium reservoir in the same manner as described in Basic Protocol 1, steps 3 and 5.

   The medium reservoir can be as small or as large as needed for each particular experiment.

   The reactor may be assembled and autoclaved the day before beginning the experiment, but it is best to perform the system-cleansing flushes (with peroxide and sterile water) on the same day as the inoculation.

2. Set up parallel silicone tubing for the desired number of experiments (usually eight or twelve, depending on the number of available channels on the pump in use).

   The tubing should be long enough to accommodate the distance from the medium reservoir to the peristaltic pump. The tubing that passes through the pump must be compatible with the pump mechanism.

3. Cut 3- or 8-in. pieces of silicone tubing (2- to 3-mm i.d.) in which the biofilms will be grown.

   The length and inner diameter of tubing used depends on the experimental goals. These lengths are simply recommendations, but the i.d. of the tubing can be ≥12 mm. Remember that changes in tube diameter will change the flow dynamics: see Reynold's equation for determining turbulent flow (step 16).

4. From the medium reservoir, run a piece of silicone tubing leading to the eight parallel pump tubes. Connect the pump tubing with barbed mini tube fittings and segments of silicone tubing to the bubble traps, which are directly connected to the biofilm tubing. Connect the biofilm tubing to another length of tubing that leads to the waste bottle.

   If preferred, use self-sealing septa placed just upstream of the biofilm tubing. The septa can be used for inoculation of the system, but it is equally straightforward to inoculate directly into the tubing (see steps 15 and 16).

   This experiment can be performed without the use of bubble traps. Be certain to seal all joints completely to avoid the introduction of air into the system.

5. Wrap loose tubing ends in aluminum foil and autoclave the entire system.

6. Prepare 500 ml of 0.6% hydrogen peroxide (10 ml of 30% H_2O_2/500 ml).

   Prepare hydrogen peroxide fresh before every use. Hydrogen peroxide is a strong oxidant, and will degrade over time, especially when exposed to light.

7. Flush the system with the 0.6% hydrogen peroxide solution for 2 hr at a pump setting of 14 rpm. Be sure to fill the bubble traps, if used.

8. Using aseptic technique, switch from 0.6% hydrogen peroxide to sterile water, and flush the system for an additional 2 hr at a pump setting of 14 rpm. Be sure to fill the bubble traps, if used.

9. Again using aseptic technique, switch from sterile water to sterile medium. Flush the system for 2 hr at a pump setting of 14 rpm, making sure to fill the bubble traps, if used.

10. Stop the flow and clamp the tubing just upstream of the inoculation site.

Prepare inoculum

11. Prepare a 5-ml starter culture as described in Basic Protocol 1, step 7. Incubate with shaking at 37°C to a culture OD_{600} of 0.1.

    At this point, it is ready to be used as an inoculum in the tube reactor.

    The medium used for this inoculum culture should be the same as that to be used in the biofilm reactor.

Inoculate biofilm reactor

12. Thoroughly clean the inoculation site with 95% ethanol.

13. Using a 1-ml syringe with a 12-G needle attached, inoculate each tube with the culture at an OD_{600} of 0.1 (step 11). For a 3-in. long biofilm tubing, inoculate with a 0.5-ml culture volume; for an 8-in. long biofilm tubing, inoculate with 1 ml culture.

    IMPORTANT NOTE: Do not inject air into the system.

    Use caution in handling the needle and syringe to avoid injury, and dispose of used needles in properly labeled sharps containers.

    Make sure that the inoculum culture and the medium in the tubing are at the same temperature and that the system remains at experimental temperature for the remainder of the experiment.

    To maintain consistency between experiments, ensure that the inocula are identical. They should each be at OD_{600} 0.1. If the OD of the inoculum is > 0.1, dilute the culture with sterile LB, using the following relation:

$$(OD_{current})(volume_{culture}) = 0.1 \times 10 \text{ ml}$$

Equation 1B.3.1

$$volume_{fresh\ LB} = 10 \text{ ml} - volume_{culture}$$

Equation 1B.3.2

14. Inoculate samples as quickly as possible, and allow the incubated tubing to sit undisturbed for 30 min at experimental temperature.

15. Adjust the rpm setting on the peristaltic pump before unclamping the tubes and starting the flow.

Some researchers suggest that the biofilm tubing should be incubated in a vertical orientation to prevent settling over the duration of the experiment. This may be of particular importance when using a low flow rate.

16. Check the flow rate after initiating the experiment, as well as before each sample harvest, by collecting the effluent in a graduated cylinder for a predetermined amount of time, then calculating the flow rate as milliliters per hour.

Although this step is not critical, it may serve as a useful gauge of the biofilm conditions, particularly at later time points.

Calculate the flow characteristics of the system (laminar, transitional, or turbulent) using the following Reynold's equation:

$$R = \rho VD / \mu$$

Equation 1B.3.3

where ρ is the fluid density, V is the fluid velocity, D is the tube diameter, and μ is the fluid viscosity. Flow is laminar at R values up to 2000. At R values >4000, the flow is turbulent. R values between 2000 and 4000 are considered to reflect transitional flow, where regions of both turbulent and laminar currents may be found.

17. When harvesting tube samples, place the end of the tubing from the bubble trap (the inflow tubing) into a petri dish containing 95% ethanol (this will help maintain sterility in the system).

Harvest tube biofilms

18. Remove the biofilm tubing from the flow path. Cut off the sections of tubing where the connectors attach. Measure the length of tubing that remains.

Steps 19 to 25 comprise a stringent method for biofilm harvesting. A more simple technique involves clamping the biofilm tubing at either end, then manually massaging the portion of tubing between the clamps. This will dislodge the biofilm from the tube walls. To remove the cells from the tubing, unclamp one end of the tube and inject 1 ml of 1× PBS or KPBS buffer through the other end. Collect the effluent cell suspension in a test tube and proceed with the experiment as described, starting from step 26.

19. Cut the tubing into two or three pieces using a sterile scalpel or razor blade. Wipe the outside of the tube with 95% ethanol, and let evaporate to dryness.

20. If using a 3-in. piece of biofilm tubing, place the cut and surface-sterilized pieces into a sterile 1.5-ml microcentrifuge tube. For an 8-in. piece of biofilm tubing, place the pieces into a sterile 50-ml conical tube. Add 800 µl of 1× PBS or KPBS.

21. Sonicate for 1 min and incubate on ice for an additional 1 min. Vortex vigorously for 1 min. Repeat this step for a total of four times.

22. Remove the biofilm tube pieces, and place them in a fresh sterile tube.

    a. If using a 1.5-ml microcentrifuge tube, centrifuge 5 min at 7500 × *g*, 4°C, to remove residual liquid or cells from the silicone tubing.

    b. If using a 50-ml conical tube, centrifuge 5 min at <7000 × *g*, 4°C, to remove residual liquid or cells from the silicone tubing.

    After centrifugation, carefully remove the biofilm tubing. Use a pipet to transfer the liquid to the tubes in step 20.

23. If the samples are for an early timepoint, combine multiple biofilm tubes into a single sample.

    This is useful if enough biomass is needed to perform OD measurements.

24. Thoroughly vortex the tube containing the cells for 1 min. Then sonicate, chill on ice, and vortex, 1 min each, for a total of two times. Make sure the culture appears homogeneous. If clumps are still visible, repeat the sonicate/chill/vortex cycle until the culture looks homogeneous.

25. Record the total liquid volume of this tube.

Analyze samples

26. Remove 10 µl of the cell suspension for cfu enumeration.

27. Prepare a 96-well microtiter plate with 90 µl of 1× PBS or KPBS in each well (eight wells/row/sample). Add the 10 µl from step 26 to the first well, and serially dilute the cells in a range from 10^{-1} to 10^{-8}.

    Fewer dilutions may be required at early time points, when the biomass coverage is low. Until the behavior of the bacterium is characterized, however, it may be best to use a broad range.

28. Spot 10 µl of each dilution, in triplicate, onto the appropriate solid medium. Incubate the plates upside down overnight at 37°C.

    Allow the spotted plates to dry before turning them over.

    Using a small volume and multiple dilutions in this manner allows for viable count assays to be performed very quickly and uses far fewer plates than the traditional viable count assay, in which larger volumes are spread, i.e., one dilution per plate. Typically, up to six 10-µl spots can fit on a standard petri plate.

    Although LB medium has been referenced throughout these protocols, choose a medium according to the needs of the organism and experimental design.

29. On the following day, count colony forming units (CFUs) for each dilution, where possible.

    For the limited spot size, ~20 organisms is an ideal number.

30. Calculate the number of organisms per tube surface area. Average the triplicate colony counts (*N*) for the dilution to be used:

$$\frac{N \times \mathrm{vol_{total}(\mu l)}}{\mathrm{vol_{spot}(\mu l)} \times \mathrm{dilution}} = \frac{\mathrm{no.\ of\ cells}}{\mathrm{SA\ (mm^2)}}$$

Equation 1B.3.4

    where $\mathrm{vol_{total}}$ is the total harvest volume (usually ≥1000 µl), $\mathrm{vol_{spot}}$ is the volume of the dilution that is plated onto the solidified medium (usually 10 µl), dilution refers to how diluted this sample is relative to the cell harvest (e.g., 10^{-5}), and SA refers to the surface area of the biofilm tube in square millimeters (this can be calculated from the tube diameter and length, recorded in step 20).

31. At the very end of the experiment, run fresh hydrogen peroxide solution through all of the tubing and bubble traps. Thoroughly wash the bubble traps and tubing with mild soap and water. Rinse thoroughly with distilled water before autoclaving.

COMMENTARY

Background Information

Spinning disc

The biofilm culture method detailed here has been used previously, with minor adaptations, to study the effects of various antibiotics and genetic mutations on biofilm formation, and on survival of biofilm populations (Pitts et al., 2001; Teitzel and Parsek, 2003; Lee et al., 2004). It is commonly referred to in the literature as a rotating disc reactor, but is not to be confused with the Rototorque and the rotating annular reactor (Characklis, 1990; Lawrence et al., 2000). The first iteration of the spinning disc reactor model was developed to study biofilm formation on ceramic surfaces under intermittent flow conditions, as found in toilets (Pitts et al., 2001). Biofilm growth and susceptibility to chlorine were studied using a rotating Teflon disc carrying six ceramic chips that was placed in an open-air reactor through which alternate flushes of medium and tap water were passed. The method was found to yield highly reproducible and statistically sound results, making it an excellent candidate for adaptation to other applications.

The Pitts protocol was adapted to the study of *Pseudomonas aeruginosa* to test the efficacy of photodynamic antimicrobial chemotherapy (PACT; Lee et al., 2004). The Teflon rotor in this case was fitted with 24 stainless steel chips and biofilms were incubated under both batch and flow conditions before samples were removed and subjected to treatment with photosensitizing agents and subsequent irradiation. As demonstrated earlier by Pitts et al., the modified model provided statistically sound, reproducible results (Pitts et al., 2001; Lee et al., 2004). The Pitt model has been further adapted for the study of *P. aeruginosa*, using discs carrying eighteen glass chips (Hentzer et al., 2001; Singh et al., 2002; Teitzel and Parsek, 2003; Boles et al., 2004).

This method utilizes both batch and continuous flow conditions to establish biofilms on small chips held in a disc fitted with a magnetic stir bar. The benefits of this method rest in its adaptability and the reproducible results it provides: rotating discs can be designed to hold any number of chips of varying sizes and materials, including glass, plastic, metal, and porcelain. Spinning disc–grown biofilms can be inoculated at any point in the growth curve, harvested in replicates at any time point, and the resulting biofilm populations can be assayed for resistance to metals, antibiotics, and biocides.

Tube biofilm culture

Culturing biofilms in tube reactors is an essentially simple method, but can be used for a wide variety of analytical tests, ranging from simple viability testing to microarray analyses, and even magnetic resonance imaging (MRI; Manz et al., 2003; Bagge et al., 2004a,b). Very fragile, thin-walled glass capillaries may be substituted for silicone tubing, allowing for powerful microscopy to be performed on the biofilms growing inside. Tube biofilm reactors are particularly relevant to the study of clinical pathogens, as many nosocomial infections are the product of microbial colonization of indwelling medical devices such as urinary catheters, endotracheal and tracheotomy tubes, and nasogastric feeding tubes (Adair et al., 1999; Leibovitz et al., 2003; Saint and Chenoweth, 2003; Perkins et al., 2004). Tube biofilm reactors can also be used to study microbes that colonize water treatment systems and dental-unit water systems (Walker et al., 2000; Smeets et al., 2003).

As with the spinning disc model, the tube model is easily adaptable. The tube model can provide a large amount of sample material and virtually unlimited replicates for subsequent testing and analysis (Bagge et al., 2004b). Tube-grown biofilms can be incubated for extended periods but, as the tubing becomes occluded with bacterial growth, flow may be obstructed. Nutrient gradients will tend to develop in some tube biofilms, depending on the flow characteristics, the metabolic rate of the bacteria, and the length and diameter of tubing used. The researcher must keep such factors in mind when analyzing data, as they may yield artifactual results. It is also important to remember that samples taken from tube biofilms represent a somewhat heterogeneous population. A number of studies have revealed the metabolic heterogeneity that arises in biofilms (Okabe et al., 1999a,b; Sternberg et al., 1999). Because of the heterogeneity inherent within a biofilm community the results of, for example, expression analyses of a tube biofilm represent an average over the whole population. Different methods and growth models must be used to study the activities of subpopulations within a complex biofilm. Heterogeneity must always be considered as a factor in biofilm research, regardless of the model system used. The key to the models described in this unit

is the reduction of variation between biofilm samples, not the variation that is inherent to any individual biofilm.

Critical Parameters and Troubleshooting

The greatest hindrance to any long-term chemostat culture is contamination. It is, therefore, critical to pay attention at every step to maintain asepsis within the biofilm culture system. Use a medium reservoir that is large enough for the entire length of the experiment without having to change out reservoirs midcourse. This is one of the most common points where contaminants are introduced. It is also critical that the medium reservoir is prepared and autoclaved properly. Seal the tubing into the reservoir and reactor ports with silicone. Both biofilm systems described here are open to the environment at specific points, and these should be protected at all times. The air inlet of the medium reservoir is protected by a filter cassette, as is the reactor vessel in the spinning disc model. The effluent tube is the only other open point of the system, and care must be taken to avoid backflow from this point. Use a clamp to prevent backflow whenever you turn off the pump. Wipe down the tubing and your gloved hands with ethanol before handling and manipulating the system once it has been autoclaved. Because both model systems described here yield samples for viable counting, sterility must be maintained throughout the harvesting and processing stages of the experiment.

It is important to avoid introducing air bubbles in the tube biofilm reactor. Not only will air bubbles interrupt the flow, but they can also interfere with the biofilm and may cause artifactual results. Prevent air accumulation by ensuring that all tubing joints are tightly secured, and that no air is introduced when injecting bacteria into the system. Many researchers use bubble traps in their biofilm reactors. A variety of bubble trap designs are available (e.g., see *UNIT 1B.2*) but they are not a guarantee that the system will remain bubble-free. It remains up to the individual whether a bubble trap is used in the tube biofilm system.

To maintain consistency between experiments, always use inocula that have been grown under similar conditions and to the same stage of growth. Typically, mid-log phase culture inocula will provide the most consistent results.

To maintain constant conditions within a tube biofilm experiment, check the flow rate before inoculation and before harvesting. If running a long time course, check the flow rate at other points during the incubation. As the biomass within the tube increases, not only will the flow rate change, but the flow dynamics as well, both of which may affect biofilm structure and behavior (Beyenal and Lewandowski, 2002; Manz et al., 2003; Leon et al., 2004).

Anticipated Results

The experiments described here will yield, at the minimum, viable counts which will reflect the amount of biomass in either the tube-grown biofilm or on the biofilm chips. In both cases, biofilms may be subjected to treatments with biocides and tested for survival. When compared with the results of planktonic cells processed in parallel, data can be obtained regarding the different susceptibilities of these bacterial populations to various treatments. The tube-grown biofilms can provide sufficient biomass to perform various expression analyses. The cells may be processed for microarray analysis to determine wide-scale differences in expression between biofilm populations and their planktonic counterparts. More simply, engineered reporter strains may be utilized in β-galactosidase assays to examine the expression levels of specific genes. Similarly, reporter genes may be fused to GFP and the bacterial populations examined microscopically for expression.

Time Considerations

A spinning disc biofilm experiment can be run in a minimum of 4 to 5 days, from the point of assembly through harvest. However, the experiment may be run for a longer period, to obtain data over a time course, or for a shorter period, if the data required are from the earliest stages of biofilm formation.

The tube biofilm experiment will take a minimum of 3 to 4 days, but may also run longer if time course data is desired.

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Preparing for Biofilm Studies in the Field

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ABSTRACT

In their natural environments, microorganisms are under constant environmental selection to form biofilms. Using aquatic biofilms as an example, this unit illustrates general concepts in field biology and practical suggestions for designing, conducting, and analyzing biofilm experiments at varying distances from the laboratory. The unit also addresses an example of a special situation (space flight) where experimentation must be done by proxy through another individual or machine. *Curr. Protoc. Microbiol.* 10:1B.4.1-1B.4.14. © 2008 by John Wiley & Sons, Inc.

Keywords: biofilm • bacteria • field studies

INTRODUCTION

Field studies of biofilms present an exciting challenge to a microbiologist. Unlike in the laboratory, microorganisms in nature exist in mixed populations and are under constant environmental selection to adhere to surfaces and form biofilms. As a result, field experimentation would offer the most rigorous and realistic conditions for the testing of materials to attract or repel biofilms or for the isolation of organisms best suited for biofilm growth. Conditions in nature are rarely predictable. Rapid changes in weather, uncooperative wildlife, or a support vehicle's mechanical condition may affect carrying out field studies, which, consequently, require a certain amount of foresight, planning, and adaptability on the part of the microbiologist.

There are a number of logistical issues that may need to be addressed. These include accessibility of the sampling site; experimental design in the field, including sample collection, storage of specimens, and transport to the laboratory; and even issues related to investigator safety. Specimen analysis should also be considered during planning. For viable counts with dilution plating, samples are generally collected and stored unaltered at lower temperatures, to restrict further growth. Ideally, samples will be analyzed as quickly as possible. In the case of other types of analyses, specimens may need to be manipulated on site for initial preservation. This includes the use of fixatives (for microscopy), addition of TE buffer for a PCR-based detection such as denaturing gradient gel electrophoresis (DGGE; *UNIT 1E.1*), or preservatives such as RNase inhibitors for RNA-based detection. Statistical analysis must also be a consideration in the choice of sampling sites and the planning and location of experimental replicates.

It would be impractical to address every conceivable field situation here. Rather, we shall assume that the investigator may have limited field experience. To that end, we have included a section on general field biology and safety. Other sections deal with the type of environment to be sampled and the proximity of the field site to the investigator's laboratory. Some field sites may have a laboratory on site, which can greatly assist in the processing of data; others may not, and so specimen storage and transport will need to be considered. To address these different considerations, this unit provides examples of three types of field studies: (1) those that are in proximity to a laboratory, (2) those in a more remote location requiring overnight accommodation, and (3) field studies by proxy, in which the investigator may need another individual or a remotely controlled device to conduct some or all of the experimentation.

TECHNIQUE REFINEMENT AND TESTING

It is often prudent to test and develop some sampling and processing techniques prior to going into the field. In the case of water experiments, aquaria offer a readily accessible microcosm for developing specific techniques and identifying equipment and supplies needed for mixed culture work and sample processing. One can also assess the potential for specimen storage conditions to alter microbial community composition. For example,

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aseptically acquire a 100- to 500-ml sample of aquarium (or pond, lake, or river) water. Mix the sample and then divide it into two aliquots, processing one immediately and the other after a delay under simulated field storage conditions.

Several strategies can be used to evaluate the influence of simulated field storage conditions on microbial survival and diversity. One fairly simple approach would be to employ dilution plating to measure bacterial survival by standard plate counts. One could also get a crude estimate of microbial diversity by the range of colony morphologies observed on the growth medium both before and after storage. DGGE (described in detail in *UNIT 1E.1*) is a PCR-based approach by which microbial diversity can be estimated by means of DNA fingerprinting. Comparing the DGGE analysis of the two samples (quickly processed samples and stored specimens) can determine whether banding patterns in the two samples are very similar (ideal situation implying no changes) or different (implying an artifact). It has been our experience that DNA templates, once prepared, can be stored at –20°C for several months without noticeable change. For DNA template preparation see, e.g., *UNIT 1.E.1* or use a kit, e.g., DNeasy (Qiagen).

GENERAL ASPECTS OF FIELD RESEARCH

Once at the field site, it is prudent to select and map sites for experimentation. If possible, photographs should also be taken of the sampling sites and sampling methodology in order to keep an accurate record. Several factors influence the final choice of sampling sites, including: site accessibility, safety of personnel, equipment limitations, and the likelihood that the chosen sites will provide a representative scientific evaluation of the study site.

Permits and Access to Study Sites

Collecting permits are very often required for field activities (addressed in part in *APPENDIX 1B*). One or more permits may be needed for the following:

Access to a site

Activities on the field site (including camping)

Sample collection

Sample transport.

Permits may be required by many organizations, including university, state, and federal agencies. For example, in the United States, national park collecting permits can be obtained through the National Park Service Research Permit and Reporting System (*http://science.nature.nps.gov/research/ac/ResearchIndex*). Collecting permits may or may not address permission for access. Some specimen transport issues are addressed by the National Park Service. However, in cases where potential human, animal, or plant pathogens are to be handled and transported, other agencies will be involved. In the United States, these include the Department of Agriculture (*http://www.aphis.usda.gov/programs/ag_selectagent/index.html*) and Centers for Disease Control (*http://www.cdc.gov/od/ohs/default.htm*). All such specific details should be checked ahead of time. Public as well as private lands often have restricted access and regulations addressing specific activities that may be conducted on the site. The importance of communication and respect for the wishes of the land owner or regulatory agency cannot be overemphasized, particularly if one wishes to have continued access to study sites.

IMPORTANT NOTE: The Web sites provided are current at the time of publication but may change.

Safety

First aid

Adequate provisions should be made for first aid and treatment of small cuts, burns, and other minor injuries. Ideally, at least one member of the field party should be trained in basic first aid (see *http://www.redcross.org/services/hss/courses*). Serious injuries must be treated by a physician or trained emergency personnel. Inquiries should be made in advance to obtain phone numbers for and directions to the nearest medical facility. If necessary, cell phones, radios, or satellite phones should be secured for emergencies.

Infectious disease safety

Although avian influenza (H5N1 influenza virus) is currently highly profiled in the media, there are a number of other infectious diseases that are also of concern for investigators in the field. These include other viral infections (e.g. hanta virus and rabies), vector-borne diseases (e.g., malaria); zoonotic (transmitted between animals and humans) bacterial infections (e.g., anthrax and tularemia); and wound infections (e.g., tetanus). The *United States Field Manual of Wildlife Diseases* (Friend and Franson, 1999), containing a number of practical suggestions,

can be downloaded from (*http://www.nwhc.usgs.gov/publications/field_manual*). Vaccination information can be obtained from the Centers for Disease Control Web site (*http://wwwn.cdc.gov/travel/contentVaccinations.aspx*).

Additional precautions for remote areas

Accidents such as sprained limbs or fractures may turn fieldwork at remote sites into life-threatening situations. As a precaution, one should take appropriate survival gear (e.g., clothing, water, food) and inform others as to travel plans, as well as anticipated date and time of return. In the event of a delay, authorities can be alerted for a potential rescue operation. Obviously, it is equally important to inform individuals or authorities as soon as possible following the safe completion of a trip to avoid the expense and hazards of unnecessary rescue operations.

Accessibility

Vehicle accessible

Equipment and personnel can be moved to research sites without problems. Emphasis can be on taking a number of supplies, backup equipment, and spare parts.

Inaccessible by vehicle

As equipment may have to be carried in by backpack, pack animal, boat, or aircraft, the emphasis must be on lightweight essentials. Carrying duplicate equipment and spare parts is usually prohibitive. These restrictions apply especially to personal equipment.

Accommodations and Personal Equipment

Accommodations

Accommodations can have a dramatic impact on field research. Even a minimum of amenities on-site will reduce the amount of personal gear required and permit more collection equipment to be included. Adverse conditions (e.g., heat, cold, precipitation) need to be addressed with personal as well as collection equipment. Batteries are especially sensitive to temperature extremes.

Food

Keep meals simple. Limit perishable items, particularly if backpacking, canoeing, or camping in an area lacking refrigeration.

Camping gear

Modern camping supplies (e.g., gas stoves, water filters) are small and take up little space. Efforts should be made to bring sufficient supplies to prepare meals at a campsite. This will avoid the unnecessary time and expense of traveling from the field to purchase a meal.

Personal gear

New field clothes, especially synthetics, provide protection from the elements (e.g., sun, heat, cold, rain) to a remarkable degree. The most important personal items are durable but comfortable field shoes or boots, a good hat for protection from sun or cold, suitable clothing (preferably layers of clothing that allow adjustments for changes in temperature), and a sleeping bag and mat.

Animal concerns

Animal interference can arise at a number of environments. In North America, nuisance animals can include raccoons, bears, or rodents. Check on these issues before going to the field. As a general precaution to avoid "unwanted guests," never store food in a tent.

Human concerns

In some instances, vandalism or some other human activity (e.g., swimming in a sampling area with possible inadvertent contact with scientific equipment) may represent a problem. Aside from the loss of scientific data, there is also concern for public safety and liability (e.g., if a swimmer should get cut on a microscope slide).

Equipment

Tables provide a general description of equipment (Table 1B.4.1) and a representative list of suppliers (Table 1B.4.2), as well as checklists for general supplies (Table 1B.4.3), microbiology supplies (Table 1B.4.4), and personal supplies (Table 1B.4.5). A brief explanation of some items is provided below.

Containers for liquids

Presterilized, lightweight containers such as 15- and 50-ml plastic centrifuge tubes with screw caps and individually wrapped, plastic urine specimen cups (~100-ml volume) can be used for a variety of purposes, including liquid collection, storage of biofilm-colonized substrata, and containers for ethanol sterilization of equipment. We also recommend bringing along sheets of Parafilm, plastic bags, and tape to use for additional protection against leakage. Aside from the loss of a

Table 1B.4.1 General Equipment for Field Work[a,b]

Item	Comments
Spare parts for repairs (generally applicable for larger pieces of equipment)	
Fuses	Variety of voltage ratings, Buss fuses, automotive style (as appropriate for equipment)
Hoses and clamps	Variety of sizes for pumps, fuel, liquids
Electrical wiring, terminals, splices	Variety of sizes for repairing connections, splicing, wiring extensions
Light bulbs	Equipment-specific variety of bulb types with different brightness and working times
Power sources	
Batteries	Variety of rechargeable batteries (e.g., gel-cell, Ni-Cad, lithium); check equipment for specific sizes and voltages
Chargers	Battery chargers that charge from vehicle electrical systems, as well as standard AC to DC
Power converters	Vehicle power converters to allow AC equipment to be run from DC automobile systems; for international studies, adapters for electrical power differences (voltage and current)
Equipment for repairs	
Duct tape	Useful for a variety of repairs
Electrician's tape	Nonconducting tape, useful for electrical repairs
Oatley or Devcon epoxy putty	Two-part pliable putty that cures in 5 min; useful for repairs to odd shapes and locations on equipment; will not drip or run
Small tool kit with assorted screwdrivers, pliers, and a set of Allen wrenches	For very small items, jeweler's screwdrivers, forceps, hemostats (very useful for clamping tubing)
Geologist's hammer	Useful for collecting rocks and may also serve as a general-purpose hammer
Measuring tape	At least 6 m
Containers	
Plastic containers	Various sizes of vials and bottles for sample collection; plastic or Nalgene will not break under rough use in the back country; individually wrapped urine specimen containers (~100 ml) and sterile centrifuge tubes (10 and 50 ml) are most useful
Reclosable plastic bags	Reclosable plastic bags (e.g., Ziploc) provide cheap waterproofing; available in a variety of sizes and designs
Waterproof/weatherproof cases and bags	Numerous designs and sizes of waterproof cases available from Pelican; waterproof gear bags of several sizes for travel in boats, canoes, and kayaks available from SealLine, Dry Pack, and others
Documentation	
Field book	Fundamental piece of equipment; vital for recording of observations and experimental protocols; paper of high rag/cotton content (e.g., Rite in the Rain); use waterproof and fade-proof ink or a pencil (not ball point or gel ink pens)
Cameras (digital and 35 mm)	Invaluable for observations; single lens reflex cameras preferable, but compact point-and-shoot cameras offer advantages of compactness, versatility, and more than adequate image quality; include coin or other object of known size in photographs for scale

continued

Table 1B.4.1 General Equipment for Field Work[a,b], *continued*

Item	Comments
GPS units	Provides precise recording of locations for documentation (often requested by many permitting and funding agencies)
Maps and compass	Obtain best maps prior to trip; topographical, digital orthophoto quarter-quadrangle (DOQQ) available from federal and state agencies, private mapping firms, or Internet sources
First aid	
Standard first aid kit	Complete units available at most outdoor and recreation supply stores
Nonstandard first aid items	Extractor venomous snake and insect kit; can be effective if administered quickly according to directions
	Topical ointments for relief from insect bites, plant allergies, and injuries
	Phone numbers and directions to the nearest local medical facility; Arizona Poison and Drug Information Center, Tucson, Arizona, is a clearinghouse for information on what to do in case of snakebite or other poisonous animals and plants (800-222-1222 or 520-626-6016; (*http://www.pharmacy.arizona.edu/outreach/poison/venom.php*); Web site accurate as of publication date
Vital medical information	Note any special medical needs of field team members and have information readily available; especially important to medical personnel in remote areas
Personal supplies	
Insect repellent	Prevents infection by arthropod vectors (e.g., ticks and mosquitoes) for diseases of current concern, such as Lyme Disease and West Nile; DEET proven effective
Sun screen	Use SPF rating 35 or higher; really works!
Backpack	Many designs and sizes for all needs
String, rope, elastic bands, or cords	Packing, securing items for transport
Head lamps, flashlights, spotlights	Do not scrimp; adequate nighttime lighting prevents mistakes and injuries; new LED lights provide bright lighting with less power drain and longer bulb life
Lanterns, light sources	Standard gas powered lanterns; new florescent and LED lanterns provide lighting without heat and fuel (batteries may be the limiting factor)
Binoculars	Useful for observing and identifying distant objects; can serve as a field microscope if one looks through them backwards
Water for drinking	Micropore filters and sterilizing tablets can be used for treatment of spring or stream water
Labeling tape	If needed for field processing
Permanent marking pen	Sharpie or equivalent (bring extras)
Knife or Leatherman tool	Handy for multiple small jobs
Hat, footwear, and other protective clothing	Will vary according to location and season (see Table 1B.4.5)

continued

Table 1B.4.1 General Equipment for Field Work[a,b], *continued*

Item	Comments
Tape measure and/or ruler	Useful for measurements
Instruments to measure environmental conditions	Portable thermometer and other instruments (e.g., pH paper or probe) very useful for collecting physical data from site
Sample storage	
Coolers	For ice or dry ice; store in shade, if possible
Preservatives	For samples to be processed for DNA or RNA, add TE buffer or a preservative such as RNAlater (Ambion); some change likely to occur to biological samples between collection and processing in the laboratory
Sample collection and field processing	
Gloves	For moving plants in desert and other environments, leather (thorn-resistant) gloves; for sterility during sample collection, latex or nitrile gloves, rinsed immediately before using with 70% ethanol
Sterile water	Very useful for dilutions and processing; autoclave and carry in plastic bottles; can also serve as emergency drinking water supply
95% ethanol for on-site ethanol sterilization	Dip object in 95% ethanol and flame with match or lighter
Sterile urine collection cups	Individually wrapped and ~100 ml
Sterile plastic tubes	50- and 15-ml presterilized tubes
Scalpel blades	Purchased sterile and individually wrapped; very useful for scraping natural biofilms from surfaces
Aluminum foil	Very lightweight and can be folded and autoclaved to provide a sterile working surface in the field when unfolded
Microscope slides	Useful as biofilm colonization substrata; can be anchored with paper clamps and string
Petri dishes, sterile	Can also function as sterile working surface
Syringes, individually wrapped sterile	For water samples, use larger syringes (e.g., 30 to 50 ml) to filter volumes of water (to collect planktonic bacteria)
Filters for concentrating planktonic samples from large volumes of water	
Filter holder units (autoclavable)	GE Healthcare (formerly Poretics/Osmonics) and SPI
Filters	0.2-μm pore size, 25-mm diameter; black polycarbonate filters available from GE Healthcare (formerly Poretics/Osmonics) or Structure Probe, great for epifluorescence or CSLM because of limited background fluorescence; other filter types OK, but not as useful; store used filters in plastic tubes
Filter tweezers	Can be ethanol sterilized in the field (see above)
Fresh filters	Purchase as individually wrapped and presterilized or, alternatively, place several in aluminum foil, label, and autoclave

[a]These items are used for a variety of purposes. Some items may not be applicable for all trips.
[b]See Table 1B.4.2 for a representative list of suppliers.

Table 1B.4.2 Representative List of Field Equipment Suppliers

Supplier	Web site
Brigade Quartermasters	*http://www.actiongear.com*
Cabela's	*http://www.cabelas.com*
CampMor	*http://www.campmor.com*
Forestry Suppliers	*http://www.forestry-suppliers.com*
REI	*http://www.rei.com*

Table 1B.4.3 General Checklist

Items	Comments
Permits: university, local, state, federal	Collecting, on-site activities, sample collecting, transport
Maps, GPS equipment	For documenting locations
First aid and emergency information	First aid supplies
	Phone numbers and locations for medical assistance
	Personal medical information for each team member (if applicable)
Cell phone and other communication equipment	Useful only if coverage is available
Vehicle	Have vehicle inspected prior to departure
	Check all fluid levels, belts, hoses, tire condition (special attention to spare tire)
	Appropriate equipment for changing tires
	Fill with gasoline before entering remote area
Sampling equipment	See Table 1B.4.4
Personal equipment	See Table 1B.4.5

valuable sample, leakage can damage clothing or field notes. Permanent markers can be used to label containers. For measuring liquids in the field, one can use markings on a container or, if necessary, sterile plastic syringes or pipets (along with a pipetting bulb). Due to weight and breakage considerations, the use of glass is strongly discouraged.

Syringes and filters

Sterile syringes (10, 30, or 50 ml) are quite useful for sampling large volumes of water and removing planktonic organisms by filtration. Typically, we have used autoclavable filter holders (e.g., 25-mm filter holder; Pall Corporation).

Unlike traditional white filters, black polycarbonate filters (0.2-μm pore size, 25-mm diameter; Structure Probe, part B0225-MB) are especially useful for fluorescence and confocal microscopy applications because they do not fluoresce under ultraviolet light. These filters and filter holders are assembled in the laboratory prior to the trip and wrapped individually in aluminum foil, labeled, and autoclaved. Labeling will permit easy identification of wrapped packages under field conditions.

Cutting and Trimming

For some sampling operations (e.g., plant-adherent bacteria), it may be necessary to cut materials in the field. One has several options. Tissue sections may be removed using individually packaged sterile scalpel blades. Larger specimens can be trimmed using scissors and tweezers (both of which can be sterilized with

Table 1B.4.4 Microbiology Checklist

Function	Item
Containers for specimen collection	Sterile specimen cups
	Sterile centrifuge vials
	Syringes
	Filter units (sterilized)
	Microscope slides
	Clips for holding microscope slides
	String for anchoring microscope slides
	Other biofilm colonization substrata (if needed)
	Reclosable plastic bags (e.g., Ziploc) or other containers for small items; autoclave bags for larger items
Specimen manipulation	Scalpel blades
	Tweezers
	Scissors (if needed)
	Ethanol (bring 95%)
	Sterile water
	Nitrile gloves
	Aluminum foil (sterilized and folded)
	Petri dishes
	Matches or lighter for ethanol sterilization
Other	Camera

in the field; see Disinfection and Field Sterilization, below). Sterile petri dishes are very useful for providing a small, sterile, working area. Should a larger sterile working area be necessary, we advise using sheets of aluminum foil of a desired size (e.g., 0.5 m^2), folding as necessary, and putting a small amount of indicator tape onto the sheet. Several of these sheets can be placed into an autoclave bag and autoclaved, prior to going to the field. In this fashion, a sterile work surface or wrapping agent is readily available. The sterilized autoclave bag can also be used as a sterile container to transport larger specimens or to gather waste.

Disinfection and Field Sterilization

Ethanol is a great disinfectant because it is quite effective, yet biodegradable. For ethanol sterilization, dip the object to be sterilized into 95% ethanol, and then light the ethanol-coated item with a match or lighter. Ethanol will burn with a fairly cool flame and provide adequate field sterilization. During this procedure, be careful that burning ethanol does not spill onto clothing or other materials. For disinfection in the field, we recommend diluting 95% ethanol with sterile water to obtain 70% ethanol. This dilution process is done easily in the field by filling a container three quarters full with 95% ethanol, filling the remainder of the container with sterile water, and mixing to achieve a final concentration of ~70%. Unlike 95% ethanol, 70% ethanol does not burn as readily and so, under field conditions, provides a measure of safety.

If sterile washing is necessary, autoclave and bring several containers of sterile water. It is usually best to pack several smaller plastic containers (e.g., 500 or 1000 ml), rather than a single 10-liter or larger container. Smaller containers are lighter and if one container of liquid accidentally gets contaminated, there are still spare containers that can be used.

Laboratory gloves are very useful if aseptic manipulation of samples is to be done. Nitrile gloves are especially useful. They are lightweight and can be easily disinfected in the field by rinsing with 70% ethanol.

Table 1B.4.5 Personal Supplies Packing List

Item	Comments
Footwear	Sturdy, comfortable, and protective (i.e., boots, water shoes); bring an extra pair
Protective clothing	Jeans/long pants, cool shirts recommended; layers for cool weather (some alpine environments can get close to freezing even in midsummer)
Rain gear (poncho)	Even in the desert it rains in July
Hat	For protection from sun or for warmth
Gloves	Cotton or leather
Swimsuit/shorts	For seining
Bedroll/sheets/pillow/cot	Most places provide a bed or mattress
Towel, toiletries, first aid	Include topical ointment for itching or irritation, band aids, medications (e.g., analgesics)
Tents	When needed; arrange to share with others
Field guides	Useful for identifying birds, plants, rocks, and wildlife
Field notebook/card catalog/index cards	Essential for recording experimental observations; cards can be useful for labeling larger specimens
Water container/canteen	Absolutely essential for carrying drinking water when away from camp
Binoculars	Bring your own; very useful for spotting distant objects, including animals and birds
Cup/mug, eating utensils	Needed for eating and drinking
Gatorade/water	*VITAL:* At least 2 quarts per day
Food	Plan for two meals per day plus snacks; orient all activities around completion of fieldwork, not the dinner table; quick breakfast at 6:00 a.m., midday snack (if back at camp), and a late evening meal; minimal supplies, no elaborate meals, simple is best: e.g., bagels, breakfast muffins, sandwiches

Tables 1B.4.1 to 1B.4.5 list equipment the field researcher should carry, as well as representative suppliers. Be sure to inventory equipment before leaving for the field. Often the item left behind is the one most needed. Carefully pack and cushion fragile equipment for transport. Check and double-check the integrity and functionality of all fittings, hoses, cables, batteries, tape, pens, and other equipment.

The closer the sampling site is to the laboratory or vehicle, the more materials are readily available. In the event of remote sites, limit supplies to essential lightweight items along with items needed for investigator camping, safety, and other needs, notably water. With respect to vehicle transport, materials may need to be secured and insulated against damage from vibration, particularly if the vehicle needs to be driven over rough terrain.

Special Issues Related to DNA and RNA-Based Studies

Few specialized pieces of equipment required for nucleic acid studies (e.g., thermal cyclers, spectrophotometers, centrifuges) are available in the field, and most such studies will be performed in the laboratory. In these cases, sample collection must be done in the context of the experimental data desired. For

example, for a nucleic acid–based community profile, the sample should be collected so as to preserve DNA or RNA. Preservation of specimen viability may not be necessary, and indeed, in some cases where viability is maintained, differential growth and survival of organisms in specimen containers (sometimes referred to as the "bottle effect") may actually be detrimental to the scientific analysis. Where feasible, the simplest approach for DNA preservation is to freeze the sample (−20°C or colder), and store frozen until processed. Should vehicle access be available, specimens can be placed in Styrofoam containers or coolers along with dry ice (frozen CO_2). Packaging materials, used to ship refrigerated or frozen biochemical reagents, are often very useful for this purpose. As a safety precaution, these containers need to be in a well ventilated place so as to avoid asphyxiation of the investigators by elevated CO_2 levels.

For RNA analysis (and even DNA analysis), a stabilizing agent (e.g., RNAlater, Ambion) is often used. This reagent stabilizes RNA and genomic DNA, but will denature many proteins (see *http://www.ambion.com/techlib/tn/113/6.html*). Once samples are placed in RNAlater, they can be stored for several days at ambient temperatures or up to one month at 4°C. Under field conditions, storage in coolers along with ice, will maintain temperatures close to 4°C. The use of fixatives such as glutaraldehyde or formaldehyde, while useful for microscopy, is strongly discouraged. These and other chemicals can be detrimental to downstream specimen processing with DNA polymerase, or reverse transcriptase.

FIELD STUDIES IN PROXIMITY TO LABORATORY FACILITIES

Biofilm studies performed in proximity to the laboratories have an advantage over those done in more remote environments in that unusual or time-sensitive procedures can be used. In addition, traditional biofilm colonization substrates such as glass microscope slides can also be used. Examples of two unusual protocols are biofilm formation on sycamore leaves (*Platanus occidentalis*; Dunn et al., 1997) and abiotic (limestone; Whiteley et al., 2001) surfaces. These studies were conducted on Texas State University property within 2 km of the laboratory, thus permitting ready access to laboratory equipment and supplies. As these studies were performed on Texas State University property, no permits were needed. The purpose of this research was to study the influence of epiphytic biofilms on leaf fossilization (Dunn et al., 1997) and identify population interactions in karst aquifer biofilm bacteria (Whiteley et al., 2001).The purpose of the discussion here is to address some practical aspects of experimentation; for the biological implications of the studies, see the references provided.

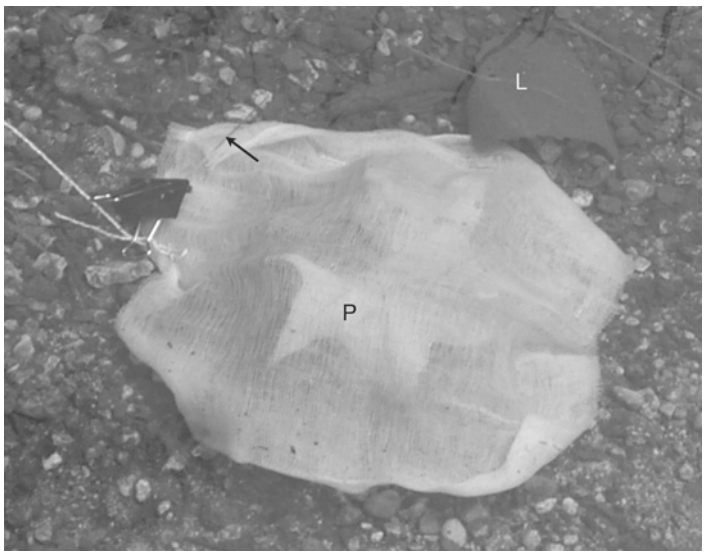

Figure 1B.4.1 Collection of biofilms in streams. During field studies of biofilm growth in streams, biological material such as a *Platanus* leaf (P) can be retained within a porous container such as cheesecloth. A glass slide (arrow) can be used as a representative abiotic surface. Note the presence of naturally occurring biofilms on substrata such as a leaf (L) and the gravel sediment.

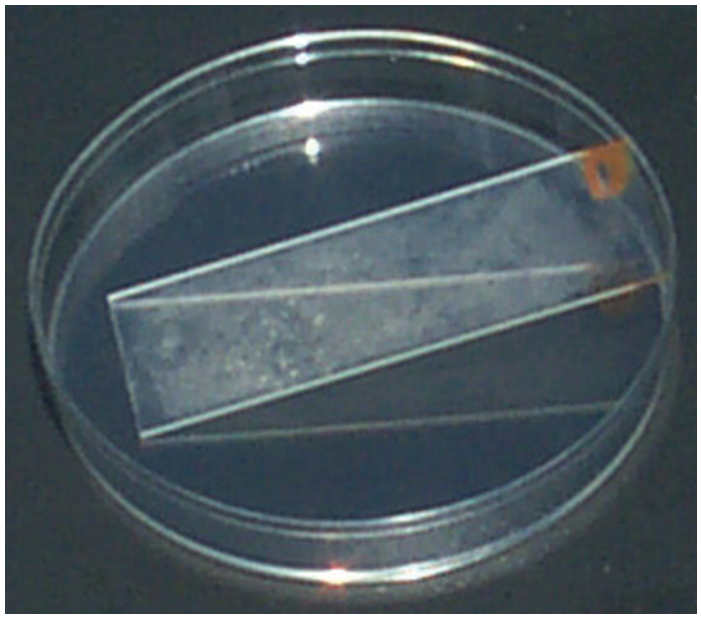

Figure 1B.4.2 Growth of biofilms on glass microscope slides. Biofilms readily form on glass microscope slides that are placed in aquatic environments.

Depending upon the environment, colonization substrata can be placed onto a surface (e.g., river sediment) or suspended at a selected depth in the water column by a paperclip or other clamping material, which is then anchored to a string as shown in Figure 1B.4.1. Such experimentally grown biofilms mimic natural biofilms on the biotic (leaf) and abiotic (rock) surfaces naturally found in that environment (also shown in Fig. 1B.4.1). While biofilms often form quite readily, surface coverage is typically quite variable. A representative biofilm grown on a glass microscope slide is shown in Figure 1B.4.2.

Collection of Epiphytic Biofilms

This study was undertaken to determine whether aquatic biofilms could enhance fossil formation in leaves (Dunn et al., 1997). The rationale was threefold: (1) leaf surfaces are typically hydrophobic due to a waxy cuticle, (2) biofilms would form readily on any submerged surface in aquatic environments (including leaves), and (3) by virtue of their anionic surface properties, bacteria and biofilms would readily bind metal ions thus enhancing mineral formation and fossilization.

Unique equipment

The local river environment presented several challenges. The river flow (typically 0.3 to 0.5 m/sec) caused leaves and other submerged materials to drift, and the grazing activities of fish and invertebrates (notably snails) would damage submerged leaves and their epiphytic biofilms. To circumvent these problems, the leaves were confined in cheesecloth bags (constructed by folding in half a piece of cheesecloth $\sim$70 $\times$ 35 cm and stapling the sides; see Fig. 1B.4.1) and the bags immobilized with rocks and string. Cheesecloth is also very useful for containing other biofilm colonization substrata, including small gravel and fiberglass. Nonbiological colonization substrata can be placed into cheesecloth, autoclaved (if desired), and then transported to the field site in sterile containers (S. Nath and R.J.C. McLean, pers. comm.).

Procedure

To maintain sterility in the field, nitrile gloves, disinfected by rinsing with 70% ethanol were used. We have found these to be much more comfortable than other materials and are also suitable for people with allergies to latex.

Approximately 50 leaves from several representative trees were collected by breaking leaves at the base of the leaf stem. At least 10 of these should be used to determine size and mass of leaves. One could also calculate area of these leaves by placing individual leaves on a scanner, obtaining an image, and then calculating the area using image analysis software (e.g., NIH Image).

For aquatic biofilm formation, one or more leaves were placed into a cheesecloth bag, which was stapled shut, attached by string to a rock, and submerged in the stream for several days (see Fig. 1B.4.1). Biofilms, largely consisting of endogenous heterotrophic bacteria, formed on the leaves.

At conclusion of field exposure, the cheesecloth bag was removed from stream, placed in a sterile container (e.g., a presterilized autoclave bag), and transported to the lab for analysis.

Growth of Epilithic Biofilm Bacteria in Groundwater

This study was undertaken in part to investigate epilithic bacteria in a karst aquifer environment (Whiteley et al., 2001).

Unique equipment

Cheesecloth bags were constructed as described above. As an alternative, nylon window screen mesh (available at any hardware store) can be substituted for added strength. These bags were filled with small rocks (~1 to 2 cm^3) as colonization substrata. A length of rope was then securely attached to the bag. The bags with the rocks were placed into an autoclave bag, fastening one end of the rope to the outside of the bag (permitting ready access at the field site, yet preserving sterility of contents) and then autoclaved 30 to 60 min. This extended autoclaving time is necessary to ensure that endolithic microorganisms within the rocks are killed. Sterilized autoclave bags keep the rocks sterile during transport to and from the field site.

Procedure

Groundwater access can be obtained by several means including wells, artesian springs, and access caves. In this particular study (Whiteley et al., 2001), access to the groundwater was obtained through an access cave named Rattlesnake Cave. Prior to entering the cave, precautions were taken to ensure this cave was not occupied by rattlesnakes (*Crotalus* spp.) or other wildlife. Samples were suspended below the water table for several days to ensure the growth and maturation of biofilms on the rocks. During specimen retrieval, autoclaved aluminum foil (to provide a sterile working surface in the field) and autoclave bags (for specimen transport) were used at the field site.

Analysis of Adherent Microbial Populations

Analysis can be accomplished by either molecular or plate-counting techniques. For plate counting assays, samples must be analyzed as quickly as possible (typically within 1 to 2 hr) because microbial numbers will change relatively quickly. Samples for DNA analysis including DGGE analysis (see *UNIT 1E.1*) can be held in TE buffer at −20°C or placed in RNAlater (Ambion) until analyzed. In either case, adherent bacteria can be removed from the substrata by bath sonication. Alternatively, if a flat surface such as a microscope slide is used for biofilm colonization, the surface can be scraped into a sterile buffer or H_2O using a sterile scalpel blade and then sonicated. The shear forces generated by sonication are used to disrupt adherent bacteria and bacterial clumps into individual organisms. We typically use a bath sonicator rather than a probe sonicator in order to minimize cell damage (*UNIT 1E.1*).

Challenges of Epiphytic and Epilithic Biofilm studies

Unlike microscope slides, rocks and leaves have very uneven surfaces, and calculating surface areas and biofilm concentrations can be problematic and will lead to considerable data variation. In addition, biofilm colonization on these substrata is typically uneven because some microorganisms preferentially colonize in crevices. The best approach for epilithic bacteria is to measure the dimensions (height length and width) of the rock and to estimate its surface area, assuming it has the shape of a cube. Most leaf surfaces are relatively flat. If necessary, 1-cm^2 sections can be cut from leaves prior to sonication.

COLLECTION OF BIOFILM SAMPLES REMOTE FROM THE LABORATORY

It may be necessary to conduct biofilm studies some distance from the laboratory. Vehicle accessibility will determine the amount and types of supplies that can be taken. With respect to microbial samples, it may be a number of hours or even days before the samples can be returned to a laboratory. Sample storage and transport are issues because changes in microbial populations will occur; these issues have been addressed in an earlier section (see Technique Refinement and Testing).

In the event of travel, especially across international boundaries, one should plan ahead (often 2 to 3 months or more) and check with appropriate government and courier agencies for permits required for travel, access to field sites, and shipment of any supplies or specimens. Given the political and security issues that have arisen in the past few years, the U.S.A. and other countries have adopted strict regulations for the transport of biological materials. Often, it is best to ship a sample, along with appropriate documentation, via a courier.

We have experience in conducting fieldwork in several remote sites involving lengthy trips by vehicle (Big Bend National Park, U.S.A.) or air (Viterbo, Italy and Yellowstone National Park, U.S.A.). For remote trips, space may be quite limited, particularly if supplies need to be transported in luggage. In these cases, the use of lightweight, sterile, plastic containers and microscope slides can be quite useful for a number of experiments. Often one will need to improvise with available local materials; bringing extra tape, pens, markers, string, magnifying glass, a knife, camera, and notebook is advisable.

Questions to Be Addressed Prior to the Trip

Site accessibility

Determine accessibility and characteristics of site as well as limitations (including dates and times of access) on sampling and experimentation, and obtain necessary permits. If necessary, coordinate sampling dates with all personnel involved.

Transportation

One must plan for transportation to and from site and the corresponding weight and volume limitations on materials to be transported. This will influence the number and types of samples that can be collected.

Materials and supplies

Decide on and prepare materials to be transported. For example, Luer-Lok filter units (for use with a syringe) can be assembled, individually wrapped in aluminum foil, and autoclaved. Small plastic (LDPE) bottles can be labeled, filled with water and autoclaved. Ethanol (95%) can also be packed into small plastic bottles for use as an on-site disinfectant.

Accommodation

Is camping gear needed? This subject is addressed under General Aspects of Field Research.

Support laboratory or system at or near site

It is prudent to assume that any necessary laboratory equipment and supplies will need to be transported.

Additional personnel

Are local support personnel, or alternatively, laboratory support personnel needed, and does scheduling need to be coordinated?

Specimen transport

Plan procedures for shipment of materials back to lab, along with necessary permits.

Tasks to be Performed at the Site

Access

Verify access to site and locations for experimentation. For example, in a spring-fed pond, one might sample as close to the spring outflow as possible, as well as at several representative sites away from the outflow.

Documentation of location

In the field book, draw a map of the site and location of experimental work. If possible, enter GPS coordinates.

Documentation of environmental conditions

Test and record physical and chemical data—e.g., temperature, pH, Eh (oxidation-reduction potential)—as well as weather, date, and time. Water samples can be collected for chemical analyses.

Scheduling

A number of factors may influence field results. These include, e.g., diurnal, seasonal, and weather-related effects. As a result, it may be necessary to modify scheduling of experiments.

In some instances, it may be desirable to get samples to the laboratory as quickly as possible. These samples should be collected immediately prior to departure and analyzed as quickly as possible upon return to the laboratory. Alternatively, they can be collected and shipped quickly for analysis by laboratory support personnel.

SAMPLING BY PROXY

For any number of reasons (e.g., safety, access to a particular environment or equipment), it may be impractical for an investigator to personally conduct an experiment. As an alternative, the experiment will need to be conducted by some other individual or device. Based on

our experience with experiments flown on the Space Shuttle (McLean et al., 2001, 2006), several issues need to be addressed:

1. Gaining access to the equipment or facilities that will enable the experiment to be conducted.

2. Planning and working within the constraints of the equipment, personnel, and scheduling involved.

3. Being flexible, as launching or landing schedules may be disrupted for a number of reasons, e.g., weather, mechanical problems, or other unforeseen issues.

In the case of spacecraft experiments, payload volume and mass are very limited, and all materials present (including organisms and experimental chemicals) must pass a rigorous scientific peer-review and safety review before being included. Due to the considerable expense, a typical space flight may carry several dozen scientific experiments. Consequently, one must design small volume experiments, capable of flying in a device that has been shown to be compatible with spacecraft. Ideally, the equipment involved would either be automated or rely on a very few manual operations because flight personnel may be simultaneously conducting multiple experiments. The commercially developed, dual materials dispersion apparatus (DMDA, Instrumentation Technology Associates) represents one such device (Cassanto and Wood, 1998). We have conducted biofilm experiments on two shuttle flights, STS-95 and STS-107 (NASA Space Transport System designation used for space shuttle flights), using this device (McLean et al. 2001, 2006). During the planning for a shuttle flight, we were initially limited to three 140-µl sample compartments. Just prior to flight, additional compartments became available through the generosity of our commercial partner, Instrumentation Technology Associates.

CONCLUSION

The techniques and approaches used for field biofilm acquisition are quite variable because they will relate to the questions being addressed and the limitations of the environment and available equipment. Nevertheless, several key issues arise which are fundamental to any scientific investigation: reproducibility, quality of data, and quality of data analysis. These issues must be addressed during all stages of experimentation from initial planning, through the actual fieldwork, to the final analysis and reports. Both successes and failures must be recorded and analyzed because they will provide invaluable information for future work. Although practical issues have been addressed in this unit, one may often need to improvise in the field when presented with unexpected obstacles or opportunities.

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Growing Oral Biofilms in a Constant Depth Film Fermentor (CDFF)

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ABSTRACT

In order to grow organisms in such a manner as to mimic their physiological growth state in vivo, it is often desirable to grow them as biofilms in the laboratory. There are numerous systems available to accomplish this; however, some are more suited to the growth of oral biofilms (dental plaque) than others. The operating parameters of one such model, the constant depth film fermentor (CDFF), are given in this unit. This model is particularly suited to studying the varied biofilms which exist in the oral cavity because environmental factors such as the substratum, nutrient source, and gas flow can be altered. *Curr. Protoc. Microbiol.* 6:1B.5.1-1B.5.18. © 2007 by John Wiley & Sons, Inc.

Keywords: constant depth film fermentor • oral biofilms • dental plaque

INTRODUCTION

The mouth provides a large number of diverse surfaces, e.g., the soft shedding tissues of the mucosa and hard nonshedding surfaces of the teeth, on which a wide variety of complex biofilms form. These biofilms (dental plaque) consist of a microbial community embedded in a matrix of polymers of bacterial and salivary origin. Dental plaque has high species diversity and contains numerous microenvironments within gradients of nutrients, oxygen, redox potential, and pH. For these reasons, mature dental plaque is a highly dynamic community. Small alterations in the environment can lead to ecological shifts and subsequent population changes, and in certain specific cases may result in predisposition to a more pathogenic microbial community. Such biofilm-related oral infections are implicated in the etiology of two of the most prevalent diseases affecting industrial societies: caries and periodontal disease. Prevention of these conditions involves constant mechanical removal of the plaque from the tooth surface, although there is often difficulty in maintaining an effective mechanical oral regime. Hence, there is considerable interest in the use of antimicrobial agents in the prevention and treatment of these diseases.

In order to study biofilm development and its perturbation, it is useful to generate steady-state systems. One approach to producing biofilms in a steady-state is to develop a constant depth reactor where the surface growth is periodically removed to maintain a constant geometry (Kinniment et al., 1996). Such a device, the constant depth film fermentor (CDFF; Fig. 1B.5.1), was first described by Coombe et al. (1982), to investigate the growth of dental plaque organisms, and was further developed by Peters and Wimpenny (1988). The CDFF consists of a glass vessel with stainless steel end-plates; the top-plate has ports for the entry of medium and gas and for sampling, while the bottom one has a medium outlet port. The vessel houses a stainless steel disk containing fifteen polytetrafluoroethylene (PTFE) sampling pans which rotate under a PTFE scraper bar that smears the incoming medium over the pans and maintains the biofilms, once formed, at a constant predetermined depth. Each sampling pan has five cylindrical holes containing PTFE plugs, which are recessed to a set depth to create a space in which the biofilms form. The sampling pans can be removed aseptically during the course of an experimental run.

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Figure 1B.5.1 The constant depth film fermentor complete with power unit and accessories.

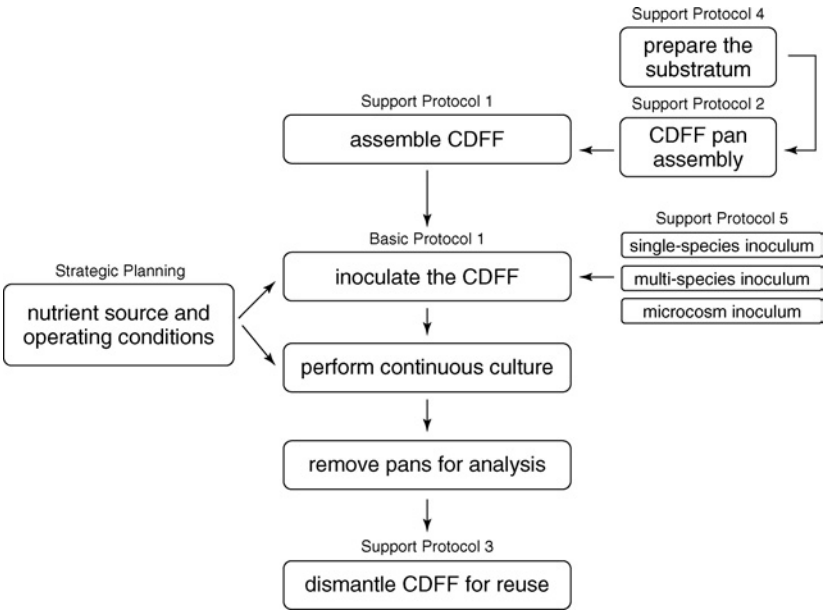

Figure 1B.5.2 Schematic representation of the interrelationship of procedures for growing biofilms in a constant depth film fermentor (CDFF) and their related protocols.

As well as being used for the study of bacterial perturbation (Pratten et al., 1998; Pratten and Wilson, 1999; Deng et al., 2005), the CDFF has been used in studies evaluating endodontic microleakage (Matharu et al., 2001), oral malodor generation (Pratten et al., 2003), corrosion potential of dental plaque (Wilson et al., 1995), and gene transfer in oral biofilms (Roberts et al., 2001). It is a sophisticated apparatus that can generate large numbers of individual biofilms and has been used extensively to investigate factors that may influence the growth of bacterial communities in the oral ecosystem. Figure 1B.5.2 provides a schematic representation of how the procedures detailed in this manuscript interrelate.

CAUTION: Follow all appropriate safety requirements relevant to the microorganism under investigation. Refer to *UNIT 1A.1* and other pertinent resources (see *APPENDIX 1B*) for instructions on safe handling of microorganisms.

STRATEGIC PLANNING

Nutrient Source and Operating Conditions

Supragingival plaque biofilms

Many studies use a simple mucin-containing artificial saliva (Pratten et al., 1998; Roberts et al., 1999) modified from the studies of Russell and Coulter (1975) and Shellis (1978; also see recipe); however, there are more defined growth media described in the literature (Wong and Sissons, 2001). The artificial saliva is delivered at a rate of 0.5 ml/min, corresponding to the resting salivary flow rate in humans (Pratten et al., 1998). Additionally, an aqueous solution of 2% (w/v) sucrose can also be pumped over the biofilms for periods of 30 min at the same speed via a second peristaltic pump. The sucrose pulsing can be carried out four times a day; during this period the artificial saliva supply should be maintained. The pH curve measured from the biofilm effluent under sucrose supplementation conditions has shown to be typical of the Stephen curve, i.e., falling and rising pH levels in response to sucrose supplementation (Zanin et al., 2005).

Subgingival plaque biofilms

Artificial gingival crevicular fluid (GCF; see recipe) is pumped into the CDFF at a rate of 50 μl/min, as an approximation of the flow rate of GCF during gingivitis. To model the periodontal region, an anaerobic atmosphere of 5% CO_2, 95% N_2 is provided by connecting a gas cylinder to the fermentor's air-inflow port. The pressure used should be 1 bar (100 kPa) and the flow rate 100 cm^3/min. Reinforced 5/16-mm i.d. PVC tubing is needed to connect the gas pressure gauge and the flow meter. Silicon tubing with an 8-mm i.d. connects the flow meter to the CDFF. The gas should be passed through air filters to maintain sterility. A Y-branch tubing connector can be used to supply gas to both the fermentor and the inoculum flask if required.

Perturbation

One of the main advantages of using this model is the ability to carry out antimicrobial and antiplaque testing over time. An agent can be pulsed in via one of the free inlet ports at appropriate times to mimic in vivo use, for example, pulsing twice daily (e.g., 9:00 a.m. and 5:00 p.m.) for 1 minute with 10 ml of 0.2% (w/v) chlorhexidine gluconate. To carry out pulsing at higher flow rates, wider bore pump tubing may be required. Additionally, a timer can be added to the power supply for the peristaltic pump to deliver a challenge to the CDFF without the operator being present.

With this setup, biofilms can also be removed from the CDFF and placed into an agent. In this case it is advisable to place the whole PTFE pan into the solution (5 ml in a 25-ml conical skirted-base tube to cover the pan) in order to reduce disruption of the biofilms. Using this method, it is possible to generate minimum inhibitory concentration (MIC)-like data for the biofilms.

INOCULATING THE CONSTANT DEPTH FILM FERMENTOR

BASIC PROTOCOL

Once the CDFF is sterilized with the pans in place (Support Protocols 1 and 2), the system is ready to inoculate with single-species or microcosm plaque inoculum (Support Protocol 5). When using a mixed-species inoculum, a direct inoculum method is used (see Support Protocol 5).

Materials

Bacterial growth medium (appropriate for the organisms being used)
10 ml single-species inoculum *or* 2 ml pooled saliva (see Support Protocol 5)
70% (v/v) ethanol
Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile

Air filters (e.g., Hepa-vent; Whatman)
Inoculum vessel: 500-ml Erlenmeyer flask with silicon rubber stopper having two metal tubes going through
3- and 5-mm bore silicone tubing (depending upon bore of stainless steel tubing), 15-cm and other lengths (depending upon setup)
Connectors and clips for silicone tubing (e.g., Sigma-Aldrich Z12, 654-3 and Z12, 651-9)
Couplers for connecting silicone tubing of different size bores (e.g., Value Plastics N220/210-6)
Nylon straps (for securing tubing)
Foil
Magnetic stir bar
Incubator, appropriate for inoculum used (see Support Protocol 5)
Magnetic stirrer
CDFF (sterilized; see Support Protocol 1), with motor and power supply unit
Peristaltic pump, precalibrated to desired flow rate
10- or 20-liter Pyrex glass bottles (for effluent collection and medium reservoir; size depending on flow rate used) with silicone rubber stoppers having two metal tubes going through
Glass grow-back traps (Hampshire Glassware)
Retort stand and clamp
Portable butane burner
Sample tool (wrapped in foil and autoclaved; provided with CDFF; see Support Protocol 1)
Forceps, sterile (e.g., flame sterilized in 70% v/v ethanol just before use or autoclaved)

Prepare CDFF inoculum vessel

1. Attach an air filter to the outside of one metal tube of the inoculum vessel stopper. To the other metal tube, attach a length of silicone tubing reaching down to the bottom of the flask (see Fig. 1B.5.3). On the opposite side of that metal tube connect a short length of pump tubing, using appropriate sized silicone tubing and couplers, as necessary to be able to pass the pump tubing through the peristaltic pump. Finally, add a connector to the end of the silicone tubing.

2. For any unused ports, place a piece of silicone tubing on the metal tubing and clamp.

3. Cover all connectors in foil. Add 500-ml medium and a magnetic stir bar to the vessel.

4. Autoclave the inoculum vessel with 500 ml bacterial growth medium 15 min at 121°C, leaving the stopper ajar. Also autoclave the effluent bottle with its attached air filter and tubing with coupling insert (with the stopper ajar).

   If the medium cannot be autoclaved, autoclave the vessel and tubing alone and then add the medium aseptically.

   The effluent bottle has two ports: one containing an air filter and the other a section of tubing and a coupling insert, which needs to be autoclaved with the stopper ajar.

   For convenience, the continuous culture medium and reservoir could also be autoclaved at this time (see steps 15 to 18).

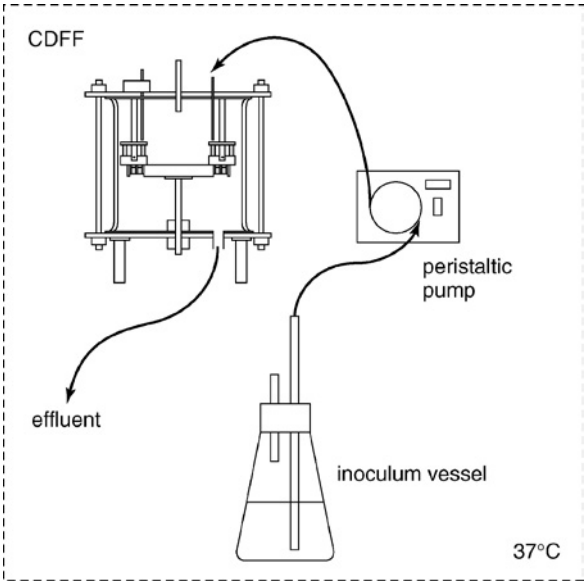

Figure 1B.5.3 Schematic of the setup required for inoculation of the constant depth film fermentor (CDFF).

5. As soon as it is safe to open the autoclave, quickly add any supplements, push the stopper firmly into place, and open the clamps to the air filters. Cool the medium to 22°C.

Inoculate the CDFF

6a. *For a single-species or microcosm inoculum:* Place either 10 ml single-species inoculum or 2 ml pooled saliva (see Support Protocol 5) into the inoculum vessel medium and proceed to step 7.

6b. *For a mixed-species inoculum:* Inoculate the CDFF as described in Support Protocol 5 and proceed to step 15 to perform continuous culture.

7. Put the inoculum vessel into the incubator on a magnetic stirrer.

8. Place the CDFF motor and power supply unit into the incubator and place the fermentor on top, ensuring the shaft for the turntable fits squarely into the motor section.

   NOTE: *This only applies to the v3 CDFF; previous versions require the motor to be attached to the CDFF turntable with four screws.*

9. Place the peristaltic pump into the incubator; feed the electric power cord through the hole in the top of the incubator.

10. Take the tubing from the inoculum vessel that has the pump tubing included and pass it through the pump before connecting to one of the inlet ports in the top-plate of the fermentor.

    It is best to loosen the foil on both, then aseptically join the two.

11. Switch on the pump at maximum speed to prime the line.

    The direction of flow can be changed once the tubing is connected.

12. When the inoculum starts to drip onto the turntable in the fermentor, switch on the turntable motor at ~3 rpm.

13. At this stage, turn the pump down to achieve the desired flow rate and pass the inoculum through the system for 8 to 24 hr at 37°C (see Support Protocol 5).

    NOTE: *The pump needs to be calibrated beforehand.*

14. Finally, attach the effluent bottle (while the fermentor is being inoculated) prepared in step 4.

    The vessel can sit on the laboratory floor (within an appropriate spill tray) to allow flow via gravity. If this is not possible an additional peristaltic pump may be required.

Set up and perform continuous culture

15. Attach a piece of silicone tubing to one of the ports in the medium reservoir reaching down to the bottom of the bottle (see Fig. 1B.5.4). On the opposite side of the stopper, attach 15-cm silicone tubing with a coupling body on the end.

16. Attach an air filter to the second port.

17. Add 8 liters bacterial growth medium to the medium reservoir and autoclave with the stopper ajar.

    Ideally, place the autoclave temperature probe (if available, set at 121°C) directly into the liquid.

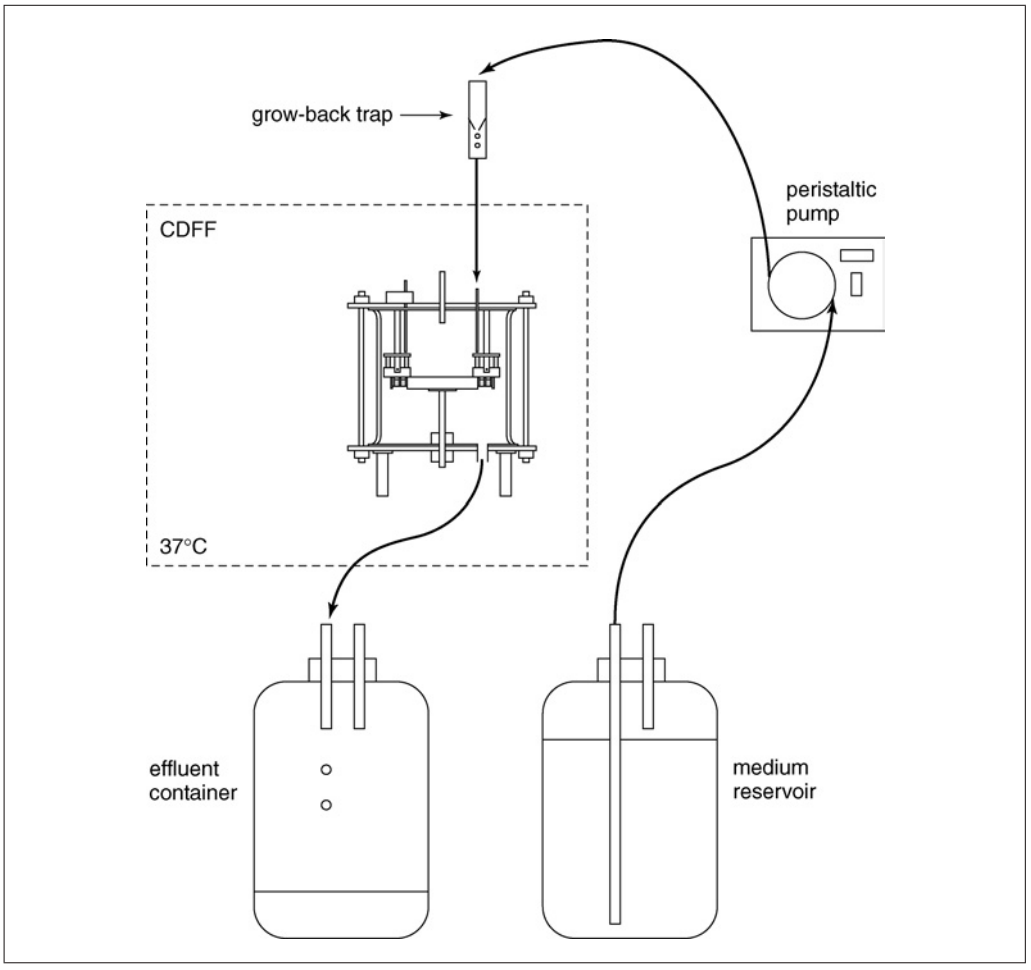

Figure 1B.5.4 Schematic of the constant depth firm fermentor (CDFF) system setup when running continuously. The dotted line represents the incubator. The feed line required from the peristaltic pump to the CDFF (including the grow-back trap) is described in the Basic Protocol.

18. As soon as it is safe to open the autoclave door, push in the stopper and unclamp the air filters.

19. Construct the feed line for the medium as follows by attaching a connector to a 30-cm piece of silicone tubing and link to a short piece of pump tubing, using appropriate couplers. Add an additional 15-cm piece of silicone tubing.

20. Add a glass grow-back trap between this piece of tubing and a further 15 cm of tubing ending with a connector to attach to the CDFF. Ensure that the grow-back trap is aligned so that the medium drips towards the CDFF.

    Further tubing may be required if different bore lengths of tube do not fit together. For example, a 5-mm bore tube will not fit the pump tubing connector, and in this case a length of 3-mm bore will be needed to join the two.

21. Cover the connectors of each feed line with foil and sterilize.

22. Switch off the pump and disconnect the inoculum vessel. Remove the pump from the incubator and place on top of the incubator together with the medium reservoir.

23. Attach the feed line to a retort stand on top of the incubator by clamping onto the grow-back trap.

24. Link the connector of the feed line (closest to the grow-back trap) to the connector of the second fermentor inflow and the second connector of the feed line to the medium vessel.

25. Pass the pump tubing of the feed line through the peristaltic pump.

26. Set the pump to maximum to prime the line until the medium starts to drip on to the rotating turntable. After this time, turn the speed of the pump down to the desired rate.

    The continuous system is fully set up.

Remove biofilm-containing pans

27. When the biofilms have developed to the desired state (typically ~2 weeks, maximum 1 month), switch off the turntable such that the desired pan comes to rest under the sample port.

28. Flame the sample port using 70% (v/v) ethanol and a portable butane burner.

29. Unscrew the port and insert the sample tool to remove a pan. Screw the sample port back into place as quickly as possible.

    During this process, the author suggests wearing a mask to avoid contamination.

30. Switch the turntable back on.

31. Remove the substratum from the sample pan by aseptically pushing each plug from below and removing the disk with sterile forceps.

32. Use biofilms either intact or place into sterile PBS. Vortex to remove the biofilm from the substratum.

33. Dismantle CDFF (Support Protocol 3).

SUPPORT PROTOCOL 1

SETUP FOR THE CONSTANT DEPTH FILM FERMENTOR (CDFF)

This protocol describes the assembly of the CDFF. Figure 1B.5.5 illustrates how the PTFE pans (see Support Protocol 2) fit into the assembled CDFF.

Materials

Spray lubricant (e.g., WD-40), optional
Silicon high-vacuum grease (VWR)

Constant depth film fermentor (CDFF; see Fig. 1B.5.1; contact Professor A. Peters for purchasing information; *APeters@UWIC.ac.uk*) including:
Motor
Power supply
15 PTFE pans, each with 5 plugs (see Support Protocol 2 for preparation)
Recess tools

8-G × 12 in. stainless steel needle tubing for CDFF inlet ports and vessels (e.g., SLS Scientific SRY700 8H)
3- and 5-mm bore silicone tubing (depending upon bore of stainless steel tubing), 15-cm and other lengths (depending upon setup)
Connectors and clips for metal tubing (e.g., Sigma-Aldrich Z12, 654-3 and Z12, 651-9)
Air filters (Hepa-vent; Whatman)
Nylon straps for securing tubing
Couplers for connecting pump tubing to 3-mm tubing (e.g., Value Plastics N220/210-6)
Foil
Autoclave tape
Incubator with ports at the top and side (for media in and effluent out) and gas tanks, as required (see Support Protocol 5) *or* 37°C room

Assemble CDFF

1. Place main fermentor tripod on the bench (Fig. 1B.5.6A).
2. Place the drive shaft of the stainless steel turntable through the hole in the base-plate of the main fermentor tripod: it should rotate freely (Fig. 1B.5.6B).

   A lubricant spray such as WD-40 can be used, if necessary.

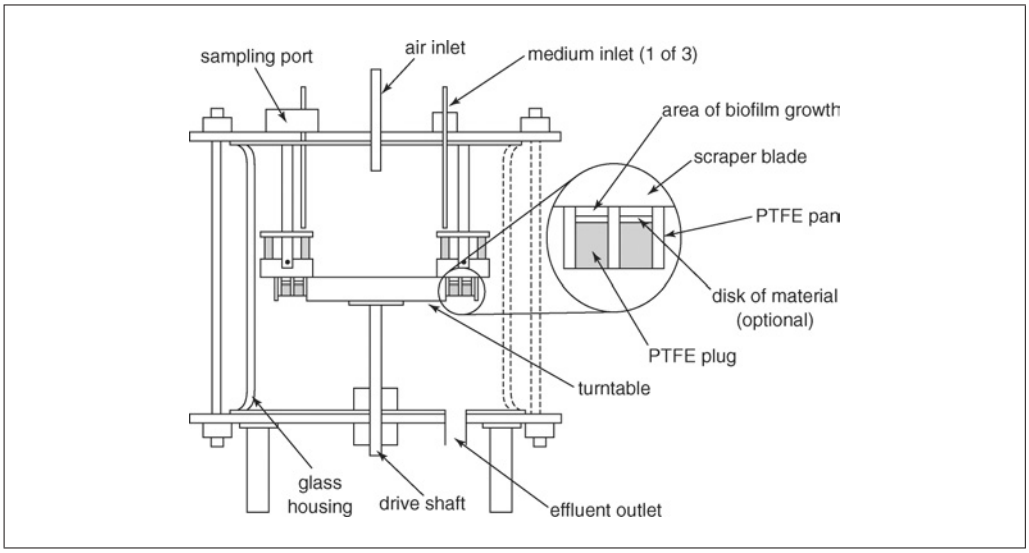

Figure 1B.5.5 Vertical schematic of the constant depth film fermentor (CDFF) highlighting one of the removable polytetrafluoroethylene (PTFE) pans.

3. *Optional:* Put PTFE pans in place.

   The PTFE pans can be assembled and put in place at this point or later.

4. Liberally smear the convex side of one PTFE seal with silicone high-vacuum grease, placing the grease downwards on the base plate of the main fermentor tripod (Fig. 1B.5.6C).

5. Liberally grease one end of the glass housing and place the greased end down onto the PTFE seal (Fig. 1B.5.6D).

6. Liberally grease the other end of the glass housing and put the second PTFE seal down on top of it (Fig. 1B.5.6E and F).

7. Liberally grease the PTFE seal and carefully place the fermentor top-plate down, ensuring that the scraper blades do not touch any of the grease. Secure the top-plate by tightening the screws using a wrench (Fig. 1B.5.6G).

8. Feed stainless steel tubing through the silicone stoppers of all three inlet ports of the top-plate of the fermentor.

9. Attach to these metal tubes 15 cm lengths of silicone tubing with a connector (coupling body) on the end (Fig. 1B.5.6H).

   It is easier to have all the connectors on the CDFF the same; this way when the tubing is connected, there will be no confusion about which connector type to use.

10. Attach silicone tubing with two air filters, with tubing between them, to the end of the port in the middle of the top-plate, which is for air-inlet (Fig. 1B.5.6H). Clamp off the filters at each end of the tubing with nylon straps before autoclaving.

    All tubing joints need to be secured using nylon straps.

11. Connect a length of silicone tubing with a connector on one end to the single drain hole on the base plate.

    The length of the tubing will depend upon the distance between the CDFF and the effluent bottle via the port in the incubator.

Sterilize CDFF

12. Cover all four connectors with tin foil and secure with autoclave tape (Fig. 1B.5.6H).

    If the connectors being used are not the self-seal type, then tubing clips will need to be added.

13. Place a piece of foil over the sample port, with the sample port open and the autoclave probe inserted into the fermentor. Autoclave the fermentor 15 min at 121°C.

14. As soon as the autoclave door can be safely opened (i.e., ideally while everything is still hot) remove the probe; quickly and aseptically screw the sample port tightly shut.

15. Remove the fermentor from the autoclave and open the clips on the filter tubing. Place into the incubator or 37°C room.

    Opening the clips will allow the air to enter the fermentor as it cools. Once cooled, the CDFF is ready for immediate use but could be stored until required.

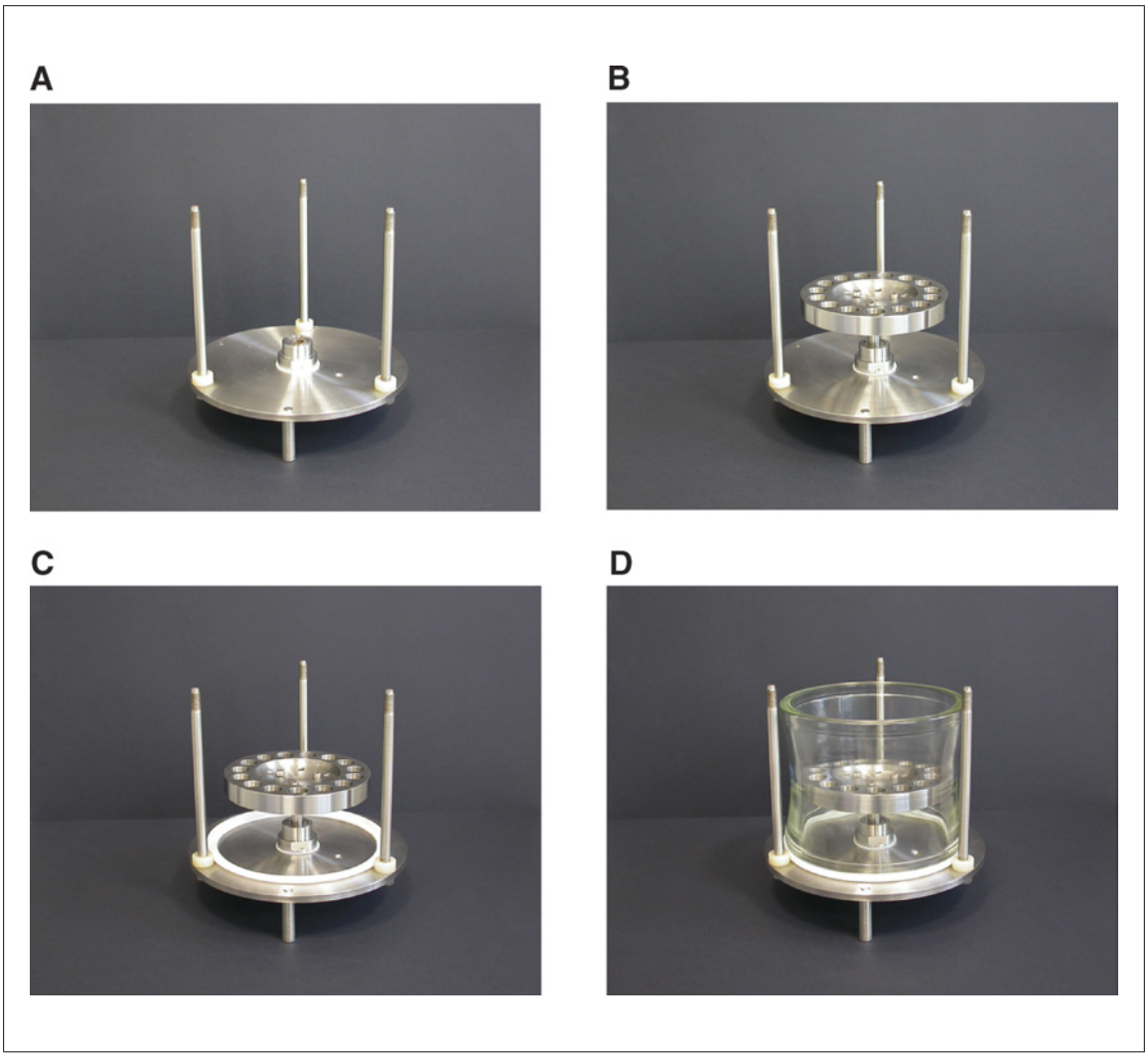

Figure 1B.5.6 (*continues on next page*) Pictures demonstrating the CDFF assembly procedure. The components are assembled in the following order: (**A**) fermentor tripod, (**B**) stainless steel turntable, (**C**) PTFE (polytetrafluoroethylene) seal, (**D**) glass housing, (**E**) application of silicone high vacuum grease, (**F**) second PTFE seal, (**G**) top plate, and (**H**) silicone tubing with connectors.

SUPPORT PROTOCOL 2

CONSTANT DEPTH FILM FERMENTOR PAN ASSEMBLY

Pan assembly can be carried out as part of the fermentor assembly, especially when using an inert substratum. However, certain materials may need to be kept wet, and it may be more convenient to assemble the fermentor and add the pans at the last moment before autoclaving. If the material cannot be autoclaved, it is possible to sterilize the CDFF as described in Support Protocol 1 and, in a sterile flow hood, remove the top-plate of the CDFF and place in pans/substratum which have been sterilized using an alternative method suitable for the material of choice. Pans should be stored placed within the CDFF. If left for a long period of time the pans can initially feel very tight, they will however loosen after an autoclave cycle. This can be carried out by autoclaving the turntable and pans alone before assembly, if necessary.

Generally the PTFE pans have five holes (each 5 mm in diameter) arranged around a central threaded hole into which the sampling tool is inserted (see Fig. 1B.5.7). When assembling the pans, it is important to have the central threaded hole uppermost. It is advisable to modify the pans by drilling a small hole (2 mm diameter) in the bottom of the threaded hole to allow liquid to pass through when sampling.

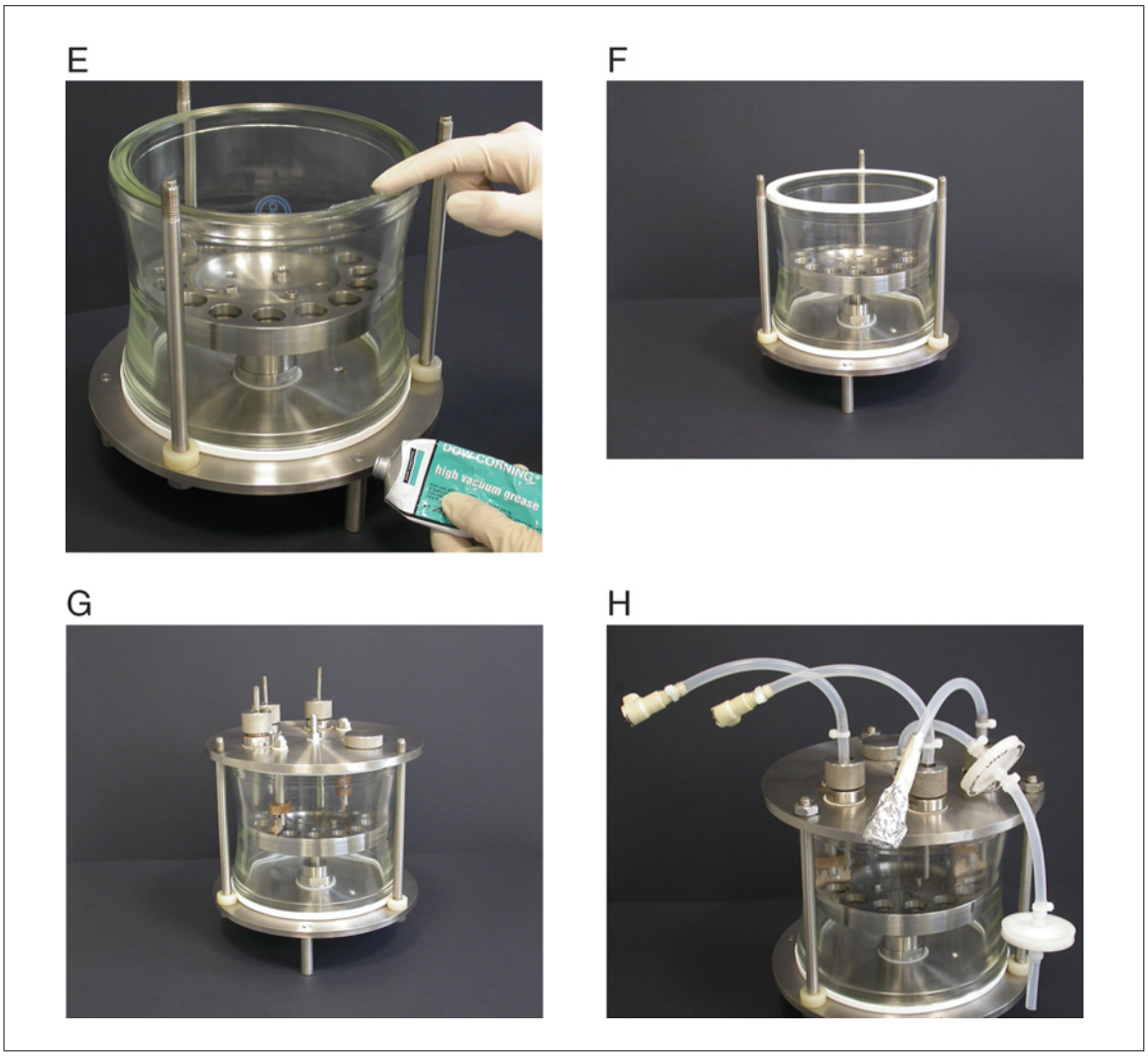

Figure 1B.5.6 (*continued*)

Materials

Silicone grease
Plugs: PTFE (supplied with CDFF; see Support Protocol 1)
PTFE pans (supplied with CDFF; see Support Protocol 1): drilling a 2-mm small hole in the bottom of the threaded hole recommended
Recess tool (supplied with CDFF; see Support Protocol 1)
Substratum (e.g., see Support Protocol 4)
CDFF turntable (supplied with CDFF; see Support Protocol 1)
Flat tool (supplied with CDFF; see Support Protocol 1)

1. Place the plugs into the holes in the pans (central threaded hole uppermost) with their flat surface uppermost.

2a. *For PTFE substratum:* Push the plugs level with the top of the pan and use the recess tool to push the plug down by the required depth.

The recess tool is simply a metal rod with a machined protrusion of known height. Two recess tools are supplied with the CDFF, allowing depths of 100, 200, 300, and 400 μm to be made; however, custom tools can also be made.

2b. *For other substratum:* Place a small amount of silicone grease between the PTFE and the chosen substratum to hold the material in place during autoclaving.

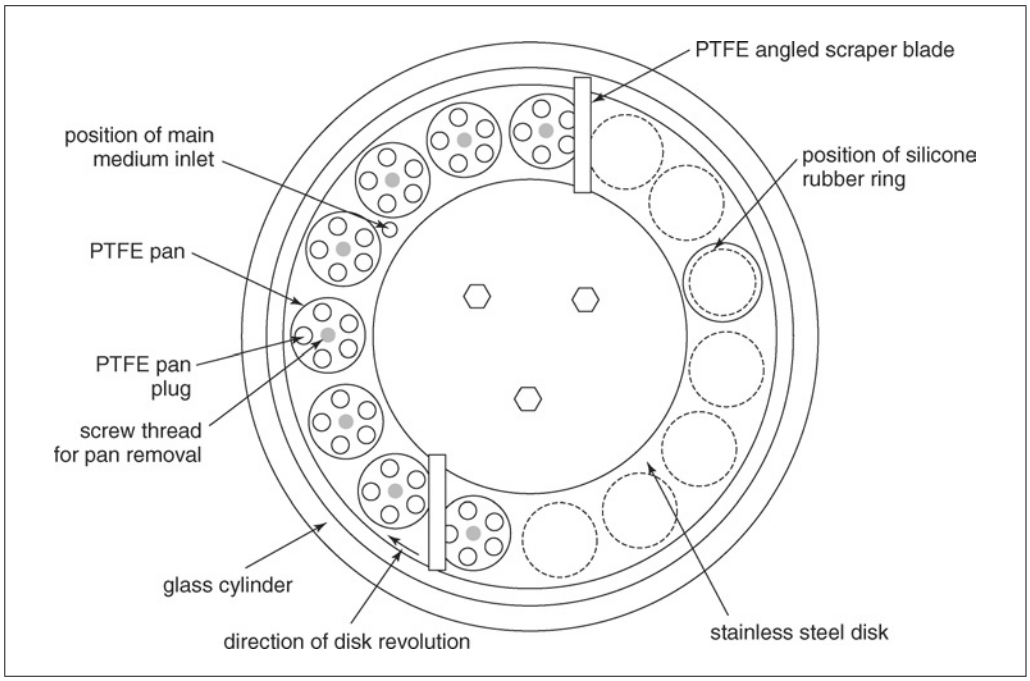

Figure 1B.5.7 Sectional schematic showing the arrangement of PTFE pans within the stainless steel turntable of the CDFF. Abbreviations: CDFF, constant depth film fermentor; PTFE, polytetrafluoroethylene.

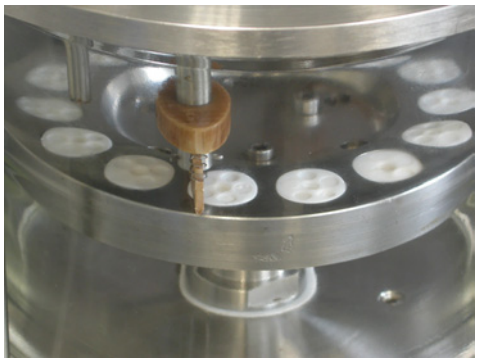

Figure 1B.5.8 Placement of pans in the CDFF.

i. Starting with the plugs slightly raised above the pan, place a small amount of silicone grease on the PTFE plug, place the disk on top of the plug, push down, and wipe off excess grease.
ii. Push the plug up again slightly from underneath and then push down with the appropriate recess tool.

3. Place the pans into the holes of the stainless steel turntable (Fig. 1B.5.8) using a flat tool to ensure they are flush.

SUPPORT PROTOCOL 3

DISMANTLE THE CONSTANT DEPTH FILM FERMENTOR AND PREPARE FOR REUSE

After the experiment has been carried out, it is important to ensure that the CDFF and effluent bottle are sterilized prior to cleaning.

Materials

CDFF with biofilm-containing pans removed (Basic Protocol)
(0.01% v/v) Decon acid rinse (Decon Laboratories)

1. Switch off the turntable motor.
2. Switch off the pump and release the tubing from the pump head.
3. Disconnect from the effluent bottle and medium inflow tubing.
4. Remove the air filter and ensure that the sample port is open.
5. Autoclave the CDFF on a kill cycle.
6. Autoclave the effluent bottle on the same cycle as above, placing the temperature probe (if available, set at 121°C) into the liquid.
7. Take apart the CDFF and leave to soak overnight in dilute (0.01% v/v) Decon acid rinse.

After autoclaving, the tubing may be reused for future experiments; however, before setting up a new system, be sure to examine the entire length of each piece of tubing for holes that could cause leaks.

PREPARING THE SUBSTRATUM

SUPPORT PROTOCOL 4

Several options are available in choosing the appropriate substratum to be used in the CDFF. Biofilms may be formed directly on the surfaces of the PTFE plugs of the sample pan. The number of plugs per pan will depend on their diameter, usually (but not necessarily) 5 mm, allowing five biofilms per pan. Custom pans can be manufactured with plugs of different diameter. Alternatively, the plugs may be used to support disks of any other material of interest to support biofilm growth, as long as the material can be machined to form a substratum of appropriate diameter and tolerance. CDFF studies have successfully used the following substrata: enamel (Zanin et al., 2005), hydroxyapatite (Pratten et al., 2003), dentine (Deng et al., 2005), glass (Allan et al., 2002), denture acrylic (Lamfon et al., 2005), intra-oral magnets (Wilson et al., 1995), dental composite materials (Leung et al., 2005) and dental amalgam (Ready et al., 2007).

Hydroxyapatite

Hydroxyapatite disks may be purchased from Clarkson Chromatography Products. Alternatively, they may be made by pressing hydroxyapatite powder into 5-mm diameter disks (3 mm depth) in a 5-mm pellet die under a pressure of 250 kg. The disks are then sintered 1 hr at 1000°C in a furnace.

Denture acrylic

Denture acrylic (polymethylmethacrylate cold-cure acrylic; e.g., Dentsply Limited) disks can be prepared, according to the manufacturer's instructions, using self-cure acrylic. In order to produce disks of the correct size, the CDFF pans can be used as 5 × 1–mm molds.

Enamel and dentine

Enamel and dentine disks can be prepared using extracted teeth (sterilized and washed). They are cut into 5-mm-diameter cylinders using a trepanning tool on a metal worker lath/drill before being sectioned using a rotary diamond saw. Further information can be found in Matharu et al. (2001).

INOCULATING THE CONSTANT DEPTH FILM FERMENTOR

Single-species inoculum

Inoculate 10-ml of medium (e.g., artificial saliva; see recipe) from a single colony (e.g., obtained from the National Collection of Type Cultures) and incubate for the desired time. Adjust the inoculum to an optical density of 1.0 at 600 nm. Carry out inoculation and culture in the CDFF as described in the Basic Protocol for a minimum of 8 hr, depending upon the expected growth rate of the particular organism.

NOTE: Some oral microorganisms may not be able to produce biofilms as single species. For example, late colonizers such as *Porphyromonas gingivalis* will not be able to adhere directly to the substrate when cultured alone.

Mixed-species inoculum

Inoculate 10-ml of medium (e.g., artificial saliva; see recipe) for each bacterial species from a single colony and incubate for the desired time for each of the species being used. Adjust the optical density of each culture to 1.0 at 600 nm and store separately in 1.0-ml aliquots up to 3 months at −80°C. Prior to inoculating the CDFF, thaw two cryovials of each bacterial suspension at room temperature, transfer the contents to a 10-ml sterile universal, and vortex mix. Using a 10-ml sterile pipet, carefully add the suspension to the rotating pans via the sample port of the CDFF. Allow to rotate an additional 1 hr. After this time, connect the CDFF to a reservoir of sterile medium and culture as described in the Basic Protocol, starting at step 15.

Microcosm plaque biofilms

Human saliva can be used as an inoculum to provide a multispecies biofilm consisting of microorganisms found in the oral cavity. Collect saliva from at least ten healthy individuals, preferably who have not taken antibiotics in the previous three months. Pool equal volumes from each of the subjects. Store in 1-ml aliquots up to 3 months at −80°C for subsequent use. To grow biofilms, proceed as in the Basic Protocol, using two 1-ml aliquots to inoculate 500 ml sterile growth medium in step 6 and culturing 8 hr in the CDFF at a flow rate of 1 ml/min in step 13.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Artificial gingival crevicular fluid (GCF)

40% horse serum (not heat-inactivated) in RPMI with L-glutamine (Sigma) containing 5 μg/ml hemin and 0.5 μg/ml menadione (see Dalwai et al., 2006). Store up to 1 month at 4°C.

Artificial saliva

1 g Lab-lemco powder (Oxoid)
2 g yeast extract
5 g proteose peptone (Oxoid)
2.5 g hog gastric mucin (Sigma)
0.35 g sodium chloride
0.2 g calcium chloride
0.2 g potassium chloride
Adjust volume to 1 liter with H_2O
Autoclave, then add 1.25 ml of 40% (w/v) urea
Store up to 1 month at 4°C

continued

The artificial saliva formulation given is the same as "complete saliva" in Pratten et al., 1998. Due to the light-weight nature of hog gastric mucin extra care should be taken when weighing.

COMMENTARY

Background Information

Models of oral biofilms, including laboratory, animal, and human in situ models, contrast noticeably in their microbiological complexity. Animal models have proven unequivocally that formation of dental caries is an infectious and transmissible disease. Use of biofilm models has enabled comparisons to be made of the cariogenic potential of different bacterial species, the role of the diet, and the effects of potential anticaries agents. In situ human models created using devices such as the Leeds in situ plaque-generating device have been designed to permit the development of "natural" plaque on standardized enamel surfaces freely exposed to the human oral environment (Robinson et al., 1997).

However, there are problems with the ethics associated with animal and human models. In addition, dental diseases are site specific, and the plaque biofilm is heterogeneous, which can lead to problems with sampling. Further, these intra-oral models suffer from the inability to use them for testing experimental products, especially in terms of safety to the patient. These restrictions have led to the development of laboratory-based models that simulate oral biofilms in vitro. Such models range from simple batch culture systems (e.g., the Zürich Biofilm Model; Guggenheim et al., 2004), to the development of artificial mouths.

In chemostat models there is a controlled fluid environment and planktonic phase, with a nutritionally limited growth rate. A number of replicate samples and a variety of surfaces can be placed within the chemostat, and properties such as the pH can be controlled. However, the large fluid phase is not consistent with the in vivo situation where shear forces and a thin film of fluid are present on the oral surfaces.

The flow cell (see *UNIT 1B.2*) is often the system of choice for short-term adhesion and colonization studies because it enables the nondestructive observation of biofilm development in real time (Busscher and van der Mei, 1995). The flow cell may be constructed in a number of ways but typically consists of a transparent chamber of fixed depth through which the growth medium passes. When mounted on a microscope stage, this system allows the biofilm development inside the chamber to be observed in real time. Other models which produce biofilms under flow conditions include the Modified Robbins Device (MRD) and the Annular Reactor. The MRD contains six or more individual ports in a linear array along a channel of rectangular cross-section. Biofilms can be removed during the course of an experiment, and the model has been used extensively to generate oral biofilms. In contrast, the Annular Reactor produces biofilms under shear conditions in a steady-state system in a similar manner as a chemostat.

Historically, so-called "artificial mouths" have been used to grow dental plaque in vitro, before the implementation of the biofilm models described previously. Artificial mouths are specifically constructed to mimic the situation in the oral cavity although most studies have focused on dental caries. In such systems, dental plaque formation is studied on a human tooth inoculated with saliva and then supplied with artificial saliva. For example, Pigman and Elliott (1952) developed a model for studying early carious lesions in order to evaluate the anticaries effects of several topical agents used as dentifrice components. The development of such models has led to sophisticated systems such as the multiple artificial mouth (MAM). This is a computer-controlled model which delivers nutrients, reagents, and gas, and continuously acquires pH data, including real-time pH changes of plaque. The system has proven to be a powerful and flexible tool for continuous monitoring of plaque growth and metabolism (Sissons et al., 1991).

All of these models are compromises between the reality of the in vivo ecosystem and the simplification and controllability necessary to gain meaningful, useful results in the laboratory. As described, there are many experimental systems available for the generation and study of in vitro dental plaques, such that a suitable system can be chosen to meet the requirements of almost any investigation. The CDFF is not principally designed to study early biofilm formation or for studies where a high shear-rate is required. However, some of the advantages of this model for experiments on oral biofilms include:

1. It represents a good simulation of the oral environment in that the fluid phase (e.g., saliva or GCF) is continuously smeared in a thin layer over the biofilms/substrata. In this

way it is an improvement over many laboratory models that involve immersing the substratum in a large excess of fluid.

2. Mechanical shearing of the biofilms by the scraper blades mimics the shearing that takes place in vivo due to tongue movements, biting, chewing etc.

3. It allows intermittent pulsing (or continuous application) of the antimicrobial agents and/or additional nutrients such as sucrose.

4. It allows easy sampling of the biofilm (with large numbers of replicates) at various intervals during the course of a run.

5. Biofilms of different thicknesses can be investigated.

6. Pure or mixed cultures of bacteria, saliva, or homogenized-plaque samples can be used as an inoculum.

7. A number of substrata can be investigated.

8. It enables the determination of the effects of long-term exposure of biofilms to antimicrobial agents.

Critical Parameters

The most debated parameter when carrying out studies on oral biofilms is the choice of microorganism or microorganisms. This is no different for the CDFF where, as already described, the operator can use single-species, multiple-species, or microcosm biofilms. Issues arise as to whether the studies are associated with biofilm formation (e.g., where primary colonizer such as *Streptococcus sanguis* is studied) or a particular disease (e.g., caries where only *S. mutans* is of interest). When considering the criteria for a community study, the organisms selected need to originate from a pool of species common to the oral environment and important components of that community. Further, the group of species should form a stable community where, under standard conditions, all members are retained over the course of the experiment. To further produce biofilms which are more representative of the in vivo scenario, the establishment of laboratory microcosms in association with a suitable substratum and nutrient source is useful.

Another important consideration is the medium to be used. Examples of an artificial saliva and artificial GCF are given in this unit. Through its chemical and physical properties, saliva exerts a profound influence on the adhesive interactions that occur in the mouth, and thus choosing a suitable artificial saliva is important. Due to the obvious difficulty in using large volumes of human saliva, a synthetic substitute will be needed to reproduce features of saliva which are considered important in cultural studies of plaque bacteria while remaining relatively inexpensive and simple to produce (see Strategic Planning).

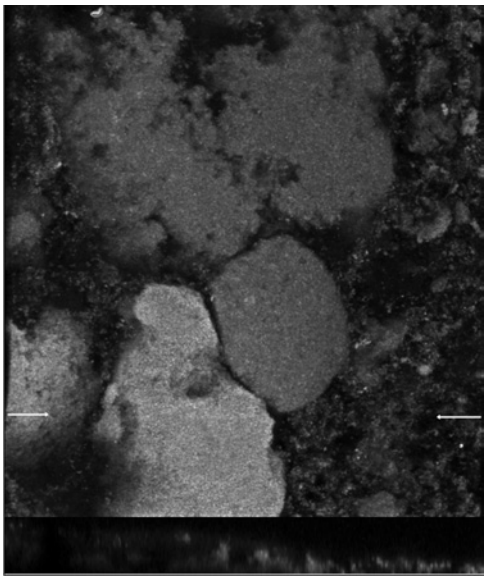

Figure 1B.5.9 Three-dimensional confocal scanning laser microscopy (CLSM) reconstruction of a supragingival plaque biofilm based on a series of *x,y* projections. The arrows indicate the position of the sagittal section (6 μm thick).

Anticipated Results

It has been shown that using the CDFF model, quasi steady-state biofilms can be generated after 4 to 7 days of growth, depending upon the organisms being studied. Such biofilms typically contain $\sim 1 \times 10^8$ cfu/mm^2. The reproducibility of the model has been shown to be good, both between pans within one experiment and between experiments. This is particularly true with work on single-species biofilms. However, as might be expected in studies on microcosms where the inoculum contains a diverse bacterial population, the resultant biofilms are far more heterogeneous. These results are less comparable, in terms of the defined bacterial population, although this depends upon the sensitivity of the technique used for quantification (McBain et al., 2003).

The microstructure of oral biofilms, as shown by confocal laser scanning microscopy (CLSM), has revealed stacks of bacteria developing over time, separated by clear channels (Fig. 1B.5.9). Additionally, the distribution of viable bacteria in biofilms grown in the CDFF has been shown to be similar to those in dental plaque in vivo. This suggests that the CDFF produces in vitro biofilms which are comparable to their in vivo counterparts in terms of both structure and the spatial distribution of viable bacteria.

When biofilms generated in the CDFF are challenged with an antimicrobial, the results are sensitive enough to be able to compare different treatment regimes. Such data has been shown to be comparable, in terms of ranking agents, to data from clinical trials.

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Internet Resources

http://www.imt.net/~mitbst/Products.html

BioSurface Technologies produces several of the biofilm reactors described, including flow cells, annular reactors, and drip flow reactors.

http://www.tylerresearch.com/instr/biofilm.shtml

Tyler research produces several biofilm-generating devices, including high-pressure and laminar devices as well as chemostats.

Methods for Analysis of Bacterial Autoinducer-2 Production

BASIC PROTOCOL

Many bacteria possess homologs of the *luxS* gene, which is responsible for the production of the quorum-sensing signal autoinducer-2 (AI-2). This unit describes a biological assay for detecting and quantifying AI-2 present in bacterial culture fluids. The procedure employs a bioluminescent bacterial reporter strain, *Vibrio harveyi* BB170, which produces light in response to AI-2. The first step of the protocol involves the collection of cell-free culture fluids. In the second step, the cell-free culture fluids are added to the *V. harveyi* reporter strain, and the resulting light production is measured using a luminometer or scintillation counter. AI-2 activity is calculated as the induction of luminescence of *V. harveyi* BB170. This protocol focuses on techniques for determining the growth conditions under which AI-2 is produced by a bacterial strain of interest. The procedure can also be used to quantify AI-2 produced by various bacterial mutants, as well as AI-2 produced in vitro. The assay is a simple and relatively inexpensive method for quantifying AI-2 and can, therefore, be used to screen a large number of samples.

Materials

Bacterial strain to be tested
Growth medium specific for bacterial strain
V. harveyi strain in BB152 (*luxM*::Tn5), frozen glycerol stock
V. harveyi strain in BB170 (*luxN*::Tn5), frozen glycerol stock
AB medium (see recipe)

Sterile syringe filters, 0.2-μm pore size
1-ml syringes
Sterile 1.5-ml microcentrifuge tubes
Luminometer, scintillation counter, or equivalent device for measuring light

CAUTION: *V. harveyi* is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: If measuring a large number of samples, use a 96-well microtiter filter plate with a sterile 96-well plate for collection rather than individual syringes, syringe filters, and microcentrifuge tubes.

NOTE: BB170 and BB152 strains (Bassler et al., 1993) are available at American Type Culture Collection (*http://www.atcc.org*) with strain ATCC #BAA-1117 and ATCC #BAA-1119, respectively.

Prepare cell-free culture fluids

1. Inoculate the bacterial strain to be tested for AI-2 production into appropriate medium and grow to saturation.

   Do not add antibiotics to the growth medium as they can inhibit the reporter strain's growth, bioluminescence, or both (see Critical Parameters).

2. Dilute the culture 1:1000 into fresh medium and resume growth.

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3. Remove 1-ml aliquots at time points of interest. Remove cells by passing through 0.2-μm syringe filters into sterile 1.5-ml microcentrifuge tubes. Use a separate syringe and filter for each sample.

   If using a 96-well filter plate, remove 0.2-ml aliquots and filter directly into a sterile 96-well plate.

   If the AI-2 production profile of the organism has not been characterized, measure AI-2 production over a time course of growth. This is important because many bacteria produce AI-2 only during a particular phase of growth. For example, maximal AI-2 accumulation in V. harveyi occurs in the stationary phase, whereas in Salmonella typhimurium, AI-2 is detectable transiently during exponential growth (Surette and Bassler, 1998). For bacteria with rapid doubling times, collect cell-free culture fluids every hour after the initial dilution, and continue until the culture reaches the stationary phase.

   In some cases, it is necessary to use the cell-free culture fluids immediately or the day after collection. The cell-free culture fluids of some bacteria such as V. harveyi can be stored at −20°C without loss of AI-2 activity, while the AI-2 activity of other bacteria such as E. coli diminishes during storage, especially with repeated freezing and thawing.

4. Collect cell-free culture fluids from a culture of *V. harveyi* strain BB152 (i.e., AI-2$^+$) grown overnight in AB medium to use as a positive control. Store this sample indefinitely at −20°C and use in future experiments.

Perform BB170 bioassay

5. Inoculate *V. harveyi* strain BB170 from a glycerol frozen stock into 5 ml AB medium. Incubate overnight (~14 hr) at 30°C with aeration until the culture is turbid (OD_{600} = ~0.7 to 1.2).

   Inoculation from a frozen stock is preferable to inoculation from a colony or liquid culture, as cultures begun from frozen stocks produce maximum light.

   Never store V. harveyi cultures in a refrigerator or cold room because this will dramatically decrease the viable cell number.

6. Verify that the culture is bright by examining in a darkroom or by using a luminometer. Dilute the BB170 overnight culture 1:5000 into fresh AB medium.

   The volume required will depend on the number of samples to be tested and the method of light detection (see step 7).

7. Set up a series of duplicate mixtures containing 10% (v/v) cell-free culture fluids prepared in step 3 and 90% (v/v) diluted BB170 culture prepared in step 6. Incubate at 30°C with aeration.

   If using a 96-well microtiter plate reader for light measurement, set up 100-μl samples per well (i.e., 10 μl cell-free culture fluids and 90 μl diluted BB170) in clear-bottomed black microtiter plates such as Corning 3603 or clear assay plates such as Falcon 353911. If using a luminometer that holds individual tubes, prepare 2- to 5-ml samples in glass test tubes.

8. In each experiment, include the following control samples in duplicate:

   Negative control: 10% AB medium
   Medium control: 10% bacterial growth medium used in step 2
   Positive control: 10% cell-free culture fluids prepared from *V. harveyi* BB152 (step 4).

9. Using a luminometer, measure the light produced in each sample at a wavelength of 490 nm (for some luminometers, this setting is the chemiluminescent mode). Take measurements every 30 min for 5 to 7 hr, i.e., until the medium control sample has produced the lowest amount of light (see Background Information; also see Anticipated Results).

If using a 96-well microtiter plate reader, measure the light output from each well. If using a luminometer that holds individual tubes, remove 100-μl aliquots from each sample and measure the light output.

Any one of a variety of light detection devices can be used in this assay, from a simple photomultiplier tube to an automated microplate reader. The optimal device for the assay will depend on the number of samples per experiment, frequency of use, and economical considerations.

REAGENTS AND SOLUTIONS

Use Milli-Q water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AB medium

Dissolve the following ingredients in 960 ml H_2O:
2.0 g Casamino acids, vitamin-free (0.2% w/v final)
12.3 g magnesium sulfate heptahydrate (50 mM final)
17.5 g sodium chloride (0.3 M final)
Adjust pH to 7.5 with potassium hydroxide
Sterilize by autoclaving
When cool, add the following sterile ingredients to give the following final concentrations:
1 mM L-arginine from 100× stock (pass stock through a 0.2-μm filter and store indefinitely at 4°C)
1% (v/v) glycerol from 50× stock (sterilize stock by autoclaving and store indefinitely at room temperature)
10 mM potassium phosphate buffer, pH 7.0 (*APPENDIX 2A*) from 100× stock (sterilize stock by autoclaving and store indefinitely at room temperature)
Store indefinitely at room temperature

Never add glucose to the medium. Glucose inhibits bioluminescence of V. harveyi (see Critical Parameters).

COMMENTARY

Background Information

Autoinducers are signaling molecules that bacteria secrete and detect to regulate the expression of specific genes based on cell population density. This process, known as quorum sensing, was initially discovered as the mechanism by which marine *Vibrio* species coordinately control bioluminescence of the population (Nealson and Hastings, 1979). Though initially considered a curious phenomenon limited to luminous marine bacteria, quorum sensing has since been shown to regulate a variety of functions in >100 different bacterial species, including virulence, sporulation, antibiotic production, and motility (Miller and Bassler, 2001; Federle and Bassler, 2003).

The autoinducer termed AI-2 was identified as one of two signaling molecules that control bioluminescence in *Vibrio harveyi* (Bassler et al., 1994). AI-2 is of particular interest because it deviates from the quorum-sensing paradigm in three significant ways. First, most autoinducer/detection systems are specific to a single species of bacteria. In contrast, the AI-2 synthase, LuxS, is broadly distributed throughout the bacterial world, and AI-2 production has been demonstrated in >50 species of bacteria (Bassler et al., 1997; Surette et al., 1999). Second, it is clear that AI-2, like other autoinducers, functions as a quorum-sensing signal in some bacteria, as AI-2 regulates virulence, biofilm formation, antibiotic production, and iron acquisition in various bacterial species (Fong et al., 2001; McNab et al., 2003; Xavier and Bassler, 2003; Sircili et al., 2004). However, AI-2 may also function in processes unrelated to quorum sensing in some bacteria (Winzer et al., 2002; McNab and Lamont, 2003). Third, in most quorum-sensing systems, the concentration of autoinducer is considered to be an indicator of cell population density, with the highest concentration of autoinducer occurring when the bacteria are most dense. In some bacteria, AI-2 production

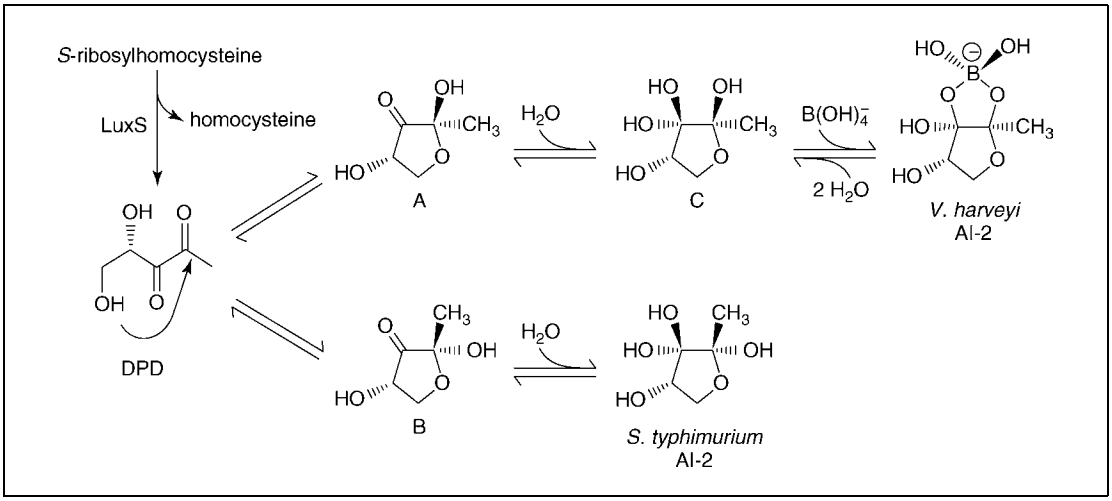

Figure 1C.1.1 Proposed pathway for formation of AI-2 molecules. DPD cyclizes spontaneously to form two stereoisomers, A and B. Spontaneous addition of water to A and B results in formation of C and *S. typhimurium* AI-2, respectively. *V. harveyi* AI-2 is formed by addition of boric acid to C (Modified from Miller et al., 2004).

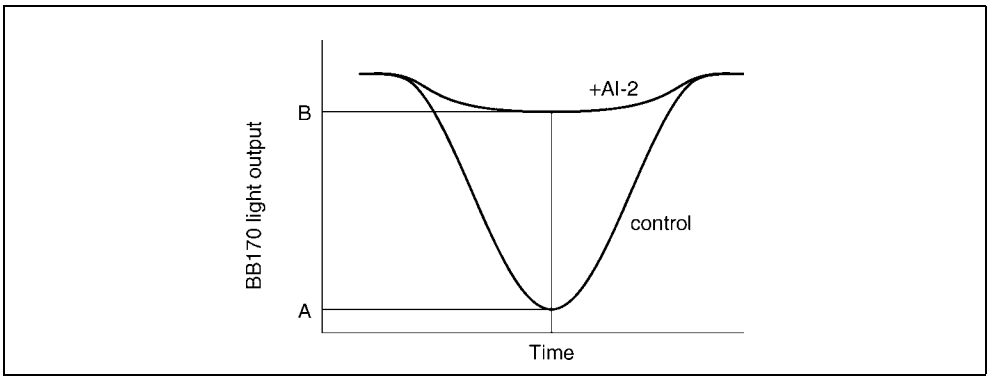

Figure 1C.1.2 Light production in the BB170 bioassay. Induction of bioluminescence is calculated at the time point marked by the vertical line.

follows this pattern, while in others, such as *S. typhimurium*, AI-2 is degraded in late exponential phase (Surette and Bassler, 1998, 1999; Taga et al., 2001).

The term "AI-2" refers to a family of molecules derived from 4,5-dihydroxy-2,3-pentanedione (DPD), the product of the LuxS reaction (Miller et al., 2004). LuxS catalyzes the formation of DPD from *S*-ribosylhomocysteine, a product of the *S*-adenosylmethionine utilization pathway (Schauder et al., 2001). DPD, in turn, cyclizes spontaneously to form an assortment of different stable compounds (Fig. 1C.1.1). Structural studies of two AI-2 receptors, *V. harveyi* LuxP and *S. typhimurium* LsrB, revealed that each AI-2 receptor specifically binds one molecule from this pool (Chen et al., 2002; Miller et al., 2004).

In BB170, the sensor for AI-1, another *V. harveyi* autoinducer, is mutated, and thus BB170 produces light exclusively in response to AI-2 (Bassler et al., 1994, 1997). Additionally, the *luxS* gene (i.e., the AI-2 synthase) is intact, so this strain produces AI-2 and induces light production at a high cell density (Bassler et al., 1994). In the absence of exogenously supplied AI-2, light production in the BB170 bioassay decreases initially for the first 3 to 5 hr. At later time points, the AI-2 produced by BB170 accumulates and induces light production (Fig. 1C.1.2, control). AI-2 added exogenously (i.e., in cell-free culture fluids) begins to induce light production in BB170 at a time point prior to self-induction by AI-2 (Fig. 1C.1.2, +AI-2; Bassler et al., 1993). The amount of AI-2 in the cell-free culture fluids being tested must be calculated at the time point immediately preceding self-induction of BB170 (Fig. 1C.1.2, vertical line), because eventually, all samples produce maximal light.

Table 1C.1.1 Troubleshooting Guide for BB170 Bioassay

Problem	Probable cause	Solution
There is no detectable light in the bioassay after several hours	The initial culture of BB170 was not bright due to contamination or a problem with AB medium	Examine the initial (undiluted) culture of BB170 in a darkroom. If the culture is turbid but no light is detectable, prepare fresh AB medium and begin the assay again.
	The luminometer is not functioning correctly	Examine the initial (undiluted) culture of BB170 in a darkroom. If the culture is bright (i.e., produces light), measure the light produced using the luminometer. If no light is detected, check the settings or contact the manufacturer.
	The temperature of the incubator is not set to 30°C	*V. harveyi* does not grow at low temperatures and does not produce light at temperatures >30°C. Perform all manipulations of *V. harveyi* at 30°C.
All samples are bright in the bioassay, including the negative control and the medium control	The bioassay has gone too long	Take readings every 30 min in order to find a time point with the lowest bioluminescence in the medium control.
	The initial dilution of BB170 contained too many cells	Grow the initial BB170 culture for no longer than 14 hr. Alternatively, perform the assay with a 1:10,000 initial dilution of BB170.
	The AB medium is contaminated with bacteria producing AI-2	AI-2-producing bacterial contaminants in the media can induce bioluminescence of BB170. Perform the assay with sterile AB medium and culture fluids.
	The wrong plates are being used (if using a 96-well plate reader)	Use clear-bottomed black microtiter plates. For best results, leave at least one empty well between samples.
The medium control alone induces bioluminescence of BB170, but the negative control does not	The bacterial growth medium includes boron or another agent that induces bioluminescence in *V. harveyi*	Reduce the amount of boric acid in the bacterial growth medium to <10 μM. If the medium control induces bioluminescence but does not contain boric acid, prepare cell-free culture fluids from bacteria grown in a different medium.
The positive control induces light production, but the cell-free culture fluids do not	The strain of bacteria being tested does not produce AI-2 at the time points examined	In step 2, use a smaller initial inoculum before collecting cell-free culture fluids to reduce the effect of AI-2 degradation. Collect cell-free culture fluids at shorter time intervals during all phases of growth. Examine the sequence of the *luxS* gene and promoter to determine whether it is likely to be expressed.
	The bacteria do not produce AI-2 under the conditions being tested	Vary the growth parameters of the bacteria being tested. For example, change the medium, temperature, aeration, and addition of host factors.

continued

Table 1C.1.1 Troubleshooting Guide for BB170 Bioassay, *continued*

Problem	Probable cause	Solution
	The bacterial growth medium inhibits bioluminescence or is toxic to *V. harveyi*	Determine whether induction of bioluminescence occurs at late time points in the medium control. If no induction occurs, prepare cell-free culture fluids from bacteria grown in a different medium.
	The bacteria produce an agent that inhibits bioluminescence or is toxic to *V. harveyi*	If the bacteria are known to produce a toxic agent, such as an antibiotic, collect cell-free culture fluids under conditions in which the agent is not produced.
		Alternatively, perform the bioassay using 1% cell-free culture fluids.
The light output is inconsistent between duplicate samples	The luminometer is not optimized for the bioassay	Examine the manufacturer's instructions and adjust the settings of the luminometer.
	The *V. harveyi* culture is not aerated properly	Always grow *V. harveyi* with aeration: the luciferase reaction requires oxygen.

V. harveyi strain BB170 is the most reliable reporter for AI-2 activity that presently exists. The difference in the timing of light production in response to the AI-2 in the culture fluids being tested versus AI-2 produced endogenously is critical for the quantification of AI-2 activity. Additionally, self-induction by BB170 is an important indicator that the assay is functioning properly and is particularly helpful in the initial characterization AI-2 production by a bacterium. Another *V. harveyi* reporter with a *luxS* mutation has been constructed (Chen et al., 2002). Because self-induction with AI-2 does not occur in this strain, it is not possible to calculate induction of bioluminescence by the method used for BB170, and therefore, it is generally used only in specific circumstances such as examining the effect of boron on light production (Chen et al., 2002; Miller et al., 2004). AI-2 reporter strains of *S. typhimurium* also exist, but these strains are ~100-fold less sensitive to AI-2 than the *V. harveyi* reporters and typically cannot detect AI-2 in cell-free culture fluids (Taga et al., 2001; Taga et al., 2003).

The AI-2 detection procedure described in this unit is a biological assay that relies on the AI-2 detection system of *V. harveyi*. BB170 is extremely sensitive to the level of AI-2, as it varies its light output over three orders of magnitude in response to changes in the amount of AI-2 present; however, because it is a biological assay, it is limited by the requirement for the *V. harveyi* reporter strain to produce light in the presence of the added agents. Any compounds that inhibit bioluminescence or are toxic to *V. harveyi* interfere with this bioassay (DeKeersmaecker and Vanderleyden, 2003).

Critical Parameters

To perform the bioassay accurately, it is important to avoid conditions that can adversely affect the growth or bioluminescence of the *V. harveyi* reporter strain. Factors that inhibit the growth or bioluminescence of *V. harveyi* include the presence of glucose, low pH, temperatures above or below 30°C, and various toxic compounds. Additionally, the presence of boric acid enhances light production of BB170 and limits its ability to distinguish between different levels of AI-2 added. Therefore, it is important to monitor the light production of the medium control samples to ensure that the reporter strain is not affected by factors within it.

In order to calculate AI-2 activity, light must be measured at the time point at which the medium control produces the least amount of light. Because the timing of the response to AI-2 varies with each assay, it is important to measure light production every 30 min to ensure that data can be obtained before all samples become bright.

Troubleshooting

A number of factors contribute to the overall success of the BB170 bioassay. A guide for resolving problems with the bioassay is presented in Table 1C.1.1.

Table 1C.1.2 Results Typical of *S. typhimurium*

Cell-free culture fluid of *S. typhimurium*	cpm at optimal time point	Induction of bioluminescence
Medium control	15,288	N/A[a]
0 hr	33,296	2.2
2 hr	92,954	6.1
3 hr	3,473,248	227.2
4 hr	8,061,968	527.3
5 hr	88,466	5.8
6 hr	26,842	1.8

[a]N/A, not applicable.

Anticipated Results

Calculate the induction of bioluminescence for each sample using data only from the optimal time point, i.e., the point at which the medium control sample produces the lowest amount of light (Figure 1C.1.2, vertical line): induction of bioluminescence = light output of the sample/light output of the control.

In the example shown in Figure 1C.1.2, induction of bioluminescence is calculated as light output (B)/light output (A).

The light output is typically measured as counts per minute (cpm). Sample data from the optimal time point of an assay of *S. typhimurium* cell-free culture fluids collected over a time course of growth are presented in Table 1C.1.2. In this example, induction of bioluminescence is calculated as cpm of the sample/15,288.

Time Considerations

The time required for the collection of cell-free culture fluids varies depending on the growth properties of the bacteria being tested, with a minimum of 6 hr for bacteria with rapid doubling times. The bioassay takes ~6 to 8 hr, including 30 min to set up the samples and 30 min for the analysis.

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LacZ-Based Detection of Acyl-Homoserine Lactone Quorum-Sensing Signals

Many bacteria synthesize and secrete small molecules to the surrounding environment, and as the cell density increases, accumulation of these molecules eventually reaches a threshold concentration which, in turn, regulates gene expression. This process is called quorum sensing. Many Gram-negative bacteria use acyl-homoserine lactones (AHL) as the quorum-sensing signal molecule. Detecting AHLs from bacterial samples is an important step in investigating and identifying novel cell-to-cell communication systems. The protocols described in this unit can help to detect and identify unknown AHLs from various spent bacterial media and environmental samples (Brelles-Mariño and Bedmar, 2001). For detection of AI-2 (a non-AHL autoinducer molecule) using a bioluminescent bacterial reporter strain, refer to *UNIT 1C.1*.

Basic Protocol 1 describes a simple method to quickly assay for AHL activity in a sample, by quantitatively measuring β-galactosidase activity of a reporter fusion gene of an AHL bioassay strain grown in various concentrations of sample filtrates (assay filtrates should first be tested at 10% to test for sensitivity and toxicity to the bioassay strain, then adjusted as needed by dilution). This includes two parts; first, the assay samples are prepared and, second, the bioassay strains with sample filtrates are incubated and the enzyme activity assay is performed.

Basic Protocol 2 describes a method using thin-layer chromatography (TLC) to identify AHL molecules. This protocol should be used if the initial AHL liquid assay is positive and the AHL content and structure needs to be determined. Performing TLC separates AHLs based on structure and polarity. Detection of AHLs on the TLC plate is performed by coating the TLC plate with bioassay cells in agar medium containing Xgal and examining for blue spots indicating *lacZ*-based Xgal hydrolysis induced by the presence of AHLs. The relative R_f of the spots will allow for AHL identification.

The Support Protocol provides instruction for preinducing *Agrobacterium tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410), which is used as the AHL bioassay strain in both basic protocols.

CAUTION: Follow all biosafety requirements relevant to the assay sample strain under investigation. Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

CAUTION: Agrobacterium tumefaciens is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

DETECTION OF ACYL-HOMOSERINE LACTONE ACTIVITY USING A BIOASSAY STRAIN

BASIC PROTOCOL 1

This protocol consists of two basic steps. The first step requires the preparation of the assay sample strains, which must be grown under the desired conditions. The culture supernatant must then be collected at various time points and filtered to remove all bacterial cells to ensure that no AHL molecules or β-galactosidase from any native *lacZ*$^+$ strains are being produced during the actual assay step. Alternatively, samples can be

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extracted with organic solvents, such as ethyl acetate, to concentrate the AHL molecules (see Basic Protocol 2).

The second part of the protocol consists of running the actual assay. First, AT medium, with various concentrations of assay filtrates, are inoculated with the bioassay strains (include necessary controls here, e.g., synthetic autoinducers and medium blanks) and are grown for 12 to 16 hr at 28°C. After sufficient growth (OD_{600} reaches 0.2 to 1), the β-galactosidase assay is ready to be performed. This is done by lysing the cells and introducing *O*-nitrophenyl-β-D-galactoside (ONPG), which is cleaved in the presence of β-galactosidase, turning the solution yellow. This assay is timed and care should be taken to record the start time and stop times of each sample. The solution is centrifuged after stopping to pellet cell debris and the OD_{420} of the supernatant is taken. Levels of *lacZ* induction by AHL are then reported in Miller units.

Materials

Bacteria of interest
Medium appropriate to organism under study (e.g,. see *APPENDIX 2C*)
Preinduced cells (Support Protocol)
AT medium (see recipe) without antibiotics
Synthetic autoinducer
Z buffer (see recipe)
0.05% (w/v) SDS solution in H_2O
Chloroform
4 mg/ml *O*-nitrophenyl-β-D-galactoside (ONPG) in H_2O
1 M Na_2CO_3
10-ml syringe and 0.2-μm syringe filter

Prepare sample

1. Grow bacteria of interest to different time points in 2 ml appropriate medium.

   Time points are best established experimentally, a good starting point would be low (early log phase), medium (mid to late log phase), and high (stationary phase) cell densities to pinpoint at what time the bacteria of interest may produce autoinducer and to test for conditions favorable for AHL stability.

   Occasionally, the production of lactonases or medium pH changes caused by some strains may degrade the AHL produced. Therefore, it is necessary to collect samples at various growth points.

2. Microcentrifuge cells 1 min at 16,000× *g*, room tempereture. Remove the pellets and filter assay supernatants using a 0.2-μm syringe filter and 10-ml syringe. Use immediately or freeze up to 1 month at −20°C until use.

   Alternatively, culture filtrates can be extracted by ethyl acetate and resuspended in the medium (see Basic Protocol 2).

Perform assay

3. Thaw one vial of preinduced cells on ice. For each filtrate to be assayed, inoculate 2 μl preinduced cells into 2 ml AT medium without antibiotics plus the desired amount (up to 10%) of assay filtrate, synthetic autoinducer, or medium used to culture the bacteria of interest. Grow ~16 hr, until OD_{600} reaches 0.2 to 1 at 28°C.

   Antibiotics are not needed as they may interfere with β-galactosidase activity. In this incubation time frame plasmids will not be lost.

   Make a stock solution of cells in AT medium; for example, make a 20-ml solution with 20 μl preinduced cells for ten assay samples to be analyzed simultaneously. This ensures that all samples have the same starting concentration of the bioassay strain.

Controls should be included at this step as well. A positive control of synthetic AHLs or cultures known to have AHL activity should be used, as well as media blanks (AT medium and the medium used to grow assay strain).

4. Measure OD_{600} of the total cell suspensions. Combine 0.2 ml preinduced bacterial cells in AT medium, 0.8 ml Z buffer, 2 drops (~10 µl) of 0.05% SDS solution, and 3 drops (~15 µl) of chloroform in 2-ml microcentrifuge tubes.

   The 2 ml culture volume is to ensure optimal conditions for growth while shaking, and to keep a consistent inoculation ratio between samples. For ten samples, 20 ml can be inoculated and divided into 2 ml volume aliquots prior to addition of the filtrate.

5. Vortex vigorously for at least 10 sec and then add 0.1 ml of 4 mg/ml ONPG. Record time (T_0).

   Add ONPG quickly to assay tubes to ensure a consistent T_0.

6. When the solution turns yellow, add 0.6 ml of 1 M Na_2CO_3 to stop the reaction, and record the time in minutes (T_s).

   If no appreciable yellow is seen, stop assay at 120 min.

7. Centrifuge cell debris 3 min at 16,000 × *g*, room temperature, and measure OD_{420} of the supernatant.

8. Calculate results in Miller units as follows:

$$\text{Miller units} = \frac{1000 \times OD_{420}}{OD_{600} \times (T_s - T_0) \times 0.2}$$

   Time is in minutes.

   For more information regarding β-galactosidase assays and Miller units, refer to Miller (1972). See Raleigh et al. (2002) for a more recent review of the Lac operon.

DETECTING ACYL-HOMOSERINE LACTONE MOLECULES BY THIN-LAYER CHROMATOGRAPHY

BASIC PROTOCOL 2

This protocol describes a method developed by S.K. Farrand and colleagues to visualize AHLs using thin-layer chromatography (Shaw, 1997; Meyers, 2001). AHLs in assay samples are first separated on a C18 reversed-phase TLC plate. Agar medium containing the *lacZ*-based bioassay strain and Xgal is then overlaid on the TLC plate. AHLs with various retention factors (R_f) on TLC plates then activate the *lacZ* reporter to produce β-galactosidase and hydrolyze the Xgal to form blue spots.

Materials

Bacteria of interest
Medium appropriate to organism under study (e.g,. see *APPENDIX 2C*)
Ethyl acetate
60:40 (v/v) methanol/water
Preinduced cells (Support Protocol)
2× AT buffer (1:10 dilution of 20× AT buffer; see recipe)
1.5% water-agar (see recipe), 45°C
20 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal) in dimethylformamide (DMF)
10-ml syringe and 0.2-µm syringe filter
2-ml glass vial
TLC tank (30-cm diameter × 10-cm width × 27-cm height)
C18 reversed-phase TLC plates (200-mm layer; Baker or Whatman)

28° and 45°C water bath
Tape (to seal TLC plates)
Sealable plastic container to accommodate TLC plate

Additional reagents and equipment for thin-layer chromatography (TLC; Meyers, 2001)

1. Grow bacteria of interest to different time points in 2 ml appropriate medium.

   Time points are best established experimentally, a good starting point would be low (early log phase), medium (mid to late log phase), and high (stationary phase) cell densities to pinpoint at what time the bacteria of interest may produce autoinducer and to test for conditions favorable for AHL stability.

   Occasionally, the production of lactonases or medium pH changes caused by some strains may degrade the AHL produced. Therefore, it is necessary to collect samples at various growth points.

2. Microcentrifuge cells 1 min at 16,000× *g*, room temperature, remove the pellets and filter supernatants using a 0.2-μm syringe filter and 10-ml syringe. Use immediately or freeze up to 1 month at −20°C until use.

3. Extract 5 ml filtered culture supernatants by adding 5 ml ethyl acetate to the supernatant in a 50-ml flask and agitate vigorously until the ethyl acetate is evenly distributed. Let stand for 5 min to separate phases.

4. Transfer upper phase to a 25-ml beaker using a pipet. Repeat the extraction to the bottom phase and add the new upper phase to the same 25-ml beaker, for a total volume of 10 ml.

5. Evaporate or vacuum dry the collected upper phase solvent inside a hood. Resuspend the dried sample in 50 μl ethyl acetate to produce a concentrated AHL solution. Store the AHL sample in a 2-ml glass vial up to 1 month at −20°C.

6. Add 200 ml of 60:40 methanol/water to the TLC tank. Close the lid and let equilibrate 2 hr.

7. Use a pencil to draw a line 2 cm away from the bottom of the TLC plates. Apply 1 to 5 μl of the AHL samples along the line with a space of at least 1.5 cm between samples, and let air dry (∼5 min).

   Apply samples one at a time, and allow them to air dry between additions. This prevents the sample from diffusing too much, creating a more contained elution.

   Standards, such as synthetic AHLs, should be used here as a positive control. Culture medium can be used as a negative control if desired.

8. Place the TLC plate in the tank and develop until the solvent front runs to the top. Remove the plate and air dry.

   Make sure the sample loading line is not immersed in the solvent.

9. Thaw one vial of preinduced cells on ice. For each filtrate to be assayed, inoculate 2 μl preinduced cells into 2 ml AT medium without antibiotics. Grow ∼16 hr, until OD_{600} reaches 0.2 to 1 at 28°C.

   Antibiotics are not needed as they may interfere with β-galactosidase activity. In this incubation time frame plasmids will not be lost.

   Make a stock solution of cells in AT medium; for example, make a 20-ml solution with 20 μl preinduced cells for ten assay samples to be analyzed simultaneously. This ensures that all samples have the same starting concentration of the bioassay strain.

10. Prepare the detecting culture mix by combining 50 ml of the overnight culture of KYC55 (pJZ372)(pJZ384)(pJZ410) with 50 ml room temperature 2× AT buffer and 50 ml of 1.5% water-agar (maintained at 45°C). Add Xgal to a final concentration of 60 μg/ml. Mix thoroughly and maintain at 45°C.

    Melt water-agar in microwave and maintain resulting solution in a 45°C water bath before addition of 2× AT medium with the bioassay strain and Xgal. Upon addition of these ingredients, immediately place solution back in 45°C water bath until use.

11. Seal the dried TLC plate with tape along edges forming a "wall" of tape ~1-cm high around the plate. Spread the culture mix over the surface evenly to a thickness of ~0.5 cm.

    Use burner flame to get rid of bubbles when overlaying the detecting agar.

12. After the agar has solidified, incubate the coated plate for 12 to 18 hr in a 28°C water bath in a sealed plastic container and then examine for blue Xgal hydrolysis.

PREPARATION OF PREINDUCED AHL BIOASSAY STRAIN

SUPPORT PROTOCOL

The bioassay strain must be prepared and grown in minimal medium. This is called preinduction and is done to ensure that these cells will grow rapidly in minimal medium during the assay, so that assay results are reproducible. Once this stock has been prepared, it can be kept indefinitely with proper storage at −80°C.

Materials

AHL bioassay strain *Agrobacterium tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410); see Zhu (2003)
LB medium (*APPENDIX 4A*) containing 1 μg/ml tetracycline, 100 μg/ml spectinomycin, and
100 μg/ml gentamycin
AT medium (see recipe) with and without 1 μg/ml tetracycline, 100 μg/ml spectinomycin, and 100 μg/ml gentamycin
15% (v/v) glycerol, sterile
28°C orbital shaker
2-ml microcentrifuge tubes

1. Grow AHL bioassay strain *Agrobacterium tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410) in LB medium supplemented with antibiotics. Incubate on an orbital shaker to late-log phase (OD_{600} of 1; ~1×10^9 cfu/ml) at 28°C.

    This bioassay strain is used as an example. It cannot produce AHLs and has three plasmids containing P_{T7}-traR, a T7 polymerase gene, and a reporter traI-lacZ fusion.

2. Inoculate 1 ml LB culture into 100 ml AT medium supplemented with antibiotics. Incubate cultures on an orbital shaker to mid-log phase at 28°C.

3. Transfer cultures into 2-ml microcentrifuge tubes and collect cells by microcentrifuging 10 min at 12,000 rpm, room temperature, and discarding supernatants.

4. Resuspend cell pellets in 15% sterilized glycerol (1/20 of original volume). Divide into 100-μl aliquots and store indefinitely at −80°C.

    These cells are now preinduced.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AT buffer, 20×

Per liter:
214 g KH_2PO_4
Adjust pH to 7.3 with KOH
Autoclave then cool to room temperature
Store up to 6 months at room temperature

AT medium

50 ml 20× AT salts (see recipe)
50 ml 20× AT buffer (see recipe)
10 ml 50% (w/v) glucose (see recipe)
890 ml H_2O
Store up to 6 months at room temperature

Autoclave salts and buffer separately, then cool solutions to room temperature. Filter sterilize glucose solution.

AT salts, 20×

Per liter:
40 g $(NH_4)_2SO_4$
1.56 g $MgSO_4$
0.152 g $CaCl_2$
0.1 g $FeSO_4 \cdot 7H_2O$
0.044 g $MnSO_4 \cdot H_2O$
Autoclave, then cool to room temperature
Store up to 6 months at room temperature

Glucose, 50% (100×)

Prepare a 50% (w/v) solution of glucose in H_2O
Filter sterilize
Store up to 6 months at room temperature

Water-agar, 1.5%

15 g agar per liter H_2O
Sterilize by autoclaving
Store up to 6 months at room temperature

Z buffer

Per liter:
16.1 g $Na_2HPO_4 \cdot 7H_2O$
5.5 g $NaH_2PO_4 \cdot H_2O$
0.75 g KCl
0.245 g $MgSO_4 \cdot 7H_2O$
2.7 ml 2-mercaptoethanol
Adjust to pH 7.0 using HCl
Store up to 6 months at 4°C

COMMENTARY

Background Information

A number of bacterial processes, such as antibiotic production, motility, and biofilm formation (see Section 1B), are regulated by the exchange of chemical signals that permit a bacterial community to coordinate its responses to environmental challenges or opportunities. The exchange of these signals enables all of the individuals in a community to participate in reaching a consensus, and is known as quorum sensing (Dunny, 1999; Miller, 2001; Whitehead, 2001). In many proteobacteria, these chemical signals consist of a set of diffusible acyl-homoserine lactones (AHLs), also called autoinducers. The key regulatory components of these signaling systems are LuxI-type proteins, which act as AHL synthases, and LuxR-type proteins, which serve as AHL receptors and AHL-dependent transcription factors (Fuqua, 1994; Zheng, 2006). All known AHLs contain invariant homoserine lactone moieties with highly variable fatty acyl groups. These acyl groups range in length from 4 to 18 carbon atoms. The C-3 carbon can be fully reduced or can bear hydroxyl or ketone substituents (Fuqua, 2001).

Detection and identification of cognate signal molecules has led to the discovery of new bacterial cell-cell signaling systems. Specifically, the detection of AHL molecules involved in quorum sensing has been facilitated by the development of a variety of bioassay strains. Such strains contain an easily assayable reporter gene and lack all AHL synthases, such that reporter activity requires exogenous AHLs for activation. Various reporter genes have been described, including *lacZ*, GFP, *lux*, and the production of an endogenous pigment. The *Agrobacterium tumefaciens* bioassay strain described here uses the T7 expression system to strongly overexpress the regulator TraR and, thus, increases sensitivity and broadens the molecule detection range in an *A. tumefaciens* AHL-synthase mutant. In the presence of exogenous AHLs, TraR can then activate a *traI-lacZ* reporter fusion, resulting in production of β-galactosidase (Zhu 1998, 2003). Because of the great sensitivity and broad range of AHL detection with this bioassay strain, it is a useful tool for detecting extremely low concentrations of AHLs, or possibly other

Table 1C.2.1 Troubleshooting ACL Detection Assays

Problem	Possible cause	Solution
Low *lacZ* activity	AHL concentration not high enough	Extract samples with ethyl acetate
High *lacZ* background	Rich medium	Change medium (the poorer medium, the better)
TLC spots diffused	AHL concentration is too high	Reduce the amount of sample loading
Xgal hydrolysis not seen over TLC plate/low *lacZ* activity in β-gal assay	Bacteria of interest do not produce AHL-type autoinducers under chosen time points	Try taking culture samples at earlier and later time points to adjust for molecule degradation or effects of cell density on AHL production
	Bacteria do not produce AHL molecules under these conditions	Change growth conditions, such as temperature and nutrient broth
	Culture conditions toxic to bioassay strains	Reduce the sample amount or extract samples with ethyl acetate before addition to assay culture
Bioassay strains fail to grow or grow too slowly	Toxicity in the sample	Reduce the sample amount or extract samples with ethyl acetate before addition to assay culture

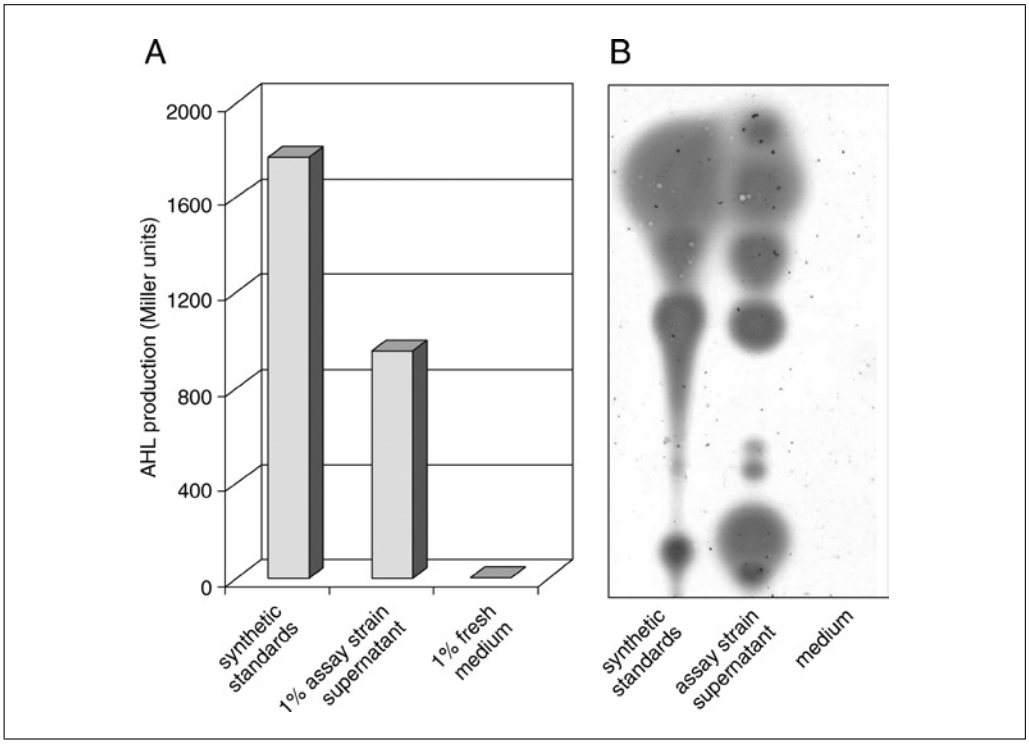

Figure 1C.2.1 An example of applying KYC55 (pJZ372)(pJZ384)(pJZ410) to detect AHL-like molecules from the spent medium of a specified Gram-negative bacterium using the liquid assay (**A**) and the TLC assay (**B**).

bioactive signal molecules. It could also have applications in AHL detection in dilute environmental samples or genomic libraries. Examples can be found in Williamson et al. (2005) and Zheng et al. (2006).

Critical Parameters

Agrobacterium strains have to grow at 28° to 30°C; they fail to grow at 37°C. Synthetic AHL standards are needed as positive controls and can be purchased from various companies (e.g., Sigma). It is important to note that many factors can affect the AHL content in experimental samples. For example, high medium pH and production of AHL lactonases may degrade AHLs in the supernatant. Thus, it may be necessary to test the AHL production under various growth conditions. For further details, consult the Troubleshooting section.

Troubleshooting

Commonly encountered problems with the procedures outlined in this unit, along with their possible causes and potential solutions, are given in Table 1C.2.1.

Anticipated Results

The bioassay for AHL-like signals described here is quite sensitive. Figure 1C.2.1 shows an example of the use of this bioassay to detect the AHL molecules from the supernatant of an unspecified bacterium. The liquid β-galactosidase activity assay showed over a 200-fold increase of *traI-lacZ* reporter induction as compared to the medium control. The same supernatant contained at least six different AHL-like molecules when assayed using the TLC plate. Practically, depending on the concentration of the sample assayed, a minimum of a ten-fold increase over the medium control is considered significant.

Time Considerations

Expect the complete autoinducer detection assay procedure to take ~2 days to complete. Sample preparation time varies depending on the rate at which the sample bacteria grow and what time points the sample cultures are removed. Expect the supernatant preparation (cell pelleting and filtration) to take ~5 to 10 min per sample. *LacZ* induction (overnight growth of bioassay strain) takes 12 to 16 hr to reach the proper concentration (OD_{600} of 0.2 to 1). Assaying for β-galactosidase using ONPG takes ~2 to 3 hr depending on the rate at which samples reach the desired endpoint.

The TLC assay will take ~2 days to complete (Meyers, 2001). Preparing the TLC assay sample (if extract is evaporated under vacuum), tank, and plate will take 2 to 4 hr. The

actual development of the TLC plate in the tank will take ~2 to 3 hr plus drying time. Overlaying the plate will take ~30 min. The coated TLC plate then needs to be incubated for 12 to 18 hr before assay results can be analyzed.

The prepared samples can be stored long term at −20°C. The preparation of the preinduced bioassay cells takes 2 days. This can be done ahead of time and the strains can be stored indefinitely at −80°C.

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Detection and Analysis of Quorum-Quenching Enzymes Against Acyl Homoserine Lactone Quorum-Sensing Signals

Quorum sensing (QS) is a microbial community genetic regulation mechanism that controls biological functions of medical, agricultural, and industrial importance. Bacteria produce, detect, and respond to QS signals to synchronize target gene expression and thereby coordinate biological activities among a local population. *N*-acyl homoserine lactones (AHLs) are one family of the widely conserved signals in Gram-negative bacteria, and they regulate a range of important biological functions, including virulence and biofilm formation (Zhang and Dong, 2004). Several groups of AHL-degrading enzymes, including AHL-lactonase, AHL-acylase, and paraoxonases (PONs), have recently been identified in a range of living organisms, including bacteria and eukaryotes (Dong et al., 2000, 2002; Leadbetter and Greenberg, 2000; Zhang et al., 2002; Lin et al., 2003; Yang et al., 2005). Given their efficiency in degrading AHLs and blocking QS signaling, these inactivating enzymes are also known as quorum-quenching enzymes (Dong and Zhang, 2005).

This unit provides methods for detecting and analyzing enzyme activity related to AHL inactivation. The Basic Protocol describes an AHL diffusion plate bioassay, which when combined with Support Protocol 1, is suitable for rapid, large-scale screening and identification of AHL-inactivating enzymes. Procedures in Support Protocol 2 aim to determine whether the AHL-inactivating enzyme is a lactonase. Support Protocol 3 provides methods that allow quantitative determination of relative enzyme activity. Support Protocol 4 describes enzyme assays using cell-free protein extracts. Support Protocols 5 and 6 use HPLC to definitively characterize AHL-inactivating enzymes as AHL-lactonases or AHL-acylases, based on the physical and chemical properties of the enzyme reaction products, which can be distinguished by the high resolution power of HPLC with a suitable chromatographic column-solvent combination.

CAUTION: Follow all biosafety requirements relevant to the microorganism under investigation. All uncharacterized bacterial isolates are potentially infectious; handle them with care and dispose of them only after sterilization. Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

CAUTION: Dimethyl sulfoxide (DMSO), dimethylformamide, methanol, and tetracycline are hazardous; see *UNIT 1A.3* for guidelines on handling, storage, and disposal.

QUALITATIVE DETECTION OF AHL-INACTIVATING ENZYMES

BASIC PROTOCOL

This protocol describes a procedure for qualitative detection of AHL-inactivating activity. The protocol is based on two principles: (1) AHL signals diffuse easily in an agar plate (Zhang and Kerr, 1991), and (2) a sensitive reporter strain (biosensor) produces blue colonies on an agar plate supplemented with X-gal when sensing AHL signals (Piper et al., 1993; Zhang et al., 1993; Lin et al., 2003).

Materials

Biosensor organism: *Agrobacterium tumefaciens* NT1(*traR*, *tra*::*lac*Z749; Piper et al., 1993)

Contributed by Hai-Bao Zhang, Lian-Hui Wang, and Lian-Hui Zhang
Current Protocols in Microbiology (2007) 1C.3.1-1C.3.15

YEB medium with and without 50 μg/ml kanamycin and 10 μg/ml tetracycline (see recipe)
Glycerol (optional)
Test organisms (e.g., bacteria from soil, water, and plant or animal tissues)
10 μM *N*-3-oxo-octanoyl-L-homoserine lactone (3OC8HSL) and other AHLs (see Zhang et al., 1993; Sigma-Aldrich; Cayman Chemical): dilute in YEB medium
Minimal medium agar plates supplemented with 50 μg/ml X-gal (see recipe)

250-ml culture flasks
28°C incubator
Rotary shaker
96-well tissue-culture plates
UV chamber (Stratagene)
Marker pen
Ruler
Scalpel, sterile
Microcentrifuge with (optional) 96-well plate rotor
Plastic film (for sealing 96-well plates)

Prepare AHL biosensor and bacterial isolates

1. Grow AHL biosensor *A. tumefaciens* NT1(*traR*, *tra*::*lacZ*749) in a 250-ml flask containing YEB medium supplemented with 50 μg/ml kanamycin and 10 μg/ml tetracycline at 28°C, with shaking at 200 rpm to an OD_{600} of ~1.5.

   A. tumefaciens NT1(traR, tra::lacZ749) harbors two plasmids expressing TraR and the reporter gene lacZ under the tra promoter, respectively (Piper et al., 1993).

   The antibiotics are used to maintain the presence of the two plasmids, which also carry genes for resistance to the antibiotics.

2. If not using the biosensor cell cultures immediately, add glycerol to 20% (v/v) and store up to 1 year at −80°C.

3. Prepare individual bacterial isolates from soil, water, and plant or animal tissues using standard techniques (see Collins et al., 1989).

4. Inoculate individual bacterial colonies into 150 μl YEB medium in 96-well plates and incubate overnight at 28°C, with shaking at 200 rpm.

   Bacteria are the rich sources of novel enzymes. Incubations are generally at 28°C, and the overnight cultures are usually ready for analysis of AHL inactivation, typically performed in 96-well plates.

   Fresh cultures of other living organisms such as fungi, plant, and animal tissues can also be prepared using an appropriate medium (see Chun et al., 2004; Yang et al., 2005).

Set up AHL-inactivating reaction

5. Add 50 μl of 10 μM 3OC8HSL into each well, then add 50 μl fresh bacterial cultures or YEB medium (as a positive control). Incubate 3 to 5 hr at 28°C.

   For screening of AHL-inactivating enzymes, the authors recommend including 3OC8HSL, which is a common substrate for all the known AHL-inactivating enzymes. It is also the most sensitive signal for the biosensor A. tumefaciens NT1(traR, tra::lacZ749), which can detect levels of 3OC8HSL as low as 10 nM on diffusion plates (5 μl sample, equal to 0.05 pmol).

Prepare AHL bioassay plates

6. After solidification, aseptically cut the agar medium in the plate into separated bars of 1-cm width by removing 2- to 3-mm slices of between the agar bars, using a marker pen, a ruler, and a sterile surgical scalpel.

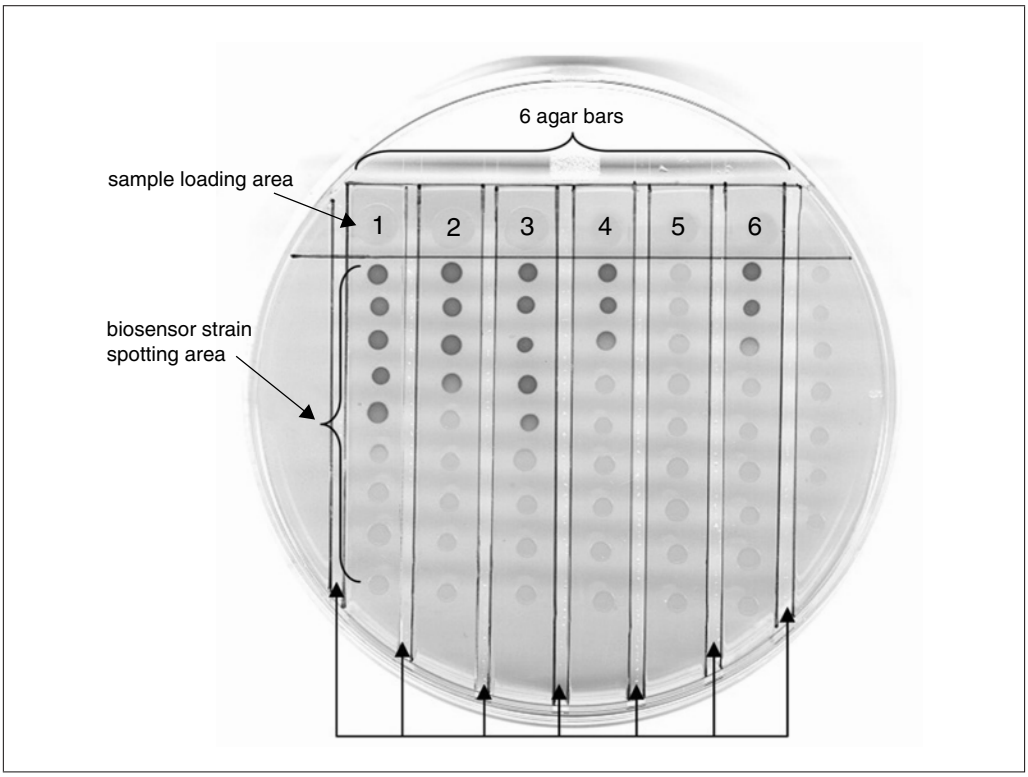

Figure 1C.3.1 The AHL bioassay plate. Blue spot indicates that the diffusible AHL signals were detected by the biosensor cells. Bar 1: the positive control (5 μM 3OC8HSL in YEB medium) was added. Bars 2 to 6: the reaction supernatants of five bacterial isolates were added, separately. The shorter distance of blue spots from the sample loading area in bars 2, 4, and 6 suggests that the bacterial samples may have AHL-inactivating activity, whereas no blue spot in bar 5 indicates a strong AHL-inactivating activity.

For the convenience of comparing different samples and measurement of distance, the agar bars in plate can be prepared as rectangles of equal length by cutting off the top and bottom parts. In general, six agar bars can be made from each 90-mm-diameter plate (see Lin et al., 2003 and Fig. 1C.3.1).

The minimal medium agar bars are used as the supporting material for both AHL diffusion and the growth of biosensor.

Perform the inactivation assay

7. Centrifuge the 96-well reaction plates (from step 4) 5 min at top speed in a microcentrifuge (or transfer the mixtures to microcentrifuge tubes for centrifugation).
8. Transfer 50 μl of each reaction supernatant to a well in a 96-well tissue culture plate and place the plate under UV light in a UV chamber for 0.5 hr to kill any remaining bacterial cells.
9. Add 5 μl reaction supernatant from each sample to one end of an agar bar.
10. Dilute the fresh culture or −80°C glycerol stock of *A. tumefaciens* NT1(*traR, tra::lacZ749*), to OD_{600} ~0.5 before use.
11. Using a micropipettor, spot ~0.5 μl drops at progressively further distances from the end of the agar bar loaded with samples. Include a positive control (the reaction mixture with only YEB medium and no bacteria) with 3OC8HSL solution; see step 4) in each plate for easy comparison.
12. Seal the plate using thin plastic film after the samples and the bacterial spots on the agar bars are absorbed and incubate 24 hr at 28°C.

13. After incubation, examine the bioassay plates for the presence of blue spots and measure their distance from the origin.

    The biosensor spot will turn blue in the presence of detectable AHL signal (e.g., see Lin et al., 2003 and Fig. 1C.3.1). Typically, the positive control with a final concentration of 5 μM 3OC8HSL produces several blue spots with more than a 2 cm diffusion distance. Any sample producing no blue spots or significantly fewer blue spots than the positive control suggests potential AHL-inactivating activity and is considered a positive bacterial isolate.

SUPPORT PROTOCOL 1

CONFIRM ENZYMATIC AHL INACTIVATION

AHL inactivation can be due to enzymes (Dong et al., 2000; Lin et al., 2003; Yang et al., 2005) or nonenzyme factors (Byers et al., 2002). This protocol describes a procedure to confirm that the AHL inactivation observed using the Basic Protocol is due to an enzymatic reaction. The procedure is based on the principle that protein enzymes are usually not heat stable and that an enzymatic reaction shows a relatively gentle progressive curve.

Additional Materials *(also see Basic Protocol)*

Bacterial isolate showing positive AHL-inactivating activity (identified using the Basic Protocol)

1. Transfer 100 μl fresh culture of each positive bacterial isolate (see Basic Protocol, step 13 annotation) into separate microcentrifuge tubes in duplicate.
2. Place one tube in boiling water bath for 10 min to kill bacterial cells and denature proteins.
3. Add the same volume of YEB medium containing 10 μM 3OC8HSL to each tube and incubate at 30°C with gentle shaking.
4. Remove 30 μl reaction mixture from both tubes at different time points (for example, 30, 60, 90, 120, min) after addition of AHL and place each into a well in a 96-well plate.
5. Assay as described in the Basic Protocol, steps 7 to 13.

   Progressive inactivation of AHL signals by the bacterial cells and loss of inactivation activity after boiling indicate the presence of a quorum-quenching enzyme.

SUPPORT PROTOCOL 2

DETERMINE WHETHER THE AHL-INACTIVATING ENZYME IS A LACTONASE

Several types of enzymes, including AHL-lactonase, PONs, and AHL-acylase, are capable of inactivating AHL signals (Dong and Zhang, 2005). This protocol describes a procedure for determining if the AHL-inactivating enzyme belongs to the lactonase group (including AHL-lactonases and PONs). It is based on the principle that the product of lactonases can be relactonized in acidic solution. This procedure can be used following the Support Protocol 1 after confirmation of the enzymatic AHL inactivation.

Additional Materials *(also see Basic Protocol)*

Bacterial isolate with confirmed AHL-inactivating enzyme (Support Protocol 1)
1 M HCl solution

1. Using bacteria confirmed as having AHL-inactivating enzymes (see Support Protocol 1), prepare a complete AHL-inactivating reaction as in the Basic Protocol, step 5, but double the volumes of 3OC8HSL and bacterial cultures.

2. Divide the reaction mixture into two 100-µl aliquots in microcentrifuge tubes.
3. To one aliquot, add 10 µl of 1 M HCl to acidify the reaction mixture (pH 1 to 2), and add 10 µl of water to the other aliquot as a control.
4. Incubate both tubes at room temperature.
5. Remove 5 µl of reaction mix from each treatment and control tube at different time points (5, 30, and 60 min after acidification) and assay as described in the Basic Protocol, steps 10 to 13.

   Progressive restoration of AHL activity in the acidified solution indicates a lactonase enzyme. Typically, >90% of degraded activity can be restored within 60 min after acidification (e.g., see Yang et al., 2005). In contrast, if the AHL activity is not restored under the acidic condition, then the enzyme that hydrolyzes AHL in the reaction mixture may be an AHL-acylase or other enzyme that has not yet been identified.

QUANTITATIVE ANALYSIS OF QUORUM-QUENCHING ENZYME ACTIVITY

SUPPORT PROTOCOL 3

This protocol describes a procedure for quantitative analysis of AHL-inactivating enzyme activity in bacterial isolates. It is based on the finding that the signal concentration-diffusion distance relationship can be accurately described by an exponential equation (Dong et al., 2000). Therefore, the amount of the AHL signal before and after the reaction can be calculated based on the diffusion distances of the signals in corresponding samples. The data are essential for determination and comparison of relative enzyme activity of different bacterial samples. The general assay procedure is the same as the Basic Protocol, with the addition of the steps on measurement of diffusion distances and calculation of a standard curve.

Additional Materials *(also see Basic Protocol)*

Methanol
Equation-fitting program (e.g., Microsoft Excel)

Establish a standard AHL diffusion curve

1. Prepare a 4 mM 3OC8HSL stock solution in methanol. Use this to produce a series of dilutions (e.g., 200, 20, 2, 0.5, 0.1, 0.05, and 0.01 µM).

   Standard AHL samples are used to determine the quantitative relationship between AHL quantity and its diffusion distance on the agar bars.

   For 3OC8HSL, the linear concentration range of detection by biosensor A. tumefaciens NT1(traR, tra::lacZ749) is from 20 nM to 200 µM using this diffusion plate method. For other AHL signal with six-carbon fatty acid side chain and more, the recommended detection range is from 200 nM to 200 µM. For detection of four-carbon fatty acid side chain signal, such as N-butyryl-L-homoserine lactone, another biosensor, Chromobacterium violaceum strain CV026, should be used (McClean et al., 1997).

   Because the methanol in solution might affect the signal diffusion as well as enzyme activity, it is recommended that the final concentration of methanol in each dilution be kept constant and <6%. For example, to prepare a series of 3OC8HSL working solutions in 5% methanol, one can prepare the high end 3OC8HSL working solution (i.e., 200 µM) in 5% methanol, then prepare the other working solutions (i.e., 20 µM, 2 µM, 0.2 µM etc) by diluting sequentially with 5% methanol (Table 1C.3.1).

2. Load 5 µl of each dilution onto a diffusion plate as described in the Basic Protocol, step 9, and incubate 24 hr at 28°C.

Table 1C.3.1 Preparation of AHL Dilutions in 5% Methanol

	AHL[a] dilutions (μM)					
	200	20	2	0.5	0.1	0.05
AHL solution	10 μl of 4 mM AHL in methanol	20 μl of 200 μM AHL	20 μl of 20 μM AHL	50 μl of 2 μM AHL	40 μl of 0.5 μM AHL	100 μl of 0.1 μM AHL
Water (μl)	195	0	0	0	0	0
5% methanol (μl)	0	180	180	150	160	100
Total volume (μl)	200	200	200	200	200	200

[a]For example, N-3-oxo-octanoyl-L-homoserine lactone (3OC8HSL).

3. After incubation, measure the distance from the last induced blue spot to the origin of the 3OC8HSL spot in each agar bar to determine the diffusion distance of 3OC8HSL.

4. Establish the relationship between the concentration of 3OC8HSL loaded on the agar bar (y, micromolar) and the 3OC8HSL diffusion distance (x, centimeters) by fitting a set of x and y values to a logarithmic equation (Fig. 1C.3.2) using Microsoft Excel.

   The relationship between 3OC8HSL concentration and diffusion distance has been empirically determined to be logarithmic, i.e., the coefficient of determination (R^2) for the logarithmic curve is higher relative to other curves (e.g., linear or exponential), indicating the best fit.

   A typical logarithmic equation describing the relationship between the concentration of 3OC8HSL loaded on agar bar (y, micromolar) and the 3OC8HSL diffusion distance (x, centimeters) is as follows:

   $\log y = 2.033x - 4.382$

   The coefficient of determination (R^2) of the above logarithmic equation is equal to 0.991, indicating a highly correlated relationship between the concentration and diffusion distance on agar plate of the signal.

   The above equation can be generally used to determine relative 3OC8HSL concentration for any sample using 5 μl of sample in the diffusion plate assay described above. However, for accurate concentration determinations, it is recommended that each experimenter build up a standard curve for each AHL signal of interest to take into account individual differences in patterns of sample handling and measurement.

   It is now possible to quantitatively determine and compare the relative enzyme activities of the positive bacterial isolates identified.

Quantify the remnant AHL in a reaction mixture

5. Follow the Basic Protocol to conduct the bioassay for detection of the remnant AHL signals at different time points after initiation of the reaction.

   The same procedures can be adopted for analysis of purified AHL-degrading enzyme (Dong et al., 2000), by scaling down the amount of purified enzyme used in each reaction (10 to 50 ng).

6. Determine the concentration of remaining AHL in reaction mixture using the standard equation. Calculate the amount of degraded AHL by deducting the remnant AHL from the total amount of AHL used in reaction.

   The enzyme activity can be expressed as AHL degraded (pmol) per minute per milliliter of culture ($OD_{600} = 1$) or per microgram total protein or per nanogram enzyme, depending on the nature of testing sample.

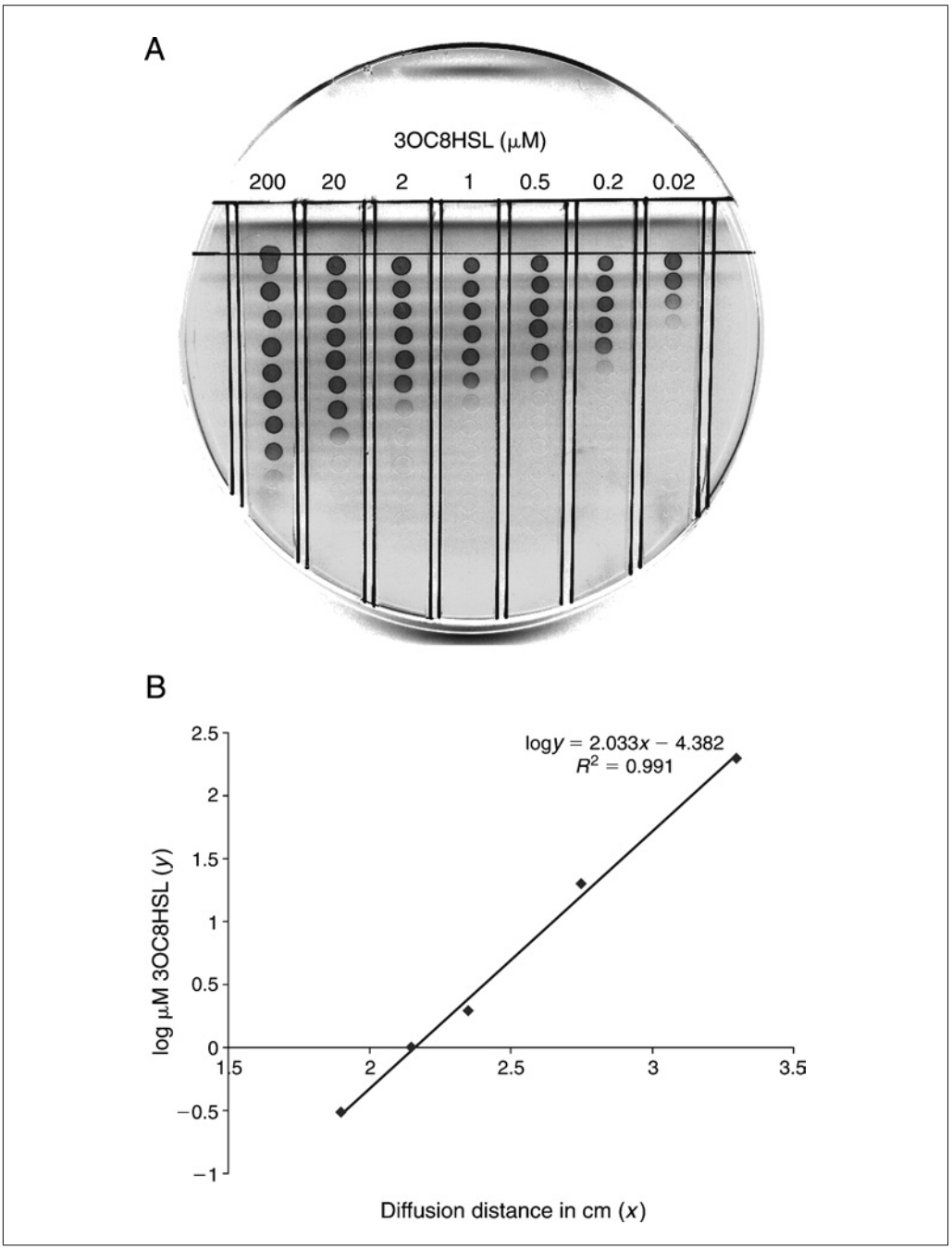

Figure 1C.3.2 Establishment of a standard curve for quantification of AHL. (**A**) Bioassay results for 3OC8HSL dilutions. (**B**) The standard curve for 3OC8HSL concentrations (μM) versus their corresponding diffusion distances in an agar bar (cm) generated by the Microsoft Excel program.

ASSAYING ENZYME ACTIVITY USING CELL-FREE TOTAL PROTEIN EXTRACTS

SUPPORT PROTOCOL 4

As an alternate to using bacterial or other organism cultures as samples for assay of AHL-inactivating activity, AHL-inactivating enzyme activity can also be assayed using cell-free protein extracts after confirming that the inactivation is enzymatic (Support Protocol 1). This protocol describes the procedures for total protein extraction from bacterial cultures and subsequent AHL-inactivation routine assay using Basic Protocol. In combination with Support Protocols 2 and 3, this protocol allows qualitative and quantitative analysis of AHL-inactivating enzymes in protein extracts.

Additional Materials *(also see Basic Protocol)*

Phosphate-buffered saline containing potassium (KPBS; *APPENDIX 2A*)
Sonicator (e.g., Digital Sonifier; Branson Ultrasonics)

1. Inoculate bacterial cells into 50 ml YEB medium. Incubate overnight at 28°C, with shaking at 200 rpm.
2. Centrifuge cultures 5 min at 10,000 × *g*, 4°C.
3. Wash the cell pellet once by adding 50 ml ice-cold KPBS and centrifuging as in step 2.
4. Resuspend the bacterial cells in 2 ml KPBS and sonicate on ice following the manufacturer's instructions. Centrifuge 20 min at 10,000 × *g*, 4°C. Retain the supernatant (cell-free total protein extracts).
5. Determination protein concentration using Bradford method (*APPENDIX 3A*) and reagents from Bio-Rad.
6. Place supernatant aliquots containing ~100 to 500 μg total proteins into two 1.5-ml microcentrifuge tubes for each sample. Keep one tube on ice. Boil the other tube for 5 min and then place on ice.
7. To each aliquot, add 3OC8HSL to a final concentration of 20 μM and mix. Incubate the reaction mixture at 37°C.
8. At different reaction time points (e.g., 0, 15, 30, 45, and 60 min), place a 20-μl aliquot of reaction mixture into a microcentrifuge tube. Heat the removed aliquots in a boiling water bath for 3 min to terminate the reaction.
9. Centrifuge the timed samples and take 5 μl supernatant from each tube to perform a diffusion plate bioassay using the Basic Protocol.
10. Using Support Protocol 3, calculate the enzyme activity in the total protein samples based on the amount of AHL inactivated, the amount of total proteins used in reaction, and the reaction time.

SUPPORT PROTOCOL 5

HPLC IDENTIFICATION OF AHL-LACTONASE HYDROLYSIS PRODUCTS

The Basic Protocol in combination with Support Protocol 1 provides a simple quantitative AHL diffusion plate assay, which is suitable for rapid and large-scale screening of bacterial strains capable of inactivating AHL. Support Protocol 2 describes an easy way to predict a lactonase as the AHL inactivating factor. If acidification of the reaction mixture described in Support Protocol 2 is able to restore the AHL activity, the enzyme of interest is likely to be a lactonase. HPLC analysis (optimized following the methods described by Dong et al., 2001) can be reliably used to confirm that the enzyme of interest is a lactonase (e.g., AHL-lactonase or PON) by showing that the enzymatic hydrolysis product has an identical retention time as 3OC8HS.

Additional Materials *(also see Support Protocol 4)*

50:50 methanol (HPLC grade, Merck)/water (HPLC grade or Milli-Q-purified)
50 mM *N*-3-oxo-octanoyl-L-homoserine lactone (3OC8HSL; Sigma-Aldrich) stock solution in methanol
10 mM *N*-3-oxo-octanoyl-L-homoserine (3OC8HS) stock solution: prepared by incubating 10 mM 3OC8HSL (Sigma-Aldrich) in 100 μl 200 mM NaOH and 100 μl DMSO for 4 hr at 25°C

HPLC column: C18 reverse-phase column, 4.6 × 250 mm, 5 μm (Symmetry)
HPLC system: Alliance 2690 separations module, and 996 photodiode array detector (Waters)
0.45-μm PTFE filter (Waters)

Additional reagents and materials for preparing bacterial cell-free extracts (Support Protocol 4)

1. Connect a C18 reverse-phase column to a Waters Alliance 2690 separation module and a Waters 996 photodiode array detector. Equilibrate by eluting isocratically with 50:50 methanol/water (v/v) at a flow rate of 1 ml/min for at least 1 hr.
2. Prepare bacterial cell-free protein extracts as described in Support Protocol 4, steps 1 to 5.

   If the gene encoding AHL-inactivating enzyme has been cloned, it is convenient to purify the enzyme by routine techniques (for references, see Dong et al., 2000; Lin et al., 2003).
3. To 0.3 ml of the cell-free protein extracts containing ~500 μg total proteins (or 50 ng purified enzyme), add 3OC8HSL to final concentration of 1 mM in a 1.5-ml microcentrifuge tube with a final reaction volume of 0.4 ml.
4. Add the same concentration of 3OC8HSL and 3OC8HS to KPBS buffer as a negative and positive control, respectively.
5. Incubate the reaction mixture 30 to 60 min at 37°C with gentle shaking.

   Typically, >80% of AHL should be degraded in the first 30 min of enzymatic reaction.
6. Denature the proteins in the reaction mixture by boiling in a water bath for 3 min.
7. Filter the reaction mixture through a 0.45-μm PTFE filter. Collect the filtrate for HPLC analysis.

   The purpose of filtration is to get rid of undissolved particles, which could block or even damage the HPLC system.
8. Inject 20 μl of filtered reaction mixture and negative and positive controls, at separate times, into the calibrated C18 reverse-phase column. Elute isocratically with 50:50 methanol/water at a flow rate of 1 ml/min.

   The retention times of 3OC8HSL and 3OC8HS are ~8.1 and 3.8 min, respectively.

   If HPLC analysis shows that the enzymatic hydrolysis product has an identical retention time as 3OC8HS, the AHL-inactivating enzyme under investigation should be a lactonase (e.g., AHL-lactonase or PON).

   The retention times of AHL and its degradation products are provided as reference only. They may vary depending on the make and age of HPLC column even when using the same solvent system.

   Under the conditions used in this protocol, all AHLs can be measured with different retention time from ~4 min to more than 30 min, depending on the acyl chain of AHLs. The retention time of these signals can be adjusted to ~10 min by altering the composition of the mobile phase, i.e., the percentage of methanol and water (see Table 1C.3.2). AHL should be dissolved in methanol as a stock solution of 50 mM, which can be diluted with water to an indicated concentration before analysis.

   Unlike gradient elutions where a continuous change in the composition of the mobile phase is employed, isocratic separations are performed with a constant concentration of the eluent or mobile phase.

Table 1C.3.2 Retention Times and Mobile Phase for Representative AHLs

AHL	Retention time (min)	Mobile phase (methanol:water)
3-HO-C4-HSL	7.2	35:65
C4-HSL	7.7	40:60
3-oxo-C6-HSL	9.3	40:60
C6-HSL	8.6	45:55
3-oxo-C8-HSL (3OC8HSL)	8.1	50:50
C8-HSL	9.6	50:50
3-oxo-C10-HSL	12.5 or 15.3	60:40 or 50:50
C10-HSL	11.8	65:35
3-oxo-C12-HSL	9.9	75:25

SUPPORT PROTOCOL 6

HPLC IDENTIFICATION OF AHL-ACYLASE HYDROLYSIS PRODUCTS

If the AHL-inactivating enzymes identified using the Basic Protocol and Support Protocol 1 are not predicted to be lactonases using the methods in Support Protocol 2 (i.e., if acidification of the reaction mixture is not able restore the AHL activity), the enzyme of interest is likely to be an AHL-acylase (Leadbetter and Greenberg, 2000; Lin et al., 2003). AHL-acylases hydrolyze the amide bond of AHL to generate two products: homoserine lactone (HSL) and a fatty acid. Because HSL is extremely hydrophilic and hard to resolve from the buffer fraction in HPLC analysis, it is difficult to detect the HSL product peak with the HPLC regime designated for AHL analysis (Lin et al., 2003). However, the HSL fraction can be resolved using a dansylation technique. Dansylation increases the hydrophobicity of HSL, making it more readily resolved during HPLC separation and enabling reliable identification of the AHL-inactivating enzyme as an acylase.

Materials

N-3-oxo-octanoyl-L-homoserine lactone (3OC8HSL; Sigma-Aldrich) in KPBS (see *APPENDIX 2A* for KPBS; for purified enzyme assay)
Purified enzyme (Lin et al., 2003)
3OC8HSL (solid; for cell debris assay)
Cell debris from 2 ml bacterial cultures (Support Protocol 4, step 4 pellet)
Homoserine lactone (HSL; Sigma-Aldrich) standard
2.5 mg/ml DANSYL chloride (Sigma-Aldrich) in acetone
0.2 M HCl

Additional reagents and equipment for HPLC (see Support Protocol 5)

1. Equilibrate a C18 reverse-phase column as described in Support Protocol 5, step 1.

2a. *When using purified enzyme:* Incubate 3 mM 3OC8HSL in 0.5 ml KPBS with ~100 ng purified enzyme 3 hr at 37°C.

The final concentration of KPBS is not critical.

2b. *When using cell debris:* Incubate 3 mM 3OC8HSL in 0.5 ml cell debris 3 hr at 37°C. Mix the reaction solution constantly by shaking until completion of the digestion (~3 hr), then centrifuge 10 min at 10,000 × *g*, and collect the supernatants for step 3.

Complete digestion can be confirmed by using the Basic Protocol. Because the free amino acids in cell-free protein extracts can interact with the dansylation reagent, i.e., DANSYL chloride, which increases the difficulty to resolve the DANSYL-HSL fraction,

it is recommended to use the purified AHL-inactivating enzyme (Lin et al., 2003) or the KPBS buffer–washed cell debris because the cell membrane fraction (cell debris) contains significantly higher AHL-acylase activity than cell-free protein extracts.

3. For DANSYL chloride derivatization, add 100 µl of the digested mixture and a 1.5 mM HSL standard to an equal volume of 2.5 mg/ml DANSYL chloride, respectively. Incubate 4 hr at 40°C.
4. After evaporation to dryness, add 50 µl of 0.2 M HCl to the sample to hydrolyze any excess DANSYL chloride.
5. Conduct HPLC analysis as in Support Protocol 5, steps 7 and 8; dilute the samples when necessary before injection.

   It is necessary to dilute the sample when the UV absorbance is >2.

   If the enzymatic digestion mixture has a product peak with identical retention time as the dansylated HSL standard (about 2.9 min under the above conditions), and shares a similar UV spectrum, the AHL-inactivating enzyme under investigation is identified as an AHL-acylase.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Minimal medium agar supplemented with 50 µg/ml X-gal

To 1 liter H_2O add:
2.0 g mannitol
2.0 g $(NH_4)_2SO_4$
10.5 g K_2HPO_4
4.5 g KH_2PO_4
0.2 g $MgSO_4.7H_2O$
5 mg $FeSO_4$
10 mg $CaCl_2$
2 mg $MnCl_2$
15 g agar
Adjust pH to 7.2 with 1 M HCl or 1 M NaOH, if necessary
Autoclave
Cool to ~50°C
Add 1 ml X-gal 50 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal; prepare in pure dimethylformamide and store up to 6 months at −20°C)
Pour into 90-mm petri dishes using 20 ml per plate
Store up to 2 weeks at 4°C

YEB medium with and without 50 µg/ml kanamycin and 10 µg/ml tetracycline

To 1 liter H_2O add:
10 g Bacto-tryptone
5 g yeast extract
5 g NaCl
5 g sucrose
0.5 g $MgSO_4.7H_2O$
Adjust pH to 7.0 with 1 M HCl or 1 M NaOH, if necessary
Autoclave
Cool to <50°C

continued

Add 50 mg/ml kanamycin (prepare stock in water and store up to 6 months at −20°C) to 50 μg/ml

Add 10 mg/ml tetracycline (prepare stock in 100% ethanol and store up to 6 months at −20°C) to 10 μg/ml

Store up to 2 weeks at 4°C

COMMENTARY

Background Information

The exciting findings that QS signals are involved in regulation of a range of important biological functions—in particular, the expression of virulence genes and formation of biofilms (Section 1B)—provide an important impetus for screening novel enzymes and inhibitors that interfere with bacterial QS. The first gene encoding an AHL-inactivating enzyme (*aiiA*) was cloned from soil bacterial isolates belonging to a Gram-positive *Bacillus* species, after screening more than 500 bacterial isolates and strains using the diffusion plate assay described in the Basic Protocol (Dong et al., 2000). The enzyme was later characterized as an AHL-lactonase (Dong et al., 2001).

Shortly after the discovery of the first enzyme, a bacterial strain (*Variovorax paradoxus*) capable of using AHL molecules as the sole sources of energy and nitrogen was reported (Leadbetter and Greenberg, 2000). Presence of homoserine lactone in the AHL metabolic mixture of *V. paradoxus* suggested that the bacterium might carry a second enzyme, an AHL-acylase. The same diffusion plate protocol helped to identify and clone the first reported AHL-acylase gene (*aiiD*) from a *Ralstonia* species as the result of collaboration by three laboratories (see Lin et al., 2003). These two groups of quorum-quenching enzymes (AHL-lactonases and AHL-acylases) have now been identified in numerous bacterial strains belonging to more than ten bacterial species (for a more recent review, see Dong and Zhang, 2005).

Intriguingly, lactonase-like enzyme activity was recently found in certain human tissues (Chun et al., 2004) and in the sera of all mammalian species tested (Yang et al., 2005). The purified human paraoxonases, especially PON2, hydrolyze several tested AHL signals (Draganov et al., 2005). Expression of mouse PON1, PON2, and PON3 enzymes in animal cells significantly boosted their ability to degrade a range of AHL signals (Yang et al., 2005). The mammalian paraoxonase family consists of three members: PON1, PON2, and PON3. PON1 was initially identified by its ability to hydrolyze toxic organophosphate and was subsequently shown to play a role in protection against atherosclerosis by hydrolyzing the derivatives of oxidized cholesterol and phospholipids (for a review, see Draganov and La Du, 2004).

The known AHL-inactivating enzymes can be grouped into two categories based on the site of action in the AHL substrates (for a review, see Dong and Zhang, 2005). AHL-lactonases and paraoxonases (PONs) belong to the lactonase group that catalyze the hydrolysis of the lactone ring of AHL signals to produce corresponding acyl homoserines (Dong et al., 2001; Zhang et al., 2002; Yang et al., 2005), whereas AHL-acylases break the amide linkage of AHLs to release homoserine lactone and the corresponding fatty acids (Leadbetter and Greenberg, 2000; Lin et al., 2003). In an acidic buffer, the acyl-homoserine can reform into AHL by restoration of the lactone ring. In contrast, the amide linkage between homoserine lactone and fatty acid cannot be restored under the same conditions (see Fig. 1C.3.3 for illustration).

Evidence is emerging regarding the roles of these quorum-quenching enzymes in microorganisms and eukaryotes. The AHL-lactonase produced by *Bacillus* species was shown to play a role in microbe-microbe interactions (Dong et al., 2004). The same enzyme produced by *Agrobacterium tumefaciens* is implicated in quorum sensing signal turnover when the bacterial pathogen encounters starvation stresses (Zhang et al., 2002; Zhang et al., 2004). However, the physiological role(s) and natural substrates of AHL-acylases and PON enzymes are far from clear (Leadbetter and Greenberg, 2000; Lin et al., 2003; Draganov and La Du, 2004; Yang et al., 2005). Microbe-microbe and pathogen-host interactions are ubiquitous in natural ecosystems, and diverse bacterial species adopt QS mechanisms to regulate a range of important biological functions relating to infection and survival. Given these observed phenomena, investigation of the roles of such enzymes in their natural hosts could provide new insights about immunity in mammalian species and about complexity and mechanisms of microbial interactions in ecosystems (Zhang and Dong, 2004).

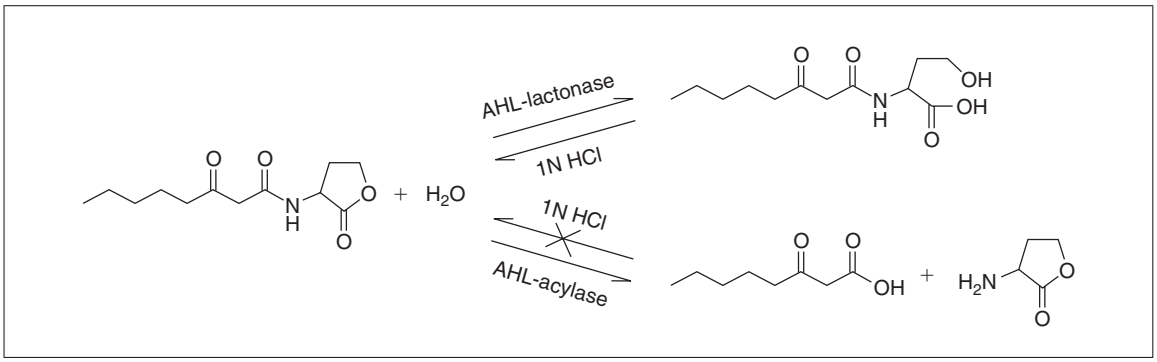

Figure 1C.3.3 Enzymatic inactivation of AHL by AHL-lactonase and AHL-acylase. Unlike the AHL-acylase hydrolysis products, the hydrolysis product of AHL-lactonase or paraoxonases (PONs) can reform AHL by acidification.

The quorum-quenching enzymes appear to have much potential in biotechnological applications. These enzymes have been used as tools for probing the biological functions of AHL signals in bacterial pathogens (Dong et al., 2000; Lin et al., 2003; Molina et al., 2005). Expression of these enzymes in transgenic plants efficiently quenches microbial QS signaling and blocks pathogenic infections (Dong et al., 2001; Zhang, 2003). Detection and analysis of quorum-quenching enzymes have become routine in microbial research in exploring new ways of controlling bacterial infections.

Critical Parameters

AHL signals are not very stable in aqueous solutions; enzymatic reactions should not proceed for more than 6 hr.

AHL biosensor strains other than those described in this unit may be used provided they are sensitive to the low level of AHL signals used in this unit.

For those biosensor strains with the reporter gene carried on a plasmid, it is important to supply appropriate antibiotics in growth medium to prevent loss of the plasmid construct during propagation of cultures.

Certain enzymes such as AHL-acylase (Lin et al., 2003) are membrane associated. When AHL-inactivating activity is detectable using bacterial cultures but not using cell-free total protein extracts, one can use the cell debris suspension to replace total protein extracts for enzyme activity assay.

Support Protocol 3 is designed for quantitative analysis of relative enzyme activity of different samples. The accuracy of analysis is dependent on the reliability of standard curve and accurate determination of diffusion distance.

Anticipated Results

The Basic Protocol is designed for rapid and reliable screening of biological samples with AHL-inactivating enzyme activities. Numerous bacterial isolates carrying either AHL-lactonase or AHL-acylase have been identified using this protocol or similar ones; however, there may be other types of AHL-inactivating enzymes. In theory, there could be at least four types of enzymes that could degrade AHL signals, including lactonases and decarboxylases that could hydrolyze the lactone ring as well as acylases and deaminases that might separate the homoserine lactone moiety and the acyl side chain (for details, see Dong and Zhang, 2005).

The lactone ring broken by a decarboxylase should not be restorable by acidification; the product of an AHL-decarboxylase should have an HPLC retention time different from that of an AHL-lactonase because the products differ in hydrophobicity. Therefore, both the acidification procedure (Support Protocol 2) and the HPLC method (Support Protocol 5) described in this unit should be sufficient for distinguishing a lactonase from a decarboxylase.

Degradation of AHL by a deaminase will produce a fatty acid amide molecule, which could also be dansylated by DANSYL chloride. The HPLC regime described in the Support Protocol 5 might not be able to resolve short chain (less than six carbons) dansylated fatty acid amide from dansylated homoserine lactone. But the potential problem could be resolved by analyzing several AHL signals of different acyl side chain lengths.

By measuring the peak area of AHL and its product using the built-in software in the HPLC equipment, Support Protocols 5 and 6 can also be used to quantify quorum-quenching enzyme activity. It is particularly

useful for analysis of enzyme kinetics against a range of AHL substrates (Wang et al., 2004).

Time Considerations

For the Basic Protocol, initiating growth of biosensor and bacterial isolates can be done in the morning of the first day; setting up the reaction and bioassay will use the morning and afternoon, respectively, of day 2; results will be obtained on day 3. Confirmation of enzymatic AHL inactivation (Support Protocol 1) and testing AHL-lactonase (Support Protocol 2) can be performed simultaneously and can be completed within 3 days. Preparation of a standard curve (Support Protocol 3) will take 2 days if the biosensor is ready. For HPLC analysis (Support Protocols 5 and 6), growth of bacterial cells and preparation of protein extracts or purification of enzyme will take ~2 days; the enzymatic reaction and HPLC analysis can be accomplished within one day for each type of enzyme.

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Example of Real-Time Quantitative Reverse Transcription–PCR (Q-RT-PCR) Analysis of Bacterial Gene Expression during Mammalian Infection: *Borrelia burgdorferi* in Mouse Tissues

Quantitative reverse transcription–PCR (Q-RT-PCR) is an important tool used to measure the expression level(s) of a gene(s) of interest in a wide variety of experimental settings. An important application of this procedure involves the detection and quantification of bacterial transcript levels within infected mammalian host tissues. This unit focuses on Q-RT-PCR analysis of the spirochete *Borrelia burgdorferi* within infected mouse tissues. As bacterial infection progresses, the number of bacteria within a tissue decreases. Q-RT-PCR is the most powerful and reliable method for detecting gene expression by a bacterial population whose numbers greatly reduce as infection time increases. Specifically, this unit details the extraction of RNA from infected mouse tissues, removal of contaminating genomic DNA from the purified RNA, preparation of cDNA and genomic DNA standards, LightCycler-based PCR, and a relative quantification analysis of the cDNA.

CAUTION: Borrelia burgdorferi is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

EXTRACTION OF RNA FROM *B. BURGDORFERI*-INFECTED MOUSE TISSUES

BASIC PROTOCOL 1

This protocol describes the pulverization of frozen *B. burgdorferi*–infected mouse tissues with a frozen RNase-free crucible and pestle, homogenization with a tissue homogenizer, and RNA extraction with a Trizol reagent (Chomczynski and Sacchi, 1987). Although the procedures described herein are for *B. burgdorferi*, the instructions given for the storage, handling, and processing of infected tissues are universally applicable. In addition, Trizol reagent has been widely used to extract RNA from numerous Gram-positive and Gram-negative bacteria cultivated under a variety of experimental settings (Chomczynski and Sacchi, 1987; Chomczynski and Mackey, 1995).

CAUTION: Trizol contains phenol, which is a poison and skin irritant. 1-Bromo-3-chloropane is also hazardous. Use in a certified fume hood and consult the Material Safety Data Sheets (MSDS) accompanying these chemicals for instructions on personal protection, use, storage, and disposal.

IMPORTANT NOTE: The use of RNase-free solutions, containers, pipettors, and benchtops is tantamount to the success of this protocol. Hands are significant sources of RNases, therefore, wear gloves at all times and change them frequently. Solutions are made RNase-free via the addition of DEPC (di-ethyl-pyrocarbonate) or RNA Secure (Ambion; also see *APPENDIX 2A*). Spray benchtops and micropipettors with RNase-Away (Fisher Scientific), and bake glassware in an oven to kill RNases. Only use sterile, RNase-free polyethylene

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or polypropylene test tubes. If possible, keep a separate set of pipettors used only for RNA-extraction procedures. See Sambrook et al. (1989) for detailed instructions on maintaining RNase-free conditions.

Materials

RNase-Away (Fisher Scientific)
Dry ice
Trizol reagent, stored at 4°C
RNAsecure resuspension solution (Ambion), stored at −20°C
Cryotubes containing *B. burgdorferi*–infected tissue, −80°C
Sterile, DEPC-treated, double-distilled water (*APPENDIX 2A*)
5% (w/v) SDS (prepared in sterile, DEPC-treated water)
1-Bromo-3-chloropane
2- Propanol (isopropanol)
75% ethanol (diluted in sterile, DEPC-treated water)

Powder-free exam gloves
Microcentrifuge tube racks
1-ml, 200-µl, and 20-µl micropipettors and corresponding RNase-free barrier tips
4 9/16–in. (~11.59-cm) blunt forceps
Ceramic crucible and pestle
Variable speed digital Dremel homogenizer (Fisher Scientific)
Ice buckets with lids
Aluminum foil cut into ~2 × 2–in. (~5 × 5–cm) squares
Sterile 12 × 75–mm, 5-ml polystyrene round-bottom conical tubes
Sterile, RNase-free 5-ml conical tubes (Ambion)
Sterile, RNase-free 1.5-ml microcentrifuge tubes (Ambion)
60°C heating block
50-ml polypropylene conical tubes, sterile
Refrigerated microcentrifuge, 4°C
Freezer boxes

Prepare equipment and reagents for homogenization

1. Wearing gloves, spray work bench, tube racks, micropipettors, forceps, crucible and pestle, and dremel portion of homogenizer with RNase-Away. Allow to air dry.

   To ensure successful RNA extraction, all equipment and work areas must remain RNase free for the duration of these experiments.

2. Fill one ice bucket with dry ice and place the 4 9/16–in. (~11.59-cm) blunt forceps, crucible, and pestle into the bucket. Place one ~2 × 2–in. (~5 × 5–cm) square of aluminum foil inside the crucible. Place lid on ice bucket and allow equipment to equilibrate.

   The items in the bucket will become sufficiently cold within 10 to 15 min. During equilibration, the remaining preparation steps listed below may be carried out.

3. Label 5-ml conical tubes (one per tissue) and 1.5-ml microcentrifuge tubes (two per tissue) with a permanent ink pen. Change gloves.

   Gloves must be changed frequently to maintain RNase-free conditions.

4. Using a 1-ml micropipettor, pipet 1 ml Trizol reagent into each labeled 5-ml conical tube.

5. Fill the second ice bucket with ice and place the 5-ml conical tubes containing Trizol reagent on the ice.

6. Remove the RNAsecure resuspension solution from −20°C and place in a 60°C heating block.

   The RNAsecure resuspension solution must be pre-equilibrated to 60°C before RNA can be resuspended in this solution.

Homogenize frozen B. burgdorferi–infected tissue samples

7. Once the equipment placed on dry ice have sufficiently equilibrated, remove one cryotube containing *B. burgdorferi*–infected tissue from −80°C, and place on dry ice. Change gloves.

   If working with spleen, proceed directly to step 10. Due to the high amount of RNase activity present within spleen tissue, this organ is directly homogenized without prior pulverization. This technique consistently results in increased quantities of high-quality RNA. For all other tissues, proceed to step 8.

8. Working quickly over the dry ice bucket, remove the frozen tissue from cryotube with forceps and wrap in the frozen aluminum foil square. Place wrapped tissue in the frozen crucible on dry ice.

9. Using the frozen pestle, quickly pulverize the foil-wrapped tissue within the crucible.

   For most frozen tissues, two to three applications of the pestle are sufficient to break the frozen tissue into pieces suitable for homogenization. Do not allow the pestle or tissue to thaw, as RNases can more easily degrade the RNA if samples or equipment become warm.

10. Quickly unwrap the foil. Using the forceps, quickly and carefully place all pulverized tissue pieces into the 5-ml conical tube containing Trizol reagent and place tube back on ice.

11. Keeping the tube on ice, use setting five on the tissue homogenizer to completely homogenize the tissue pieces within the Trizol reagent.

    This setting is sufficient to completely homogenize most tissues with only three to four strokes. Due to the presence of bone, joints require an initial homogenization with the seven setting. The five setting can then be used to complete the homogenization.

Clean tissue homogenizer

12. To clean the tissue homogenizer, pour ∼30 ml sterile, DEPC-treated water into a 50-ml centrifuge tube. Place the Dremel end of the homogenizer into the water and homogenize on setting five for 5 to 10 sec. Remove the homogenizer.

    The homogenizer must be thoroughly cleaned after the homogenization of each individual tissue sample to prevent cross-contamination of RNAs isolated from different tissues.

13. Dispense ∼30 ml of 5% SDS solution into a 50-ml centrifuge tube. Place the Dremel end of the homogenizer into the SDS solution and homogenize on setting five for 5 to 10 sec. Remove the homogenizer.

14. Repeat step 12 three more times. Wipe off excess water from the Dremel end of the tissue homogenizer with a Kimwipe.

15. Spray Dremel end of tissue homogenizer with RNase-Away. Wipe off excess RNase-Away with a Kimwipe.

16. To process another tissue sample, go back to step 7 and proceed with the remaining protocol steps.

Extract RNA

17. Change gloves. Using the 1-ml micropipettor, transfer each homogenized sample from its 5-ml conical tube to a 1.5-ml microcentrifuge tube. Incubate 5 min at room temperature.

18. Change gloves. Add 100 µl of 1-bromo-3-chloropane to each sample. Shake each tube vigorously to mix contents. Incubate 3 min at room temperature.

    Alternatively, 200 µl of chloroform can be added to each sample. RNA extracted using chloroform is suitable for northern blot analyses. However, chloroform inhibits the reverse transcriptase reaction used to synthesize cDNA from RNA (Chomczynski and Mackey, 1995). For this reason, 1-bromo-3-chloropane is used in this protocol.

    Do not vortex the sample as the RNA will shear.

19. Centrifuge samples in a refrigerated microcentrifuge 15 min at 12,000 × *g*, 4°C.

20. Change gloves. Using the 200-µl micropipettor, carefully transfer the aqueous phase of each sample into a new 1.5-ml microcentrifuge tube. Leave a bit of the aqueous phase behind to ensure that the interphase is not disturbed during pipetting.

    The addition of 1-bromo-3-chloropane to the Trizol-containing samples results in a phase-separation reaction. The top phase is the aqueous phase and contains the RNA. The next two phases, the milky interphase and the pink organic phase, contain DNA and protein (Chomczynski, 1993). Tubes containing the interphase and organic phases may be discarded. Follow all institutional guidelines for the proper handling and disposal of hazardous chemical materials.

21. Change gloves. Add 500 µl isopropanol to each tube. Shake vigorously and incubate 10 min at room temperature. Centrifuge samples 10 min at 12,000 × *g*, 4°C.

    Isopropanol is added to precipitate the RNA out of solution. Following centrifugation, a small white pellet containing the RNA is usually visible at the bottom of the tube. For some very small tissues, such as bladder, this pellet may barely be visible.

22. Following centrifugation, carefully decant the supernatant into an appropriate waste container. Change gloves. Add 1 ml of 75% ethanol to each tube to wash the pellet. Centrifuge samples 5 min at 7500 × *g*, 4°C.

    The application of 75% ethanol helps wash impurities away from the pellet and makes it easier to resuspend.

23. Carefully decant the supernatant from the pellet. Change gloves. Allow the pellet 5 min to air-dry by placing the open 1.5-ml microcentrifuge tube upside-down onto a clean Kimwipe. Gently tap out all remaining drops of supernatant.

    It is critical that the pellet does not over-dry, as it becomes extremely difficult to resuspend. Do not allow drying time to exceed 10 min.

24. Change gloves. Using the appropriate micropipettor, resuspend each pellet in a suitable volume of RNAsecure resuspension solution that has been pre-warmed to 60°C (Table 1D.3.1). Gently flick the bottom of the tube to aid resuspension of the pellet. Immediately incubate all samples 10 min at 60°C.

Table 1D.3.1 Recommended Resuspension Volumes for RNA Pellets Obtained from Select *Borrelia burgdorferi*–Infected Mouse Tissues

Tissue	Resuspension volume (µl)
Bladder	10–30
Heart	50–60
Joints	50–60
Spleen	100
Ears	50–60

Alternatively, RNA pellets can be resuspended in sterile, DEPC-treated double-distilled water and then heated. The RNAsecure resuspension solution inactivates RNases when heated 10 min at 60°C, improving overall RNA quality and yield. Incubation of the samples at 60°C is instrumental in facilitating complete resuspension of the pellet in solution.

25. Store all RNA samples in freezer boxes up to ~1 year at −80°C.

Avoid repeated freeze-thaw cycles, and dispense RNA aliquots into several tubes, if necessary. Always thaw and work with RNA samples on ice. Alternatively, proceed to Basic Protocol 2 without freezing the RNA.

QUALITY CONTROL: REMOVAL OF CONTAMINATING GENOMIC DNA AND ASSESSMENT OF RNA QUALITY AND YIELD

BASIC PROTOCOL 2

In Basic Protocol 1, RNA was extracted from *B. burgdorferi*–infected mouse tissues. However, the RNA samples used in the protocol are not pure and are contaminated with both protein and DNA. Before cDNA can be reversely transcribed from the RNA samples, the contaminating genomic DNA must be removed. After the successful removal of contaminating DNA, the concentration of each RNA sample is determined spectrophotometrically, and the relative amount of contaminating protein present in each RNA sample is assessed. The quality of the extracted RNA is determined via agarose gel electrophoresis. Although the protocol described herein has been tailored for RNA samples extracted from *B. burgdorferi*–infected mouse tissues, they are broadly applicable to a wide variety of experimental systems.

CAUTION: Ethidium bromide is a mutagen. Wear gloves and avoid contact with skin.

CAUTION: Wear goggles or other suitable eye protection when using the UV light box. Avoid prolonged exposure and wear protective face shield, if necessary.

NOTE: Refer to Sambrook et al. (1989) for 5× TBE and 5× agarose gel electrophoresis loading dye recipes.

IMPORTANT NOTE: The use of RNase-free solutions, containers, pipets, and benchtops is tantamount to the success of this protocol. Hands are a significant source of RNase, therefore, wear gloves at all times and frequently change them. Solutions are made RNase-free via the addition of DEPC (di-ethyl-pyrocarbonate) or RNAsecure (Ambion). Benchtops, and micropipettors are sprayed with RNase-Away (Fisher Scientific), and glass containers are baked in an oven to kill RNases. Use only sterile, RNase-free polyethylene or polypropylene test tubes and conical tubes. If possible, keep a separate set of pipets used only for RNA-extraction procedures. See Sambrook et al. (1989) for detailed instructions on maintaining RNase-free conditions.

Materials

RNase-Away (Fisher Scientific)
RNA extracted from *B. burgdorferi*–infected tissues, –80°C
10× Turbo DNA-free buffer (Turbo DNA-free kit) treated with RNAsecure (Ambion)
25× RNAsecure solution (Ambion), stored at −20°C
DNase inactivation reagent (Turbo DNA-free kit) treated with RNAsecure (Ambion)
10 U/μl RNase-free DNase I (Roche Applied Science)
Agarose
1× TBE: dilute from 5× TBE stock (*APPENDIX 2A*; also see Sambrook et al., 1989) in sterile, DEPC-treated water (*APPENDIX 2A*) and treat with RNAsecure
Ethidium bromide staining solution (*APPENDIX 2A*)

5× DNA loading dye (prepared in sterile, DEPC-treated water and treated with RNAsecure; see Sambrook et al., 1989)
100-bp DNA ladder (New England Biolabs)
Takara 10× dNTPs (Fisher Scientific)
Takara 10× PCR buffer (Fisher Scientific)
30 μM *B. burgdorferi* Fla1 oligonucleotide primer (Miller et al., 2005)
30 μM *B. burgdorferi* Fla2 oligonucleotide primer (Miller et al., 2005)
Takara *Taq* polymerase (Fisher Scientific)

10-μl micropipettor and barrier tips
37° and 60°C heating blocks
Sterile, RNase-free 1.5-ml microcentrifuge tubes
Refrigerated microcentrifuge
Agarose gel casting apparatus, gel trays, and combs
Agarose gel electrophoresis gel box
Electrophoresis power supply
UV light box
RNase-free, sterile, thin-walled PCR tubes
Thermal cycler

Remove contaminating genomic DNA

1. Wearing gloves, spray work bench and micropipettors with RNase-Away. Let air-dry.
2. Remove RNA samples from −80°C freezer and thaw on ice.
3. Thaw 10× Turbo DNA-free buffer and DNase inactivation reagent on ice. When thawed, incubate 10 min in a 60°C heating block. Cool 5 min on ice.

   Incubation at 60°C activates the RNAsecure (Ambion) previously added to these reagents and inactivates any RNases present in these vials.

4. Change gloves. Remove 2 μl of each RNA sample and place into labeled, sterile, RNase-free 1.5-ml microcentrifuge tubes. Set these tubes aside on ice.

   These "dirty" RNA samples will be compared with the corresponding "clean" (DNA-free) samples by agarose gel electrophoresis, providing a method whereby the success of the DNase I treatment is evaluated.

5. Change gloves. Add 0.1 vol of 10× Turbo DNA-free buffer and 3 μl DNase I to each RNA sample. Gently flick the bottom of each tube to mix its contents. Pulse spin each sample in a refrigerated microcentrifuge so that all contents settle to the bottom of the tube.

   Alternatively, 1 μl DNase I may be used. However, for the majority of samples, this is not enough enzyme to digest all of the contaminating genomic DNA present. As a result, repeated DNase I treatments are necessary. Repeated 37°C incubations increase the potential for damaging RNase activity. For most samples, using 3 μl enzyme ensures that only one DNase I treatment is performed.

6. Incubate the samples 30 min in a 37°C heating block.
7. Remove the samples from the heating block and place on ice. Change gloves. Vortex the DNase inactivation reagent and add 10 μl to each tube. Flick the bottom of the tube to mix the contents. Incubate 2 min at room temperature, periodically flicking the tube to keep the contents mixed.

   The DNase inactivation reagent also removes any divalent cations that are present within the samples. This is important because some divalent cations act as co-factors for the activation of RNases.

8. Centrifuge the samples 5 min at 5000 × *g*, 4°C, to pellet the inactivation reagent. Change gloves and transfer the RNA to new microcentrifuge tubes. Place the tubes back on ice.

   It is necessary to transfer the RNA to new tubes because the DNase inactivation reagent could inhibit RT-PCR reactions. After this step, samples can be stored up to ∼1 year at −80°C or proceed directly to step 9.

Analyze extracted RNA by agarose gel electrophoresis

9. Spray all surfaces of the agarose gel casting apparatus, gel tray, comb, and gel box with RNase-Away. Set up gel tray and comb on casting apparatus, and place the lid on the gel box. Let the RNase-Away air-dry.

   Do not spray RNase-Away on the electrodes on the lid of the gel box. Instead, using a Kimwipe, carefully apply RNase-Away to the gel box lid.

10. Prepare a 1% agarose gel in RNase-free 1× TBE. When the agarose solution has cooled to ∼60°C, add ethidium bromide staining solution (2 µl for small gels, 4 µl for large gels). Gently swirl the flask to mix contents. Pour the solution into the gel tray set up in the casting apparatus and allow the gel to solidify.

    Refer to Sambrook et al. (1989) for instructions on the preparation and casting of agarose gels.

11. While waiting for the agarose gel to solidify, transfer 2 µl of each DNase I–treated RNA sample to labeled, sterile, RNase-free 1.5-ml microcentrifuge tubes and place on ice.

12. Add 8 µl RNase-free 1× TBE to each of the "dirty" RNA samples (set aside in step 4) and DNase I–treated ("clean") samples.

13. Add 2 µl RNase-free 5× DNA loading dye to each RNA sample. Flick the bottom of each tube to mix its contents and pulse spin to collect sample at the bottom of each tube. Place all tubes on ice.

14. Place the solidified agarose gel and its gel tray into the gel box. Add enough RNase-free 1× TBE buffer to completely cover the gel.

15. Pipet 5 µl of the 100-bp DNA ladder into one well of the gel.

16. For RNA-extracted samples, first pipet a "dirty" sample into a well, then add a "clean" sample into the next well.

    Applying the pre- and post-DNase I–treated aliquots of each RNA sample to the gel in this specific order allows a side-by-side comparison of each "dirty" and "clean" aliquot, therefore, assessing the efficacy of the DNase I treatment. In addition, if RNase-mediated degradation of the RNA has occurred, this method allows the scientist to determine whether this degradation occurred before or during DNase I treatment of the RNA.

17. Connect the electrodes of the gel box to the power supply and run the gel for 45 min to 1 hr at 125 (small gels) or 145 V (large gels).

    For more detailed instructions on the proper voltage to use for electrophoresis of agarose gels of varying sizes, consult Sambrook et al. (1989).

18. After electrophoresis has concluded, check the integrity of each RNA sample by illuminating the gel on a UV light box.

    As illustrated in Fig. 1D.3.1, at least two sharp discrete bands should be visible for each sample. Since RNA was extracted from mouse tissue, these two bands represent the mammalian 28S and 18S ribosomal RNAs (rRNAs). If one or both of these bands are missing or smeared, then RNase-mediated degradation of the RNA has occurred. Under

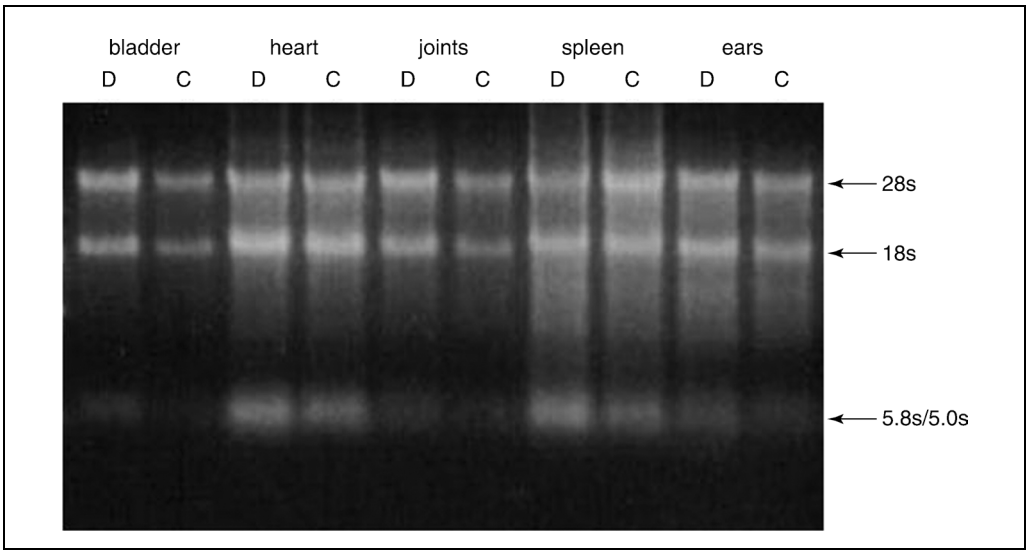

Figure 1D.3.1 Assessment of RNA integrity prior to, and following DNase I treatment. RNA was isolated from bladder, heart, joint, spleen, and ear tissues obtained from a *B. burgdorferi*-infected mouse. The integrity of each RNA sample was then assessed before (D = dirty) and after DNase I treatment (C = clean). A volume of 2 μl of each RNA sample was analyzed by agarose gel electrophoresis and resultant bands were visualized with ethidium bromide. Mouse 28S, 18S, and 5.8S/5.0S rRNA bands are indicated by arrows. Note the presence and clarity of all three rRNA bands.

these circumstances, the integrity of the sample is not good enough for Q-RT-PCR analysis. A third, higher molecular weight band is sometimes visible in the "dirty" samples, which represents the contaminating genomic DNA. This band should be absent in the "clean" samples if the DNase I treatment was successful.

Occasionally, the 5.8S/5.0S rRNAs are visible on the gel. If visible, they will appear as one very low molecular weight band, due to the limited resolution of the 1% agarose gel. However, due to their small size, these rRNAs are often not visible on a 1% agarose gel.

19. Determine RNA concentration by spectrophotometry (see Support Protocol 1).

Determine RNA purity by PCR analysis

20. Change gloves. To set up 100-μl PCR reactions, pipet 8 μl dNTPs and 10 μl of 10× PCR buffer into each clean, RNase-free, thin-walled PCR tube. Add 5 μl of 30 μM Fla1 and 5 μl of 30 μM Fla2 oligonucleotide primers to each tube. Place the tubes on ice.

This protocol is designed for 100-μl PCR reactions but can easily be modified for other volumes. In addition, a mastermix containing everything but RNA and DEPC-treated water can also be used. Add the RNA and water to each tube after the mastermix is dispensed into each tube. Fla1 and Fla2 are oligonucleotide primers designed to amplify the B. burgdorferi housekeeping gene, flagellin B (flaB). Primers specific for other bacterial housekeeping genes may also be used.

21. Change gloves. Add 1 μg of a DNase I–treated RNA sample and the appropriate volume of sterile, DEPC-treated water to each tube (to bring volume up to 100 μl). Place the tubes back on ice.

For each sample of RNA, 1 μg is used as the template for PCR reactions because this is the amount of RNA that will later be reverse-transcribed into cDNA.

22. Pipet 1 μl of *Taq* polymerase into each PCR tube.

23. Place the tubes into the thermal cycler. Using the following parameters, program the thermal cycler according to the manufacturer's instructions.

40 cycles:	1 min	94°C (denaturation)
	1 min	50°C (annealing)
	1 min	65°C (extension)

For this PCR reaction, 40 cycles are used because the LightCycler-based protocol (see Basic Protocol 3) used for cDNA amplification requires 40 cycles.

24. Change gloves. Pipet 10 µl of each completed PCR reaction and 2 µl of 5× DNA loading dye into clean, RNase-free microcentrifuge tubes. Analyze each PCR amplicon on a 1.5% agarose gel containing ethidium bromide. First load 5 µl of the 100-bp DNA ladder into one well of the gel, then load each PCR reaction. Run the gel for 1 hr at 135 V (for small gels) or 150 to 160 V (for large gels).

25. After electrophoresis, place the gel on a UV light box to examine the PCR results. If the DNAse I treatment was successful, only the DNA ladder should be visible. However, if genomic DNA is still present in the RNA samples, a band at ~450 bp will be visible. If such band is present, repeat the DNase I treatment. If no bands are present, either store the RNA up to 1 year at −80°C or proceed directly to the production of cDNA (see Basic Protocol 3).

SPECTROPHOTOMETRIC DETERMINATION OF RNA CONCENTRATION

SUPPORT PROTOCOL 1

To accurately assess the purity and concentration of the extracted RNA samples, both the A_{260} and A_{280} must be determined. The absorbance at 260 nm is used to quantify nucleic acids, whereas 280 nm is used to quantify protein. Therefore, the A_{280} is measured as an indicator of the amount of protein contamination present within the RNA sample (Chomczynski and Sacchi, 1987).

Materials

RNAsecure resuspension solution (Ambion), stored at −20°C
DNase I–treated RNA sample (see Basic Protocol 2)

RNase-free 1.5-ml microcentrifuge tubes
Spectrophotometer
Cuvettes

1. Using the RNA resuspension solution as diluent, prepare a 1:100 dilution of each DNase I–treated RNA sample. Dispense into clean, RNase-free 1.5-ml microcentrifuge tubes. Flick the bottom of each tube a few times to mix its contents and pulse spin to collect contents at the bottom of the tube. Place tubes on ice.

2. Pipet a suitable volume of RNA resuspension solution into a clean, RNase-free 1.5-ml microcentrifuge tube as the blank used to zero the spectrophotometer. Place the tube on ice.

   The blank and the diluent should always be the same solution in which the RNA was resuspended.

3. Set the wavelength for absorbance at 260 nm on the spectrophotometer. Carefully pipet the blank solution into a cuvette and place in the spectrophotometer. After the wavelength reading has stabilized, strike the zero key (or equivalent), where the A_{260} should read 0.00 nm. Remove the cuvette and set it aside.

4. Carefully pipet the first diluted RNA sample into a cuvette and place in spectrophotometer. Record the stabilized wavelength. Remove the cuvette and set it aside.

5. Change the wavelength on the spectrophotometer to 280 nm. Insert the blank cuvette and strike the zero key, where the A_{280} should read 0.00 nm. Remove the cuvette and set it aside.
6. Place the cuvette containing the first diluted RNA sample in the spectrophotometer. Record the stabilized wavelength. Remove the cuvette and discard, if disposable. If not disposable, rinse the cuvette out with water, followed by ethanol, and set it aside to dry.
7. Repeat steps 3 to 6 for all additional samples.
8. To determine the purity of each extracted RNA sample, divide the A_{260} value by the A_{280} value to obtain the A_{260}/A_{280} ratio. If the number obtained is ≥ 1.7, then the RNA is of sufficient purity for RT-PCR (Sambrook, et al., 1989).
9. To calculate the concentration of each RNA sample, multiply the A_{260} value by 100 (the dilution factor), and then by 40 (the value of 1 μl single-stranded RNA at an A_{260} of 1.0 is 40 ng). The resultant number is the concentration of RNA in nanograms per microliter (Sambrook, et al., 1989).

   The pathlength of the cuvette must also be considered in these calculations. Often, this value is 1 cm, so no multiplication factor is necessary. If the pathlength is not 1 cm, then the A_{260} must also be multiplied by this number. After the concentration has been determined, the RNA can be stored at −80°C.

BASIC PROTOCOL 3

QUANTITATIVE RT-PCR AND ANALYSIS

Basic Protocol 1 detailed the extraction and purification of RNA from *B. burgdorferi*–infected mouse tissues, and Basic Protocol 2 described the quality control procedures needed to ensure that the resultant RNA samples are of sufficient quality and purity for RT-PCR analyses. This protocol provides the step-by-step instructions necessary to synthesize cDNA from RNA and to assess the cDNAs for transcription of *B. burgdorferi* genes using a LightCycler-based approach. In addition, a method for determining the average relative expression of a gene of interest is also discussed. Although the procedures and examples described within this protocol have been adapted to examine *B. burgdorferi* gene expression, they are also appropriate for the analysis of genes from other bacteria.

IMPORTANT NOTE: The maintenance of RNase-free conditions is critical when handling RNA that will be converted to cDNA. DNA-free conditions are essential to prevent DNA contamination of cDNA or LightCycler PCR reagents. To maintain DNA-free conditions, spray all work areas and pipets with DNA-Away (Fisher). If possible, keep a separate set of pipets used only for Q-RT-PCR experiments. Also use separate boxes of RNase-free, DNA-free, sterile, barrier pipet tips. Hands are a significant source of DNA. Wear gloves at all times, and change them frequently.

NOTE: SYBR green is light-sensitive, therefore, do not expose to direct light for more than a few seconds. It is unstable or short-lived, therefore, do not subject to freeze-thaw cycles. Once SYBR green has been thawed and diluted, do not refreeze. Small, aliquoted volumes can be purchased to avoid this problem. SYBR green is more stable if diluted in TE buffer, than if diluted in water. Store small volume aliquots of diluted SYBR green suitable for single use. To avoid significant loss of activity, use all diluted aliquots of SYBR green within 3 months.

Materials

RNase-Away (Fisher Scientific)
DNA-free RNA sample
Sterile, DEPC-treated water (*APPENDIX 2A*)

DNA-Away (Fisher Scientific)
First-strand cDNA synthesis kit for RT-PCR (AMV; Roche) containing the following:
10× reaction buffer
25 mM $MgCl_2$
40 mM (10 mM each) dNTPs
0.04 A_{260} U/μl random primer p(dN)6
50 U/μl RNase-inhibitor
AMV reverse transcriptase
Enzyme diluent (Idaho Technology; also see recipe for SED in *APPENDIX 2A*)
10× dNTPs containing 2 mM each dNTP (Idaho Technology; also see *APPENDIX 2A*)
10× PCR buffer containing 30 mM $MgCl_2$ (Idaho Technology; also see *APPENDIX 2A*)
20 μM each of housekeeping gene primers Fla1 and Fla2 (Miller et al., 2005) *or* 20 μM of each oligonucleotide primer used to amplify gene of interest
Platinum *Taq* polymerase (Invitrogen)
Sterile TE buffer (made with sterile, DEPC-treated water; *APPENDIX 2A*)
10,000× SYBR green (Molecular probes) diluted 1000× in TE (final concentration 10×) in amber-colored, sterile, siliconized tubes
Genomic DNA standards (see Support Protocol 2)
2-, 10-, 20-, and 200-μl, and 1-ml micropipettors and RNase-free, DNA-free barrier pipet tips
Clear, sterile, RNase-free, DNA-free, siliconized 0.5-ml flat-top microcentrifuge tubes (Fisher Scientific)
42° and 99°C heating blocks
LightCycler centrifuge adapters in an aluminum cooling block (Roche), 4°C
20-μl LightCycler capillary tubes (Roche)
LightCycler thermal cycler with carousel (Roche)
Computer equipped with LightCycler software v. 3.5.3 (Roche)
Color printer

Prepare cDNA

1. Wearing gloves, spray work bench and RNA micropipettors with RNase-Away. Let air-dry.
2. Change gloves. Add 1 μg of each DNA-free RNA sample to sterile, RNase-free, DNA-free 0.5-ml microcentrifuge tubes and place tubes on ice.
3. Pipet the appropriate volume of sterile, DEPC-treated water into each tube such that the total volume of RNA plus water equals 8.2 μl. Set the tubes aside on ice.
4. Spray work bench and Q-RT-PCR micropipettors with DNA-Away. Let air-dry.
5. Change gloves. Prepare a master mix of the first-strand cDNA synthesis kit reagents needed to convert each RNA sample to cDNA in a sterile RNase-free, DNA-free 0.5-ml microcentrifuge tube. To establish the volume needed, multiply the following volumes, appropriate for one sample, by the number of samples being processed:

   2 μl 10× reaction buffer
   4 μl 25 mM $MgCl_2$
   2 μl 40 mM (10 mM each) dNTPs
   2 μl 0.04 A_{260} U/μl random primer p(dN)6
   1 μl 50 U/μl RNase-inhibitor
   0.8 μl AMV reverse transcriptase

   In this experimental setting, single-stranded cDNA is generated by the binding of the random primer to numerous nonspecific RNA sequences (Sambrook et al., 1989).

6. Flick the bottom of the tube a few times to mix its contents. Pulse centrifuge to collect the contents at the bottom of the tube. Change gloves. Aliquot 11.8 μl master mix into each tube containing 8.2 μl of RNA and DEPC-treated water (step 3).

   The total volume in each tube should now equal 20 μl.

7. Flick the bottom of each tube a few times to mix its contents. Pulse centrifuge the samples to collect the contents at the bottom of the tubes. Incubate 10 min at room temperature.

   This first incubation step is performed to allow the random primer to anneal to the RNA.

8. Incubate tubes 1 hr in a 42°C heating block.

   RNA is reverse transcribed into cDNA during this incubation period.

9. To inactivate the reverse transcriptase, incubate samples 5 min in a 99°C heating block. Remove tubes from the heating block and place 5 min on ice.

   If the reverse transcriptase is not heat-inactivated, it can interfere with PCR-based assays using the newly created cDNA (Kawasaki, 1990).

10. Store the cDNA up to 1 month at −20°C in a non-frost-free freezer. Alternatively, keep cDNA on ice and proceed directly to the LightCycler-based PCR (step 11).

Amplify genomic DNA standards and cDNA by LightCycler-based PCR

11. Change gloves. Spray work bench and Q-RT-PCR micropipettors with DNA-Away. Let air-dry.

12. Prepare a master mix of the reagents needed to conduct LightCycler-based PCR amplification of the genomic DNA standards and the cDNA synthesized from RNA extracted from *B. burgdorferi*-infected mouse tissues in a sterile, RNase-free, DNA-free, siliconized 0.5-ml flat-top microcentrifuge tube. To establish the needed volume, multiple the following volumes, appropriate for one sample, by the number of samples being processed:

    0.9 μl enzyme diluent
    1.0 μl 10× dNTPs
    1.0 μl 10× PCR buffer
    0.05 μl each 20 μM Fla 1 and 20 μM Fla 2 primers (final concentration 0.1 μM)
    or 0.2 μl of each 20 μM gene of interest primer (final concentration 0.4 μM)
    3.9 μl (or 3.6 μl) TE buffer
    0.1 μl Platinum *Taq* polymerase
    1.0 μl 10× SYBR green

    SYBR green binds double-stranded DNA. The LightCycler detects the amount of dye that is being incorporated into the newly synthesized PCR product and records this data as a function of the fluorescence incorporated versus temperature. The amount of SYBR green binding the DNA is decreased as the temperature increases. For more detailed information, consult the LightCycler operator's manual, version 3.5, and "Principles and Applications of the LightCycler" available online at http://www.Roche-applied-science.com/lightcycler-online.

13. Flick the bottom of the tube a few times to mix its contents. Pulse centrifuge to recollect the contents at the bottom of the tube. Set the tube aside on ice.

14. Remove the LightCycler centrifuge adapters located within the aluminum cooling block from 4°C. Change gloves. Place a 20-μl LightCycler capillary tube into the first adapter and aliquot 8 μl master mix into it.

15. Add 2 µl TE buffer (a negative control to ensure no DNA contamination of reagents), genomic DNA, or cDNA to the capillary tube. Place the cap firmly over the top of capillary tube to seal it.

    A minimum of ten samples will initially be analyzed with each primer set. A negative control and at least six genomic DNA standards are included. In addition, each cDNA sample is assessed in triplicate. The negative control should be the first sample prepared, followed by the genomic DNA standards and then each cDNA triplicate. Traditionally, each of these samples is first analyzed using the primers for the housekeeping gene (flaB in this example). Subsequent PCR assays are then set up using primers specific for a gene of interest. The data are then analyzed by comparing the values obtained for the gene of interest and the housekeeping gene (described in more detail below).

16. Repeat steps 14 and 15 for each sample to be analyzed.

17. After all of the samples have been aliquotted into the capillary tubes, carefully remove each adapter from the aluminum cooling block and place into a microcentrifuge. Collect the contents of each sample at the bottom of the capillary tube by centrifuging 1 min at 3000 × *g*, 4°C.

18. Carefully remove each adapter from the microcentrifuge and place it back into the cooling block. Carry samples over to the LightCycler thermal cycler using the cooling block.

19. Double click the LightCycler software icon on the computer desktop to open version 3.5.3 module.

20. Double click Run.

    This action opens a `new experiment`.

21. To set up the PCR reaction conditions that will be used for these samples, double click the Add button.

    The reaction conditions for these experiments have been previously reported (Gilmore et al., 2001; Miller et al., 2005).

22. When prompted for a program name, enter `denaturation`. Enter Type as `none`. Click the Ins button to insert the following cycling parameters:

    Cycles: `1`
    Temperature Target: `95°C`
    Hold Time: `120 sec`
    Slope: `20`
    2° Target Temp: `0`
    Step Size: `0`
    Acquisition Mode: `none`

    This program is needed to inactivate the hot-start enzyme that is bound to the Taq polymerase.

23. Double click the Add button and enter `amplification`. The Type is `quantification`. Prepare the program containing the cycling parameters for the PCR reaction in the following manner.

    a. Click Ins and enter the following information:
    Cycles: `40`
    Temperature Target: `94°C`
    Hold Time: `5 sec`
    Slope: `20`
    2° Target Temp: `0`

Step Size: 0
Acquisition Mode: none

b. Click Ins again and enter:
Temperature Target: 55°C
Hold Time: 5 sec
Slope: 20
2° Target Temp: 0
Step Size: 0
Acquisition Mode: none

c. Click Ins to add a third segment and enter:
Temperature Target: 72°C
Hold Time: 30 sec
Slope: 20
2° Target Temp: 0
Step Size: 0
Acquisition Mode: none

d. For *flaB* analyses, click Ins and enter a fourth segment with the following parameters:
Temperature Target: 82°C
Hold Time: 1 sec
Slope: 20
2° Target Temp: 0
Step Size: 0
Acquisition Mode: single

To conduct PCR using primers specific for a gene of interest, delete this fourth segment and change the acquisition mode on the third segment to single.

24. Double click the Add button and enter melting. The Type is melting curves. Enter the following segemnents

a. Click the Ins button and insert the following cycling parameters:
Cycles: 1
Temperature Target: 95°C
Hold Time: 0 sec
Slope: 20
2° Target Temp: 0
Step Size: 0
Acquisition Mode: none

b. Click Ins and insert a second segment with the following parameters:
Temperature Target: 65°C
Hold Time: 15 sec
Slope: 20
2° Target Temp: 0
Step Size: 0
Acquisition Mode: none

c. Click Ins again and enter a third segment with the following parameters:
Temperature Target: 95°C
Hold Time: 0 sec
Slope: 0.1
2° Target Temp: 0
Step Size: 0.2
Acquisition Mode: continuous

This program is necessary so that the software will perform a melting curve analysis of the data. One reason a melting curve analysis is important is because it indicates whether the primers selected for analysis are specific, and whether the peak(s) present correlates with the expected PCR amplicon(s) (visualized via agarose gel electrophoresis). Consult Wittwer (2001) for a more detailed explanation.

25. Double click the Add button and enter `cooling`. The Type is `none`. Click the Ins button to insert the following cycling parameters:

    Cycles: `1`
    Temperature Target: `40°C`
    Hold Time: `30 sec`
    Slope: `20`
    2° Target Temp: `0`
    Step Size: `0`
    Acquisition Mode: `none`

26. Double click Edit Samples to be directed to the module where sample information is entered. Type the sample name and indicate whether it is a `negative`, `standard`, or `unknown`. Earmark cDNA replicates and enter the concentration of each genomic DNA standard. At the bottom right of the screen, enter `ng` for concentration units. Click Done.

27. Double click on Save Experiment File and name the file.

    The protocol will now be saved in the `protocol` *folder.*

28. Carefully insert each capillary tube into the corresponding slot of the LightCycler carousel. Place the carousel into the LightCycler and gently close the lid.

29. Click Run. When prompted, name and save the experiment to the `Data folder`.

    The PCR assay will now be conducted.

    Roche Applied Science publishes several references that provide more detailed information on the LightCycler and the use of LightCycler software. The LightCycler operator's manual, version 3.5, and "Principles and Applications of the LightCycler" can be found online (http://www.Roche-applied-science.com/lightcycler-online). Another description of the methods and applications of LightCycler-based PCR can be found in print (Wittwer, 2001).

Analyze cDNA amplicons by LightCycler-based quantitative analysis

30. When the LightCycler assay has concluded, the Complete light will be illuminated on the thermal cycler and a data screen will be visible on the computer. Two analysis modules (quantification and melting curve analysis) will be applied to the data. Double click on Select a Program, located in the top left corner of the data screen. Select `amplification is a 40 cycle program...` Click the Quantification button on the data screen. The quantification module is now open.

31. There are two quantification modes available, the second derivative maximum and fit points methods. Select the second derivative maximum method.

    The second derivative maximum module automatically quantifies each sample based on the point at which the fluorescence curve first turns.

    This phenomenon is known as the crossing point of the sample and represents the beginning of the log-linear phase of PCR product amplification. The turning point is equal to the first maximum of the second derivative curve, which is how this analysis mode received its name. The fit points method allows the user to define the crossing points of the samples via experimental manipulation of the noiseband of the data (Roche Molecular Biochemicals, 2000; Wittwer, 2001). Under certain special circumstances, this analysis module is useful.

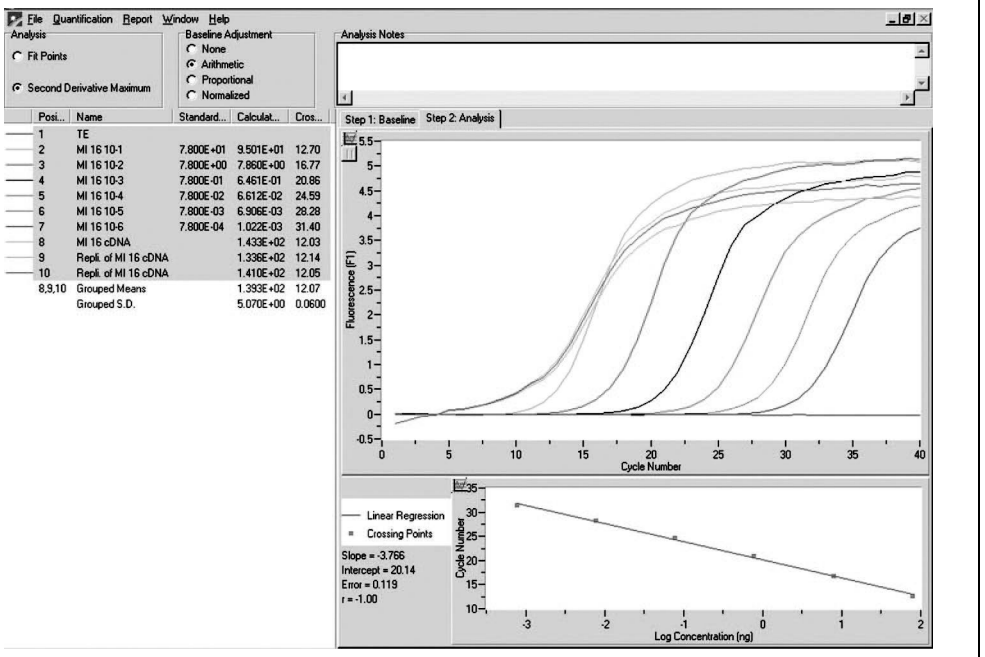

Figure 1D.3.2 Quantitative analysis of *B. burgdorferi flaB* gene expression within cultured bacteria. cDNA was obtained from *B. burgdorferi* B31-MI-16 bacteria cultivated to mid-logarithmic phase and 2 μl was analyzed for expression of the housekeeping gene *flaB*. The fluorescence curves (fluorescence versus the PCR cycle number) are displayed in the top right graph. A standard curve (bottom right corner) generated using known quantities (ng) of B31-MI-16 DNA (top left panel) was used by the LightCycler software to calculate the amount of *flaB* transcript present in each cDNA triplicate (top left corner). Note the ideal regression value of −1.00 obtained in this assay.

However, due to the inherent user bias present with this module, the automated second derivative maximum module is preferentially used to avoid this problem.

32. Three analysis steps will be now be present. Select `step 3: analysis`.

    Two graphs will be shown. The top graph displays fluorescence curves represented as the fluorescence versus the PCR cycle number. The bottom graph is the standard curve, which is represented as the crossing point number for each genomic DNA standard versus the log of the concentration (in ng) of each genomic DNA standard. Plotting of these data yields a linear regression line, from which the concentration of each unknown cDNA sample is calculated by the software. The slope of the line and an error value for the plotted data are also reported (Fig. 1D.3.2). A regression (r) value of −1.00 is ideal, and values ≤−0.95 are acceptable.

    The other two analysis steps, `step 1: baseline` *and* `step 2: noise band`*, individually display the baseline and noise band lines determined for the fluorescent curve graph by the software. As these features are not user-defined in the second derivative module, and the baseline is also shown in the analysis step, these first two steps are not as useful as the third step.*

33. Although the quantification results for each sample are displayed in the panel to the left of the graphs, not all the results are visible in the printable window. To view a summary of all results, including the average and standard deviation values obtained for each cDNA triplicate, click on Window and select Print Summary Report.

LightCycler-based melting curve analysis of cDNA amplicons

34. To exit the quantification mode and return to the main data analysis menu, click on Window and select `close window`.

35. Double click on Select a Program, and select Melting Curve is a . . . . Click the Melting Curve button on the data screen.

The melting curve module is now open and two graphs should be visible. The top graph displays the fluorescence versus the temperature and shows that the amount of SYBR green binding to the DNA is decreased as the temperature increases. The bottom graph replots this data as fluorescence (−d(F1)/dt) versus temperature such that the data is now displayed as a peak(s). Note how many major and minor peaks are present. If one PCR product is expected then only one major peak should be present (Fig. 1D.3.3A).

Melting curve analysis is important as the major peak(s) represents a melting temperature(s) that is unique for a specific PCR product(s) (Roche Molecular Biochemicals, 2000; Wittwer, 2001). Thus, melting curve analysis indicates whether a PCR amplicon of interest has been obtained and whether the PCR reaction was specific (minor peaks could indicate primer-dimers, other major peaks imply the primers or other PCR conditions utilized were not specific (see Troubleshooting).

36. To print the melting peaks, click on File and select Print Window.

Quantify expression for a gene(s) of interest

37. For each sample, divide the average computed for each cDNA triplicate for a gene of interest by that obtained for the *B. burgdorferi* housekeeping gene *flaB* (Gilmore et al., 2001; Miller et al., 2005). Refer to the average value computed for each cDNA triplicate, found on the "Quantification Summary" printout obtained in step 33. Note that the resultant number represents the average relative expression value for a gene of interest whose transcription levels are being measured within an infected tissue sample.

This is only one relative quantification method for tabulating and expressing the transcription data obtained for a particular gene(s) of interest. Other relative quantification approaches have also been utilized. In addition, absolute quantification methodologies have also been employed (http://www.Roche-applied-science.com/lightcycler-online, (Hodzic et al., 2002; Wittwer, 2001).

PREPARATION OF GENOMIC DNA STANDARDS

SUPPORT PROTOCOL 2

In Basic Protocol 3, the average relative expression for a gene of interest was calculated based on values obtained from standard curves generated using serial ten-fold dilutions of genomic DNA. The success of this quantification mode is critically dependent on the quality and integrity of the prepared genomic DNA. This protocol provides an easy method by which high-quality genomic DNA may be obtained.

Materials

DNA-easy tissue kit (Qiagen)
5-ml mid-logarithmic phase culture of *B. burgdorferi* strain of interest (*UNIT 12C.1*)
0.7% agarose gel
RNase-free TE buffer (*APPENDIX 2A*)
Spectrophotometer
Gel electrophoresis equipment
Sterile, RNase-free, DNA-free siliconized 0.5-ml microcentrifuge tubes

1. Using the DNA-easy tissue kit (Qiagen), extract genomic DNA from a 5-ml *B. burgdorferi* mid-logarithmic phase culture. Follow the manufacturer's directions. Remember to change gloves frequently to prevent DNA contamination of the kit reagents.

Other methods for extracting genomic DNA can also be used (Sambrook et al., 1989), as long as the resultant DNA is of high quality and purity. It is important that the B. burgdorferi strain used to generate the genomic DNA standards is the same as the strain used to infect the mice.

2. Use a spectrophotometer to determine the concentration of the extracted DNA.

   The concentration of double-stranded DNA at an A_{260} of 1.0 is 50 ng/μl. For more detailed information, consult Sambrook et al. (1989).

3. Analyze the quality of the extracted DNA via 0.7% agarose gel electrophoresis.

   One distinct band should be easily visualized on the agarose gel. If no band is present, or the band is smeared, then DNase-mediated degradation of the DNA has occurred. DNA that is partially or completely degraded is not suitable for LightCycler-based PCR analyses.

4. Change gloves. Using sterile, DNA-free TE buffer as diluent and sterile, RNase-free, DNA-free siliconized 0.5-ml microcentrifuge tubes, prepare at least six ten-fold serial dilutions of the extracted genomic DNA. Set these tubes aside on ice.

   To detect candidate genes which may be expressed at very low levels within B. burgdorferi–infected mouse tissues, a concentration range of 10 ng to 100 fg is recommended. These samples will be the genomic DNA standards that will be used to generate a standard curve during LightCycler PCR assays.

   Other concentration ranges may also be used as the LightCycler is sensitive enough to amplify templates of a wide range of concentrations. However, the level of detection achievable with the LightCycler is dependent on numerous other reagents, including the oligonucleotide primers utilized (see Critical Parameters and Troubleshooting and Table 1D.3.2).

5. Change gloves. Aliquot the remainder of the undiluted extracted genomic DNA into small volumes and place in sterile, DNA-free microcentrifuge tubes.

6. For long-term storage (weeks to years), keep the DNA in a frost-free −20°C freezer. If the DNA is to be used within 1 or 2 weeks, store at 4°C.

   Avoid freeze-thaw cycles. These cycles can result in broken, degraded DNA samples, which are not suitable for LightCycler-based PCR analyses.

BASIC PROTOCOL 4

VERIFICATION OF SPECIFICITY OF LIGHTCYCLER-BASED PCR REACTIONS

Although melting peak analysis will indicate whether a specific amplicon has been obtained, there are occasions when verification that a melting peak is a specific PCR product is necessary. An example of such an occasion would be to test if new primers are specific prior to using them on precious cDNA samples obtained from *B. burgdorferi*–infected mouse tissues. Alternatively, it may be necessary to confirm that amplification of a particular cDNA amplicon(s) is specific prior to scientific publication of the data. One method is to analyze the LightCycler reaction products via agarose gel electrophoresis for size and purity by comparing the reaction product to a positive control PCR product obtained using the same primers via conventional PCR analysis. Ideally, the positive control has already been sequenced to confirm that is the amplicon of interest. The second method is to sequence the LightCycler amplicons to confirm that they are the PCR products of interest.

Materials

Capillary tubes containing the LightCycler amplicons of interest (Basic Protocol 2)
1.5% agarose gel containing ethidium bromide
5× loading dye
100-bp DNA ladder
1.5-ml microcentrifuge tubes
UV lightbox
Microcon-100 microconcentrator filter (Ambion)
Spectrophotometer

1. Carefully remove the capillary tubes of interest from the LightCycler carousel and place each into an open 1.5-ml microcentrifuge tube.
2. Carefully remove the cap from each capillary tube. Place the open capillary tube into the microcentrifuge tube, with the cup portion of the capillary facing down into the tube.
3. Centrifuge each sample 2 min at 1000 × *g*, room temperature, to collect the sample at the bottom of the microcentrifuge tube.
4. Discard empty capillary tubes into proper waste containers.

For agarose gel analysis, proceed to step 5a. For instructions on preparing the amplicon for sequencing, go to step 5b.

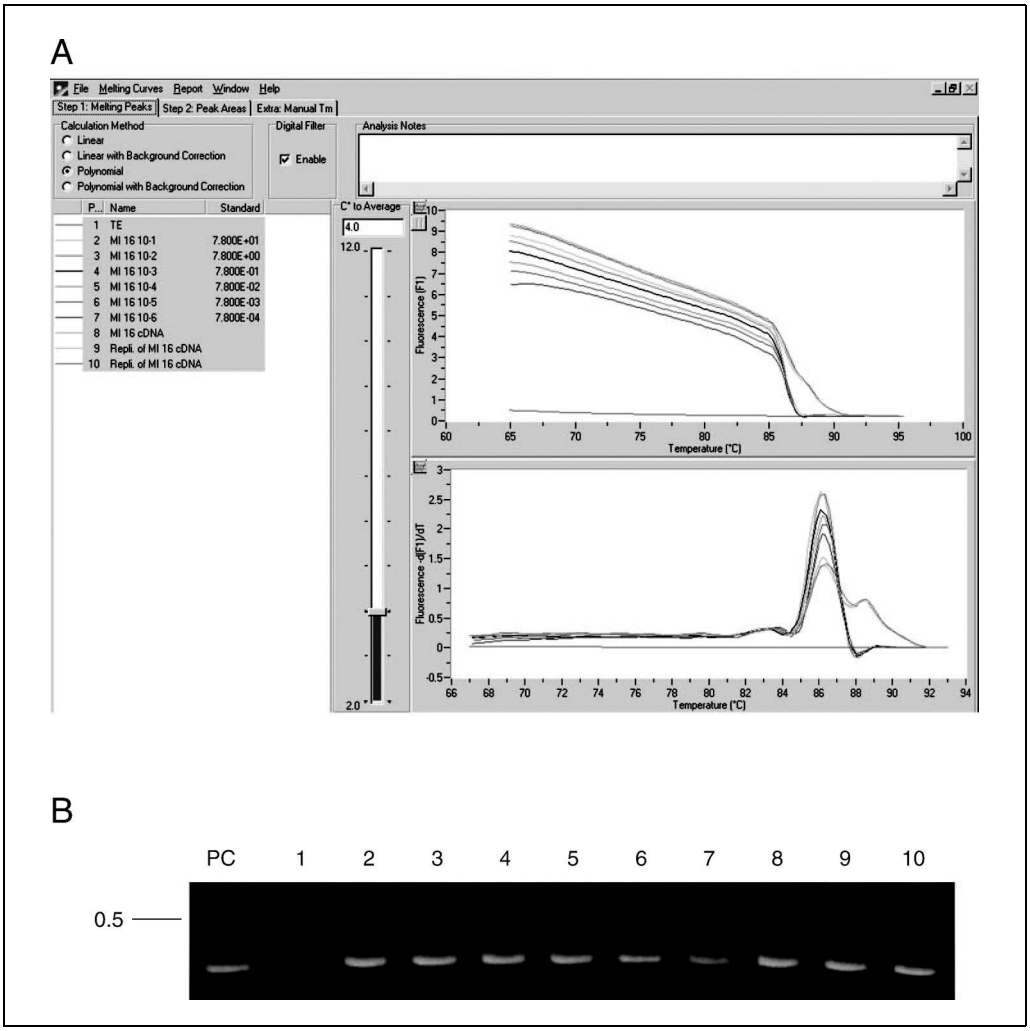

Figure 1D.3.3 Melting curve analysis of *B. burgdorferi flaB* gene expression within cultured bacteria. (**A**) The fluorescence versus temperature is displayed in the top graph. This graph demonstrates the decrease in SYBR green binding to the DNA that occurs as the temperature increases. The bottom graph replots this data and displays it as a peak(s). Note that one major peak, corresponding to the specific *flaB* amplicon, was obtained for each sample (except the negative control, TE). The minor peak obtained at 89°C for the three cDNA samples is non-specific and does not represent an amplification product (see Fig. 1D.3.3B). (**B**) Agarose gel electrophoresis analysis of the LightCycler reaction products obtained in the above experiment (Figs. 1D.3.2 and 1D.3.3A). Note that only one amplicon of the expected size was obtained for each sample (except the negative control, sample 1). Numbers above the lanes correspond with the numbers and samples listed in the top left panel of **A**. PC = positive control *flaB* PCR product, 0.5 = 0.5-kb DNA size marker.

To verify by gel electrophoresis

5a. For agarose gel electrophoresis analysis, prepare a 1.5% agarose gel containing ethidium bromide.

6a. Pipet 5 to 10 μl of each LightCycler PCR product, along with the appropriate amount of 5× loading dye (1 to 2 μl), into sterile microcentrifuge tubes. Next, pipet 10 μl positive control along with 2 μl loading dye into a sterile microcentrifuge tube.

7a. Load 5 μl of a 100-bp DNA ladder into the first lane of the gel, load the positive control into the second lane, and load each LightCycler amplicon into the remaining lanes of the gel.

8a. Run the gel for 1 hr at 135 V (small gels) or 150 to 160 V (large gels). When electrophoresis is complete, use a UV lightbox to visualize the bands on the gel.

For more detailed instructions on the proper voltage to use for electrophoresis of agarose gels of varying sizes, consult Sambrook et al. (1989).

If one specific LightCycler amplicon is expected, then there should be one band on the agarose gel of the same size and clarity as the band corresponding to the positive control. In turn, this should correlate with the presence of one major peak obtained during the melting curve analysis (Fig. 1D.3.3A,B).

To verify by sequencing

5b. Before the LightCycler amplicon can be sequenced, remove PCR buffers and other reagents by using a Microcon-100 microconcentrator filter. Follow the manufacturer's directions to obtain purified DNA.

6b. Determine the concentration of the DNA using a spectrophotometer.

7b. Sequence DNA.

COMMENTARY

Background Information

The Lyme disease spirochete *Borrelia burgdorferi* is maintained in nature within an enzootic cycle involving hard ticks of the genus *Ixodes* and mammals. If the appropriate antibiotic therapy is not administered, *B. burgdorferi* will persist within the mammalian host for at least several years (Schwan et al., 1999). Infected juvenile ticks feed upon rodents and other small mammals, making the mouse a useful model for studying *B. burgdorferi* gene expression within warm-blooded hosts. The borrelial genome consists of a small, ~900 kb linear chromosome and numerous extrachromosomal linear and circular plasmids (Fraser et al., 1997; Casjens et al., 2000). To date, all identified infection-associated genes are located on the plasmids, indicating that these minichromosomes assume an important role in the pathogenesis of *B. burgdorferi* (Casjens et al., 2000; Stevenson et al., 2001; Stewart et al., 2005). While expression of several well-characterized virulence genes by cultured borrelia is regulated by numerous factors including temperature, pH, and soluble chemicals (Schwan et al., 1995; Stevenson et al., 1995, 1996, 1998; Carroll et al., 1999, 2000; Porcella et al., 2000; Carroll et al., 2001; Babb et al., 2001; Hefty et al., 2001; Roberts et al., 2002; Yang et al., 2003), less is known about gene expression within infected mammalian hosts.

Initial studies focused on the examination of serum samples obtained from *B. burgdorferi*–infected hosts for the presence of antibodies that recognized certain proteins hypothesized to be involved in spirochetal pathogenesis (Akins et al., 1995; Suk et al., 1995; Wallich et al., 1995; Das et al., 1997; Stevenson et al., 1998; Miller et al., 2000a,b; Hefty et al., 2001, McDowell et al., 2001; Hefty et al., 2002; Miller and Stevenson, 2003; Miller et al., 2003). Results of such experiments indicate whether a particular protein is antigenic, but offer no information on gene transcription. The dialysis membrane chamber (DMC) model (see *UNIT 12C.4*) in which cultured *B. burgdorferi* are placed within dialysis tubing and then implanted into the peritoneum of a rat has often been used to analyze

bacterial gene expression, as the spirochetes adapt to the host environmental conditions (Akins et al., 1998). The drawback to this model system is that the spirochetes are contained within the dialysis tubing, preventing dissemination to other anatomical sites, which is a key feature of *B. burgdorferi* infection. Although northern blot analysis is the oldest method utilized to examine bacterial gene transcription, the technique is not very sensitive. Large amounts of RNA (a minimum of 10 μg) are needed (Sambrook et al., 1989) for successful execution of the experimental protocol, and less abundantly expressed genes may not be detected. Qualitative RT-PCR partially circumvents these problems, as it is a more sensitive technique that requires significantly less RNA (a maximum of 1 μg) (Sambrook et al., 1989; Kawasaki, 1990). In addition, RT-PCR is less time-consuming and messy than northern blot analysis. However, there are two significant limitations to qualitative RT-PCR: (1) differences in transcript levels cannot be quantified without a densitometer, and (2) it lacks the sensitivity required to detect non-abundant messages.

Quantitative RT-PCR (Q-RT-PCR) was developed to overcome the limitations of qualitative RT-PCR. It is much more sensitive than either northern blot analysis or qualitative RT-PCR. In addition, the reactions are carried out in sealed, thin-walled glass capillary tubes placed into a PCR thermal cycler called the LightCycler. This allows 32 samples to be assayed simultaneously, enabling high-throughput analysis. Q-RT-PCR utilizes a system of dyes. One example is SYBR green, which binds double-stranded DNA. The LightCycler detects the amount of SYBR green that is being incorporated into the newly synthesized PCR product and records this data as a function of the fluorescence incorporated versus temperature. This data can then be analyzed utilizing a melting curve module which provides detailed information about the number and specificity of the resultant PCR amplicons. Quantification results are calculated with either a relative or absolute quantification module, which determines the level of expression for a gene of interest based on results obtained from a standard curve (*http://www.Roche-applied-science.com/lightcycler-online*; Wittwer, 2001). Q-RT-PCR is routinely used to quantify the message levels of poorly expressed genes, and to assess the very small differences in expression that can exist between a set of genes. For these reasons, this technique is well-suited for the analysis of *B. burgdorferi* gene expression within infected mice, as the number of bacteria residing within tissues after dissemination is small, and the transcription of infection-associated genes by these bacteria is often several orders of magnitude below housekeeping gene expression.

Gilmore and colleagues (2001) were the first investigators to utilize Q-RT-PCR to analyze *B. burgdorferi* gene expression. In this landmark report, the transcript levels of several pathogenesis-associated genes were assessed within engorged larval and nymphal ticks harboring a *B. burgdorferi* infection. Shortly thereafter, the message levels of a few select genes were measured within infected mouse tissues (Hodzic et al., 2002, 2003). Following in the footsteps of these initial publications, several additional laboratories have recently utilized Q-RT-PCR to analyze *B. burgdorferi* gene expression within mouse tissues and cultured bacteria (Caimano et al., 2004; Lederer et al., 2004; Liang et al., 2004; Miller et al., 2005; Zhang et al., 2005).

Critical Parameters

Prior to beginning any experiment, the primers to be used for LightCycler-based PCR analysis should be designed and ordered. It is recommended that each primer length not exceed 24 bp, and that the target amplicon be between 350 and 500 bp. Amplicons that are 200 to 250 bp in size can be easily accommodated, but products approaching 1 kb (or larger) will require significant modification of the amplification protocol and/or $MgCl_2$ concentrations used for PCR amplification. In addition, oligonucleotide primers should be free of secondary structures, and should not exhibit melting temperatures (T_m) below 60°C. T_m lower than 60°C will require modifications of the annealing temperature used during the amplification program. Integrated DNA technologies (IDT) has a Website that is very useful for assessing the T_m and secondary structures of potential PCR primers (*http://www.Idt.com*).

If RNA is not to be extracted from *B. burgdorferi*–infected mouse tissues on the day of animal euthanasia, the infected tissues must be removed aseptically and immediately snap-frozen in liquid nitrogen. Tissues stored in this manner are good for ~9 months. It is extremely important that RNase-free conditions are maintained throughout the duration of all RNA-based protocols. In addition, frozen tissues must be kept cold on dry ice while being pulverized to prevent RNase-mediated degradation of the RNA. These experimental

Table 1D.3.2 Troubleshooting Guide for Commonly Encountered Problems

Protocol	Problem	Possible cause(s)	Solution(s)
RNA Isolation	RNA pellet difficult to resuspend and/or is gelatinous	Overdrying of pellet	Only allow pellet to air dry for 5–10 min
		Due to incomplete homogenization, proteinaceous particles may be present resulting in a gelatinous pellet	Homogenize samples more thoroughly or increase incubation time to 15–20 min at 60°C and flick tube every few minutes to facilitate pellet resuspension
	A_{260}/A_{280} <1.7	High level of protein contamination, due to disruption of the interphase between the aqueous and organic layers	Leave a bit of aqueous phase behind to ensure that the interphase is not breached
		Pellet not completely resuspended	Increase incubation time to 15–20 min at 60°C and flick tube every few minutes to facilitate pellet resuspension
		Too small a volume of Trizol used for tissue homogenization	Increase volume of Trizol used for tissue homogenization
	Concentration of RNA much lower than expected	Frozen tissues are older than 9 months	Only use frozen tissues <9- months old
		Improper storage/handling of frozen tissues	Tissues must be removed aseptically, immediately snap-frozen in liquid nitrogen, and stored at −80°C. Tissues must not be allowed to thaw during the homogenization process.
		Incomplete homogenization of tissue	Homogenize tissues thoroughly, breaking up all tissue fragments
		Too small a volume of Trizol used for tissue homogenization	Increase volume of Trizol used for tissue homogenization
		Insufficient removal of aqueous layer	Only leave a small amount of the aqueous layer behind
		RNase-mediated degradation of RNA	Ensure all benchtops, reagents, and equipment are kept RNase-free
	No RNA obtained	Frozen tissues are older than 9 months	Only use frozen tissues <9 months old
		Improper storage/handling of frozen tissues	Tissues must be removed aseptically, immediately snap-frozen in liquid nitrogen, and stored at −80°C. Tissues must not be allowed to thaw during the homogenization process.
		RNase-mediated degradation of RNA	Ensure all benchtops, reagents, and equipment are kept RNase-free

continued

Table 1D.3.2 Troubleshooting Guide for Commonly Encountered Problems, *continued*

Protocol	Problem	Possible cause(s)	Solution(s)
DNase I treatment/ agarose gel electrophoretic analysis of RNA	RNA remains contaminated following DNase I treatment	Sample heavily contaminated with genomic DNA	Increase incubation time to 1 hr at 37°C. Increase amount of DNase I and inactivation reagent used for each sample.
	RNA is no longer present, is smeared, or only one rRNA band is visible following DNase I treatment	RNase contamination of Turbo DNA-free buffer and/or inactivation reagent	Treat buffer and inactivation reagent with RNAsecure. Heat to inactivate RNases before and after use.
		DNase I contaminated with RNases	Maintain RNase-free conditions. Use only RNase-free DNase I and aliquot into small volumes to prevent contamination of entire stock vial.
	Prior to DNase I treatment, RNA is no longer present, is smeared, or only one rRNA band is visible	RNase-mediated degradation of RNA during Trizol extraction	Maintain RNase-free conditions. Only allow pellet to air dry for 5–10 min, by inverting tube over KimWipes used only for RNA extractions.
		Agarose gel electrophoresis reagents contaminated with RNases	Maintain RNase-free conditions. Use RNase-free solutions. Treat solutions with RNAsecure.
	Both rRNA bands are intact and well defined, but RNA has an overall smeared appearance	Sample is overloaded on gel (commonly seen with spleen tissue)	Load <5 μg of each sample into gel wells
		RNA is heavily contaminated with protein (commonly seen with spleen tissue)	Digest the homogenized sample with 20 μg/ml proteinase K for 1–3 hr at 55°C, prior to the addition of chloropane
LightCycler-based analysis	Capillary tube breaks while inserting into instrument carousel	Excessive force used to insert tube into carousel slot	Gently push tube down through slot. If resistance is encountered, stop and check the carousel slot for pieces of broken tube. Remove clog with a paperclip. Collect broken capillary pieces (if the tube did not completely shatter). Place in a microcentrifuge tube. Invert the cup portion in the bottom of the microcentrifuge tube and place the broken stalk on top of the inverted cup. Centrifuge briefly to collect the contents in the eppendorf tube. Pipet contents into a new capillary tube.

continued

Table 1D.3.2 Troubleshooting Guide for Commonly Encountered Problems, *continued*

Protocol	Problem	Possible cause(s)	Solution(s)
	Amplification of the "no-template" negative control occurs	Benchtop and/or LightCycler reagents are contaminated with DNA	Use DNA-Away to maintain DNA-free conditions. Change gloves frequently. Prepare reagents in DNA-free vessels that have never been used for bacterial cultivation.
	Melting peak analysis shows two large peaks, but only one was expected	Nonspecific template binding by oligonucleotide primers, resulting in nonspecific amplicons	Analyze reaction products on an agarose gel. (1) If the diagnostic gel electrophoresis indicates only the specific amplicon is present, decrease the primer concentration, and/or add 1–5 sec to the amplification step at the temperature where the nonspecific peak occurs. Be sure to modify the program to set the data acquisition to occur at this final step. (2) If the diagnostic gel electrophoresis indicates more than one PCR product is present, alter the annealing temperature of the amplification module and/or the Mg^{2+} concentration or design more specific oligonucleotide primers.
	Melting peak analysis shows the expected specific peak, but a much smaller peak is also present at a lower temperature	The primers are binding to each other, forming primer dimers	Decrease the primer concentration, and/or add 1–5 sec to the amplification step at the temperature where the nonspecific peak occurs. Be sure to modify the program to set the data acquisition to occur at this final step.
	The quantification curves are very flat and the fluorescence is extremely low	The SYBR green is no longer active	Order and use fresh SYBR green
	No melting peaks are obtained and the quantification curves are crooked straight lines	Reagents incorrectly added to the master mix: either two of the same primer were added or too much SYBR green was added	Add the correct volumes and concentrations of reagents to the master mix
	All reagents were correctly added to the master mix, and the SYBR green is fresh, but the quantification curves and melting peaks are flat for the more dilute standards and cDNAs	An inhibitor of the PCR reaction is present. Potential inhibitors: DEPC-treated Tris buffer (never DEPC-treat Tris containing compounds); DNase inactivation reagent (always move DNase-treated RNA into clean tubes); chloroform (always use chloropane instead of	Identify and remove inhibitor

continued

Table 1D.3.2 Troubleshooting Guide for Commonly Encountered Problems, *continued*

Protocol	Problem	Possible cause(s)	Solution(s)
		chloroform during RNA extraction); ethanol (always remove ethanol and dry RNA pellets for 5–10 min to allow residual ethanol to evaporate); protein contamination of RNA samples (too much protein inhibits RT-PCR)	
	The cDNAs and more concentrated standards are amplifying, but the more dilute standards are not	The DNA standards are not fresh	Prepare freshly diluted standards
		The genomic DNA is old and beginning to degrade	Extract more genomic DNA from cultured bacteria

precautions are absolutely critical, as the key to achieving good results from all of these protocols is the extraction and purification of high-quality RNA.

Once suitable RNA has been obtained, the appropriate protocol steps must be followed to completion to ensure that all contaminating genomic DNA is removed. RT-PCR data obtained using the LightCycler is useless if the RNA used to synthesize cDNA is contaminated with genomic DNA. Another critical quality control parameter involves the genomic DNA used to generate the standard curves during LightCycler-based PCR. This DNA must also be of high quality, and dilutions should be freshly prepared on a weekly basis. The sensitivity and accuracy of all LightCycler-based results is dependent on the genomic DNA standards. If the standards are not high quality, the assay will not work. Prior to cDNA synthesis and PCR amplification, DNA-free conditions must be maintained to prevent DNA contamination of reagents. Finally, the SYBR green utilized for LightCycler-based PCR must be diluted in TE buffer and properly stored in light-protected containers in a non-frost-free freezer. Most importantly, do not use SYBR green that is older than 3 months, as suboptimum results will be obtained.

Troubleshooting

There are several problems that can be encountered with each of the protocols described within this unit. Possible causes and solutions for each of these common problems are summarized in Table 1D.3.2.

Anticipated Results

The following yields can be expected upon extraction of RNA from *B. burgdorferi*–infected mouse tissues: 700 to 800 ng/μl from bladder, 2.0 to 2.2 μg/μl from heart, 1.2 to 1.4 μg/μl from joints, 5.0 to 7.0 μg/μl from spleen, and 2.0 to 2.3 μg/μl from ear. The conversion of RNA to cDNA by reverse transcriptase has a minimum efficiency of 30%, resulting in at least 300 ng of cDNA being synthesized from 1 μg of RNA (Sambrook et al., 1989).

Time Considerations

RNA can be feasibly extracted from a maximum of twelve tissues per day, processed in two groups containing six tissues each. If a small number of samples are being analyzed, the DNase I treatment, agarose gel electrophoresis analysis, and diagnostic PCR reactions can all be performed in 1 day. RT-PCR reactions and LightCycler-based PCR analysis of a small number of samples can be accomplished in 1 day, as the first-strand cDNA synthesis reaction takes just over 1 hr and LightCycler-based analyses only require 2 hr (1 hr to prepare 32 samples, and 1 hr for LightCycler-based PCR of the samples). To simultaneously analyze numerous samples, more time will be required to complete each phase of the protocol. Extracted RNA samples are stable for ~1 year at −80°C, and cDNA

samples can be maintained at −20°C in a non-frost-free freezer for ∼1 month, allowing the researcher weeks to months to complete the entire protocol, if necessary.

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Key References

Gilmore et al., 2001. See above.

Gilmore and colleagues were the first investigators to utilize Q-RT-PCR to analyze B. burgdorferi gene expression.

Miller et al., 2005. See above.

This report utilizes all the protocol steps outlined in this unit to examine Borrelia burgdorferi gene expression within tissues of chronically infected non-human primates.

Internet Resources

http://www.Roche-applied-science.com/lightcycler-online

Excellent resource offering detailed technical notes on the LightCycler and its applications. The

LightCycler Operator's Manual Version 3.5 is also available for downloading free of charge at this site.

http://www.IDT.com

Free Website offers online tools for the analysis of oligonucleotide primer sequences.

Contributed by Jennifer C. Miller
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Characterization of Bacteria in Mixed Biofilm Communities Using Denaturing Gradient Gel Electrophoresis (DGGE)

Organisms within mixed culture biofilms are inherently difficult to study. Due to the wide range of microenvironments within biofilms (Costerton et al., 1994), the distribution of some organisms may be restricted to a few cells within a specialized microniche, whereas others may be more broadly distributed. Although traditional culturing techniques are widely used, they have an inherent bias in selecting those organisms capable of growing on a particular laboratory medium. Molecular-based detection methods are frequently used to detect bacteria in complex environments since they are unaffected by the limitations of bacterial culturability (Amann et al., 1992; Muyzer and Smalla, 1998).

Several molecular tools are currently available to aid in the quest to describe and identify the consortia within environmental biofilms. Fingerprinting assays like terminal restriction fragment length polymorphisms (t-RFLPs), which use PCR and restriction enzyme digestion, create a banding pattern unique to each environmental sample, enabling descriptions of changes in bacterial community structure with time or treatment (Diez et al., 2001). Cloning and sequencing of the PCR product of the 16S small ribosomal subunit rRNA gene is typically used for sequence-based identification of bacterial community members (Amann et al., 1992). However, fingerprinting assays are limited because they fail to identify community members; cloning assays are likewise limited because the number of clones that can be sampled and sequenced may fail to describe the overall diversity (Dunbar et al., 2002). Denaturing gradient gel electrophoresis (DGGE; see Basic Protocol) both reveals a community fingerprint and provides sequence information for identifying community members. This sequence information can then be used for fluorescence in situ hybridization (FISH) to determine the spatial distribution of individual bacteria (Tonolla et al., 2005).

CAUTION: Follow all biosafety requirements relevant to the microorganisms and biofilm substrates under investigation. Although the majority of organisms used with these protocols are environmental isolates, classified as biosafety level-1 (BSL-1) organisms, additional safety precautions would be needed for BSL-2 and BSL-3 organisms. Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

DENATURING GRADIENT GEL ELECTROPHORESIS

BASIC PROTOCOL

Denaturing gradient gel electrophoresis (DGGE) is an excellent molecular tool for examining biofilm bacteria in mixed communities, particularly where traditional culturing techniques are not possible (Muyzer et al., 1993; McLean et al., 2005). In brief, ~200- to 500-bp DNA fragments are amplified using PCR (Support Protocol 1), typically targeting the 16S rRNA gene with specially modified primers using as a template the total bacterial DNA extracted from an environmental sample (Support Protocol 2). This community template PCR product, containing fragments of differing guanine and cytosine (G+C) content, is then separated into individual bands by electrophoresis through an acrylamide gel with an increasing gradient of formamide and urea. Electrophoresis through the denaturing gradient causes partial denaturation of the PCR duplexes. Ideally, each unique sequence in the community PCR product will partially denature at a different spot in the gel. The banding pattern generated can be used as a bacterial community fingerprint, and

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Table 1E.1.1 Examples of General Eubacterial Primers Suitable for PCR and DGGE Analysis[a]

Primer name	Sequence 5′-3′	Reference
GC 357F	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	Muyzer et al. (1993)
907R	CCG TCA ATT CMT TTG AGT TT	Amann et al. (1992)

[a]Many other general and specific primers are available and described in the literature.

bands excised from the gel can give sequence-based identification of organisms detected in the biofilm (see Critical Parameters and Troubleshooting).

DGGE procedures should be performed according to the instructions provided by the equipment's manufacturer; the following procedure was designed for the Bio-Rad DGGE system. Successful DGGE requires careful optimization of the ideal denaturant concentration, time of electrophoresis, and voltage for each set of biofilm samples (Muyzer et al., 1993; Lyautey et al., 2005). The conditions described below should work well for biofilms from typical aerobic sites using the GC357F and 907R universal bacterial primer set (Table 1E.1.1).

Materials

6% acrylamide solutions containing 30% ("LO") and 50% ("HI") denaturant (see recipe)
0.5× TAE buffer: mix 100 ml 50× TAE (*APPENDIX 2A*) in a final volume of 10 liters H_2O for at least 1 hr; store up to 3 months at room temperature
Ammonium persulfate (APS): store up to 1 year at 4°C
N,N,N′,N′-tetramethylethylenediamine (TEMED): store up to 1 year at 4°C
PCR-amplified samples and standards (Support Protocols 1 and 3)
2× DGGE gel loading dye (see recipe)
Ethidium bromide staining solution (see recipe)
TE buffer (*APPENDIX 2A*)

DGGE System, e.g., DCode Universal Mutation Detection System (Bio-Rad) for 16-cm parallel gels, including:
  Glass plates with spacers (grooved, 1 mm), two pairs
  Sandwich clamps
  Sponges
  Casting stand
  Alignment card
  30-ml syringes, syringe sleeves, syringe cap screws, luer syringe locks, and luer couplings
  Tygon tubing
  Model 475 gradient former
  Y fitting
  16-well combs
  DCode lid stand
  Electrophoresis/temperature control module
  Electrophoresis tank
  Sandwich core
Absorbent bench paper
50-ml conical polypropylene tubes
20-G needle
250-ml beakers
Micropipettor with gene sequencing tips
Power supply (e.g., Bio-Rad Power Pac 300)

Plastic mesh shelf liner (available from many stores, e.g., Wal-Mart)
UV transilluminator
Scalpel, sterile
1.5-ml microcentrifuge tubes, sterile
Digital or other camera for gel documentation

Additional reagents and equipment for sequencing DNA (e.g., Slatko et al., 1999; optional)

Prepare gel sandwich equipment and denaturant gel solutions

1. Select one long plate (18 × 20 cm) and one short plate (16 × 20 cm) for each gel. Wash the plates using deionized water and allow to air dry.

   The Bio-Rad system is designed to fit two gels in the sandwich core to be run simultaneously. The authors typically run duplicates of the same samples on both gels for data redundancy. It is possible to run only one gel at a time. In that case, the second position in the sandwich core should hold the two glass plates with no spacer (see step 19 below). The following protocols are for running two gels.

2. Cover the bench with absorbent paper. Pipet 20 ml "HI" denaturant (50%) acrylamide solution into each of two labeled 50-ml conical tubes and allow it to come to room temperature in the dark. Pipet 20 ml of "LO" (30%) solution into each of two additional labeled 50-ml conical tubes and allow it to come to room temperature in the dark.

   CAUTION: *Unpolymerized acrylamide is a potent neurotoxin. Proper safety precautions such as gloves and lab coat should be worn throughout the DGGE protocol (also see UNIT 1A.3).*

   Bio-Rad recommends degassing the acrylamide solutions immediately before casting the gel. The authors have eliminated this degassing step and have noticed no difference in the time or quality of gel polymerization.

3. Assemble the gel sandwich.

   a. Lay the large plate down first and then place the spacers (1.0 mm thick) on the plate so that the notched opening of the spacers faces the sandwich clamps and grooves are down.
   b. Place the short glass plate on top of the spacers so that it is flush with the bottom edge of the long plate.
   c. Position a sandwich clamp on each side of the gel sandwich.

4. Assemble the gel-casting apparatus.

   a. Place the sandwich assembly in the alignment slot of the casting stand. Loosen the clamps.
   b. Insert the alignment card. Align the plates and spacers.
   c. Remove the alignment card and remove the sandwich assembly from the casting stand. Check that the plates and spacers are flush at the bottom.
   d. When good alignment is obtained, tighten the clamp screws finger-tight.

      The gel sandwich will leak if the glass plates and spacers are not aligned carefully and if the clamps are too loose.

5. Place the aligned sandwich assembly on a sponge in the casting stand and lock the assembly in place by securing the sandwich clamps in the stand.

6. Repeat steps 3 and 4 for the second gel to be cast, but set it aside. Do not place the second sandwich assembly into the casting stand until the first gel has been cast.

Prepare syringes, gradient former, and catalysts

7. Prepare two 30-ml syringes in the following manner:

   a. Label one syringe "LO" (for the 30% solution) and the other one "HI" (for the 50% solution).

   b. Slide a syringe sleeve on each such that the volume markings on the syringe can be seen in the front.

   c. Place a syringe cap screw on the plunger end of each syringe such that the catch screw on the cap is facing the back.

   d. Screw a luer syringe lock with ~8 in. (~20 cm) of Tygon tubing fitted on it onto each syringe.

      The catch screw will fit into the screw slide on the gradient former.

8. Place the Model 475 gradient former on the covered lab bench with the cam wheel on your right side. Rotate the cam wheel counterclockwise to the vertical or start position and tape the wheel in place so that it doesn't move when attaching the syringes.

   When first using this gradient former, filling both syringes with water, practicing putting them on and off the former, and delivering the water into a beaker is highly recommended. Getting a good feel for the gradient former will be critical to delivering the acrylamide solutions quickly in later steps of this protocol.

9. Attach a 20-G needle to the luer coupling on ~12 in. (~30 cm) of Tygon tubing which has a Y-fitting on the other end.

10. Place the needle (pointing down) at the top center of the sandwich assembly in the casting stand and insert it between the two glass plates ~1/4 in. (~0.63 cm) beveled side facing away. Tape the needle in place.

11. Prepare catalysts immediately prior to use:

    a. In a 1.5-ml microcentrifuge tube, prepare a 10% ammonium solution (APS) by adding 0.1 g APS and 1.0 ml deionized water. Vortex to dissolve.

    b. Aliquot ~90 µl TEMED into another 1.5-ml microcentrifuge tube.

       TEMED solution should be clear; if it is yellow replace it.

12. On the covered lab bench, place the rack with the 50-ml tubes with acrylamide, gradient former, and casting stand with sandwich assembly such that the Tygon tubes from the gradient former can attach to the Y-fitting on the needle. Prepare a beaker of water to rinse the tubes and an empty beaker for waste water.

    Steps 13 to 16 are time sensitive and need to be completed within 5 to 7 min. Ensure that the sandwich assembly, syringes, and gradient former are completely ready before proceeding.

Cast DGGE gels

13. Move one "HI" and one "LO" acrylamide tube to the front of the rack. Add the catalysts: 18 µl TEMED and then 180 µl 10% ammonium persulfate. Cap and mix by inverting several times.

    These steps will be repeated for the second gel to be cast.

    The final concentrations (each) of APS and TEMED are 0.09%.

14. With the syringe connected to the tubing, withdraw all of the high-density solution into the "HI" syringe. Carefully release any air and a bit of solution back into the tube with 19 ml remaining in the syringe. Place the syringe in the gradient former.

Parallel gels are a top-filling method, so the "HI" syringe is placed in the back position on the gradient former farthest from you. The cap screw on the syringe slides into the black groove on the gradient former, and the sleeve on the syringe is tightened into place with the white plastic screw on the gradient former.

15. Repeat step 14 for the low-density solution into the "LO" syringe and place in the front position on the gradient former.

16. Connect the tubing from each syringe to the Y-fitting with the needle. Rotate the cam wheel slowly and steadily to deliver the gel solution.

17. When finished, remove the needle from between the glass plates. Carefully insert the comb (16-well, 1.0 mm thick).

18. Place the tubing and needle into a beaker of water and reverse the cam to rinse any gel solution. Rinse tubing and fittings thoroughly. Discard all acrylamide-contaminated liquids into hazardous waste.

    The residual casting mixture in the conical tube solidifies fairly quickly (within minutes). If it remains liquid, or semisolid, there will be a problem with the gel. If the sandwich assembly leaks there will be puddles of acrylamide in the corners of the sandwich assembly, and air bubbles will form along the edges of the comb. Leaking disrupts the gradient and the well integrity; the gel will need to be cast again.

19. Place the second gel sandwich assembly in the casting stand carefully. Prepare and cast the second gel as in steps 7 to 18 above.

20. Let gels polymerize at least 60 min (up to 24 hr). If the gels are left overnight to polymerize, place a layer of damp paper towels soaked in 0.5× TAE buffer on top, then surround the top half of the sandwich assemblies in the casting stand with plastic wrap (go around two or three times). Place in a dark cupboard.

Prepare DGGE apparatus for electrophoresis

21. Fill the electrophoresis tank with ~7 liters of 0.5× TAE buffer (to the line that says "Fill"). Put the electrophoresis/temperature control module into the tank. Place the clear loading lid on the module.

    The authors place the DGGE tank on a rolling lab cart to allow easy access to the 10-liter carboy of buffer, to both sides of the core for loading samples and for moving the entire tank to the sink to dispose of used buffer.

22. Set the temperature controller to 60°C and set the ramp rate to 200°C/hr to allow the buffer to reach the desired temperature most quickly. Turn on both the heater and pump.

23. After the gels have polymerized, carefully remove one sandwich assembly from the casting stand. Remove the comb.

24. Rinse the wells two or three times with buffer using narrow gene sequencing tips that can slide between the glass plates or the 20-G needle and tubing on a syringe. Repeat for the second gel.

25. Assemble the upper buffer chamber of the sandwich core while it lies flat on the bench. Make sure the white core gaskets are clean and in place and lubricate the entire gasket with running buffer to obtain a good seal.

26. Snap the sandwich assembly onto the core by sliding the grooved sandwich clamps into the white posts on the core. Turn the core over and repeat with the other sandwich assembly, forming an upper chamber dam.

    The shorter gel plate should face the inside of the core buffer chamber.

If only one gel is to be run, assemble a set of glass plates without the spacers. Ensure that the bottoms of the plates are flush. Attach it to the other side of the core to form an upper chamber dam.

27. Pour 350 ml of 0.5× TAE running buffer into the upper buffer chamber. Check for leakage. If it leaks, remove both sandwich assemblies from the core and repeat steps 25 and 26.

28. Turn off the power on the electrophoresis/temperature module and remove it to the DCode lid stand. Place the sandwich core in the tank with the red electrode post on the right side of the tank and module. Replace the electrophoresis/temperature module and turn on once again.

Load and run DGGE gels

29. Thaw on ice the PCR-amplified samples and standards from Support Protocol 1.

30. Add one vol 2× gel loading dye to each DGGE sample and standard immediately prior to loading (i.e., for 10 μl PCR product add 10 μl dye). Mix contents of tube by flicking, and centrifuge briefly.

31. Remove the clear loading lid from the electrophoresis/temperature module.

    Look down into the upper buffer chamber and note the wells of the gel in the sandwich assembly on the opposite side of the tank. The samples are loaded through this opening while the temperature control module of the electrophoresis unit is running to maintain temperature.

32. Load the samples into the wells. Load one or two wells with the DGGE standards.

    Careful aim is critical while loading the samples and requires some practice. The sample will rush out of the tip of the pipet upon contact with the hot buffer in the tank. The authors find it helpful to allow a small air bubble at the tip of the pipet to allow a bit of time to position the tip before the sample flows out.

33. Place the clear loading lid on top of the temperature control module. Attach the electrical leads to the Bio-Rad Power Pac 300 power supply. Run the gel at 180 V for 7 hr at 60°C.

Remove and stain DGGE gels

34. After electrophoresis is complete, turn the power supply and system off. Disconnect the power cord and electrical leads. Remove the temperature control module and place it on the DCode lid stand. Remove the core and pour the buffer from the upper chamber into the sink.

35. Lay the core with sandwich assemblies on a covered laboratory bench. Remove the sandwich assembly from the core by pressing firmly on the black clamps on the core and pulling toward you. Turn the core over and remove the second sandwich assembly.

36. For each sandwich assembly, remove the clamps from the sandwich. Slide out the spacers. Carefully pry off the shorter glass plate.

    The gel is extremely thin and fragile but should stick to one plate or the other. (It doesn't matter which.)

37. Cut a piece of plastic mesh shelf liner that is slightly larger than the gel. Lay the mesh on top of the gel. Invert so that the mesh is now underneath and the gel is above, still attached to the glass plate.

38. Release the gel from the plate using a spacer damp with buffer to pull one corner of the gel loose from the plate while lifting that edge of the glass plate slowly.

39. Place each gel on its mesh into a plastic tub containing 500 ml ethidium bromide staining solution. Stain 20 to 30 min in the dark. Destain in another tub with 500 ml of deionized water 20 to 30 min in the dark.

    CAUTION: *Wear gloves for protection from DNA stains. See UNIT 1A.3 for more information regarding ethidium bromide, including methods of disposal.*

40. While staining and destaining, clean the DCode equipment. Pour out the running buffer in the electrophoresis tank and fill the tank with deionized water. Place the electrophoresis/temperature module back in the tank, turn on the pump, and run for 10 min. Rinse core, clamps, and other equipment with water and air dry.

41. Place the gel on a UV transilluminator using the mesh for transport, and photograph.

    CAUTION: *Wear UV eye protection and gloves.*

Extract DNA from DGGE gels

42. Excise the gel from the middle of the band using a sterile scalpel while the gel is on the UV transilluminator and place into a sterile 1.5-ml microcentrifuge tube. Minimize the amount of time the UV lamp is on and use protection from the UV light.

43. Add 20 µl TE buffer to each tube, crush the gel slice with the pipet tip, and soak overnight at 4°C.

44. Use 2.5 µl of the gel slice supernatant as template in a 50-µl PCR reaction, and concentrate the PCR product as if a pure culture to 100 to 200 ng (see Support Protocol 1, but reduce the reaction volume to 50 µl).

45. Perform DGGE to verify the presence of only one band.

    DGGE may promote heteroduplex formation (where two heterologous DNA strands anneal together). In subsequent DGGE analysis, heteroduplexes typically result in two or three bands as opposed to just one (see Critical Parameters and Troubleshooting for possible solutions to this problem).

46. If one band is confirmed, use another 2.5 µl of the gel slice supernatant from step 43 as template in a 50-µl reaction (see Support Protocol 1) to generate PCR product for sequencing.

    The 357F primer without the GC clamp should be used with the 907R primer. The GC-clamp will interfere with the sequencing reaction.

47. Sequence the amplified DNA, e.g., as described by Slatko et al. (1999), or submit samples to a sequencing laboratory.

PCR OF BIOFILM SAMPLES FOR DGGE

SUPPORT PROTOCOL 1

PCR parameters must be carefully optimized for DGGE samples to avoid artifacts in banding patterns and to maximize bands detected. There are several well established universal bacterial primers for DGGE targeting the 16S rRNA gene (Watanabe et al., 2001); however, group or even species-specific primers can be used with DGGE provided either the forward or reverse primer has a GC clamp (Muyzer et al., 1993; Diallo et al., 2004). The GC clamp is a string of alternating guanines and cytosines added to the 5′ end of the forward or reverse primer used in DGGE (see Table 1E.1.1). The addition of a 40-base-pair GC clamp to one primer prevents the duplex PCR product from completely

denaturing in the DGGE gel (Muyzer et al., 1993). DGGE can not resolve PCR fragments >600 bp (Muyzer and Smalla, 1998). To describe overall bacterial diversity, the authors recommend using universal bacterial primers GC357 F and 907 R targeting the V3 to V6 regions of the 16S rRNA gene (Table 1E.1.1; Watanabe et al., 2001).

Materials

Ampli*Taq* GOLD Polymerase, 5 U/μl (Applied Biosystems)
10× PCR buffer II (Applied Biosystems)
25 mM $MgCl_2$ (Applied Biosystems)
10 mM dNTP blend (Applied Biosystems)
Primers (Table 1E.1.1): dilute to 20 μM
DNA template: extracted from biofilm sample (Support Protocol 2)
Nuclease-free PCR water (Applied Biosystems)
Low mass DNA ladder (Invitrogen)
100% isopropanol
3 M sodium acetate pH 5.2 (*APPENDIX 2A*)
70% ethanol
TE buffer (*APPENDIX 2A*)

0.2-ml PCR tubes, sterile
Thermocycler with block for 0.2 ml tubes
−80°C freezer
Vacuum centrifuge (Savant; optional)

Additional reagents and equipment for performing agarose gel electrophoresis and ethidium-bromide staining (see Voytas, 2000)

Carry out PCR

1. Prepare the mix for PCR amplification as 100-μl reactions in sterile 0.2-ml PCR tubes containing the indicated final concentration of the components:

   0.5 μl 5 U/ml Ampli*Taq* Gold polymerase (2.5 U)
   10.0 μl 10× PCR buffer (1×)
   12.0 μl 25 mM $MgCl_2$ (3.0 mM)
   8.0 μl 10 mM dNTP blend (200 μM each)
   2.5 μl 20 μM GC 357F primer (0.5 μM; see Table 1E.1.1)
   2.5 μl 20 μM 907R primer (0.5 μM; see Table 1E.1.1)
   2.5 μl DNA template (2.5% v/v)
   62 μl nuclease-free PCR water.

   Volumes given are specific for 100-μl reactions. If less product is needed (e.g., in re-amplification of an extracted DGGE band or preparation of a particularly concentrated biofilm DNA extraction), the reaction volumes can be reduced to 25 or 50 μl. The primary reason to reduce the volumes of some reactions is to save reagents and avoid wasting Taq polymerase. In addition, the authors have found that contaminants in the biofilm matrix interfere with PCR less when the least amount of template is added to the PCR reaction.

   The performance of the AmpliTaq Gold polymerase has been consistent in the authors' experience.

   This recipe is optimized with low stringency for the universal primer set. With a more specific primer set it may be desirable to increase the stringency of the reaction by decreasing the $MgCl_2$ concentration or altering the primer concentration (Kramer and Coen, 2001).

2. Carry out PCR using the following amplification cycles:

Initial step:	10 min	96°C	(denaturation)
30 cycles:	45 sec	96°C	(denaturation)
	45 sec	46°C	(annealing)
	60 sec	72°C	(extension)
Final step:	30 min	72°C	(final extension)
	indefinitely	4°C	(hold).

Thermocycler parameters with universal eubacterial primers should include: a low annealing temperature to maximize universal primer binding, a low number of cycles to reduce bias caused by differential template binding, and a 30-min final extension step at 72°C to reduce heteroduplex formation.

3. Confirm the presence of PCR product using an ethidium bromide–stained agarose gel loaded with low mass DNA ladder (e.g., Voytas, 2000).

4. Estimate the amount of PCR product by comparing the brightness of sample bands with the brightness of the ladder bands to determine how many PCR reactions will need to be pooled to get 700 to 1000 ng of DNA per well with replicate DGGE runs, for sample consistency.

Typically 100 to 200 μl of PCR product will need to be pooled to obtain 700 to 1000 ng of DNA.

Concentration of the PCR product can also be estimated by measuring the A_{260}/A_{280} ratios on a spectrophotometer (Gallagher and Desjardins, 2006).

Concentrate sample

5. Pool all the PCR product required from one sample into a 1.5-ml centrifuge tube.

6. Add 1 vol isopropanol and 0.1 vol 3 M sodium acetate, pH 5.2. Place in a –80°C freezer for 10 min. Centrifuge 15 min at 16,100 × *g*, room temperature.

The PCR product should be in a tight white pellet.

7. Remove the isopropanol by decanting or aspirating with a pipet. Wash the pellet with 70% ethanol by adding ~500 μl to the tube.

8. Centrifuge 3 min at 16,100 × *g*, room temperature. Remove the ethanol with a pipet. Repeat wash (steps 7 and 8).

The pellet may be loose and easily lost in decanting. The pellet should be nearly colorless now.

9. Allow the pellet to air dry or place in a vacuum centrifuge to dry any remaining ethanol.

10. Resuspend the pellet in 10 μl TE if preparing sample for one gel and in 20 μl TE if preparing sample for two gels. Store the resuspended PCR product up to 3 months at –20°C.

It is very difficult to load more than 40 μl into a DGGE well. Loading dye should be mixed with PCR product in a 1:1 ratio (see Basic Protocol). Therefore, precipitated PCR product should be resuspended in a very small amount of TE (10 to 15 μl).

SONICATION OF FIELD SAMPLES AND EXTRACTION OF DNA

SUPPORT PROTOCOL 2

Field experiments offer the opportunity to examine microbial communities for biofilm growth under conditions of natural selection. For example, this protocol describes procedures that have been used in studies of aquatic biofilm formation on dialysis tubing

(McLean et al., 2005) and wastewater biofilms (A.K. Welsh and R.J.C. McLean, unpub. observ.). DNA extraction methods may need to be modified for biofilms in other environments. To this end, one strategy employed for technique development is to add known quantities of a test microorganism to an environmental sample and then use different methods to extract and amplify 16S rRNA from the test microorganism. Ideally, the test organism should not occur naturally in the particular environment being tested. In this context, the organism will not be detected in negative control samples and will be detected in positive control samples.

Pipets, dilution tubes, and media are used in large quantities during a field experiment. The authors recommend preparing agar plates and tubes for dilutions at least 1 to 2 days in advance, and having ~5% extra materials available in the event of unforeseen circumstances. In this manner, any plates or tubes that become contaminated can be identified and discarded. Types of substrata used in biofilm field studies are widely variable and can be adapted to each unique environmental biofilm studied.

Materials

Biofilm sample (from the field)
95% or 100% ethanol
Sterile deionized H_2O
R2A agar medium (Difco): prepare agar plates according to manufacturer's instructions
TE buffer (*APPENDIX 2A*)
DNeasy tissue kit (Qiagen)
Enzymatic lysis buffer: 20 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/2 mM EDTA (*APPENDIX 2A*)/1.2% Triton X-100
Lysozyme (Fisher Scientific)

Writing paper
Petri dishes, sterile
Scalpel, sterile
Glass scintillation vials: Fisher brand 20-ml borosilicate vials with polypropylene caps recommended, sterile
Bath sonicator (e.g., Branson Ultrasonic Cleaner Model 1510MT): fill to a depth of ~1 cm prior to use; drain and clean after use
50-ml centrifuge tubes
Vacuum filter unit with 0.2-μm filter

1. *Optional:* To cut a sample (e.g., a leaf) into a section prior to analysis, trace a 1-cm square (or desired area) on a piece of writing paper. Lay a sterile petri dish on top of the paper. Place the sample to be trimmed onto the petri dish and cut with a sterile scalpel.

   In this fashion one has an accurately measured area, yet can maintain aseptic technique.

2. Sterilize a pair of forceps by dipping in 95% to 100% ethanol and passing through a flame. Use the sterile forceps to aseptically place the sample into a sterile glass scintillation vial with enough sterile deionized water to completely submerge the biofilm material.

   Process biofilm samples from the field for molecular analysis as soon as possible (typically within one week of collection).

   The recommended glass vials are particularly useful for small objects because they are inexpensive and can be readily autoclaved. Other similar type vials are perfectly suitable. For larger objects, glass beakers covered with aluminum foil or bottles can be substituted.

The use of plastic containers is strongly discouraged because the elastic walls dampen the shear forces generated by the sonicator, which drastically reduces biofilm cell yield.

The containers used for sonication should be partially filled with a desired volume of dilution medium (e.g., 10 ml water or physiological buffered saline in scintillation vials) and autoclaved prior to use.

3. Place vials with biofilm material into a bath sonicator filled with water to just above the level of liquid in the vials. Close sonicator lid tightly and sonicate for the optimal time.

In the authors' experience 10 to 15 min is optimal for most samples; however, optimizing sonication conditions for the colonization substrata that are to be used is recommended. For this purpose, perform a preliminary experiment by growing biofilms on a surface, and then subjecting that particular surface to varying periods of sonication while periodically removing aliquots of liquid for dilution plating (McLean et al., 1999). As the plate counts are plotted against sonication time, the time giving the maximum counts is to be selected for the samples. Shorter times will not disrupt bacterial clumps (each of which will plate as a single colony), whereas longer times may result in cell damage and loss of viability, both conditions resulting in lower plate counts.

Some samples, notably limestone rock or plant leaves, will become cloudy or discolored during this process. This cloudiness or discoloration should not cause any problems in subsequent steps.

4. Decant the liquid containing the released biofilm bacteria from the scintillation vials into 50-ml centrifuge tubes. Use the sonicated samples to perform plate counts (step 5) and to extract DNA (step 7).

Ideally, the researcher should immediately proceed with DNA extraction. If necessary, samples may be stored overnight at −20°C before proceeding with extraction.

Perform plate counts

5. Serially dilute an aliquot of the sonicated material 10^{-2} to 10^{-5} in sterile water, and surface plate the dilutions on R2A agar.

For many freshwater heterotrophs, the authors have found sterile water to represent a suitable dilution buffer. R2A agar is an excellent general purpose medium that will grow a wide variety of heterotrophic bacteria commonly found in freshwater environments.

6. Incubate for the appropriate time and at the appropriate temperature for the particular environmental sample. Count colony forming units (cfus).

Time and temperature of incubation for these plates greatly depends upon the environment from which the biofilm sample is obtained. Freshwater heterotrophs typically require incubation for 72 hr at 25°C.

The number of cfu can give an overall indication of the concentration of heterotrophic bacteria present in the biofilm. The data can be used to compare replicate samples or samples with different treatments, in terms of the overall numbers of bacteria present. DGGE can then be used to describe the overall diversity of bacteria present.

Extract DNA

7. Centrifuge the sonicated samples containing the bacteria 10 min at 5900 × *g*, room temperature. Decant or pipet off the supernatant.

8. Suspend the sample in 1 ml TE buffer to preserve nucleic acids during storage.

9. Extract the bacterial community DNA using 250 µl of this sample and the DNeasy Tissue kit following the manufacturer's instructions for extracting Gram-positive bacterial DNA, which includes an enzymatic lysis step using enzymatic lysis buffer with 20 mg/ml lysozyme (see Internet Resources). Store the remaining sample at 4°C.

The Qiagen kit uses an enzymatic lysis protocol, which will target active bacterial cells (not dormant cells or spores) in the biofilm samples. The Gram-positive protocol will readily lyse both Gram-positive and Gram-negative bacteria.

The authors have found the DNeasy Tissue kit to be a straightforward and reliable DNA extraction kit. Other DNA extraction methods such as a freeze-thaw protocol (Gillan et al., 1998) can certainly be used to extract bacterial DNA for DGGE analysis.

The authors have found that substances present in biofilm samples may interfere with PCR. A DNA extraction and purification method is recommended, e.g., a Qiagen kit, that captures DNA on a column and removes it from the biofilm debris.

SUPPORT PROTOCOL 3

PREPARATION OF DGGE STANDARDS

Unlike normal gels, commercially available standards are not available for DGGE. Standards may be prepared from a mixture of PCR products from several organisms such as *Escherichia coli* MG1655, *Pseudomonas aeruginosa* ATCC 10145, and *Chromobacterium violaceum* ATCC 12472. Any organisms can be used to create a DGGE standard. Standard will need to be prepared in large quantities, because it will be included in every DGGE gel.

Additional Materials *(also see Support Protocol 1)*

Bacterial strains for use in the standard—e.g., *Escherichia coli* MG1655 (ATTC #47076), *Pseudomonas aeruginosa* (ATCC #10145), and *Chromobacterium violaceum* (ATCC #12472)
Appropriate medium for bacteria used (*APPENDIX 2C*)
DNeasy tissue kit (Qiagen)

CAUTION: *E. coli, P. aeruginosa, and C. violaceum are* Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

1. Extract DNA from an overnight broth culture of each of the bacterial cultures for the standard using the DNeasy Tissue kit.
2. Carry out PCR, isopropanol precipitation, and sample concentration on the extracted bacterial cultures as described in Support Protocol 1, with the following modifications.
   a. Because these are pure cultures, use a concentration of 100 to 200 ng per well.
   b. Prepare enough of each culture for at least 20 DGGE gels.
   c. Rehydrate dried PCR product from the pure cultures in TE such that the final volume of the mixture of organisms is not more than 15 µl per DGGE well.
3. Electrophorese each pure culture PCR product in individual wells of a DGGE gel using the same denaturant concentration, time, and voltage as optimized for the environmental biofilm samples to determine the melting point of each. Adjust the concentration as required.

   The amount of each pure culture added per well will need to be optimized for each strain. For example, Escherichia coli MG1655 will make one nice bright band and three other weak bands due to multiple copies of its 16S rRNA gene. Reducing the amount of E. coli PCR product added will reveal only one primary band.

4. Mix the appropriate concentrations of PCR product for each of the strains in the standard for use in 20 DGGE gels. Divide into ~60-µl aliquots (for four gels) and store up to 1 year at –20°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acrylamide solution (6%) containing denaturant (30% or 50%)

15.0 ml 40% (37.5:1) acrylamide/bisacrylamide
2.0 ml 50× TAE buffer (*APPENDIX 2A*)
12.0 ml (for 30% denaturant) *or* 20.0 ml (for 50% denaturant) formamide, molecular-biology grade
12.6 g (for 30% denaturant) *or* 21.0 g (for 50% denaturant) urea
Adjust volume to 100 ml with water
Store in a dark bottle up to 3 months at 4°C

CAUTION: *Wear gloves because unpolymerized acrylamide is a neurotoxin. It is very helpful to cover lab benches with an absorbent paper in order to contain any spills.*

The authors typically use a 6% polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gel with a 30% to 50% denaturant concentration where 100% denaturant contains 7M urea and 40% formamide. See (Muyzer et al., 1993) for more information.

DGGE gel loading dye, 2×

Add 0.25 ml 2% bromophenol blue and 0.25 ml xylene cyanol to 7.0 ml 100% glycerol. Add 2.5 ml water. Mix well. Sterilize by passing through a 0.2-μm filter. Divide into 1-ml aliquots. Store up to 5 years at room temperature.

100% glycerol is very difficult to move with a pipet. The authors typically pour the glycerol into a 10-ml graduated cylinder then add all the other ingredients into the cylinder. Cover the graduated cylinder with Parafilm and invert several times to mix. Pour the dye mixture into a sterile petri dish and draw it up into a 10-ml Luer-Lok syringe. Attach a 0.2 um filter by screwing it onto the Luer-Lok syringe with dye in it. Push the dye mix through the filter into a sterile 10-ml centrifuge tube. Dispense aliquots into ten 1.5-ml sterile tubes.

Ethidium bromide gel staining solution, 3μg/ml

Prepare 500 ml of 0.5× TAE by combining 495 ml water and 5 ml 50× TAE buffer (*APPENDIX 2A*) in a plastic tub. Add 3 drops (~50 μl per drop) concentrated (10 mg/ml) stock solution of ethidium bromide (Bio-Rad) using the dropper provided, to obtain a final working concentration of 3 μg/ml. Mix by rocking tub gently. Place cover on tub. Store dilute stain in the dark and use within 1 week.

COMMENTARY

Background Information

Denaturing gradient gel electrophoresis (DGGE), originally developed to identify point mutations, has now been successfully used for evaluating microbial diversity in a number of environments (Muyzer and Smalla, 1998). The banding patterns revealed by DGGE for each biofilm sample yields two kinds of information about the sample. First, the banding pattern from DGGE can be used as a community fingerprint which can be compared to other samples with similar or different treatments to evaluate overall community structure (Emtiazi et al., 2004; Sigler and Zeyer, 2004; Webster et al., 2004). Unlike other popular community fingerprinting techniques, bands can be extracted directly from the DGGE gel and sequenced to reveal the identity of dominant bacterial community members in the biofilm sample (Emtiazi et al., 2004; Webster et al., 2004).

Interpretation of DGGE community fingerprints and identification of community members from excised bands from DGGE gels have several possible limitations, e.g., PCR products with similar GC content can migrate to similar places in the gel, one organism can have generated more than one band due to multiple copies of the 16S rRNA gene, and DGGE has been shown to reveal only organisms that are >1% of the population, (reviewed in Muyzer and Smalla, 1998). In spite of its limitations, DGGE is an excellent tool for disclosing microbial diversity in complex

environments like biofilms (Emtiazi et al., 2004; Webster et al., 2004).

Critical Parameters and Troubleshooting

The procedures in this unit have been successfully carried out using the Bio-Rad DGGE system; the photographs in the DCode manual showing the different steps are very helpful. The only problems encountered have been with the gradient former, which is prone to rust and inaccuracy in the gradient delivery volume. Although there are other DGGE system manufacturers (e.g., C.B.S. Scientific), the authors have no experience with their equipment.

Lack of DGGE bands in a gel

The major critical issue that will compromise DGGE success is insufficient DNA loaded onto a gel. Typically 700 to 1000 ng of PCR product is required from environmental samples (Lyautey et al., 2005).

Time of electrophoresis and voltage will also affect the clarity of bands revealed in the DGGE. First, decide on a time of electrophoresis and a voltage. This has as much to do with scheduling as it does the resolution required. For the GC 357F and 907R primer set, the authors use 180 V for 7 hr. Roughly 1000 to 1200 volt hr should be sufficient and can be achieved with short high voltage runs or long low voltage runs (Diez et al., 2001). Time of electrophoresis should not exceed 16 to 18 hr because the denaturant dissolves into the buffer and can affect banding patterns (Sigler and Zeyer, 2004). Additionally, Bio-Rad recommends not exceeding 200 V with the DCode system. To determine whether gels are running long enough, carry out a time trial DGGE in which samples are loaded every hour for 7 or 8 hr, and examine the banding patterns. The time trial DGGE should show smears at the first time points as well as the migration progression and eventual melting points of the samples. Ideally, the samples should have melted and ceased migrating fully in the last two time points. If not, then the time of electrophoresis should be increased or decreased to match this pattern of migration (see Muyzer et al., 1993).

Another cause of lack of clear bands is an improper gradient. The most challenging aspect of applying DGGE for environmental samples, particularly with species-specific primers, is optimization of the appropriate denaturing gradient. An incorrect gradient will reveal smears, one tight band at the very top of the gel, or no bands at all. Bio-Rad sells a program, WinMelt or MacMelt, which will predict the optimal gradient for a sequence of interest, but this is of little use in biofilms from the environment where there is probably no sequence information.

Bio-Rad also recommends running a perpendicular gel to determine the ideal gradient for new, unknown samples (Fig. 1E.1.1A). The direction of electrophoresis (top to bottom) is perpendicular to the direction of the gradient (right to left). However,

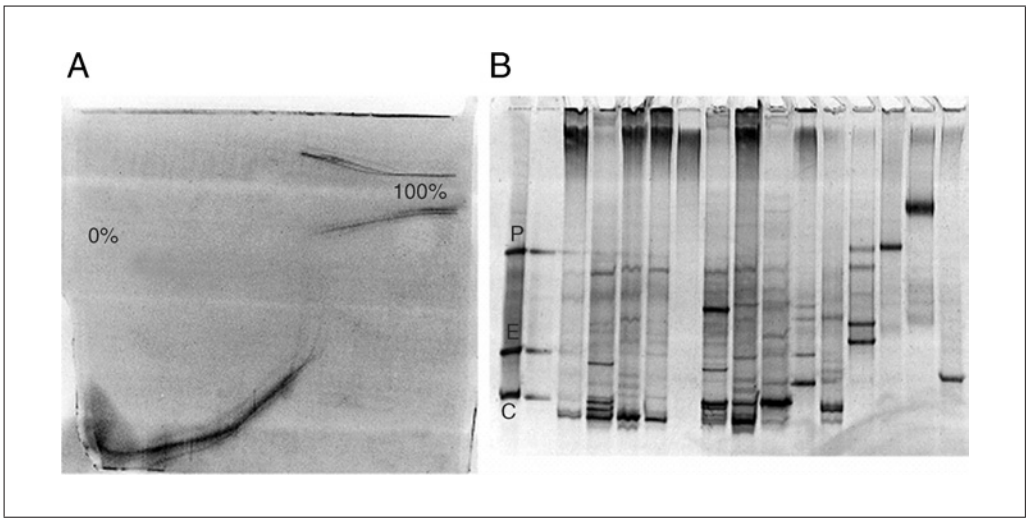

Figure 1E.1.1 Representative DGGE gels. (**A**) Perpendicular DGGE gel of seven wastewater biofilm organisms used to establish gradient range for further analysis. Here changes in band migration occurred between 45% and 60% concentration of urea/formamide. **(B)** A parallel DGGE gel analysis of mixed aquatic biofilm communities. PCR product from three organisms: *Pseudomonas aeruginosa* (P), *Escherichia coli* (E), and *Chromobacterium violaceum* (C) was used as standards. The number of bands approximates the number of different organisms in each environment.

perpendicular gels are extremely hard to cast because the sandwich assembly is filled from the side rather than the top. Instead of running perpendicular gels, it is more advisable to run a parallel gel (Basic Protocol) with a broad gradient of 30% to 70% and then adjust the gradient by 10% to find the appropriate one. For example, in aquatic biofilm samples with the GC 357F and 907R primer set, 20% to 40% denaturant resulted in a few smears and maybe one band, 30% to 50% denaturant gave a beautiful display (Fig. 1E.1.1B) and, 40% to 60% denaturant resulted in smears.

Poor gel polymerization

Replacing the necessary reagents, TEMED and ammonium persulfate, can rectify poor or slow gel polymerization. The authors replace DGGE reagents after one year.

Casting DGGE gels

This procedure must be done quickly yet safely as the reagents will polymerize within 5 to 10 min once the catalysts have been added. Typically the authors use two syringes that are labeled "HI" or "LO" (reflecting a high or low concentration of urea/formamide), labeled with two different colors of tape. Other containers used for mixing the acrylamide should be labeled with the same color tape. Often working with a second individual greatly helps here. One person will actually cast the gel, the second will quickly rinse the syringes and tubing used for casting with water, so that they can be reused.

Staining

Both 1% ethidium bromide and various SYBR stains produced by Molecular Probes (now Invitrogen), have been used for DGGE imaging. While any of these stains will work, the authors prefer ethidium bromide. The SYBR stains may be too sensitive and will also stain single-stranded DNA and generate other smears. (On a precautionary note, it is best to assume that any chemical that stains DNA is a potential mutagen and presents a significant health hazard; see *UNIT 1A.3*.) It is recommended that staining solutions be prepared on a weekly basis, and destaining solutions fresh before each gel.

Imaging

There are a number of protocols used to image gels, ranging from Polaroid pictures to electronic imaging and documentation. Typically, the authors acquire electronic images of all gels and then optimize contrast with a computer program (e.g., Adobe Photoshop). In the authors' experience, producing negative images of DGGE gels (using the Invert Image command of Photoshop) increases resolution.

Removal of bands for subsequent sequencing

Speed is important. The DGGE gel should be imaged quickly and then bands cut out and processed. The ultraviolet light from the transilluminator will interfere with DNA structure through the generation of thymine dimers.

Heteroduplex formation and multiple bands upon reamplification of DGGE bands

Heteroduplexes have been a chronic problem with the DGGE technique, particularly when using universal primer pairs (Muyzer and Smalla, 1998). Heteroduplex formation is caused during PCR when two heterologous strands form a duplex at the primer initiation sites. Following band excision and reamplification, heteroduplexes result in multiple bands upon DGGE electrophoresis of this re-amplified band. If heteroduplexes are a problem, try decreasing the annealing temperature of the PCR reaction, increasing the $MgCl_2$ concentration, and increasing the primer concentration (Muyzer and Smalla, 1998). More than one band in a DGGE gel from one reamplified band may also result from an improper gradient or not running the gel long enough. What may appear to be one band can actually still be a mixture of sequences. Resolving individual bands on DGGE upon reamplification may require changing the gradient, e.g., from a 30% to 50% gradient to a 35% to 40% gradient.

Anticipated Results

Figure 1E.1.1 shows the two types of gels that are commonly used with DGGE: a perpendicular gel (Fig. 1E.1.1A) in which the urea-formamide gradient is perpendicular to the direction of electrophoresis, and the more commonly seen parallel gel (Fig. 1E.1.1B). Although perpendicular gels are sometimes used for identifying suitable gradients for analyzing a particular community, the authors have found it to be more practical to use a parallel gel with a wide gradient range (e.g., 30% to 70%) for range-finding, and a smaller gradient range (e.g., a 35% to 45% gradient) to enhance resolution of DNA fragments of similar G+C content.

Time Considerations

Certainly transportation and other issues will affect the time needed for field work. Table 1E.1.2 provides a general guide for time

Table 1E.1.2 Time Considerations for Field Sampling and DGGE Analysis of Field-Grown Biofilms

Procedure	Time required
Preparation of all supplies and samples needed for experiment and analysis	1-2 days
Transportation to the field site and back to lab	varies with location
Sonication of sample	30 min[a]
DNA extraction and purification	1 hr
PCR	2-3 hr
Agarose Gel analysis of PCR product	1-2 hr
DGGE setup and specimen loading	1-2 hr
DGGE run	4-16 hr[b]
Gel staining and photography	1 hr

[a]Sample can be stored overnight under suitable conditions (e.g., in appropriate buffer at −20°C).
[b]Specimens can be run at lower voltage for longer times (e.g., 16 hr at 80 volts, 60°C) or higher voltages for shorter times (e.g., 7 hr at 180 volts, 60°C).

requirements in obtaining biofilm growth on a glass microscope slide and its subsequent analysis by DGGE. The most time-consuming aspect of applying DGGE for biofilm samples from the environment is optimizing the DGGE conditions. The conditions described in this unit should work well for biofilms from typical aerobic sites using the GC357F and 907R universal bacterial primer set. However, field sites conducive to growing high GC-containing Gram-positive bacteria or a specific primer set targeting sulfate-reducing bacteria, for example, will require careful optimization of the DGGE conditions to reveal the overall bacterial diversity. Optimization of the DGGE conditions should be done prior to the final assay with, if possible, biofilm samples from the same or a similar field site.

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Internet Resources

http://www1.qiagen.com/literature/handbooks/PDF/GenomicDNAStabilizationAndPurification/FromAnimalAndPlantIssues/DNY_BloodTissue/1037951_HB_DNY_Blood_Tissue_062006_Web.pdf

The Qiagen DNeasy tissue kit manual gives directions for extraction of DNA from Gram-positive and Gram-negative bacteria.

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Biological Sand Filters: Low-Cost Bioremediation Technique for Production of Clean Drinking Water

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ABSTRACT

Approximately 1.1 billion people in rural and peri-urban communities of developing countries do not have access to safe drinking water. The mortality from diarrheal-related diseases amounts to ~2.2 million people each year from the consumption of unsafe water. Most of them are children under 5 years of age—250 deaths an hour from microbiologically contaminated water. There is conclusive evidence that one low-cost household bioremediation intervention, biological sand filters, are capable of dramatically improving the microbiological quality of drinking water. This unit will describe this relatively new and proven bioremediation technology's ability to empower at-risk populations to use naturally occurring biology and readily available materials as a sustainable way to achieve the health benefits of safe drinking water. *Curr. Protoc. Microbiol.* 9:1G.1.1-1G.1.28. © 2008 by John Wiley & Sons, Inc.

Keywords: biofiltration • bioremediation • biosand filter • water treatment • microbiological contamination • water quality • developing countries

INTRODUCTION

The burden of microbiologically contaminated water is borne most heavily by the rural (largest, 80%) and peri-urban (fastest-growing) populations without access to safe water in developing countries—all need microbiologically clean water to sustain their lives and secure their livelihoods.

There is conclusive evidence that biological sand (biosand) filters are capable of dramatically improving the microbiological quality of drinking water. Biosand filters are based on a centuries-old bioremediation concept: water percolates slowly through a layer of filter medium (sand), and microorganisms form a bacteriological purification zone atop and within the sand to efficiently filter harmful pathogens from microbiologically contaminated water. Household-scaled biosand filters are a small adaptation of traditional large, slow sand filters such that they can uniquely be operated intermittently.

To use the simple, yet effective, on-demand biofiltration intervention, a person simply pours contaminated water into the household biosand filter and immediately collects treated water.

The purpose of the following comprehensive protocols is to facilitate knowledge transfer with the goal to empower vulnerable, poorest-of-poor populations in rural and peri-urban communities of developing countries, and to also promote using naturally occurring biology and readily available materials that they already possess as a cost-effective practical approach to combat poverty and inequality and achieve the health benefits of safe water by developing their own household water security solutions.

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CAUTION: Under ideal circumstances, a biosand filter can produce drinking water of high quality. However, this cannot always be assured or guaranteed due to variations in the construction and installation of the filter. This also applies to the consumption of water from the biofilter. It should be noted that a biosand filter cannot be relied upon to remove certain or all forms of water contamination.

CAUTION: If it is suspected that the community water source is contaminated with organic and inorganic industrial and agricultural toxicants, or in regions where the ambient air reaches freezing temperatures, biosand filtration should not be recommended as an appropriate water treatment technology.

STRATEGIC PLANNING

The performance efficiency of the biosand filter is limited by excessive turbidity, especially during the monsoon or rainy season where the performance of the filter will be compromised with excessive clogging, requiring frequent recovery of flow rate (see Basic Protocol 8). It is essential to obtain turbidity readings (see Basic Protocol 1) on the raw water source where biofiltration is being proposed as a potable water solution.

When working in partnership with village health workers among the rural poor with the challenge of providing affordable safe drinking water in every household using biosand filters, it is recommended to implement a comprehensive community-based multiple barrier approach (Nath et al., 2006). Education and training, emphasizing environmental awareness, hygiene, and sanitation are thus first necessities. Reduction of excessive turbidity and particulates by settlement or prefiltration or flocculation establishes a second barrier (Basic Protocol 2). Removal of parasites, protozoal cysts, bacterial pathogens, and in some situations removal of chemical contaminants (e.g., arsenic) by biosand filtration is the third barrier (Basic Protocol 3). The fourth and final barrier, safe storage in a closed, spigotted container and providing a disinfectant residual (see Basic Protocol 9) are all key aspects to strengthen local capacity to carry out sustainable community-based primary health care to prevent preventable illnesses and deaths caused by microbiologically contaminated water.

Selecting the Type of Biosand Filter

The three most common biological sand filters, as illustrated in Figure 1G.1.1, are based on the 60 liter (15 gallons) "BioSand" design developed by Dr. David Manz at the University of Calgary, Canada, in the 1990s.

As long as adaptations do not contravene the principles and protocols put forth that safeguard the bioremediation processes and functions of the filter, it may also be suggested that highly effective 15 to 20 gallon (60 to 80 liters) volume per day biological sand filters can be constructed by the household using readily available materials in their own community.

As illustrated in Figures 1G.1.1 and 1G.1.2, there are various materials that biosand filters can be constructed from, ranging from concrete, plastic, metal, or indigenous containers—each with its own benefits and drawbacks.

Strategically, the local availability of appropriate media (sand and gravel), parts, tools, and their respective costs will be critical predetermining factors in making decisions on the choice of construction method and corresponding materials used; therefore, stepwise one-fits-all construction protocols cannot be standardized. This flexibility of construction choice based on locally available resources will also provide the lowest cost—an important parameter for community acceptability and to ensure sustainability.

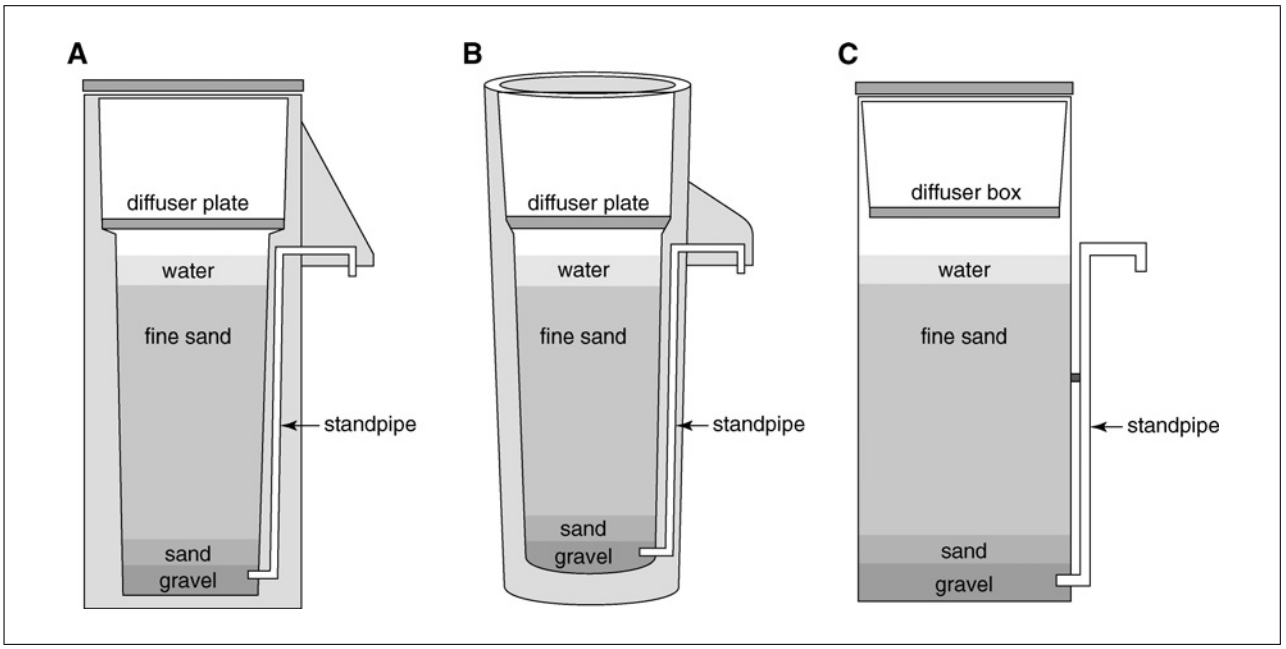

Figure 1G.1.1 Schematic diagrams of three common filters. (**A**) CAWST's concrete (BioSand) filter—high rates of user acceptance (over 300,000 filters worldwide). (**B**) BushProof's Concrete (BioSand) Filter—round shape provides additional strength and requires less materials. (**C**) Barrel Filter—locally built biosand filters made from plastic barrels or metal drums.

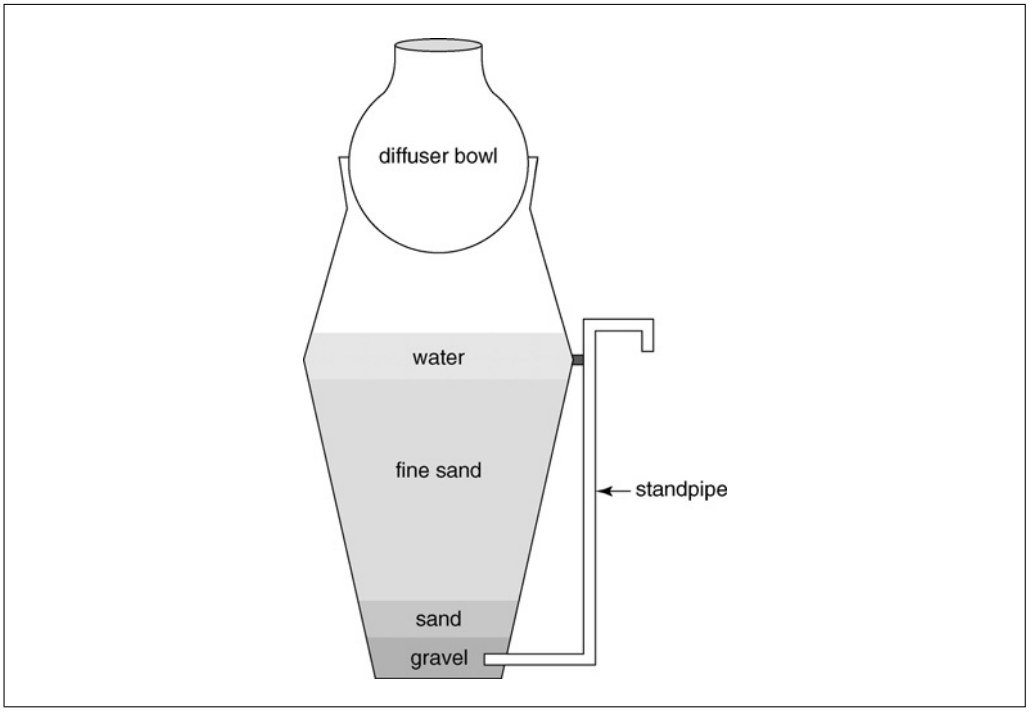

Figure 1G.1.2 Illustration featuring an indigenous biofilter constructed of clay jars.

Constructing the Biofilter

In an attempt to demystify biosand engineering to the essential principles, all on-demand household-scaled biological filters share five simple but critical design parameter commonalities featured in Figure 1G.1.3:

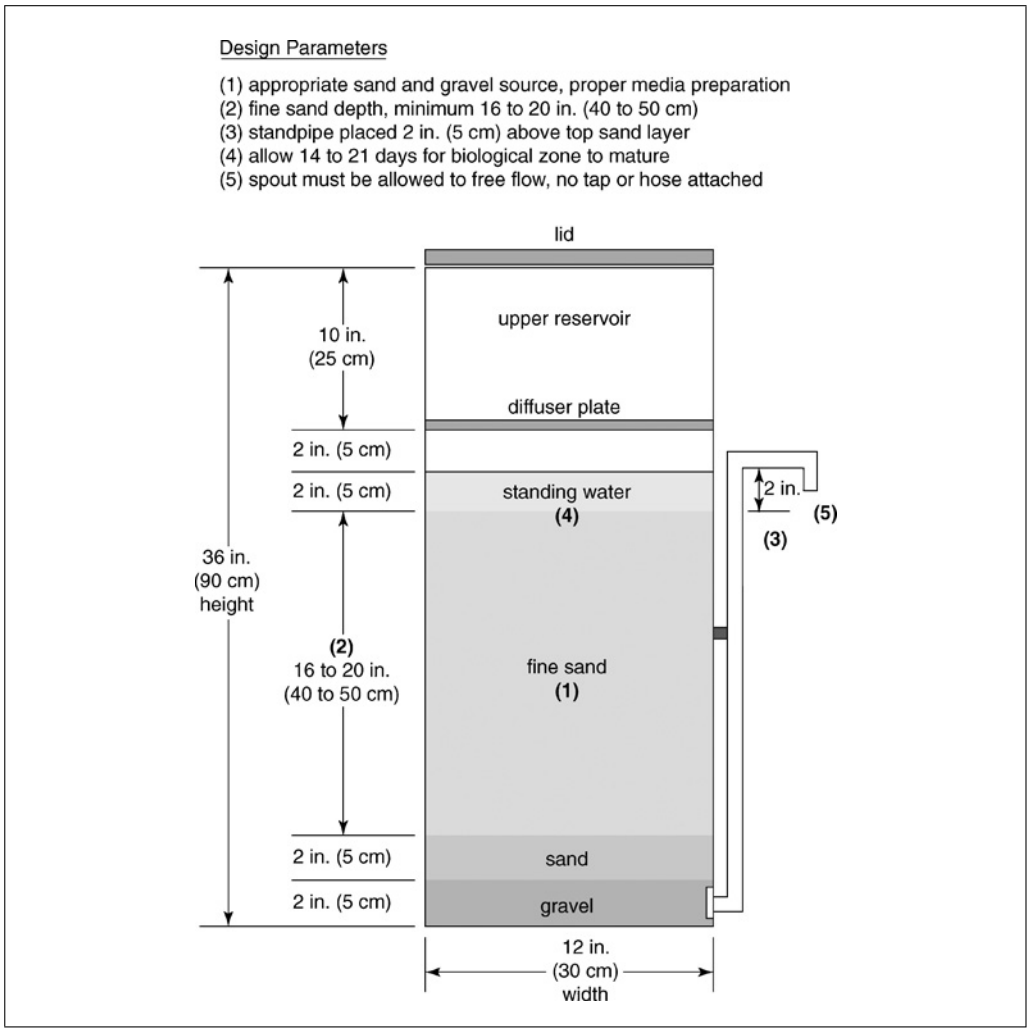

Figure 1G.1.3 Illustration highlighting major principles and generic size dimensions.

1. Biosand filters can be built wherever there is a good source of sand and gravel and where proper media preparation is undertaken (see Basic Protocol 4).

2. The most widely used version of the biosand filter is a concrete container filled with ~16 to 20 in. (40 to 50 cm) of fine sand. This is the absolute minimum fine sand requirement to ensure the best quality of water possible.

3. A layer of static standing water (the supernatant) is automatically maintained by placing the outlet pipe 2 in. (5 cm) above the level of the top sand layer.

4. Allow 14 to 21 days for the biological zone to mature.

5. Water must be allowed to flow freely from the filter—never plug or put a hose or tap on the outlet spout.

Further discussion of specific detailed methods of construction are not required as there are numerous methods and associated protocols readily available from various organizations for constructing the various featured configurations of biosand filters (see Key References).

Major Components of Biological Filters

Container

Durable water-tight "water safe" containers can typically be purchased or constructed in various shapes (square or round) and materials (e.g., concrete, metal, plastic, ferrocement, brick, or clay jars). The key is to find an appropriate-sized container that is readily available or can be constructed at a reasonable price in your area.

Lid

The lid can be made out of any material, but it must be clean and ideally fit tightly onto the container. A filter lid is essential to prevent excess biofilm growth by blocking sunlight and guarding against insects and other contaminants entering the filter.

Diffuser plate

The diffuser plate can also be made out of various common materials, such as plastic or metal. If made from sheet metal, ensure it is constructed out of good quality galvanized metal, or it will rust, either prematurely plugging the diffuser holes with rust or gradually increasing the diffuser hole size. A larger hole will result in disturbance of the schmutzdecke—a word derived from the German schmutz (dirt) and decke (covering)—or sand media.

Avoid wood, as it will attract mold growth and tend to shrink or warp, ultimately not fitting tightly inside the filter container, allowing potential disruption of the top sand layer.

A drip grid is a required feature of all diffusers to evenly distribut the water without disturbance of the schmutzdecke or sand media. On the bottom of the diffuser plate, measure and mark a 1-in. × 1-in. (2.5 cm × 2.5 cm) grid. At each intersection on the grid, pound a $^1/_8$-in. (3 mm) diameter hole through the diffuser material, using a hammer and nail. Smaller holes will restrict the flow through the filter; larger holes will result in disturbance of the schmutzdecke or sand media.

The primary functions of the diffuser plate are: (1) protecting the surface zoogleal biofilm—the schmutzdecke—and top layer of sand by dispersing the energy of water as it enters the filter, and (2) facilitating the addition of critical oxygen to the supernatant water through aeration process.

Filtrate standpipe and the standing water level (supernatant)

The standpipe is the essential component in all biosand filters. This simple but key design component automatically maintains the standing water level (the supernatant) to a constant depth when installed 2 in. (5 cm) above the top of the filtering sand. As illustrated, see Figure 1G.1.1, household-scaled biological filters can be made in various ways, but each configuration share this one simple but important design commonality.

The standpipe can be made out of $^1/_4$-in. (6 mm) i.d. tubing 3 feet (1 meter) long. The materials can vary from plastic or metal, such as copper, PVC pipe fittings, polyethylene, or vinyl tubing. The primary function of the supernatant, as set by the standpipe placed 2 in. (5 cm) above the filtering stand, is to keep the biological layer alive during pause periods. The schmutzedecke requires an aquatic environment and a constant influx of food and oxygen. If the static water level rises above 2 in. (5 cm), oxygen will not diffuse, creating a thinner biological zone. If the static water level drops below 2 in. (5 cm), then the inflowing water will disturb the sand and the biolayer may dry out due to excessive evaporation in high ambient temperatures.

Media (sand and gravel) bed

The media bed is composed of the following matrix of sand and gravel. Also refer to the discussion below: Media Selection and Preparation.

Filtering layer (fine sand)

Upper fine sand (filtering) layer—$^1/_8$ in. (3.15 mm) or less diameter sand. The depth of the filtering sand bed is 16 to 20 in. (40 to 50 cm). This is the absolute minimum fine sand requirement to ensure the best quality of water possible.

The actual volume of fine sand required is 25 quarts (~25 liters). The upper fine sand (filtering) layer is responsible for removal of pathogens and the establishment of the biological zone, including the schmutzdecke.

Support layer (coarse sand)

Support layer—$^1/_8$- to $^1/_4$-in. (3.125 to 6.25 mm) diameter sand, depth 2 in. (5 cm). Coarse sand volume required is 3 quarts (~3 liters). The purpose of the middle support layer is to prevent the sand mixing with the underdrain layer.

Underdrain layer (fine gravel)

Underdrain layer—$^1/_4$- to $^1/_2$-in. (6.25 to 12.5 mm) diameter gravel, depth should cover standpipe inlet [may be a depth of 2 in. (5 cm) or more]. Gravel volume required is 3 quarts (~3 liters). The purpose of the lower gravel layer is to allow unrestricted water flow out of the filter via the standpipe.

Adapting Biosand Construction for Available Materials and Community Needs

Again, as long as adaptations do not contravene basic construction parameters for the major components of biological filters, it is advocated that highly effective biosand filters can be constructed by the household using readily available materials in their own community.

Locating a source of appropriate sand and gravel

The effectiveness and efficiency of the filters is dependent on the community locating a source of uncontaminated sand and gravel.

Media Selection and Preparation

All biosand filters share a common feature, correct media (sand and gravel) selection and preparation.

Good sources of sand and gravel

Clean crushed rock from a quarry or gravel pit is the material of choice. The sand grains will have more surface area and the rough edges provide different ionically charged surfaces causing contaminants to be attracted to the sand grains.

If crushed rock is absolutely not available, the next choice would be sand from high on the banks of a river (that have not been submerged in water).

Poor sources for sand and gravel

Avoid riverbed sand and gravel. The grains are too smooth, round, and uniform in size. River sand is often contaminated with bacteria and organic material.

Sand and gravel should never come from a beach area. The grains are also too smooth, round, and uniform in size. It may also contain salts that dissolve into the filtered water.

Indicators the sand is appropriate for use in a biofilter

When you pick up a handful of the sand, the grains should be of different sizes and shapes and you should be able to feel the coarseness of the grains.

When you squeeze a handful of dry sand, the sand should all pour smoothly out of your hand.

Indicators the sand is NOT appropriate for use in a biofilter

When you squeeze a handful of dry sand, it should not ball up in your hand. If it does, it probably contains dirt or clay.

It should also not contain any very fine sand, silt, or organic material (e.g., leaves, grass, sticks, loam, clay, or dirt).

It should not contain microbiological contamination (avoid areas that have been used frequently by people or animals).

Selection criteria for appropriate sand and gravel

Grain size and quality of the sand are crucial to the effectiveness of a filter's performance. Grain size is important since larger or nonuniform sizes result in removal of less contaminants from the water. Alternatively, a finer grain size, by filling in the voids between larger grains, may render the media bed to be so compact as to offer an unacceptable flow rate due to high resistance. Quality of sand refers to the absence of fine silt or clay, which causes turbidity in the effluent.

As a last resort, a filter can be installed with contaminated river or beach sand. Initially, this will create a situation where the supposedly treated water will contain a greater density of indicator pathogens than the source water originally poured into the filter. Over time (up to 3 months), this situation will stabilize when all the food on the contaminated media has been consumed by the filter's normal biological processes. During this time period, it is highly recommended that filtered water from the biofilter be treated by an additional process such as household chlorination or SODIS (see Basic Protocols 9 and 10).

The Importance of the Schmutzdecke Layer

While locating and preparing proper sand and gravel are of great importance, the formation of the biological zone is perhaps the single most important component within the filter. Newly installed or recently cleaned filters do not effectively remove pathogens.

The biologically active zoogleal film develops on the surface of the sand filter and helps water purification by breaking down pathogens into inorganic compounds through chemical, microbiological oxidation, and predatory activity. The effectiveness of the biofilm relies on the following critical points: (1) a constant aquatic environment; (2) the biological zone needs food, therefore raw water should be intermittently fed within a consistent daily regimen [at least one 5-gallon (20 liter) bucket of water every day with minimum of 1 hr and maximum of 48 hr pause periods, a recommended pause period of 6 to 12 hr in-between feeding is suggested (CAWST, 2008)]; (3) oxygen is required for the metabolism process, and (4) sufficient water temperature are all essential to keep the biofilm microorganisms alive.

The schmutzdecke merges with the deeper distinct biological zone, a continuation of the area of biological action where microorganisms living below the schmutzdecke also help to consume and trap other microorganisms. The bacterial activity is most pronounced in the upper part of the filter bed and gradually decreases with depth as oxygen and food becomes scarcer to sustain life.

The biofilm and biological zone typically develop within two to three weeks (it may take up to 30 days) depending on the temperature and the biological content of the raw water. The water from the filter can be used during the first few weeks while the schmutzdecke is being established if a safer water source is not available, but chlorination is recommended at least during this time period. Over time the filter flow rate may decrease when the schmutzdecke becomes too thick and dense, requiring periodical maintenance (see Basic Protocol 8).

Guidelines for Operating a Biosand Filter

The combined use of the following recommended guidelines for operating a biosand filter will ensure the best quality of treated water.

1. Use a designated dirty container for collecting raw water from source.

2. Use the best sources of water (least contaminated) available: the better the source of water is, the better the treated water will be. A biosand filter can use any water source such as rain water, shallow wells, rivers, lakes, or surface water, but it should be taken from the same source consistently. Using the same source of water every day will improve the filter effectiveness. The water source should be the cleanest available since biosand filters cannot remove 100% of biological contaminants.

3. Over time, the microorganisms in the biological zone adapt to the "food" available in the untreated water source. If different water sources are used for each pour, that may result in an increased level of a contaminant that the microorganisms of the schmutzdecke are unable to consume or destroy. Several days may be required for the schmutzdecke to adapt to a new water source.

4. The diffuser plate must always be in place when pouring water into the filter: **never pour water directly into the filter without using the diffuser plate.**

5. The filter lid should always be kept on the filter.

6. Water must always be allowed to flow freely from the filter—never plug or put a hose or tap on the outlet spout. Plugging the outlet pipe could increase the water level in the filter, which could kill the biolayer due to lower oxygen diffusion into the standing water layer (supernatant) and/or resulting in a thinner biological zone that becomes anaerobic. Putting a hose on the outlet spout can drain (siphon) the water in the filter, dropping the water level below the sand layer, also, killing the biolayer.

7. Use a separate designated safe storage container container to safely store the filtered water. (See below for considerations when storing filtered water.)

8. Food should never be stored in the filter. It will attract insects. Since the water in the top of the filter is contaminated, it will in turn contaminate the food.

9. The treated water should be chlorinated after it passes through the filter to ensure the highest quality of water and to prevent recontamination. See Basic Protocol 9.

10. An ideal flow rate is 0.6 liters per min.

11. Having a pause period between filter usages is important. The in-between time when the filter is not actively filtering contaminated water is the pause period. A pause period of 6 to 12 hr is a suggested time, which allows the biological zone to remain vibrant by consuming the pathogens that have been introduced daily. The percentage removal of biological contaminants is inversely proportional to the flow rate through the filter because the greater biological removal of contaminants takes more time. However, if the pause period is extended for too long, the microorganisms will eventually consume all of the food supply and then die off. This will reduce the filter's treatment efficiency when it is used again.

12. One 5-gallon (20 liter) bucket of water should be poured into the filter every day to maintain the schmutzdecke and the biological zone. A biofilter is most efficient when operated consistently. The maximum volume of daily water that can be treated amounts to a total of 15 to 20 gallons or 60 to 80 liters (CAWST, 2008) depending on the schedule of the household, for instance, filter feeding can occur once in the morning, noon, evening, and later with 6 hr pause periods in-between each feeding.

Storing Filtered Water Safely

It is important to remember that water collected and stored from a filter can be recontaminated before being consumed by the household. To prevent water from becoming contaminated again, follow these recommendations.

1. Use a designated safe storage container for collecting and storing treated water. A safe container has a lid and a narrow opening to prevent recontamination due to dipping with dirty cups or hands. A container with a tap or spigot to access the water is ideal.

2. Keep the container off the ground and away from insects and animals.

3. If possible, do not treat more water than required for daily use.

4. At regular intervals (only when dirty), clean the outside of the storage container.

READING SOURCE WATER TURBIDITY

Biological filters have limits to the amount of turbidity of the raw water source being poured into the filter. High amounts of suspended particles present in the turbid water settle in the top sand layer, leading to rapidly diminishing flow rates. In turn, requiring the filter to be cleaned frequently involves disturbing the biological layer, leading to diminished filter performance for several days thereafter. The preferred turbidity rate is <50 NTU (Nephelometric Turbidity Units). Higher turbidity levels (>50 NTU) will require prefiltration (CAWST, 2008).

Quantitative Estimate of Turbidity

BASIC PROTOCOL 1

The following straightforward procedure provides a reasonably accurate quantitative estimate of turbidity.

Materials

Turbidity gauge (see Support Protocol)

Additional reagents and equipment for turbidity treatment (Basic Protocols 2 and 10)

1. Fill the tube with water until the black and white disk on the bottom of the newly constructed gauge is no longer visible.

2. Read the gauge at the nearest marked water level.

   A simpler alternative test to measure the turbidity is to use a 2-liter clear plastic soft drink bottle filled with water. Place this on top of a black and white disk. Look down through the bottle from the top, if you can see the black and white disk the water probably has a turbidity of <50 NTU.

3. Perform the appropriate treatment based on the following turbidity guidelines:

   a. *If the turbidity reading is 50 NTU or greater before filtration*: Perform turbidity pretreatment as recommended (see Basic Protocol 2) to eliminate the inconvenience of frequent declogging of top layer of fine sand.

   b. *If the turbidity reading is >5 after filtration*: Use a disinfection process such as chlorine addition or solar disinfection (SODIS) in conjunction with the sand biofilter (see Basic Protocol 10).

   No health-based guideline value for turbidity has been proposed within WHO Drinking Water Guidelines *(http://www.who.int/water_sanitation_health/dwq/gdwq3rev/en/index.html)*. Generally, water with a turbidity of <5 NTU is usually acceptable in households, although this may vary with local circumstances.

SUPPORT PROTOCOL

Building a Simple Turbidity Gauge

The following simple do-it-yourself turbidity gauge can be easily constructed at a low-cost.

Materials

1-in (2.5 cm) diameter PVC end cap
Waterproof marking pen
Glass or clear plastic tubing, 1-in (2.5 cm) diameter, 28-in. (70 cm) length
PVC cement (glue)
Measuring tape

1. On the inside of the PVC end cap, draw a secchi disk using the waterproof marking pen by splitting the PVC circle into 4 equal quadrants using the marking pen (Catherman, 2006) as illustrated in Figure 1G.1.4.

2. Close one end of glass or plastic tube with the PVC end cap and cement in place.

   This end should be leakproof.

3. With the marking pen and measuring tape, mark the levels of turbidity, as illustrated in Figure 1G.1.4 (Rau, 2003).

OPTIONS FOR PRETREATING SOURCE WATER

Highly turbid water (>50 NTU) will require pre-filtration procedures to ensure the best quality of water possible. If the raw water source exceeds the previously stated design parameter for turbidity (50 NTU), the filter will clog up rapidly and may produce filtrate (outlet water) exceeding the intended filter performance values for turbidity (1 NTU), see Anticipated Results.

BASIC PROTOCOL 2

Alleviating High Turbidity

A particular challenge for most household-based water treatment technologies is high turbidity, i.e., >150 NTU. The efficiency of the filter is limited by the turbidity of the water source, especially during monsoon season where performance of the filter will be compromised. You can make a pretreatment filter out of old sari cloth, linen, or other fabrics.

Materials

Collected water
Filter container
Sari cloth or other fabrics

1. Let collected water settle (sedimentation) in the container so that solids sink to the bottom

   In some situations, this may be all that is necessary, especially if water is allowed to sit overnight. If so, settled water can be poured directly in the filter. If not, proceed with fabric straining.

2. Remove the lid from the filter container.

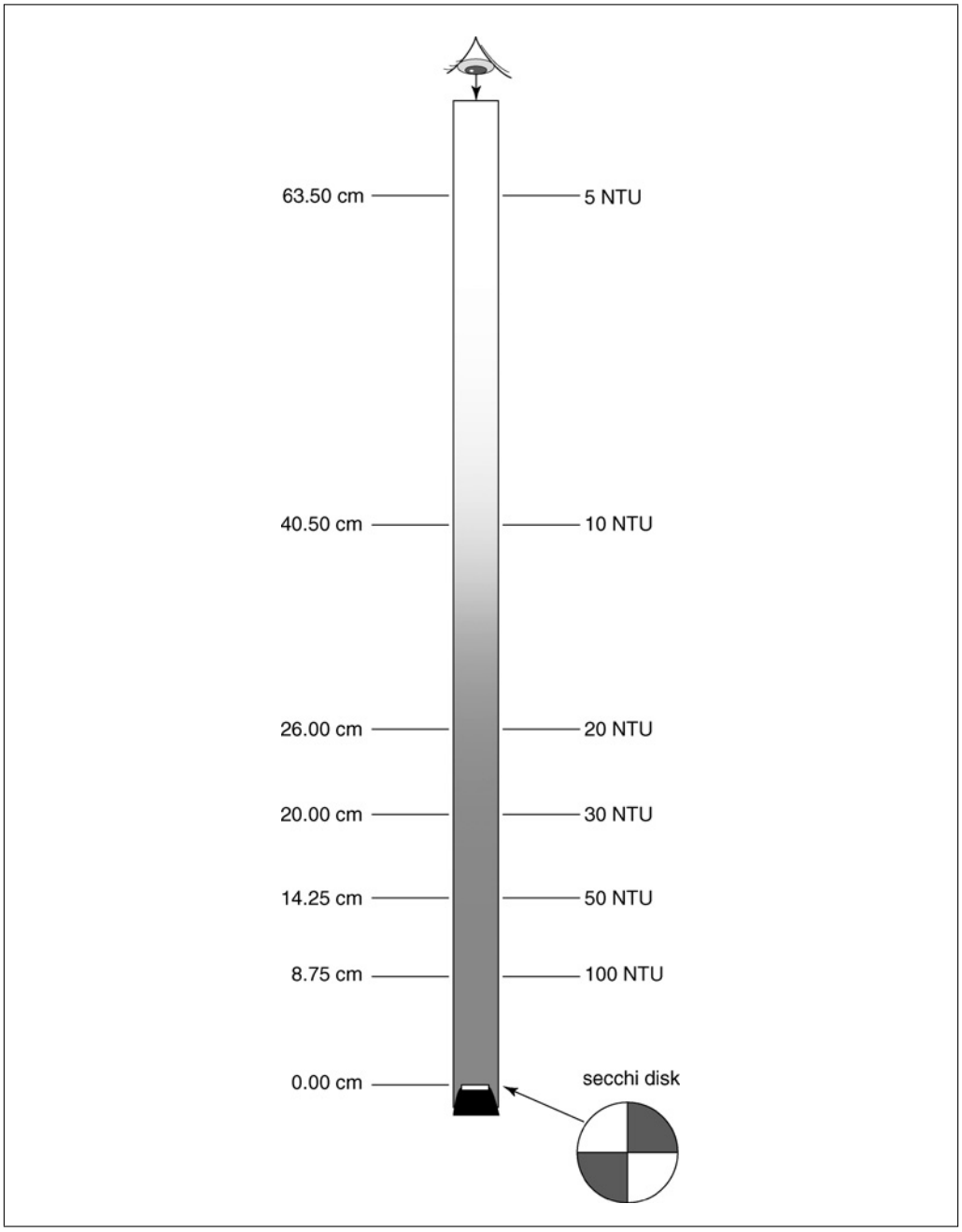

Figure 1G.1.4 Diagram of a home-brew turbidity gauge.

3. Fold the old sari cloth or other fabric four times and tie it over the mouth of the filter container.

   Old cloth is more effective because the worn fibers make the pores smaller and better for filtering.

4. Slowly pour the high turbidity water through the cloth and into the filter.

5. When finished pouring the untreated water through the cloth, remove by untying the folded cloth from the filter, wash the cloth, and expose to sunlight to dry.

   Alternatively, in many countries, there are also indigenous coagulation solutions using native plants, such as moringa seeds, known as drumstick or horseradish tree in India, or benzolive tree in Haiti and the Dominican Republic, or malunggay in the Philippines.

BASIC PROTOCOL 3

Adapting the Biosand Filter for Arsenic Removal

Arsenic is a natural element found in groundwater and is an important public health concern; fecal-contaminated drinking water poses immediate risks to human health for the majority. In countries such as Bangladesh, India, Vietnam, and many others, biosand technology can be modified to reduce both waterborne pathogens and certain toxic chemicals, such as arsenic, to acceptable concentrations with very little additional cost and a simple modification.

The simple modification of adding nails to a sand biofilter affects arsenic removal, as illustrated in Figure 1G.1.5. The nails, when exposed to air and water, rust very quickly, producing iron oxide (common red rust) which is an excellent adsorbent for effectively filtering out arsenic contaminants. The arsenic-loaded iron particles are flushed through the diffuser plate and trapped on top of the fine sand. The purpose of the stones and brick chips is to disperse the water over the nails to promote further absorption.

This ingenious arsenic depletion solution was developed by researchers at Massachusetts Institute of Technology (MIT), Environment and Public Health Organization (ENPHO)

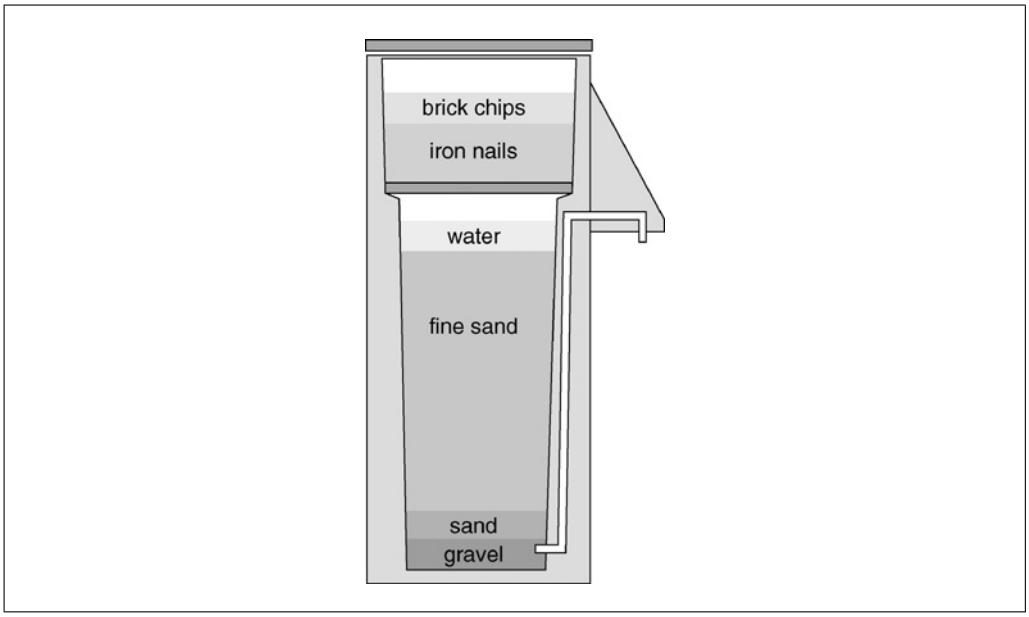

Figure 1G.1.5 Arsenic filter—a simple adaptation for removing both pathogens and arsenic based on biosand remediation and iron hydroxide adsorption principles.

of Nepal, and Rural Water Supply and Sanitation Support Programme (RWSSSP) of Nepal, based on slow sand filtration and iron hydroxide adsorption principles.

Anticipated results for the modified biosand filter are best summarized in the Centre for Affordable Water and Sanitation Technology (CAWST) document entitled: A complete summary of field and laboratory testing for the biosand filter is available for download at the following link: *http://www.cawst.org/assets/File/BSF_Literature_Brief.pdf.*

Materials

Water (from the best source possible)
11 lb (5 kg) of nongalvanized iron nails, length $<^3/_4$ in. (20 mm)
Filter containing diffuser box (see Strategic Planning)
Small broken brick chips or stones, 2- to 4-in. (5 to 10 cm) diameter

1. Wash the nongalvanized iron nails.
2. Place all nongalvanized iron nails in the diffuser box that should already be in place.

   It's important that the iron nails are evenly distributed and cover the diffuser.
3. Wash brick chips or stones with the best available water.
4. Add brick chips or stones on top of iron nails.

   The purpose of the brick chips is to protect the underlying nails from dispersing due to the force of incoming source water to be treated.
5. The filter is now ready to remove arsenic from source water poured into the filter.

   Over time the holes in the diffuser may clog due to exfoliated rust. If so, make the diffuser holes bigger using a $^1/_4$-in. (0.6 mm) nail.
6. Once a year, remove the iron nails, break apart, wash thoroughly, and return the nails back into the diffuser basin, covering again with the brick chips or stones.

   The nails' arsenic adsorption capacity will last years before new nails will need to be added. The hypothesis is that as the iron nails get rusted, the rusted iron particles become exfoliated and fall into the fine sand layer. This exfoliation exposes new iron surface, allowing more arsenic to be adsorbed. Annual washing of the iron nails can help to expose additional iron surface for improved adsorption capacity (Ngai et al., 2006).

CONSTRUCTING THE SAND BIOFILTER

Preparing Media for Use in a Biosand Filter

BASIC PROTOCOL 4

The following protocol describes the steps in preparing sand and gravel for use in a sand biofilter. As the first step, mixed sand and gravel must be separated into its different-sized grain sizes by passing through a series of constructed sieves. *The rate of filtration is influenced by grain size, so this is an important protocol for the operation of a biofilter.*

Next the sand and gravel will be washed to remove fine silt, clay, and other impurities that the media may contain.

Refer to Strategic Planning for factors important in selecting a source of sand and gravel. As a last resort, if biologically contaminated water has been used to wash the sand and gravel, place the media in the sun to dry—the solar radiation will inactivate any possible attached pathogens. If this is not possible, remember that pathogens within the water or attached to sand grains will be consumed by the filter's normal biological processes or, when exposed to an anaerobic environment within the filter, will not survive.

Materials

Mixed sand and gravel
Water (from the best source possible)

Materials for building three wooden sieves including:
- 1-in. × 4-in. (2.5 cm × 10.00 cm) lumber to construct three sieves, three 8 foot (2.4 m) lengths
- 1-in. × 1-in. (2.5 cm × 2.5 cm) wood strapping, three 8 foot (2.4 m) lengths
- $\frac{1}{2}$-in. (12 mm) screen, $\frac{1}{4}$-in. (6 mm) screen, and $\frac{1}{16}$-in. (~1.5 mm) mosquito screen, screens must be metal, not nylon or fiberglass
- Tape measure
- Hammer
- Nails
- Saw

Shovels
Tarp
5-gallon (20 liter) buckets

Sieve the mixed sand and gravel media

1. If sand and gravel is wet, dry in sun.

   The solar radiation will inactivate many possible attached pathogens. Also, sieving is a lot easier if the media is dry.

2. Construct three sieves, using the lumber and the $\frac{1}{2}$-in. (12 mm), $\frac{1}{4}$-in. (6 mm) screen, and the $\frac{1}{16}$-in. (~1.5 mm) mosquito screen (see Fig. 1G.1.6).

   The suggested size is ~16-in. (40 cm) × 22-in. (56 cm) for the three sieves. For the mosquito screen it is necessary to add a piece of $\frac{1}{2}$-in. (12 mm) screen under the finer screen for additional support. The 1-in. × 1-in. (2.5 cm × 2.5 cm) strapping is measured and cut to the same lengths as sieve frames. The strapping will be used to cover the screen edges where the screens were nailed to the frames.

3. Using a shovel, pass the mixed sand and gravel through the constructed $\frac{1}{2}$-in. (12 mm) sieve. Discard the media that doesn't pass through.

   For a simplified understanding of size grading that is going to take place using the screens, please reference Figure 1G.1.7.

4. Using a shovel, pass the mixed sand and gravel that passed through the $\frac{1}{2}$-in. (12 mm) sieve through the $\frac{1}{4}$-in. (6 mm) sieve. Keep the media that doesn't pass through the $\frac{1}{4}$-in. (6 mm) sieve.

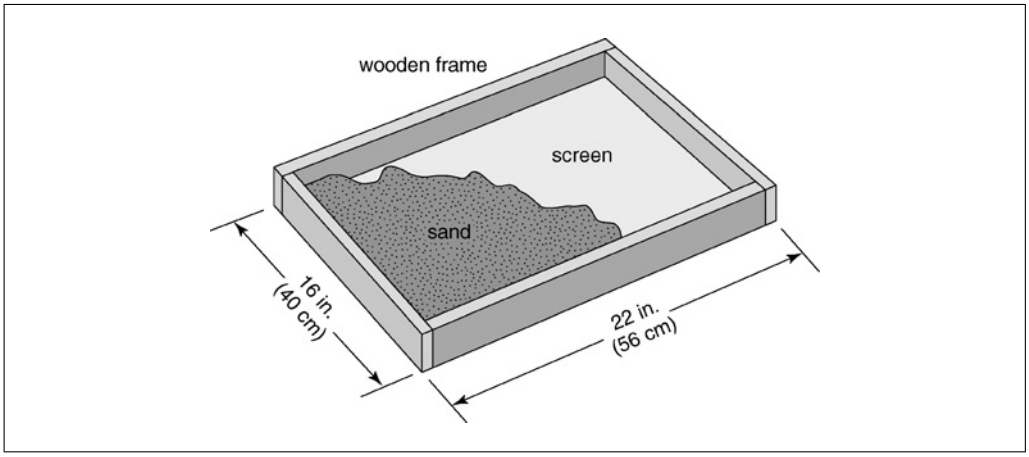

Figure 1G.1.6 Diagram of constructed wooden sieve.

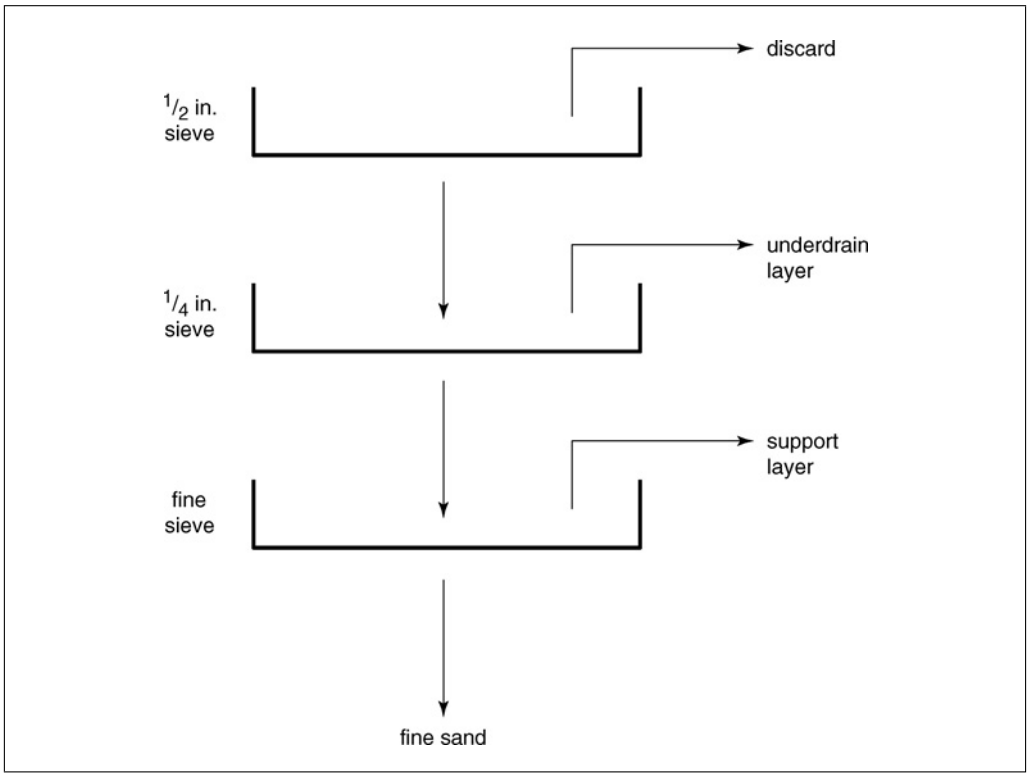

Figure 1G.1.7 Steps needed to sieve the three different grades of media.

The underdrain (gravel) media layer is composed of gravel that passed through the ½-in. (12 mm) sieve but was held back by the ¼-in. (6 mm) sieve. The purpose of this media layer is to allow the treated (effluent) water unrestricted flow out of the filter container and into the standpipe.

5. Using a shovel, pass the mixed sand and gravel that passed through the ¼-in. (6 mm) sieve through the mosquito sieve. Keep the media that doesn't pass through the mosquito sieve (fine gravel), as well as the sand that passed through the mosquito sieve.

   The support (fine gravel) media layer is composed of sand particles that passed through the ¼-in. (6mm) sieve but were held back by the mosquito screen sieve. The purpose of this media layer is to prevent the fine filtering sand from mixing with the underdrain layer.

   The filtering media layer is composed of fine sand that has been sieved through the fly or mosquito mesh. It is this layer that is responsible for removal of pathogens and the establishment of the biological zone, including the schmutzdecke. Please note that it takes substantial sieving to reach the required volume of fine sand as this is the deepest layer of the filter.

6. Store the sand and gravel in a protected dry area away from possible human or animal contamination.

7. Cover the sieved sand and gravel with the tarp until needed.

Wash the underdrain gravel

8. Place a small amount (½-in., or 12 mm) underdrain gravel in a bucket.

9. Place double the amount of water in the same bucket.

10. Using your hand, swirl the underdrain gravel a few times around the bucket.

11. Pour the dirty water out of the bucket. To help conserve water, pour into a waste water container and allow the water to settle for reuse.

12. Repeat steps 8 to 11 until the water in the bucket remains clear and contains no fine particles.
13. Clean the remaining underdrain gravel using steps 8 to 12.

Wash the support gravel

14. Place a small amount of $^1/_4$-in. (6 mm) middle support gravel in a bucket.
15. Place double the amount of water in the same bucket.
16. Using your hand, swirl the middle support gravel a few times around the bucket.
17. Pour the dirty water out of the bucket. To help conserve water pour into a waste water container and allow the water to settle for reuse.
18. Repeat steps 14 to 17 until the water in the bucket remains clear and contains no fine particles.
19. Clean the remaining middle support gravel using steps 14 to 18.

Wash the upper filtering sand

20. Now place an even smaller amount of upper filtering sand in a bucket.
21. Place double the amount of water in the same bucket.
22. Using your hand, swirl the upper filtering sand ~10 times around the bucket.

    This time, do not wash the fine sand until the water is clear; the residual water should be mildly dirty in the bucket.

23. Quickly decant the mildly dirty water out of the bucket.

    As you wash, count the number of times that you decant the bucket. It takes time and practice with the following flow rate test (see Basic Protocol 5) to determine how much the fine sand has to be washed.

24. Repeat steps 20 to 23 until all the remaining fine sand has been washed.

    Once you have initially determined how much the fine sand has to be washed, this will be used as a guideline for future media washes. The filtering sand is washed to ensure an effective size (ES) of 0.10 to 0.25 mm and uniformity coefficient (UC) of 1.5 to 2.5 (CAWST, 2008).

Dry the media

25. If biologically contaminated water has been used to wash the sand and gravel, place the media in the sun to dry.

    The solar radiation will inactivate many possible attached pathogens. If this is not possible, remember that pathogens within the water or attached to sand grains will be consumed by the filter's normal biological processes or when exposed to an anaerobic environment within the filter and will not survive.

26. Once dried, store the sand and gravel in a protected dry area away from possible human or animal contamination. Cover the sieved sand and gravel with the tarp until needed.

BASIC PROTOCOL 5

Installing the Media

Incorrect sand media installation can result in flow rates that are too low or too high, with subsequent problems developing.

Materials

Water
Household chlorine bleach solution (see Basic Protocol 9)

Filter (see Strategic Planning)
Materials for correct media installation including:
2 in. (5 cm), 3 quarts (~3 liters) of washed $^1/_2$-in. (12 mm) gravel
2 in. (5 cm), 3 quarts (~3 liters) of washed $^1/_4$-in. (6 mm) gravel
20 in. (50 cm), 25 quarts (~25 liters) of washed fine sand
Stick, 40 in. (100 cm) long
Measuring tape
Black magic marker pen
Diffuser plate or box (see Strategic Planning)
Materials for testing filter flow rate including:
Measured container (1 liter pop bottle is adequate)
Stop watch
Materials to disinfect the standpipe including:
3-foot (1 meter) garden hose
Hose clamps
Funnel
Bleach-soaked cloth

Place the media in the filter container

1. First, place the empty filter inside the home in an appropriate place (i.e., its final location).
2. Make sure the drain hole, standpipe, or outlet pipe is clear and unobstructed.
3. Place a stick inside the filter.
4. Using a black magic marker, draw a mark across the stick where it meets the top edge of the filter.
5. Measure and mark a second line 2 in. (5 cm) down from the first. Remove the stick.
6. Fill the filter container half full of water.

   The water doesn't need to be uncontaminated; it can be from a raw water source that eventually will be treated. Water is required to be inside the filter before adding sand media to prevent pockets of air from being trapped within the media. If air pockets do develop while placing media, this has the potential to slow the flow rate results.

7. Pour or place the lower underdrain gravel into the filter, level out the surface.
8. Place the stick back into the filter, placing the bottom of the stick on the newly placed lower underdrain gravel.

   When the second line matches up with the top edge of the filter container, enough gravel has been added.

9. Measure and mark a third line 2 in. (5 cm) down from the second line.
10. Pour or place the middle support gravel into the filter, level out the surface.
11. Place the stick back into the filter, placing the bottom of the stick on the newly placed middle support gravel.

    When the third line matches up with the top edge of the filter container, enough middle support gravel has been added.

12. Place a bucket under the standpipe.

13. Ensuring that there is always water above the sand surface, quickly pour the majority of washed upper filtering sand into the filter. Continue adding sand until water starts pouring out of the standpipe.

    A random distribution of different sand grain sizes is critical to the proper operation of the filter. Adding sand quickly maintains the random distribution by not allowing the different sizes of grains to settle into layers.

14. Wait until the water stops pouring out of the standpipe.

    When the water stops pouring out of the spout, the water level is equalized.

    The water level in the filter is determined by the spout. Due to a siphoning effect, the water will stop coming out of the filter when the water is at the same level as the bottom of the spout.

15. Level out the surface of the upper filtering sand.

16. Measure the depth of the water above the upper filtering sand.

17. If the water depth is <2 in. (5 cm), remove sand until the water depth equals 2 in. (5 cm).

18. If the water depth is >2 in. (5 cm), continue to add washed upper filter sand into the filter until the water depth is 2 in. (5 cm).

19. When the water depth equals 2 in. (5 cm), once again level out the surface of the sand.

    This 2 in. (5 cm) of standing water will be the supernatant. Any changes in the water depth above the sand surface will cause a change in the biological zone disrupting the efficiency of the filter. A water depth of >5 cm results in lower oxygen diffusion and consequently a thinner biological zone. A high water level can be caused by a blocked outlet spout or by an insufficient amount of sand media. As the water depth increases, the oxidation and metabolism of the microorganisms within the biological zone decrease. Eventually the layer dies off and the filter becomes ineffective.

Flushing the filter

When all three layers of media have been installed, and the supernatant water depth equals 2 in. (5 cm) perform the following steps.

20. Place the diffuser plate into the filter container.

    The diffuser plate must be above but not touching the surface of the water at its resting level. That would greatly reduce the amount of oxygen in the standing water layer, affecting the survival of the schmutzdecke.

21. Place a waste water container (bucket) under the outlet pipe or standpipe.

    The waste water captured can be reused.

22. Start pouring the cleanest water available into the filter (<50 NTU), continue pouring water until the water starts running clear out of the outlet or standpipe.

    This may take 10 to 20 gallons (40 to 80 liters).

    If the water doesn't run clear after 25 gallons (100 liters), the gravel or sand was too dirty to start with. It is probably easiest to take the media out, wash in pails, and then place back in.

Testing filter flow rate

The amount of water that flows through the biosand filter is controlled by the size of sand media contained within the filter. If the rate is too fast, the efficiency of bacterial removal may be reduced. If the flow rate is too slow, there will be an insufficient amount of treated water available from the filter to meet the needs of the users. The flow rates of

a biofilter are found from measuring the time it takes to fill up a container of a known volume with water.

23. Place the measured 1-liter container under the outlet or drainpipe spout.

24. Fill the filter container completely to the top with water.

    The flow rate through the filter decreases as the height of the water added into the influent reservoir drops.

25. With the stopwatch, time how long it takes the 1 liter container to be filled completely with filtered water—ideally, it should be 0.6 liter per min (or 100 sec per liter).

    Experimentation may be necessary to achieve the desired flow rate.

    If the flow takes a longer time (>100 sec) to fill the 1 liter container, the flow rate is too slow. The filter is still functional, but it will require more maintenance than normally required due to frequent clogging. Since flow rates are controlled by the screening and washing of sand, the sand should be washed more to achieve the desired flow rate. The slow flow rate can sometimes be improved by disturbing the upper sand layer with your fingers and scooping out the dirty water. If this doesn't work, scoop out a few inches of upper filtering sand which contains too many fine particles and rewash, and replace back into the filter.

    If the flow takes a shorter time (<100 sec), the flow rate is too fast, and the efficiency of treatment will be compromised. Since flow rates are controlled by the screening and washing of sand, the sand should be washed less to achieve a slower flow rate. In this instance, the fine media should be taken out and replaced with finer media (less washed).

    It can take up to 45 min for the 5 gallons (20 liters) of poured water to completely pass through the filter. The flow rate of 0.6 liters per min is based on the top reservoir being full of water. The actual flow of water will drop off as the water level (hydraulic head) drops.

Disinfecting the standpipe

The following steps are required to disinfect the underdrain gravel and standpipe of any possible contamination. This procedure is only to be implemented during the initial commissioning of the filter.

26. Fit and clamp the garden hose over the spout.

27. Fit the funnel at the free end of the garden hose.

28. Hold the funnel higher than the spout, and pour ~1 quart (1 liter) of bleach solution into the funnel.

    The bleach solution equals 3 drops per quart (liter)—see Basic Protocol 9.

29. Hold the funnel higher than the spout for 2 min.

30. Remove the garden hose from the biofilter and drain the bleach solution from the hose.

31. Wipe the spout with a bleach-soaked cloth.

32. Add 5 gallons (~20 liters) of water to the top of the filter.

    Never pour chlorine bleach solution into the top of the filter as chlorine will kill important, purifying organisms.

33. Wait 30 min for the bleach to be flushed out.

    The flushed water containing chlorine is not suitable for drinking or cooking.

34. Place the lid on the filter.

OPERATING THE BIOSAND FILTER

Educate all of the filter users, including children on the correct operation for daily use of the filter.

Materials

Raw (untreated) water
Biosand filter including a diffuser plate (see Strategic Planning)
Buckets

1. Use a dedicated "dirty" bucket for fetching raw source water.
2. Place a dedicated "safe" storage container under the spout.

   Place the container as close to the spout as possible to reduce dripping noise and prevent recontamination.

3. Remove the lid on the filter.
4. Make sure the diffuser plate is installed.

   The diffuser must always be in place when pouring water into the filter never pour water directly onto the sand layer.

5. Slowly pour raw (untreated) water into the filter daily, at least 5 gallons (20 liters), twice per day to assist in the establishment of the biolayer [first 14 to 21 days (may take up to 30 days)].

   Based on the recommended flow rate of 0.6 liters per min and the time required for pause periods, the biosand filter can effectively treat 15 to 20 gallons (60 to 80 liters) per day (CAWST, 2008).

   Thereafter, the family can establish a rate of usage that fulfills the family's daily water needs. For example, the filter can be used in the morning, noon, and evening with 6 hr pause periods in between each 5 gallon (20 liter) raw source water input.

   The pause periods are very important because they allow time for the microorganisms in the biological layer to consume the pathogens contained in the water, thereby increasing the hydraulic conductivity of the filter. Consequently, the biosand filter is most effective and efficient when operated intermittently.

   Use the best source of water (least contaminated) available—the better the raw water, the better the treated water will be. Using the same source of water every day will establish a constant biolayer and improve the filter's effectiveness.

6. Replace the filter lid onto the filter.

   Water must be allowed to flow freely out of the standpipe—never plug the spout or connect a hose to end of spout. Doing so will alter the standing water level in the filter, thus potentially harming the biolayer.

   During the first few weeks while the schmutzdecke (biolayer) is being established, the filtrate water can be used if a safer water source is not available, but chlorination is recommended (see Basic Protocol 9) at least during this time.

MAINTAINING THE SAND BIOFILTER

Once the household biological filter has been installed and is operational, periodically the following two primary maintenance protocols will be required. These are periodical disinfection of filter container and cleaning the biolayer when the flow rate is insufficient.

Periodic Disinfection (Cleaning) of Filter Container

BASIC PROTOCOL 7

Clean the spout regularly (every day) with soap and clean water or a chlorine cleaning solution. Regular cleaning of the filter spout will be required due to exposure to insects, animals, and dirty hands of children and other family members.

Clean the entire outside surface of the filter regularly (only when dirty) with soap and water or a chlorine cleaning solution.

IMPORTANT NOTE: Never pour chlorine cleaning solution into the top of the filter.

Recovering the Flow Rate

BASIC PROTOCOL 8

Over time the flow rate of the filter will decrease with usage because of increased accumulation of inorganic and organic material on the filter bed surface. This is a naturally occurring process and recovery of the flow rate can easily be restored with the following simple cleaning protocol.

Better water quality is actually produced at a reduced flow rate due to increased contact time with the ripened biological zone. Thus, this cleaning procedure should only be undertaken when the flow rate has become inconvenient to the family's daily needs.

Materials

Soap
Water
BioSand filter with a diffuser plate (see Strategic Planning)

1. Remove the lid, making sure that the diffuser plate is in place.
2. Pour enough water into the influent reservoir to the half-full mark, then remove the diffuser plate.
3. Gently disturb the surface of the upper filtering sand layer with fingers, ever mindful not to disturb the surface any deeper than 1 cm.
4. Remove suspended turbid water.

   The water in the influent reservoir will now be holding inorganic and organic materials in suspension that can be easily removed with a small container or cup.
5. Discard the turbid contaminated water in an appropriate location.
6. Return the diffuser plate and lid onto the filter.
7. Pour one 5-gallon (20 liter) bucket filled with water.
8. If the recovery of the flow rate is unsuccessful upon first attempt, repeat steps 1 to 7 as many times as necessary to regain the desired flow rate.

   Be mindful that after disturbing the surface, the removal efficiency declines somewhat, but increases very quickly (may take up to 1 week) to its previous level as the biofilm is re-established.
9. Wash hands with soap.

DISINFECTING EFFLUENT WATER

It is recommended that a disinfection process such as chlorine addition or solar disinfection (SODIS) be used in conjunction with the biosand filter as a post barrier.

There are limitations to SODIS and chlorine disinfection. SODIS limitation is the need to have numerous bottles for each household. Chlorination is ineffective against pathogens

such as the zoonotic (referring to pathogens of animal origin that also infect humans) protozoan Cryptosporidium. Furthermore, both SODIS and chlorination disinfection are ineffective with higher turbidity in the water. A benefit of the SODIS method is that it's relatively inexpensive. Alternatively, chlorination requires the household to incur additional costs. The following techniques can be used for effluent disinfection. If the disinfection protocols are done properly, the filtered water will be of the highest quality. Investigators will have to determine the most appropriate method consistent with their purpose and needs and with the availability of resources.

BASIC PROTOCOL 9

Bleach (Free Chlorine) Disinfection of Effluent Water

Residual "free chlorine" disinfection is used to provide a safeguard against recontamination of treated water within the safe storage container. Household bleach is the most common affordable form of free chlorine. The following protocol will demonstrate how to disinfect water with household bleach.

Materials

Household bleach
Clean water
Clean empty container

1. Add 1 cup (240 ml) of household bleach to a clean empty container.

   Household bleach can contain different amounts of chlorine, 3.5% and 5% being the most common. To get the correct measure of chlorine needed to disinfect water, a 1% (final) stock solution is prepared (Conant, 2005).

2. Fill the container with the clean water.
3. Cover the top and shake for ~30 sec.
4. Let the container sit for 30 min. The stock solution is now ready.

   Add the following amounts of stock solution to the water that has been filtered through the sand biofilter:

   3 drops stock solution to 1 quart (~1 liter)
   12 drops to 1 gallon (4 liters)
   1 teaspoon (5 ml) to 5 gallons (20 liters)

   It is important to add enough stock solution to not only kill pathogens in the water, but, to have some chlorine still available in the water as a last barrier to ensure water quality until the time of consumption. The excess chlorine is called "free chlorine" and can easily be identified by the slight chlorine smell and taste of the disinfected water which is still safe to consume.

   If the filtered water is cloudy, twice as much of the stock solution will be required.

   Highlighting an additional benefit of biosand intervention—filtration reduces organic carbon in the water; thus, the filters also reduce the formation of chemical by-products. WHO states that "... the risks to health from these by-products are extremely small," posing no significant health risk.

   Instead, post-chlorine disinfection has to be recognized as potentially being a deterrent towards household acceptance due to lack of product accessibility or the unpleasant odor, taste, and the additional costs that will need to be incurred by the household.

Solar Disinfection (SODIS) of Effluent Water

SODIS is a low-cost, effective way to improve the microbiological quality of drinking water using solar radiation (sunlight) to destroy pathogenic microorganisms. The mechanism of disinfection is heat plus UV-A (wavelength 320 to 400 nm) radiation.

Recent laboratory and field experiments indicate that a number of low-cost additives are capable of accelerating the SODIS process in both sunny and cloudy weather. These additives included 100 to 1000 mM hydrogen peroxide (both at room temperature and at elevated temperatures), 0.5% to 1% lemon or lime juice, and copper metal or aqueous copper plus ascorbate (with or without hydrogen peroxide; Fisher et al., 2008).

Materials

Biosand filtered water
1- to 2-liter clear bottles (e.g., soda bottles)

1. Obtain 1- to 2-liter clear bottle(s).

   1- to 2-liter soda bottles have a better surface/volume ratio.

2. Wash the bottles well if it's the first time using the bottles.

3. Fill one or many 1- to 2-liter clear plastic bottles ¾ full with biosand filtered water.

   Over time replace old or scratched bottles. Do not use green- or brown-colored plastic bottles—they do not transmit UV-A radiation. Use transparent bottles only.

4. Shake the bottle(s) for 20 sec.

   Shaking adds air bubbles to the water, which will assist in disinfecting the water faster.

5. Lay the bottle(s) horizontally in the sun.

   Pick a location where the bottles will not be disturbed by shade, people, or animals.

6. Leave the bottle(s) in the sun for at least 6 hr; 2 days if the sky is cloudy. If the water temperatures rises >50°C, the disinfection process is 3× faster.

7. Consume the water.

   The UV radiation intensity is reduced by turbidity and water depth in the containers. Water should have a low turbidity (<30 NTU) and the water depth in containers should not exceed 10 cm (EAWAG, undated).

   Solar disinfection is most effective in countries close to the equator.

   Alternatively, in many countries, using UV lamp disinfection may be another option where a source of electricity is available (see Internet Resources).

COMMENTARY

Background Information

Household-sized biological filters are simple in design; what is surprising are the complex purification processes that combine and take place inside the filter to provide water treatment. The principle processes are sedimentation, mechanical straining (filtering), adsorption (electrostatic), and most interestingly, a biological process (NSFC, 1997).

Biological filtration

The bioremediation process starts when raw turbid water is poured onto the filter's porous sand bed. Suspended particles and organic colloidal (viscous, gelatinizing) substances are deposited and absorbed (Ellms, 1928) within the top 400 mm of sand (Muhammad et al., 1996). The more organic matter (e.g., algae, diatoms, bacteria, protozoa, and worms) contained in the raw water, the quicker the sand grains become gelatinously coated, in turn decreasing the pore size between sand grains.

As the jelly-like density gradually increases over a 2- to 3-week period, the greatest density forms on top [2 to 4 in. (5 to 10 cm)] of

the sand layer. The filter matures or "ripens" with the creation of a surface zoogleal film (schmutzdecke) at the $\frac{1}{2}$-in. (1.5 cm) sand-water interface, while the bottom level of the media is a particularly hostile environment starved of oxygen, nutrients, and temperature required to sustain life for bacteria such as intestinal bacteria (i.e., *Escherichia coli*) and other disease-causing pathogens.

The fundamental importance of the self-purifying power of the biological ecosystem is illustrated by the living microorganisms naturally contained within the biological zone. Algae and diatoms use photosynthesis to take in carbon dioxide and release oxygen which becomes available for oxidizing organic particles and for beneficial bacterial predation activities (Ellms, 1928). In turn, protozoa and worms feed on pathogenic bacteria and other disease-causing pathogens.

A filter's microbiological ecosystem is aerobic and requires oxygen to thrive (Buzanis, 1995). The increased presence of dead cell material further decreases the pore sizes which increases filtration efficiency, but, also requires additional oxygen to thwart the possibility of anaerobic conditions (Smethurst, 1992). Depending on increased oxygen solely through photosynthesis is limited by: (1) the turbidity (cloudiness) of the filtrate water, and; (2) the secondary function of the filter lid, which is to inhibit clogging (maintenance) caused by possible exponential schmutzdecke growth by the naturally occurring photosynthesis process. Instead, the additional critical oxygen is acquired through oxygen diffusion between the influent reservoir and the intermittently refreshed standing water (supernatant) layer. The finely balanced equilibrium between consistent oxygen and nutrient influx and microorganisms' metamorphosis results in a living system providing extremely efficient pathogen (disease-causing organisms) removal from filtrate water.

Emerging biosand technology

The HydraAid Filter—International Aid recently introduced a plastic biosand water filter (Fig. 1G.1.8). The HydraAid Filter will provide clean, safe drinking water at the rapid rate of up to 12 gallons (48 liters) per hr.

The JAL Filter—Mr. Brett Gresham created a unique biofiltration pathogen removal design (Fig. 1G.1.9) while serving in Afghanistan during the early 1990s. This filter produces the same controlled environment based on biosand principles using just one layer of fine sand, and no standpipe is required.

Importance of bioremediation dissemination

Biosand filters have gained acceptance by the World Health Organization (WHO) as a viable household water treatment (HWT) technology. The WHO is presently "developing guidelines that will establish microbial reduction benchmarks and propose minimum criteria for protocols to verify HWT system performance (WHO, 2007)." In the meantime, "There is now conclusive evidence that simple, acceptable, low-cost interventions at the household and community level are capable of dramatically improving the microbial quality of household stored water" and will reduce the attendant risks of "diarrheal and other enteric diseases by 6 to 50% (Sobsey, 2002)." "A preliminary health impact study (to be published in 2008) estimates a 30% to 40% reduction in diarrhea (CAWST, 2008)" within at-risk populations. The real challenge now is to facilitate the rapid dissemination of this proven bioremediation technology to developing countries.

Critical Parameters

Multiple indicator tools required

"Between 1972 and 1999, 35 new agents of disease were discovered (WHO, 2003)." Waterborne pathogens of importance, such as *E. coli* O157:H7 and Cryptosporidium are amongst this group. A single traditional microbial indicator (i.e., *Escherichia coli*) primarily used for water quality monitoring is inadequate to determine Cryptosporidium cysts, Giardia, and additional emerging water-related diseases of unknown etiology. There is a need for accurate, low-cost multiple indicator tools that can be applied in remote areas of developing countries to extend the multiple barrier approach to the potential risks of emerging water-related microbial pathogens.

Further research

Two groups amongst the major causes of diarrhea worldwide and a significant cause of mortality amongst children are small round-structured waterborne viruses (caliciviruses) and rotaviruses. Presently, there is concern that biosand filters may have a low rate of virus inactivation, therefore further research is required.

Affordability

Variations in regional conditions and availability of local materials contribute to the variability of trying to provide construction cost estimates which are beyond the scope of the provided protocols. As a general guideline, concrete biosand water filters range from $12

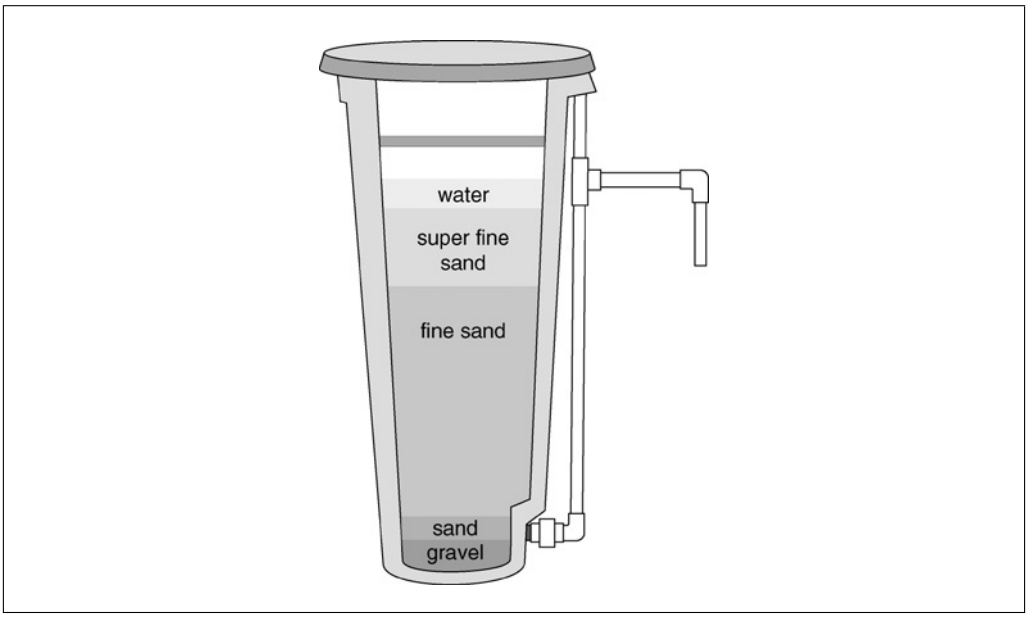

Figure 1G.1.8 International Aid's plastic HydraAid (BioSand) filter—lighter weight, stores and filters up to 47 liters (15 gallons) per hour.

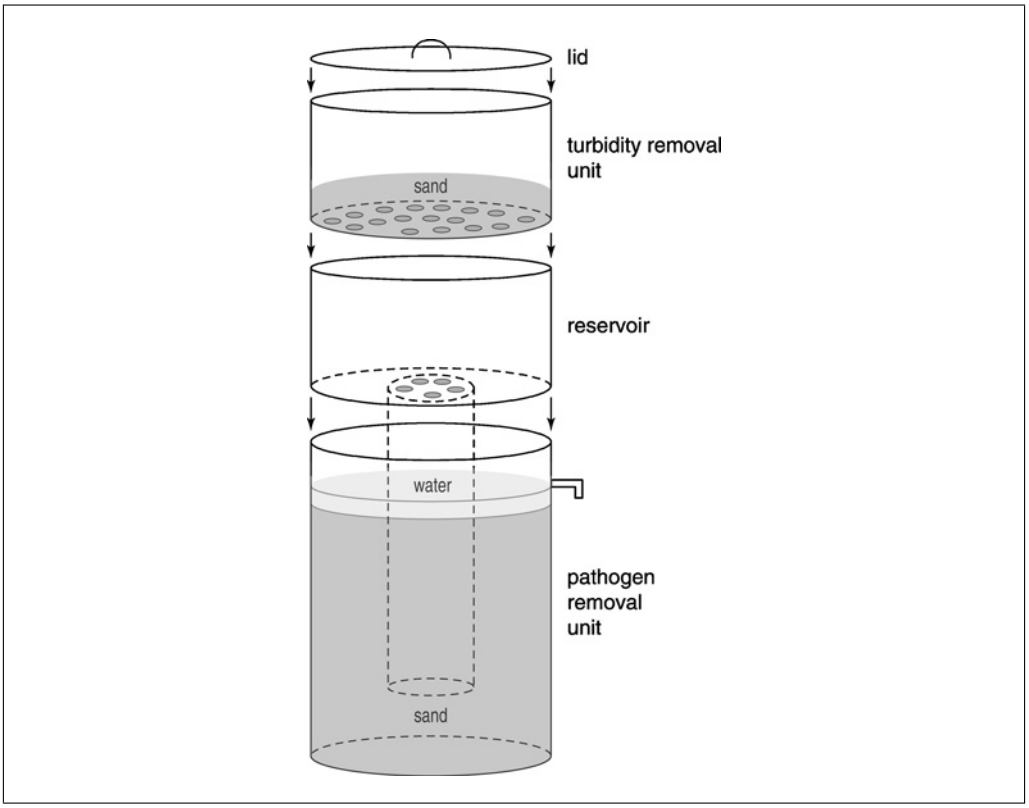

Figure 1G.1.9 Schematic diagram of the JAL filter. No standpipe and only one layer of sand required; lightweight and inexpensive to build.

to $30 US. A multibarrier approach as described increases costs and may limit use by some of the world's poorest people.

Drawbacks

There is no one-size-fits-all biosand filter. Each has its own drawbacks, for example, concrete-made filters require a welding shop to construct a steel mold. Plastic drums of consistent size are hard to locate. Metal filters require a tin smith. Ceramic containers tend to break easily. Industrial produced plastic filters may experience challenging logistics.

Table 1G.1.1 Troubleshooting Guide for Biofilter Implementation

Problems	Solutions
Preparing sand and gravel media	
Unsieved material was mixed with pile of sieved media	Prepare the media by re-sieving mixed pile
Uncertain whether the sand has been washed clean	Place a small amount of sand, and stir in a clear glass of water, allow to settle—if you can't see the sand surface within 3-5 sec, the sand needs to be washed further
Suspicion that dissolved salts exist in filtered water	Ensure that media is not beach sand
Uncertain of gauge size for sieving screens.	Ensure that screen sizes are 2, 4, and 14 gauge
Assembling the biofilter	
The diffuser plate or box floats when water is poured into the filter	Placing a rock or other weight on top of diffuser will stop it from floating
Metal diffuser plate or box is rusting	Ensure that sheet metal is of good quality galvanizing
Uncertain whether to build diffuser plate or box	A diffuser box is will be required for arsenic treatment
Using the biofilter	
Water isn't running clear after filter flush	Ensure that media is taken out of filter and rewashed
The diffuser plate is touching the standing water during pause period	Ensure that the diffuser plate is positioned at least 2 in. (5 cm) above the standing water level
Experiencing problems with air pockets in the filter media	Re-installation of sand and gravel will be necessary. The schmutzdecke and biological zone will need to be re-established with a ripening time of 14 to 21 days.
The top layer of sand is uneven	Ensure that the diffuser plate fits snuggly against the container walls
The flow rate slows down when water in the influent reservoir drops	This is a normal occurrence due to drop in hydraulic head
The flow rate is too low	Most likely caused by top layer of sand being clogged, flow rate recovery maintenance required or check to make sure diffuser plate holes are not too small or are plugged
Chlorine bleach was accidentally poured into the top of the filter	Re-installation of sand and gravel will be necessary after being thoroughly washed and dried in the sun to ensure sand is clear of chlorine. The schmutzdecke and biological zone will need to be re-established with a ripening time of 14 to 21 days.
The standing water depth is no longer 2 in. (5 cm)	Add or remove sand. Initially the sand may settle over time and require more sand to be added.
Maintaining the filter	
Water inside the filter has drained away	Ensure that a hose hasn't been attached to spout that initiated siphon action
Noticed higher turbidity during rainy season	Ensure a turbidity pretreatment process is initiated
There is an irritating dripping noise from the spout	Ensure that the safe storage container has a small opening and is positioned as close as possible to the spout
Removal of arsenic	
The holes in the arsenic diffuser box are plugged with iron rust	Make the holes bigger (~3/16 in.)
Purchased nails for arsenic treatment seem to be oily	Ensure that nails are uncontaminated and nongalvanized

Greatest obstacle

In most countries the greatest obstacle is procuring sand and gravel to meet the media specifications for the filter, and to a lesser degree the availability of components from local suppliers (see Strategic Planning).

Troubleshooting

Table 1G.1.1 outlines some of the more common problems that may be experienced in performing the basic and supportive protocols from this unit. This is not an exhaustive list; others may be encountered.

Anticipated Results

A fully established (ripe) schmutzdecke will perform at 90% to 99% efficiency (CAWST, 2008), removing biological pathogens, but, it will take ~14 to 21 days (ripening period) of daily use to establish the bioremediation zone and the schmutzdecke (may take up to 30 days), depending on the temperature and turbidity (biological content) of the source water. Until the development of the schmutzdecke, the filter will only be performing between 30% to 70% efficiency (CAWST, 2008). The water from the filter can be used during the 14 to 21 day start-up period, but, effluent disinfection (Basic Protocols 9 and 10) is highly recommended during this time period to ensure pathogen free water quality.

Anticipated results under field conditions with fully established schmutzdecke should be: (1) *E. coli* bacteria removal of >97%; (2) protozoa and helminths removal of >99%; (3) removal of 50% to 90% of organic and inorganic toxicants; (4) removal of 90% to 95% of iron; and (5) removal of 85% to 90% arsenic with design modification known as Kanchan Arsenic Filter (see Basic Protocol 3).

Under field conditions, the biosand filter limitations are: (1) cannot remove some dissolved contaminants (e.g., hardness, salt, calcium, and magnesium); (2) cannot remove some organic chemicals (e.g., pesticides and fertilizers or color); and (3) cannot guarantee 100% pathogen-free water—it is recommended to disinfect the water after it has passed through the biosand filter (see Basic Protocols 9 and 10).

Additional laboratory and field testing results are best summarized in the Centre for Affordable Water and Sanitation Technology (CAWST) document entitled: A complete summary of field and laboratory testing for the biosand filter (see Key References).

Time Considerations

The simple design facilitates procedures in the unit to be completed in a timely manner. In a developing country context, the longest period required will be invested in procuring uncontaminated sand and gravel and in finding local suppliers for specific filter components to ensure sustainability.

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Internet Resources

http://www.who.int/household_water/network/en/

The International Network to Promote Household Water Treatment and Safe Storage.

http://manzwaterinfo.ca/

Dr. David Manz's internet-based cooperative discourse for the Biosand Water Filter.

http://www.who.int/water_sanitation_health/monitoring/jmp2005/en/index.html

Link to the report Water for life: Making it happen. This report, prepared by the WHO/UNICEF Joint Monitoring Program, makes it clear that achieving the International Decade for Action Water for Life 2005–2015 target of access to safe drinking water and basic sanitation will bring health and dignity to millions of the world's poorest people.

http://www.medrix.org/water.html#UVWaterTreatment

Medical Education and Development of Resources through International Exchange (MEDRIX)—provides a UV lamp water treatment (with optional sand filter) system handbook available upon request.

http://www.biosandfilter.org

A very useful Website, which contains detailed technical information (guidelines) on how to build the metal mould, how to produce the round concrete, and provides description and drawings for plastic or metal drum biosand filter construction.

http://web.mit.edu/watsan/worldbank_summary.htm

Massachusetts Institute of Technology-Kanchan Arsenic Filter (KAF). Project promotes KAF technology.

http://www.hydraid.org/

International Aid's new plastic HydrAid BioSand Water Filter initiative.

http://www.jalmandir.com/filtration/biosand/biosand-filters.html

Clearinghouse for Low-cost Household Water Treatment Technologies—provides overview of biosand filtration technology.

http://www.jalfilter.org

Provides description, photographs, and schematics drawings for JAL filter construction.

http://www.safewaterintl.org/

Safe Water International's development initiative toward a lightweight collapsible filter container and safe storage pouch combination.

http://www.cawst.org

The Centre for Affordable Water and Sanitation Technology (CAWST) is a Canadian humanitarian organization that provides training, education, and technical consulting in water and sanitation to organizations working with the poor in developing countries.

http://www.biosandfilter.org/biosandfilter/index.php/item/229

BushProof, Biosand Filter home page. (n.d.). Source of Round Concrete and Metal Drum Biosand Filter Construction Manuals. Retrieved March 15, 2007. Presents the most comprehensive online reference material.

Proper Alignment and Adjustment of the Light Microscope

Optical microscopes can be powerful tools in biomedical research and diagnosis if properly aligned and adjusted. This is essential for optimal image quality and accurate quantitative measurements. Video cameras, digital cameras, and electronic image processing can improve visibility of structural detail resolved by the microscope optics in comparison to viewing by eye. However, the quality and accuracy of the image still depends critically on proper microscope alignment and adjustment.

This unit presents protocols for alignment and adjustment of a typical research compound light microscope for transillumination and epi-illumination imaging modes typically used today in microbiology research. The transillumination light modes include bright field, phase contrast, dark field, and differential interference contrast (DIC). The primary epi-illumination mode is fluorescence microscopy (*UNIT 2A.2*).

The described procedures are for alignment of a research upright microscope (Fig. 2A.1.1). The procedures are also applicable to inverted microscopes which have similar imaging and illumination light paths to the upright microscope. In either case, the specimen image is produced by an objective lens and the image is projected either to the eye with an eyepiece or to a camera with (and sometimes without) a projection lens. One lamp attached to the back of the microscope provides light for transillumination of the specimen through a condenser lens (Fig. 2A.1.2 and Fig. 2A.1.3). Another lamp attached to the back of the microscope provides light for epi-illumination of the specimen through the objective using a mirror in a filter cube to bring the illuminating light into the objective light path (Fig. 2A.1.5). For both the transillumination and epi-illumination paths, there are field diaphragms for controlling the specimen region illuminated and condenser diaphragms for controlling illumination from the condenser. The lamps, the field diaphragm, and the condenser diaphragm, as well as the condenser and the objective, must be properly focused and centered for the best image formation. In addition, each mode of image formation has special optical components that also require alignment and adjustment for optimal performance.

The first section in this unit (see Major Components of the Light Microscope) describes the location and basic functions of the most important features of the upright compound microscope. The second section (see Basic Imaging and Köhler Illumination Light Paths for Bright-Field, Fluorescence, and Dark-Field Microscopy) introduces the imaging and illumination light paths of the microscope, and describes the principles of Köhler illumination, which is the alignment used typically for both transillumination and epi-illumination in the light microscope. Basic Protocol 1 lists steps in microscope alignment for transmitted-light Köhler illumination. Basic Protocol 2 discusses alignment of the eyepieces. Basic Protocol 3 lists steps in microscope alignment for epifluorescence Köhler illumination. Basic Protocols 4 and 5 provide brief descriptions of the principles of image formation and microscope alignment for phase-contrast and differential interference contrast (DIC) microscopy; these optical modes produce contrast of transparent specimens using transmitted-light illumination. The section entitled Alignment for Dark-Field Microscopy provides a brief description of the principles of dark-field microscopy and details how a bright-field microscope can be easily converted for dark-field microscopy at low- (see Basic Protocol 5) or high- (see Basic Protocol 6) power magnification. Support Protocol 1 deals with mating cameras to the microscope and Support

Contributed by Edward D. Salmon, Kate von Lackum, and Julie C. Canman
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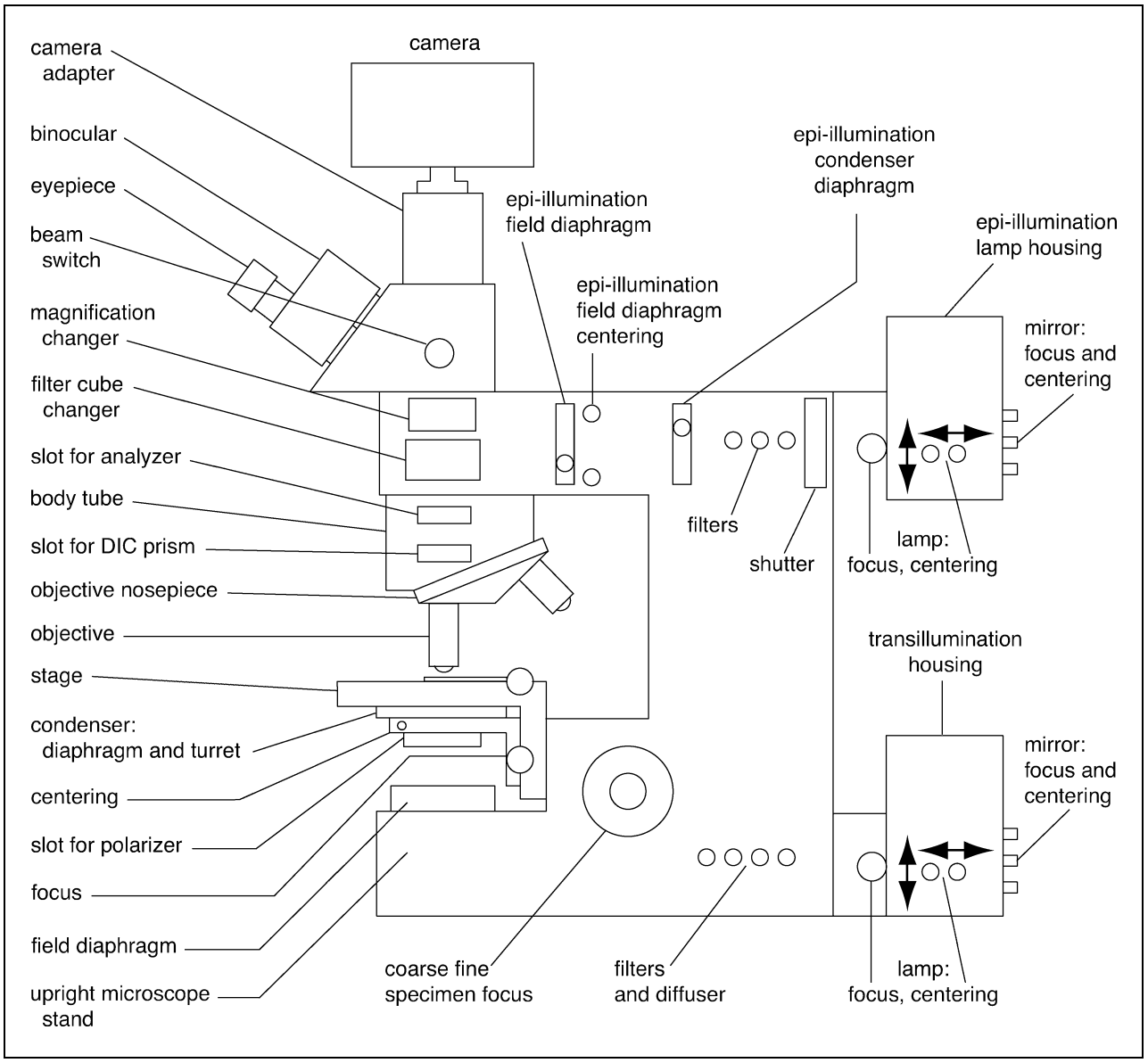

Figure 2A.1.1 Diagram of the major component parts and centering screws for a research upright light microscope.

Protocol 2 deals with calibrating image magnification. Several procedures (see Support Protocols 3, 4, and 5) are given for testing the optical performance of the microscope; these also describe test specimens for microscope performance and their sources. Finally, Support Protocol 6 deals with the care and cleaning of microscope optics. The Commentary refers the reader to references that provide more comprehensive treatments of the basic modes and methods of light microscopy as well as advanced microscope imaging methods like multiwavelength, confocal, or multiphoton imaging, and electronic imaging techniques, including video and digital microscopy.

MAJOR COMPONENTS OF THE LIGHT MICROSCOPE

Proper Alignment and Adjustment of the Light Microscope

Familiarizing oneself with the components of the light microscope is best done using the manual for the microscope as reference. Figure 2A.1.1 provides a diagram of an upright research compound light microscope equipped for both transmitted light and epifluorescence microscopy. Locate the following components on the microscope, identify their

adjustment screws (if applicable) and movements (e.g., condenser focus), and ascertain that they appear correctly mounted. In this and the following sections, *z* is a direction along the microscope axis; *x* and *y* are perpendicular directions.

Image-Forming Components

Specimen stage and focus

A mechanical carrier on the stage holds the specimen slide. Knobs control movement in the *x*–*y* direction. Look for the vernier scales that mark the *x* and *y* positions. Rotatable stages are typically used for DIC microscopy. The objective is usually fixed and the specimen is focused by moving the stage along the *z* axis using coarse- and fine-focus knobs on the microscope body. Check the scale on the fine focus; on research microscopes, this is usually is 1 μm/unit.

Objectives and revolving nosepiece

Examine the different objectives on the nosepiece. Each objective is usually labeled with the following designations: magnification (e.g., 60×); the degree of optical correction (Apochromat is better than Fluor which is better than an Achromat); the label Plan (if both the center and edges of the field are in focus); numerical aperture (NA), which measures the half-angle of the cone of light from the specimen accepted by the objective (Fig. 2A.1.3); immersion medium to be used (e.g., air, oil, water, or glycerin between the objective front lens and the specimen); coverslip thickness; optical tube length (previously 160 mm, currently infinity); and other special features like phase contrast, DIC, or long working distance. Working distance refers to the distance from the front element of the objective to the specimen, and usually decreases with increasing objective magnification and NA. Check that the objectives are mounted in series from the objective of the lowest magnification to the objective of the highest magnification. It is usually best to find the specimen or region of a specimen with a low-magnification objective, and then swing in objectives with higher magnifications and shorter working distances. The high-NA, short-working distance oil-immersion objectives often have the ability to lock up the nosepiece when changing objectives. This prevents running the front element of the objective into the mounting medium on the edges of the coverslip. Check that the nosepiece is down for imaging.

Specimen slides and coverslips

Microscope condenser lenses are usually corrected optically for 1-mm-thick glass slides. The objectives are corrected optically for coverslips with a thickness of 0.17 mm; no. 1.5 coverslips are on average this thick. The image quality decreases for thinner (e.g., no. 1) or thicker (e.g., no. 2) coverslips, particularly for non–oil immersion ("dry") objectives with high NA. For oil-immersion objectives, this problem is not critical when the refractive index of the oil and coverslip (∼1.515 to 1.52) match. Coverslips thinner than no. 1.5 are often used with oil immersion, to allow a greater range of focus.

Body tube

In the modern research light microscope, the imaging light leaving the objective and nosepiece is unfocused and the image is projected to "infinity" (Fig. 2A.1.2, left). This infinity space above the objective allows insertion of filters without changing the focal position of the image at the intermediate image plane. A positive focusing lens, the tube lens, above the infinity space, is used to bring the imaging light into focus for the eyepieces or camera.

Check for the following possible inserts in the infinity space of the microscope.

1. DIC prisms for the DIC objectives. These are usually located right above the objective.
2. Analyzer. This is used for DIC microscopy and should be removed from the light path for bright field, phase-contrast, and fluorescence microscopy.
3. Epi-illuminator filter-cube changer. These devices usually hold two to four filter cubes for fluorescence microscopy, each of which contains an excitation filter, an emission filter, and a dichroic mirror (Fig. 2A.1.5) designed specifically for different fluorophores. Check the numbers on the filter cubes and mark the corresponding positions on the outside of the filter changer to identify the proper cube position for a given fluorophore.
4. Magnification changer and Bertrand lens. Body tube magnifications are selected from a turret, and possible values are 1.0×, 1.25×, 1.5×, and 2.0×. Often one position of the turret contains a Bertrand lens. This lens is used in combination with the eyepieces to produce a telescope view of the objective back focal plane (Fig. 2A.1.2, far right). This is an important device for checking that the lamp image is centered and in focus during alignment for Köhler illumination, and for adjustment of the condenser diaphragm (see Transillumination Components, below).

Beam switch

This device switches the light between the binocular and the camera port. Check for the percentage of light in each direction. For fluorescence microscopy, it is important to be able to send 100% of the imaging light either to the eye or to the camera.

Eyepieces

Eyepiece magnification to the eye is marked on the barrel (e.g., 10×). Note that there are two and maybe three adjustments. The interpupillary distance for the eyes is adjusted by grasping the bases of the eyepiece tubes and moving them closer or further apart. At least one of the eyepieces is adjustable so that the eyes are parfocal.

Camera adapters

Check the type of camera adapter and whether it matches the detector. There are several different types of camera adapters. One type uses an eyepiece tube (not the binocular tube) and a projection eyepiece combined with a camera lens to project an image onto the camera detector. This method is common in photographic film cameras (which use ≥35-mm film) and for some video cameras with large-size detectors (e.g., 1 in., equivalent to 2.54 cm). The recent video and cooled slow and progressive scan charge-coupled device (CCD) cameras have small detectors (≤ $\frac{2}{3}$ in., equivalent to 1.7 cm). These cameras require smaller projection magnifications or none—in the latter case the detector is mounted at the intermediate image plane, the focal plane of the objective (Fig. 2A.1.2), and no eyepiece or projection lens is used.

Transillumination Components

Lamp and housing

The lamp is typically a low-voltage 100-W quartz halogen bulb with a tungsten-filament light source and variable control. Some lamp housings have no adjustments for centering the bulb; the socket is prefixed. Most lamp housings have *x*–*y* adjustments for the bulb

socket, while an advanced lamp housing also has a mirror in the back for reflecting an image of the lamp back along the microscope axis. This mirror usually has adjustments for *x*, *y*, and *z* positions of the mirror image of the lamp. There is usually a knob on the side of the lamp housing for moving the collector lens back and forth along the *z* axis to focus the tungsten-filament image onto the condenser diaphragm plane.

Diffuser and filters

A diffuser and other filters are often inserted in slots in the base of the microscope. The diffuser helps spread the image of the source at the condenser diaphragm plane in order to uniformly fill the condenser aperture. This is important for achieving high resolution. A heat-reflecting filter (e.g., BG 58) blocks infrared light: video cameras, but not the eye, are often sensitive to this wavelength of light. A green filter is frequently preferred for illuminating living cells. High-efficiency interference filters with a 40-nm bandwidth around 540 nm are often best. Various neutral-density (ND) filters, which are not wavelength selective, are useful for attenuating light to cameras.

Field diaphragm

This is usually located just above or beneath the mirror that deflects the light up to the condenser lens. It controls the specimen area illuminated by the condenser (Fig. 2A.1.2, left).

Polarizer

This is inserted below the condenser diaphragm for polarization and DIC microscopy. Otherwise, it is removed from the light path.

Condenser focus knob

This translator moves the condenser along the microscope *z* axis to focus an image of the field diaphragm on the specimen.

Condenser centering screws

There are usually two screws on the condenser carrier which move the condenser in an *x*–*y* plane. These screws are used to center the image of the field diaphragm on the *z* axis.

Condenser diaphragm

The condenser diaphragm is located on the bottom of the condenser. It controls the angle (NA) of the condenser cone of illumination of the specimen (Fig. 2A.1.3).

Condenser turret

Condensers have turrets with inserts for special image-contrast techniques such as phase-contrast, DIC, and dark-field microscopy. Each insert matches certain objectives. In phase-contrast microscopy, the inserts are annuli of different diameters designed to match the phase ring in phase-contrast objectives (Fig. 2A.1.6). For DIC, the inserts are typically DIC prisms designed for certain objectives (Fig. 2A.1.8). For dark-field microscopy, the NA of the condenser must exceed that of the objective. To see these inserts, as well as the opening and closing of the condenser diaphragm, remove the condenser from the condenser carrier by loosening the locking screen and turning it upside down.

Condenser lens

The condenser's main function is to provide bright, uniform illumination of both the specimen field (Fig. 2A.1.2, right) and objective aperture (Fig. 2A.1.3) for objectives with different NAs. Check the markings on the condenser to see if it is designed for air (dry) or oil immersion with the glass slide. Dry (no oil-immersion) condensers have NA values of $\leq$0.9 and should not be used with immersion oil. Oil-immersion condensers usually have NA values of 1.0 to 1.4 when using immersion oil.

Epi-illumination Components

Lamp and housing

The light source for the epifluorescence illumination is typically an HBO 50-W or HBO 100-W mercury arc lamp, or a xenon lamp of similar wattage. These lamps need to be handled with care because they can blow up if mistreated. Their glass envelopes should be carefully cleaned with 70% ethanol before installation to prevent fingerprints or other materials from inducing hot points that may result in fracturing of the bulbs. Look for the adjustment screws to adjust the *x*–*y* position of the lamp. Look also for the rear mirror and its *x*, *y*, and *z* adjustment screws. Identify the focusing knob for the lamp collector lens. Do not turn the lamp on until the bulb has been installed according to the manufacturer's instructions, the collector lens has been inserted properly, and the lamp housing has been installed on the back of the microscope. These lamps produce intense light, and the mercury bulbs have large peaks in the UV range. Therefore, UV-protective glasses should always be worn when handling and installing these lamps. The light intensity decreases and the probability of explosion increases with the number of hours of operation and lamp starts. Check the timer on the lamp power supply to see that the recommended limits are not exceeded.

Shutter

Photobleaching is a major problem in fluorescence microscopy. A shutter is used to block the light from the specimen when not taking exposures on a camera or viewing by eye.

Filters

A heat-reflecting filter is used to prevent infrared illumination of the specimen and to keep infrared scattered light from reaching the camera. Neutral-density (ND) filters are used to reduce the light intensity of fluorescence illumination by the amount indicated on each filter.

Epi-illumination condenser diaphragm

This diaphragm provides variable adjustment of the illumination intensity. Some microscopes lack this diaphragm.

Epi-illumination field diaphragm

This diaphragm controls the area of the specimen illuminated. Look for the centering screws that control the *x*–*y* position of the field diaphragm.

Filter cubes

See Transillumination Components, above.

Dark-Field Illumination Components

Turret condenser with dark-field stop included

A bright-field microscope can be converted for dark-field illumination by adding a special dark-ground condenser or a multifunction condenser with dark-ground facilities. Dark-field stops are included in specially designed dark-ground condensers such that the stops can block the light on its way to or through the condenser. Make sure to fully open the aperture diaphragm of the condenser. A turret condenser (Fig. 2A.1.4) is optimal because the dark-field stop is positioned in exactly the same location as the condenser aperture. If one can outfit their microscope with a turret condenser with a dark-field stop included, then dark-field studies can begin at once. The most sophisticated means of dark-field microscopy configuration makes use of a dark-field condenser/objective pair called a cardioid dark-field condenser. Mirrored surfaces in the cardioid condenser concentrate light onto the specimen as depicted in (Fig. 2A.1.4B).

Multifunctional condenser fitted with patch stops

Alternatively, any condenser can be used and artificial patch stops can be created out of opaque material. The stop is placed below the substage condenser where it blocks out the center of the beam of light coming from the base of the microscope forming the hollow cone of light needed for dark-field illumination. Each lens will have its own stop size requirement based on its NA. For a 10× objective of NA 0.25, the diameter of the patch stop should be ~16 to 18 mm and ~22 to 24 mm for 20× and 40× objectives of NA near 0.65.

BASIC IMAGING AND KÖHLER ILLUMINATION LIGHT PATHS FOR BRIGHT-FIELD, FLUORESCENCE, AND DARK-FIELD MICROSCOPY

The second major step in learning proper alignment of the light microscope is to understand the basic image-forming and Köhler illumination light paths and the functions of the key optical components and diaphragms. These are outlined in Figure 2A.1.2, Figure 2A.1.3, and Figure 2A.1.5.

Imaging-Ray Paths

The upper left section of Figure 2A.1.2 shows ray paths for the image-forming light from the objective. The objective, in combination with the tube lens, produces a real, magnified image of the specimen at the intermediate image plane. The eyepiece provides a second stage of magnification to the eye. The magnification to the eye is the product of objective magnification and eyepiece magnification multiplied by any magnification provided by additional lenses in the body tube of the microscope between the objective and the eyepieces.

When a camera detector—film, video, or charge coupled device (CCD)—is mounted at the intermediate image plane, the magnification to the camera is the magnification produced by the objective and body tube. When a camera is mounted above an eyepiece, the unfocused light leaving the eyepiece must be focused onto the camera detector by a camera lens, which modifies the total magnification to the detector. Special adapters (see Image-Forming Components, Camera Adapters) are also available for projecting the objective image onto a camera detector with magnification, but without the need for an eyepiece.

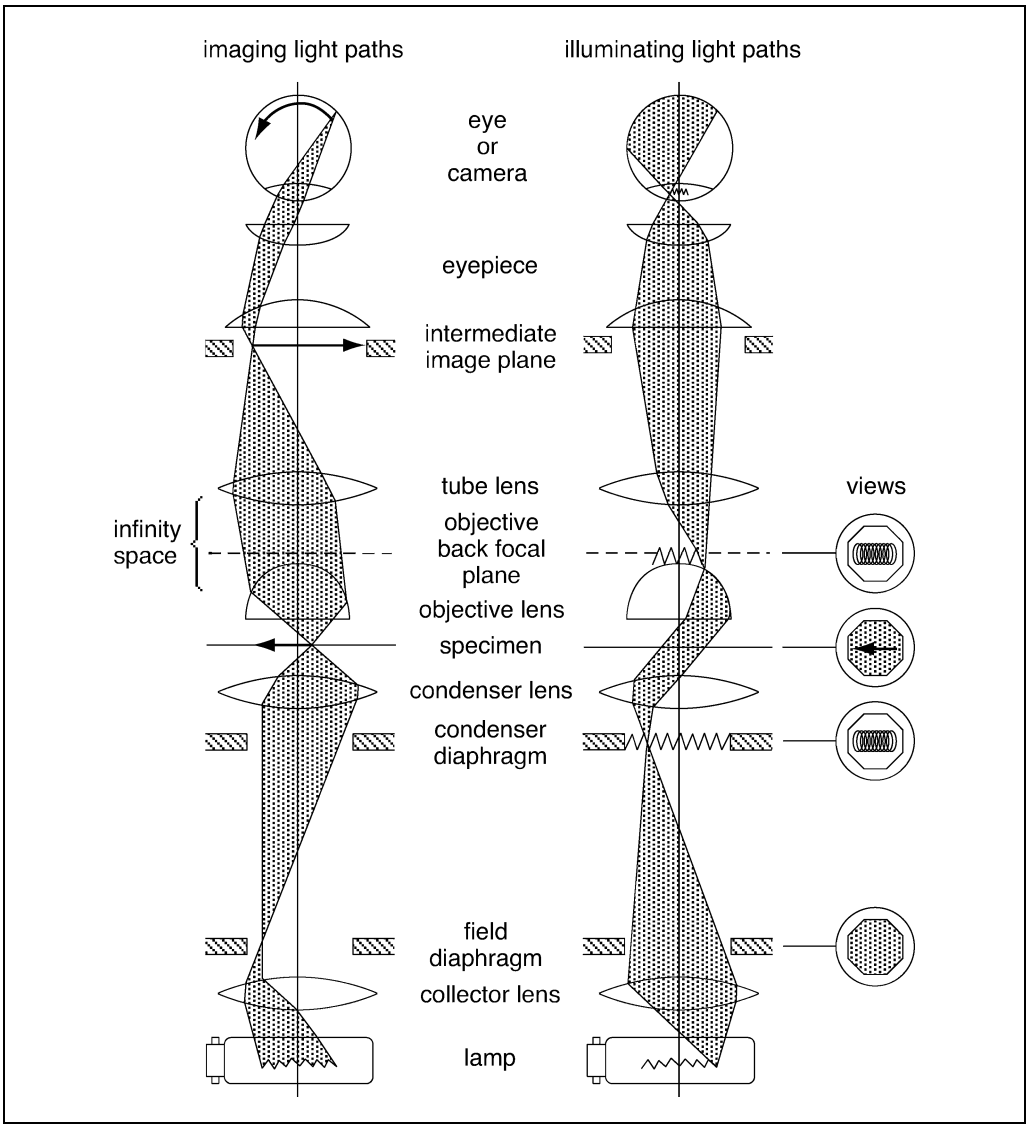

Figure 2A.1.2 The imaging and illuminating light paths for a bright-field microscope aligned for transmitted light Köhler illumination. Modified from Keller (1998).

Transillumination Ray Paths

In the illustration of the standard Köhler method (Fig. 2A.1.2), trace the illumination ray paths for a bright-field microscope aligned for Köhler transillumination (right side). A point on the light source is focused by the lamp collector lens onto the front focal plane of the condenser lens, where the condenser diaphragm is located. This light is projected, out of focus, through the specimen by the condenser lens, producing uniform illumination of the specimen. The objective collects the unfocused illumination light and brings an image of the light source into focus at its back focal plane, which is at the back aperture of the objective (Fig. 2A.1.3). The light source is again out of focus at the intermediate image plane and at the retina of the eye or the detector of the camera. In between these two points, the light source is in focus at the exit pupil of the eyepiece, at a position ~15 mm above the eyepiece. This position is also called the eyepoint, the position of eye placement above the eyepiece (Fig. 2A.1.2, right).

The condenser diaphragm controls the NA (cone angle) of specimen illumination by the condenser lens (Fig. 2A.1.3). Opening the diaphragm increases the aperture of illumination, which increases both the light intensity and resolution in bright-field light

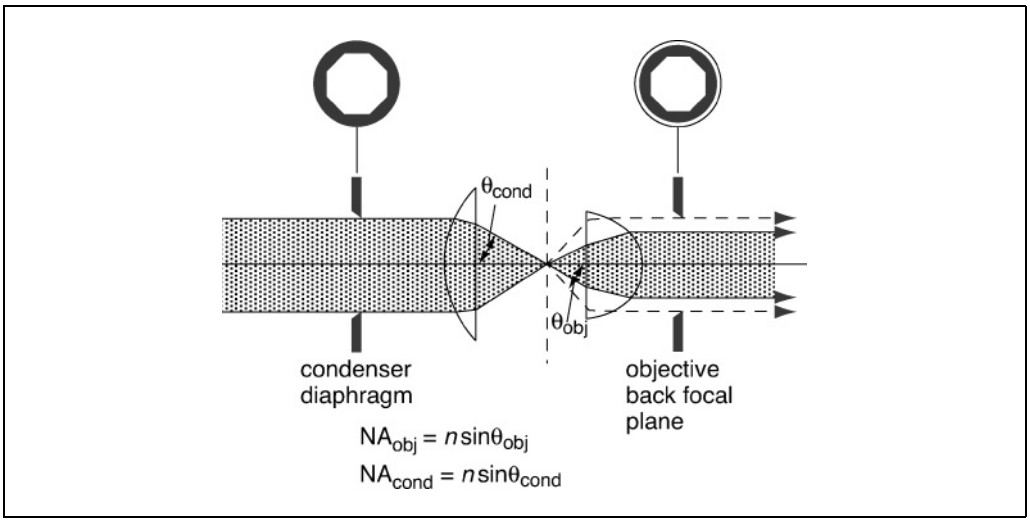

Figure 2A.1.3 Numerical aperture (NA) of objective light collection and condenser illumination. The objective numerical aperture (NA_{obj}) depends on the angle of the cone of light from the specimen, which is accepted by the objective aperture while the numerical aperture of condenser illumination (NA_{cond}) is controlled by the condenser diaphragm and limited by the maximum NA of the condenser when the condenser diaphragm is wide open.

microscopy (see Support Protocol 3). Note on the right side of Figure 2A.1.2 that the image of the condenser diaphragm is in focus where the lamp image is in focus along the microscope axis, at the objective back focal plane, and the exit pupil of the eyepiece.

Trace the imaging light rays from the field diaphragm through the microscope (Fig. 2A.1.2, left). Note that the field diaphragm controls the specimen area illuminated by the condenser. Note also that the field diaphragm is placed between the lamp collector lens and the condenser lens in a plane where the lamp image is out of focus (compare Fig. 2A.1.2, left and right). When the condenser lens has focused the image of the field diaphragm onto the specimen, then the field diaphragm will be in focus with the specimen at the intermediate image plane and at the eye or detector.

Epi-illumination Ray Paths

Figure 2A.1.5 shows the optical alignment for an epi-illuminator and Köhler illumination through the objective. As in transillumination (Fig. 2A.1.2), the light source is focused by a collector lens onto the condenser diaphragm of a condenser lens in the light path. The condenser lens illuminates a field diaphragm. Another lens collects this light and projects it off a reflective mirror into the objective. The image of the light source is focused at the back focal plane of the objective so that it is out of focus at the specimen plane. In epifluorescence microscopy, filter cubes containing dichroic mirrors are used in combination with excitation and emission filters to efficiently reflect the excitation light into the objective and transmit to the eyepiece or camera only the longer-wavelength fluorescent light emitted from the specimen (Taylor and Salmon, 1989; Herman, 2002).

BASIC IMAGING FOR DARK-FIELD MICROSCOPY

Dark-Field Ray Paths

Figure 2A.1.4 shows the optical alignment for a dark-field microscope. The light source is focused by the collector lens onto the condenser diaphragm of the condenser lens as in both transillumination and epi-illumination. Similarly, the condenser lens illuminates the field diaphragm. The majority of the light passing through the condenser aperture is blocked by a dark-field stop within the plane of the condenser aperture. The dark-field

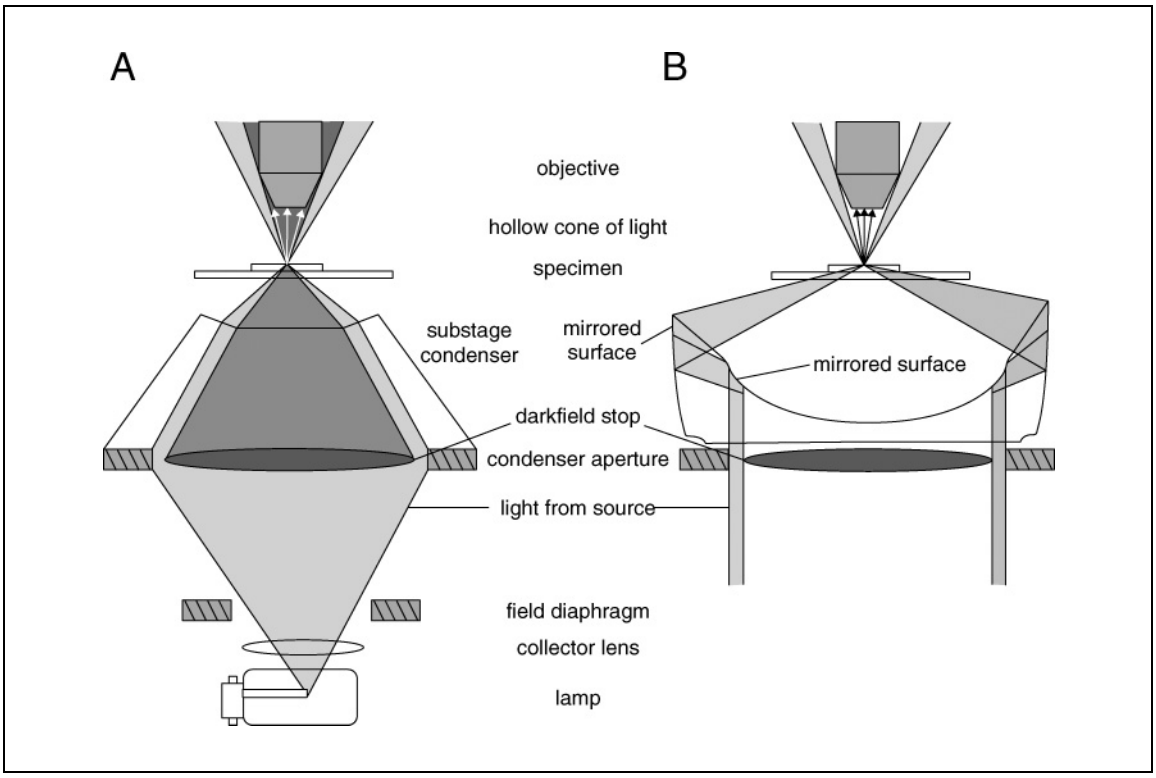

Figure 2A.1.4 The illuminating light paths for dark-field microscopy using (**A**) a multifunctional condenser fitted with a dark-field stop or (**B**) a cardioid condenser.

stop must be of a specific diameter to block the direct light, but it is designed to be open around the outer perimeter, letting light pass around the dark-field stop's circumference. The aperture diaphragm of the condenser must be fully opened so that some light can pass through the dark-field stop. These rays are refracted in the condenser at an oblique angle such that they form a hollow cone of light that passes just outside the objective lens. Compare this to the illuminating light path for a bright-field microscope in Fig. 2A.1.2, in which all of the direct light passes through the specimen and up into the objective lens.

When a mounted specimen is placed on the stage, it is located at the apex of the hollow, inverted cone of light. As the light passes through the specimen it is scattered in all directions. Some of the light is reflected or refracted up into the objective lens, so the specimen appears as a luminous object against a dark background. Only the rays of light reflected, refracted or diffracted by the specimen will enter the objective. The remaining light forms a hollow cone of light that passes outside the objective (Fig. 2A.1.4).

BASIC PROTOCOL 1

ALIGNMENT FOR KÖHLER ILLUMINATION IN BRIGHT-FIELD, TRANSMITTED LIGHT MICROSCOPY

The following procedures assume that the positions of the quartz-halogen tungsten filament and collector lens are adjustable. Inexpensive, nonresearch, compound microscopes may have the illuminator built into the stand, with no adjustment for the lamp. Such microscopes depend on a ground-glass filter for even illumination. For these microscope stands, skip steps 2, 3, 4, and 8.

Focus the light source

Proper Alignment and Adjustment of the Light Microscope

1. If possible, remove the diffusion filter in the transillumination pathway during alignment so that a crisp image of the light source can be viewed at the condenser diaphragm plane and at the objective back focal plane. When alignment is complete, reinsert the diffusion filter.

2. Center and focus the lamp filament near the condenser diaphragm plane. Remove the condenser lens. Place a sheet of lens paper on the microscope stage. Close down the field diaphragm and adjust the intensity of the lamp so that the lens paper is moderately illuminated. Use the lamp-focusing knob to move the condenser lens (or lamp) along the *z* axis until an image of the filament is in focus on the lens paper. Roughly center the lamp on the microscope axis with the adjustment screws on the lamp housing, then adjust the mirror image (if there is a mirror) using its adjustment screws on the lamp housing.

3. Place the lens paper on the condenser carrier and raise the condenser carrier until it is ~20 mm from the top of the stage.

   This is the approximate position of the condenser diaphragm when a condenser is installed and in focus.

4. Refocus the lamp and mirror images at this position of the lens paper.

   If there is no mirror image, center the lamp image. If there is a mirror image, then position the images so that they sit side by side to fill the condenser aperture. Remove the lens paper and replace the condenser.

Focus for low-power viewing

5. Obtain an in-focus image of a specimen with the low-power objective (10× or 16×) by placing a test specimen that absorbs light on the stage (e.g., stained muscle section; see Table 2A.1.1).

   The focus position can be estimated from the working distance of the objective lens. For a standard 10× lens, this is −4 mm. So, use the coarse focus to bring the specimen to ~16 mm from the coverslip by viewing the objective position from the side of the microscope.

6. Move the condenser lens up close to the lower surface of the slide and open the field and condenser diaphragms all the way. Look down the eyepieces, and use the coarse- and fine-focus knobs to bring the specimen into sharp focus.

7. Focus the image of the field diaphragm centered on the specimen by adjusting the condenser *x*–*y* screws and the condenser focus. Initially, close down the field diaphragm until an edge of the image can be focused by the condenser, then close the field diaphragm further as the image is centered, using the *x*–*y* translation screws.

   Usually, the field diaphragm is opened just enough to match the field of view in the eyepiece or the camera. However, the best image contrast is obtained if the field diaphragm is opened just enough to illuminate the region of interest. This eliminates the presence of scattered light from outside the region of interest.

8. Center an in-focus image of the lamp and mirror images at the objective back focal plane using the focus and adjustment screws on the lamp housing. View the objective back aperture (the position of the back focal plane; Fig. 2A.1.3) by using the Bertrand lens in the magnification changer in combination with the eyepieces, by replacing one eyepiece with a telescope, or by simply removing one eyepiece and peering down the body tube. Open the condenser diaphragm all the way. Readjust the collector lens and the mirror so that the lamp images are in focus and sit side by side to fill the objective aperture.

   This is a rough adjustment with the low-power objective and will need to be fine-tuned using the objective with the highest NA.

9. Adjust the opening of the condenser diaphragm so that the diameter of its image at the objective back focal plane (see far right of Fig. 2A.1.2; also see Fig. 2A.1.3) is slightly less than the diameter of the objective back aperture.

   Some research condensers also have centering adjustments for the condenser diaphragms. The image of the condenser diaphragm should be centered in the objective back aperture.

10. Adjust the light intensity for comfortable viewing of the specimen by adjustment of the light-source intensity, either using the power-supply rheostat or by inserting neutral-density filters in the illumination light path.

    The microscope is now adjusted properly for the low-power objective.

Adjust for 40× viewing

11. Switch the turret to the 40× high-dry objective.

    Note that because of parfocal objective design, the specimen is almost in focus. Focus on the specimen with the fine focus.

12. Focus and center the field diaphragm with the condenser focus and the condenser-carrier *x*–*y* adjustment screws.

13. View the objective back aperture (Fig. 2A.1.3).

    Notice that the image of the condenser diaphragm adjusted for the low-power objective is only about half the diameter of the 40× back aperture. The ratio of the diameter of this image to the diameter of the objective back aperture is equal to the ratio of the NA of the condenser illumination to the NA of the objective (Fig. 2A.1.3). When the condenser illumination NA equals the objective NA, the aperture of the objective is filled with light and maximum resolution will be achieved for the objective NA (see Commentary).

14. Adjust the condenser diaphragm so that the condenser illumination NA just about matches the objective NA.

    Each time objectives are changed, these procedures should be repeated.

Adjust for high-power viewing

15. Rotate the nosepiece so that the high power (60× to 100×, NA 1.25 to 1.4) oil-immersion lens is coming into place, but stop before it clicks into place. Place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the point upon which the condenser beam is focused.

    This oil drop must be free of air bubbles and dirt. The refractive index of the immersion oil should be close to that of the glass coverslip.

16. Complete the rotation of the nosepiece so that the oil-immersion objective clicks into place. Lower the objective nosepiece if appropriate.

    IMPORTANT NOTE: *Use only the fine adjustment knob when working with the oil-immersion lenses.*

    The space between its front lens and the coverslip is now filled with immersion oil.

17. Remove the eyepiece (or use the telescope or Bertrand lens) and inspect the back aperture of the objective. Open the condenser diaphragm as much as possible to try and match its image with the objective aperture (Fig. 2A.1.3).

    This will be impossible if the condenser is dry and not designed for oil immersion, since dry condensers have NA ≤ 0.9 and the relative size of the image of the condenser aperture or condenser diaphragm seen in the objective back focal plane is given by the following ratio (see Fig. 2A.1.3).

$$\frac{NA_{cond}}{NA_{obj}}$$

18. View the objective back aperture, and tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

19. Replace the eyepiece (or remove the Bertrand lens) and examine the specimen. Adjust the field diaphragm until its margins just match the field of view.

    With inexpensive condensers, the image of the edge of the field diaphragm will not be in good focus, even at the optimum position. If a condenser designed for oil immersion is being used, enormous improvement in the field iris image can be achieved by oil immersion.

20. Oil the condenser (if possible) to achieve the highest resolution and image quality using the following procedure:

    a. Rotate the objective out of the way and remove the slide.

    b. Oil the condenser.

    c. Replace the slide and refocus the objective.

    Before oiling the condenser, make sure it is not designed to be a "dry" condenser by checking that it is marked with an NA > 0.9. It takes much more air-bubble-free oil than the objective.

    The image of the field diaphragm should now be much sharper when in focus and centered.

    Before oiling the condenser, make sure it is not designed to be a "dry" condenser by checking that it is marked with an NA >0.9.

    The image of the field diaphragm should now be much sharper when in focus and centered.

21. Look at the objective back aperture. Again, tune up the focus and position of the lamp images so that they fill the objective aperture with light as uniformly as possible.

    After oiling, the aperture should be filled by the condenser illumination when the condenser diaphragm is wide open.

    Notice also that viewing the objective back aperture is the best way to see if air bubbles have become trapped in the oil. If so, carefully wipe off the oil with lens paper and repeat steps 15 through 21.

22. Reinsert the diffusion glass in the illumination light path.

    This will reduce light intensity, but make the illumination of the objective aperture more uniform.

23. When finished for the day, use lens paper to remove excess oil from the objective and condenser lens surfaces to prevent dripping on specimens.

    It is not necessary, however, to completely clean oil from the optics after every use (see Support Protocol 6).

ALIGNMENT OF THE EYEPIECES

BASIC PROTOCOL 2

The binocular usually has adjustments for the inter-eyepiece distance and visual acuity in each eye. The goal is for the image to be in focus for each eye without any eyestrain or discomfort. Only the left eyepiece tube or eyepiece is adjustable on a typical microscope. However, if the microscope has a target reticle in the microscope stand that can be rotated into view, then usually both eyepieces are adjustable.

1. With a low-power objective (10× or 16×), focus on a stained specimen placed on the stage of the microscope (e.g., stained muscle section, see Table 2A.1.1) and align the microscope for Köhler illumination.

2. Move the bases of the eyepiece tubes together or apart to set the proper interpupillary distance for your eyes.

3a. *If the microscope has a target reticle:* Rotate the reticle into the field of view. Close the left eye and use the diopter-adjustment ring on the right eyepiece until the target is in sharp focus for the right eye. Then close the right eye and use the diopter-adjustment ring on the left eyepiece until the target is in sharp focus for the left eye. Remove the target to view the specimen.

3b. *If the microscope has no target reticle:* Set the right eyepiece tube to the inter-eyepiece setting (if possible). Close the left eye and bring into sharp focus a structural detail near the center of the field of view with the right eye. Close the right eye and use the diopter-adjustment ring on the left eyepiece tube to bring the specimen detail into sharp focus for the left eye. With both eyes open, fine tune the focus of the left eyepiece tube.

BASIC PROTOCOL 3

ALIGNMENT FOR KÖHLER ILLUMINATION IN EPIFLUORESCENCE MICROSCOPY

The eye is most sensitive to green light. Hence, the following protocol is best done using a filter cube that produces green excitation light (e.g., a filter cube for rhodamine).

Focus the lamp

1. Remove one objective, and rotate the nosepiece so the open position is centered on the microscope axis. Place a white card on the microscope stage, on top of supports that position the card at ~2 to 3 cm from the nosepiece.

   This is approximately the position of the objective back focal plane, where the images of the light source should be in focus and centered after alignment (Fig. 2A.1.5).

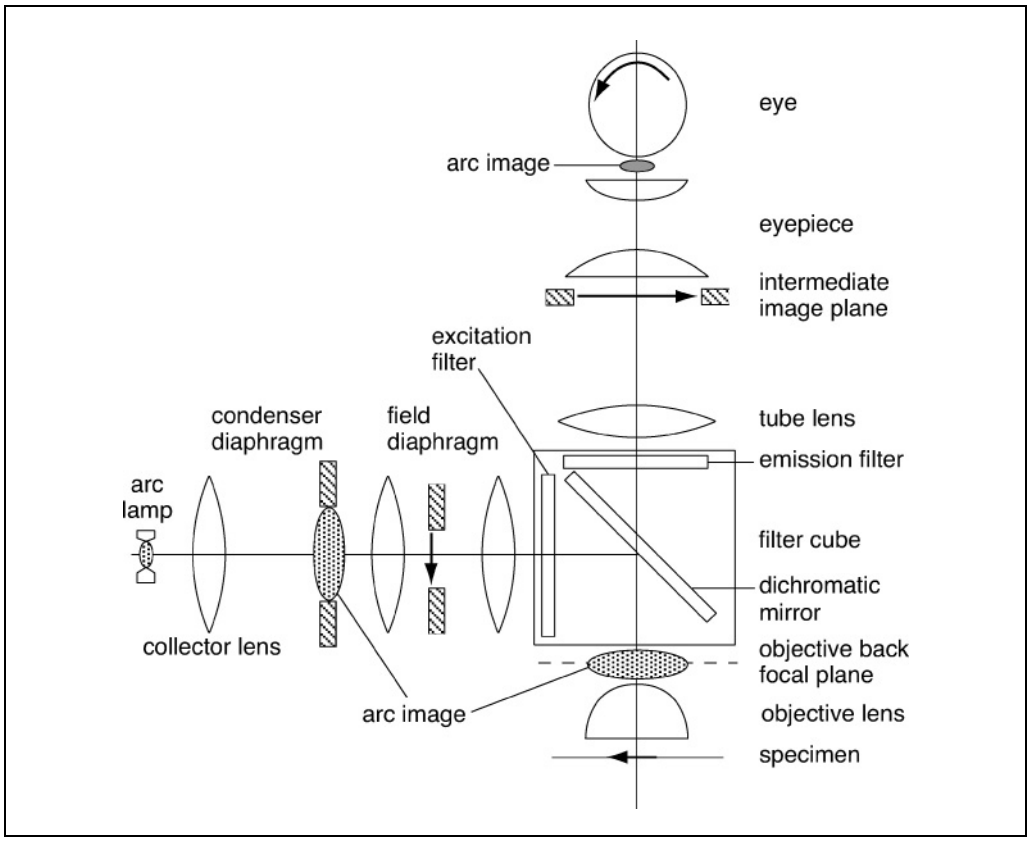

Figure 2A.1.5 Microscope alignment for epifluorescence Köhler illumination.

Some microscopes have a special device for lamp alignment. It screws into the objective nosepiece and projects an image of the epi-illumination arc and electrodes onto a small diffusion screen within the barrel of the device.

2. Install the mercury bulb in the lamp housing and attach the lamp housing on the back of the microscope according to the manufacturer's instructions.

3. Turn on the power source and ignite the lamp.

   CAUTION: *It is usually a good idea to make sure computers in the vicinity are turned off before starting the lamp because the high voltage pulse used to ignite the arc plasma may damage the electronics. Laboratory personnel should protect their eyes against UV light from the lamp.*

   It takes ~10 min for the arc to brighten.

4. Close down the field diaphragm and open the condenser diaphragm (if there is one) all the way.

5. On the diffusion glass screen or on the white card on the stage, the image of the arc between the tips of the two electrodes should be visible. Use the lamp focusing knob to obtain an in-focus image of the lamp arc and electrodes. Use the lamp *x–y* adjustment screws to approximately center the image of the arc and electrode tips (see Figure 2A.1.2).

6. If there is a lamp mirror, use its adjustment screws to focus the mirror image of the arc and electrode tips, centered on the target.

   Often the primary image and the mirror image of the arc are adjusted side-by-side and slightly overlapping, but centered on the microscope axis.

Focus the field diaphragm

7. Place a test fluorescent specimen (see Table 2A.1.1) on the stage and rotate the objective nosepiece to a low-power objective. Select the appropriate filter cube for the fluorescent specimen. Open up the epi-illuminator field diaphragm all the way. Open the shutter and view the specimen.

8. Focus the specimen, then close down the field diaphragm until an edge comes into the field of view. Use the *x–y* adjustment screws for the field diaphragm to center the field diaphragm image within the field of view.

9. Close down the field diaphragm until only the region of interest is illuminated.

   This prevents photobleaching of areas outside the region of interest and reduces the amount of scattered fluorescent light in the image from outside the region of interest.

10. Switch to a high-power objective. For an oil-immersion objective, place a small drop (~2 mm diameter) of immersion oil directly onto the coverslip above the region of

Table 2A.1.1 Suppliers for Items Used in Light Microscopy

Test specimen	Supplier
Hematoxylin/eosin-stained skeletal muscle	Carolina Biological Supply
Diatom test plate	Carolina Biological Supply
Squamous cheek cells	Freshly prepared (see Support Protocol 4)
Stage micrometer	Fisher
Red, green, and blue fluorescent tissue culture cells	Molecular Probes

interest. Carefully swing in the objective. Open the shutter and focus on the specimen. Readjust the size and centration of the field diaphragm.

This oil drop must be free of air bubbles and dirt.

11. To prevent photobleaching, be sure to close the epi-illumination shutter when not viewing or taking camera exposures.

BASIC PROTOCOL 4

ALIGNMENT FOR PHASE-CONTRAST MICROSCOPY

Phase-contrast microscopy is often used to produce contrast for transparent, non-light-absorbing, biological specimens. The technique was discovered by Zernike in 1942, who received the Nobel prize for his achievement (Zernike, 1942, 1955, 1958). The last of these references describes the principles of phase contrast and provides an excellent introduction to the wave optics of image formation, resolution, and contrast in the microscope.

The phase-contrast microscope is a bright-field light microscope with the addition of special phase-contrast objectives (Fig. 2A.1.6) containing a phase plate or ring and a condenser annulus instead of a diaphragm; the annulus is usually located on a condenser turret because it has to be selected for different objectives. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method. However, there is no condenser diaphragm to adjust. Instead, the phase annulus must be selected and adjusted properly. Modern phase-contrast objectives have a phase plate containing a ring in the back focal plane within the barrel of the objective. This ring absorbs and advances the phase of the light passing through it by $\frac{1}{4}$ wavelength in comparison to light passing through the rest of the objective aperture. For each phase objective, there is a corresponding annulus in the condenser turret that has about the same relative size as the phase ring in the objective. Light passing through this annulus

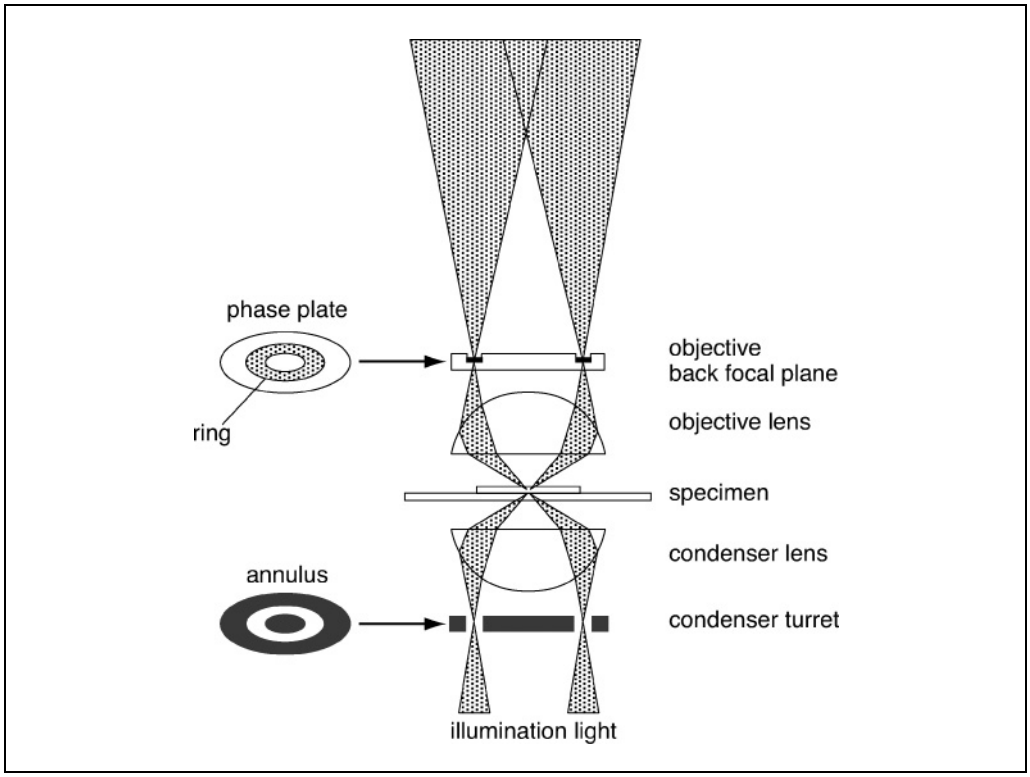

Figure 2A.1.6 Illumination light path through the condenser annulus and objective phase ring in a microscope aligned for phase-contrast microscopy.

passes through the phase ring in the objective when the annulus is selected and aligned properly. Light scattered by the specimen mainly passes through the objective aperture outside the phase ring. Light scattered from a thin transparent specimen is $-\frac{1}{4}$ wavelength retarded from the unscattered illumination light. The additional $\frac{1}{4}$ wavelength retardation between the scattered and illumination light produced by the objective phase ring makes the scattered and illumination light 180° out of phase. They destructively interfere with each other at the image plane to produce the "dark" contrast of structural detail typical of phase contrast images of biological specimens.

Because the phase annulus and the phase ring reduce the intensity of the background light, a bright illuminator—e.g., a 100-W quartz-halogen illuminator—is necessary at high magnifications (because image intensity decreases as 1/magnification^2). For living cells, heat reflection and green illumination filters should be used.

To examine the objective phase ring and matching condenser phase annulus, remove the low- and medium-power phase objectives and the phase condenser from the microscope. View the phase ring (phase plate) within the objective by looking in the back end. The phase ring is located at the position of the objective back focal plane; its diameter is usually $\sim\frac{2}{3}$ that of the objective aperture. Note that it is situated within the objective body and is visible because it absorbs light. Locate the annulus for each objective in the condenser turret. The annulus is located at the condenser diaphragm plane, which is situated at the condenser front focal plane. Notice that as the objective NA increases, the diameter of the corresponding annulus in the condenser turret increases. The NA of condenser illumination from an annulus is designed to match that of the phase ring in the corresponding objective.

Replace the condenser on the microscope and illuminate with white light. Hold a piece of tissue paper near the top of the condenser and examine the illumination cone. Notice that it is an annular cone of illumination. Change the annulus from the setting for the low-power objective to the 40× setting. Note that the NA or angle of illumination increases. Points of illumination further from the central axis in the condenser diaphragm plane produce higher-aperture rays passing through the specimen.

Perform alignment using the following steps.

1. Align the microscope for bright-field Köhler illumination using the low-power phase objective (10× or 16×) and a cheek cell preparation for the specimen (see Support Protocol 4).

   The cheek cell specimen is an excellent transparent test specimen for alignment of the microscope for phase contrast, dark field, and DIC. The cheek cells are transparent and only barely visible by bright-field illumination. To find the plane of focus in bright field, initially close down the condenser diaphragm and look for the edges of air bubbles in the preparation. These edges scatter much light and appear dark in the image. When using dark-field configuration, the cheek cells will appear white against a dark background.

2. Align the condenser annulus with the phase ring by rotating the condenser turret to the position where an annulus matches the phase ring in the objective; use the telescope, your eye, or the Bertrand lens to view the objective back aperture.

   As the condenser turret is rotated, notice in the objective back focal plane the images of the different-diameter annuli designed for the different numerical aperture objectives. The correct annulus may not be properly aligned with the objective phase ring.

3. Use the adjustment screws in the condenser (and the special tools if necessary) to move the annulus in the *x*–*y* plane to achieve alignment with the phase ring in the objective.

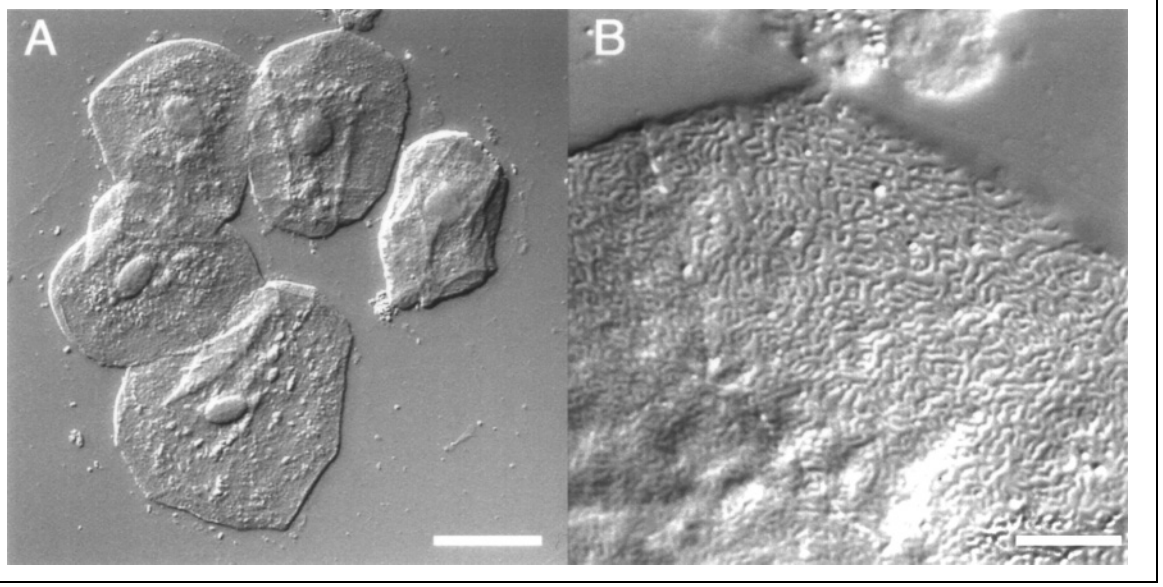

Figure 2A.1.7 DIC images of a human cheek cell test specimen. (**A**) Low magnification of cheek cell preparation with a 20× objective. Bar = 20 μm. (**B**) High-resolution image of the surface of the cell at the top of (A) using a 60×/(NA = 1.4) Plan Apochromat objective and matching condenser ilumination. The ridges on the cell surface are often diffraction limited in width. Bar = 5 μm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

Note that the phase ring is slightly wider than the image of the correct annulus. It is critical that the image of the annulus be within the phase ring, but it is not critical if it is very slightly off center.

4. When the objective phase ring and condenser annulus are aligned, view the specimen and properly adjust the focus and centration of the field diaphragm.

   Notice that if the phase ring and annulus are slightly misaligned (rotate the turret slightly), the background light intensity goes up. This is because the phase ring is designed to absorb much of the illumination light. The unscattered illumination light becomes closer to the intensity of the light scattered by the specimen, which passes through the objective aperture outside of the phase ring. Minimizing the background light intensity while viewing the specimen image can also be used to align the annulus with its phase ring or to touch up the alignment done by viewing down the body tube.

5. Switch to the 40× phase objective and then the high-power 60× or 100× phase objective (NA 1.25 to 1.4) and repeat steps 1 to 4.

   It is necessary to increase light intensity at the higher magnifications. It should be possible to see the ridges on the cell surface (see Fig. 2A.1.7 for a DIC image of the ridges). Notice the "phase halos" at discrete edges, a problem that limits conventional phase contrast in high-resolution imaging.

BASIC PROTOCOL 5

ALIGNMENT FOR DIC MICROSCOPY

Since its introduction in the late 1960s (e.g., Allen et al., 1969), DIC microscopy has been popular in biomedical research because it highlights edges of specimen structural detail, provides high-resolution optical sections of thick specimens—including tissue cells, eggs, and embryos—and does not suffer from the "phase halos" typical of phase-contrast images. See Salmon and Tran (1998) for details of image formation and video-contrast enhancement.

Proper Alignment and Adjustment of the Light Microscope

The DIC microscope is a bright-field light microscope with the addition of the following elements (Fig. 2A.1.8, middle): a polarizer beneath the condenser; a DIC beam-splitting

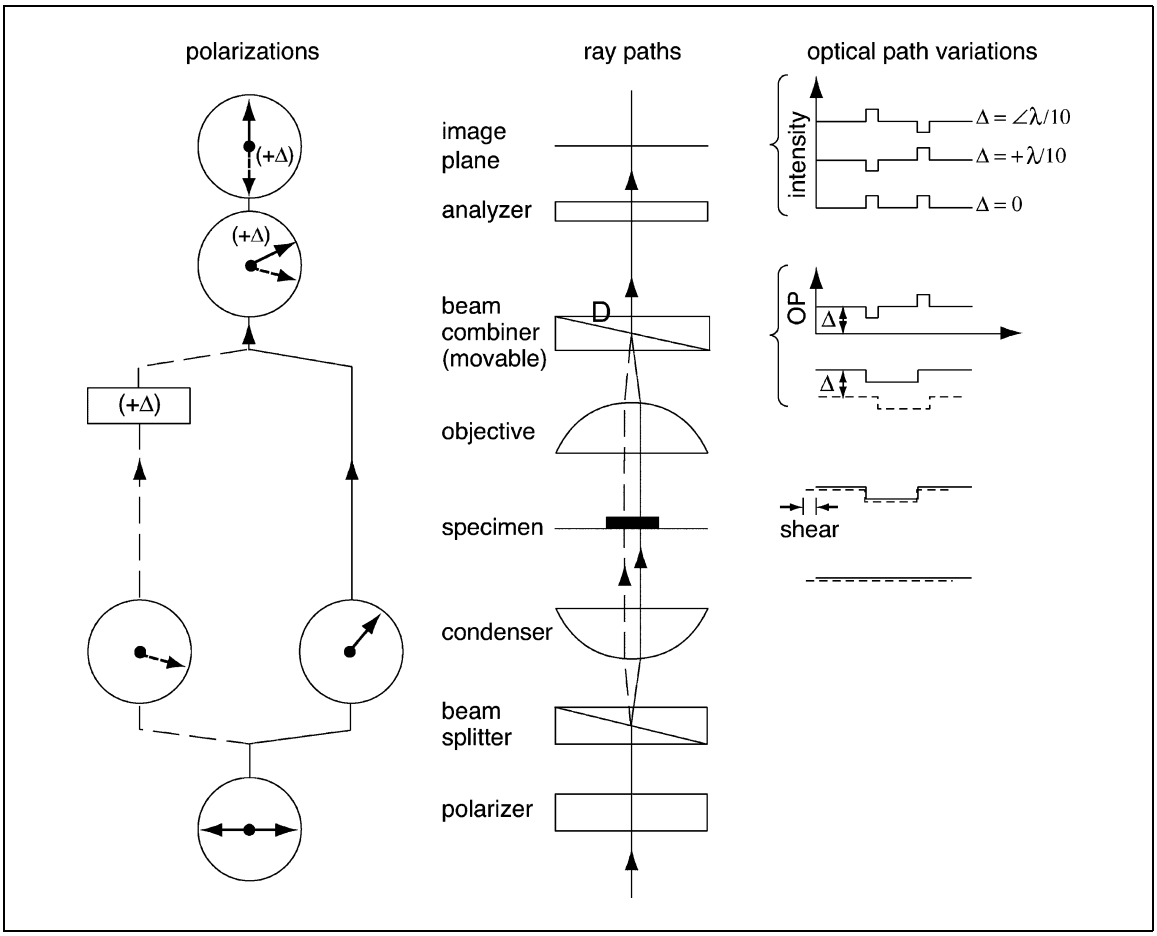

Figure 2A.1.8 The optical system for DIC microscopy. From Salmon and Tran (1998), reprinted with permission from Academic Press.

prism (Nomarski or Wollaston) in the condenser turret; a DIC beam-combining prism (Nomarski) just above the objective; an analyzer above the objective prism in the infinity body tube space; a compensator after the polarizer or before the analyzer in some microscopes (not shown in Fig. 2A.1.8); and a rotatable stage. The microscope optics are usually aligned for bright-field specimen illumination by the standard Köhler method.

The polarizer, which produces plane-polarized light (Fig. 2A.1.8, left), is typically oriented with its transmission azimuth in an east-west direction facing the front of the microscope. Polarizers with high transmission efficiency are preferred. Polarizers are usually high-quality polaroid material held between thin optical glass flats. Another polarizer is used as an analyzer. The transmission azimuth of the analyzer is oriented north-south at 90° with respect to the polarizer azimuth to produce extinction of the illumination light in the absence of the DIC prisms.

The specimen is held on a rotating stage. Contrast depends on orientation and frequently specimens must be reoriented to achieve maximum contrast of the structures of interest. Either the stage or the objective and condenser must be centerable.

For the highest sensitivity, the objective and condenser lenses should be selected free of birefringence (Inoué and Spring, 1997). Rectifiers can be used to correct for the rotation of the plane of polarization of light which occurs at the periphery of lens surfaces (Inoué and Spring, 1997).

Figure 2A.1.8 outlines the principles of DIC image formation and contrast. The DIC beam-splitting prisms are located at the condenser diaphragm plane, just above the polarizer in the condenser turret. Objectives of different magnification and/or NA usually require condenser prisms of different optical characteristics.

The condenser prism splits the light coming from the polarizer into divergent polarized light wavefronts whose planes of polarization are orthogonal to each other and at 45° with respect to the initial plane of polarization (Fig. 2A.1.8, left). The divergent beams are converted by the condenser into two wavefronts, which pass through the specimen separated laterally from each other in the direction of the prism wedge (the shear direction, Fig. 2A.1.8, middle) by a tiny amount that is usually less than the resolution limit of the condenser-objective lens combination (Fig. 2A.1.8, right). These two wavefronts are recombined just above the objective by a beam-combining prism. Often, each objective has its own prism so that it accurately matches the action of the condenser prism. In some microscopes, there is one beam-combining prism for all objectives and a different condenser prism for each objective. Check which is the case for your microscope.

DIC image contrast depends on the "compensation" or "bias retardation" (Δ) between the two wavefronts along the microscope axis (Fig. 2A.1.8, right). When the objective beam-combining prism is perfectly aligned with the condenser beam-splitting prism and there is no compensation ($\Delta = 0$), the background light is extinguished and the edges of objects are bright against a black background (Fig. 2A.1.8, upper right). When one wavefront is retarded relative to the other by Δ, this increases the optical path (OP) between the wavefronts (Fig. 2A.1.8, middle right) and brightens the background light. One edge of an object becomes brighter than the background while the opposite edge becomes darker (Fig. 2A.1.8, upper right). This produces the "shadow cast" appearance of DIC images. Reversing the sign of retardation reverses the contrast of the specimen edges.

In some microscopes, the objective beam-combining prism (Fig. 2A.1.8, middle) is used as a compensator by translating the prism in the direction of prism wedge away from the position of background light extinction. One direction produces positive while the other produces negative retardation (Δ). In other microscopes, both the objective and condenser prisms are fixed at positions that produce background light extinction, and typically a deSenarmont compensator (a birefringent quarter-wave retarder in combination with a rotatable polarizer or analyzer; Inoué and Spring, 1997) is inserted just above the polarizer or beneath the analyzer.

Bright illumination sources are required for high magnification because of the crossed polarizer and analyzer; at least the 100-W quartz-halogen illuminator is usually needed.

To examine the DIC prisms, remove the objective prisms and the DIC condenser from the microscope. Examine the objective prism. Notice that it is very thin and wide enough to cover the objective aperture. Also notice if there is a screw that can be used to translate the prism back and forth in one direction across the objective aperture. Examine the prisms in the condenser turret. Notice that there are different prisms for low- and high-NA objectives. The highest-resolution (NA) objectives often have special prisms designed only for the optical properties of that objective. In aligning the microscope for DIC, be careful to use the condenser Wollaston prism that matches the objective in use. Reinstall the condenser.

Proper Alignment and Adjustment of the Light Microscope

Steps 1 to 6 in the following procedure are for the initial alignment of the microscope. Once this is done, begin at step 7 for routine use.

Perform initial alignment

1. Align the microscope initially for Köhler illumination using the low-power objective and the cheek cell preparation. Again look for the bright air-bubbles in the preparation to find the specimen plane.

2. Check that the polarizer is oriented with its transmission azimuth in an east-west direction as determined facing the front of the microscope (look for the line or double-headed arrow on the polarizer mount). Rotate the deSenarmount compensator (if there is one) so that it is at its zero position (no compensation).

3. Remove the objective prism and rotate the condenser turret to an open position. Make sure that the analyzer transmission azimuth is crossed to the polarizer by checking that the background light is at extinction.

   This exercise is best done with the brightest light position of a 100-W quartz-halogen illuminator.

4. Insert the objective DIC prism (observe caution as the image will be very bright). Observe the extinction fringe in the middle of the prism (you must use the telescope, remove an eyepiece, or insert the Bertrand lens to focus on the objective focal plane). Make a drawing of your view of the fringe in the back aperture.

   The fringe should be at 45° with respect to the analyzer-polarizer transmission azimuths.

5. Remove the objective DIC prism and rotate into place the condenser prism that matches the objective. Observe the objective back focal plane.

   The orientation of the fringe should match the orientation of the extinction fringe for the objective prism in your drawing.

6. Insert the objective prism and observe the objective back focal plane. Rotate in the other condenser prisms and notice that the extinction fringe is no longer spread across the objective aperture. Rotate in the correct condenser prism.

   If the condenser and objective prisms are properly matched and oriented in the same direction, the fringe should become spread across the objective aperture (a dark cross will still occur in high-power, unrectified objectives; Inoué and Spring, 1997).

Align for specimen viewing

7. View the specimen with the correct objective and condenser prisms in place. Translate the objective prism (or rotate the deSenarmount compensator if the objective prism is fixed) to induce a retardation (Δ) between the two wavefronts to brighten the background light and make the edges of the cell appear shaded. If possible, rotate the specimen and observe that contrast is directional—45° with respect to the analyzer-polarizer orientations.

   As the upper prism is translated (or the deSenarmount compensator is rotated) to compensation of opposite sign, the initially bright edges become darker and the initially dark edges become brighter than the background.

8. Adjust for proper bright-field, transmitted-light Köhler illumination.

   The quality of the DIC image depends critically on the field diaphragm being sharply focused on the specimen (Fig. 2A.1.2, left), since this puts the condenser prism in the proper place. The highest possible resolution is achieved when the condenser diaphragm is adjusted to match the diameter of the objective aperture (view the objective back focal plane during this adjustment).

   Optimum edge contrast is produced when the retardation between the wavefronts is adjusted to extinguish the light coming from one set of edges in the specimen. Further retardation increases light intensity, but not contrast to the eye. When using video cameras, it is often important to view the specimen by eye and adjust the compensation for best

contrast, then change illumination intensity to provide the camera with enough light for good image quality.

9. Repeat steps 2 to 8 for the other objectives.

Once you are sure of the correct prisms and their orientation, then only steps 7 and 8 are necessary for each objective.

ALIGNMENT FOR DARK-FIELD MICROSCOPY

Dark-field microscopy, also referred to as dark-ground illumination, is a special optical technique used to observe subjects that have a refractive index similar to that of the medium in which they are mounted and that are difficult to image using conventional bright-field illumination. A major advantage of this technique is that low-power objectives can be used to produce good observation of a specimen that would require higher power magnification using bright-field illumination. Dark-field microscopy does not provide the best resolution, but it can resolve objects clearly against a dark background. The technique was developed by Dupouy to examine bacterial flagella (Dupouy et al., 1969) and bacterial cell walls (Dupouy, 1973). Ideal candidates for dark-field microscopy include living aquatic organisms, diatoms, unstained bacteria, and cells in tissue culture. It is recommended that the specimen be a thin, homogeneous preparation in order to eliminate diffraction artifacts that can interfere with image formation. For this reason, the standard method for observation of such biomedically important bacterial species as *Borrelia burgdorferi* (Fig. 2A.1.9), *Leptospira interogans*, *Treponema denticola*, and *Treponema pallidum*, among others, is dark-field microscopy. Dark-field microscopy detection of spirochetes from patient samples is often critical for diagnosis.

Dark-field conditions are created by blocking bright-field light that normally passes through the specimen and into the objective. The light path is blocked by the application of dark-field stops to the plane of the condenser aperture (Fig. 2A.1.4). To examine this plane, remove an ocular from the microscope and look down the tube. The condenser aperture can be seen at the point of the back objective focal plane. To aid in locating this plane, close and open the condenser aperture while looking down the tube. The diaphragm can be seen moving. The diameter of the stop must be sufficient in size to block the majority of the light passing through the condenser, but open at the perimeter of the condenser diaphragm to allow some light outside the edges of the stop. The apex of the inverted cone of light is centered in the specimen plane, the result of which is a bright image of the specimen seen against a black background. The hollow, inverted cone of light can be visualized using a demonstration created by Faine (1961). This simple but informative experiment involves placing a dish of agar on the stage of the microscope set up for dark-field configuration using a low-power dark-field condenser. As the light source is turned on, the observer can visually reproduce the two inverted cones of light that can be seen from a side view of the agar.

When a mounted specimen is placed on the stage, the specimen is aligned at the apex of the hollow cone of light. All of the light that escapes the dark-field stop is passed through the specimen. The light is then scattered in every direction, such that some of the light is reflected into the objective lens. The result is a bright image of the specimen against a dark background (Fig. 2A.1.9).

Due to the blockage of the majority of the light passing through the condenser of a microscope configured for dark-field microscopy, a 100-W tungsten-halogen bulb is required as a minimum for both low- and high-power magnification. Microscope slides must be $\sim 1 \pm 0.1$ mm thick, and slides used for preparation of specimens for dark-field illumination should not be reused. All slides and optical surfaces in the microscope

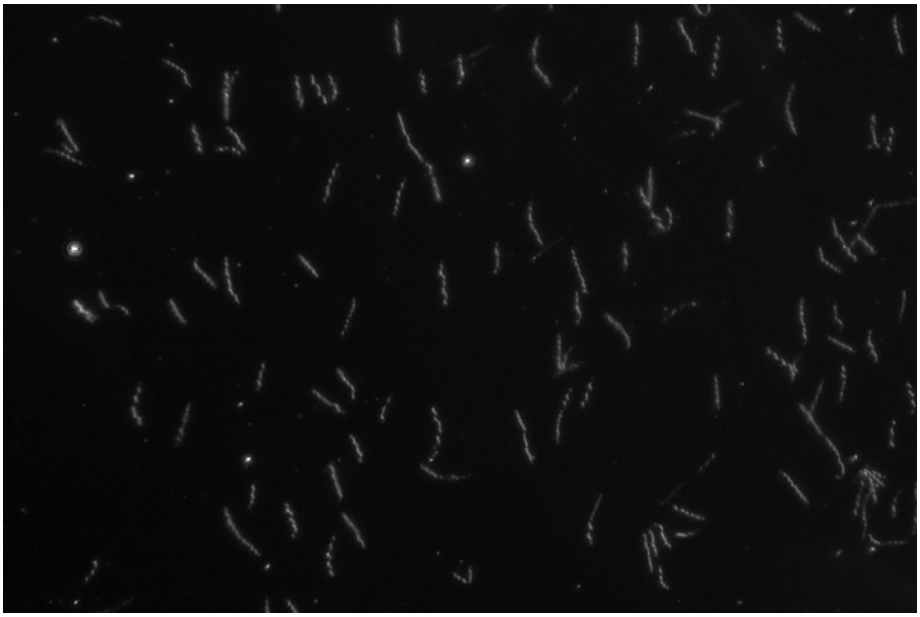

Figure 2A.1.9 Dark-field image of the Lyme disease spirochete, *Borrelia burgdorferi* with a 20× objective and a turret condenser with dark-field stop included.

must be spotlessly clean since dark-field microscopy will detect every speck of dust and particulate matter. Dilute particulate preparations if necessary.

A bright-field microscope can easily be converted for use in dark-field microscopy with the addition a turret condenser with a dark-field stop included. If a turret is not available, any multifunction condenser will work. Dark-field stops can be purchased or handmade. Hayden (2002) provides an excellent resource for instructions on how to devise dark-field patch stops.

ALIGNMENT FOR LOW-POWER MAGNIFICATION DARK-FIELD MICROSCOPY

BASIC PROTOCOL 6

1. Align the microscope as for bright-field Köhler illumination using the lower-power phase objective, but ignore the step used to optimize the opening of the condenser aperture.

   Dark-field conditions require that the condenser aperture be fully opened to its maximum setting to let in as much light as possible. Köhler illumination provides a default position for the user to reference and return to while changing the optics for optimal dark-field settings.

2. Align the condenser turret for dark-field settings using the default dark-field stop. If the turret does not include a dark-field stop, insert the appropriate diameter patch stop (16 to 18 mm stop for use with the 10× objective) by either positioning it into the substage condenser filter holder or by taping it onto the underside of the condenser.

   The turrets usually possess five or six openings, one opening for bright-field, one opening has a dark-field stop (labeled DF), and the remaining openings house several phase stops.

3. Open both the field and the substage condenser aperture iris diaphragms fully to their maximal setting.

   This allows the condenser to operate at its highest NA.

4. Check the dark-field stop diameter by removing the eyepiece and looking down into the focal plane of the objective.

   The dark-field stop diameter should eclipse the bright disk of light that can be normally viewed at the objective rear focal plane under bright-field conditions. If the dark-field stop does not completely mask the bright disk, use the substage screws to center the condenser until the bright disk is fully blocked by the stop. Replace the eyepiece.

5. Place a low-contrast specimen that is suitable for dark-field illumination on the stage.

   The specimen image should be bright against a black background view. Dust and small contaminants on the slide may create artifacts that could obscure the object. Be sure to use premium quality microscope slides and clean them thoroughly with ethanol and Kimwipes Delicate Task Wipers before observing specimens under dark-field illumination. If the specimen image is too dark, increase the light source to its maximum voltage.

6. Focus the condenser by adjusting up or down until a single bright ring of light is central in the field of view.

   When using the condenser dry, the top of the lens should almost come into contact with the bottom of the microscope slide. If a small dark region lies within the bright ring of light, then the condenser is too low. The bright ring of light will appear off-center in the field of view if the microscope is not properly aligned for Köhler illumination.

7. When the condenser is properly adjusted, view the specimen and properly adjust the focus.

   If the field of view has a dark spot in the center obscuring the image, but objects in the periphery appear normal, then the substage condenser may need to be re-positioned or the inappropriate diameter stop is being used or the NA of the objective. Re-establish alignment for Köhler illumination (see Basic Protocol 1) and repeat the steps for dark-field illumination.

BASIC PROTOCOL 7

ALIGNMENT FOR HIGH-POWER MAGNIFICATION DARK-FIELD ILLUMINATION

1. Repeat step 1 of Basic Protocol 6 for low magnification dark-field illumination.
2. Insert a high NA condenser into the substage holder and secure into place.

   Both dry and immersion dark-field condensers are commercially available, but the best results can be obtained with an immersion dark-field condenser due to its internal mirrored surfaces that eliminate chromatic aberration. Use of an oil immersion condenser will also produce the blackest background. The dry dark-field condenser is ideal for objectives with an NA below 0.75. Paraboloid and cardioid immersion condensers are designed for use with objectives with NA up to 1.4.

3. Adjust the condenser until it is <2 mm from the bottom of the specimen slide.
4. Place a low-contrast specimen that is suitable for dark-field illumination on the stage.
5. Use the $10\times$ objective to center the high NA dark-field condensers.

   While viewing the specimen with the $10\times$ objective, focus the condenser as described in step 6 for low magnification dark-field illumination.

6. If using a high NA immersion condenser, apply oil to the lens of the condenser by first lowering the condenser below the mechanical stage and placing a drop of oil on the lens. Next, slowly raise the condenser until it makes contact with the underside of the specimen slide. Make sure to eliminate any air bubbles trapped between the slide and the condenser top lens.

   Note that when an oil immersion condenser is used without oil, regardless of the magnification of the objective used, light will not emerge from the condenser.

7. Switch to the 40× DF objective and then the higher power 100× objective. Position the objective to be used for viewing the specimen.

   Some objectives can be used dry or with oil immersion. For oil immersion–only objectives, place a drop of oil on the specimen slide's cover slip before positioning the objective. Observe the back focal plane of the objective to check for air bubbles. If bubbles are detected, move the objective to the next detent stop on the microscope and remove the oil with lens paper. Then re-apply oil again and re-position the objective.

8. View the specimen and properly adjust the focus.

MATCHING MICROSCOPE MAGNIFICATION TO DETECTOR RESOLUTION

SUPPORT PROTOCOL 1

This procedure uses the diatom test slide (see Table 2A.1.1 for supplier information). For more details on matching a camera to the microscope see Hinsch (1998) or Inoué and Spring (1997).

1. Look down the eyepieces and obtain an in-focus image of the frustrule pores of the diatom *Pleurosigma angulatum* (Fig. 2A.1.10, number 6 in panel A) using 40×/(NA −0.7) phase-contrast or DIC and proper Köhler illumination.

   The pores should be clearly visible by eye.

2. Project the diatom image onto the faceplate of a video or CCD camera.

3. Adjust the gain and contrast camera controls for optimum image brightness and contrast.

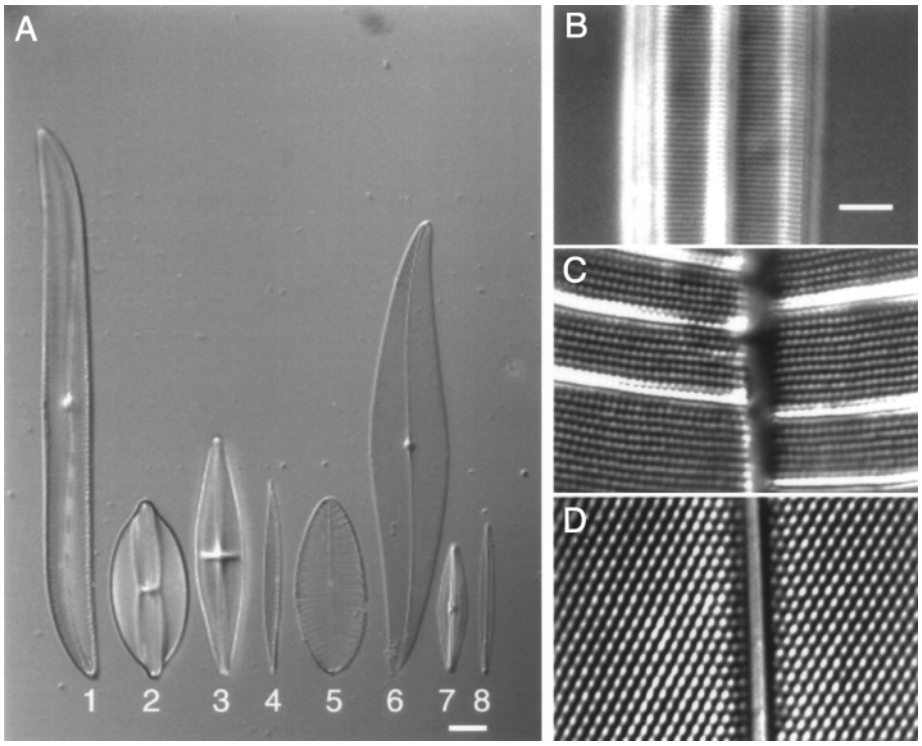

Figure 2A.1.10 (**A**) The diatom test plate. The rows of pores are spaced in the silica shell by ~0.25 μm in *Amphipleura pellucida* (panel **B**; also number 8 on panel A), 0.41 μm in *Surrella gemma* (panel **C**; also number 5 on panel A), and 0.62 μm in *Pleurasigma angulatum* (panel **D**; also number 6 on panel A). Bar: panel A, 10 μm; panels B, C, and D, 2.5 μm. From Salmon and Tran (1998), reprinted with permission from Academic Press.

As you increase contrast, you will need either more camera gain or brighter illumination. Usually, a better signal-to-noise ratio is achieved by leaving the gain control in the middle of its adjustment and increasing illumination intensity.

If the projection magnification to the detector is too small, the frustrule pore lattice will be invisible in the video image, although it will be clearly visible when viewing the image by eye.

4. Increase the magnification to the camera as much as possible.

   As magnification increases, resolution in the image becomes less limited by the resolution of the camera. However, the size of the field of view decreases inversely with magnification. In addition, the intensity of light in the image decreases as magnification^{-2}. The image will become noisy at low light intensities. It will be necessary to increase illumination intensity as much as possible at high magnification or increase the integration period for the camera exposure.

SUPPORT PROTOCOL 2

CALIBRATING IMAGE MAGNIFICATION WITH A STAGE MICROMETER

1. Insert the stage micrometer (see Table 2A.1.1) on the microscope stage.
2. With the low-power objective, find the region of the micrometer with 10-μm scale intervals.

   The larger intervals are 100 μm.

3. Use the 100- or 10-μm scales to calibrate distance in images taken with your objectives.

   The magnifications on the objective barrel and projection lenses are only approximations; accurate measurements of distances in images require a calibration scale.

4. Acquire images of the scale in both the horizontal and vertical directions to check if the camera has square pixels.

TESTS FOR THE OPTICAL PERFORMANCE OF THE MICROSCOPE

Test slides are used to evaluate the performance of the microscope under different conditions.

SUPPORT PROTOCOL 3

Testing Phase-Contrast and DIC Using Diatom Testing Slide

Diatoms have silica shells shaped like pillboxes. There are pores in the shell arranged in a lattice pattern specific for each diatom species. Figure 2A.1.10 shows a low-magnification view of the eight diatoms on the test slide (panel A) plus higher-magnification views of the lattices of three diatoms most useful in testing the resolution performance of microscope optics using phase-contrast or DIC. Number 6 in panel A of Figure 2A.1.10, *Pleurosigma angulatum*, has a triangular pore lattice with spacing of ~0.61 μm between rows (illustrated in Fig. 2A.1.10, panel D). Number 5, *Surrella gemma*, has rows of pores where the rows are separated by ~0.41 μm (illustrated in Fig. 2A.1.10, panel C). Number 8 is *Amphipleura pellucida*, which has horizontal rows of pores separated by ~0.25 μm (illustrated in Fig. 2A.1.10, panel B). In transmitted light, the diffraction limit to lateral resolution, r, is given by (Inoué, 1989):

$$r = \frac{\lambda}{(NA_{obj} + NA_{cond})}$$

where λ is the wavelength of light, NA_{obj} is the objective NA, and NA_{cond} is the condenser NA (see Fig. 2A.1.3). The lateral resolution, r, is equal to 0.195 μm for the highest objective NA, which is equal to 1.4, with $NA_{cond} = NA_{obj}$ and 546 nm green light. As

seen in Figure 2A.1.10B, this objective is capable of resolving the rows of pores in the shell of *Amphipleura*, but not the individual pores, which are slightly <0.19 μm apart.

Use the above equation to calculate the diffraction limit of resolution for your other objectives and use the diatoms to test whether they achieve their theoretical limits.

Testing Phase-Contrast, Dark-Field, and DIC Microscopes Using a Squamous Cheek Cell Test Slide

SUPPORT PROTOCOL 4

Cheek cells are a convenient specimen for testing the performance of phase-contrast, DIC, and dark-field microscopes. As seen in the low-magnification view (Fig. 2A.1.7A) they are large and flat, ≤3 μm thick except near the cell center which contains the nucleus. The upper and lower surfaces have fine ridges which swirl around much like fingerprints. Many of the ridges are <0.2 μm in width and separated by <0.5 to 1.0 μm.

To prepare the cheek cells, gently scrape the inside of your mouth with the tip of a plastic pipet or similar soft tool and spread the cells and saliva on the center of a clean no. 1.5 coverslip. Invert the coverslip quickly onto a clean slide and press down to spread the cell preparation into a thin layer. Seal the edges with a thin layer of nail polish.

Testing Fluorescence Using a Red, Green, and Blue Fluorescent Tissue Culture Cell Test Slide

SUPPORT PROTOCOL 5

The cells in Figure 2A.1.11 are triple labeled: DAPI stained nuclei and chromosomes (blue fluorescence; Fig. 2A.1.11A); Alexa 488-phalloidin labeled actin filaments (green fluorescence; Fig. 2A.1.11B); and X-Rhodamine immunofluorescently labeled microtubules (red fluorescence; Fig. 2A.1.11C). The microtubules and fine actin filamentous arrays are <100 nm in width, but they should appear sharp and in high contrast in the microscope. There should be no "bleed-through" from one fluorescence channel to another if the filters are chosen properly (Taylor and Salmon, 1989).

In fluorescence microscopy, the diffraction-limited lateral resolution is given by (Inoué 1989; Inoué and Spring, 1997):

$$r = \frac{0.61\lambda}{NA_{obj}}$$

and the intensity of the image, I_{image}, is given approximately by:

$$I_{image} \approx \frac{I_{ex} NA_{obj}^4}{M_p^2}$$

where I_{ex} is the excitation light intensity entering the objective and M_p is the projection magnification from the objective to the eye or camera. The rate of photobleaching, R_p, of a specimen depends on:

$$R_p \approx \frac{I_{ex} NA_{obj}^2}{M_{obj}^2}$$

where M_{obj}^2 is the magnification of the objective.

Note from these equations that resolution depends inversely on NA_{obj} (r = 0.24 μm for NA_{obj} = 1.4 and 546 nm green light), image intensity depends on the fourth power of NA_{obj}, while loss of intensity by photobleaching of the fluorophore increases with the

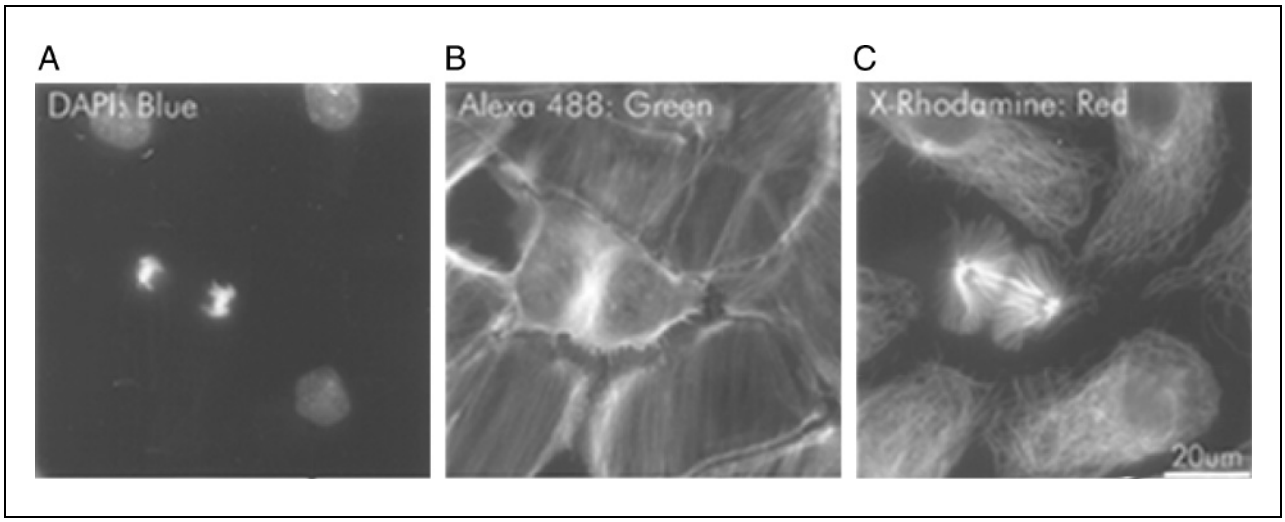

Figure 2A.1.11 Epifluorescent images of fixed tissue culture cells stained with (**A**) DAPI, making DNA fluorescent blue; (**B**) Alexa 488 bound to phalloidin to label actin filaments fluorescent green; and (**C**) X-rhodamine labeled antibodies against tubulin to label microtubules fluorescent red. Bar = 20 μm. Images recorded with a 40×/(NA = 1.4) Plan Fluor objective, 1.5 magnification, to a cooled CCD camera and the multi-modem multiwavelength microscope described by Salmon et al. (1998).

square of objective NA (a measure of the excitation light collected by the objective) and magnification (which concentrates the excitation light on the specimen).

Verify these equations by imaging the fluorescent specimen (the microtubules in the spread cells are a good choice) for objectives with different magnifications and numerical apertures. It will quickly be seen why in fluorescence one wants to use the minimum total magnification necessary to resolve the structures of interest in the detector, as well as use the maximum NA_{obj} in order to maximize light intensity for the least amount of excitation light (and the least amount of photobleaching).

SUPPORT PROTOCOL 6

CARE AND CLEANING OF MICROSCOPE OPTICS

Keeping the microscope optics clean is important for high-quality imaging. Dust, fingerprints, excess immersion oil, or mounting medium on or in a microscope causes reduction in contrast and resolution. DIC is especially sensitive to contamination and scratches on the lens surfaces. Below are steps for keeping the microscope clean.

1. Always keep microscopes covered when not in use.
2. Make sure that all ports, tubes, and unoccupied positions on the lens turrets are plugged.

   Plastic plugs are usually supplied with the microscope.

3. Store objectives in screw-top containers when not on the microscope. Keep accessories—e.g., condensers and compensators—in plastic bags or boxes. Keep slides and coverslips covered.
4. Be careful with salt water, corrosive liquids, and all solvents.
5. When cleaning lens surfaces, avoid touching the lens surface with anything (even lens paper if possible).

   IMPORTANT NOTE: *Never use Kimwipes or commercial facial tissue, because they may contain a filler that is part diatomaceous material (glass). One pass of a Kleenex could ruin an objective.*

6. Remove dust by gently brushing with an oil-free (ether-washed) camel's hair brush or by using a low-velocity stream of purified air.

7. Remove water-soluble contamination using distilled water with a small amount of detergent, such as Kodak Photoflow solution.

   Much (modern) immersion oil can also be removed by washing with detergent.

8. Remove most immersion oil by passing a high-quality lens tissue over the objective or condenser front element.

   IMPORTANT NOTE: *Do not rub. No area on the tissue should come in contact with the lens twice. This prevents dust and dirt removed from the lens from coming back and possibly scratching it. This is easily accomplished by passing the tissue over the lens in a "Z" pattern or by making parallel passes.*

9. Clean objective lenses by holding a piece of doubled lens paper over the objective and placing a few drops of solvent on the paper. Draw the paper across the lens surface so that the solvent flows rapidly in a circular pattern over the recessed lens surface (see Inoué and Spring, 1997). Finish the stroke with a dry portion of the paper. Repeat as necessary.

   In this way, the solvent contacts the lens but the paper does not, because the lens is recessed. For solvent, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface. Avoid soaking a lens with solvent, to prevent damage to lens cements.

10. To clean recessed front elements of dry objective lenses or to remove stubborn dirt, use a cotton-tipped applicator that has been soaked in cleaning solution and then shaken to remove excess fluid. Rotate the cotton tip over the lens surface to clean. Again, first use a 1% solution of Kodak Photoflow in distilled water to remove much of the oil and water-soluble material. Then use a small amount of oil solvent like ethyl ether or xylene to clean all the oil from the surface.

11. Use a detergent solution or ethanol to clean the surfaces of the eyepiece lenses.

    Do not use xylene as it may solubilize enamel surfaces.

COMMENTARY

The development of lasers, electronic cameras, digital image analysis, and specific fluorescent molecular probes have recently made light microscopy an enormously powerful tool in the biomedical sciences. There are a number of excellent books and review articles about these applications, as well as the optical principles of light microscope design and image formation. Listing of all these references is beyond the scope of this unit; only a few of these books and reviews are given here, and these can be used to find other useful references.

Spencer (1982), Zernike (1958), and Keller (1995, 1997, 1998) provide excellent introductions to the principles of image formation and contrast in the light microscope for beginners, while Pluta (1988, 1989) provides a more comprehensive treatment. Inoué and Oldenbourg (1995) also review the basic concepts and principles of microscope design and different methods of image formation. The abovementioned references describe the many modes of transmitted-light imaging in the microscope, including bright-field, phase-contrast, and DIC, which are described in this unit, as well as dark-field, polarization, interference contrast, Hoffman modulation, and reflection interference contrast methods. Taylor and Salmon (1989) and Herman (2002) give clear introductions to fluorescence microscopy. *Video Microscopy* by Inoué and Spring (1997) is a comprehensive introduction to the principles and practical aspects of light microscopy, video, and digital imaging. The *Video Microscopy* volume of *Methods in Cell Biology* edited by Sluder and Wolf (1998) also has contributions that cover many basic concepts and practical aspects of light

microscopy in the biomedical sciences; it also includes chapters on multiwavelength, multimode digital imaging methods, camera selection, ion ratio imaging, and specimen chambers. Salmon and Tran (1998) review the principles of video-enhanced DIC methods used to image macromolecular complexes invisible in the microscope when viewed by eye.

Photography with a Microscope by Rost and Oldfield (2000) provides a detailed overview of dark-field illumination and devising patch stops. Hayden (2002) provides an excellent introduction into the creation and use of dark-field illumination using a bright-field microscope. This reference also points out a comprehensive dark-field microscopy primer located online at *http://www.microscopy.fsu.edu/primer/techniques*. This website is an excellent source for dark-field information, microscope configuration, and troubleshooting. Taylor et al. (1992), Salmon et al. (1998), and Rizzuto et al. (1998) review microscope design for multiwavelength, multimode digital imaging of fluorescent specimens, and four-dimensional microscopy. Advanced quantitative fluorescence methods like ratio imaging, photobleaching, photoactivation, resonance energy transfer, chromophore-assisted laser ablation, and fluorescence lifetime imaging are also reviewed in the *Methods in Cell Biology* volumes edited by Taylor and Wang (1989), and Sluder and Wolf (1998), as well as in Herman and Jacobson (1990), and Inoué and Spring (1997). Reviews on imaging cells containing expressed protein coupled to green fluorescent protein (GFP) include Heim and Tsien (1996), and Sullivan and Kay (1999). Agard et al. (1989), Carrington et al. (1995), and Rizzuto et al. (1998) review how deconvolution of three-dimensional image stacks can produce super resolution in the light microscope. The recent edition of *Handbook of Biological Confocal Microscopy* edited by Pawley (1995) is an excellent reference on this important method for imaging fluorescent structures in thick specimens (Smith, 1997). The new technique of multiphoton laser scanning microscopy is described by Denk et al. (1994), while biological applications of optical traps to manipulate organelles and measure molecular forces is reviewed by Svoboda and Block (1994).

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Total Internal Reflection Fluorescence (TIRF) Microscopy

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ABSTRACT

Total internal reflection fluorescence (TIRF) microscopy represents a method of exciting and visualizing fluorophores present in the near-membrane region of live or fixed cells grown on coverslips. TIRF microscopy is based on the total internal reflection phenomenon that occurs when light passes from a high-refractive medium (e.g., glass) into a low-refractive medium (e.g., cell, water). The evanescent field produced by total internally reflected light excites the fluorescent molecules at the cell-substrate interface and is accompanied by minimal exposure of the remaining cell volume. This technique provides high-contrast fluorescence images, with very low background and virtually no out-of-focus light, ideal for visualization and spectroscopy of single-molecule fluorescence near a surface. This unit presents, in a concise manner, the principle of operation, instrument diversity, and TIRF microscopy applications for the study of biological samples. *Curr. Protoc. Microbiol.* 10:2A.2.1-2A.2.22. © 2008 by John Wiley & Sons, Inc.

Keywords: total internal reflection fluorescence microscopy

INTRODUCTION

Over the past 25 years, researchers from physics and biology have shown significant interest in developing optical techniques for investigating nanostructures (e.g., single molecules) beyond the diffraction limit of light. Total internal reflection fluorescence (TIRF) microscopy is such a technique. TIRF was introduced in 1965 by Hirschfeld (1965) as a technique for selective surface illumination at a solid/liquid interface. This microscopy technique was further developed and refined by Axelrod (see Axelrod et al., 1983, 1984), with later theoretical contributions from Reichert, Truskey (see Reichert, 1989; Reichert and Truskey, 1990), and Burmeister (see Burmeister et al., 1998).

TIRF microscopy represents a method of exciting and visualizing fluorophores present in the near-membrane region of live or fixed cells grown on coverslips. If the microscope is thoroughly calibrated, the separation distance between cell membrane and substrate can be measured (Reichert, 1989; Truskey et al., 1992). In addition, any movement of fluorescent-tagged proteins in focal adhesion contacts, at the membrane level or in its immediate proximity, can be monitored quantitatively in real time.

TIRF microscopy is based on the total internal reflection phenomenon that occurs when light passes from a high-refractive index medium (e.g., glass) into one with a lower refractive index (e.g., water, cell; see Table 2A.2.1). Due to the difference between the refractive indices at the interface, only a short-range electromagnetic disturbance, called the evanescent field, will pass into the low-refractive medium. This type of excitation can be used to obtain high-contrast fluorescence images, with very low background and virtually no out-of-focus light. While resolution in optical microscopy is usually diffraction limited (see Table 2A.2.1) by the wavelength of light, the TIRF optical imaging technique allows imaging of cellular structures at the coverslip-cell interface with a higher z-resolution ($<$100 nm) than confocal microscopy ($\sim$300 nm) in a wide-field approach (see *UNIT 2C.1* for more on confocal microscopy).

This unit presents, in a concise manner, the principle of operation, instrument diversity, and TIRF microscopy applications for the study of biological samples.

PRINCIPLE OF OPERATION

TIRF microscopy is based on the total internal reflection phenomenon that occurs when

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Table 2A.2.1 Glossary of Optical Terms

Reflection	The return of light by a surface, in the same medium, without a change of wavelength (the angle of incidence equals the angle of reflection)
Refraction	The bending of oblique incident light as it passes an interface between media of different refractive indices, which implies a change of speed (Snell's law; see Equation 2A.2.1)
Refractive index	A characteristic of a material (medium) that represents the ratio of the velocity of light in vacuum to the velocity of light in that medium
Diffraction	Refers to various phenomena associated with the bending of waves when they interact with obstacles in their path
Diffraction limit	The effect in an optical microscope where a beam of light of a finite size undergoes diffraction and spreads in diameter, limiting the minimum diameter d (i.e., the diffraction limit) of the spot of light formed at the focus of a lens: $d = 1.22 \times \lambda \times f/a$, where λ is the wavelength of light, f is the focal length of the lens, and a is the diameter of the beam of light, or (if the beam is filling the lens) the diameter of the lens

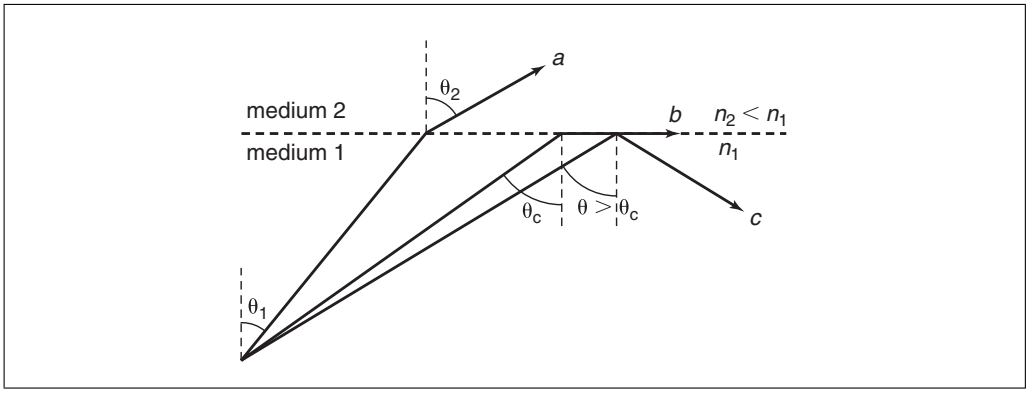

Figure 2A.2.1 Light path at the interface between two media. (***a***) Refracted light at an angle of incidence smaller than the critical angle; (***b***) light at an angle of incidence equal to the critical angle of light, traveling along the interface between the two media ($\theta_2 = 90^\circ$); and (***c***) total internal reflection occurring at incident angles higher than the critical angle. θ_1, incident angle; θ_2, refractive angle; θ_c, critical angle; n_1 and n_2, refractive indices of the two media.

light passes from a medium with a high refractive index into a medium with a low refractive index (n_1 and n_2, respectively, in Fig. 2A.2.1). At the interface, the light will bend and travel along the interface if the incident angle is equal to the critical angle. If the incident angle is higher than the critical angle, the light will turn back into the high refractive medium and only a short-range electromagnetic disturbance called the evanescent field will pass into the low refractive medium. The evanescent field intensity decreases exponentially with distance from the interface (Fig. 2A.2.2), confining the eventual fluorescence excitation to the near-field region (excitation decays over ~λ/4 to λ/5; Axelrod, 2001a). This imaging method provides high-contrast images of the near-membrane area, with a useful maximum depth of penetration of 100 to 200 nm. Due to minimal exposure of cells to light in any other planes in the sample, TIRF images have very low background, with virtually no out-of-focus fluorescence (Fig. 2A.2.3) and low phototoxicity.

TIRF Parameters

To better understand the characteristics and the usefulness of this microscopy technique, we will briefly discuss the theoretical TIRF parameters. See Table 2A.2.1 for definitions of terminology related to optics.

The relationship between the angles of incidence (θ_1) and refraction (θ_2) and the indices of refraction of the two media—n_1 and n_2 are the indices of refraction of the incident and refractive medium, respectively—is known as Snell's Law. This law will apply to the refraction of light in any situation, regardless of what

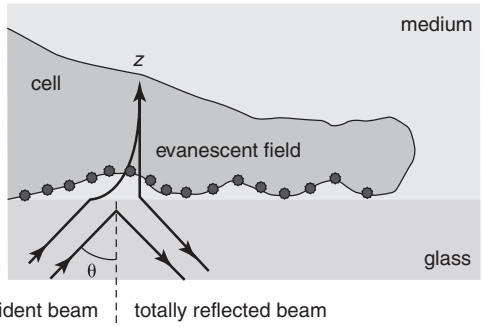

Figure 2A.2.2 Schematic representation of the TIRF effect. The evanescent field produces selective excitation of the fluorophores at the cell-coverslip interface. θ, angle of incidence; *z*, distance from the interface.

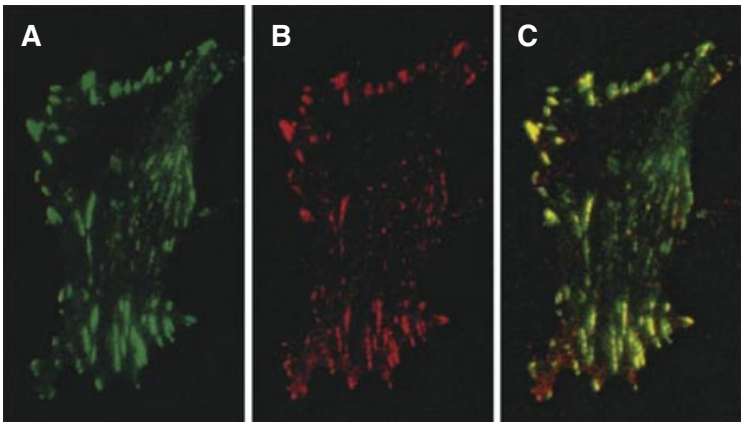

Figure 2A.2.3 A fixed vascular smooth muscle cell labeled with vinculin-Alexa 488 was imaged by epifluorescence (**A**) and TIRF (**B**; pseudo-colored red). The TIRF image was overlapped with the epifluorescence image (**C**) to emphasize the differences between the two imaging modes (yellow represents good overlap between the two images). The discrete pixilation of vinculin-Alexa 488 in the TIRF image (B) is due to the fact that it was obtained by optically sectioning only the very bottom of the cell at a maximum depth of 100 nm, compared to the epifluorescence image which was obtained by collecting the fluorescence through the whole cell volume. Image size is 150 × 110 μm. For the color version of this figure go *to http://www.currentprotocols.com.*

the two media are.

$$n_1 \sin\theta_1 = n_2 \sin\theta_2$$

Equation 2A.2.1

The critical angle of incidence (θ_c in Fig. 2A.2.1) represents the incident angle at which the light travels along the interface between the two different media:

$$\theta_2 = 90^\circ \rightarrow \sin\theta_{critical} = \frac{n_2}{n_1}$$

Equation 2A.2.2

For incidence angles higher than the critical angle, the light will turn back into the high-refractive medium, and only a short-range electromagnetic disturbance called the evanescent field will pass into the low-refractive medium. The total internal reflection (TIR) effect is obtained for a ratio $n_2/n_1 < 1$, with the maximum incident angle (Axelrod, 2001a):

$$\sin\theta_{max} = \frac{NA}{n_1}$$

Equation 2A.2.3

where NA represents the numerical aperture of the microscope objective.

The evanescent field intensity $I(z)$ decreases exponentially with the distance z from the interface of the two media, depending on both

the incident angle and the polarization of the incident light (Fig. 2A.2.4):

$$I(z) = I_o \exp\left(-\frac{z}{d}\right)$$

Equation 2A.2.4

with the depth of penetration given by:

$$d = \frac{\lambda}{4\pi} \frac{1}{\sqrt{n_1^2 \sin\theta_1^2 - n_2^2}}$$

Equation 2A.2.5

This depth of penetration d is independent of the polarization of light and represents the depth of field at which the intensity of evanescent light is $1/e$ of the boundary intensity I_o. The sensitivity of the method is directly related to fluorescence intensity, which is maximal at the interface and decreases exponentially with increasing distance from the interface.

In a simple way, one can consider the ideal case of an interface between two optically transparent media, where no light is reflected back and the fluorophores are homogeneously distributed and randomly oriented in a thin layer (Mathur et al., 2000). The fluorescence intensity in TIRF illumination is proportional to the local electric field energy, which means that it exponentially decreases with the relative separation distance (z) from the interface of the two media. These assumptions allow determination of the distance z from the logarithmic transformation of the fluorescence intensity (Fig. 2A.2.5).

In actual experiments, the interface may be stratified, being approximated with a thin multilayer system rather than an interface between two media (Burmeister et al., 1998; Axelrod, 2001b). The presence of an intermediate layer of dielectric material with a mismatched refractive index deposited on the glass coverslip will affect the evanescent field intensity and depth of penetration. A detailed presentation of the expressions for the evanescent intensities of stratified interfaces is presented in Gingell et al. (1987).

Diversity of TIRF Experimental Configurations

There are different optical configurations for carrying out TIRF in an optical microscope. In the early 1980s, Axelrod described TIRF experimental configurations for use on the stage of an inverted (Axelrod et al., 1983) or an upright (Axelrod et al., 1984) microscope. This optical instrumentation was based on the use of a glass prism placed in optical contact with the coverslip of the cell culture dish (Axelrod, 2001b). Figure 2A.2.6 presents schematic drawings for prism-based TIRF configurations for an inverted microscope.

In the early 2000s, very high–NA objectives became commercially available. The new 1.45- to 1.65-NA objectives are more suitable for live cell work than the former prism configurations. In the "prismless" or "through-the-lens" TIRF (Axelrod, 2001c) the total internal reflection condition is achieved in the microscope with off-center illumination from the periphery of the back focal plane of the objective. Figure 2A.2.7 shows schematic optical paths for through-the-lens TIRF

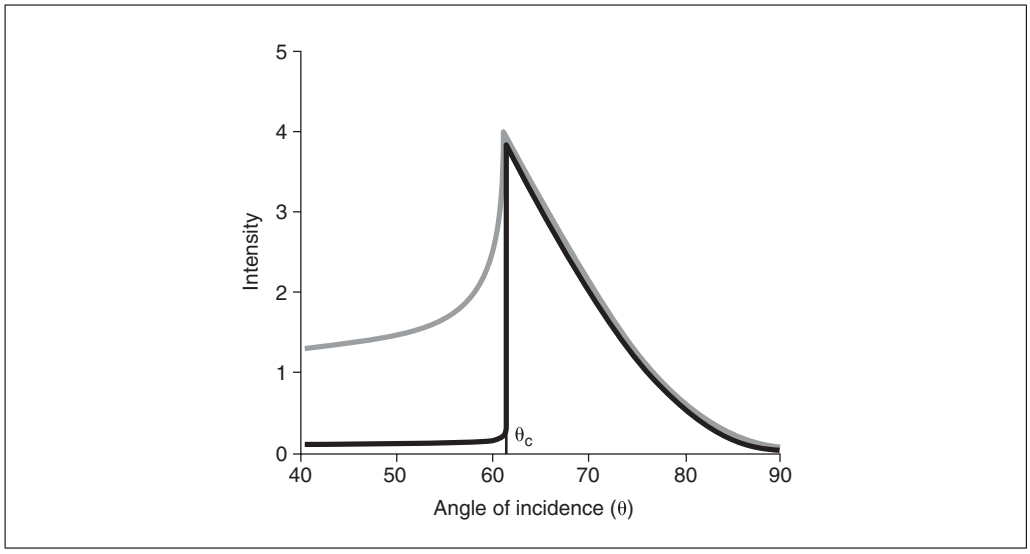

Figure 2A.2.4 Evanescent field intensities of the two planes of polarization parallel (gray line) and perpendicular (black line) to the sample plane as a function of the incident angle.

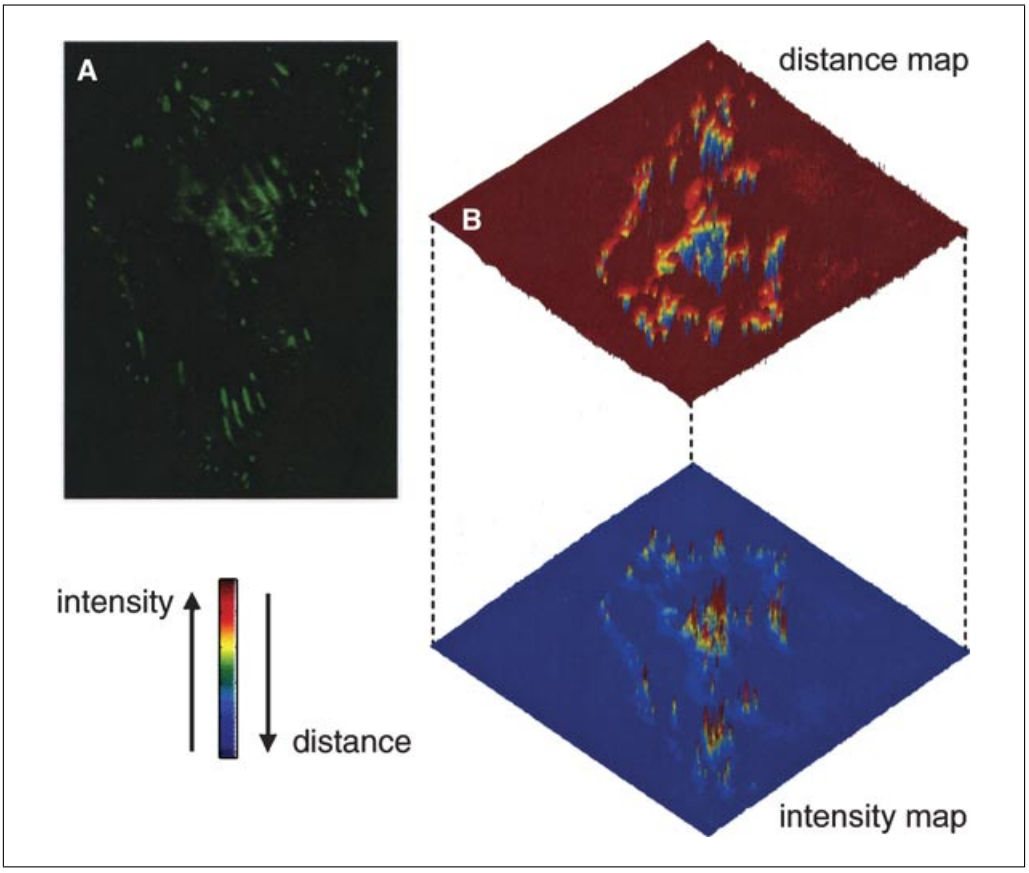

Figure 2A.2.5 (**A**) A vascular smooth muscle cell transiently transfected with GFP-vinculin was imaged in TIRF mode. (**B**) A three-dimensional representation of the fluorescence intensity and the corresponding linear logarithmic transformation of the intensity that generates the separation coverslip-cell distance map. In the distance map, blue indicates a position close to the coverslip and red indicates a position distant from the coverslip. In the intensity map, blue indicates low intensity and red indicates high intensity. The rainbow scale in the lower left shows the colors associated with intermediate levels of distance or intensity. Image size is 70 × 110 μm. For the color version of this figure go to *http://www.currentprotocols.com*.

Table 2A.2.2 Commercially Available TIRF Systems

Manufacturer	Web site
Leica	*http://www.leica-microsystems.com/products/am_tirf_mc*
Nikon	*http://www.nikon-instruments.jp/eng/page/products/tirf.aspx*
Olympus	*http://www.olympusmicro.com/primer/techniques/fluorescence/tirf/olympusaptirf.html*
TILL Photonics	*http://www.till-photonics.com/Applications/tirf.php*
Zeiss	*http://www.zeiss.de*

configurations in an inverted microscope, using side or rear microscope ports for introducing the laser light or conventional arc lamp illumination. Table 2A.2.2 lists the present manufacturers of TIRF modules as turn-key systems.

Introduction of the laser illumination through the side port requires a special dichroic mirror cube facing the side, which is available for the Olympus IX-70 microscope. The beam is focused at the back focal plane (BFP) of the objective at a radial position sufficient to lead to supercritical angle propagation into the coverslip. Moving the lens (L) transversely changes the angle of incidence at the sample and allows switching between subcritical (epi) and supercritical (TIR) illumination (Fig. 2A.2.7a).

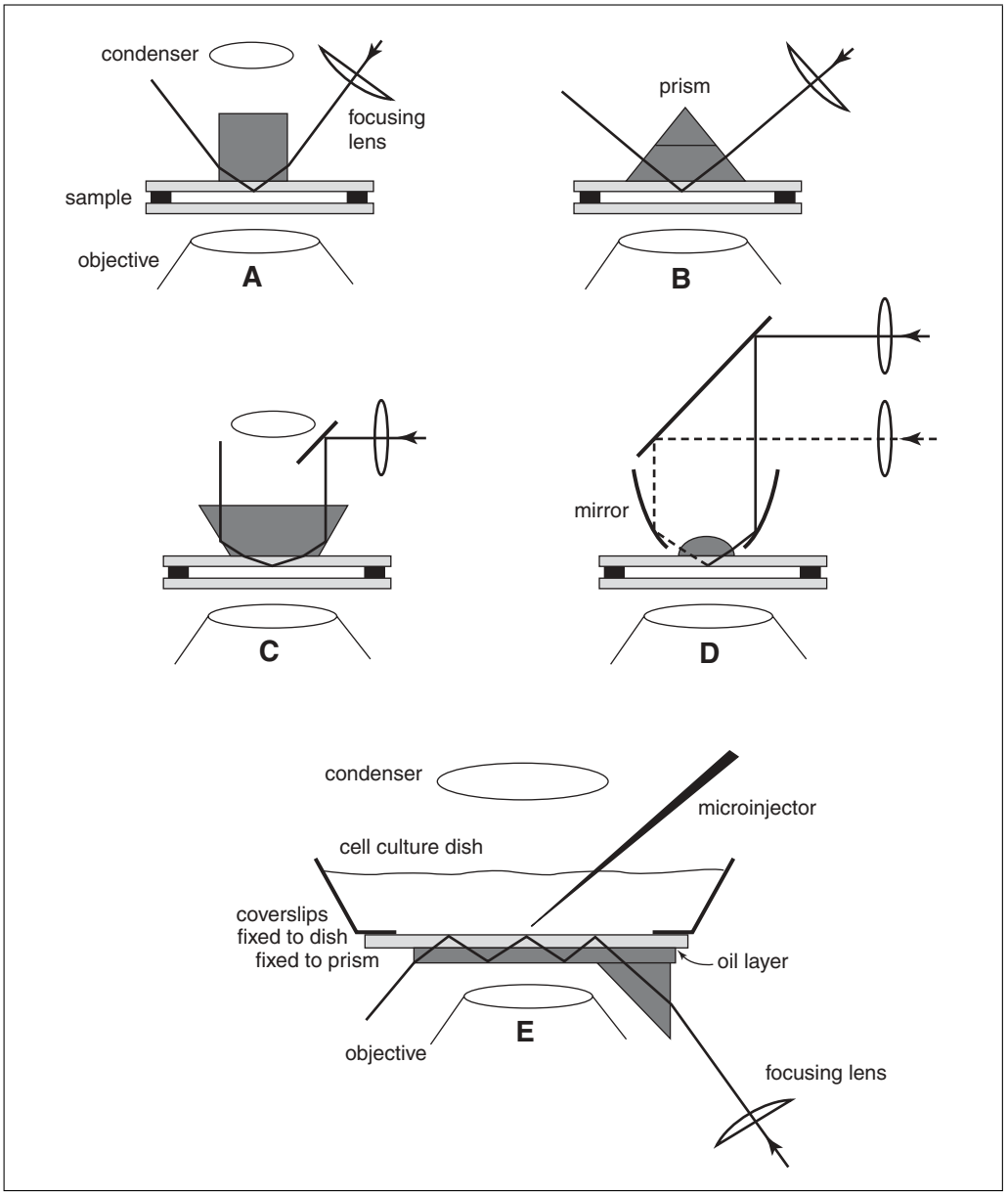

Figure 2A.2.6 Schematic drawings for prism-based TIRF in an inverted microscope (laser illumination). Configurations **A** to **D** use a TIR prism above the sample. In these configurations, the buffer-filled sample chamber consists of a lower bare glass coverslip, a spacer ring (Teflon) and the fluid cell coverslip inverted so the cells face down. The upper surface of the fluid cell coverslip is put in optical contact with the prism by a layer of immersion oil or glycerol. The lateral position of the prism is fixed but the sample can be translated while still maintaining optical contact. In configuration D, two incident beams split from the same laser and intersect at the TIR surface, thereby setting up a striped interference pattern on the sample which is useful in studying surface diffusion. Configuration **E** places the prism below the sample and TIR effect depends on multiple internal reflections in the substrate. This configuration thereby allows complete access to the sample from above for solution changing and/or electrophysiology studies. However, only air or water immersion objectives may be used because oil at the substrate lower surface will thwart the internal reflections. The vertical distances are exaggerated for clarity. Reproduced from Axelrod (2001b) with permission from Blackwell Publishing.

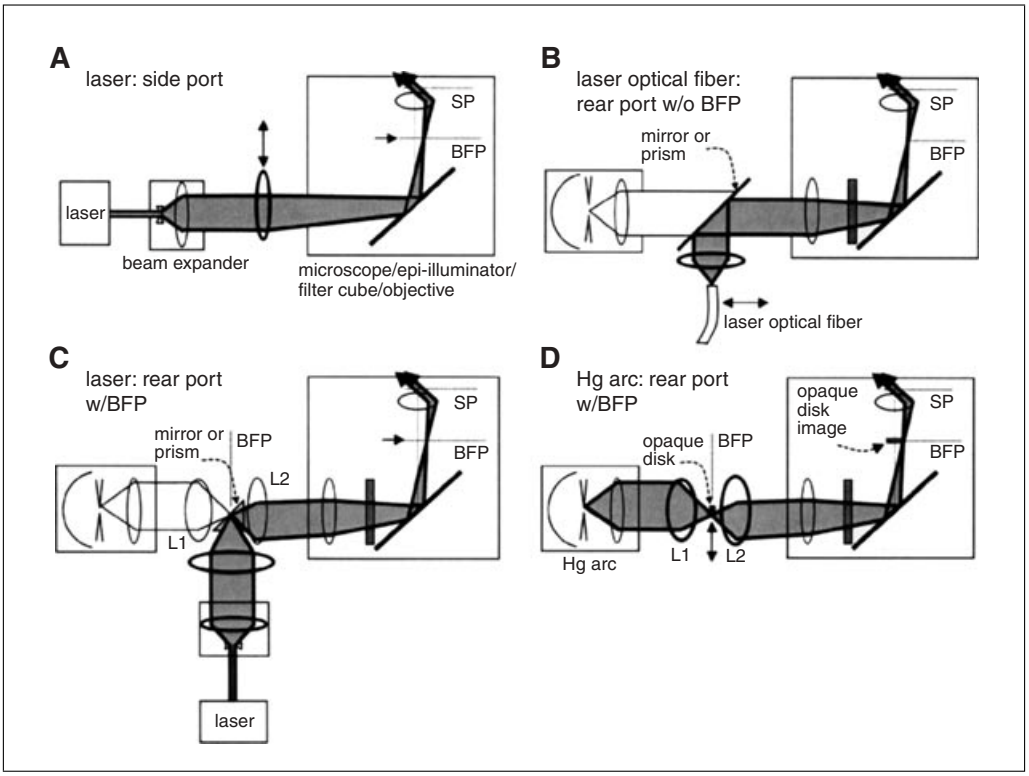

Figure 2A.2.7 Schematic drawings for prismless TIRF in an inverted microscope. (**A**) Laser illumination through a side port. (**B**) Laser illumination introduced by an optical fiber through the rear port normally used by the arc lamp. (**C**) Laser illumination in microscope systems containing an equivalent back focal plane (BFP) in the rear path normally used by an arc lamp. (**D**) Arc lamp TIR illumination. If (as in the Olympus IX-70) an aperture plane does not exist in the indicated position, it can be created with a pair of lenses L1 and L2 (C and D). The illumination at the BFP is a circular annulus; it is shown as a point on one side of the optical axis for pictorial clarity only. In all these configurations. SP refers to sample plane and BFP refers to the objective back focal plane or its equivalent planes (i.e., aperture planes). Components drawn with thick lines need to be installed; components in thin lines are possibly preexisting in the standard microscope. The BFP "aperture plane" (L1 and L2) in panels C and D is also provided by the Nikon TE 2000-U microscope. Reproduced from Axelrod (2001b) with permission from Blackwell Publishing.

Laser illumination introduced by an optical fiber through the rear port of the microscope shares the same port used by the arc lamp. This scheme is employed by the commercial Olympus TIRF device (Fig. 2A.2.7b). Laser illumination in microscope systems containing an equivalent BFP in the rear path normally used by an arc lamp will require that the laser beam be focused at BFP where the arc lamp would normally be imaged. The Zeiss Axiovert 200 provides this BFP "aperture plane" (Fig. 2A.2.7C).

In arc lamp TIR illumination, the goal is to produce a sharp-edged image of an opaque circular disk at BFP such that only supercritical light passes through the objective (Fig. 2A.2.7D). The actual physical disk (aluminized coating on glass) must be positioned at an equivalent upbeam BFP, which in Kohler illumination also contains a real image of the arc. The Zeiss Axiovert 200 provides this BFP, marked as an "aperture plane." The through-the-lens arc-lamp TIRF configuration (Fig. 2A.2.7D) can be easily switched to the laser TIRF configuration (Fig. 2A.2.7C) by insertion of the reflecting mirror into the arc-lamp path.

TIRF Objective Lenses

There are two types of high-NA objectives for TIRF applications currently available on the market: Olympus, Nikon, and Zeiss offer 1.45-NA oil immersion objectives, and Olympus offers a 1.65-NA. The 1.45-NA objectives require glass coverslips (n = 1.51) and standard immersion oil and are the objectives of choice for versatile TIRF microscopy. For special applications that use arc lamp illumination (Stout and Axelrod, 1989) or very dim samples, the 1.65-NA

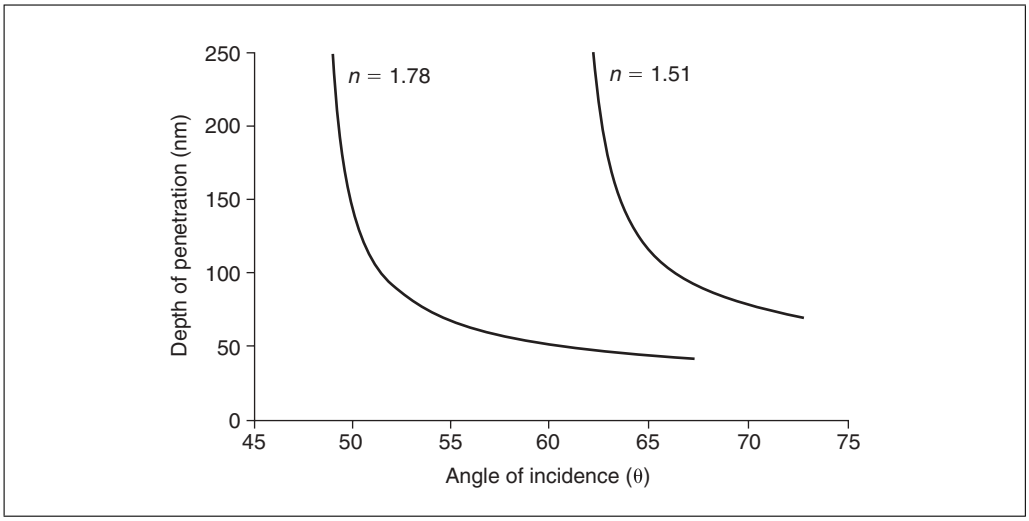

Figure 2A.2.8 Depth of penetration of the evanescent field versus angle of incidence (θ) for the two different refractive indices (*n*) of the objective-coverslip combination used in TIRF.

Table 2A.2.3 TIRF Parameters

NA	$\theta_{critical}$[a]	θ_{max}[a]	*d* (nm)[a]
1.45	~61°	~72°	<100
1.49	~61°	~80	<100
1.65	~48°	~67°	<220

[a]Theoretical values calculated for $\lambda = 488$ nm, $n_1 = 1.516$ (or 1.788 for the 1.65-NA lense), $n_2 = 1.333$.

Table 2A.2.4 TIRF Objectives

Manufacturer	Objective	Numerical aperture (NA)
Olympus	APO 60×	1.45
	PLAN APO 100×	1.45
	APO 100×	1.65[a]
	U APO 150×	1.45
Zeiss	Plan Fluor 100×	1.45
Nikon	Plan APO 60×	1.45
	APO 60×	1.49
	Plan APO 100×	1.45
	APO 100×	1.49
Leica	HCX PL Apo 100×	1.46

[a]Requires high refractive–index coverslips (see Axelrod, 2001c): Sapphire (birefringent) or Schott glass LAF21 and SF11 (*http://www.us.schott.com*) and high refractive index oil ($n = 1.78$) from Cargille Laboratories (*http://www.cargille.com/immeroil.shtml*).

objective might be a better choice. This particular objective requires higher refractive–index coverslips and immersion oil ($n = 1.78$; Cargille Laboratories). The high refractive–index oil is volatile and leaves a crystalline residue in a short period of time. There are several choices for the high refractive–index coverslip, but all are expensive, and they cannot be easily cleaned (Axelrod, 2001c). Figure 2A.2.8 and Tables 2A.2.3 and 2A.2.4 present generic characteristics for these objectives.

PRACTICAL GUIDELINES

Control of the Angle of Incidence

Figure 2A.2.9 presents, in detail, the optical path for a through-the-lens TIRF configuration in an inverted microscope. The laser beam used for excitation is collimated, and then focused at the back focal plane of the objective at a chosen angle of incidence θ with respect to the optical axis of the microscope. The point of focus in the back focal plane is off-axis (black solid line). There is a one-to-one correspondence between the off-axis radial distance (δ), which is dictated by the distance between the optical axis of the microscope and the fiber position at the entrance of the TIRF illuminator (*d*), and the angle of incidence θ. By moving the micrometer that changes the fiber position with respect to the optical axis, the axial radial distance (δ) will increase, and the critical angle of incidence can be exceeded to allow supercritical angles to be reached in a reproducible manner. This adjustment reaches its limit when the laser light is damped into the edge of the objective (Axelrod, 2001c).

Microscope Stability

For obtaining reproducible TIRF images, the microscope focus should be as stable as possible. For quantitative or time-lapse measurements, the focus stability is of great importance. The microscope room should be kept at a constant temperature, and the microscope might require a warm-up time that allows for thermal equilibrium to be achieved (see *UNIT 2C.5*). The new autofocus lock-in systems commercially available from Olympus, Applied Scientific Instrumentation, and Nikon, may improve the focus stability performance.

Sample Preparation

Best TIRF images are obtained on cells cultured on good quality glass coverslips. Due to the small penetration depth of the evanescent field, the quality and thickness of the coverslip are important. Usual TIRF images are obtained by fluorescent labeling of the molecules at the cell level. In this case, TIRF microscopy will provide high-intensity fluorescence images of the labeled molecules on a dark background (Fig. 2A.2.3 and Fig. 2A.2.5). The contact

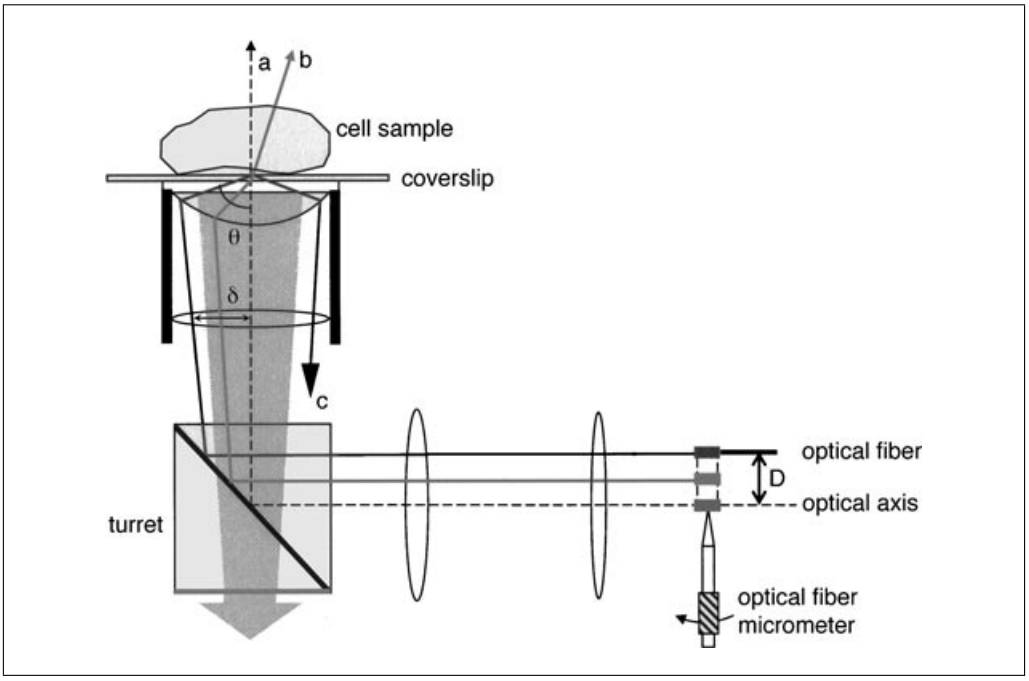

Figure 2A.2.9 Schematic representation of the TIRF optical path in a through-the-lens microscope configuration. The TIRF illuminator provides a micrometer that allows the controlled movement of the optical fiber off-axis to achieve the total internal reflection (TIR) condition at the sample level. Ray a is obtained with the fiber optic centered on the optical axis. Ray b is obtained for an incident angle at the sample plane smaller than the critical angle. Ray c is obtained when TIR effect takes place. The fluorescence light reflected from the sample plane is collected through the same microscope objective and is further sent to the camera where the image is recorded. θ, angle of incidence at the sample; *d*, distance between the optical axis and the fiber position; δ, off-axis distance of the laser beam in the back focal plane of the objective.

regions between cell and substrate could also be investigated by "negative" TIRF. In this case, the cells are not labeled, but a membrane-impermeant dye is added to the medium outside of the cells. The contact regions will appear as dark areas on a fluorescent background.

For some TIRF experiments, it is useful to biofunctionalize the substrate (Fulbright and Axelrod, 1993; Burmeister et al., 1994). This involves pretreatment of the glass substrate before culturing the cells. One should take into consideration the thickness of any pretreatment when coating the glass substrate. If the coating is too thick, it will prevent the evanescent light from reaching the cell membrane and its close proximity.

Artifacts

The oil used between the objective and the coverslip is another important aspect in obtaining good TIRF images. Air bubbles or contaminants in the oil will introduce a mismatch into the refractive indices, producing scattered light. If the oil does not form a film of uniform thickness between the two glass elements, a nonuniform illumination of the sample plane could cause a random striped pattern of light and dark bands (Temple, 1981), and the stripes will move when the stage moves. Dust on the microscope objective will produce similar light and dark stripes in the field of view, but the stripes will not follow the stage movement. Dust on the optics inside the turret could produce a concentric interference fringe pattern. The best way to clean these optical elements is to blow dust away, avoiding wiping the optical surfaces.

TIRF Applications

The unique features of TIRF make this microscopy technique an invaluable tool for biological studies that is finding increasing applications. TIRF can be further combined with other techniques to cover a much larger range of applications, for instance: visualization and spectroscopy of single-molecule fluorescence near a surface by combining TIRF with fluorescence correlation spectroscopy (FCS; Lieto et al., 2003); measurement of the kinetic rates of binding of extracellular and intracellular proteins to cell surface receptors and artificial membranes by combining TIRF with fluorescence recovery after photobleaching (FRAP; Axelrod et al., 1986); measurements of intermolecular distances between fluorescent surface-bound molecules by combining TIRF and Förster resonance energy transfer (FRET, also known as fluorescence resonance energy transfer; Seong and Almers, 2004); observation of turnover of the cytoskeleton and remodeling of focal adhesions in real time by combining TIRF with fluorescence speckle microscopy (FSM; Adams et al., 2004); study of Ca^{2+} channels activity by patch-clamp technique combined with TIRF (Demuro and Parker, 2004); single-molecule force and fluorescence measurements by a combined optical trap and TIRF (Wallace et al., 2003; Lang et al., 2004); monitoring of modifications in the micromorphological structures and dynamics of live cells by combining AFM with TIRF (Sund et al., 1999; Mathur et al., 2000; Wallace et al., 2003; Trache and Meininger, 2005); and making long-term fluorescence movies of cells during development in culture under TIRF illumination (Wang and Axelrod, 1994).

TIRF Applications to Microbiology

Single molecule detection using TIRF imaging

DNA mapping is an important tool in genomic sequence assembly, medical diagnostics, and microbial pathogen identification (van Belkum, 1994; Ried et al., 1997; Wong et al., 1997). A novel method for optical DNA mapping has been developed by Xiao et al. (2007), using TIRF imaging. Direct imaging

Figure 2A.2.10 *(at right)* Direct imaging of DNA molecules and localization of multiple sequence motifs of lambda phage DNA. (**A**) The predicted Nb.BbvC I map of lambda DNA. Positions of the nicking sites are indicated by arrows. Nicking sites 2 to 4 and 5 to 6 are closely clustered and are not resolvable due to the limits of optical diffraction. (**B**) In the intensity-scaled composite image of linear lambda DNA, the Nb.BbvC I sites (labeled with Tamra-ddUTP) are shown as green spots, and the DNA backbone (labeled with YOYO) is shown as blue lines. Due to the diffraction limits of the microscope, only four labels can be fully resolved. In this field, two DNA fragments (a and b) are fully labeled while one fragment (**c**) has three labels. Red arrows point to clustered sites; some of them are brighter than others because of the presence of multiple labels. (**C**) The sequence motif map in the bottom graph was obtained by analyzing 61 single molecular fluorescence images. The solid line is the Gaussian curve fit, and the peaks correspond well to the predicted locations of the sequence motifs. Reproduced from Xiao et al., (2007) with permission from Oxford University Press. For the color version of this figure go *to http://www.currentprotocols.com.*

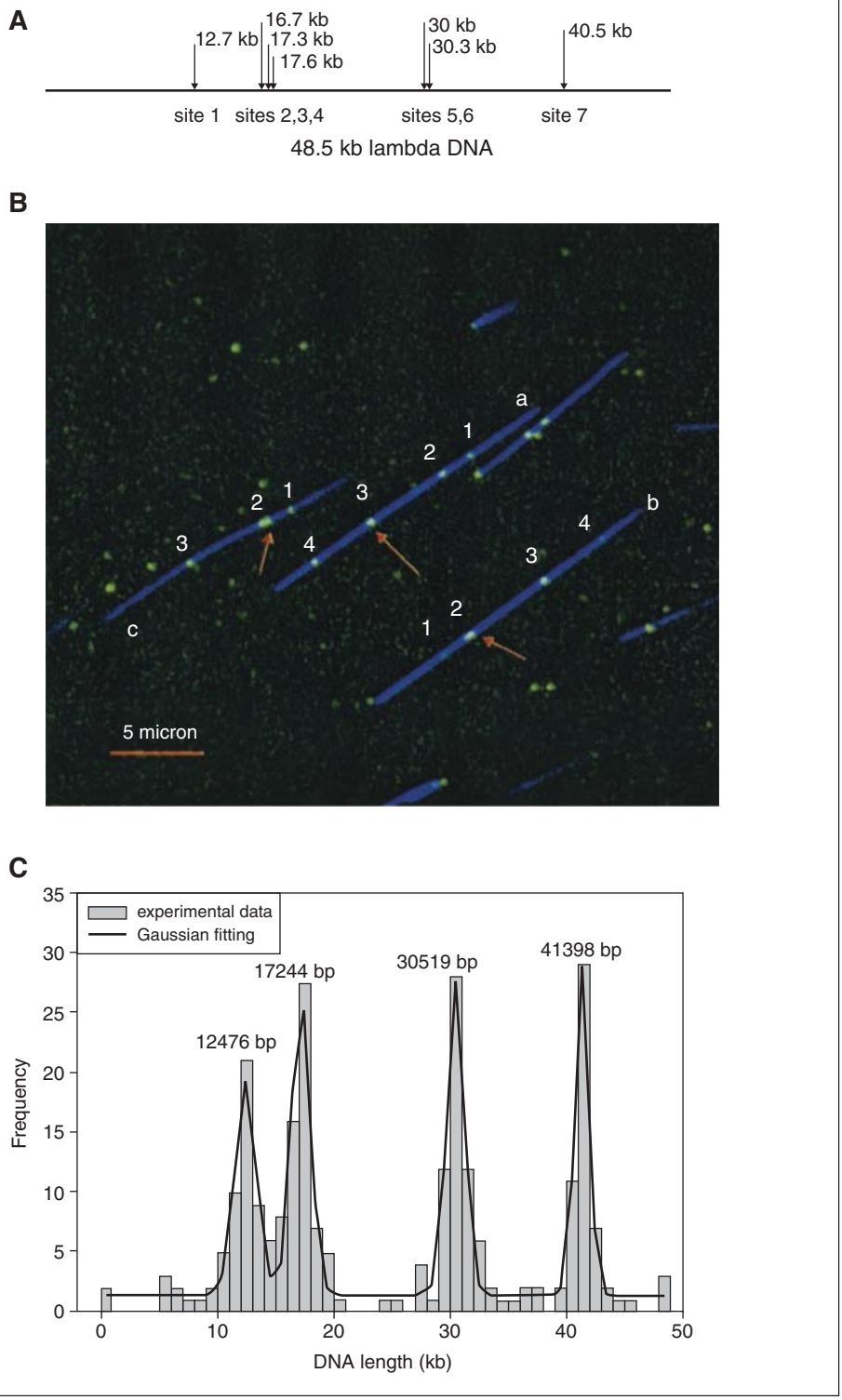

Figure 2A.2.10 *(legend at left)*

of individual DNA molecules and localization of multiple sequence motifs is possible by fluorescently labeling DNA molecules at specific sequence motifs by the action of nicking endonuclease, followed by the incorporation of dye terminators with DNA polymerase (Sanger and Coulson, 1975). After labeling, the DNA molecules were stretched into linear form on a modified glass surface and individually imaged using multicolor TIRF microscopy with nanometer accuracy (Yildiz et al., 2003). This approach has been applied to the construction of sequence motif maps of lambda-phage (Fig. 2A.2.10), a strain of human adenovirus (Fig. 2A.2.11), and human rhinovirus (Fig. 2A.2.12).

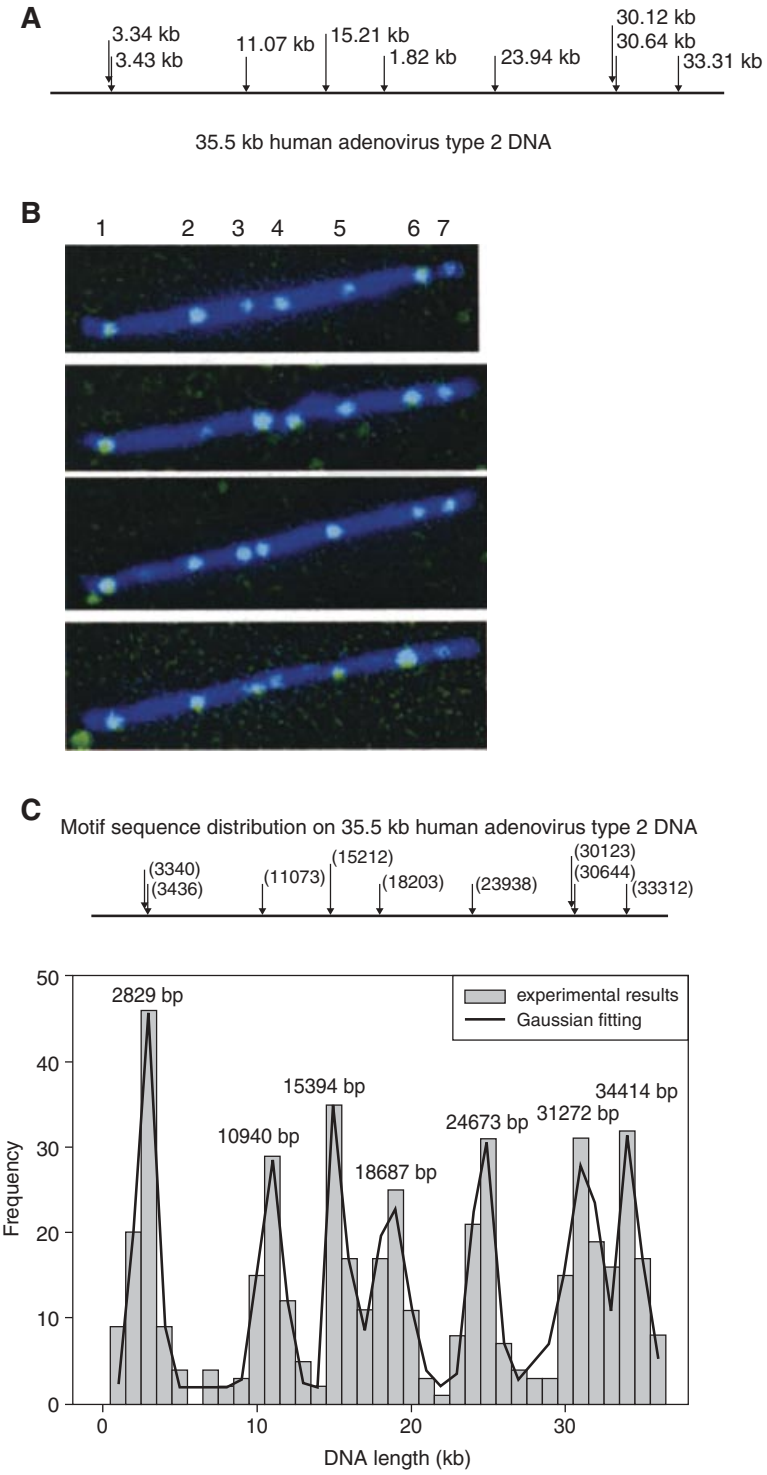

Figure 2A.2.11 Direct imaging of DNA molecules and localization of multiple sequence motifs of human adenovirus type 2. (**A**) Predicted Nb.BbvC I map of human adenovirus type 2. Nine sites are found on the 35.5-kb viral DNA with two sets of clustered sites (1 to 2 and 7 to 8), leading to seven resolvable labels. (**B**) In the intensity-scaled composite images of four fully labeled human adenovirus type 2 DNA, the Nb.Bbvc I sites (labeled with Tamra-ddUTP) are shown as green spots, and the DNA backbone (labeled with YOYO) is shown as blue lines. Due to the diffraction limits of the microscope, only seven labels can be fully resolved. Labels 1 and 6 are generally brighter than the other labels due to clustering. (**C**) The sequence motif map in the graph was obtained by analyzing 63 single molecule fluorescence images. The solid line is the Gaussian curve fitting and the peaks correspond well to the predicted locations of the sequence motif. Reproduced from Xiao et al. (2007) with permission from Oxford University Press.

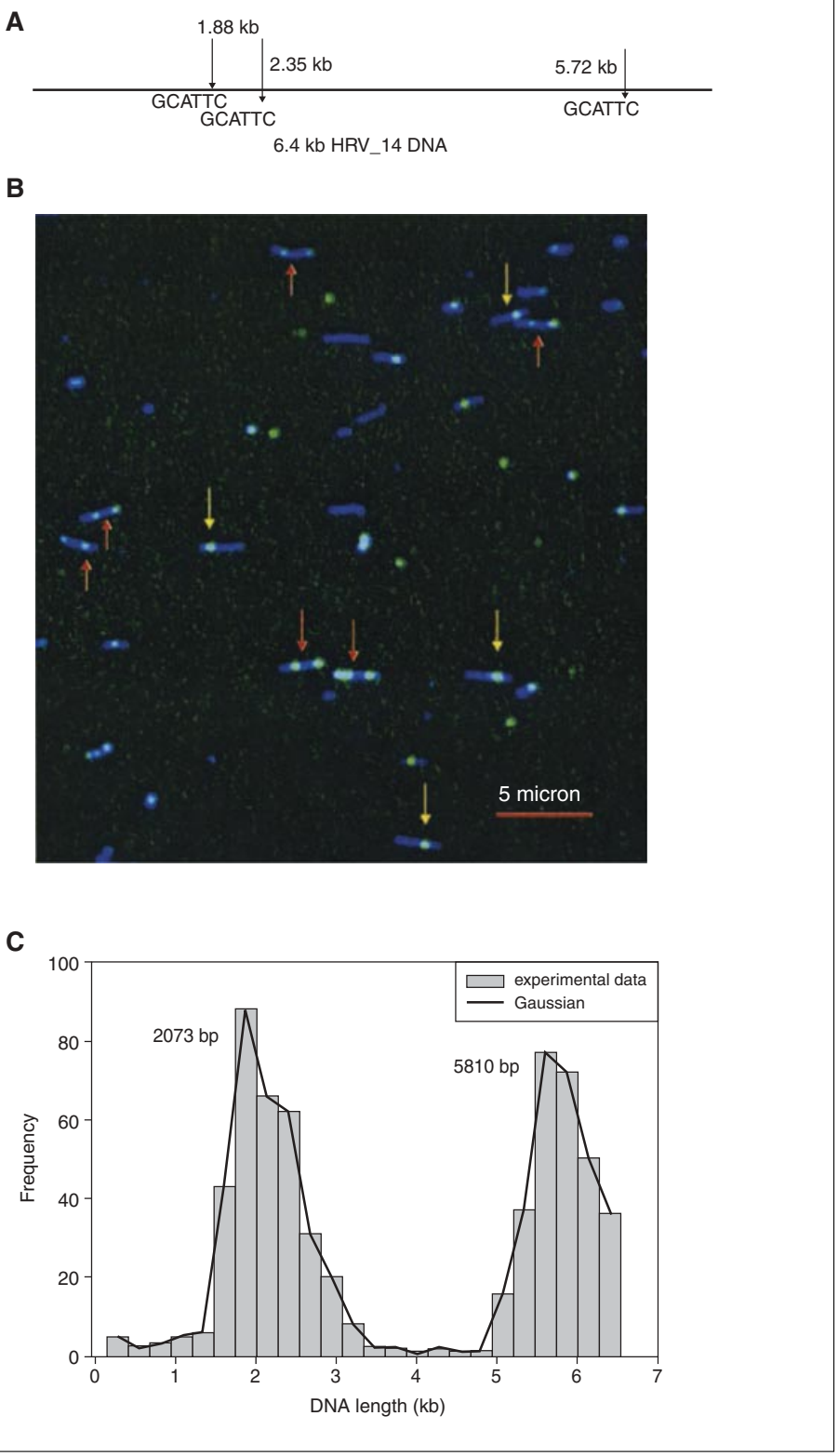

Figure 2A.2.12 Direct imaging of DNA molecules and localization of sequence motifs of human rhinovirus 14 DNA. (**A**) The predicted Nb.Bsm I (GCATTC) map of human rhinovirus 14 DNA. Only two sites are present in this small RNA virus. (**B**) In the large field of the intensity-scaled composite image, numerous molecules are found. Some molecules have two green labels (red arrows) and some have only one green label (yellow arrows). (**C**) The sequence motif map in the graph was obtained by analyzing 56 single molecule fluorescence images. The solid line is the Gaussian curve fitting and the peaks correspond well to the predicted locations of the sequence motif. Reproduced from Xiao et al. (2007) with permission from Oxford University Press. For the color version of this figure go *to http://www.currentprotocols.com.*

Membrane-trafficking perturbations induced by the dynamic interaction of HIV-1 Nef with the clathrin-mediated endocytic pathway

The *nef* gene product of HIV-1 associates with cell membranes, being essential for maximal viral replication in vivo, and markedly contributes to the pathogenesis of AIDS. The positive influence of Nef on viral replication and infectivity is a multifactorial process that affects trafficking of membrane proteins within the endocytic pathway. Endocytosis internalizes plasma membrane–associated proteins through membrane-bound vesicles, supporting various cellular functions (Conner and Schmid, 2003). One of the best characterized endocytosis pathways relies on the protein clathrin (Bonifacino and Traub, 2003). Clathrin adaptors link clathrin to cytoplasmic determinants of endocytic cargo during the formation of plasma membrane invaginations known as clathrin-coated pits, which then bud into the cell to form a coated vesicle in the cell cytoplasm. A dynamic analysis of Nef behavior was necessary to clarify the action of Nef at the plasma membrane, in a model in which Nef uses the clathrin-dependent endocytic pathway to induce internalization of some membrane proteins from the surface of HIV-1 infected T cells (Burtey et al., 2007).

The dynamic behavior of Nef has been observed and analyzed in transiently transfected HeLa cells expressing both Nef-GFP (wild-type HIV-1 Nef fused to GFP green-fluorescing protein) and dsRed-Clathirin (clathrin light chain fused to DsRed) by both epifluorescence (Fig. 2A.2.13) and TIRF microscopy (Fig. 2A.2.14). The epifluorescence image shows that Nef-GFP spots were barely distinguishable from background fluorescence, which precluded the use of this method to follow the dynamics of Nef at the

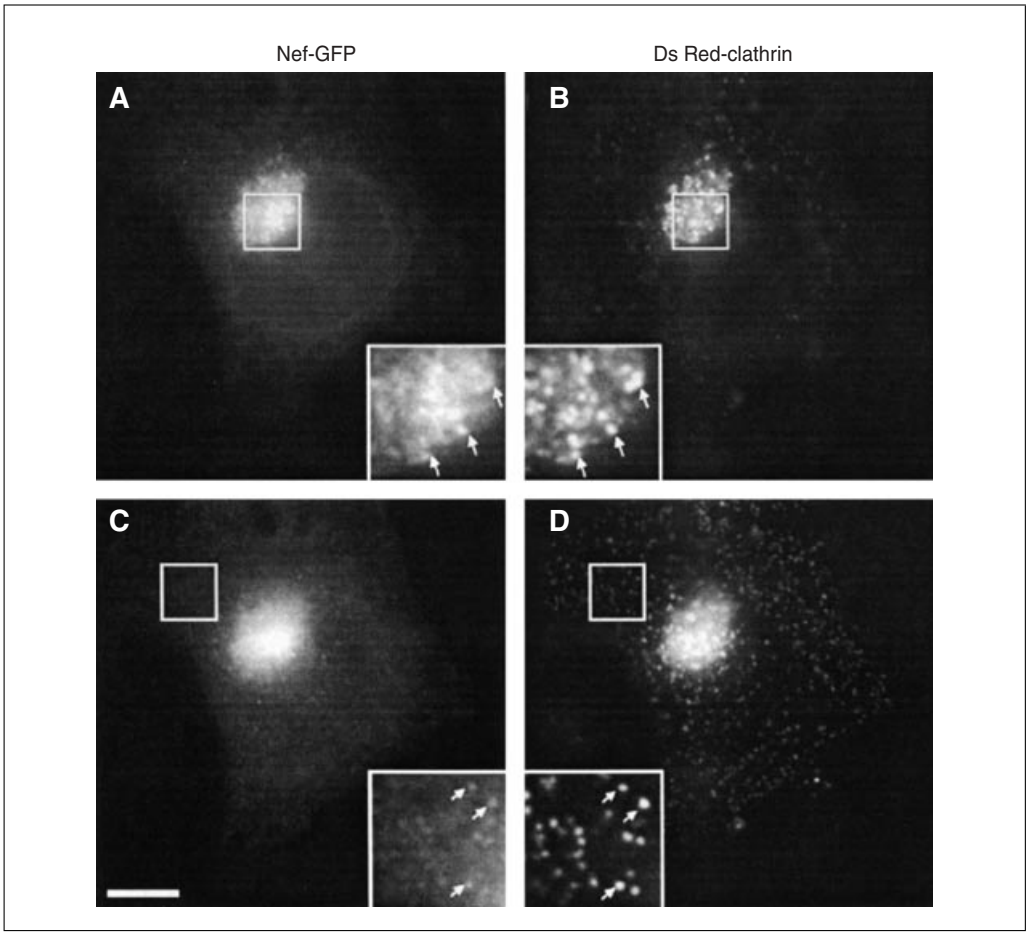

Figure 2A.2.13 Distribution of HIV-1 Nef protein in HeLa cells observed by epifluorescence. HeLa cells were transfected with plasmids encoding wild-type HIV-1 Nef fused to green-fluorescing protein (Nef-GFP, **A** and **C**) and clathrin light chain fused to DsRed (**B** and **D**). Cells were then fixed and observed by epifluorescence. The focus was made on a medial region (**A** and **B**) and on the adherent surface (**C** and **D**) of the same cells. Insets show higher magnification (2.5×) of representative areas. Arrows point out colocalization of both markers. Scale bar, 10 μm. Reproduced from Burtey et al. (2007) with permission from Blackwell Publishing.

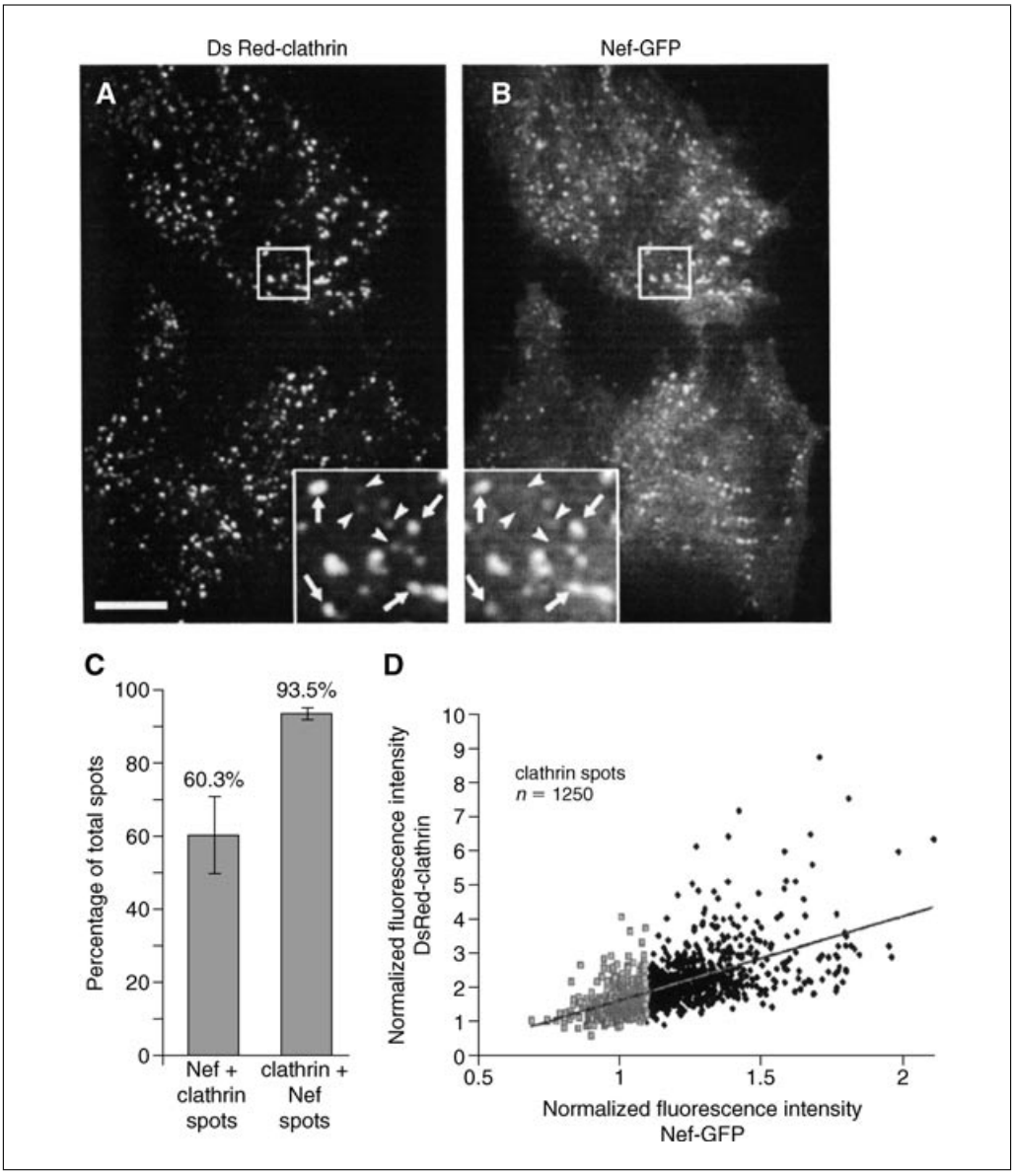

Figure 2A.2.14 Colocalization of Nef with clathrin at the plasma membrane revealed by TIRF microscopy. Live HeLa cells expressing DsRed-clathrin (**A**) and Nef-GFP (**B**) were imaged by TIRF microscopy. Insets show higher magnification (3×) of a representative area. Arrows point out colocalizing spots, and arrowheads show clathrin spots (CS) in which Nef is not concentrated. Scale bar, 10 μm. (**C**) Colocalization of Nef-GFP within CS was quantified in more than seven cells. One thousand spots were quantified and GFP- and DsRed-associated fluorescence intensities were measured and normalized to the local background. The presence of Nef within CS was then quantified by selecting Nef spots with a normalized fluorescence intensity above 1.1, a value corresponding to a detectable increased fluorescence relative to background (a "spot") by direct observation (data not shown). Conversely, the presence of clathrin with Nef spots was similarly quantified. (**D**) Spots analyzed in **C** were plotted as function of their normalized fluorescence for both clathrin and Nef. Spots with a normalized fluorescence below 1.1 appear in gray. Reproduced from Burtey et al. (2007) with permission from Blackwell Publishing.

plasma membrane. In contrast, TIRF imaging shows very clearly that Nef is concentrated within the majority of the clathrin spots (CS) observable at the plasma membrane.

CS at plasma membrane can be categorized into three different populations over a 1 min observation: 80% remain static, 15% disappear from the surface, and 5% move laterally. In the analysis performed on 40 spots from each CS category (Fig. 2A.2.15), Nef was detected in the large majority of static CS (91.5%), in approximately half of the disappearing CS (50.6%), and in a minority of laterally mobile CS (22.1%). Altogether,

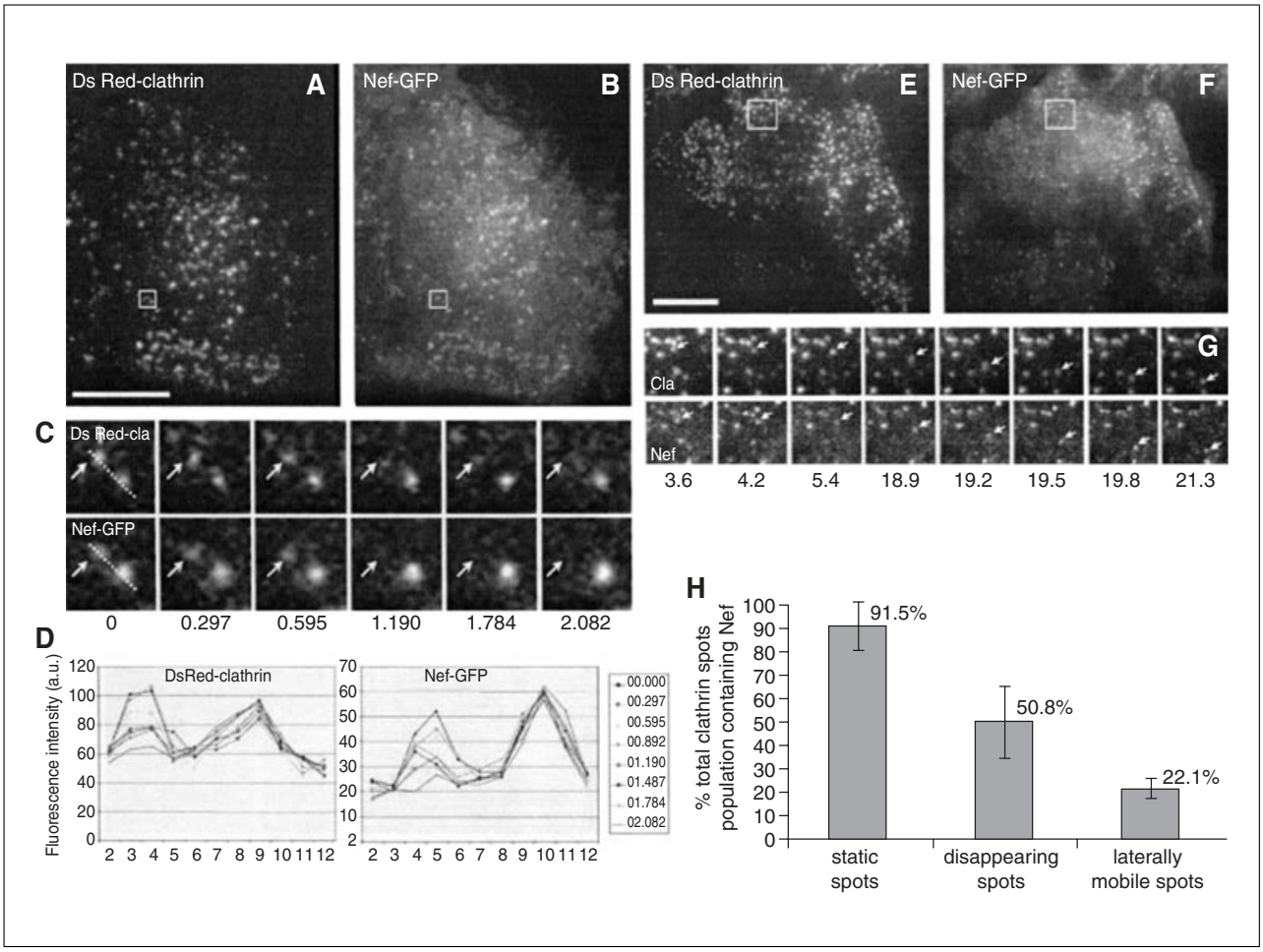

Figure 2A.2.15 Distribution of Nef in dynamic clathrin spot (CS) populations. Live HeLa cells expressing DsRed-clathrin (**A** and **E**) and Nef-GFP (**B** and **F**) were imaged using TIRF microscopy acquired at ∼300 msec/frame for 30 sec. (**A** to **D**) Distribution of Nef in disappearing CS. A disappearing spot was identified on the clathrin frame, green and red images were separated, and the corresponding regions were magnified (5×). (**C**) The fluorescence intensity of each marker was analyzed over time along a line drawn between two spots, a disappearing spot (arrow in C) and a static spot (D). The Nef- and clathrin-associated fluorescence decreased with the same kinetics in the disappearing spot while remaining stable in the neighboring static spot. (**E** to **G**) Distribution of Nef in laterally mobile CS. A laterally mobile spot was identified on the clathrin frame, then green (F) and red images (E) were separated, and the corresponding region was magnified (5×, G). The upper panel in G corresponds to the clathrin images where a laterally mobile CS containing Nef-GFP (lower panel) was identified (arrows). Scale bars 10 μm. (**H**) The presence of Nef-GFP inside 40 CS (from more than five cells) from each CS population (static, disappearing and laterally mobile) was determined. Results are expressed as the percentage of CS of each population that contain Nef-GFP (normalized fluorescence >1.1). Reproduced from Burtey et al. (2007) with permission from Blackwell Publishing.

these results indicate that HIV-1 Nef is present in the clathrin-coated pits (static spots) and in the developing clathrin-coated vesicles (disappearing spots) because it disappeared from the evanescent field together with some CS.

Single particle tracking of murine polyoma virus-like particles in live cells

Murine polyoma virus (Py) is a nonenveloped DNA tumor virus (Stehle et al., 1994) that uses gangliosides GD1a and GD1b as receptors (Tsai et al., 2003) and relies on clathrin-independent, cholesterol-dependent endocytosis to deliver its genome into the cell for replication (Gilbert and Benjamin, 2004). The virus-like particles (VLP) that resemble the virus structurally but do not contain the DNA genome (Gleiter and Lilie, 2001), were labeled with Alexa 568 or fluorescein isothiocyanate (FITC). Their lateral mobility was studied by single fluorescent particle tracking using TIRF microscopy (Ewers et al., 2005). The particle trajectories analyzed in terms of diffusion rates and modes of motion on 3T6 mouse fibroblasts revealed three distinct modes of mobility: rapid random motion, confined movement in small

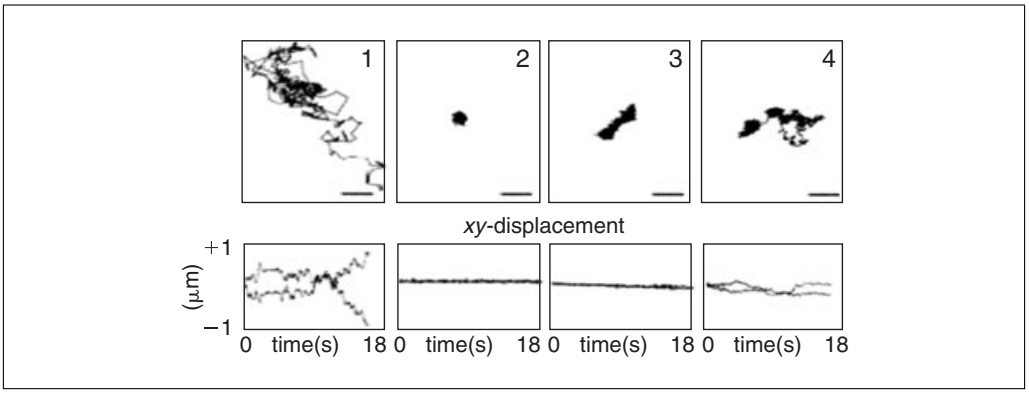

Figure 2A.2.16 Analysis of VLP trajectories at the bottom surface of live cells. All harvested trajectories are plotted irrespective of the recorded length and whether the particles were bound or free at the start of the recording. Every point represents one trajectory, and every trajectory is at least 100 steps (5 sec) long. The longest trajectories are 2000 steps (100 sec) long. Representative trajectories (numbered 1, 2, and 3) and one typical outlier (trajectory 4) represent rapid and random movement (trajectory 1), confinement (trajectory 2), and confinement with a slow drift (trajectory 3). Trajectory 4 represents a VLP that changes its mode of motion during the acquisition time. Below each of these trajectories are the respective analytical plots for absolute displacement in μm for *x* and *y* direction versus time. (Adapted from Ewers, H., Smith, A.E., Sbalzarini, I.F., Lilie, H., Koumouysakos, P., and Helenius, A. Copyright 2005. Single-particle tracking of murine polyoma virus–like particles on live cells and artificial membranes. *Proc. Natl. Acad. Sci. U.S.A.* 102:15110-15115 with permission from the National Academy of Sciences, U.S.A.).

zones (30 to 60 nm in diameter), and confined movement in zones with slow drift. The confinement zones (CZ) fell into a size range of 500 to 3000 nm^2.

For particle tracking, live cells with several VLP already bound were imaged in TIRF at 20 frames/sec for a total of 50 to 100 sec. Trajectories of particle were extracted from digital images by linking particle positions from frame-to-frame using an algorithm developed by the authors (Sbalzarini and Koumoutsakos, 2005) and were classified by computational analysis (Fig. 2A.2.16).

TIRF imaging of constitutive exocytosis

Constitutive secretion, the process that delivers newly synthesized proteins to the plasma membrane and external medium, occurs in all eukaryotic cells. The final stage of secretion is fusion of a Golgi transport vesicle with the plasma membrane. Schmoranzer et al. (2000) applied TIRF microscopy to imaging the fusion of single post-Golgi carriers with the plasma membrane. Quantitative analysis with a time resolution of 30 frames/sec allowed measurements of diffusion constants for the spread of vesicular stomatitis virus glycoprotein (VSVG, Wehland et al., 1982) into the plasma membrane. For easy tracking in TIRF, COS-1 cells were transiently transfected with VSVG fused to its cytoplasmic tail with GFP (Presley et al., 1997; Toomre et al., 1999).

Each carrier (i.e., discrete VSVG-GFP single particle visible in TIRF in proximity to cell membrane) was characterized by its movement, total fluorescence intensity, peak intensity, and the width of its intensity profile. They were classified into three groups: (1) docked carriers that stopped moving and remain stationary adjacent to the membrane, without fusing during the 1 min observation time; (2) carriers that exhibited a synchronous increase and decrease in the total peak intensity due to their movement in and out of the evanescent field, without fusing to the membrane; and (3) carriers that showed a synchronous increase in the total peak and width of the fluorescence and, finally, fusion with the plasma membrane (Fig. 2A.2.17).

When a carrier fuses with the plasma membrane, the total fluorescence intensity will increase as the vesicle flattens to the membrane, whereas the peak intensity will transiently increase and then decrease as VSVG-GFP diffuses laterally into the plasma membrane. In addition, the width will increase as a result of this diffusion. Figure 2A.2.17B shows the parameters of the fusion event plotted over time. Table 2A.2.5 shows a qualitative summary of the kinetic behavior of the characteristic parameters for a fusing carrier. Thus, three temporal phases can be distinguished along this process: (1) stationary (movement of the carrier towards the plasma membrane before

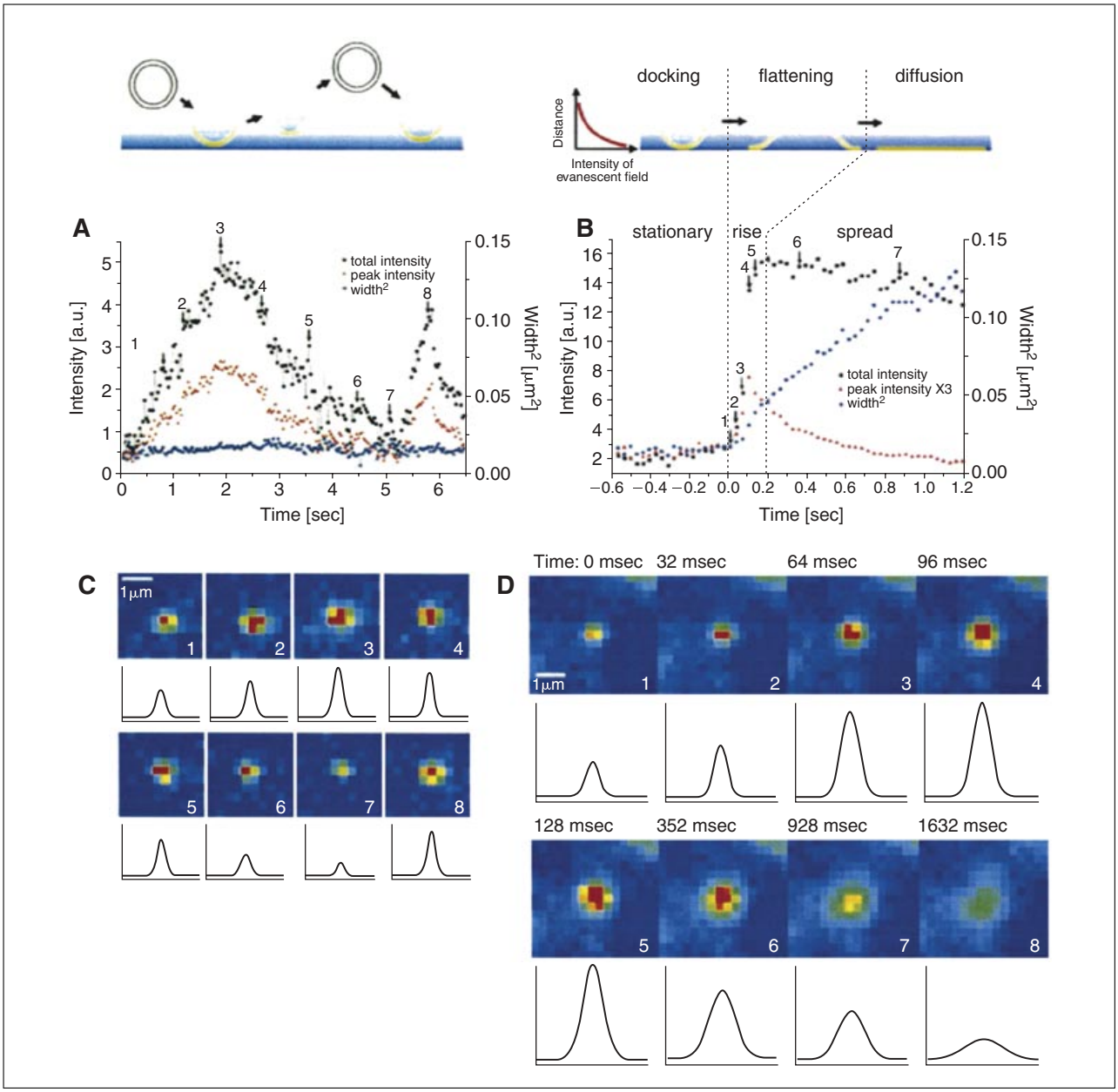

Figure 2A.2.17 Analysis of carriers. The vesicular stomatitis virus glycoprotein/green fluorescent protein (VSVG-GFP) fluorescence was imaged for carriers close to the plasma membrane. The total intensity, peak intensity, and square of the Gaussian width were plotted over time in panels **A** and **B** for the carriers shown in **C** and **D**, respectively. Selected frames are shown from a sequence in panel C for a carrier that moved perpendicular to the coverslip, without fusing to the plasma membrane and in panel D for a carrier which fused to plasma membrane. The intensity of the VSVG-GFP in frames C and D is shown in pseudo-color. Each sequence was processed with a running average in time of ±1 frame, and thresholded separately to aid visualization. The radically symmetric Gaussian fit of the carrier fluorescence is shown below each frame. The numbered arrows refer to the frames from sequences C and D. In panel B the three phases (stationary, rise, and spread) are separated by dotted lines. Times are marked relative to the start of the rise phase. (Reproduced from Schmoranzer et al., *The Journal of Cell Biology*, 149:23-31. Copyright 2000 The Rockefeller University Press. For the color version of this figure go to *http://www.currentprotocols.com.*

Table 2A.2.5 Qualitative Summary of the Kinetic Behavior of the Characteristic Values for a Fusing Carrier

Phase	Stationary	Rise	Spread
Total intensity	Constant	↑↑	Relatively constant
Peak intensity	Constant	↑↑	↓↓
Width	Constant	↑	↑

fusion), (2) rise (carrier was diffusing into the plasma membrane), and (3) spread (carriers were neither moving closer to or further away from the plasma membrane). This study shows that in constitutive exocytosis, there are vesicles that fully fuse and integrate with the plasma membrane.

Toomre et al. (2000) observed the fusion of single carriers with the plasma membrane, and they were able to isolate a distinct analytical signature in fluorescence spectra over time. TIRF and epifluorescence microscopy were used to visualize the exocytic pathway of vesicular stomatitis virus glycoprotein tagged with yellow florescent protein (VSVG3-SP-YFP) from Golgi exit to plasma membrane fusion (Fig. 2A.2.18). The time-lapse microscopy allowed for monitoring the carrier exit from the Golgi and its approach to the cell surface. Figure 2A.2.18C shows a red carrier that exited the Golgi turned yellow as it approached the surface, where it paused for 45 sec before becoming partially fused. In Figure 2A.2.18D another carrier is shown entering the field, approaching the surface, pausing, and completely fusing with the plasma membrane. At fusion, the signal observed by epifluorescence dropped as the carrier diffused into the membrane (Fig. 2A.2.18E, red trace). In contrast, the corresponding TIRF signal rapidly increased at fusion as the carrier was more excited near the coverslip (Fig. 2A.2.18E, green trace).

ADVANTAGES OF TIRF

The prism-based TIRF configurations are inexpensive and offer a variety of prism shapes that can be coupled with the coverslip. In addition, the prism-based TIRF excitation produces less scattered light than through-the-lense TIRF (Axelrod, 2001b). The drawback of the prism setup is that it restricts sample access, and the entire TIRF path must be custom assembled in the laboratory.

The through-the-lens TIRF configuration represents the simplest method for TIRF excitation. The sample is fully accessible, does not require any customized items, and the angle of incidence adjustment is controlled through a micrometer.

The main advantages of TIRF are:

1. It is a wide-field approach (as opposed to sectioning methods) in the sample plane and is compatible with other microscopy techniques (see Chapter 2, section 2A), e.g., epifluorescence, differential interference contrast (DIC), and interference reflection microscopy (IRM). Switching among these microscopy techniques is straightforward, accomplished simply by sliding different optical components and objectives in and out of the optical path, without moving the sample.

2. Compared to IRM, which is used for the visualization of attachment sites of unstained cells due to the contrast obtained from interference of monochromatic polarized light, TIRF is able to offer biochemical specificity when coupled with fluorescence labeling of specific molecules.

3. In some applications (e.g., photosensitive samples, time-lapse recordings, and FRAP), TIRF offers the advantage of illuminating only a thin section at the bottom of the sample, while the rest of the sample is not affected.

4. Considering that TIRF is an optical sectioning technique in a thin plane parallel with the sample, it is comparable to confocal microscopy *(see UNIT 2C.1)*. As mentioned earlier, TIRF has a higher z-resolution ($<$100 nm) than confocal microscopy ($\sim$300 nm).

The main limitation of TIRF microscopy is its specific localization; TIR effect occurs only at an interface between two media with different refractive indices. Compared to confocal microscopy, where optical sectioning is possible at any plane of the sample, TIRF is effective only at the cell-substrate interface.

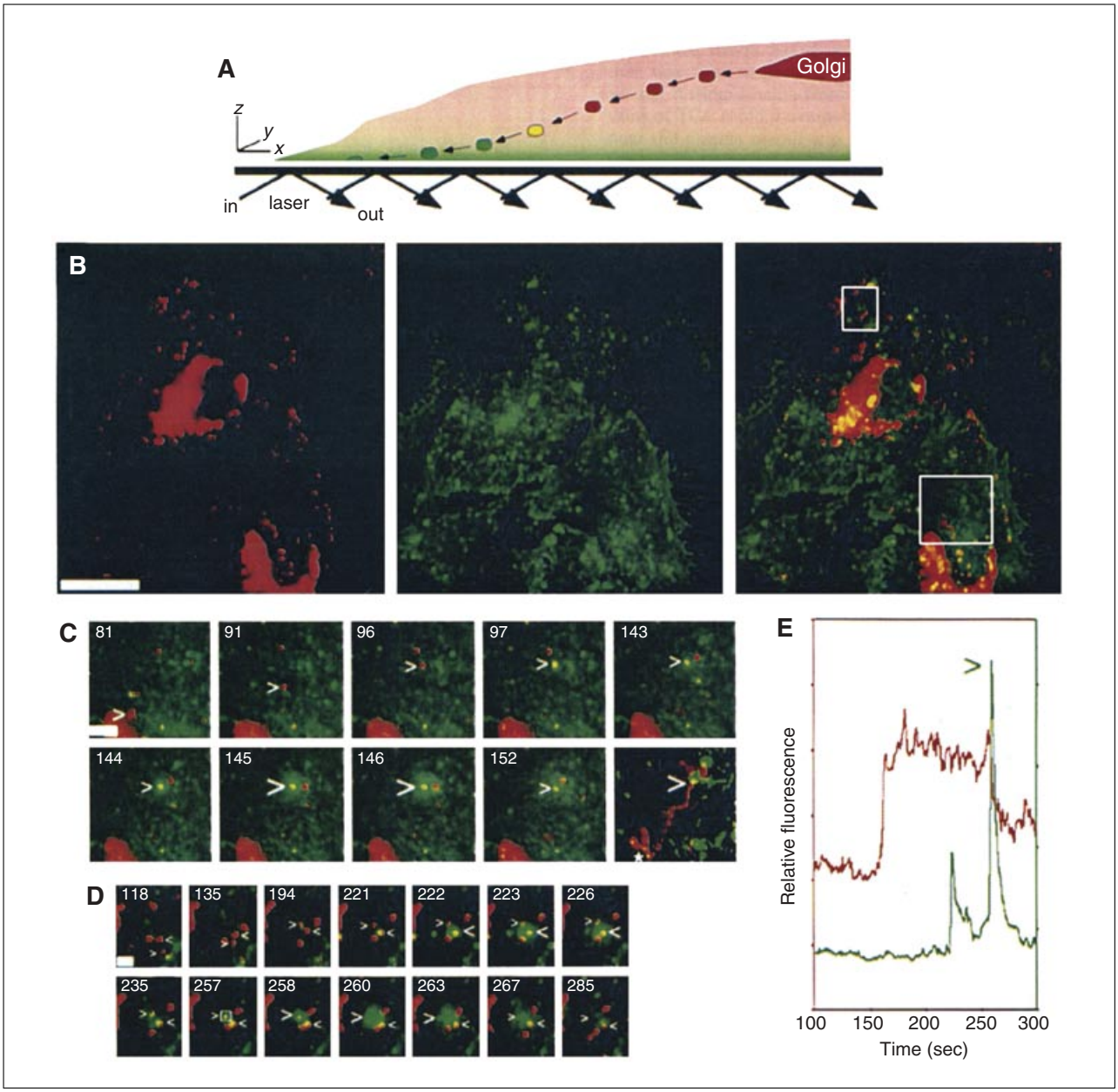

Figure 2A.2.18 Live cell visualization of the late exocytic pathway by combined epifluorescence and TIRF. (**A**) Organelles and carriers distant from the plasma membrane (>100 nm) are visible only by epifluorescence (red). Once carriers approach the plasma membrane, they also become visible by TIRF (green). In an overlay of the two channels, originally red carriers turn yellow and subsequently green as they approach the plasma membrane and later fuse. (**B**) Two live cells imaged by epifluorescence (left) and TIRF (center) as individual channels are merged on the right, where boxes indicate areas enlarged in subsequent images. (**C**) A carrier exits the Golgi complex and moves to the plasma membrane (small >), where it docks and partially fuses (large >). A running subtraction of successive frames tracks the movement of the carrier from the Golgi complex to the apparent docking site (last frame). (**D**) Two carriers that undergo successive fusion in close proximity. Only the second one (>) undergoes complete fusion. The time is indicated in seconds. (**E**) Plot of the relative fluorescence intensity of the second fusion event boxed in panel D. Note that the decrease in epifluorescence (red) is accompanied by a large, sharp increase in TIRF signal (green) when fusion occurs (>). The small TIRF peak is due to an earlier (222 sec) nearby fusion event. Bars: (B) 20 μm; (C) 4 μm; (D) 2 μm. (Reproduced from Toomre et al., *The Journal of Cell Biology*, 149:33-40. Copyright 2000 The Rockefeller University Press.). For the color version of this figure go to *http://www.currentprotocols.com*.

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INTERNET RESOURCES

http://www.micro.magnet.fsu.edu/primer/index.html

Molecular Expressions Microscopy Primer. Excellent presentation of optical microscopy.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) has long been an important tool in the expanding field of microbiology. A major virtue of TEM is the potential resolution provided by electron beams accelerated at high voltage with effective wavelengths that are shorter by a factor of 10^5 than visible light. In principle, electron microscopes are capable of molecular resolution if only the wavelength (diffraction) limitation is considered; in practice, however, other factors limit the achievable resolution, including the sample itself. Other limitations of electron microscopy result from the fact that samples must be thin, dry, and capable of scattering electrons in a vacuum. Typically, this necessitates fixation or stabilization of the sample, and the introduction of electron-scattering heavy metal stains to generate contrast for imaging.

This unit describes two types of TEM preparation techniques for examining particulate samples as well as samples presenting more complex ultrastructural considerations that require analysis in thin sections. Negative staining is a simple but valuable technique that is useful for routine examination of particulate samples in suspension ranging from bacteria to viruses to purified macromolecules. Strategies are presented for delivering sample and stain sequentially onto a grid for TEM analysis (Basic Protocol 1), the direct deposition of sample and stain together onto the grid (Alternate Protocol 1), the delivery of spray droplets of sample and stain to the grid (Alternate Protocol 2), and the preparation of grids for negative staining (see Support Protocol 1). Combination of negative staining with immunolocalization of antigens using protein A– and/or protein G–gold is presented in Basic Protocol 2, along with the alternative procedure using gold-conjugated secondary antibodies in place of protein A/G–gold (Alternative Protocol 3). In order to investigate the relationships between microbes and the environments with which they interface, more complex specimen preparation methods are required. Viruses and bacteria on or in various substrates, including cells and tissues, can be identified by imaging in fixed samples prepared for analysis in sections of 60- to 90-nm thickness. Due to the many steps in sample preparation for ultrastructural analysis of thin-sectioned samples, the major steps in the process are divided into Basic Protocols 3 and Alternate Protocols 4, 5, 6, and 7. In Basic Protocol 3, the fixation and initial processing of solid tissue or biopsy samples is considered; Alternate Protocol 4 describes the variant procedure for pelleted cells, Alternate Protocol 5 describes the procedure for bacterial suspensions, Alternate Protocol 6 describes the procedure for cell monolayers; and Alternate Protocol 7 describes the fixation and initial processing of samples to be subjected to immunogold labeling. Basic Protocol 4 then addresses the process of embedding tissues and cell pellets into plastic resin for sectioning, while Alternate Protocol 8 deals with embedding procedures for monolayers grown on glass slides and Alternate Protocol 9 considers embedding procedures for thin-section immunogold labeling. Once tissues are embedded, the cutting (using an ultramicrotome) of uniform thin sections, free from defects, that adhere to each other as straight ribbons, is an extremely challenging manual skill requiring a high level of dexterity. Ultramicrotomy is not a technique that can be effectively communicated or learned using a protocols manual, and therefore only an overview is presented in this unit (Basic Protocol 5). It is assumed that the reader has access to ultramicrotomy support service or hands-on training in this key element of specimen preparation for analysis of thin sections. Once thin sections are obtained and collected onto a TEM grid, the sample is usually stained with heavy metals as a final step before examination in the electron microscope (Basic Protocol 6). This can be preceded by immunolocalization of antigens in thin sections as discussed in Alternate Protocol 10.

Contributed by Robert C. Burghardt and Robert Droleskey

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SAFETY

Aside from the biohazards posed by handling and disposing of pathogenic microbes (refer to *UNIT 1A.1*), many of the chemicals used in the processing of samples for electron microscopy are either volatile, inflammatory, toxic, carcinogenic, and/or radioactive. It is important to understand the nature of health and environmental hazards posed by these chemicals and to handle and dispose of them according to appropriate safety regulations (see *UNITS 1A.1, 1A.3 & 1A.4*). Expose electron microscopy grids containing negatively stained pathogenic specimens to UV radiation or chemical fixation before examination and store in a suitable container for safe temporary storage. Discard grids into a small wide-based container with appropriate decontamination reagents upon completion of the analysis. Rinse forceps used to handle grids with pathogenic samples in alcohol and flame before and after use. Specimens processed for embedding and thin sectioning are typically chemically stabilized in a fresh aldehyde-containing fixative, which will inactivate/kill the majority of viruses and bacteria. Autoclave other disposable preparative materials that come into contact with pathogens (e.g., Parafilm, filter paper) in appropriate containers prior to disposal.

BASIC PROTOCOL 1

NEGATIVE STAINING FOR TEM USING THE DROP APPLICATION METHOD

The following protocols describe the basic steps necessary for the setup and execution of the negative staining procedure. The goal of these procedures is to deliver a suspension of dispersed particles onto a grid coated with a support film such that particle density is sufficiently high and adequately dispersed in order to detect individual particles whose structure can be discerned using TEM. The negative stain surrounds particles, forming an opaque background so that the particles appear translucent. The ideal sample for negative staining is one of high concentration and purity that poses no biohazard, and which is suspended and well dispersed in a solute that can be easily replaced by the negative staining solution during processing. Since few samples meet these ideal criteria, the procedures and protocols necessary to produce adequately stained samples require a considerable amount of flexibility. Ideally, virus samples should contain about 10^9 particles/ml and bacterial samples derived from the desired growth phase should contain at least 10^6 cfu/ml. Highly fragile and/or pathogenic microbes may require chemical fixation (e.g., using 2.5% buffered glutaraldehyde) and washing prior to staining.

In general, a reasonable starting point for the negative staining of viruses and/or bacteria is the use of the versatile neutral 2% phosphotungstic acid, due to the compatibility of the stain with a wide variety of samples. The method chosen for the delivery of the sample onto the grid can affect the population of particles to be viewed. In the following basic negative staining protocol, the drop method is described. This method is used to deposit a population of the sample onto a grid; adsorption of the sample is affected by both the concentration and the affinity of the sample for the coated grid. A similar procedure, described in Alternate Protocol 1, involves direct application of both sample and stain onto the grid. When components within a particulate sample have different affinities for the coated grid surface, there is a need to employ Alternate Protocol 2, the spray droplet (nebulizer) method. The latter technique should result in the deposition of a more representative sample onto the grid surface for analysis.

Each of the negative stains (see Reagents and Solutions) can be prepared with 1% (w/v) carbohydrate (glucose or trehalose) to protect samples that are sensitive to air drying. However, inclusion of carbohydrate involves additional considerations, as it reduces the overall density of the stain, requiring higher stain concentration; also, the electron-beam instability of the carbohydrates necessitates use of lower beam intensities and/or specimen cooling if possible. Lower concentrations of these stains can be prepared from stock solutions if reduced specimen contrast is needed for any particular application.

Negative staining can be at times a relatively dirty procedure with respect to microscope vacuum, due to the generation of a corrosive sublimate, and in some cases can degrade the sample itself. It is always best to limit contamination by using appropriate anticontamination devices such as ion-getter pumps and liquid nitrogen cold traps, if they are available on the TEM instrument.

Materials

Samples: bacterial or viral suspensions
Negative staining solution: phosphotungstic acid staining solution (see recipe), uranyl acetate staining solution (see recipe), or ammonium molybdate staining solution (see recipe)
37% formaldehyde (optional)
Desiccant

Several sets of self-locking fine-point forceps
Formvar- or carbon-coated Formvar hexagonal 300- or 400- mesh copper grids (see Support Protocol 1)
Whatman no. 4 filter paper
E-Series Germicidal Ultraviolet lamp (Spectroline) or equivalent, with UV monitor and UV goggles (optional)
35-mm petri dish (optional)
Grid box (Electron Microscopy Sciences or Ted Pella)

1. Cut a piece of Parafilm from a roll (~6 to 8 cm in length) along with its paper overlay and place wax-side-down on a clean bench top. Prior to removal of the paper overlay, score corners of the Parafilm or score parallel lines into the Parafilm with a blunt object to loosely attach the Parafilm to the bench top.

   This will provide the surface upon which to place individual droplets of sample, wash, and staining solution in rows for sequential transfer of grids during the staining process.

2. Pipet in a row, with a spacing of ~1.5 cm, a 15- to 25-µl droplet of sample, two or three droplets of Milli-Q-purified water (or equivalent) and one droplet of the negative staining solution onto the wax surface of the Parafilm.

   Several rows of these solutions can be prepared to process several grids at one time. The number of water droplets needed depends on the concentration of solute to be removed from the sample, as the presence of salt crystals will degrade and/or obscure the sample. Optimization of the sample is largely by trial and error. If the sample is too concentrated, it can be diluted in solvent and deposited on the Parafilm for further processing.

3. Using several pairs of self-locking forceps, place a Formvar-coated or carbon-coated Formvar grid onto the sample droplet (coated side towards the sample) for a period of time ranging from 5 to 60 sec. Remove the grid from the sample surface and carefully remove the excess liquid by allowing the grid to touch the edge of a wedge of Whatman no. 4 filter paper. Do not let the grid dry completely (Fig. 2B.1.1B and E).

   Grids with support films tend to be hydrophobic; in order to increase the hydrophilicity of the support film, a brief glow discharge treatment can be applied to increase sample adhesion and stain distribution. This involves placing grids into a vacuum evaporator with a glow discharge unit (Emitech Products, Inc.; http://www.emitech.co.uk), under reduced pressure (~0.1 Torr) in air, thereby exposing the grids to a plasma of gas electrons moving toward an electrode through the gas, resulting in deposition of a negative charge on the surface of the grid which lasts about half a day. Alternatively, the inclusion of surfactants such as 0.01% to 0.1% poly-L-lysine, 0.05% bacitracin, or 0.01% bovine serum albumin in the negative stain solution, or floatation of grids on a solution of one of the abovementioned surfactants for a few seconds prior to the addition of the sample, may improve sample spreading on grid.

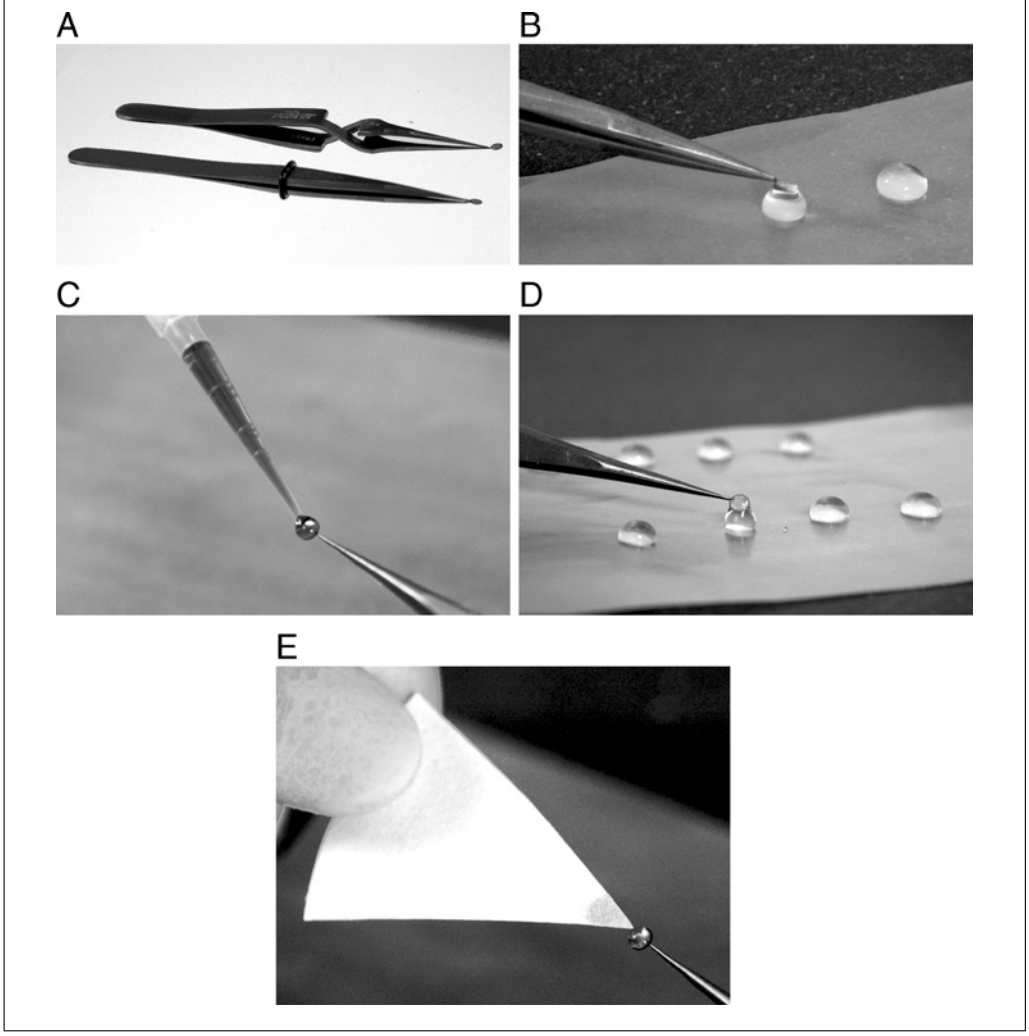

Figure 2B.1.1 Aspects of the negative staining procedure. (**A**) Grids can be conveniently handled using either self-closing forceps (top) or conventional fine-tipped forceps with a rubber O-ring to keep the jaws closed (bottom). Specimen to be examined can be applied either by (**B**) floating grid on a drop of the specimen or by (**C**) direct application to the grid. Stain can be applied as illustrated in B or C. Washing (**D**) and fluid removal (**E**) are performed in subsequent steps of the procedure.

4. Before the sample has air dried on the grid, transfer the grid to the surface of the water droplets sequentially, carefully removing the excess water between each transfer using the edge of a wedge of filter paper.

   This step is necessary if the sample has high concentrations of buffer salts or other solutes which may interfere with uniform deposition of stain. If the sample is suspended in water, the number of washing steps can be reduced or washing can be eliminated.

5. Transfer the grid to the surface of the negative stain solution for 5 to 30 sec, then remove the grid and remove the excess staining solution by again allowing the grid to touch the edge of a filter paper wedge.

   The rate of removal of stain can be adjusted by changing the orientation of the filter paper wedge, since the pointed edge adsorbs less liquid than the broader edges. Thereby, it is possible to increase or decrease the amount of stain removed if stain density requires modification.

6. Allow the grid to air dry completely before TEM analysis to avoid damage to sample and contamination of the microscope.

   The grid with the sample can be placed in a container with a desiccant to accelerate drying.

   Several iterations of this basic procedure are usually required to optimize the concentration and distribution of the sample particles on the grid (see Critical Parameters and Troubleshooting).

7. *Optional:* When dealing with pathogenic microbes, inactivate in one of the following ways:

   a. Expose the grids to UV irradiation (900 μW/cm^2) at 15 to 20 cm for at least 10 min.

   b. Expose grids to 37% formaldehyde fumes in a 35-mm petri dish for at least 30 min.

      The formaldehyde can be placed in the lid from a screw-top fixation vial and the grids can be distributed on filter paper surrounding the lid and exposed to the fumes for at least 30 min.

8. Store grids in a grid box with desiccant at room temperature until ready for further processing or TEM analysis.

NEGATIVE STAINING FOR TEM USING DIRECT DEPOSITION OF SAMPLE AND STAIN ONTO GRIDS

ALTERNATE PROTOCOL 1

Excessive handling of coated grids can be eliminated by the use of the direct deposition technique whereby both the sample and stain are dropped onto the coated grid. This reduces grid manipulation and can speed up the staining procedure once an optimal ratio of sample to stain is determined. When examining pathogenic organisms, it is often necessary to inactivate them with glutaraldehyde prior to staining. If this is the case, it is possible to directly add the negative stain to the sample and drop the mixture in a one-step procedure similar to that outlined below.

For materials, see Basic Protocol 1.

1. Using several sets of self-locking forceps, pick up grids by their edges and place them with support film facing upward in a row on top of plastic-backed bench protectors (“diapers”).

2. Deposit a 10-μl sample droplet on top of the first grid with a pipet, let sample adhere to the grid for 30 to 60 sec, then add a 10-μl droplet of negative stain directly to the grid with a pipet.

   This adsorption step should distribute the sample and stain uniformly over the surface of the grid (Figure 2B.1.1C).

   When dealing with large volumes of sample, it is possible to combine samples, stain together in a microcentrifuge tube, and apply them to the grid simultaneously. Several dilutions of stain and sample can be prepared and run at the same time.

3. Briefly touch the edge of the grid to a wedge of filter paper to remove excess sample.

4. In the case of samples containing high salt concentration, wash twice, each time by placing a 10- to 15-μl drop of distilled water on the grid for a few seconds and then removing it with a clean wedge of filter paper.

   In some instances this extra washing step may cause inadequate sample contrast and will require another brief application of the negative stain solution.

5. Allow the grid to air dry. Inactivate biohazardous organisms as in Basic Protocol 1, step 7, if necessary. Store grids in a grid box with desiccant at room temperature until ready for further processing or TEM analysis.

ALTERNATE PROTOCOL 2

NEGATIVE STAINING FOR TEM USING SPRAY DROPLET APPLICATION

The spray droplet technique is useful for examining specimens or constituents of heterogeneous samples that adsorb poorly to the support film, and/or if there is a need to for a more representative analysis of the sample constituents. Very small droplets of sample and negative stain can be atomized and distributed onto the coated grid with a hand-held nebulizer.

CAUTION: Spray application is not suitable for highly pathogenic organisms even when using an appropriate laminar flow hood. In those instances, one of the drop-staining methodologies (Basic Protocol 1 or Alternate Protocol 1) should be used.

Additional Materials *(also see Basic Protocol 1)*

Hand-held glass nebulizer (Electron Microscopy Sciences or Ted Pella, Inc.)
Stand that can support self-locking forceps holding grid while sample spray is applied

1. Mix appropriate volumes of sample and negative stain in a hand-held glass nebulizer.

   An equal mixture of sample and negative stain is a reasonable starting point, although the ratio of sample and negative stain can be adjusted to yield suitable results.

2. Hold grid in front of the nebulizer using a set of locking forceps inserted into a suitable stand so that the grid projects into the path of the spray.

3. Apply small droplets of sample onto the grid by atomizing the sample.

   CAUTION: *If potentially pathogenic organisms are being examined, appropriate safety precautions must be taken (see UNIT 1A.1). These can include fixation of the sample with glutaraldehyde before use of the nebulizer, spraying the sample onto the grid in a biological safety hood, and/or spraying the sample onto a grid secured in front of a disinfectant-coated towel.*

   The size of droplets can be adjusted by the pressure applied to the nebulizer bulb. The density of sample on the grid can be regulated by varying the distance of the grid from the nebulizer.

4. Allow sample to air dry without washing.

   This prevents differential removal of sample with lower affinity to the substrate.

SUPPORT PROTOCOL 1

PREPARATION OF GRIDS FOR NEGATIVE STAINING

Negative staining requires the use of grids coated with a support film. The preparation of grids with support films is a technique that requires some experience and some specialized equipment (e.g., vacuum evaporator and glow discharge unit), and involves more than following a stated protocol. There are a variety of materials used for support films, which can be cast using a variety of methods. A thorough description of the different materials used for the preparation of support films, their formulations, and casting procedures is given in Hayat (2000). Due to the number of procedural details and considerations in preparing support films, grid preparation is presented here as an overview rather than a step-by-step protocol in this unit. If the laboratory itself does not prepare coated grids on a regular basis, uniform-quality coated grids can be purchased from electron microscopy supply vendors such as Ted Pella, Inc., Electron Microscopy Sciences, Structure Probe, Inc./SPI Supplies (see ***SUPPLIERS APPENDIX*** for these first three suppliers), Canemco Inc.,

and Marivac Inc. (the last two suppliers are accessible through a common Web site at *http://www.canemco.com*). If commercial suppliers are used, it is recommended that freshly prepared support films be purchased in relatively small lots, as the films lose stability under the electron beam when stored for several months.

Support films are commonly prepared from Collodion (nitrocellulose), Formvar, and carbon due to their relatively high transparency to electrons and strength under electron irradiation. Collodion or Formvar can be cast on glass slides or a water surface. Collodion is soluble in ethyl or amyl acetate and acetone, and films are prepared from solutions ranging from 0.5% to 2% (w/v). Formvar is soluble in ethylene dichloride or chloroform and can be prepared from solutions that range in concentration from 0.1% to 0.5% (w/v). A film can be deposited by dipping slides into Coplin jars containing these solutions at a depth of ~10 cm. Once the slide is dipped, the edge is drained onto filter paper and the slide dried in a dust-free area. The dried film is then floated off of the slide by dipping it into a bowl of water at a 30° to 40° angle after the edges of the slide are scraped with a razor blade to allow the hydrophobic film to float off. Once the rectangular film floats on the water surface, a number of grids that are precleaned in acetone can be carefully distributed over the surface of the floating film. A strip of Parafilm slightly larger than the area of the floating support film is then gently laid on top of the grid-containing support film and immediately lifted off of the water surface. The Parafilm with grids on the upper surface is then placed in a covered petri dish or desiccator where the grids remain until needed. Plastic films can be strengthened/stabilized by depositing a thin film of carbon in a vacuum evaporator.

Carbon films are strong and very stable under the electron beam and are useful for high-resolution electron imaging. Preparation of carbon films is a relatively laborious procedure requiring a vacuum evaporation device with appropriately sharpened carbon electrodes. Preparation of carbon support films as well as carbon deposition on plastic films are described in detail in Hayat (2000).

PROTEIN A–GOLD STAINING AND NEGATIVE STAINING FOR IMMUNOELECTRON MICROSCOPY

BASIC PROTOCOL 2

The following protocol describes basic steps necessary for immunolocalization of antigens in negatively stained preparations utilizing colloidal gold preparations conjugated to either secondary antibodies or to protein A–gold, protein G–gold, or protein A plus protein G (protein A/G)–gold, which are used as electron-opaque reporters of primary antibodies that bind to surface antigens on viruses or bacteria. The conjugates interact with mammalian immunoglobulins; however, their individual affinity for immunoglobulins is not equivalent for all species or for all antibody subclasses. There are many alternatives and variations on immunoelectron microscopy approaches, only two of which are outlined below. In these examples, it is assumed that the sample is stable without fixation and that optimal conditions for sample concentration and delivery onto a grid, as well as for negative staining of the sample, have already been determined. If it is suspected that the stability of the sample will be jeopardized by the number of steps and duration of the protocol, the samples can be fixed in appropriately buffered, freshly prepared, 1% paraformaldehyde. This is followed by washing in a glycine-containing buffer to remove residual free aldehydes resulting from fixation prior to immunolocalization of antigens and negative staining.

Materials

Samples: bacterial or viral suspensions
Primary antibody against antigen of interest and irrelevant primary antibody of same species/class as control
Immunogold antibody dilution buffer (see recipe)

Protein A–gold or protein A plus protein G–gold solution: e.g., Structure Probe/SPI Supplies or Nanoprobes (see ***SUPPLIERS APPENDIX***) or Aurion (*http://www.aurion.nl*)

96-well microtiter plate

Humidified chamber: e.g., Tupperware or Rubbermaid plastic container with sealable lid, containing water-saturated paper towels

Several sets of self-locking fine-point forceps

Formvar-coated 300- or 400-mesh nickel grids.

Additional reagents and equipment for negative staining (see Basic Protocol 1)

1. In a well of a microtiter plate, mix 25 µl sample suspension with an equal volume of a suitable dilution of primary antibody in immunogold antibody dilution buffer. Incubate 1 hr at 25° to 37°C in a humidified chamber.

   The antibody dilution needs to be empirically determined. Initial tests should include a serial dilution of antibody that increases by factors of 10. This will help determine optimum conditions for labeling with minimum background. A 1/100 and 1/1000 dilution provide a reasonable starting point.

   To streamline the process, controls can be processed at the same time and should include an irrelevant primary antibody of the same species and/or class, and another preparation without primary antibody.

2. Add 25 µl/well of protein A–, protein G–, or protein A/G–gold solution, depending on the primary antibody subclass and species used to generate the primary antibody, and incubate for another 30 min to 1 hr at 25° to 37°C in a humidified chamber.

   The dilution of the protein complex should be based upon recommendations of the vendor. Note that the size of the gold particles can affect the affinity of the protein A–gold complex for the primary antibody (see Critical Parameters, below). Typically, ≥10-nm-sized gold particles are used with negative staining preparations.

   There are a number of commercial suppliers of colloidal gold products in useful size ranges from 5 to 20 nm and larger (e.g., Structure Probe, Inc./SPI Supplies or Nanoprobes in the U.S. or Aurion in the Netherlands).

3. Using several sets of self-locking forceps, pick up grids by their edges and place them with support film facing upward in a row on top of plastic-backed bench protectors (“diapers”).

4. Deposit a 10- to 15-µl droplet of the mixture from step 2 directly onto the grid with a pipet and allow to stand 5 min.

5. Cut a piece of Parafilm from a roll (~6 to 8 cm in length) along with its paper overlay and place wax-side-down on a clean bench top. Prior to removal of the paper overlay, score corners of the Parafilm or score parallel lines into the Parafilm with a blunt object to loosely attach the Parafilm to the bench top.

6. Pipet, in a row, with a spacing of ~1.5 cm, two drops of immunogold antibody dilution buffer and one drop of distilled water onto the wax surface of the Parafilm. Before the sample dries on the grid, wash by sequential immersion in the two drops buffer and the drop of distilled water. Carefully remove the excess liquid between transfers by allowing the grid to touch the edge of a wedge of Whatman no. 4 filter paper.

7. Perform negative staining as in Basic Protocol 1, using 2% phosphotungstic acid and bringing the sample-containing grid into contact with staining solution for 30 sec.

STAINING FOR IMMUNOELECTRON MICROSCOPY USING GOLD-CONJUGATED SECONDARY ANTIBODIES

There are many variations on immunogold labeling of negatively stained particulate samples. Secondary antibodies generated in a different species against the Fc fragment of the primary antibody can be used to indirectly label the primary antibody, in lieu of the protein A and/or G employed in Basic Protocol 2. The following protocol describes the use of commercially available secondary antibodies conjugated with colloidal gold particles of different sizes.

Additional Materials *(also see Basic Protocols 1 and 2)*

Colloidal gold-conjugated secondary antibody directed against primary antibody

1. Deposit sample onto grids either by floating the Formvar-coated grid on a drop of sample (see Basic Protocol 1) or by direct deposition of the sample onto a grid (see Alternate Protocol 1).
2. Float grid on a drop of the immunogold antibody dilution buffer using the technique described in Basic Protocol 1.
3. Blot excess fluid from grid with Whatman no. 4 filter paper, but do not allow sample to dry completely onto the grid. Immediately transfer grids to a drop of primary antibody diluted in immunogold antibody dilution buffer for 30 min to 1 hr. Cover with a petri dish lid to prevent evaporation.
4. Rinse grids by transferring them to four successive drops of immunogold antibody dilution buffer using the technique described in Basic Protocol 1.
5. Transfer grid to a drop of colloidal gold–conjugated secondary antibody diluted in immunogold antibody dilution buffer.

   The working antibody solution is prepared by diluting the stock with immunogold antibody dilution buffer to yield a solution in which the color of the colloidal gold solution is barely perceptible. Cover and incubate 30 min at room temperature.
6. Rinse grids by sequentially transferring them through four successive drops of immunogold antibody dilution buffer followed by one drop of distilled water using the technique described in Basic Protocol 1. Use a clean wedge of filter paper at the edge of the grid to wick off the water.
7. Perform negative staining as described in Basic Protocol 1.

FIXATION AND INITIAL PROCESSING OF SAMPLES FOR THIN SECTIONING

Biological materials processed for TEM examination of thin sections afford the greatest potential for exploiting the resolving power inherent in the wavelengths of electron beams accelerated at high voltage. Assessment of ultrastructural information within microorganisms, as well as the relationships between microbes and the environments with which they interface, can be analyzed in samples that are chemically stabilized and embedded in a plastic resin. This permits the cutting of sections on the order of 50- to 90-nm in thickness. Such samples are for examination by TEM following deposition of heavy metals on macromolecules to scatter electrons and generate contrast in the sample (see Basic Protocol 6).

The materials to be processed may be particulates such as viral or bacterial suspensions (Alternate Protocol 5), bacterial or cultured cell samples concentrated by centrifugation into pellets (Alternate Protocol 4), monolayer cell cultures (Alternate Protocol 6), or tissue pieces (e.g., biopsies or autopsy tissue samples; Basic Protocol 3). The processing

of these preparations may involve a variety of different strategies. The following protocols describe the basic steps necessary for the chemical fixation and initial processing of tissue pieces, cell pellets, bacterial suspensions, cells in monolayer culture, and samples that are to be immunolabeled in thin sections, each outlined separately.

CAUTION: Due to the volatility and toxicity of the fixatives used in the following protocols (i.e., glutaraldehyde, paraformaldehyde, and osmium tetroxide), these items should be handled in a fume hood using latex or nitrile gloves. Protective eyeware should also be worn.

BASIC PROTOCOL 3

Fixation and Initial Processing of Tissue Samples

Biopsy or autopsy samples are often used to diagnose viral diseases and to study bacterial invasion of epithelial tissues or phagocytosis by macrophages. The following protocol provides the basic steps for processing small pieces of tissue for thin sectioning and electron microscopy. The critical consideration is the size of the tissue sample, which must be reduced to 1 mm^3 or smaller. This critical dimension must not be exceeded, in order to ensure adequate penetration of fixative during the initial processing, post-fixation, en bloc staining, and subsequent infiltration steps that involve incorporation of liquid plastic resin into tissues polymerized into a hardened plastic necessary to permit the cutting of thin sections with glass or diamond knives.

Materials

Tissue sample
TEM primary fixative 1 (see recipe)
Phosphate/sucrose rinse buffer (see recipe), 4°C
Osmium tetroxide post-fixative (see recipe)
Uranyl acetate staining solution (see recipe)
Graded ethanol series: 50% and 75% ethanol (4°C) and 95% and 100% ethanol (room temperature)
Acetone or propylene oxide

Polypropylene cutting board
Single-edged razor blades
Fixation vials: dram vials with caps
Platform rocker or orbital shaker (optional)

Additional reagents and equipment for embedment (Basic Protocol 4)

Fix tissue

1. Obtain a fresh piece of tissue and, as quickly as possible, slice tissue on a polypropylene cutting board with a razor blade into pieces <1 mm in thickness (or smaller than 1 mm^3).

   It is preferable to cover the tissue with a small amount of TEM primary fixative 1 during slicing.

2. Place several small pieces of tissue in a fixation vial containing freshly prepared TEM primary fixative 1 at a volume at least 20 times greater than that of the tissue pieces.

3. Incubate tissue at room temperature for 60 min, them move to a 4°C ice bath for an additional 60 min. Alternatively, fix tissues overnight at 4°C.

4. Decant fixative from vials and replace with phosphate/sucrose rinse buffer at 4°C. Replace the buffer with fresh phosphate/sucrose rinse buffer several times over a 30-min interval to wash the tissue.

Perform post-fixation and en bloc staining

5. Post-fix tissue pieces in osmium tetroxide post-fixative for 90 min at 4°C. Rock tissue pieces frequently during post-fixation or place vials on a platform rocker operating at low speed to facilitate penetration of osmium into the tissue.

6. After post-fixation, decant osmium solution into a waste container in a fume hood. Using the technique described in step 4, rinse tissue pieces twice in chilled phosphate/sucrose rinse buffer, then six times in cold distilled water to remove phosphate buffer residue.

7. Stain tissue en bloc in uranyl acetate staining solution for 90 to 120 min at 4°C with periodic agitation. Alternatively stain in 0.5% aqueous uranyl acetate overnight at 4°C.

   Heavy metal stains are most often deposited after fixation and sectioning. En bloc staining is a supplemental or alternative method for depositing uranyl ions onto subcellular structures in pieces of tissue or tissue pellets after fixation and prior to embedding and section.

   One disadvantage of en bloc staining with uranyl acetate is that glycogen is either extracted or rendered unstainable. If preservation of glycogen in the tissue is of importance, this step may be bypassed. Alternatively, see de Bruijn, (1973)

8. Using the technique described in step 4, wash tissue three times in cold distilled water to remove uranyl acetate.

Dehydrate and embed tissue

9. Dehydrate through ethanol series by sequentially replacing the solution in the vial with the following solutions for 10 min each:

   50% ethanol, 4°C
   75% ethanol, 4°C
   95% ethanol, room temperature
   100% ethanol, room temperature.

10. Rinse tissue three times, each time for 10 min, in acetone or propylene oxide.

11. Embed in epoxy resin (see Basic Protocol 4).

Fixation and Initial Processing of Cell Pellets

ALTERNATE PROTOCOL 4

The following alternate protocol provides a general approach for the preparation and analysis of samples presented as individual cells in a suspension that must be concentrated by centrifugation into pellets. Samples can range from cell cultures used for the isolation and propagation of viruses to pure bacterial cultures in growth medium. The quantity of cells and the type of sample tube will determine the type of pellet to be fixed and subjected to initial sample processing. There are numerous methods to prepare cells in culture for electron microscopy ranging from scraping monolayers with a cell scraper to generating monodispersed populations by trypsinization. These cells are then concentrated by centrifugation and fixed. Once the initial stabilization of the sample is accomplished, the pelleted material must be removed from the centrifuge tube and, depending on the size and stability of the pellet, it can be processed as a solid piece of tissue or it can be encased in agar to ensure its integrity during subsequent steps of the sample preparation process.

Additional Materials *(also see Basic Protocol 3)*

Cell suspension in buffered saline or serum-free culture medium
2.5% molten agar, between 40°C and 60°C

Obtain pellet

1. Harvest bacteria or cultured cells in buffered saline or serum-free culture medium and centrifuge in either hard- or soft-walled microcentrifuge tubes.

   A variety of microcentrifuge tubes can be used for pelleting samples. When using hard-walled tubes such as polypropylene microcentrifuge tubes, pellets can be overlaid with a glutaraldehyde-based fixative for stabilization of cells. Polyethylene microcentrifuge tubes have soft walls and the tips of these tubes containing the pellet can be cut off with a razor blade and dropped directly into fixative for initial tissue processing.

Fix cells

2a. *If the sample is collected in a hard-walled microcentrifuge tube:* Carefully remove supernatant from pellet. In a fume hood, slowly add freshly prepared TEM primary fixative 1 using a volume at least 20 times greater than that of the pellet. Fix for 1 hr at 4°C.

2b. *If the sample is collected in a soft-walled microcentrifuge tube:* Cut off the tip of the microcentrifuge tube containing the sample pellet and drop the pellet along with the end of the tube into a fixation vial containing freshly prepared TEM primary fixative 1 at a volume at least 20 times greater than that of the pellet. Fix for 1 hr at 4°C.

3. Replace fixative with phosphate/sucrose rinse buffer and carefully remove the pellet from the microcentrifuge tube or cut tip of tube.

Encase cells in agar and fix again

4a. *If pellet is <1 mm in thickness:* Carefully transfer pellet to 2.5% molten agar (maintained at 40° to 60°C) deposited on Parafilm, and keep suspended in the agar until solidified.

4b. *If pellet is >1 mm in thickness:* Slice the pellet, from top to bottom, into ~1-mm-thick slices. To maintain pellet integrity, carefully transfer slices of the pellet to a droplet of molten agar deposited on Parafilm and keep slice suspended in the agar until it solidifies.

5. Remove excess solidified agar with a razor blade to enhance fixative and solvent exchange.

6. Transfer pellet or slices to fixation vials containing fresh fixative and incubate an additional 90 min at 4°C.

7. Following fixation, decant fixative from pellet and replace with phosphate/sucrose rinse buffer at 4°C. Incubate 10 min at 4°C, then replace with fresh phosphate/sucrose rinse buffer and incubate an additional 10 min at 4°C.

Perform post-fixation and en bloc staining

8. Remove phosphate/sucrose rinse buffer, replace with osmium tetroxide post-fixative, and incubate 90 min at 4°C. Rock pellet frequently during post-fixation, or place vials on a platform rocker operating at low speed to facilitate penetration of osmium into the tissue.

9. After post-fixation, decant osmium solution into a waste container in a fume hood. Using the technique described in step 7, rinse pellet twice in chilled phosphate/sucrose rinse buffer, then six times in cold distilled water to remove phosphate buffer residue.

10. Stain tissue en bloc in 1% aqueous uranyl acetate for 90 to 120 min at 4°C with periodic agitation.

    Alternatively stain in 0.5% aqueous uranyl acetate overnight at 4°C.

11. Using the technique described in step 7, rinse pellets three times in cold distilled water to remove uranyl acetate.

Dehydrate and embed pellet

12. Dehydrate through ethanol series by sequentially replacing the solution in the vial with the following solutions for 10 min each:

    50% ethanol, 4°C
    75% ethanol, 4°C
    95% ethanol, room temperature
    100% ethanol, room temperature.

13. Rinse tissue three times, each time for 10 min, in acetone or propylene oxide.

14. Embed in epoxy resin (see Basic Protocol 4).

Direct Fixation and Initial Processing of Bacteria in Suspension

ALTERNATE PROTOCOL 5

When organisms, whether in pure or mixed culture, are prepared in growth medium and presented in suspension for preservation, it is frequently easier and more beneficial to add fixative directly to cells in medium, instead of to pelleted preparations. The objective of this procedure is to preserve the ultrastructure of the organisms while concentrating them sufficiently for embedding and sectioning to prepare a representative sample. The use of direct fixation eliminates potential centrifugation artifacts that may be encountered during initial centrifugation of unfixed preparations and helps to ensure that all constituents of the suspension will uniformly fixed and concentrated.

Additional Materials *(also see Basic Protocol 3)*

TEM primary fixative 2 (see recipe)
Bacteria in suspension
2.5% molten agar, between 40° and 60°C
15-ml conical centrifuge tubes
Horizontal mixer (e.g., Adams Nutator Single Speed Orbital Mixer)
Tabletop centrifuge
Polyethylene microcentrifuge tubes

Fix cells

1. In a fume hood, add an equal volume of TEM primary fixative 2 to bacterial cells suspended in culture medium or buffer in a 15-ml conical centrifuge tube.

   This will result in a final fixative concentration of 2% glutaraldehyde.

2. Immediately invert mixture and place on a horizontal mixer 60 min at room temperature.

3. After initial room temperature fixation, transfer cell suspension to a 4°C ice bath for an additional 1 hr of fixation.

4. Centrifuge cell suspension 10 min at 1000 × *g*, 4°C, and discard supernatant.

5. Resuspend cells in phosphate/sucrose rinse buffer and incubate at 4°C for 10 min.

6. Centrifuge cells 10 min at 1000 × *g*, 4°C, and discard supernatant.

Encase cells in agar and fix again

7. Resuspend cells in enough 2.5% molten agar to cover pellet, mix with a warmed transfer pipet, and transfer to polyethylene microcentrifuge tubes.

8. Centrifuge the agar and cell suspension 5 min at 5000 × *g*, room temperature, to pellet cells.
9. Chill tubes briefly on ice to solidify agar.
10. Using a single-edged razor blade, cut through the microcentrifuge tube just above the cell pellet in the agar to remove the pellet. Slice the removed agar encased pellet into thin slices, ~0.5 mm wide, and place in fixation vials containing chilled phosphate/sucrose rinse buffer.

Perform post-fixation and en bloc staining

11. Replace buffer with osmium tetroxide post-fixative and incubate 90 min at 4°C. Rock pellets frequently during post-fixation or place vials on a platform rocker and rock at low speed to facilitate penetration of osmium into the tissue.
12. Rinse pellets twice in chilled phosphate/sucrose rinse buffer.
13. Rinse pellets six times over the course of 1 hr with chilled distilled water to remove phosphate buffer residue.
14. Stain pellets en bloc in uranyl acetate staining solution for 90 to 120 min at 4°C with periodic agitation.

    Alternatively, stain in 0.5% aqueous uranyl acetate overnight at 4°C.

15. Wash three times in cold distilled water to remove uranyl acetate.

Dehydrate and embed pellet

16. Dehydrate through ethanol series by immersing successively for 10 min each in the following solutions:

    50% ethanol, 4°C
    75% ethanol, 4°C
    95% ethanol, room temperature.

Transfer to 100% ethanol.

17. Rinse tissue three times, each time for 10 min, in acetone or propylene oxide.
18. Embed in epoxy resin (see Basic Protocol 4).

ALTERNATE PROTOCOL 6

Fixation and Initial Processing of Monolayer Cells in Culture

Tissue culture cell preparations are frequently used for studying the propagation of viruses, the evaluation of viral cytopathic effect, and the evaluation of bacterial attachment and cell invasion. There are numerous methods that can be used to prepare cells in culture for electron microscopy, ranging from scraping monolayers with a cell scraper followed by centrifugation into a pellet to methods that permit evaluation of the monolayer in situ. In the following alternate protocol, a useful method for preserving and embedding cell monolayers by TEM is presented. The method involves use of cells cultured in Nunc Lab-Tek Chamber Slides. These slides consist of a plastic culture chamber that is attached to a glass microscope slide and sealed by means of a silicone gasket. The chamber is available in various configurations, ranging from a single chamber up to eight separate chambers on one slide. The protocol outlined below should produce embedded cell monolayers that can be sectioned either en face or in cross sections.

Additional Materials *(also see Basic Protocol 3)*

Cultured cells grown in Nunc Lab-Tek chamber slides
TEM primary fixative 2 (see recipe)

1. In a fume hood, slowly remove medium from the cultured cells and add TEM primary fixative 2 to the chamber so as to cover the monolayer with ~3 to 5 mm of fixative (~6 ml fixative per slide). Cover, and incubate cells in fixative for 30 min at room temperature followed by an additional 30 min at 4°C.

   The plastic chamber should not removed until after the fixation and uranyl acetate staining are completed.

2. Remove fixative and replace with an equal volume of cold phosphate/sucrose rinse buffer. Cover and incubate 10 min.
3. Remove phosphate/sucrose rinse buffer and replace with an equal volume of osmium tetroxide post-fixative. Incubate at 4°C for 60 min.
4. Remove post-fixative solution and rinse once in phosphate/sucrose rinse buffer.
5. Rinse chambers with cold distilled water six times over the course of 1 hr.
6. Add 1 ml uranyl acetate staining solution to the chamber and incubate 1 hr at 4°C.
7. Rinse chambers twice with cold distilled water.
8. Dehydrate and embed cells on the chamber slide (see Alternate Protocol 8).

Fixation and Initial Processing of Samples for Immunogold Labeling of Thin Sections

ALTERNATE PROTOCOL 7

The fixation and processing of samples that will permit labeling of antigens in thin-sectioned materials pose substantial challenges. Methods commonly used for optimal ultrastructural morphology must usually be modified to ensure that antigens are not only retained but available for labeling. This often involves optimization, with compromises between the fixation, processing, and embedding steps; however, careful selection of preparation methods can yield high-quality combinations of structural detail and immunochemical labeling.

Additional Materials *(also see Basic Protocol 3)*

Tissue or cell sample
TEM primary fixative 3 (see recipe)
Aldehyde quenching solution (see recipe)

1a. *For tissue specimens:* In a fume hood, fix tissue specimens by incubating with TEM primary fixative 3 for 12 to 24 hr at 4°C.

1b. *For free cells or cell suspension:* In a fume hood, fix cells or pellet by incubating with TEM primary fixative 3 for 2 to 4 hr at 4°C.

2. Rinse in phosphate/sucrose rinse buffer three times for at least 10 min each at 4°C.
3. Incubate in aldehyde quenching solution for 30 min at 4°C to remove residual free aldehydes.
4. Wash in phosphate/sucrose rinse buffer two times for 15 min each at 4°C.
5. Dehydrate through ethanol series by immersing successively in the following solutions for the indicated amounts of time:

50% ethanol, 4°C	10 min
75% ethanol, 4°C	10 min
95% ethanol, room temperature	10 min
100% ethanol, room temperature	30 min
100% ethanol, room temperature	30 min.

6. Directly embed in resin (see Alternate Protocol 9).

   Note that this procedure omits the acetone or propylene oxide infiltration step included in the previous tissue fixation and processing protocols.

BASIC PROTOCOL 4

EMBEDDING OF TISSUES AND CELL PELLETS FOR THIN SECTIONING

The following basic protocol is a continuation of Basic Protocol 3 and Alternate Protocols 4 and 5. All material to be thin sectioned for TEM examination must be embedded in a hardened plastic matrix. The unpolymerized plastic matrix must be fluid enough to completely infiltrate the specimen. Once polymerized, the plastic must not alter the ultrastructure of the specimen (i.e., as a result of specimen shrinkage, swelling, or extraction of cellular components) while being able to withstand the stress of thin sectioning. The resulting sections should remain stable in the electron beam and impart adequate contrast to the sample to allow for the visualization of ultrastructural detail. An additional consideration is the preservation of antigenicity in the case of thin-section immunogold labeling; a separate procedure is provided to deal with this issue (see Alternate Protocol 9).

In general, the goal is to introduce a plastic resin into the sample over time by the gradual replacement of dehydrating agent and resin solvent (usually ethanol and acetone, or ethanol and propylene oxide) with increasing concentrations of resin and solvent over time. Tissue pieces and cell pellets are eventually transferred to resin-filled embedding molds for polymerization. Cells in polymerized resin can then be prepared for thin sectioning.

CAUTION: Some resin components are possible carcinogens and others can produce allergic reactions in certain individual. Their preparation may therefore require a fume hood, a suitably vented oven, and other adequate measures for personal protection (also see *UNIT 1A.3*).

Materials

Fixed and processed tissue (Basic Protocol 3), cell pellet (Alternate Protocol 4), or bacterial sample (Alternate Protocol 5)
Luft's epoxy mixture (see recipe)
Acetone or propylene oxide
Orbital shaker
Transfer forceps or applicator stick
Embedding molds or capsules (available from Ted Pella; BEEM flat embedding molds, cat. no. 111-2, PTFE flat embedding molds, cat. no. 10509, or BEEM embedding capsules, size 00, cat. no. 13)
60°C oven

1. Pipet out the last rinse of acetone or propylene oxide from the fixation vial and replace with a mixture of 50% (v/v) Luft's epoxy mixture and 50% acetone or propylene oxide. Place the vial on an orbital shaker and rotate sufficiently to suspend the tissue pieces or pellet in the resin.

   Upon initial introduction of the resin, the tissue pieces or pellets will float to the top. With time, they will sink as they become infiltrated.

2. Incubate at room temperature for 30 min with rotation on the orbital shaker to aid infiltration.

3. Remove all of the 50-50 resin mixture and replace with a mixture of 75% (v/v) Luft's epoxy mixture and 25% acetone or propylene oxide. Incubate at room temperature for 30 min with rotation to aid infiltration.

   The pellets will again float to the top of the suspension and will slowly sink as the resin infiltrates the pellets.

4. Remove the resin from step 3 and replace it with 100% Luft's epoxy mixture. Rotate on an orbital shaker for 1 hr at room temperature, then remove the epoxy mixture and replace with freshly prepared 100% Luft's epoxy mixture.

5. Remove the resin and, using transfer forceps or an applicator stick, carefully transfer pellets to suitable embedding molds containing freshly prepared 100% Luft's epoxy mixture. Transfer molds to a vented 60°C oven and incubate overnight.

   A variety of embedding molds and capsules are commercially available that allow tissue to be optimally positioned for sectioning once released from the mold. For pelleted material, polyethylene caps for snap-cap sample vials (Electron Microscopy Sciences #64257-30) work well for this purpose. They come in a variety of sizes, allow for easy placement of the sample, have plenty of surface area for outgassing of residual solvent if necessary, and are reusable; in addition, the polymerized resin is easily removed. Pellets or tissue can be excised from the resin using a jeweler's saw or a single-edged razor blade if the resin is slightly heated and mounted on a blank BEEM capsule or Plexiglas pegs (Ladd Research #21830) using superglue. Small pieces of tissue can be directly placed in resin-filled BEEM capsules for polymerization. However, when BEEM capsules are used for direct embedding of tissue, it is often advantageous to employ flat embedding molds for embedding extra pieces of tissue if the need arises.

RESIN EMBEDDING OF MONOLAYER CELLS ON GLASS CHAMBERED SLIDES

ALTERNATE PROTOCOL 8

The following is a continuation of Alternate Protocol 6, describing the dehydration, resin infiltration, and polymerization steps for cultured monolayer cells.

Materials

Fixed and processed monolayer cells in chamber slides (Alternate Protocol 6)
50%, 75%, 95%, and 100% ethanol
Mollenhauer's no. 2 resin mixture (see recipe)
Single-edged razor blade

1. Dehydrate the cell monolayer in the chamber slide by sequentially adding the following solutions at room temperature to the chamber, leaving for the indicated length of time, and then draining and filling with the next solution:

50% ethanol	5 min
50% ethanol	5 min
75% ethanol	5 min
75% ethanol	5 min
95% ethanol	5 min
95% ethanol	5 min
100% ethanol	5 min
100% ethanol	5 min
100% ethanol	5 min.

   Each dehydration step is performed multiple times to insure adequate exchange of fluid around the corners of the chambers.

2. Remove the last change of ethanol and then quickly invert chamber onto an absorbent paper towel to remove excess ethanol, being careful not to allow cells to dry out or touch the paper towel. Quickly refill chambers with a mixture of 50% (v/v) Mollenhauer's no. 2 resin mixture/50% ethanol.

3. Rotate the chamber slide gently to mix contents and decant mixture. Invert over a fresh paper towel and then refill the chamber with fresh resin mixture of the same composition that was used in step 2. Cover and incubate for 30 min, rotating the slide occasionally.

4. Repeat the procedure used in steps 2 and 3 with a mixture of 75% Mollenhauer's no. 2 resin mixture/25% ethanol, then again with 100% Mollenhauer's no. 2 resin mixture. Replace the 100% resin with fresh resin after an initial 30 min incubation, for a total incubation time of 60 min in 100% resin.

5. Using a pair of forceps, carefully pull up the plastic chamber from the slide, leaving the silicone gasket intact on the glass. Carefully refill each gasket-outlined chamber with fresh 100% resin to just below the top of the gasket surface. Place in a level 60°C oven to polymerize.

6. After resin polymerization, remove the gasket from the slide and then remove the embedded cells from the slide using a single-edged razor blade.

   The application of heat to the slide may be necessary for the removal of the embedded chambers. If that is the case, set the slide on an 80°C hot plate for 30 to 45 sec.

   Pieces of the embedded monolayer can be cut out and mounted on plastic pegs so that they are sectioned either en face or in cross-section. When sectioning across the monolayer, the use of grids with Formvar support will provide stability to the section.

ALTERNATE PROTOCOL 9

RESIN EMBEDDING FOR THIN SECTION IMMUNOGOLD LABELING

Preservation of sample antigenicity is of paramount importance when choosing an embedding resin for thin section immunogold labeling. Dehydration and embedding protocols must minimize loss of potential antigenic sites. This generally requires the use of ethanol alone as the dehydrating agent, an altered infiltration schedule, and the use of a more hydrophilic embedding resin coupled with room-temperature or cold polymerization. Several specific resins are available, all acrylic based, that offer good antigen protection, although some require elaborate cooling chambers in order to optimize their superior antigenic preservation qualities during polymerization. The resin protocol described below utilizes a one-part resin, which only needs an accelerator for room temperature polymerization and yields consistent results.

Materials

Fixed and processed tissue or cell sample (Alternate Protocol 7)
LR White resin (Polysciences)
LR White accelerator (Polysciences)
Embedding molds (available from Ted Pella; BEEM flat embedding molds, cat. no. 111-2, PTFE flat embedding molds, cat. no. 10509, or BEEM embedding capsules, size 00, cat. no. 13)
Cooled water bath or cooling block (optional)
Jeweler's saw
Plexiglas pegs (Ladd Research, cat. no. 21830; *http://www.laddresearch.com/*)
Cyanoacrylate glue (e.g., Superglue, Krazy Glue)

1. Following the last two washes of tissue in 100% ethanol described Alternate Protocol 7, infiltrate the sample with LR White resin as follows. Remove the ethanol and replace with 100% LR White resin, incubate 1 hr at room temperature, then replace with fresh 100% LR White resin and incubate an additional 1 hr at room temperature. Finally, replace again with fresh 100% LR White resin and incubate an additional 1 hr to overnight at room temperature.

   See technical data sheet for LR White resins at http://www.polysciences.com/shop/assets/datasheets/305A.pdf.

2. To 10 ml LR White resin, add 20 µl LR White accelerator and mix well. Partially fill embedding mold(s) with the resin/accelerator mixture. Remove LR White resin–infiltrated tissue pieces or pellets from their vials using fine-tipped forceps

or a wooden stick with a fine tip. Deposit the tissue pieces into the resin-containing embedding mold(s). Allow molds to polymerize for 48 hr at room temperature.

If flat embedding molds are used, it may be necessary to exclude air from the surface of the resin and overlay the mold with a piece of ACLAR film (Ted Pella, cat. no. 10501-10) to exclude as much air as possible from the surface of the mold. Embedding in BEEM or gelatin capsules is preferred over the use of flat embedding molds, as the surface area exposed to air is minimized and the capsules do not require the film overlay.

3. Remove polymerized block(s) from embedding mold(s) or capsule(s). If the tissue has been flat-embedded, cut out the desired area with a jeweler's saw and mount on Plexiglas specimen-mounting pegs (that fit into the specimen chucks of the ultramicrotome) using cyanoacrylate glue.

OVERVIEW OF ULTRAMICROTOMY

BASIC PROTOCOL 5

As noted previously, the goal of ultramicrotomy is to prepare thin sections between 50 and 90 nm thick. The generation of sections that adhere to one another as straight ribbons using an ultramicrotome is an extremely challenging manual skill that requires a high level of dexterity. Ultramicrotomy is not a technique that can be effectively learned using a protocols manual and is therefore given as an overview rather than a method in this unit.

While it is assumed that the reader has access to ultramicrotomy support service or hands-on training in this key element of specimen preparation for analysis of thin sections, a brief description of ultramicrotomy follows. In order to thin-section embedded tissue that has been placed in an embedding mold, it must be mounted in a holder for trimming. Some ultramicrotomes have different types of holders that can grip flat embedded tissue or hold a cylinder of plastic with a tapered end (pyramid shape) containing the tissue. The tapered end must be trimmed further to form a small trapezoid of ~200 μm^2. This is usually done by hand with a single-edged razor blade under a stereo microscope and requires considerable dexterity and practice. Some microtomes have specimen holders, which permit rapid trimming of the block to form the trapezoid with a highly polished face.

Thin sections are cut with either a glass or diamond knife. Glass knives are normally made freshly with a knife-maker instrument, which breaks ~1-in. (~2.5-cm) squares of glass from high-quality glass strips. The squares of glass are scored diagonally and carefully broken to form triangular pieces, one edge of which contains the knife edge. A commercially made plastic trough is sealed with dental wax. Alternatively, a trough or "boat" is fashioned around the knife edge with Mylar or other tape that will provide a distilled water–filled reservoir that reaches the edge of the knife and that will allow sections to float away from the edge of the knife from the block during the cutting stroke of the microtome arm. The wide side of the trapezoid-shaped block face makes first contact with the knife edge during the cutting stroke of the microtome. Prior to collecting sections, the block face must be "polished" in preparation for thin sectioning by cutting initial sections until the block face appears to have a mirrored surface. Thin sections are then cut, and individual trapezoid-shaped sections adhere to the knife edge until the subsequent section displaces the previous section during the cutting stroke of the microtome arm. With each consecutive section, a ribbon resembling the shape of a tapeworm floats into the boat (Fig. 2B.1.2). These ribbons are typically manipulated into parallel rows with a fine eyelash attached to the end of a small applicator stick just prior to picking up the sections on a 3-mm-diameter electron microscope grid. Sections are adhered to the grid by lowering it with a pair of forceps above the sections, gently touching the grid to the surface of the water, and then lifting the grid off the water surface in a smooth motion. Residual water is removed from the edge of the grid with a wedge of filter paper as is done for negative staining.

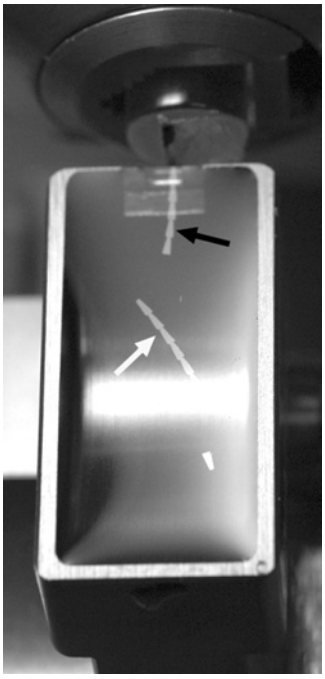

Figure 2B.1.2 Thin sections (~70 to 80 nm) prepared on a diamond knife as seen through a stereo microscope. Trapezoid-shaped sections are cut sequentially, adhering to the knife edge and forming ribbons during sectioning (black arrow). They are visible as the result of reflected light from above, which generates an interference pattern that can be used to estimate section thickness. As sections accumulate, they can be separated into smaller ribbons, which float on the water surface and can be aligned for collection onto a grid. The width of the trough of the diamond knife shown is ~10 mm.

Copper grids are routinely used for most biological samples; nickel grids are usually used with samples prepared for immunogold electron microscopy. The 50- to 90-nm thickness of each section can be estimated by adjusting the microtome stereomicroscope viewing and illumination system to detect interference colors reflecting from the sections. Generally, gray sections indicate a thickness of less than 60 nm, while silver sections range from 60 to 90 nm (the typical section thickness for most biological samples), and gold sections range from about 90 to 150 nm. The diamond knife, which contains a high-quality diamond with a cleaved knife edge mounted in an aluminum holder fashioned into a trough for floating sections, is a considerably more expensive and longer-lasting alternative to disposable, freshly made glass knives.

BASIC PROTOCOL 6

HEAVY METAL (AND IMMUNOGOLD) STAINING OF THIN SECTIONS

Heavy metal staining of thin sections is normally required to impart contrast in thin-sectioned biological materials. Two of the most widely used heavy metal post-staining solutions (i.e., uranyl acetate and lead citrate) are described. It is also possible to immunolocalize antigens in thin sections using colloidal gold particles of various sizes (5 to 20 nm) that effectively scatter electrons, which can be easily seen even when sections are post-stained with uranyl acetate and lead citrate.

Materials

Thin sections collected on copper grids (Basic Protocol 5)
Uranyl acetate staining solution (see recipe)
Lead citrate staining solution (see recipe)

Whatman no. 4 filter paper
Spray bottle with distilled H_2O
Grid box

1. Place 50-µl drops of uranyl acetate staining solution onto a clean Parafilm surface using the technique described in Basic Protocol 1.
2. Using forceps, place grids containing sections face-down onto uranyl acetate drops using the technique described in Basic Protocol 1, and incubate for 3 to 10 min at room temperature.
3. Remove grids from uranyl acetate drops and carefully direct a gentle stream of Milli-Q or deionized distilled water from a spray bottle onto the section side of the grid for 10 to 15 sec, slowly wetting the back side of the grid (i.e., the side without the section).
4. Remove excess water with a wedge of Whatman no. 4 filter paper (see Basic Protocol 1); do not let section dry.
5. Place 50-µl drops of lead citrate staining solution onto a clean sheet of Parafilm and deposit the grid, section-side-up, into the drop. Incubate 2 to 5 min at room temperature. Use a new drop of stain for each grid.
6. Remove grids and gently run a stream of Milli-Q or deionized distilled water from a spray bottle down the tips of the forceps onto the grid to remove residual stain.
7. Remove excess water with a filter paper wedge and ensure that no water remains between the tips of the forceps by sliding a piece of filter paper down between them while releasing the grid from the forceps onto a clean piece of filter paper.
8. Ensure that grids are completely dry and place them into a grid box. Store in a desiccator until ready to image in the electron microscope.
9. Place lead and uranyl waste in appropriate containers for disposal.

IMMUNOGOLD POST-EMBEDDING STAINING OF THIN SECTIONS

ALTERNATE PROTOCOL 10

Localization of antigens in thin sectioned material usually requires special fixation procedures to limit the extent of macromolecular cross-linking, as well as embedment in a plastic resin which preserves antigenic sites. The following is one of many alternative procedures that can be used to localize antigens with immunogold reagents.

Materials

Thin sections on 300-mesh Formvar-coated nickel grids (Basic Protocol 5)
Immunogold blocking buffer (see recipe)
Primary antibody against antigen of interest and control (irrelevant) primary antibody of same Ig class
TBS-Tween (see recipe)
Reagents for colloidal gold labeling—one of the following:
  Colloidal gold-labeled secondary antibody against species from which primary antibody was obtained
  Biotinylated secondary antibody against species from which primary antibody was raised, and streptavidin-conjugated colloidal gold
  Colloidal gold-labeled protein A and/or protein G
TEM primary fixative 2 (optional; see recipe)
Whatman no. 4 filter paper
Spray bottle
Grid box

Additional reagents and equipment for etching of epoxy embedding resin (optional; see Support Protocol 2) and uranyl acetate/lead citrate staining of thin sections (see Basic Protocol 6)

1. If the sample is not embedded in LR White resin, perform Support Protocol 2.

Block sample

2. Place an ~50-µl drop of immunogold blocking buffer onto Parafilm and float sample-containing grid on top of it for 30 min as a blocking step.

Treat with primary antibody

3. Prepare appropriate dilution of primary antibody in TBS-Tween, place 50-µl droplets of the diluted antibody onto Parafilm, then place grids on droplets and incubate at room temperature for 2 hr.

   Controls can be processed at the same time and should include both an irrelevant primary antibody of the same class and another preparation without primary antibody.

4. Remove grid and draw off excess solution with a filter paper wedge (see Basic Protocol 1) and gently rinse with a stream of TBS-Tween from a spray bottle.

Label with colloidal gold

5a. *To use colloidal gold–conjugated secondary antibody:* Place appropriate dilution of colloidal gold-conjugated secondary antibody onto Parafilm in 50-µl droplets, then transfer grids onto droplets and incubate at room temperature for 60 min.

5b. *To use biotinylated secondary antibody:* Place appropriate dilution of biotinylated secondary antibody onto Parafilm in 50-µl droplets, then transfer grids onto droplets and incubate at room temperature for 1 hr. Wash grids briefly in 50-µl droplets of distilled water, then transfer to 50-µl droplets of streptavidin-conjugated colloidal gold and incubate at room temperature 60 min.

5c. *To use protein A and/or protein G:* Place appropriate dilution of colloidal gold–conjugated protein A, protein G, or protein A/G onto Parafilm in 50-µl droplets, then transfer grids onto droplets and incubate at room temperature for 60 min.

6. Rinse grids 1 min in a succession of four or five 50-µl droplets of TBS-Tween on Parafilm.

Post-fix (optional) and stain with heavy metals

7. *Optional:* Post-fix by transferring grid to a 50-µl droplet of TEM primary fixative 2 for 10 min, then rinse in a 50-µl droplet of TBS-Tween for 1 min.

8. Wash grid with a stream of Milli-Q or deionized distilled water from a spray bottle.

9. Stain grids with uranyl acetate and lead citrate as described in Basic Protocol 6.

10. Rinse grids in a gentle stream of distilled water and remove excess water with a filter paper wedge.

11. Ensure that grids are completely dry and place them into a grid box. Store in a desiccator until ready to image in the electron microscope.

ETCHING OF EPOXY EMBEDDING RESIN TO INCREASE IMMUNOGOLD STAINING

The following steps may precede Alternate Protocol 10 if the tissue is not embedded in LR White resin. Tissues subjected to optimal fixation for immunolocalization with colloidal gold reagents embedded in an epoxy resin may be etched to increase access of primary antibody to antigenic sites in the sectioned tissue.

Materials

Plastic etching solution: 5% (w/v) sodium metaperiodate (prepare fresh)
Thin sections on 300-mesh Formvar-coated nickel grids (Basic Protocol 5)
TBS-Tween (see recipe)
Spray bottle

1. Place 50-µl drops of plastic etching solution onto clean Parafilm.
2. Place grids section-side-down on etching solution for 10 min at room temperature.
3. Gently run a stream of TBS-Tween from a spray bottle down the tips of the forceps onto the grid to wash off etching solution.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified or deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Aldehyde quenching solution

Prepare 100 mM glycine by dissolving 0.75 g in 100 ml phosphate/sucrose rinse buffer (see recipe). Alternatively, prepare 100 mM ammonium chloride by dissolving 0.53 g ammonium chloride in 100 ml phosphate/sucrose rinse buffer. Store up to 1 month at 4°C.

Ammonium molybdate staining solution

Prepare a 1% to 4% (w/v) solution of ammonium molybdate tetrahydrate (e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies) in water. Adjust with 1 M KOH or NaOH to pH 7.5. Store up to several months at 2° to 8°C.

Ammonium molybdate is slightly more hydrophilic than phosphotungstic acid and may result in a more even distribution of sample and stain for some specimens.

Immunogold antibody dilution buffer

To 180 ml Milli-Q-purified water or distilled H_2O add:
1.04 g sodium phosphate, monobasic ($Na_2PO_4 \cdot H_2O$)
8.70 g sodium phosphate, dibasic, heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)
4 g bovine serum albumin (BSA), fraction V
0.6 ml Tween 20
Milli-Q-purified or deionized distilled water to 200 ml
Store up to 2 months at 4°C

Final concentrations 1 mM PBS containing 2% BSA and 0.3% Tween 20.

Immunogold blocking buffer

To 100 ml TBS-Tween (see recipe) add:
1 g bovine serum albumin (BSA), fraction V
3 ml normal serum from species in which secondary antibody was generated
Stir to dissolve
Store up to 6 months at 4°C

Final concentrations: 1% (w/v) BSA, 3% (v/v) serum in TBS-Tween.

Lead citrate solution

Add the following items to 30 ml distilled H_2O in the following order:
1.33 g lead nitrate
1.76 g sodium citrate, dihydrate
5 ml of 1 N NaOH
Stir 10 min to dissolve
Add an additional 15 ml H_2O
Store solution for 3 to 6 months at 4°C

The solution becomes cloudy when sodium citrate is added and then clear when NaOH is added.

Luft's epoxy mixture

Combine the following components in a plastic disposable beaker and mix thoroughly while avoiding the generation of bubbles in the resin:
12 g Epon 812 (e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
7.0 g nadic methyl anhydride (NMA; e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
6.0 g dodecenyl succinic anhydride (DDSA; e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
0.5 g 2,4,6-tri-dimethylaminomethyl phenol (DMP-30; e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
Prepare fresh

Mollenhauer's no. 2 resin mixture

Combine the following components in a plastic disposable beaker and mix thoroughly while avoiding the generation of bubbles in the resin:
6.2 g Epon 812 (e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
8.1 g Araldite 506 (e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
10.0 g dodecenyl succinic anhydride (DDSA; e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
0.5 g 2,4,6-tri-dimethylaminomethyl phenol (DMP-30; e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies)
Prepare fresh

Osmium tetroxide post-fixative

In a fume hood and wearing nitrile gloves, combine the following in a 25-ml graduated cylinder:
2.5 ml of 4% aqueous osmium tetroxide stock from sealed ampule (see recipe for TEM fixative stock solutions)
5 ml phosphate/sucrose buffer stock (see recipe)
2.5 ml Milli-Q-purified or distilled water
Mix and keep on ice for immediate use
Prepare fresh each time

Final concentrations: 1% (w/v) osmium tetroxide in 100 mM phosphate buffer containing 100 mM sucrose, pH 7.4.

Phosphate/sucrose buffer stock solution

To 150 ml of Milli-Q-purified water or distilled water add:
1.04 g sodium phosphate monobasic ($Na_2PO_4 \cdot H_2O$)
8.70 g sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)
13.68 g sucrose
Milli-Q-purified or deionized distilled water to 200 ml
Store up to 2 months at 4°C

Final concentrations: 200 mM sodium phosphate plus 200 mM sucrose, pH 7.4.

Phosphate/sucrose rinse buffer

Combine 50 ml phosphate/sucrose buffer stock solution (see recipe) with 50 ml Milli-Q-purified or deionized distilled water. Store up to 2 months at 4°C.

Final concentrations: 100 mM sodium phosphate plus 100 mM sucrose, pH 7.4.

Phosphotungstic acid staining solution

Prepare 1% to 2% (w/v) solution of phosphotungstic acid (e.g., Electron Microscopy Sciences, Ted Pella, or SPI Supplies) in water. Stable for months (pH 7.0) at 2° to 8°C.

Solution is acidic and should be adjusted to a pH ranging from 5.0 to 7.0 with 1 M KOH or NaOH. A pH of 7.0 is most often used.

This is a versatile stain for viruses, bacteria, subcellular structures, and macromolecules.

TBS-Tween

To 1 liter of distilled water add:
6.1 g Trizma base
9 g NaCl
Mix to dissolve
Adjust pH to 7.6 using 1 N HCl
Add 0.5 ml Tween 20
Store up to 6 months at 4°C

Final concentrations: 0.5 M Tris-buffered saline, 0.05% Tween 20.

TEM fixative stock solutions

The most convenient source for EM-grade fixative stocks is from commercial electron microcopy suppliers (e.g., Electron Microscopy Sciences, Energy Beam Sciences, Structure Probe/SPI Supplies, or Ted Pella). For example, stock solutions of 16% paraformaldehyde, 8% or 25% glutaraldehyde, and 4% aqueous osmium tetroxide can be purchased in 10-ml quantities in sealed ampules individually or in boxes of 10 or more units. These stocks typically have a shelf life of 12 months if unopened, but once opened must be used in fixative solutions on the same day, preferably within hours of preparation.

Uranyl acetate staining solution

Dissolve 1 g uranyl acetate (Polysciences, cat. no. 21447) in 100 ml Milli-Q-purified or distilled water by stirring overnight at room temperature. Stable for months at 2° to 8°C in the dark.

Uranyl acetate can produce an image with higher contrast than phosphotungstic acid. Because uranyl acetate has smaller grain size than phosphotungstic acid, it has the potential for producing an image with higher resolution. Uranyl acetate is a useful stain for viruses, (especially since it tends to inactivate them) and macromolecules; however, structures sensitive to low pH may be damaged. Specimens stained with uranyl acetate may exhibit both positive and negative staining. Uranyl acetate should not be used with samples prepared in phosphate buffer unless they are first rinsed either in distilled water, or, in the case of unfixed biologically sensitive organisms, HEPES buffer, as uranyl ions form an insoluble uranyl phosphate.

COMMENTARY

Background Information

Negative staining TEM

Negative staining is one of the simplest yet most useful methods for imaging small particles in suspension. Introduced in the late 1950s for the study of viruses, it has been widely used for diagnostics in infectious diseases and for structural analysis of particulates ranging from bacteria to viruses to macromolecules. Because of the simplicity of the technique,

it is also a very practical method for rapid screening and quality assessment of fractionated cellular constituents and protein preparations and for screening contaminants of cell cultures. In recent years, bioterrorism threats have increased awareness of the utility of negative staining for identifying infectious disease agents, e.g., for assisting in poxvirus diagnosis and/or ruling out other causes of illness involving rash. Electron microscopy techniques, including negative staining, can provide relevant information in conjunction with other investigative techniques to identify and/or further the understanding of infectious diseases. Useful information regarding sample collection and preparation, and particle enrichment in the application of electron microscopy for identification of infectious disease agents has been reviewed by Hazelton and Gelderblom (2003). Other considerations for application of negative staining in the ultrastructural analysis of proteins and macromolecular complexes have been reviewed by Ohi et al. (2004).

By embedding particulate samples in an air-dried heavy metal solution, which is thought to occupy the hydrated sample surface and associated structures, the transparent (i.e., electron translucent) sample is surrounded by a dark halo of stain that scatters electrons, resulting in a negative image of the sample. Quite a range of different negative-staining salts are available (see Bremer et al., 1992), each exhibiting a variety of interactions with the sample due to properties of the stain (e.g., size of the metal salt crystals affecting resolution, as well as pH of the stain and other considerations) that can affect sample-stain interactions. These interactions can damage some fragile structures and are an important consideration when choosing a suitable negative stain. While there are numerous negative stains, those listed for use in Basic Protocol 1 are widely used and considered reliable for a wide variety of applications. Phosphotungstic acid and ammonium molybdate yield negatively charged metal oxide ions and have the advantage that the pH of the stains can be adjusted. Uranyl acetate is a positively charged stain that produces high contrast and has fixative properties; however, the staining solution is acidic (pH ~4.5) and pH adjustment without precipitation of the stain is limited. The above-referenced stains have been selected as general-purpose negative stains; however, other formulations may be advantageous for specific applications (see Hayat and Miller, 1990). For example, uranyl formate, despite its relative instability, is the highest-density uranyl salt with smallest grain size, and is often required for structural analysis of macromolecules, particularly those of smaller size (Ohi et al., 2004).

It is important to note that the utility of negative staining can be significantly enhanced in conjunction with immunoelectron microscopy methods to improve sensitivity of sample (usually virus) detection, for enrichment of specific sample components, for rapid detection and diagnosis of infectious agents, and/or for localization or structural identification of antigens within a sample. For example, rapid immunologic methods such as solid-phase immunoelectron microscopy, which involves attachment of antibodies or protein A plus antibodies to the grid (Pegg-Feige and Doane, 1983) and the serum-in-agar method, which employs pooled human immunoglobulins or specific antibodies in agar (Anderson and Doane, 1973), are both used to concentrate antigens by immune capture and are useful to search for specific and/or unknown infectious agents prior to negative staining. Many of these applications are described in Doane and Anderson (1987).

Immunoelectron microscopy

In general, immunoelectron microscopy applications involve the direct electron microscopy imaging of antigen-antibody complexes. A wide range of applications have been developed to improve the sensitivity of microbe detection and to reveal the ultrastructure of elusive organisms. When used to increase detection sensitivity, antibodies serve to aggregate viruses or bacteria in solution or onto a grid for negative staining. Immunogold staining can be combined with negative staining to provide a sensitive technique that enables the identification and visualization of individual antigens by TEM, and which can be useful in the identification of the biologic agent itself.

Fixation techniques for TEM

Ultrathin sections of samples provide insights regarding the internal structure of chemically stabilized samples. Individual sections that are used to provide images in the TEM represent two-dimensional views of three-dimensional objects; therefore, considerable efforts must be made to understand the three-dimensional ultrastructure of a sample. It is also important to appreciate that the appearance of the thin-sectioned sample is the result of the multiple steps in the protocol, including isolation of the sample, one or more chemical fixation steps, dehydration and embedding, sectioning, and staining.

Embedding techniques for TEM

The term epoxy resin, as used in electron microscopy, usually denotes a mixture of at least three and sometimes four components: resin, hardener (frequently a mixture of two hardeners to yield polymerized resin with the correct characteristics), accelerator, and sometimes a resin modifier. The resin formulations provided herein yield reproducible results for the preparations mentioned in this unit.

The component Epon 812 is used in two of the formulations outlined above and come from their original citations (Luft, 1961; Mollenhauer, 1964). This particular component is no longer produced by the original manufacturer, but suitable substitutes are available. These include Poly/Bed 812 from Polysciences, EMbed 812 from Electron Microscopy Sciences, SPI-Pon 812 from SPI Supplies, LX-112 from Ladd Research, and Eponate 812 from Ted Pella.

TEM of thin sections

Contrast in thin-sectioned biological materials is generated in the electron microscope as the result of scattering of electrons by macromolecular constituents of the sample that have bound heavy metal stains. In several of the tissue-processing protocols given above, both osmium tetroxide and uranyl acetate were used because of their combined properties as postfixatives and heavy metal stains. Additional electron-scattering stain is usually deposited on thin-sectioned samples shortly after collecting sections onto a grid and is usually the last step of sample preparation prior to viewing sections on the TEM.

Commercially available colloidal gold conjugated to protein A/G, secondary antibodies, or streptavidin can also be used as an electron-scattering agent for immunolocalization of antigens in thin sections. Many of the suppliers of these products have recommended protocols and specific reagents for use with their products, although the protocol provided here is fairly generic.

Critical Parameters and Troubleshooting

Negative staining TEM

Several iterations of this basic procedure are usually required to optimize the concentration and distribution of the sample particles on the grid. The sample dilution is usually determined empirically. Stain density can be varied by adjusting the amount of excess stain during removal with the filter paper wedge, by additional washing steps, and/or by reducing the concentration of the staining solution. As with any technique, technical problems can arise with the sample itself (e.g., concentration, purity, stability, and solute composition), the substrate used to support the sample (stability of support film under the electron beam, hydrophilicity of the support film affecting sample distribution, etc.), and the stain selected (stability of sample in stain, grain size, etc.). A list of some of the technical problems that can occur with negative stain preparations, some of their causes, and possible solutions are summarized in Table 2B.1.1.

Immunoelectron microscopy

Adequate controls must be incorporated into the procedure for the proper analysis of experimental results. Controls should include a grid in which the primary antibody incubation is omitted and a grid incubated with a nonspecific IgG of the same species as the primary antibody to identify nonspecific binding. Adjustments to increase labeling and decrease nonspecific binding may include, for example, use of different or additional blocking agents and/or concentrations and blocking times, changing concentration or incubation time of primary antibody, or addition of more rinses (see Table 2B.1.2).

When considering the use of the protein A–, protein G–, or protein A/G–gold procedure, it is important to understand that, while these conjugates interact with mammalian immunoglobulins, their individual affinity for immunoglobulins is not equivalent for all species or for all antibody subclasses. For example, in the case of polyclonal antibodies, protein A or protein G can be used for human, pig, rabbit, or mouse. Horse and cow immunoglobulins have relatively high affinity for protein G, while sheep, goat, chicken, hamster and rat antibodies will bind protein G weakly. Guinea pig immunoglobulins will bind protein A. For use with monoclonal antibodies, human isotypes IgG_1, IgG_2, and IgG_4 have good affinity for protein A or protein G, while human IgG_3 has good affinity for protein G only. Protein G is used with rat isotypes, whereas mouse isotypes $IgG_{1,}$ $IgG_{2a,}$ $IgG_{2b,}$ and IgG_3 all bind protein G, and mouse IgG_{2a} and IgG_{2b} also bind protein A. The use of protein A/G can therefore be useful if the antibody isotype is unknown.

Another important consideration is size and affinity of the conjugated gold particles for the primary antibody. In general, the smaller the gold particle, the lower the steric hindrance to

Table 2B.1.1 Troubleshooting Guide for Negative Staining

Problem	Possible cause	Solution
Sample not uniformly dispersed on grid	Grids with support films tend to be hydrophobic	Grids with support films can be glow discharged shortly before staining.
		Wetting agents can be used make grids more hydrophobic. Incubate grids for 30-60 sec on a drop of bacitracin (50 μg/ml), poly-L-lysine (1 μg/ml), or BSA (0.05% to 0.005% w/v).
		Alternatively, wetting agents can be added directly to the sample to aid spreading.
Stain not uniformly dispersed on grid	Grids with support films tend to be hydrophobic	Can add wetting agents at lower concentrations than those mentioned above directly to the stain
		If using phosphotungstic acid, switch to a more hydrophilic stain such as ammonium molybdate
Stain density too dense to discern structural details	Too much stain retained on the grid	Allow more stain to absorb onto the filter paper before removing grid during staining
	Stain concentration too high	Reduce stain concentration
	Adjust accelerating voltage on TEM	Increasing acceleration voltage can result in the reduction of image contrast
Structural details of sample surface are not resolved	Grain size of negative stain may be too large	Phosphotungstic acid grain size is ~0.9 nm whereas uranyl salts are approximately half of this. Molecular dimensions of the negative stain must be smaller than dimensions of structures in the hydrated sample surface.
Stain precipitate	Buffer containing phosphate forms insoluble salts with uranyl salts	Wash sample with additional distilled water to remove phosphate
		Resuspend sample in a non–phosphate containing buffer such as HEPES or PIPES
		Change stain
Unstable coating during examination in the electron microscope	Heating of the film due to interaction of the electron beam with the film and the specimen	If using Formvar-coated grids, switch to those with a carbon coating
		Stabilize the film by slowly increasing the intensity of the electron beam over time as it crosses the grid
		Coat grid with an additional layer of indirect carbon using a vacuum evaporator

Table 2B.1.2 Troubleshooting Guide for Negative Stain Immunoelectron Microscopy

Problem	Possible cause	Solution
No gold labeling	The antigen may be present in very low amounts	Use longer incubation times and more concentrated primary antibody
	The primary antibody may be bad, e.g., due to poor titer, age, improper storage, improper dilution, or excessive freezing and thawing	If available, run a positive control to check
	The pH of solutions may be excessively acidic or alkaline	Adjust the pH
	Heavy negative staining may mask gold particles	Reduce the concentration of negative stain and/or use larger gold particles. Use higher magnification to visualize gold particles (e.g., 200,000× for 5 nm; 100,000× for 10 nm; 80,000 for 15 nm; 50,000 for 20 nm).
	The section may not have been exposed to solutions (as a result of being wrong-side-up) if on a plastic film	Be careful when transferring and washing grids that the side of the grid containing the sample is kept facing up
	The antigen may be destroyed by preparative procedures	Use a different procedure. Consider brief fixation in 1% paraformaldehyde.
Excessive background gold particles	Ionic concentration of solutions may be too low	Use increased salt concentration (up to 2.5%). Add ovalbumin, BSA, or normal goat serum (not for protein A) to ~1% in incubation solutions.
	Sections may have been inadequately washed between incubations	Increase washing steps
	Nonspecific charge attraction of antibody can cause background	Use 1% detergent (e.g., Tween 20) in all solutions. Include normal goat serum (not for protein A) in all solutions. Increase concentration of normal goat serum before primary antibody incubation.
	Free aldehyde groups in fixed tissue may be a source of background	Reduce by exposing sections to 0.5 M ammonium chloride for 1 hr before incubations.
	The primary antibody concentration may be too high	Dilute by orders of magnitude
	The gold conjugate concentration may be too high	Dilute further
Clustering of gold particles	Clumped primary antibody.	Use fresh antiserum
	Clustering can be caused by the natural amplification factor of the gold conjugate. For IgG–gold conjugates, up to 10 conjugated gold particles may attach to the Fc component of the primary antibody, producing the appearance of clusters on the section. This does not occur with protein A conjugates.	Use higher dilution of gold conjugates if desired
Gold particles over surface of support film	Nonspecific binding	Incorporate additional blocking steps prior to delivery of primary antibody

antigen detection; however, a confounding consideration is that the smaller the gold particle, the fewer the molecules of protein A bound per gold particle. For example, it has been estimated that a 5-nm gold particle can bind about four protein A molecules, whereas a 20-nm particle can bind ~48 protein A molecules. Similarly, a 5-nm gold particle can bind about three secondary antibody molecules, while 10-nm gold particles can bind ~12 antibody molecules and 20-nm gold particles can bind ~48 antibody molecules. Therefore, the affinity of the gold particle can increase with increasing size. An additional consideration when immunogold staining is combined with negative staining is that the smallest gold particles (e.g., 5 nm) are much more difficult to detect in negatively stained preparations; therefore, the recommended range of gold particle sizes is from 10 to 30 nm.

The use of colloidal gold-tagged secondary antibodies described above is an alternative to protein A/G and involves a secondary antibody generated in a different species against the Fc fragment of the primary antibody. A third alternative involves the use of biotinylated secondary antibodies that are subsequently rendered visible in the TEM by binding of colloidal gold–conjugated streptavidin. Immunolocalization of two antigens in the same preparation is also possible if compatible primary antibodies are available (e.g., primary antibodies generated in different species). This procedure involves the sequential indirect labeling of two primary antibodies with secondary antibodies conjugated to gold particles of different size.

Fixation techniques for TEM

Preparation of the sample and the choice of fixative, stains, and plastic resin can affect the quality of the preparation. Unfortunately, there is no optimal fixative for all biological samples, although freshly prepared fixatives containing glutaraldehyde are considered among the best primary fixatives currently available. The useful property of glutaraldehyde, a five-carbon dialdehyde, is its ability to cross-link proteins. Freshly prepared glutaraldehyde at neutral pH will also polymerize into longer chain dialdehydes, which provide cross-links of variable size that efficiently stabilize protein structure. Paraformaldehyde is commonly combined with glutaraldehyde to better stabilize biological samples. In general, when fixatives are identified as containing paraformaldehyde, it usually means that they contain formaldehyde generated from the paraformaldehyde polymer of formaldehyde. The advantages of this freshly prepared one-carbon monoaldehyde, formaldehyde, which reacts in an aqueous solution as methylene glycol, are its rapid penetration properties and ability to polymerize and cross-link proteins and nucleic acids, and also modify the chemical properties of lipids. While not included here, acrolein is another highly reactive fixative that is often used in combination with other aldehydes such as glutaraldehyde and/or paraformaldehyde for fixing very dense specimens.

The primary aldehyde-fixation step is frequently combined with a post-fixation step involving osmium tetroxide, which acts as a fixative and electron stain and also as a mordant to enhance staining of the sections with lead. The primary fixatives and post-fixatives described in these protocols are generally considered to be good starting points for the preservation of solid tissues and cells in either suspension or pellets. More specific protocols are available in the literature that address specific fixation requirements of specific organisms and cells (Fassel et al., 1997; Karlyshev et al., 2001). Variations on fixative concentrations, fixation times, buffer types and osmolarity are all important considerations for optimal preservation of any particular sample.

The preparation of individual cells or aggregates of cells poses special challenges. Generating a sample of adequate size for processing usually requires concentration of cells by centrifugation into pellets. Maintenance of pellet integrity is also an important consideration during primary fixation, post-fixation, post-staining, dehydration, and embedding. If cells are not sufficiently concentrated during centrifugation before addition of fixative, as described in Alternate Protocol 5, there can be a tendency for the pellet to disintegrate during subsequent processing. If the pellet is of sufficient size to yield multiple slices for processing, some cell loss can be tolerated. If not, then it is important to encase the slices of pellet in a thin layer of agar to preserve the integrity of the pellet. Proper temperature management of the agar used to encase suspension fixed cells is also important during the preparative procedure. The agar must be warm enough to remain molten when the cells are introduced while at the same time not being hot enough to damage tissue and/or to allow the cells to effectively spin out of the agar during centrifugation. It is advisable, if the sample size permits, to subdivide the sample and perform multiple runs to ensure preservation of the pellet.

Preparation of tissues and cells for immunogold labeling requires a balance between preservation of tissue/cell ultrastructure and the preservation of antigens. The goal is to optimize these compromises in order to combine adequate structural detail and adequate immunochemical labeling. Concentrations of glutaraldehyde that are normally used for ultrastructure most often destroy or cross-link antigens. Therefore paraformaldehyde alone or in combination with dilute glutaraldehyde (0.1% to 1%) is a useful starting point for fixation. Tissues processed for immunolabeling in thin sections require special embedding procedures, as discussed below.

The protocols presented in this section can be classified as representing classical approaches to the preservation of tissue for examination by thin-section electron microscopy. Other techniques are available that offer the advantages of potentially better preservation of specimen ultrastructure and possibly shorter processing times at the expense of having specialized processing equipment, additional safety concerns, and special modifications of the electron microscope itself. Freeze substitution, accomplished by the formation of vitreous ice within the sample by either plunging the sample into cooled liquid propane or ethane or exposing samples to a jet of liquid propane or to high-pressure freezing, offers the advantage of better preservation of ultrastructure, and usually better antigen preservation as well. Microwave processing techniques offer the advantage of rapid specimen fixation and embedding times. All of these techniques require specialized preparation protocols adapted to suit the specific equipment on hand, and are beyond the scope of this chapter.

Embedding techniques for TEM

By their nature, epoxy resins and their components are viscous, and this viscosity tends to increase with time after the individual components of the resin are mixed. Because of this, it is advisable to prepare resins in small batches just prior to use. Dilutions of complete resin mixtures in dehydration agents are prepared by dispensing the resin components into new disposable beakers. Preparations of resin and dehydration agent are kept capped to prevent evaporation of the agent over time, an especially important consideration when working in a fume hood. In the case of epoxy resin, accurate weighing of all components and dilution of resins is of critical importance in their preparation to ensure a resin mixture that will give the proper characteristics. It is also important that the individual components of the resin be completely mixed. Inadequate mixing of resin components can result in poorly infiltrated samples that section poorly, that have poor specimen contrast, and that do not hold up in the electron beam. Shaking or vigorous stirring results in well mixed resin. Any resulting entrapped air is liberated upon standing or can be removed by use of a vacuum chamber for 5 to 10 min or until the generation of gas bubbles stops (the vacuum provided by a one-stage rotary pump is sufficient). Caution must be used when degassing the resin mixture, as it can boil out of the disposable beaker; careful monitoring of the mixture in the vacuum chamber is necessary. Furthermore, the ability to introduce air into the chamber to minimize initial boiling of the resin during degassing is also necessary.

The decision to process cultured cells as pellets or as intact monolayers warrants additional comment. While scraping and pelleting monolayers provides a useful approach for collecting and concentrating a sufficient number of cells for processing, the disadvantage of this approach is that scraping of monolayers can damage some cells and disrupt the cell-substrate interactions, and often cell-cell interactions as well. Figure 2B.1.3 shows an example of virus-infected cultured chicken spleen cells prepared as a pellet.

When there is a need to evaluate the polarity of cells and/or cell-cell and cell-substrate interactions, processing of monolayers in chambered slides is a useful, albeit more complicated alternative that poses some special challenges. Figure 2B.1.4 shows an example of HeLa cells prepared as a monolayer in chamber slides along with the appearance of the sample at different stages of the processing steps, ending with the embedment of the entire monolayer for thin sectioning.

The individual epoxy resin–filled chambers with embedded cells, outlined by the silicone gasket, should come free cleanly from the glass microscope slide surface. Each individual chamber can be further subdivided and mounted for sectioning purposes. It is important when processing monolayers to ensure that there is an adequate exchange of fluids during dehydration and resin infiltration. Areas of the monolayers near the edges of the gasket on chambered slides tend to retain fluid during exchanges, which can inhibit uniform dehydration and infiltration of the monolayer. If the monolayer being prepared is not confluent, identification of the areas of embedded chambers where cells are located can be aided

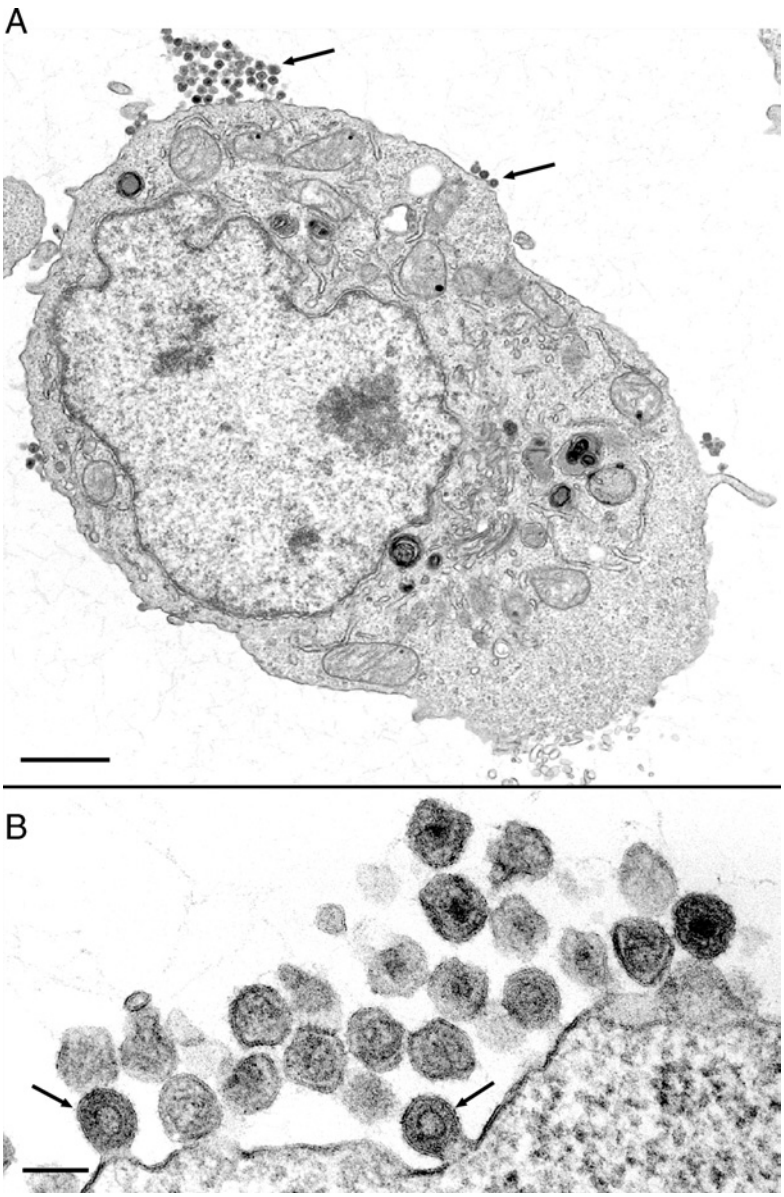

Figure 2B.1.3 Transmission electron micrograph of cultured chicken spleen cells infected with avian reticuloendotheliosis virus, embedded using Basic Protocol 4. (**A**) Clusters of virus particles (arrows) are seen adjacent to the plasma membrane of an individual spleen cell. (**B**) Higher magnification of an area of plasma membrane with shedding virus (arrows) similar to that shown in A. Bars = 1.0 μm (A) and 0.1 μm (B).

by the addition of a staining step just prior to the initiation of dehydration. A stain composed of toluidine blue (0.365 g) and basic fuchsin (0.135 g) in 50 ml of 30% ethanol can be used to stain cells just prior to the first step in the dehydration sequence. The stain is diluted 1:10 in distilled water and the cells are stained for 10 min. The cells will destain to some degree during the subsequent steps of the dehydration process, but there will be enough residual stain in the cells after resin polymerization to enable areas of high cell density to be easily visualized. En face sectioning of the embedded monolayers allows for the observation of cells from the attachment surface of the cells up through the cells to the apical surface of the cell. While positioning the attachment surface of the monolayer parallel to the knife edge while sectioning will allow one to serially section through the cells, sectioning on an axis slightly off parallel will enable both the apical and basal surfaces of the monolayer to be visualized simultaneously as the block is sectioned. If cross-sections of the monolayer are

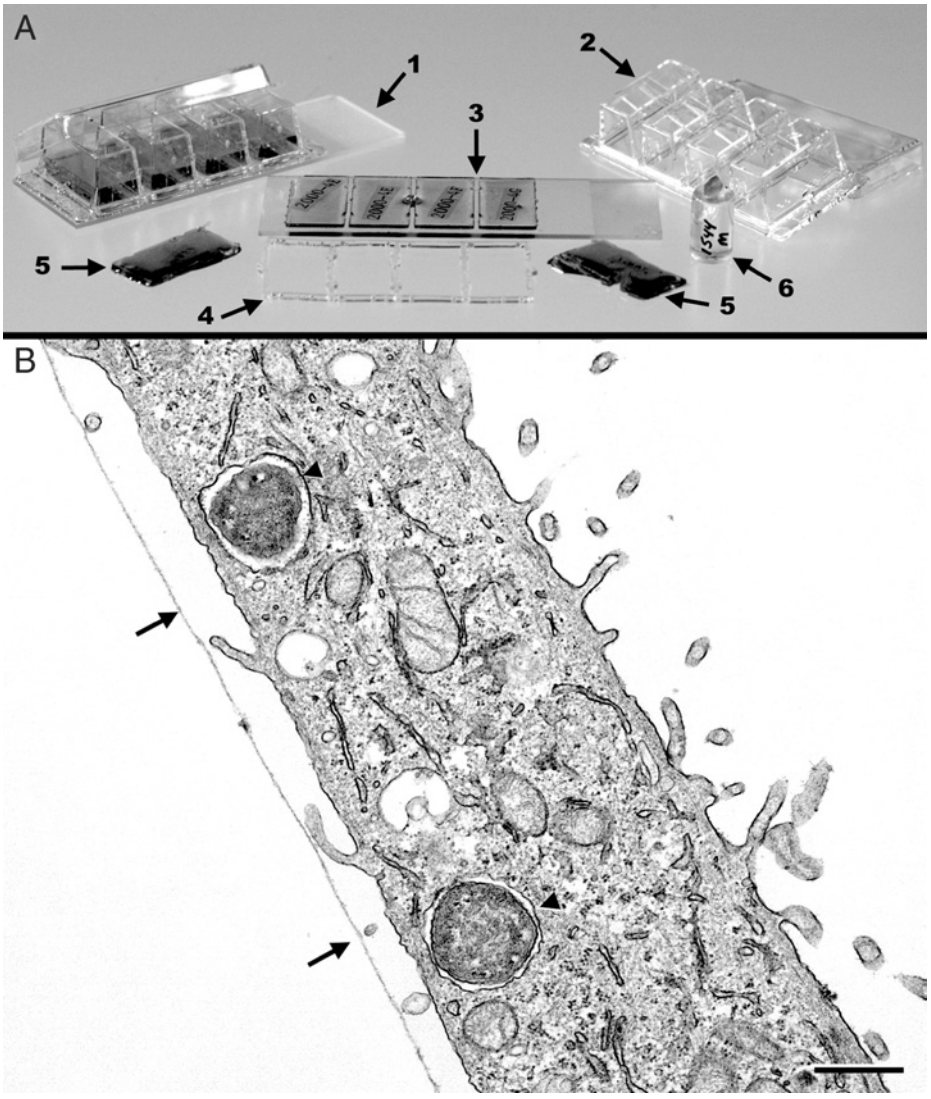

Figure 2B.1.4 Monolayer processing in chambered slides. (**A**) The 4-well chambered slide consists of plastic wells mounted onto a glass slide (1) with a silicone gasket. When the plastic chamber is removed (2), a thin layer of unpolymerized epoxy resin is added (3) at the thickness of the silicone gasket (4). Embedded wells containing cells are removed from the glass slide (5) and pieces of the resin embedded wells are mounted onto plastic pegs for sectioning (6). (**B**) Transmission electron micrograph of a HeLa cell monolayer grown and processed for electron microscopy on a 4-well chambered slide. In this preparation, a cross-section of the monolayer was prepared following removal of the embedded monolayer from the glass slide, and an additional layer of fresh resin (5 in panel A) was added to the substrate side. A piece of the monolayer was then cut with a jeweler's saw and mounded on plastic pegs (6 in panel A) for sectioning. Bar = 0.5 μm.

desired, the side of the embedded monolayer in contact with glass, upon removal of the slide, can be coated with epoxy resin which is then allowed to polymerize. This additional layer of resin material should support the edge of the monolayer for cutting cross-sections.

The cells of embedded monolayers frequently display less contrast than similar cells that have been fixed, scraped off the tissue culture vessel, pelleted, and embedded. This reduced contrast is thought to result from a more complete infiltration of the resin components compared to what is achieved when embedding larger pieces of tissue (Mollenhauer and Droleskey, 1997).

Embedding molds are available from commercial electron microscopy suppliers in a variety of shapes and sizes. In selecting embedding molds, consideration should be given to the relative size of the tissue piece or cell

pellet, the number of tissue pieces, the tissue orientation, and the tissue's ability to be easily mounted in the specimen holder of the ultramicrotome. In general, use of flat embedding molds offers the most options for sectioning of tissues or cell pellets, as they allow for specific portions of the pellet to be excised for subsequent mounting and sectioning. These embedded portions can be removed by gently heating the polymerized resin and cutting it with a single-edged razor blade or by sawing the selected area out using a jeweler's saw.

For embedding of material in LR White resin, it is important to remember that dehydration in acetone is omitted along with dilution of the resin in a solvent. For optimal antigen preservation, it is advisable to cool this resin during polymerization to dissipate the heat generated. It is possible to polymerize LR White resin using heat alone, without the accelerator, but this can be detrimental to antigen preservation and can lead to incomplete curing of the block unless additional steps are taken.

TEM of thin sections

Staining with uranyl acetate and lead are relatively straightforward procedures. Occasionally, electron-dense precipitates occur that are the result of a staining problem. Care is taken with lead citrate to minimize the exposure to CO_2, which can come from one's breath and result in the precipitation of insoluble lead carbonate. Precipitates can be formed from contaminants in glutaraldehyde and osmium tetroxide, and, as noted previously, uranyl phosphate forms an insoluble precipitate. For valuable samples, post-staining precipitates can sometimes be removed by using the plastic etching solution for 10 min, washing in a stream of distilled water, and then restaining (see Aldrich and Mollenhauer, 1986).

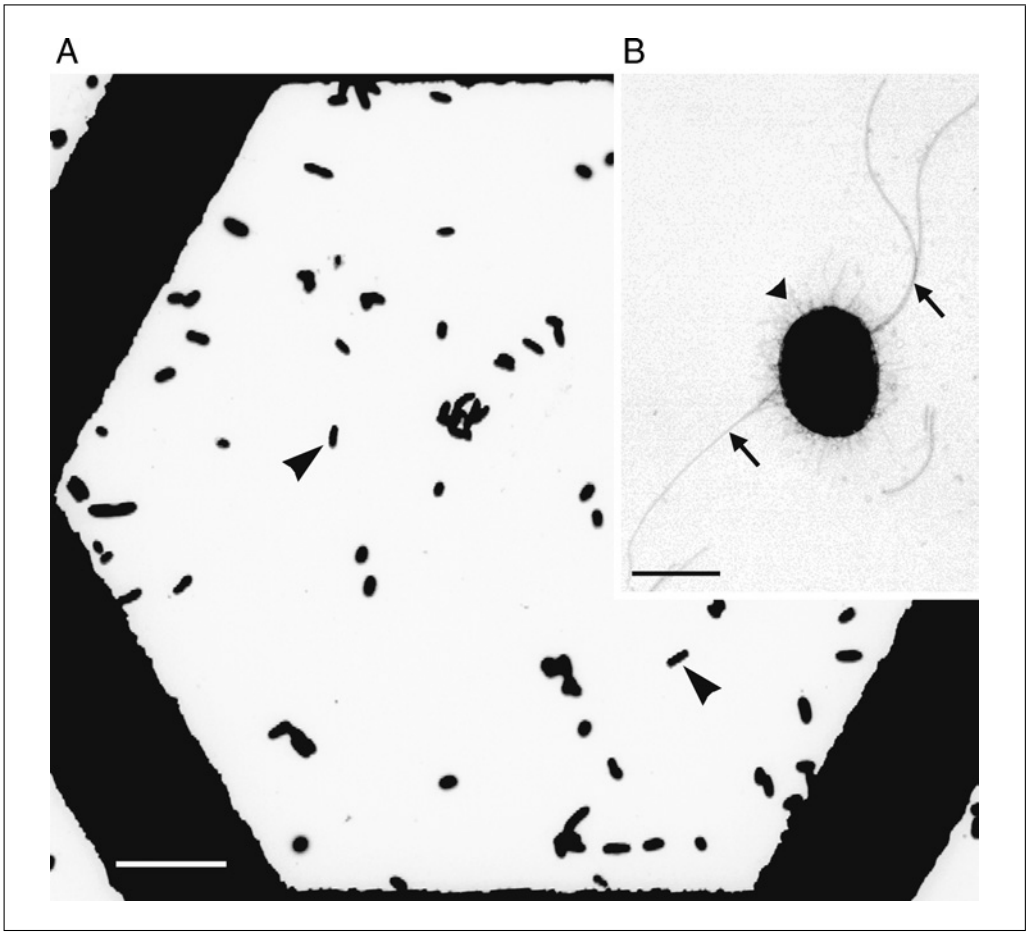

Figure 2B.1.5 Transmission electron micrographs of *Salmonella typhimurium* negatively stained using the direct deposition of sample and stain procedure (Alternate Protocol 1). (**A**) An example of an even distribution of bacteria (arrowheads) over the surface of a 300-mesh Formvar-coated hexagonal grid. Grid stained with 0.25% ammonium molybdate. (**B**) Higher magnification of a bacterium from the same grid. Peritrichous flagella are visible radiating from the cell (arrows) while individual fimbrae are barely evident around the bacterium (arrowhead) at this magnification. Bars = 10 μm (A) and 0.5 μm (B).

Troubleshooting suggestions for immunogold staining of negatively stained preparations are summarized in Table 2B.1.2 and are generally relevant to immunogold staining of thin sections. It is important that a suitable nonspecific binding control be included for all samples. This should at least consist of a section blocked according to the standard procedure described above and then incubated on the colloidal gold suspension, eliminating the primary antibody incubation step. The addition of a grid incubated with a purified IgG from the same species as that in which the specific primary antibody was raised and used at the same concentration as the primary antibody is also appropriate. Before any meaningful assessment of labeling is performed, these controls must be evaluated for labeling. In addition, the degree of labeling in the portions of the grid containing only support film and, if present, plastic without sample, provides an index of background labeling. While it is not absolutely necessary to use Formvar-coated grids in this procedure, they greatly aid the retention of sections on the grid, as the numerous wash steps in this procedure increase the chances of losing sections from uncoated grid surfaces.

LR White embedding resin was chosen for the above-referenced protocol due to its good antigen-preservation characteristics. Tissues embedded in epoxy resins may need to be etched to expose antigenic sites. Etching involves basic attack on the end-linked epoxide rings. Some proteins and peptides and many amino acids have been successfully deplasticized by etching methods to restore immunoreactivity lost in the embedding process.

Anticipated Results

Negative staining TEM

A successful negatively stained preparation has sample distributed on all Formvar-coated grid squares; aggregates of sample and stain can be easily detected at low magnification in the electron microscope. Figures 2B.1.5A and B show low and higher magnification view of a grid in which a relatively uniform distribution of sample and stain. Figures 2B.1.6A and B provide representative examples of negatively stained bacteria and bacteriophage.

Immunoelectron microscopy

When successfully labeled and stained, the negatively stained organisms should be dispersed onto the grid with colloidal gold

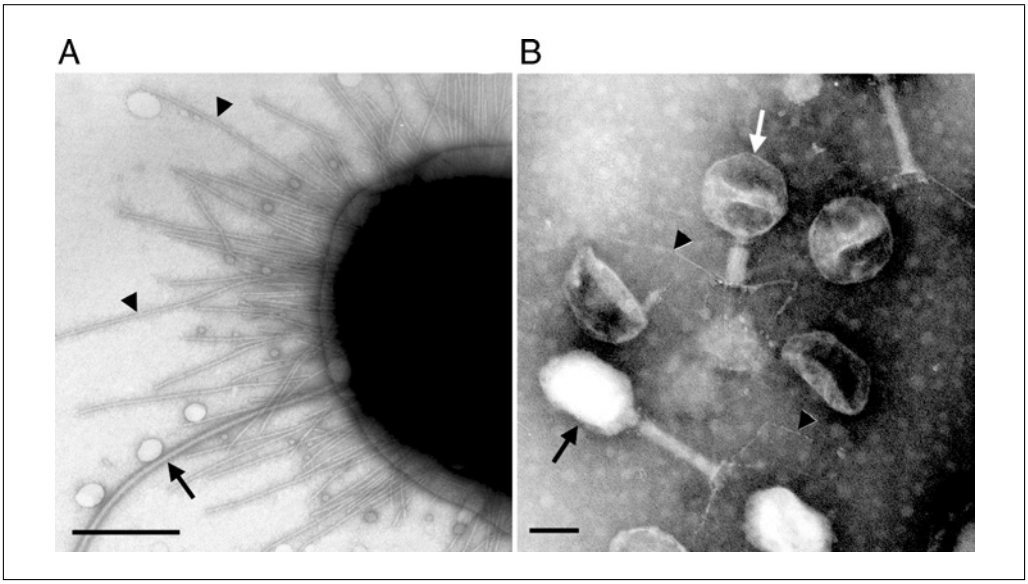

Figure 2B.1.6 Higher-magnification transmission micrographs of negatively stained organisms showing structural detail obtainable with negative stain. (**A**) *Salmonella typhimurium* stained with 0.5% ammonium molybdate by direct deposition of sample and stain showing type 1 fimbrae (arrowheads) and a single flagellum in the field of view. Stain is evenly distributed around the cell, which allows these structures to be easily discernible. (**B**) Bacteriophage (T-even type) isolated from the intestinal contents of a sheep stained with 0.2% phosphotungstic acid. Stain is distributed to allow for the recognition of tail fibers (arrowheads). Phage with intact (black arrows) and empty heads (white arrows) are present in the preparation. In the background of the preparation are globular aggregates of protein from the growth medium used to culture the phage and its host bacterium. Bars = 250 nm (A), 50 nm (B).

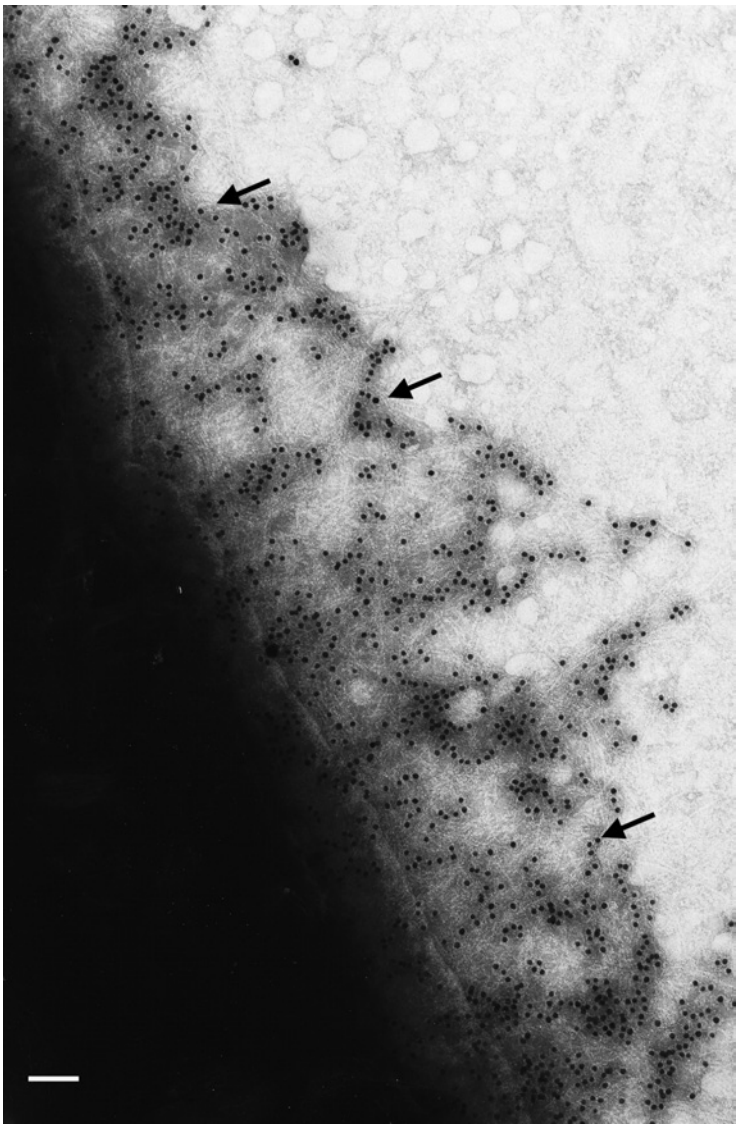

Figure 2B.1.7 Immunogold-labeled and negatively stained preparation of *Salmonella typhimurium* prepared using Basic Protocol 2. Bacteria were deposited on the grid and incubated with an anti-fimbrial primary antibody; this was followed by incubation with protein G–gold and negative staining with 1% ammonium molybdate. The 10-nm gold particles (arrows) decorate the fimbrial structures as seen in Figure 2B.1.5 A.

particles bound to antigenic sites located on the surface of the sample. The support film lacking any organisms should be free of gold particles.

Figure 2B.1.7 is an example of an immunogold labeled, negatively stained preparation of *Salmonella typhimurium*. Table 2B.1.2 lists some common problems with immunoelectron microscopy on negatively stained samples and some possible solutions.

Fixation techniques for TEM

At the conclusion of the fixation and initial processing of samples for thin-sectioning procedures, samples sufficiently stabilized to preserve ultrastructural organization are now ready for the step of embedding in plastic. This step is necessary to stabilize tissues in a matrix that is uniformly hardened to allow ultrathin sectioning with glass or diamond knives.

Embedding techniques for TEM

At the conclusion of the embedding process, tissues are infiltrated with a hardened matrix which is able to withstand the stress of ultrathin sectioning (i.e., ultramicrotomy) with glass or diamond knives.

Time Considerations

Negative staining TEM

The protocol for negative staining is relatively simple, and, when dealing with an ideal sample (i.e., high concentration of particulates in an easily diluted solvent, with a relatively stable structure in the negative staining solution), grids can be ready for viewing in a TEM within 30 min to 1 hr. Similarly, when viewing grids with the TEM, recording of sample images can begin relatively soon after initial evaluation of the preparation. It is usually necessary to survey the grid at low magnification to assess the quality of the preparation and to select areas for higher-magnification examination. Typically, areas with moderate stain that outlines aggregated sample and/or sometimes debris should be carefully screened. Suitable specimens for image acquisition can usually be found within minutes at higher magnification. Prior to image acquisition, it is sometimes necessary to stabilize the area of the grid to be photographed with the electron beam to reduce movement of the Formvar-coated film. Sample dilution and stain density can be adjusted empirically; technical problems (e.g., poor spreading of the negative stain, structural damage to sensitive samples caused by low pH in samples stained with aqueous uranyl acetate, or unstable support films) require thoughtful adjustment with subsequent preparations, which can significantly extend the duration of the specimen preparation and examination process.

TEM of thin sections

Successful heavy metal staining of thin sections results in sections that are relatively easy to visualize on the phosphor screen of the electron microscope. The deposition of sufficient stain is required to record images, as identification of optimal focus in a TEM is another procedure that requires considerable care and practice. Figure 2B.1.3 and Figure 2B.1.4 show examples of samples suitably stained with uranyl acetate and lead. Figure 2B.1.8 provides an example of the use of immunogold

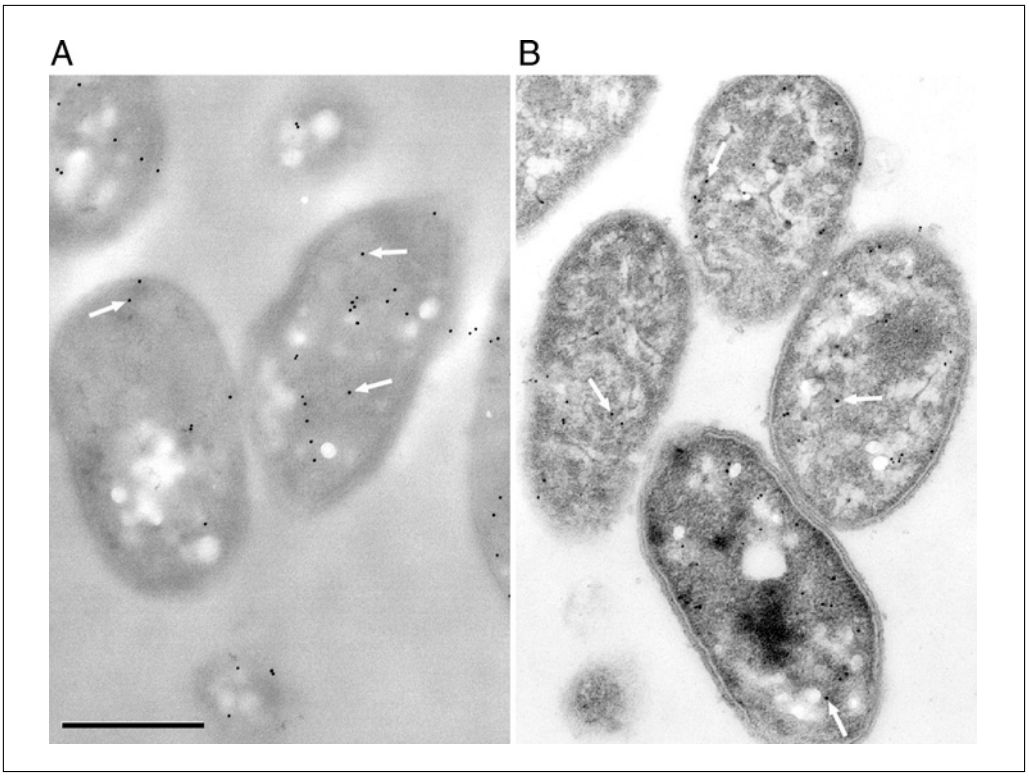

Figure 2B.1.8 *Propionibacterium* fixed and initially processed for immunogold labeling and embedded in LR White resin. In panels (**A**) and (**B**) shown at the same magnification, the LR White–embedded sections were labeled first with a primary monoclonal antibody specific for this particular isolate of *Propionibacterium*; this was followed by secondary labeling with 10-nm protein G–gold (white arrows). Panel A shows inherent specimen contrast without staining uranyl acetate and lead staining of the section, whereas panel B shows the added contrast obtained by employing those stains. Note that while the ultrastructural detail of cells is improved with heavy metal staining, the visualization of gold particles can be more difficult and may require examination of sections at higher magnification.

localization of antigens followed by heavy metal staining. In this figure, differences between the electron scattering of 10-nm gold particles and heavy metal stains that generate contrast in the TEM can be appreciated.

Immunoelectron microscopy

These procedures require extensive manipulation of grids and several incubations, which will require several hours to complete. It is therefore desirable to process several girds simultaneously, not only to speed up the process of evaluating samples, but to ensure that a sufficient number of samples are observed along with appropriate controls. It is also possible to run several dilutions of antibody and gold conjugate simultaneously to expedite the procedure.

Fixation techniques for TEM

Preservation of cells for examination by thin sections is an extensive protocol that requires multiple steps to accomplish. If a sample is presented early in the work day, it is possible to have the infiltrated sample polymerizing in the oven by the end of the day. However, it is more likely that these procedures will be performed over several days. Fortunately, there are several points at which the processing can be arrested until such time that it can be resumed without doing damage to the sample, all of which involve storage at 4°C. Some potential points at which it is possible to halt the procedure include the primary fixative stage (if the tissue is being fixed overnight at 4°C), after the first buffer rinse following primary fixation, after the buffer rinse following post-fixation, or when the sample is in the 100% resin mixture.

Embedding techniques for TEM

In the embedding process, it typically takes several hours to replace the resin/solvent mixture with unpolymerized resin, which is then usually hardened in an oven overnight. On the following day, blocks are removed from the oven and either trimmed in preparation for ultramicrotomy or stored in appropriate containers until samples are needed for preparation of thin sections. Embedded tissue will last many years when kept at normal room temperature and humidity.

TEM of thin sections

Staining with uranyl acetate and lead usually takes about 30 min. Immunogold staining of antigens on thin sections typically takes 4 to 6 hr to complete.

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A good general reference with some protocols.

Internet Resources

http://www.ncbi.nlm.nih.gov/ICTVdb/#ICTVdB

The Universal Virus Database, ICTVdB, is a resource that provides an Index of Viruses and includes searchable of virus isolates, species, genera, families, orders, and images of many viruses, as well as links to genomic and protein databanks.

http://www.polysciences.com/shop/assets/datasheets/305A.pdf

Polysciences, Inc. Technical Data Sheet no. 305A for LR White resin.

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Basic Confocal Microscopy

INTRODUCTION

Confocal microscopy is a powerful tool for visualizing fluorescent specimens. The principal advantage of confocal microscopy over conventional wide-field microscopy is that it can reveal the three-dimensional structure of the specimen. Fluorescent specimens viewed with a conventional wide-field fluorescent microscope appear blurry and lack contrast because fluorophores throughout the entire depth of the specimen are illuminated, and fluorescence signals are collected not only from the plane of focus but also from areas above and below. A confocal microscope selectively collects light from a thin (<1 μm) optical section at the plane of focus in the specimen (Fig. 2C.1.1). Structures within the focal plane appear more sharply defined than with a conventional microscope because there is essentially no flare of light from out-of-focus areas. A three-dimensional view of the specimen can be reconstructed from a series of optical sections at different depths (Fig. 2C.1.2).

Several types of confocal microscopes are available. The most common type is the laser scanning confocal microscope (LSCM), which captures images by scanning the specimen with a focused beam of light from a laser and collecting the emitted fluorescence signals with a photodetector. LSCMs sometimes are referred to as "spot-scanning" confocal microscopes, to distinguish them from microscopes that scan the specimen with a slit of light (slit-scanning) or multiple spots of light (spinning-disk or Nipkow disk). Spot-scanning LSCMs have slower image acquisition rates than slit-scanning or spinning-disk microscopes (<1 frame/sec versus 30 frames/sec or higher). However they are more versatile in a number of ways. They can accommodate lasers with a wide range of wavelengths (from the UV to the infrared) and can be configured to image multiple fluorophores either simultaneously or sequentially. Some include spectral detectors that can capture the entire spectrum of the fluorescence emitted at each pixel in the image. The most sophisticated LSCMs allow the user to control the illumination wavelength and intensity on a microsecond time scale. This feature makes it possible to perform experiments that require selectively illuminating fluorophores in a defined region of interest in order to photobleach (Fig. 2C.1.1D) or photoactivate them. Measurement of fluorescence recovery after photobleach (FRAP) or fluorescence loss in photobleach (FLIP) can provide information about molecular mobility and binding (Cole et al., 1996; McNally and Smith, 2002; Lippincott-Schwartz et al., 2003; Sprague and McNally, 2005). Photosensitive molecules include certain fluorescent proteins (for example, see Patterson and Lippincott-Schwartz, 2002, for the fluorescent protein PaGFP and see Ando et al., 2002, for the photosensitive protein Kaede), "caged" molecules such as caged Ca^{2+} chelators, neurotransmitters, and second messengers (Nerbonne, 1996). Confocal microscopy also can be used to measure fluorescence resonance energy transfer (FRET; Wouters and Bastiaens, 2000).

In microbial research, confocal microscopy is widely used for applications that require visualizing microorganisms within their hosts (Roux et al., 2004; Fig. 2C.1.3A,B). Topics that have been addressed include the mechanisms of microbial adhesion and entry into host cells (Elphick et al., 2004; Ferrari et al., 2003), mechanisms of intracellular motility (Satpute-Krishnan et al., 2003; Viachou et al., 2004; Forest et al., 2005), and the reactions of host cells to infection (Perrin et al., 2004; Shaner et al., 2004). Confocal microscopy also has been used to characterize the growth and physical properties of biofilms (Drenkard and Ausubel, 2002; Rani et al., 2005; Daims et al., 2006; Fig. 2C.1.3C,D). The current generation of confocal microscopes, particularly those that utilize charge-coupled devices (CCDs) as photon detectors, are sufficiently sensitive to detect weak fluorescence signals and potentially could be used for imaging structures within individual microorganisms.

The purpose of this unit is to provide background information and practical tips for optimizing confocal imaging. The first section (Basis of Optical Sectioning) explains the basic principle of confocal imaging as implemented in a LSCM. The second section (Configuration of an LSCM) describes the components and light path in a typical LSCM and compares this with the light paths of spot-scanning microscopes and a new type of slit-scanning confocal microscope. The third

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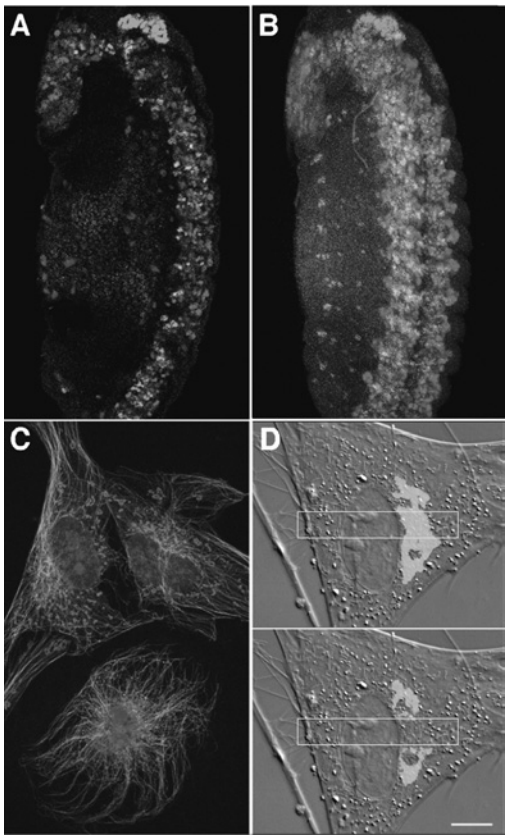

Figure 2C.1.1 Applications of laser scanning microscopy. (**A, B**). Imaging in thick specimens. Neurons in a *Drosophila* embryo were immunolabeled with antibodies against three different transcription factors (images provided by Dr. Ward Odenwald of the National Institutes of Health, Bethesda, Md.; reproduced from Kamabadur et al., 1998). (**A**) A single optical section (~2.5-μm) captured with 25×, 0.8-NA objective. Labeled neurons in the plane of focus appear sharply defined, while those outside it are not visualized. (**B**) A maximum projection of 65 optical sections collected at 2-μm intervals in the *z* axis. (**C**) Imaging intracellular structures. Dissociated rat fibroblasts were immunolabeled with anti-tubulin antibodies to visualize microtubules (green) and stained with fluorescent probes for mitochondria (Mitotracker, red) and DNA (DAPI, blue). The image is a projection of 20 optical sections (0.3-μm intervals) captured with a 100×, 1.4-NA objective. (**D**) Measuring molecular mobility in living cells. In a living fibroblast expressing a Golgi membrane protein (galactosyltransferase) fused to GFP (S65T), GFP fluorescence (green) is localized in the Golgi complex, shown superimposed on a DIC image of the cell. After the first image was collected, the boxed region (yellow) was scanned with full laser power to photobleach the GFP in the boxed area. The second image was collected 2 sec later. Subsequent images (not illustrated) showed that the GFP-galactosyltransferase rapidly diffused back into the photobleached area. Images were captured with a LSM410 (Carl Zeiss, Inc.). For the color version of this figure go to *http://www.currentprotocols.com*.

section (Practical Guidelines) provides guidelines for preparing specimens and configuring the critical parameters for confocal imaging. The Commentary provides references to sources of additional information.

BASIS OF OPTICAL SECTIONING

Confocal microscopes accomplish optical sectioning by scanning the specimen with a focused beam of light and collecting the fluorescence signals emitted by the specimen via a pinhole aperture. The pinhole aperture blocks signals from out-of-focus areas of the specimen whereas light from the focal plane passes through the pinhole to reach the detector. The physical basis of optical sectioning is illustrated in Figure 2C.1.4. The microscope objective focuses light from a point source (a laser) to a diffraction-limited spot in the specimen. The irradiation is most intense at the focal spot, but areas of the specimen above and below the focal spot are also illuminated. Fluorescent molecules excited by the incident light

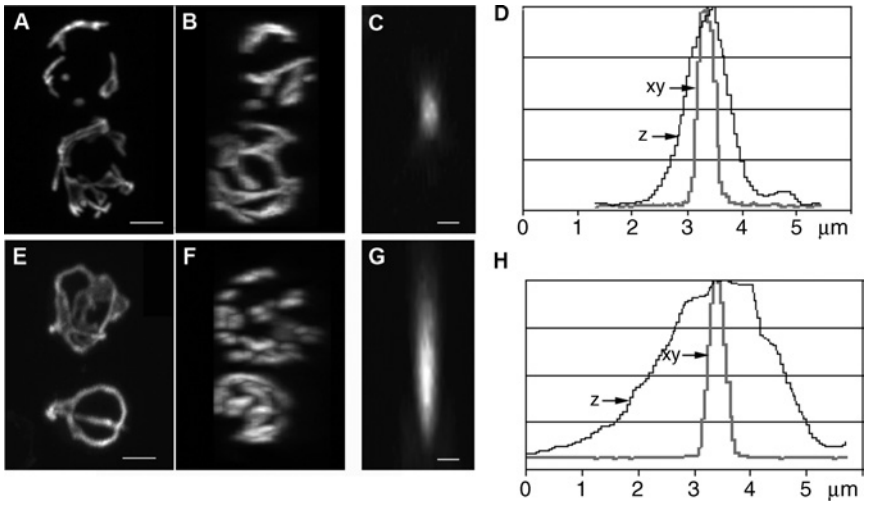

Figure 2C.1.2 3-D Imaging in living specimens. Comparison of water- and oil-immersion objectives. (**A,B,E,F**) Living yeast cells expressing a GFP construct that targets to the mitochondrial matrix were visualized with C-APO 63× 1.2 NA (water) objective (**A,B**) or a Planapochromat 100 × 1.4 NA (oil) objective (**E,F**). The images show *xy* and *yz* projections of stacks of 40 images collected at 0.2-µm intervals along the optical axis. The *xy* projections appear sharper than the *yz* projections because the resolution is higher in the focal plane of the objective than along the optical axis. The yeast were embedded in an aqueous solution with 0.2% agarose. Panels C and G are *yz* projections of images of 0.19-µm fluorescent beads captured with a 63× (water; **C**) or 100× (oil; **G**) objective. The beads were embedded in an aqueous solution with 2% agarose. **D** and **H** are intensity profiles along the horizontal and vertical axes of the beads. A 63× 1.2 NA (water) objective provides better axial resolution than an 100× 1.4 NA objective (oil) in specimens in an aqueous solution. Scale bars = 5 µm (**A,B,E,F**); 0.5 µm (**C,G**). Images were captured with a LSM510 (Carl Zeiss, Inc.).

emit fluorescence in all directions. The objective captures a portion of the emitted light. The objective projects light from the focal spot in the specimen to a conjugate spot in an "image" plane. The pinhole aperture is positioned in the image plane so as to be centered on this spot. The light that passes through the aperture is detected by a photomultiplier tube (PMT). Light from out-of-focus areas of the specimen is spread out at the image plane and is largely blocked by the pinhole aperture.

The diameter of the pinhole determines how much of the fluorescence emitted by the illuminated cone in the specimen is detected, as well as the thickness of the optical section. From wave optics, it is known that a point light source in the plane of focus of an objective produces a three-dimensional diffraction pattern in the image plane. The cross-section at the image plane is an Airy disk, a circular diffraction pattern with a bright central region. The radius of the bright central region of the Airy disk in the reference frame of the specimen is given by $R_{\text{Airy}} = 0.61\lambda/\text{NA}$ where λ is the emission wavelength and NA is the numerical aperture of the objective (Inoué and Spring, 2002). At the image plane, the radius of the central region is R_{Airy} multiplied by the magnification at that plane (Wilson, 1995).

Adjustment of the pinhole to a diameter slightly less than the diameter of the central region of the Airy disk allows most of the light from the focal point to reach the detector and reduces the background from out-of-focus areas by ~1000-fold relative to wide-field microscopy (Sandison et al., 1995). The separation of the in-focus signal from the out-of-focus background achieved by a properly adjusted pinhole is the principal advantage of confocal microscopy for examination of thick specimens.

Point illumination and the presence of a pinhole in the detection light path also produce improved lateral and axial resolution relative to conventional microscopy (Table 2C.1.1). The actual extent of improvement depends on the size of the pinhole. Near-maximal axial resolution is obtained with a pinhole radius of $\sim 0.7 \times R_{\text{Airy}}$, whereas optimal lateral resolution is obtained with a pinhole smaller than $0.3 \times R_{\text{Airy}}$ (Wilson, 1995). However, a pinhole smaller than $\sim 0.7 \times R_{\text{Airy}}$ significantly

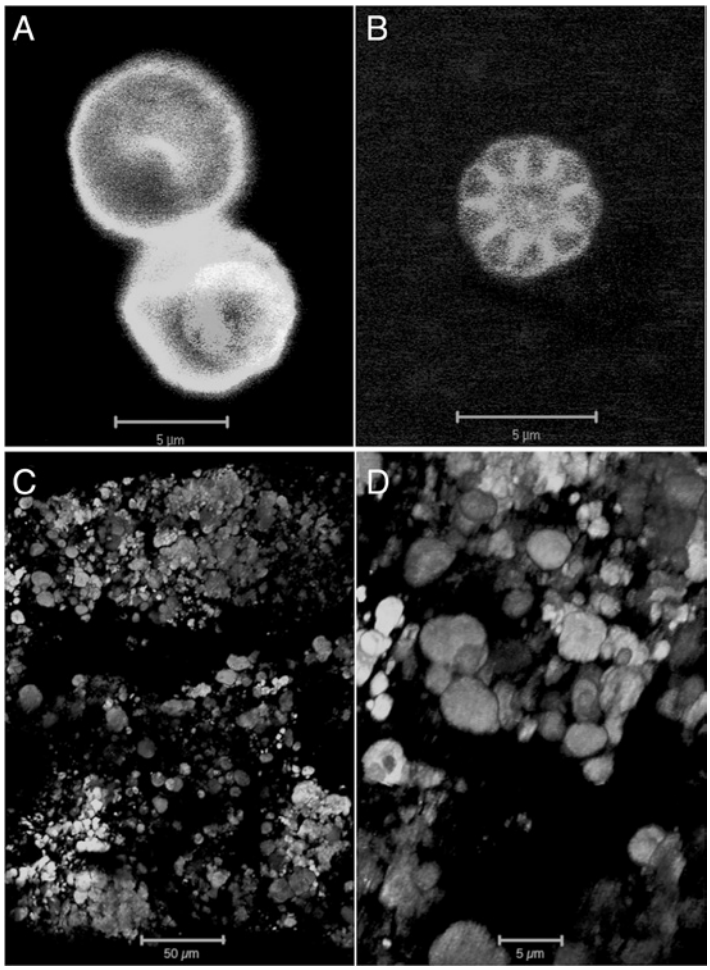

Figure 2C.1.3 Applications of confocal microscopy in microbial research. (**A, B**). Human red blood cells (RBC) infected with malaria parasites (*Plasmodium falciparum*; 3D7 strain). Biotinylated human RBC were labeled with streptavidin-conjugated Quantum Dots 525 (CA; green color; *http://www.qdots.com*) and 0.5- to 4-μM FM-64 (Molecular Probes, red color). The cells were injected into chambers (HybriWell HBW20 from Grace Bio-Labs) and were examined with an LSM 510 confocal microscope (Carl Zeiss, Inc.) with a 100× 1.4-NA oil objective. Panel A shows two RBC, one containing a parasite at the trophozoite stage; panel B shows a parasite at the schizont stage. The schizont was extruded from the RBC for better imaging of individual parasites (red). The green signal in the center of the schizont represents autofluorescence from hemazoin in the digestive vacuole. Images were provided by Dr. Svetlana Glushakova (National Institutes of Health, Bethesda, Md.; methods described in Glushakova et al., 2005). (**C, D**) Biofilm composed of microcolonies of nitrifying bacteria (ammonia oxidizers; *Nitrosomonas sp.*) and nitrite oxidizers (*Nitrospira sp.*). Both populations were labeled by fluorescence in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes. *Nitrosomonas* colonies are green; *Nitrospira* colonies are red. Images are 3-D reconstructions created using the *Daime* imaging program (see Daims et al., 2006, and Internet Resources) from stacks of optical sections collected with an LSM 510 (Carl Zeiss, Inc). Panel C shows an overview of the projected image of the biofilm, while panel D shows a smaller region at higher zoom. Images were provided by Dr. Holger Daims (Universität Wien, Vienna, Austria). For the color version of this figure go to *http://www.currentprotocols.com*.

reduces the total signal, a sacrifice that may not be worth the gain in resolution, especially when imaging dim samples. In fluorescence imaging, resolution is also influenced by the emission and excitation wavelengths (Table 2C.1.1).

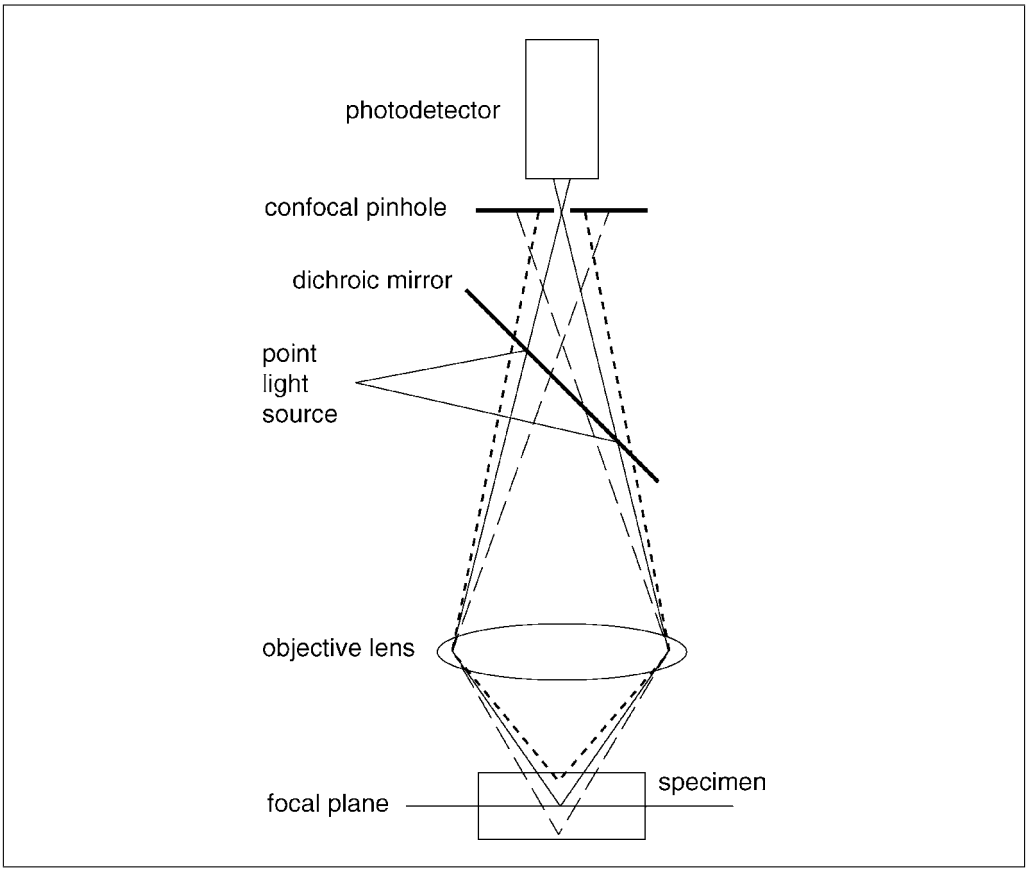

Figure 2C.1.4 The basis of optical sectioning. Illumination from a point light source is reflected by a dichroic mirror into the back aperture of a microscope objective. The objective lens focuses the light to a diffraction-limited spot within the specimen. Fluorophores at the focal spot and within the cones of illumination above and below it are excited, emitting fluorescence in all directions. The fluorescence captured by the objective passes through the dichroic mirror because the fluorescence is at a longer wavelength than the excitation. The confocal pinhole allows fluorescence from the focal spot to reach the photodetector and blocks fluorescence from out-of-focus areas. Redrawn from Shotton (1993).

Table 2C.1.1 Theoretical Resolutions of Confocal and Conventional Microscopes[a,b]

λ_x/λ_{em}	Objective					
	10×, 0.4 NA, air		40×, 0.85 NA, air		60×, 1.4 NA, oil	
	Lat. res.	Ax. res.	Lat. res.	Ax. res.	Lat. res.	Ax. res.
Confocal fluorescence microscope						
488/518	0.55	4.50	0.26	0.99	0.16	0.56
568/590	0.64	5.17	0.30	1.09	0.18	0.64
647/677	0.72	5.88	0.34	1.28	0.21	0.72
Conventional fluorescence microscope						
518	0.79	6.48	0.37	1.43	0.24	0.93
590	0.90	7.38	0.42	1.63	0.28	1.06
680	1.04	8.50	0.49	1.88	0.32	1.22

[a]Data reprinted from Brelje et al. (1993) by permission of Academic Press.

[b]λ_{ex} and λ_{em}, excitation and emission wavelengths; lat. res. and ax. res., lateral and axial resolutions.

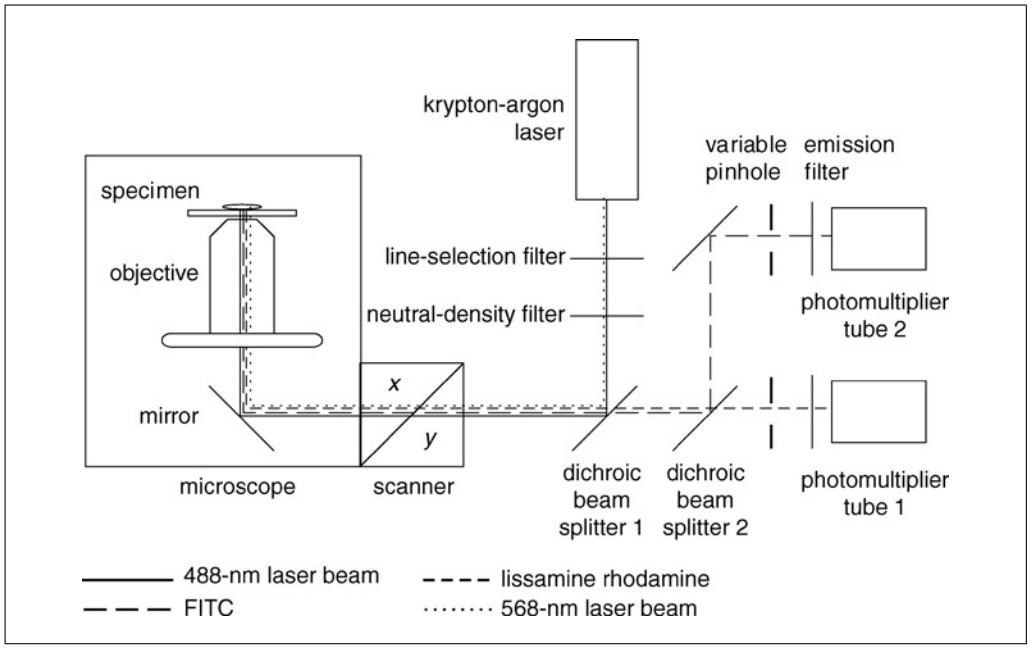

Figure 2C.1.5 The light path of a laser-scanning confocal microscope. The diagram illustrates the light path of a LSCM set up for simultaneous imaging of FITC and lissamine rhodamine. The 488- and 568-nm lines of a krypton-argon laser are reflected by dichroic beam splitter 1 into the optical axis of the microscope. The beam is reflected by a mirror into the microscope objective, which focuses the beam to a diffraction-limited spot in the specimen. The scanner consists of a pair of galvanometer mirrors that deflect the laser beams so as to scan the spot across the specimen in a raster pattern. Fluorescence emitted as each point is illuminated travels the reverse path through the scanning system. The FITC fluorescence (peak at 520 nm) and lissamine rhodamine fluorescence (peak at 590 nm) pass through dichroic beam splitter 1 to dichroic beam splitter 2, which transmits the lissamine rhodamine fluorescence to photomultiplier tube 1 and reflects the FITC fluorescence to photomultiplier tube 2. A variable pinhole in front of each photodetector blocks light from out-of-focus areas of the specimen while allowing light from the focal plane to reach the detector.

CONFIGURATION OF AN LSCM

Confocal microscopes use lasers for illumination because they provide intense excitation within a narrow range of wavelengths. Mixed krypton-argon gas lasers are popular for multicolor confocal microscopy because they emit at three wavelengths (488, 568, and 647 nm) that excite many commonly-used fluorophores—e.g., FITC, rhodamine, Cy3, Cy5, Alexa 488/555/568/647, green fluorescent protein (GFP), and red fluorescent protein (mRFP or DsRed). The disadvantage of krypton-argon lasers is that their life spans are short (~2000 hr). Another way to achieve multiwavelength excitation is to combine the outputs of multiple lasers. Many of the confocal microscopes currently on the market combine an argon laser (488 nm) with a green helium-neon (HeNe) laser (543 or 594 nm) and a red HeNe laser (633 nm). The argon laser also may provide 458 and 514 nm lines, which can be used to excite the cyan and yellow variants of GFP (CFP and YFP). Some confocal microscopes can accommodate a 405-nm diode laser. The 405-nm laser is more optimal for excitation of CFP than the 458 line of the argon laser and also excites photosensitive GFP (PaGFP). It can even be used to visualize some UV fluorophores such as DAPI and Hoechst DNA dyes, although 405 nm is not the optimal excitation wavelength for these dyes. UV argon lasers (351/364 nm) also are available. Inclusion of a 405 nm or UV argon laser adds considerably to the cost of the confocal microscope system due to the requirement for additional optical components to handle these wavelengths.

The light path in a simple confocal microscope is illustrated in Figure 2C.1.5. The output of the laser (or the combined output of multiple lasers) is reflected into the optical axis of the microscope by the primary dichroic beam splitter (splitter 1 in Fig. 2C.1.5). Wavelength-selection filters are inserted into the light path to block specific laser lines, and neutral-density filters may be inserted to attenuate the illumination. In current, high-end confocal

systems, the line selection and neutral-density filters have been replaced with an electronically controlled acousto-optical tunable filter (AOTF). An AOTF can alter its transmission characteristics so as to pass selected wavelengths, while completely blocking others. An AOTF also provides precise control over the attenuation of the individual laser beams.

The scanner deflects the laser beam into the objective at varying angles in order to scan the laser beam across the specimen. Several different technologies for scanning have been devised. The most common method employs a pair of galvanometer mirrors. One mirror oscillates rapidly to excite sequential spots along the *x*-axis of the specimen, and the second mirror oscillates more slowly to move the illumination from line to line in the *y*-axis.

The fluorescence emissions that are collected by the objective follow the reverse path through the scanner to the primary dichroic beam splitter, and thereby are "descanned" (Fig. 2C.1.5). The fluorescence signals (which are at a longer wavelength than the excitation due to the Stokes shift; are transmitted through the beam splitter. To simultaneously image fluorescence from multiple fluorophores requires selection of a primary dichroic beam splitter that reflects each of the required excitation wavelengths and transmits the emissions of all of the fluorophores. Secondary dichroic mirrors split the fluorescence emissions from different fluorophores for detection by separate detectors. Emission filters are inserted in the light path to the detectors (Fig. 2C.1.5) to block backscattered excitation light and to reduce bleedthrough of signals between channels. Current high-end confocal microscopes use more sophisticated technology for emission discrimination; descriptions of the designs of specific systems are available from the vendors (see *SUPPLIERS APPENDIX*).

The fluorescence captured by the objective focuses to a stationary spot (Airy disk) in the image plane (Fig. 2C.1.4). The pinhole aperture is positioned in the image plane so as to be centered on the Airy disk. The diameter of the pinhole aperture can be adjusted to allow optimization for different Airy-disk sizes, which vary with the objective's numerical aperture and the emission wavelength. Adjustment of the diameter of the pinhole to a value of 0.7 to 1.0 Airy Unit allows most of the in focus light to reach the detector and blocks most of the out-of-focus light. In systems with a separate pinhole aperture for each detector, the pinhole apertures are located immediately in front of the detectors. Incorporation of a separate pinhole for each detector allows the user to optimize the pinhole settings for different wavelengths.

The photodetectors in LSCMs are photomultiplier tubes (PMTs), which generate electrons at a rate proportional to the intensity of the incoming fluorescence signal (Art, 1995). The PMT output is converted to a digital image that can be displayed on a computer monitor and stored as a digital file for later analysis. Digitization may be at 8-bit (256 gray levels), 10-bit (1024 gray levels) or 12-bit resolution (4096 gray levels). Confocal microscopes typically have two to four PMTs for reflected light/epifluorescence imaging and may have, in addition, a photodetector for transmitted light.

In spinning disk confocal microscopes, the illumination from a laser or white light source passes through pinholes in the Nipkow disk so as to excite fluorescence at multiple (~1000) sites within the specimen. The disk revolves rapidly (1000 to 5000 rpm) causing the illuminating spots to sweep across the specimen as uniformly-spaced scan lines (Inoué and Inoué, 2002). Fluorescence emitted by the specimen that is collected by the objective returns through the same pinholes in the Nipkow disk that provided the excitation light before it is detected by a full-field CCD camera. In this way, point light sources and detector pinholes to block out-of-focus fluorescence are provided, with the advantage of higher collection speeds than a spot scanner. Drawbacks of this approach include decreased illumination to the specimen from light loss through the pinholes and the inability to change the pinhole diameter. This means that, unlike the case with a spot scanner, optimal confocality is achieved only for one objective magnification, and the thickness of the optical section cannot be changed. Although the reduced specimen illumination generates a smaller fluorescent signal, scientific-grade CCD cameras have significantly higher quantum efficiencies than the PMTs used for fluorescence detection in LSCMs and are able to more than adequately detect these levels of fluorescence. Fluorescent specimens have been reported to undergo less photobleaching during examination with a spinning disk confocal microscope than with a LSCM. The lower rate of photobleaching is thought to be due to the lower illumination levels (Inoué and Inoué, 2002).

A new type of slit-scanning confocal microscope (LSM 5 Live; Carl Zeiss, Inc.) that allows images to be acquired at rates as fast

as or faster than can be achieved with a spinning disk confocal microscope and with as low or lower rates of photobleaching has recently been introduced. The system adopts principles from both the spot scanner and the spinning disk in that it uses a single scanning galvanometer to move an illumination line that is combined with a sensitive single-line CCD detector. The point source of light from the laser is optically converted to a narrow line, which is reflected onto the specimen by a novel beam splitter consisting of a mirrored line on transparent glass. The line illumination is scanned across the specimen. The emitted fluorescence from the specimen that is collected by the objective passes through the beam splitter and is detected by a linear CCD detector. A slit aperture in front of the detector blocks out-of-focus light, analogous to the pinhole aperture in an LSCM. The LSM 5 Live has somewhat poorer resolution than a spot-scanning LSCM, and the initial version is less versatile but can capture images much more rapidly.

PRACTICAL GUIDELINES

Sample Preparation

The preeminent goal in preparing samples for imaging with a confocal microscope is to maximize the fluorescence signals while preserving the three-dimensional structure of the specimen. Ideally, the sample should be less than ~50 μm in thickness, although thicker samples can be visualized. Guidelines for preparing fixed and living samples are described below.

Fixation

A standard fixative for fluorescence microscopy is 2% to 4% formaldehyde in PBS. Formaldehyde penetrates cells rapidly and preserves the antigen-recognition sites for many antibodies. However, formaldehyde cross-links proteins slowly and may cause vesiculation of membranes. Some commercial preparations of formaldehyde (formalin) contain methanol, which shrinks cells. Techniques for optimizing formaldehyde fixation are described by Bacallao et al. (1995). Fixatives containing a small amount of glutaraldehyde (0.125% to 0.25%) in addition to formaldehyde preserve cellular morphology better, but glutaraldehyde destroys the epitopes for some antibodies. Glutaraldehyde fixation induces autofluorescence but autofluorescence can be reduced by treating the sample after fixation with $NaBH_4$ (1 mg/ml in PBS, pH 8.0, using two treatments of 5 min each for dissociated cells, longer for thicker samples). An alternative procedure for preparing specimens for immunocytochemistry is to immerse them in cold (−20°C) methanol or acetone but fixation by this method causes severe shrinkage.

Choices of fluorophores

The choice of fluorophores should take into account the available laser lines and the detector channels of the confocal microscope. Excitation is most efficient at wavelengths near the peak of the excitation spectrum of the fluorophore, but a precise match is not required. For experiments that require imaging multiple fluorophores with standard photodetectors (PMTs), it is best to select fluorophores that are excited by different laser lines, in order to minimize spectral crossover (bleed-through) between the channels (Fig. 2C.1.6). Excitation and emission spectra for many fluorophores are available via the Internet (see Internet Resources). A recommended combination of fluorophores for excitation at 405 nm, 488 nm, 543/561 nm, and 633 nm would comprise Marina Blue, Alexa 488, Alexa 555, and Alexa 647 (all available from Molecular Probes/Invitrogen). The nucleic acid stain DAPI can be excited by illumination at 405 nm, although ultraviolet excitation (350 nm) is more optimal. The cyanine dyes Cy2, Cy3, and Cy5 (available from Jackson ImmunoResearch Laboratories) are also suitable for confocal microscopy. For multiwavelength imaging with a spectral detector and spectral unmixing, it is important to select fluorophores that have distinct emission spectra, but there is no advantage in using fluorophores that have differing excitation spectra. Indeed, it is best to use fluorophores that have similar excitation maxima, so that they can be excited with a single laser line reflected into the microscope with a single-wavelength dichroic mirror. Other important criteria to consider in selecting fluorophores for confocal microscopy are the quantum efficiencies and rates of photobleaching. In addition, the staining protocol should be designed so as to produce similar signal intensities in each channel. More information about selecting fluorophores for confocal imaging is available at the Molecular Expressions Web site (see Internet Resources).

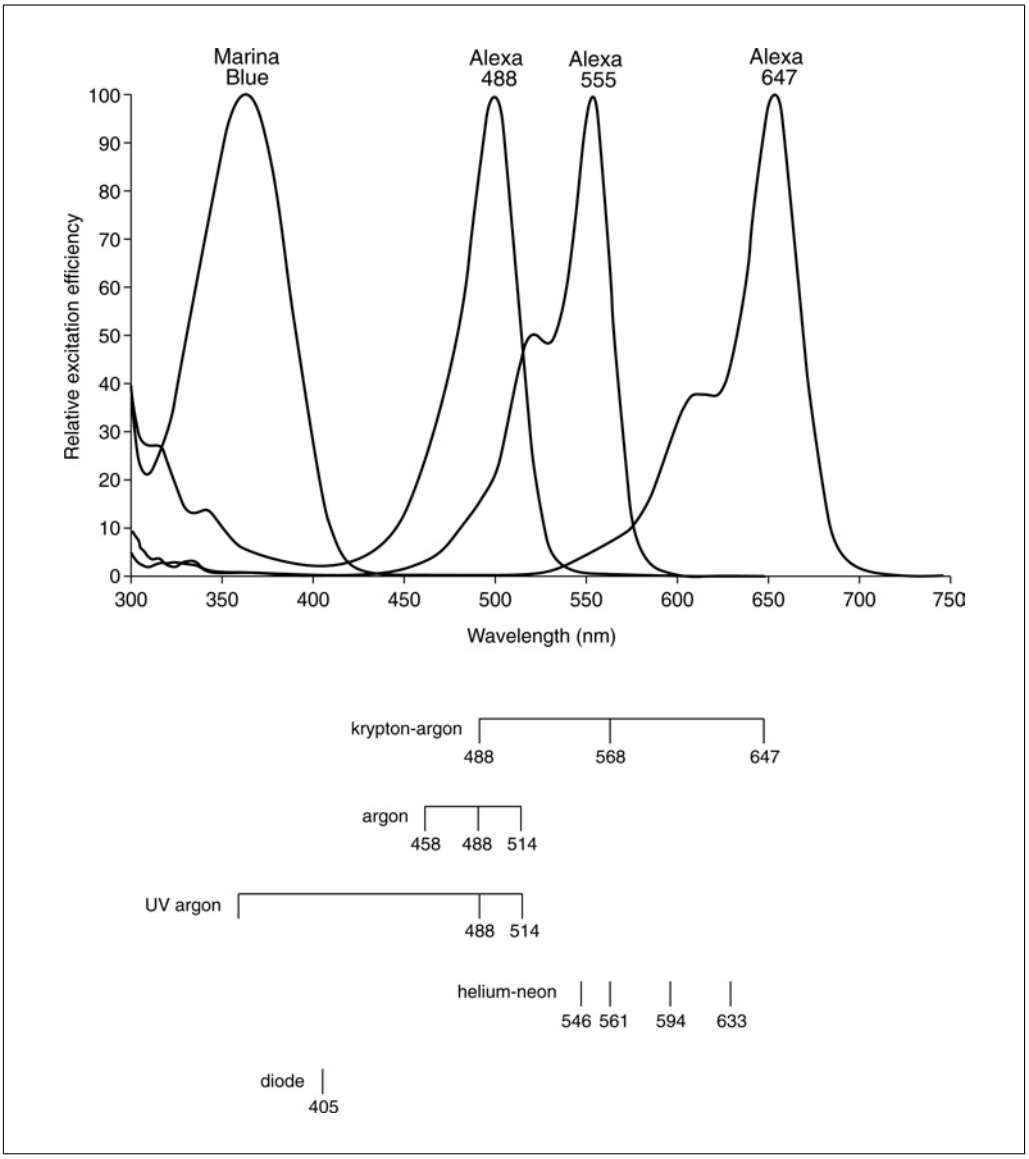

Figure 2C.1.6 Excitation spectra of representative fluorophores and emission wavelengths of lasers for confocal microscopy. The graph at the top shows the excitation spectra of Marina Blue, Alexa 488, Alexa 555, and Alexa 647 (Molecular Probes). The emission wavelengths of lasers commonly used for confocal microscopy are shown below. Data for the excitation spectra are from Molecular Probes.

Control samples

Confocal microscopes rely on electronic image enhancement techniques that can make even dim autofluorescence signals or non-specific background staining look bright. In order to distinguish a real signal from background, it is essential to prepare and examine appropriate control samples. For immunofluorescence experiments with one primary antibody, the appropriate control samples are unstained specimens and specimens treated with the secondary antibody but no primary antibody. Other control experiments may be required to verify the specificity of labeling. Experiments with two primary and secondary antibodies require additional controls to test whether the secondary antibodies cross-react with the "wrong" primary antibody. Singly stained samples also should be prepared and imaged to determine the extent of spectral cross-over between the channels.

Mounting the specimen

Selection of a mounting medium should take into account the type of microscope objective that will be used to observe the specimen (see section on Microscope Objectives). In order for an objective to perform optimally, the mounting medium should have the same refractive index as the objective immersion

Table 2C.1.2 Refractive Indexes of Common Immersion and Mounting Media

Medium	RI
Immersion media	
Air	1.00
Water	1.338
Glycerol	1.47
Immersion oil	1.518
Mounting media	
50% glycerol/PBS/DABCO	1.416[a]
5% *n*-propyl gallate/0.0025% *p*-phenylene diamine (PPD) in glycerol	1.474[a]
0.25% PPD/0.0025% DABCO/5% *n*-propyl gallate in glycerol	1.473[a]
VectaShield (Vector Labs)	1.458[a]
Slow Fade (Molecular Probes)	1.415[b]
ProLong (Molecular Probes)	1.3865[b,c]

[a]Data from Bacallao et al. (1995).
[b]Data from Molecular Probes.
[c]Refractive index (RI) for liquid medium. (RI for solidified medium will be higher.)

medium. Mismatches in the refractive indices produce spherical aberration leading to loss of light at the detector, as well as decreased *z*-axis resolution and incorrect depth discrimination. Image deterioration caused by spherical aberration increases with depth into the specimen; therefore, matching the immersion and mounting medium refractive indices is particularly important for thick specimens. The refractive indices of some commonly used mounting media are listed in Table 2C.1.2.

Mounting media that have refractive indices close to that of immersion oil (RI = 1.51) include DPX (RI = 1.5; ProSciTech) and Permount (RI = 1.52; ProSciTech). However, specimens must be dehydrated prior to mounting in these media, and dehydration causes shrinkage and distortion. Moreover, some fluorophores cannot withstand dehydration. Cells retain their three dimensional shapes when they are kept in physiological saline (PBS) or a mixture of PBS and glycerol (Bacallao et al., 1995). If the specimen is to be mounted under a coverglass, it may be necessary to support the coverglass to avoid damaging the specimen.

Addition of an antioxidant (antifade agent) to the mounting medium helps to alleviate photobleaching of synthetic fluorophores such as those used for immunocytochemistry. One of the best antifade agents is 100 mg/ml 1,4-diazabicyclo[2,2,2]octane (DABCO; Sigma; Bacallao et al., 1995). *n*-propyl gallate (Giloh and Sedat, 1982) and *p*-phenylenediamine (PPD; Johnson et al., 1982) are also effective antifade agents, but the former may cause dimming of the fluorescence while the latter may damage the specimen (Bacallao et al., 1995). A wide variety of mounting media are available from commercial sources (Biomeda, Electron Microscopy Sciences, ProSciTech, Molecular Probes, Vector Laboratories) and many of these contain antifade agents. It is wise to check a the fluorophore provider for recommendations about which mounting medium and antifade agents to use. Antioxidants do not reduce photobleaching of fluorescent proteins.

Living specimens

Microscopy on living specimens grown in vitro is most conveniently performed with an inverted microscope, because the specimens can be viewed through the bottom of the culture chamber and the top can be opened for access. To allow imaging with an oil- or water-immersion objective, the culture chamber substrate should be a coverglass. The glass coverslip can be coated with poly-L-lysine (using a 1 mg/ml solution; Sigma) to promote adhesion of the specimens (either eukaryotic cells or microorganisms). Nonadherent specimens can be immobilized by embedding them in a thin layer of low-melting-point agar (0.2% for eukaryotic cells, up to 2% for smaller organisms). Motile specimens

such as *Paramecium* can be attached to the substrate with CellTak (BD Biosciences; W. Bell, pers. comm.). Culture chambers with coverglass substrates can be made from standard plastic petri dishes by boring holes in their bottoms and affixing coverglasses to the holes with Silgard (Dow-Corning). Culture chambers with coverglass substrates are also available from commercial sources (Labtek coverglass chamber, Fisher Scientific; MatTek glass-bottom culture dish, MakTek Corp). Alternatively, cells may be grown on a coverglass that can be mounted in a chamber for observation on a microscope. A simple chamber can be constructed from a gasket cut from a sheet of silicon rubber or a soft plastic ruler and affixed to a glass microscope slide with silicon grease. The well formed by the gasket is filled with medium, and then the coverglass with cells attached is sealed onto the well. More elaborate chambers, some having built-in heaters and/or ports for perfusion, are available from commercial sources (see Internet Resources for a list of suppliers).

Specimens that need to be kept warm during observation pose a particular challenge because temperature transients can make it difficult to maintain focus. Probably the best way to keep specimens warm is to place the entire microscope in a temperature-controlled enclosure. Alternative strategies include warming the microscope stage with heated air (using an air stream incubator or hair dryer) or infrared lamps, or using a temperature-controlled specimen chamber (Terasaki and Dailey, 1995). If an oil or water immersion objective is used, heating the objective helps to maintain the specimen at the desired temperature. Microscope enclosures, stage warmers, temperature-controlled chambers, and objective heaters are available from suppliers of microscopes and microscope accessories.

Living specimens should be kept in a medium that is buffered to maintain the correct pH. Many commonly used culture media are buffered with bicarbonate and require an atmosphere with 5% to 10% CO_2 to maintain the correct pH. For microscopy, it is more convenient to use a buffer that maintains the correct pH in air. Many types of cells can be maintained for several hours in a balanced saline solution or culture medium that is buffered with HEPES (10 to 20 mM). Use of a medium that contains phenol red should be avoided because phenol red adds background fluorescence and can produce oxygen radicals when exposed to intense illumination. Addition of 0.3 U/ml Oxyrase (Oxyrase, Inc.) to the medium can help to alleviate photobleaching of synthetic fluorophores (Waterman-Storer et al., 1993).

Optimizing Imaging Parameters

Microscope objectives

High-NA objectives are optimal for fluorescence microscopy because they collect more light than low-NA objectives (brightness is proportional to NA^4). Oil-immersion objectives have the highest numerical apertures (NA = 1.4 or 1.45). However, oil-immersion objectives have short working distances (100 to 200 μm). Moreover, they work optimally only with specimens mounted in a medium with a refractive index the same as that of immersion oil ($n = 1.51$). Mismatch of the refractive indices leads to a deterioration of image quality that becomes increasing severe with depth into the specimen. When a high-NA oil objective is used to image a specimen mounted in an aqueous medium, image quality and signal brightness decline noticeably at distances of 5 to 10 μm from the coverglass. Mismatch of the refractive indices also leads to spatial distortion in the *z*-axis. The actual movement of the focal plane in the specimen (d_s) produced by a movement of the objective (d_{obj}) depends on the ratio of the refractive indexes: $d_s/d_{obj} = \eta_s/\eta_{obj}$ (Majlof and Forsgren, 2002).

A water-immersion objective is useful for imaging living specimens that are more than a few microns thick. Water-immersion objectives with numerical apertures of 1.2 are available. These objectives are designed for viewing specimens mounted under a coverglass (0.17 μm; no. 1.5) and have fairly short working distances (130 to 220 μm). "Dipping" objectives, which are intended for use without a coverglass, have lower numerical apertures (NA = 0.9) and longer working distances (1 to 2 mm).

Objectives differ in their transmission efficiency and degree of correction for spherical and chromatic aberration and flatness of field. Plan Apochromat objectives provide the flattest fields of view and color correction for three wavelengths. Plan Apocromat objectives generally transmit efficiently throughout the visible spectrum (400 nm to 700 nm), but may transmit poorly in the UV (<400 nm) or infrared (>700 nm; Keller, 1995). Some objectives that are less highly corrected (Fluar, Plan NeoFluar, Plan Fluor) provide higher transmission at visible, UV, and infrared wavelengths. For additional information about objectives for confocal microscopy see

the *UNIT 2A.1*, Keller (1995), Benham (2002), and the Molecular Expressions Web site (see Internet Resources).

Pinhole size

As explained in the section on the Basis of Optical Sectioning, the size of the detector pinhole has a critical influence on image quality. A pinhole with a diameter slightly less than or equal to the diameter of the bright central region of the Airy disk will let most of the light from the plane of focus reach the detector, while blocking most of the out-of-focus flare. The lateral resolution will be ~10% better than that obtainable by conventional microscopy with the same optics (Centonze and Pawley, 1995), although not as good as can be achieved with a smaller pinhole. Lateral resolution continues to improve as pinhole radius is decreased down to a pinhole size of ~0.2 × Airy disk radius, but a pinhole this small excludes ~95% of the signal (Wilson, 1995). Axial resolution improves as pinhole size decreases, down to ~0.7× Airy disk radius, then levels off. The best trade-off between signal intensity and resolution will depend on the characteristics of the sample and the required resolution.

Scan zoom

The scan zoom determines the dimensions of the area in the specimen that is scanned. Increasing the zoom reduces the dimensions of the scan area. The pixel number remains the same; consequently, individual pixels represent a smaller area. For example, the scan area at zoom 2 is one quarter the scan area at zoom 1 and the pixel dimensions are half as large in each dimension. That is, if the pixel dimensions represent 0.25 μm × 0.25 μm at zoom 1, then dimensions are 0.125 × 0.125 μm at zoom 2.

For each objective, there is an optimal zoom setting that yields pixel dimensions sufficiently small to take advantage of the full resolution of the objective but large enough to avoid oversampling. In order for the minimum resolvable entity to be visible on the display monitor, the pixel dimension needs to be smaller than (less than one-half) the optical resolution. However, if the pixel size is made too small by using a higher-than-optimal zoom factor, the specimen is subjected to more irradiation than necessary, with an increased risk of photobleaching. The rate of photobleaching increases proportionally to the square of the zoom factor (Centonze and Pawley, 1995). A guideline for selecting an appropriate zoom factor derived from information theory (the Nyquist Sampling Theorem) states that the pixel dimensions should be equal to the optical resolution divided by 2.3 (see Webb and Dorey, 1995). The optical resolution in confocal imaging depends on the numerical aperture of the objective, the refractive index of the immersion medium, the excitation and emission wavelengths, and the diameter of the pinhole aperture. Values calculated for different objectives and wavelengths using the point-spread functions (PSF) for wide-field and confocal microscopy are given in Table 2C.1.1. The lateral resolution for confocal microscopy can be approximated by: $\text{Resel}_{x,y\ \text{confocal}} = 0.4\lambda/\text{NA}$, where λ is the wavelength of the illumination and NA the numerical aperture of the objective (see Webb and Dorey, 1995). The above equation assumes the use of an infinitesimal pinhole; $\text{Resel}_{x,y}$ will be larger with a pinhole of 0.7 to 1 Airy unit.

z-axis sectioning interval

In order to study the three-dimensional structure of a specimen, a series of images are captured at fixed intervals throughout the entire depth of the specimen. The interval between focal planes needed to achieve optimal resolution in the *z*-axis is not as small as the *x,y* pixel dimensions because the axial resolution is poorer than the lateral resolution (see Table 2C.1.1). The optimal interval (according to the Nyquist Sampling Theorem) is equal to the axial resolution divided by 2.3. The axial resolution for an objective in confocal imaging can be approximated by: $\text{Resel}_{z\ \text{confocal}} = 1.4\lambda\eta/\text{NA}^2$ where n is the refractive index (see Webb and Dorey, 1995). Collecting images at shorter intervals results in oversampling, with an increased risk of photobleaching.

Illumination intensity

Fluorescence emission increases linearly with illumination intensity up to a level at which emission saturates. Optimal signal-to-background and signal-to-noise ratios are obtained with illumination levels well below saturation (Tsien and Waggoner, 1995). The illumination intensity on a laser-scanning microscope can be adjusted by operating the laser at submaximal power and by inserting neutral-density filters into the light path or varying the transmission through the AOTF. In general, the best images are obtained with illumination levels that are as high as possible without producing unacceptable rates of photobleaching.

PMT black level and gain

The contrast and background of confocal images are determined by the gain and black-level settings of the photomultiplier tube (PMT) amplifiers. To obtain maximal information, the black level and gain should be adjusted to take advantage of the full dynamic range of the PMTs. The appropriate black-level setting can be found by scanning while the light path to the PMT is blocked. The image that appears on the display monitor should be just barely brighter than the background, which is black (gray level = 0). To set the gain, scan the specimen and adjust the gain so that the brightest pixel in the image is slightly below white (gray level = 255, for 8-bit images). Ensuring that all signals fall within the dynamic range of the PMT is especially important for quantitative imaging experiments. Confocal imaging software typically includes a pseudocolor image display mode ("range indicator") that facilitates selection of appropriate black level and gain settings by highlighting pixels with intensity values near 0 or 255.

Averaging

Confocal images of dimly fluorescent specimens captured at the fastest scan rate on a typical LSCM (~0.5 sec/frame) appear noisy because of the small numbers of photons collected from each spot. Improved signal-to-noise can be attained by scanning the specimen at a slower rate or by scanning multiple times and averaging the signals. Current LSCMs allow individual lines in the image to be repeatedly scanned and averaged. Line averaging generally produces sharper images than frame averaging (which averages full frames) because there is less risk of blurring due to movements or changes in the specimen.

Imaging multiple fluorophores

Confocal microscopes can typically be configured to capture images of two or more fluorophores simultaneously or sequentially. Each approach has advantages and disadvantages. For simultaneous imaging, the specimen is scanned with all of the required excitation wavelengths and the emissions of the different fluorophores are split for detection by separate photodetectors (Fig. 2C.1.5). The drawback of this approach is that spectral cross-over between channels may occur if the emission spectra of the fluorophores overlap. If each fluorophore is excited by only one laser line, then exciting them sequentially will avoid spectral cross-over. The disadvantage of sequential excitation is that there may be misalignment of the signals in different channels, particularly if the specimen is alive and moving. A third way of imaging multiple fluorophores is available in confocal systems in which the laser excitation is controlled with an AOTF; such systems can scan each line of the specimen sequentially with different excitation wavelengths with a time delay between scan lines of less than a millisecond. Line-by-line wavelength switching provides rapid acquisition of fluorescence signals from each spot in the specimen while avoiding the spectral cross-over between channels that may occur when the fluorophores are excited simultaneously.

Image display

Confocal images are typically displayed as 8-bit grayscale or 24-bit RGB (red/green/blue) color images. Each channel of an RGB image can represent a different fluorophore (Fig. 2C.1.1A to C). Color mixtures indicate colocalization of fluorophores within a pixel. A RGB fluorescence image can be merged with a grayscale transmitted light image by adding the transmitted light image to each channel of the RGB image (Fig. 2C.1.1D; fluorescence in green channel merged with DIC image).

The three-dimensional dataset obtained by capturing a series of optical sections through the specimen can be used to compute views of the specimen from different viewing angles. Commercial confocal microscopes typically include the capability to generate orthogonal views of the specimen (*xy*, *xz*, and *yz*) and may permit views from arbitrary angles. An *xy* projection or "*z*-series projection" is a two-dimensional display formed by merging multiple image planes (Fig. 2C.1.1B,C, Fig. 2C.1.2A,E). The most common type of projection is a "maximum" projection in which each pixel represents the intensity of the brightest pixel in the *z*-axis. Another type of projection, referred to as "surface render," displays the most superficial pixels with intensities above a defined threshold. Projections also can be created for different viewing angles (Fig. 2C.1.2B, F). Projections of the specimen from different viewing angles can be combined to create an animation in which the specimen appears to rotate in space. Such animations give the viewer a striking impression of the three-dimensional geometry of the specimen. Generating two projections at azimuths differing by 4° to 10° creates stereo pairs that can be visualized with a stereo viewer or color-coded and merged to form a stereo anaglyph. Volume-rendering

imaging software is available that provides additional options for three-dimensional visualization and measurements (see Internet Resources).

COMMENTARY

Effective use of a confocal microscope requires understanding of the principles of image formation and knowledge of how to set up and use a microscope. The unit on Proper Alignment and Adjustment of the Light Microscope (*UNIT 2A.1*) describes the components of a light microscope and provides protocols for setting up a microscope for transmitted light and epifluorescence imaging. This unit also lists references to literature on light microscopy. *The Handbook of Biological Confocal Microscopy* (edited by V. Centonze and J. Pawley, 1995, new edition planned for March 2006) is a comprehensive reference book on confocal microscopy. It includes chapters on the fundamental principles, instrumentation, image acquisition and display, sample preparation, and much more. *Confocal Microscopy* (Wilson, 1990) provides a thorough discussion of the principles behind confocal imaging. *Cell Biological Applications of Confocal Microscopy* (edited by B. Matsumoto, 2002) discusses the performance of different types of confocal microscopes and contains practical information about common applications such as imaging immunofluorescence and calcium ion indicators. Additional applications are described in *Confocal and Two Photon Microscopy: Foundations, Applications and Advances* (Diaspro, 2002). The Molecular Expressions Web site also is an excellent source of information about light microscopy, including confocal microscopy (see Internet Resources).

Confocal microscopy is only one of several available techniques for capturing optical sections in fluorescent specimens. An alternative to confocal microscopy is computational "deconvolution" of images captured by wide-field epifluorescence microscopy (McNally et al., 1999; Boccacci and Bertero, 2002). Computational deconvolution makes use of all of the fluorescence captured by the objective, in contrast to confocal microscopy, which discards fluorescence from out-of-focus areas. In addition, wide-field microscopy can employ CCD cameras that have higher quantum efficiencies than the photodetectors used for confocal microscopy. For these reasons, wide-field microscopy and deconvolution can be superior to confocal microscopy for imaging dim specimens or specimens that are susceptible to photobleaching or photodamage. However, computational deconvolution of images is time-consuming and does not work well in specimens with high levels of dispersed fluorescence. Confocal microscopy allows direct visualization of optical sections and is applicable to a wider range of specimens.

Another technique for confocal imaging takes advantage of the optical phenomenon known as multiphoton excitation. Multiphoton microscopy allows deeper penetration into tissue than either wide-field microscopy or conventional (single-photon) microscopy, and is particularly useful for imaging in thick specimens such as tissue slices or multicellular organisms. However, this method suffers loss of resolution due to the longer illumination wavelength and absence of a detector pinhole.

Troubleshooting

Test samples are useful for monitoring the performance of a confocal microscope. A micrometer slide should be used to check the spatial calibration of each objective. Fluorescent microspheres with mixtures of fluorophores (FluoSpheres; Molecular Probes) are useful for checking the *x,y* and axial alignment of images acquired at different excitation wavelengths. Misalignment of the images in the *xy* plane may indicate that the pinholes are not centered or that the lasers need to be aligned; misalignment in the *z* axis may be due to incorrect setting of a collimating lens, misalignment of pinholes, or chromatic aberration in the objective. The optical resolution of the microscope can be measured by capturing images of submicroscopic (<0.2 nm) fluorescent microspheres (Fig. 2C.1.2C,G). The images of the microspheres should be radially symmetrical in the *xy* plane and elliptical in the *z*-axis (Fig. 2C.1.2C). Horizontal and axial resolutions are defined by the full width at half maximal intensity (FWHM) of intensity profiles along the horizontal and vertical axes of the beads (Fig. 2C.1.2D,H).

Anticipated Results

Confocal microscopy provides sharp images of fluorescent structures in thick specimens (Fig. 2C.1.1, Fig. 2C.1.2, Fig. 2C.1.3C, D). The maximum depth at which adequate images can be obtained depends on the objective and the optical properties of the specimen. With a high-NA immersion objective, it may be possible to capture images at depths of >100 μm in a specimen that is transparent and not heavily stained (Centoze and Pawley, 1995). However, if the specimen scatters light, both the illumination intensity and the proportion

of the emitted fluorescence that is captured by the objective decline with increasing focal depth. Mismatch of the refractive indexes of the immersion medium and specimen will further reduce the depth at which adequate signal can be obtained. A low-NA objective can capture images at greater depths but provides much poorer axial resolution.

A three-dimensional reconstruction of the specimen can be generated from a series of optical sections at appropriately spaced intervals along the optical axis. The reconstruction can be viewed from any angle, but the view along the optical axis of the objective will appear sharper than off-axial views, because the lateral resolution of the objective is better than the axial resolution (Fig. 2C.1.2). The axial distortion can be corrected by computational deconvolution (Wouterlood, 2005).

Confocal imaging in living specimens is feasible although care must be taken to avoid phototoxicity and photodamage. Robust fluorophores such as the fluorescent proteins EGFP and EYFP can be imaged hundreds of times with minimal photobleaching and no apparent phototoxicity, provided that the illumination is kept at a low level. Synthetic fluorophores, such as organelle-specific dyes (Molecular Probes), generally are more photosensitive, although in some applications the rate of bleaching can be reduced by the addition of Oxyrase. The maximum rate at which images can be collected will depend on the scan speed, resolution, and area. Typical scan times for a 512 × 512 image are 1 to 4 sec/frame.

A common application of confocal microscopy is to determine the relative distributions and extent of colocalization of the molecules tagged with different fluorophores (Brelje et al., 2002). As many as four different fluorophores can be discriminated on a confocal microscope with laser excitation at 350/405, 488, 546/568, and 633/647 nm, and standard photodetectors, provided that the excitation spectra of the fluorophores are well separated and matched to the laser lines. Confocal microscopes with spectral detectors can discriminate larger combinations of fluorophores on the basis of their emission spectra. Spectral detection and linear unmixing allows discrimination of fluorophores with highly overlapping emission spectra, such as GFP and YFP (Dickinson, et al., 2001).

Confocal microscopy also is well suited for visualizing variants of the green fluorescent protein. CFP and YFP can be visualized with minimal cross-talk between channels on a confocal microscope with 405 nm and 514 nm excitation. The overlap of the excitation and emission spectra of CFP and GFP or GFP and DsRed may result in cross-talk between channels in experiments with these combinations of fluorophores. New fluorescent proteins have been developed that potentially could provide more optimal combinations for multi-color imaging (Shaner et al., 2004). Fluorescent proteins have also been incorporated into biochemical reporters for measuring intracellular calcium, kinase activity, and other signaling molecules (Zhang et al., 2002).

Photosensitive fluorescent proteins are available that undergo a change in spectral properties upon photoactivation. Photactivatable GFP (PaGFP; Patterson and Lippincott-Schwartz, 2002) exhibits little fluorescence under 488 nm illumination prior to activation but undergoes a 100-fold increase in fluorescence after photoactivation at 400 to 430 nm. An LSCM with a 405- or 413-nm laser can be used to photoactivate PaGFP within a user-defined region of interest within the specimen and thereby selectively "highlight" GFP fluorescence within that region. The activated GFP retains its fluorescence indefinitely and, importantly, manifests these properties even when fused to another protein. PaGFP fusion proteins provide a useful tool for studying the intracellular dynamics of proteins and organelles (Karbowski et al., 2004).

LSCMs that incorporate an AOTF to control the illumination wavelength and intensity can be configured to perform various types of photobleach experiments. Measurement of fluorescence recovery after photobleach (FRAP) or fluorescence loss in photobleach (FLIP) can provide information about molecular mobility and binding (Cole et al., 1996; McNally and Smith, 2002; Lippincott-Schwartz et al., 2003). In FRAP, fluorescence in a small region of the specimen is photobleached by scanning with high-intensity illumination, and recovery of fluorescence into the bleached area is then monitored by scanning with low-intensity illumination. The rate of return of fluorescent molecules into the bleached area may be governed by diffusion, binding interactions with other molecules, or a combination of both, and appropriate mathematical models have been developed to analyze these responses (Sprague and McNally, 2005). In FLIP, a region of the specimen is photobleached several times with a delay between the bleach scans and images are collected during this process to monitor the distributions of bleached and nonbleached fluorescent molecules. Observation of FLIP can

show whether there is exchange of fluorescent molecules between two compartments of a cell or whether a fluorescent structure is a single organelle or a network of contiguous but independent organelles (Cole et al., 1996).

The spatial precision by which two fluorophores can be said to colocalize on the basis of light microscopy is limited by the optical resolution (~0.2 μm in the *xy* plane and 0.6 μm in the *z*-axis). The phenomenon of fluorescence resonance energy transfer (FRET) can potentially reveal whether two fluorophores are within <10 nm proximity. FRET (Wouters and Bastiaens, 2000) is the nonradiative transfer of energy from a fluorescent donor molecule to an acceptor molecule. Energy transfer occurs only if the molecules are within a distance of less than ~10 nm, and only if the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. One application of FRET is to determine whether two populations of molecules undergo binding interactions. One population is labeled with donor fluorophores (e.g., CFP) and the second is labeled with acceptor fluorophores (e.g., YFP). Several techniques for measuring FRET have been devised (Jares-Erijman and Jovin, 2003) and many of these can be carried out with current LSCMs.

Current LSCMs are much superior to their predecessors in sensitivity, speed of image acquisition and versatility. Although they are expensive ($200,000 to $600,000) and require costly service contracts to ensure optimal performance, their many benefits justify these costs.

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Covers the basics of light microscopy, video microscopy, and much more.

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Good source of practical information about confocal imaging.

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Guide to digital image processing.

INTERNET RESOURCES

http://www.microbial-ecology.net/daime

Daime Web site, from which the Daime software application can be downloaded. Daime (digital image analysis in microbial ecology) is an open-source software program for 2-D and 3-D image analysis developed by Holger Daims, Sebastian Lücker, and Michael Wagner (Universität Wien, Vienna, Austria). The features of Daime and its application to analysis of biofilms are described in Daims et al. (2006).

http://rsb.info.nih.gov/ij

ImageJ is a public domain image analysis program developed by W. Rasband (Research Services Branch, National Institute of Mental Health, NIH) for operating systems running Java (including Windows/PC and OSX/Macintosh). ImageJ has many useful tools for analysis of confocal images.

http://www.uhnres.utoronto.ca/facilities/wcif/imagej/

A manual written by Tony Collins that describes the use of ImageJ to visualize and analyze confocal images.

http://www.molecularexpressions.com

The Molecular Expressions Web site is a rich source of information about all aspects of light microscopy, including confocal microscopy. It includes sections on the basic principles of confocal imaging, instrumentation, sample preparation, and choices of fluorophores. An interactive tutorial "Choosing fluorophore combinations for confocal microscopy" allows the user to determine the extent of spectral cross-over that will occur when imaging different combinations of fluorophores with specific laser lines and filter sets.

Web sites of vendors of confocal microscopes

These provide product descriptions, manuals, tutorials and literature.

http://www.zeiss.com

Carl Zeiss, Inc.

http://www.leica-microsystems.com/company

Leica Microsystems.

http://www.nikonusa.com

Nikon, Inc.

http://www.microscopyu.com

For information about light microscopy and confocal microscopy.

http://www.olympusconfocal.com/

Olympus, Inc.

http://www.perkinelmer.com/

PerkinElmer, Inc.

http://www.solameretech.com/

Solamere Technology.

Spectra of fluorophores

http://fluorescence.nexus-solutions.net/frames6.htm

Biorad Microsciences fluorochrome database and charting application.

http://home.earthlink.net/~fluorescentdyes/

George McNamara Multiprobe Microscopy.

http://www.probes.com/

Molecular Probes.

http://www.molecularexpressions.com

Molecular Expressions.

http://www.olympusfluoview.com/resources/specimenchambers.html

Sources of chambers for maintaining living specimens during observation by microscopy.

http://listserv.buffalo.edu/user/sub.html

Many topics of interest to confocal microscopists are discussed on the confocal listserver operated by the listserver at the University at Buffalo. To subscribe to the lis,t go to the URL and type "confocal" in the box that asks which list one wishes to join.

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Atomic Force Microscopy (AFM)

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ABSTRACT

The atomic force microscope (AFM) is an important tool for studying biological samples due to its ability to image surfaces under liquids. The AFM operates by physical interaction of a cantilever tip with the molecules on the cell surface. Adhesion forces between the tip and cell surface molecules are detected as cantilever deflections. Thus, the cantilever tip can be used to image live cells with atomic resolution and to probe single molecular events in living cells under physiological conditions. Currently, this is the only technique available that directly provides structural, mechanical, and functional information at high resolution. This unit presents the basic AFM components, modes of operation, useful tips for sample preparation, and a short review of AFM applications in microbiology. *Curr. Protoc. Microbiol.* 8:2C.2.1-2C.2.17. © 2008 by John Wiley & Sons, Inc.

Keywords: AFM • imaging • force curves

INTRODUCTION

Understanding the function of microbial cell surfaces requires knowledge of their structural and physical properties. Most of the classical methods used to investigate these structures (e.g., electron microscopy, X rays) require cell drying, which may compromise the accuracy of the analysis by denaturing the surfaces (Dufrêne, 2002, 2003). Because of microorganisms' small size, the information is generally obtained from a large number of cells and not at the individual cell level. Thus, there is a need for new, nondestructive technologies capable of probing single cell surfaces at high resolution. The atomic force microscope (AFM) has emerged as a nondestructive technique, offering resolution comparable to electron microscopy but allowing the study of biological material in its native environment. AFM was invented in 1986 by Binnig et al. (1986) as a novel technique that allows imaging of surfaces and force measurements at the atomic scale, whether the sample is in air or under fluid. This last property makes it very attractive as a tool for biological applications for live cell imaging (Radmacher et al., 1992; Lal and John, 1994) and single molecule force spectroscopy (Willemsen et al., 2000; Wojcikiewicz et al., 2004; Trache et al., 2005), providing structural, mechanical, and functional information with high resolution under physiological conditions. Biologists are using the AFM in an increasing number of creative ways, and the referenced literature is vast (see Key References). This unit presents the basic AFM components and modes of operation, gives useful tips for sample preparation, and provides a short review of AFM applications in microbiology.

PRINCIPLE OF OPERATION

The principle of operation of the AFM is very similar to that of a stylus profilometer: a sharp cantilever tip interacts with the sample surface, sensing the local forces between the molecules of the tip and sample surface (Fig. 2C.2.1). This instrument is not a conventional microscope that collects and focuses light. The word microscope has been

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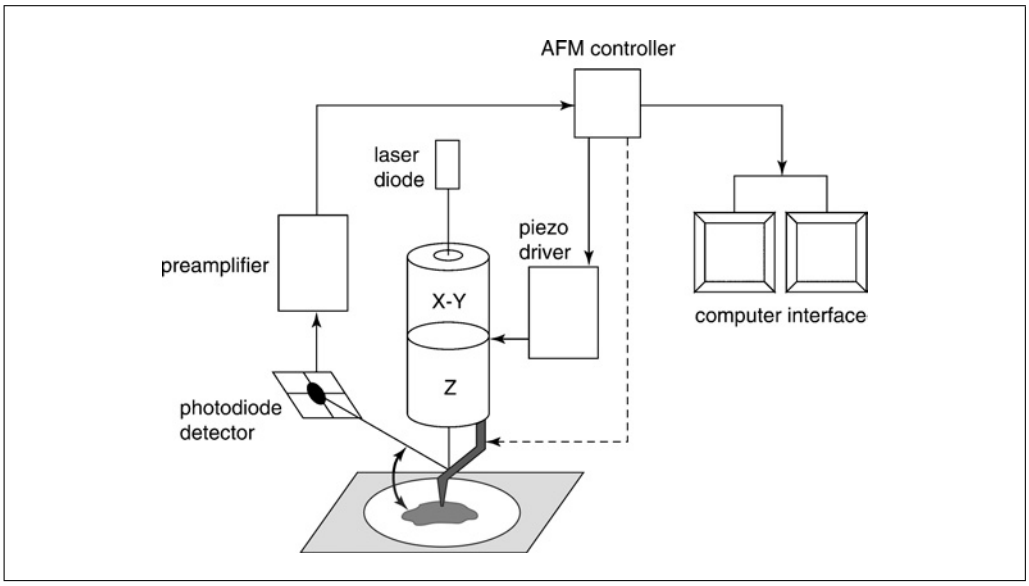

Figure 2C.2.1 Schematic illustration of the AFM system. A flexible cantilever with a tip at the end is rigidly connected to an *xyz* piezoelectric element. The optical lever consists of a laser diode beam that is focused on the back of the cantilever and bounces off, reaching the quadrant photodetector. In contact mode the *xyz* position of the cantilever is given by recording the corresponding position of the piezoelectric element, and the detector reading is proportional to the cantilever deflection. In tapping mode the AFM cantilever is vibrated at its resonant frequency under an external electrical excitation (dashed line).

associated with this instrument because it is able to measure microscopic features of the sample. The most characteristic property of the AFM is that the images are acquired by "feeling" the sample surface without using light. A common analogy is to imagine a blindfolded person who perceives the characteristics of a surface just by touching it. In this way, not only the sample topography can be recorded with atomic resolution, but also material characteristics and the eventual strength of the interaction between the sample surface and the AFM tip. Due to the fact that no light is involved in acquiring the sample properties, the resolution achieved by the AFM is not limited by the wavelength of the radiation that investigates the sample, as in classical light microscopy. Thus, the AFM reaches a resolution far below the diffraction limit offered by light microscopy, being limited only by the tip radius and the spring constant of the cantilever.

The main components of an AFM (Gad and Ikai, 2001) consist of the AFM probe (a sharp tip mounted on a soft cantilever), the optical lever that allows for measuring the cantilever deflections, the feedback loop that allows for monitoring the interaction forces between the molecules on the tip with the ones on the cell surface, the piezoelectric scanner that moves the tip in respect with the sample in a 3D pattern, and a conversion system from raw data acquired by the instrument into an image or other useful display. A short overview of the system components is presented below.

The most sensitive part of an AFM is the tip that interacts directly with the sample surface. The most common probes are constructed from silicon or silicon nitride using microfabrication techniques (Binnig et al., 1987). Each probe can have multiple integrated cantilevers (Fig. 2C.2.2). The properties and dimensions of the cantilever and tip play a major role in determining the sensitivity and resolution of the AFM (Morris et al., 1999). For special applications the pyramidal tip can be replaced with glass or polystyrene beads with a diameter of 1 to 5 μm. The AFM tip must be chosen carefully depending on the specific application. In general, the cantilever should be soft enough to be deflected at

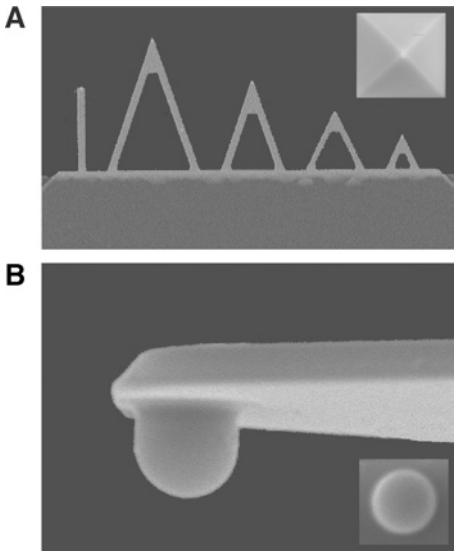

Figure 2C.2.2 Scanning electron microscope images of AFM cantilevers. (**A**) Typical AFM probe for force measurements (magnification 45×). At the very end of the cantilever is a pyramid-shaped tip with a 4-μm base, 35° half angle of the tip, and a radius of 50 nm (inset, magnification 16,000×). (**B**) Typical AFM probe for mechanical stimulation measurements (magnification 10,000×). The cantilever is V-shaped, with a spring constant of ∼0.06 N/m and a 2-μm glass bead attached at its end (inset, magnification 10,000×). Images were taken at the Microscopy & Imaging Center of TAMU, College Station, Texas. Reproduced from Trache and Meininger (2005) with permission of the International Society for Optical Engineering.

Table 2C.2.1 Most Common Cantilever Parameters

Cantilever material	Silicon nitride[a]		Silicon[a]
Spring constant (k)	0.58, 0.32, 0.12, 0.06		20-100 N/m
Resonant frequency			200-400 kHz
Cantilever configuration	V-shaped		Single beam
Reflective coating	gold		Uncoated, aluminum
Tip shape	Pyramid	Sphere[b] glass/polystyrene	Pyramid
Tip radius of curvature/diameter	20-60 nm	0.6-45 μm[b]	5-10 nm
Sidewall angles	35° on all four sides	NA	35° on all four sides

[a]Adapted from SPM Training Notebook (2003) with permission of Veeco Instruments.
[b]Parameters from Novascan Technologies.
Abbreviation: NA, not applicable.

very small forces, and the tip radius should be comparable with the features of interest. Table 2C.2.1 presents the properties of the most common cantilevers.

Use of the optical lever is the most common technique for detecting the cantilever deflection. A laser beam coming from a laser diode is reflected off the back of the cantilever onto a photodetector. The photodetector consists of a split photodiode that senses the change in light intensity from the reflected beam due to changes in the deflection of the cantilever following tip-sample interaction. The sensitivity of such a system is in the range of 0.1 nm (Meyer and Amer, 1990).

The AFM scanners are made from piezoelectric material, which expands or contracts proportionally with an applied voltage. The piezoelectric scanner can move very precisely with very good reproducibility for small displacements, but for displacements >70% of the full-scale displacement, the piezoelectric response is nonlinear. The commercially available AFM featuring closed-loop feedback systems independently monitors the scanner movement and corrects its motion for nonlinearity. There are two types of scanner configurations: scanned tip AFM (where the piezoelectric scanner is rigidly attached to the probe and is moved over the sample surface, which stands still) and scanned sample AFM (where the scanner is attached to the sample and is moved under the tip). Both designs could be integrated with optical microscopes or used as stand-alone AFM systems.

A dual monitor computer interface allows for display of data (images or force curves) on the display monitor and the parameters of the system on the control monitor.

OPERATION MODES OF AFM

Contact Mode Imaging

The most common imaging mode is the constant force mode. In this case, the AFM tip is brought in contact with the sample surface and set to scan the sample in an *xy* raster pattern. The feedback loop maintains a constant deflection (force) of the cantilever with respect to the sample surface by moving the *z* scanner for each *xy* coordinate. This change in *z* axis corresponds to the topographical height of the sample at each given point. The height image preserves the true height information of the sample. Also, a "deflection" image can be recorded by monitoring the cantilever deflection from a straight line. This image is not recorded at constant force, the tall features in the image representing regions of higher force (i.e., higher degree of cantilever bending). This type of imaging loses the true height information, but it presents more fine details of the sample than the height image because the feedback loop response is faster for correcting the position of the small cantilever, in comparison with correcting the position of the piezoelectric element.

Tapping Mode Imaging

For very soft samples, contact mode might not be the best choice due to the friction between the AFM tip and the sample surface. In tapping mode, the cantilever vibrates at its resonant frequency (bounces up and down) under an external electrical excitation. While rastering the sample in *xy* the AFM tip briefly touches the sample at the bottom of each swing, producing a decrease in the oscillation amplitude. Similar to the constant force in contact mode, the feedback loop keeps this decrease in oscillation amplitude at a constant value by moving the piezoelectric tube, and a height image can be recorded (Putman et al., 1994). In addition, a "phase" image can be recorded in tapping mode. When the tip touches the sample at the bottom of its swing, the phase of oscillation is disturbed, inducing a phase difference between the tip and the electrical oscillator that is driving it (Morris et al., 1999). The contrast of a phase image is directly dependent on the elastic properties of the sample. This is not a preferred mode for imaging cells because the elastic properties of the cell surface do not vary enough from point-to-point to offer a good phase-contrast image.

Dynamic Force Spectroscopy Mode

For force measurements the AFM is operated in force mode. The piezoelectric scanner is set to drive the cantilever to touch and retract over a predefined distance in the *z* axis at a fixed *xy* position, in such a way that the *z* axis movement of the piezoelectric element and the deflection signal from the cantilever are recorded in a force curve. In this way,

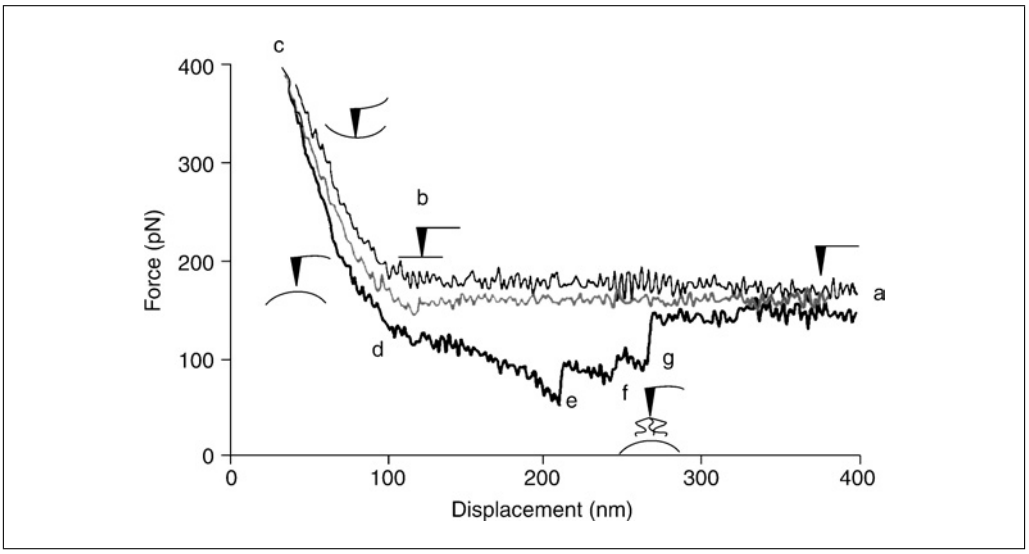

Figure 2C.2.3 Diagram of generic force curves. Approach (black thin line) and retraction force curves with (black thick line) and without (middle line) adhesions are presented. The *x* axis represents the piezoelectric element displacement, and the *y* axis represents the force (see text for details).

measurements of the relative stiffness of the cell surface (approach curve) and adhesion force measurements between the biologically functionalized AFM tip and the cell surface (retraction curve) are acquired (Fig. 2C.2.3). When the probe is extended towards the cell surface (a), a contact point is established with the cell (b), and thereafter the cell surface is indented. Due to cell stiffness, further probe extension causes an opposing force of increasing magnitude to be generated along with increasing indentation in the cell membrane (b to c). The upward deflection of the cantilever as it bends in response to this force, results in an increasing deflection signal. When the probe retracts from the sample (c to d), the force between probe and sample gradually decreases until the cantilever returns to the original position, at which time the deflection signal returns to the original value (a). However, if adhesions occurred between the probe and sample surface, the force causes the cantilever to bend downward, and the deflection signal will be lower than the original value (e to g). When all adhesions are broken, the cantilever returns to the original position (a), and the deflections (unbinding of adhesion events) are recorded on the retraction force curve. The adhesion force is calculated as the product of the deflection height associated with the unbinding event and the spring constant of the cantilever.

On soft samples such as cells, the force application results in an indentation of the cell that is a measure of the local elastic properties of the cell membrane. Due to this elasticity of the cell membrane, only a portion of the piezoelectric element movement causes deflection of the probe; the remainder results in indentation of the cell membrane. The membrane indentation part of the approach curve could be analyzed using different models (Sneddon, 1965; Costa and Yin, 1999) in order to determine the local values of the Young modulus of elasticity as a measure of the local apparent elasticity of the cell membrane.

PRACTICAL GUIDELINES

Although AFM has great potential in microbiological studies, it is important to understand that accurate data collection is sometimes difficult due to the technical limitations associated with the technique itself. Images produced by AFM always represent a convolution of the tip shape with the sample features. It is important to correlate the tip geometrical

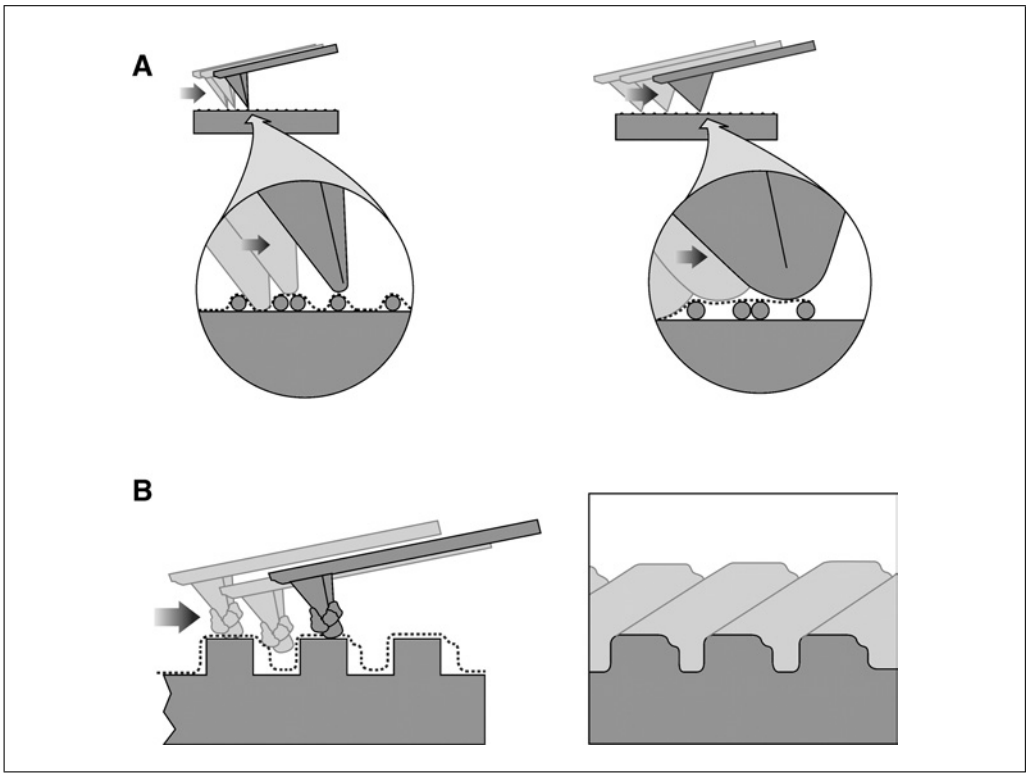

Figure 2C.2.4 Schematics of tip artifacts. (**A**) The smaller the tip radius of curvature, the smaller the sample feature that can be resolved. A dull or dirty (**B**) tip will affect the image profile. Adapted from SPM Training Notebook (2003) with permission of Veeco Instruments.

features with the expected features of the sample to obtain the best images. A tip that is too large will not be able to record features in the sample smaller than the tip parameters (Fig. 2.C2.4A). Also, a contaminated, broken, or blunt tip will introduce distortions into an image, affecting its sharpness or even introducing noise, with the geometry of the tip dominating the geometry of the sample (Figure 2.C2.4B). A detailed description of AFM artifacts is presented at *http://www.pacificnanotech.com/afm-artifacts_single.html*.

Sample Immobilization

One of the most important aspects of imaging live cells under physiological conditions using the AFM is sample immobilization. If the sample is not firmly attached to the bottom of cell culture dish, the AFM tip can easy lift the sample. In this case the tip will break or get contaminated in the process, and no measurements can be performed. Most live cells (e.g., smooth muscle cells, endothelial cells, fibroblasts) attach well to classical substrates like polystyrene or glass. Some cells (e.g., myocytes) can be problematic for AFM measurements due to their intrinsic movement or poor attachment to the substrate. Coating the cell culture dish with gelatin or an extracellular matrix protein (e.g., fibronectin, laminin) can considerably enhance the cell attachment. Live cell imaging can be performed at room temperature or 37°C depending on the experimental requirements. For imaging single bacteria, yeast, or fungal cells under physiological conditions, a concentrated cell suspension is absorbed through an isopore polycarbonate membrane with a pore size comparable to the cell size (Kasas and Ikai, 1995; Dufrêne et al., 1999). Imaging of reconstituted microbial layers or DNA is performed by absorbing the layers of interest on a flat substrate, i.e., fresh cleaved mica or glass (Müller et al., 1997; Colton et al., 1998). Other immobilization methods involve air-drying the sample (a droplet of a concentrated cell suspension is placed on a mica or glass substrate and

allowed to dry in air; Amro et al., 2000) or covalently bonding bacteria to silanized glass slides (Camesano et al., 2000).

Labeling AFM Tips

For force spectroscopy measurements, the AFM tips are typically biofunctionalized with ligands of interest to allow the study of ligand-receptor or protein-protein interaction. This process consists of cross-linking a protein of interest on the AFM tip that has the role of ligand, while its specific receptor is situated on the cell surface. The conjugation can be performed in-laboratory or by using specialized services for more complicated conjugation processes. Two simple ways of cross-linking fibronectin on the AFM tip are presented here. These methods can be applied for other proteins as well.

Coating silicon nitride cantilever probes with fibronectin

For adhesion force measurements, the silicon nitride cantilevers probes (ThermoMicroscope) can be coated (Lehenkari and Horton, 1999) with 1 mg/ml fibronectin (FN). Polyethylene glycol (Sigma) at 10 mg/ml is used to cross-link fibronectin onto probes at room temperature (Hinterdorfer et al., 2000). After the tip is mounted on the glass holder and washed, it is incubated 5 min with polyethylene glycol, washed five times with deionized water, and then incubated 1 min with fibronectin. The tip is then washed again five times with phosphate-buffered saline (*APPENDIX 2A*) and mounted on the AFM scanner.

Coating modified silicon nitride cantilevers with avidin-biotin/fibronectin

A more robust coating that can be used for mechanical cell stimulation consists of avidin (Sigma) cross-linked with biotin/fibronectin (Pierce) onto functionalized glass beads attached to silicon nitride cantilevers (Novascan Technologies). The cantilevers are customized by Novascan Technologies, such that glass beads are glued to the cantilever and then prefunctionalized with biotin. The probes are first incubated 5 min with avidin (1 mg/ml), washed with phosphate-buffered saline five times, and then incubated 5 min with biotin/fibronectin. The tip is washed again with phosphate-buffered saline five times.

The coating should be performed only at the very end of the cantilever to avoid altering its spring constant that is assumed to be unchanged after protein labeling.

AFM APPLICATIONS SPECIFIC TO MICROBIOLOGY

AFM is the only technique available that directly provides structural, mechanical, and functional information at high resolution of cells under physiological conditions. In comparison to electron microscopy, which requires cell fixation and drying, AFM offers the possibility of acquiring high-resolution data in living specimens in real time. Tables 2C.2.2 and 2C.2.3 provide examples of AFM studies relevant to microbiology, focusing on imaging cell surface layers using different immobilization procedures and imaging living cells under physiological conditions (Dufrêne, 2002), respectively. To avoid air-drying techniques that may induce protein or cellular surface denaturing, the physiological imaging techniques are preferred when appropriate. Table 2C.2.4 shows a list of microorganisms for which molecular interactions or local elasticity measurements have been performed. Combining force measurements with high-resolution imaging of cells will be an important challenge, as this will make possible to correlate molecular interactions with structural changes. The examples presented here are just a few of many applications that can be developed in applying AFM to microbiology.

Table 2C.2.2 Imaging the Ultrastructure of Cell Surface Layers[a]

Organism	Sample	Immobilization procedure	Observations[b]	Reference
Bacillus coagulans	S-layer	Covalent linkage to glass/mica	Oblique lattice; $r \approx 10$ nm (study of antibody binding)	Ohnesorge et al. (1992)
		Recrystallization on silanized silicon	Oblique lattice; $r = 1$-2 nm (agreement with electron microscopy)	Pum and Sleytr (1996)
Bacillus sphaericus	S-layer	Covalent linkage to glass/mica	Square lattice; $r \approx 12$ nm (agreement with electron microscopy)	Ohnesorge et al. (1992)
		Recrystallization on silanized silicon	Square lattice; $r = 1$-2 nm (agreement with electron microscopy)	Pum and Sleytr (1996)
		Recrystallization on supported lipid bilayers	Square lattice; $r = 1$-2 nm (novel S-layer/lipid bilayer structure)	Wetzer et al. (1997)
Bacillus stearothermophilus	S-layer	Recrystallization on various silicon surfaces	Oblique lattice; $r \approx 1.5$ nm (study of recrystallization process)	Pum and Sleytr (1995)
Deinococcus radiodurans	Hexagonally packed intermediate layer	Covalent linkage to glass	Ring-shaped hexamers; $r = 1$ nm (correlation with electron microscopy)	Karrasch et al. (1994)
		Adsorption on mica	Conformational change of central pores	Müller et al. (1996)
		Adsorption on HOPG	Tapping mode; $r = 1$-1.5 nm	Möller et al. (1999)
Halobacterium laobium	Purple membrane	Adsorption on mica, silanized glass, and supported lipid bilayers	Hexagonal symmetry; $r =$ 1.1 nm	Butt et al. (1990)
Halobacterium salinarum	Purple membrane	Adsorption on mica	Conformational changes; $r < 1$ nm	Müller et al. (1997[b])
			Tapping mode; $r = 1$-1.5 nm	Möller et al. (1999)
			Trigonal lattice; $r = 0.5$ nm (antibody labeling; complementarity with X-ray and electron crystallography)	Müller et al. (2000)
Escherichia coli	Porin OmpF	Assembly on mica in the presence of lipids	Porin trimers; $r = 1$ nm (comparison with X-ray structure; detection of two conformations)	Schabert et al. (1995)
			Voltage and pH-dependent conformational changes	Müller and Engel (1999)
Escherichia coli	Aquaporin Z	Assembly on mica in the presence of lipids	Tetramers; p42(1)2 and p4 symmetry, $r<1$ nm (proteolytic cleavage force-induced conformational changes)	Scheuring et al. (1999)

[a]Reproduced from Dufrêne (2002) with permission of the American Society for Microbiology.
[b]r-values are lateral resolutions determined directly from the images or after image processing.

Table 2C.2.3 Imaging the Surface Structure of Living Cells[a]

Organism	Observations	Reference
Saccharomyces cerevisiae	Low resolution, bud scars	Kasas and Ikai (1995); also see Figure 2C.2.8
	In situ investigation of cell growth process	Gad and Ikai (1995)
	High resolution; smooth surface	Dufrêne (2002)
Phanerochaete chrysosporium	High resolution; 10 nm; rodlet structures, changes upon germination	Dufrêne et al. (1999)
Aspergillus oryzae	High resolution; 10 nm; rodlet structures, changes upon germination	Van der Aa et al. (2001)
Lactococcus lactis	Sample deformation and imaging artifacts (holes, grooves)	Boonaert et al. (2002)
Streptococcus salivarius	Smooth surface vs surface appendages (fibrils)	Van der Mei et al. (2000)
Pseudomonas aeruginoas	Surface morphology of biofilms; extracellular polymeric substances	Steele et al. (1994)
Pseudomonas putida	Surface morphology of biofilms; extracellular polymeric substances	Auerbach et al. (2000)

[a]Reproduced from Dufrêne (2002) with permission of the American Society for Microbiology.

Investigating *Actinomyces* Species

Tang et al. (2004) used AFM to image and directly detect interactive forces between the AFM tip and *Actinomyces* species that are predominant early colonizers of the oral cavity and prime mediators of interbacterial adhesion. Figure 2C.2.5 shows the two-dimensional and three-dimensional reconstruction of a height image of *A. naeslundii genospecies 1* adherent to mica substrate. In their paper, several sets of experiments were performed in which the interaction forces between the AFM tip and different locations on the same or different *Actinomyces* species were measured. This study was the first to quantitatively evaluate interactive forces at cell surfaces of *Actinomyces* species at the nanoNewton (nN) force range.

Imaging Tipula Iridescent Virus

The largest virus investigated by AFM is the insect virus tipula iridescent virus (TIV). Figure 2C.2.6 shows such a virus, dried from water onto a mica substrate, forming hexagonal arrays that have some degree of crystalline order. Although not properly crystalline, the constraints introduced by association into an array make it possible to measure center-to-center distances that agree well with height measurements and dimensions determined by other methods (Williams, 1998; Kuznetsov et al., 2001).

Imaging Paramecium Bursaria Chlorella Virus Type 1 (PBCV-1)

Paramecium bursaria chlorella virus type 1 (PBCV-1) is a member of a large group of viruses that infect certain unicellular, eukaryotic, chlorella-like green algae and is common in fresh water bodies worldwide (Van Etten, 2000). Kuznetsov et al. (2005) performed an AFM study to complement the electron microscopy data for this type of virus. AFM can reveal structural anomalies and individual defects of single particles.

Table 2C.2.4 Probing Cell Surface Properties, Molecular Interactions, and Elasticity[a]

Organism	Method	Results	References
Escherichia coli	Force-distance curves with cell-coated probes	Attractive hydrophobic interactions; repulsive steric interactions; hydrophobic and electrostatic interactions	Lower et al. (2000), Ong et al. (1999), Razatos et al. (1998)
Shewanella oneidensis	Force-distance curves with cell-coated probes	Adhesion between cells and mineral surfaces; adhesion peaks suggested to reflect unfolding of a an outer membrane protein	Lower et al. (2001)
Burkholderia cepacia, Pseudomonas putida	Force-distance curves with silicon nitride probes	Electrostatic and steric interactions	Camesano and Logan (2000)
Phanerochaete chrysosporium	Force-distance curves with chemically functionalized probes	Hydrophobic and hydration interactions, mapping of surface hydrophobicity	Dufrêne (2000)
Magnetospirillum gryphiswaldense	Approach force curves	Elasticity of cell wall, estimate of turgor pressure	Arnoldi et al. (1998) Arnoldi et al. (2000)
Streptococcus salivarius	Approach force curves	Softness of surface fibrils	Van der Mei et al. (2000)
Methanospirillum hungatei	Depression technique	Elasticity of proteinacious sheath	Xu et al. (1996)
Escherichia coli, Pseudomonas aeruginosa	Depression technique	Elasticity of murein sacculi	Boulbitch et al. (2000) Yao et al. (1999)
Deinococcus radiodurans	Retraction force curves	Stretching single hexagonally packed intermediate protomers, images of single-molecule defects	Müller et al. (1999)
Aspergillus oryzae	Retraction force curves	Stretching surface macromolecules	Van der Aa et al. (2001)

[a]Reproduced from Dufrêne (2002) with permission of the American Society for Microbiology.

Figure 2C.2.7 shows high-magnification images of individual virions that exhibit a surface appearing to be an open network with large spaces of almost hexagonal shape.

Visualizing Dynamic Events of *Saccharomyces cerevisiae*

Figure 2C.2.8 represents a three-dimensional reconstruction of an AFM height image under fluid from a *Saccharomyces cerevisiae* cell trapped in a porous membrane (Dufrêne, 2002). The cell surface is smooth and shows a circular protrusion attributed to a bud scar. The main advantage of using AFM at low resolution (as done here) is that dynamic events such as the cell growth and budding process (Gad and Ikai, 1995; Kasas and Ikai, 1995) and the change in cell surface morphology resulting from treatment with external agents (e.g., enzymes or antibiotics) can be explored in real time.

Force Spectroscopy Measurements on *S. cerevisiae* at the NanoNewton Level

As mentioned earlier, AFM can also be used in the force spectroscopy mode to measure molecular interactions and physical properties by functionalizing the AFM tip with chemical coatings (Ahimou et al., 2002; Dufrêne, 2003). Such an example is shown in Figure 2C.2.9 where the AFM tip functionalized with carboxyl groups maps the electrostatic properties of *S. cerevisiae* at the nanometer level. Changes in adhesion forces were measured as a function of pH, providing information on local isoelectric points.

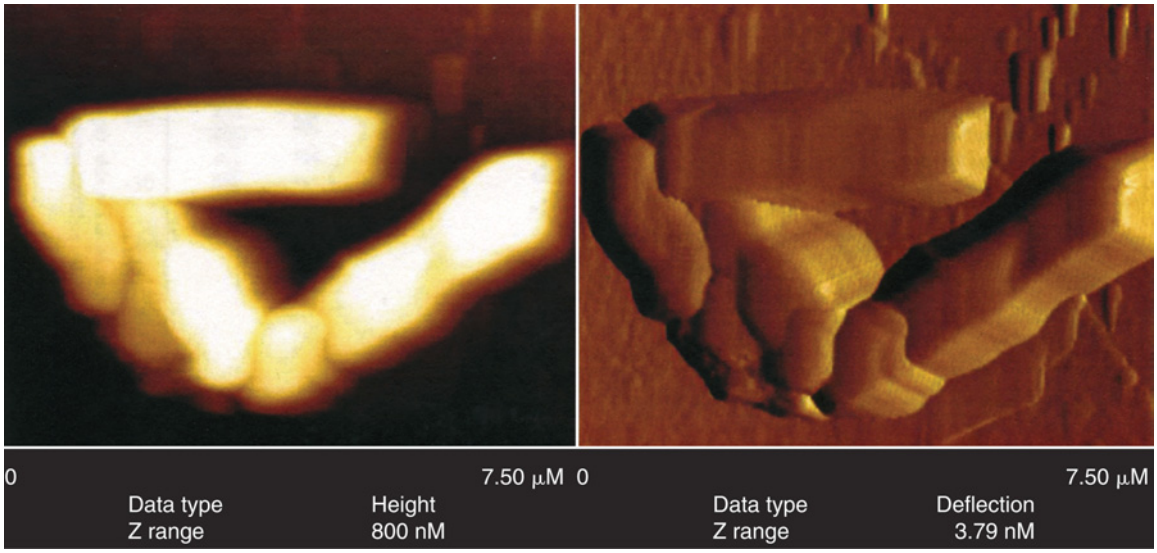

Figure 2C.2.5 Height and deflection images of *A. naeslundii genospecies 1* (ATCC #12104) adherent to the mica substrate after a 5-day period of anaerobic growth. The shape of the outer bacterial surface and the typical diptheroidal arrangements (V, Y, or T forms and palisades) of *Actinomyces* species are seen in these images. Reprinted from *Archives of Oral Biology*, vol. 49. Tang, G., Yip, H.K., Samaranayake, L.P., Chan, K.Y., Luo, G., and Fang, H.H.P. Direct detection of cell surface interactive forces of sessile, fimbriated, and nonfimbriated *Actinomyces* spp. using atomic force microscopy. pp. 727-738. Copyright 2004, with permission from Elsevier.

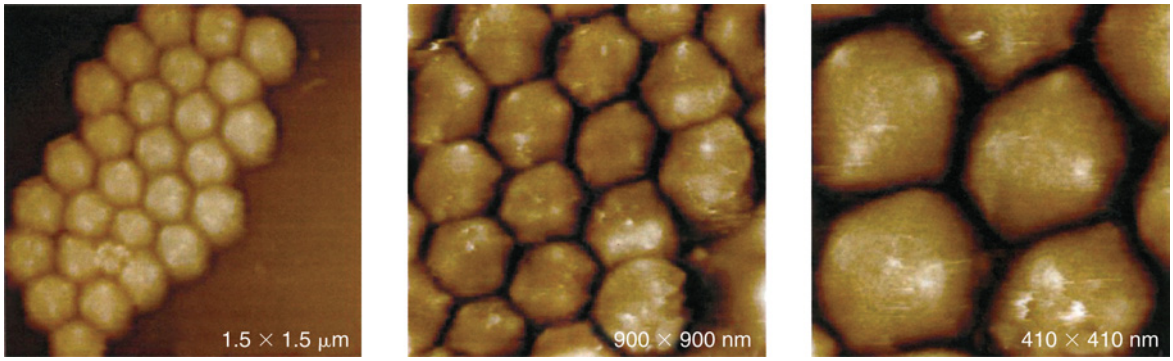

Figure 2C.2.6 When virus particles are clustered into two-dimensional arrays on the mica substrate, they are more firmly immobilized and, therefore, yield better AFM images. The progressively magnified hexagonal arrangement seen here is of the insect tipula iridescent virus (TIV). The little capsid structure is visible, and the polygonal structure of the capsid is evident. Reproduced from Kuznetsov et al. (2001) with permission of the Society for General Microbiology.

Effect of BMS Biosurfactant on Dental Plaque Interactive Forces

Dental plaque can develop and maintain its position on the tooth surface only if the interactive forces between the organism and the tooth surface are sufficiently strong to withstand oral shear forces. Using an adhesion force spectroscopy approach, van Hoogmoed et al. (2006) showed that *Streptococcus mitis* BMS biosurfactant changes the interactive forces between mutant streptococci and enamel, explaining the effects of biosurfactant on adhesion. This study showed for the first time direct measurements of interactive forces between enamel and oral bacteria. The AFM probes were functionalized by gluing enamel particles onto silicon nitride cantilevers. Upon approach of the enamel particles toward each of the mutant streptococci strains trapped in an isopore polycarbonate membrane filter, force-distance curves were acquired, and the data are presented in Table 2C.2.5.

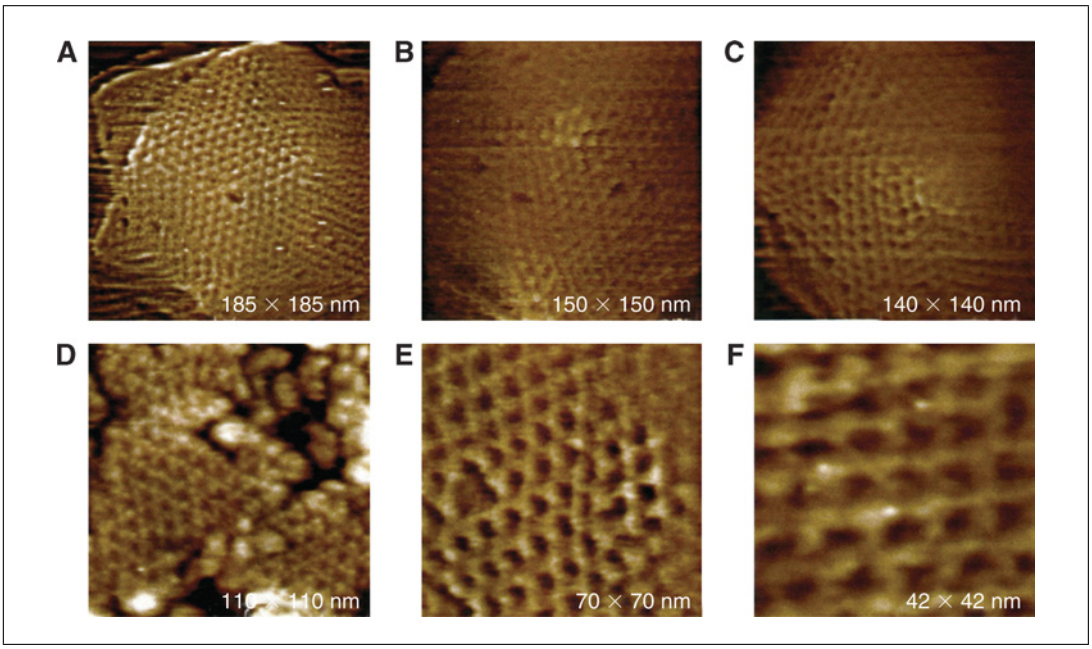

Figure 2C.2.7 At higher magnification, the honeycomb appearance of the surface lattice of Paramecium bursaria chlorella virus type 1 (PBCV-1) is evident. Scars and defects are common on the virion faces, and the unique pentagonal clusters at the vertices can be seen in some cases. In panel **D** a large fragment of a trisymmetronis flattened on the mica substrate, thereby allowing high-resolution imaging. In panels **E** and **F** are two higher-magnification images of the network of trimeric proteins making up the honeycomb arrangement. Reprinted from *Journal of Structural Biology*, vol. 149, Kuznetsov, Y.G., Gurnon, J.R., Van Etten, J.L., and McPherson, A. Atomic force microscopy investigation of a chlorella virus, PBCV-1. pp. 256-263. Copyright 2005, with permission from Elsevier.

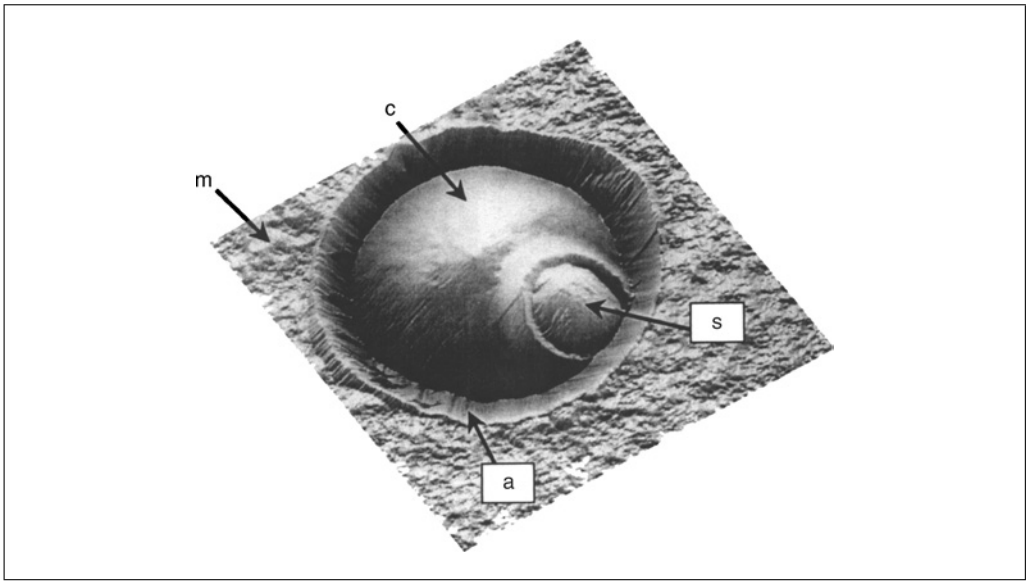

Figure 2C.2.8 AFM height image (6 × 6 μm, *z* range 1.5 μm), in aqueous solution, of a single *Saccharomyces cerevisiae* cell trapped in a porous membrane. The cell (c) is located at the center of the image, while the surrounding flat area represents the polymer membrane (m). The ~1 μm circular protrusion is attributed to a bud scar. Note that the cell is surrounded by an artifactual structure (a) resulting from the contact between the AFM probe and the pore edges Reproduced from Dufrêne (2002) with permission from the American Society for Microbiology.

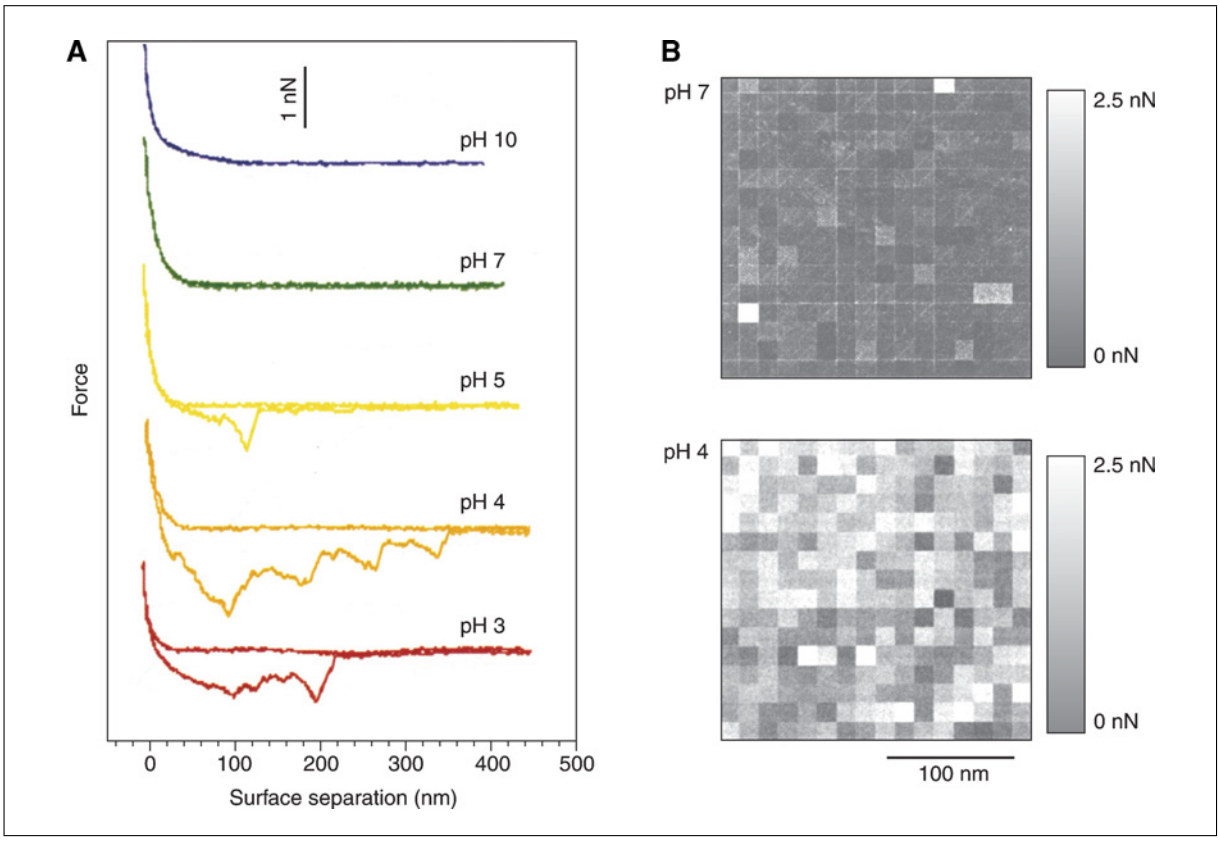

Figure 2C.2.9 Mapping cell surface charges using chemically functionalized probes. (**A**) Force-distance curves recorded in solutions of varying pH between the surface of *Saccharomyces cerevisiae* and an AFM probe functionalized with carboxyl groups. (**B**) Adhesion force maps recorded at pH 7 and pH 4. The differences in adhesion forces observed with pH were related to a change of ionization state of the cell surface. Abbreviation: nN, nanoNewton. Reprinted from *Current Opinions in Microbiology*, vol. 6. Dufrêne, Y.F. Recent progress in the application of atomic force microscopy imaging and force spectroscopy to microbiology. pp. 317-323. Copyright 2003, with permission from Elsevier.

Investigating the Elastic Modulus of the *Methanospirillum hungatei* GP1 sheath

For the first time, AFM force spectroscopy measurements have allowed for probing mechanical properties of microbial cell-surface layers on a local scale. In a pioneering investigation, Xu et al. (1996) measured the elastic modulus of the sheath of the archeon *Methanospirillum hungatei* GP1. The large values obtained for the Young modulus of elasticity showed that this layer of unusual strength could withstand an internal pressure of 400 atmospheres. Other investigators applied AFM to measure the local elasticity of various cell-wall layers (Dufrêne, 2002). Touhami et al. (2003) used force mapping to demonstrate that the cell wall elasticity of the *S. cerevisiae* varies significantly across the cell surface. A 10-fold increase in the Young modulus of elasticity was measured for the bud scar, in agreement with the accumulation of chitin in this region of the cell wall.

CONCLUSION

The AFM is the only instrument capable of nondestructively imaging specific molecules in real time with a resolution comparable to electron microcopy. The exploration of biological applications of the AFM, although technically challenging, are destined to be of great importance. The new approaches that integrate AFM with classical optical methods lead towards a deeper understanding of the dynamics of biological processes, allowing a wide range of experiments, from discerning single molecule interactions to monitoring the live cell responses. In this respect, AFM can be further combined with optical methods to cover a much larger range of applications, including Forster

Table 2C.2.5 Characteristics of Force-Distance Curves Measured by AFM between Two Strains of Mutants Streptococci and Enamel Particles with and without a Salivary Pellicle in the Absence and Presence of a Biosurfactant Coating[a,b]

Bacteria	Enamel coating	Approach curve characteristics		Retraction curve characteristics		
		F_0 (nN)[c]	Λ(nm)[d]	Adhesion (%)[e]	D_{max} (nm)[f]	F_{max} (nN)[g]
S. sobrinus HG 1025		3.9 ± 1.7	53 ± 45	77	80 ± 78	-0.9 ± 0.9
	Biosurfactant	14.3 ± 9.5	253 ± 110	0		
	Saliva	4.6 ± 1.4	83 ± 14	10	107 ± 44	-0.3 ± 0.2
	Saliva + biosurfactant	4.3 ± 1.4	315 ± 90	8	178 ± 89	-2.0 ± 0.4
S. mutans ATCC 25175		0.9 ± 0.7	30 ± 11	78	39 ± 19	-0.8 ± 0.7
	Biosurfactant	0.8 ± 0.4	59 ± 20	10	106 ± 110	-0.2 ± 0.1
	Saliva	5.5 ± 1.2	186 ± 62	0		
	Saliva + biosurfactant	1.3 ± 0.8	333 ± 58	0		

[a]Results are means of 50 force-distance curves, taken over five different organisms on ten randomly selected locations per organism, and including four different enamel particles standard ± deviations.

[b]Reproduced from van Hoogmoed et al. (2006) with permission of the International and American Associations for Dental Research.

[c]F_0 the repulsive force of zero separation distance.

[d]Λ, the decay length of the repulsive force upon approach.

[e]Percentage of the force-distance curves showing adhesion.

[f]D_{max}, average maximum distance at which a local maximum in adhesion forces occurred upon retraction.

[g]F_{max}, average local maximum in adhesion forces upon retraction.

resonance energy transfer (FRET; Ebenstein et al., 2004), surface plasmon resonance (SPR) measurements (Chen et al., 1996), total internal reflection fluorescence (TIRF) microscopy and/or internal reflection microscopy (IRM; Hugel et al., 2002; Trache and Meininger, 2005), or confocal microscopy (Horton et al., 2000; Noy and Huser, 2003; *UNIT 2C.1*).

The future should bring advances to the challenging aspects of the present technology, most importantly the development of instrumentation for increasing acquisition speed to allow for real time imaging of fast biological processes, an improved sample preparation and immobilization technology, and better technology for preparing AFM functionalized bioprobes.

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Internet Resource

http://www.pacificnanotech.com/afm-artifacts_single.html

Good presentation of imaging artifacts.

Laboratory Maintenance of *Ehrlichia chaffeensis* and *Ehrlichia canis* and Recovery of Organisms for Molecular Biology and Proteomics Studies

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ABSTRACT

Tick-borne illnesses are emerging as a major concern for human health in recent years. These include the human monocytic ehrlichiosis caused by the *Amblyomma americanum* tick-transmitted bacterium, *Ehrlichia chaffeensis*; human ewingii ehrlichiosis caused by *Ehrlichia ewingii* (also transmitted by *A. americanum* ticks); and human granulocytic anaplasmosis caused by the *Ixodes scapularis* tick-transmitted pathogen, *Anaplasma phagocytophilum*. Likewise, tick-borne rickettsial pathogens are also a major concern to the health of various vertebrates including dogs, cattle, and several wild animals. In vitro–cultured pathogens grown in a vertebrate host cell and a tick cell culture system will be useful in studies to understand the pathogenic differences as well as to perform experimental infection studies and to generate large quantities of purified antigens. In this unit, methods for culturing *E. chaffeensis* and *Ehrlichia canis* (a canine monocytic ehrlichiosis pathogen) in cell lines to represent vertebrate and tick hosts are described. The unit also includes methods useful in purifying bacteria from the host cells and to evaluate proteins by 2-D gel electrophoresis and western blotting. *Curr. Protoc. Microbiol.* 9:3A.1.1-3A.1.21. © 2008 by John Wiley & Sons, Inc.

Keywords: *Ehrlichia* • culture • macrophage • tick cell • 2-D gel electrophoresis • IFA • polychromatic staining • cell-free *Ehrlichia*

INTRODUCTION

Several emerging tick-borne rickettsial infections have been reported in recent years and have been shown to significantly impact the health of humans and other vertebrates (Paddock et al., 1993; De Silva et al., 1996). These include infections by *Ehrlichia chaffeensis*, a human monocytic agent; *E. ewingii*, a granulocytic ehrlichiosis agent; and *Anaplasma phagocytophilum*, a human granulocytic anaplasmosis agent (*UNIT 3A.2*; Dawson et al., 1991; Chen et al., 1994; De Silva et al., 1996; Caspersen et al., 2002). *Ehrlichia canis* has been identified as the major cause of tick-borne infections in dogs in nearly all parts of the world for several decades (Haas and Meyer, 1986). *E. chaffeensis* and *E. canis* may have evolved common strategies in host-pathogen adaptation based on the fact that they share extensive genetic similarity, contain several immunogenic antigens with shared epitopes, and are macrophage-tropic pathogens (Dawson et al., 1991; Palmer et al., 2000). Understanding the biology of parasitism and host-pathogen interactions requires the growth of these organisms under in vitro conditions.

This unit presents protocols for culturing the *Ehrlichia* species *E. chaffeensis* and *E. canis*. In addition, it includes methods for identifying *Ehrlichia* organisms in culture or tissue samples through the use of polychromatic staining and IFA methods. Protocols are also included for purifying bacteria from host cells from in vitro cultures in support of isolating

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Ehrlichia genomic DNA, RNA, and total protein. Proteome analysis protocols, such as 2-D gel electrophoresis and western blotting, are also discussed.

CAUTION: E. chaffeensis and *E. canis* are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of these pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: The protocols described in this unit involve the use of human and/or other animal blood or tissue samples that may also be infected with *Ehrlichia* species or other potential pathogens. Follow all appropriate guidelines and regulations for the use and handling of human- and animal-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: When preparing solutions, use deionized or double distilled water and all of the highest quality reagents that are available from a vendor. Sterilization by filtration through a 0.22-μm filter or by autoclaving is recommended for most solutions and is essential for cell culture media. Discard any reagent that shows evidence of contamination, precipitation, or discoloration.

BASIC PROTOCOL 1

CULTURING *EHRLICHIA* SPECIES IN A MACROPHAGE CELL LINE

This protocol outlines the culturing of *Ehrlichia* species in the canine macrophage cell line, DH82 (Dawson et al., 1991). The step-by-step protocol described here is intended to help users in culturing *Ehrlichia* isolates currently available at ATCC or at various research laboratories to sufficient quantities for use in experimental infection studies as well as to isolate nucleic acids and proteins for molecular characterization.

Materials

E. chaffeensis and *E. canis* strains of interest (ATCC)
Complete MEM medium (see recipe)
Canine macrophage cell line, DH82 (ATCC # CRL-10389), grown to ~60% confluence in 25-cm^2 tissue culture flask (see Support Protocol 1)
Minimum essential medium with Earle's salt (MEM; Mediatech cat. no. 15-010-CV)

25-cm^2 sterile tissue culture flasks
37°C, 5% CO_2 humidified incubator

1. Obtain frozen stocks of *E. chaffeensis* or *E. canis* from ATCC or from a laboratory stock stored in a liquid nitrogen tank.
2. Thaw a vial of frozen culture by warming to 37°C in a water bath until culture is thawed. Transfer the contents of the vial into a 15-ml centrifuge tube containing 10 ml complete MEM medium using a sterile 1-ml pipet. Centrifuge 10 min at 200 × *g*, 4°C.
3. Discard the medium and resuspend the culture pellet in 1 ml complete MEM medium. Transfer into a 25-cm^2 tissue culture flask containing at least ~60% confluent monolayer DH82 cells (prepared as in Support Protocol 1).
4. Transfer the flask to a 37°C, 5% CO_2 humidified incubator.
5. Check the infectivity two times a week by making a Cytospin slide and evaluating under a light microscope as described in Basic Protocol 5.
6. If the infectivity is less than 50% (as estimated by calculating the ratio of infected cells to total cells counted under a microscope), change half of the medium (see Fig. 3A.1.1 for identification of *Ehrlichia*).

7. When the infectivity reaches 80% to 90%, subculture by inoculating 10% to 20% of the infected culture into a new 25-cm^2 tissue culture flask having at least 60% confluence of DH82 cultures. Alternatively, harvest the cultures for protein preparations (as described in Basic Protocol 7) or for nucleic acid isolation as described in UNIT 3A.3.

   This culture procedure can also be used to inoculate a specimen (such as a blood or a tissue sample) suspected to be positive for Ehrlichia in an effort to recover an isolate. For recovering Ehrlichia species from a specimen, refer to UNIT 3A.3.

MAINTAINING HEALTHY DH82 CELL CULTURES

SUPPORT PROTOCOL 1

Healthy DH82 (macrophage) culture is the prerequisite for growing *Ehrlichia* species. The DH82 cell line is established from neoplastic progenitor cells from a dog with malignant histiocytosis (Wellman et al., 1998). This cell line is widely used for growing *E. chaffeensis* and *E. canis* and to serve as an in vitro model system representing a vertebrate host (Singu et al., 2006).

Materials

70% (v/v) ethanol
Complete MEM medium (see recipe)
Fetal bovine serum (FBS; Atlanta Biologicals cat. no. S11550)
L-Glutamine (Mediatech cat. no. 25005CI)
Canine macrophage cell line, DH82 (ATCC # CRL-10389 or a previously stored frozen stock)

Biosafety Class II (Type B2) cabinet
37°C water bath
15-ml centrifuge tubes (Falcon)
25- and 75-cm^2 tissue culture flasks
37°C, 5% CO_2 humidified incubator
Inverted microscope
4-mm glass beads (Fisher Scientific cat. no. 11-312 A), sterile

1. Sterilize the biosafety cabinet by turning on the UV light for at least 30 min prior to initiating the culture work.
2. Disinfect the work surface area inside the biological safety cabinet with 70% ethanol.
3. Prepare complete MEM medium inside the biosafety cabinet by mixing 500 ml MEM, 6 ml L-glutamine, and 35 ml heat-inactivated FBS (heat inactivate 30 min at 56°C).
4. Thaw a vial of frozen DH82 culture by warming to 37°C in a water bath until cultures are thawed, transfer the contents of the vial into a 15-ml centrifuge tube containing 10 ml complete MEM medium, and centrifuge 10 min at 200 × *g*, 4°C.
5. Discard the medium and resuspend the culture pellet in 5 ml of complete MEM medium.
6. Transfer the contents into a 25-cm^2 tissue culture flask and incubate in a 37°C, 5% CO_2 humidified incubator.
7. Change 50% to 100% of the medium every 3 to 4 days. Replace 50% of the medium if the cell confluence is ≤50% (as assessed by viewing under inverted microscope at 40× magnification) and replace the entire medium if the confluence is ≥50%.
8. When the cells reach 90% to 100% confluence, replace the entire volume of medium and detach the cultures by gently rocking the culture flask by hand after adding ~15 sterile 4-mm glass beads (typically takes ~15 to 30 sec).

9. Dilute the culture in complete MEM medium two- to ten-fold depending on the need to expand the cultures, then transfer 5 or 15 ml each to new 25- or 75-cm^2 tissue culture flasks, respectively, and continue growing the cultures in a 37°C, 5% CO_2 humidified incubator.

BASIC PROTOCOL 2

PREPARATION OF UNINFECTED AND *EHRLICHIA*-INFECTED DH82 CELL LIQUID NITROGEN STOCKS

Preparation of frozen stocks of cultures on a regular basis (preferably once every month) should be done to prevent the loss of cell lines due to accidental contamination or other incidents. Always store stocks in two separate liquid nitrogen tanks. Both the uninfected and *Ehrlichia*-infected cultures should be stored by following a slow freezing protocol done overnight to reach −80°C (described below). Culture vials should then be transferred to a liquid nitrogen tank for long-term storage.

Materials

70% ethanol
E. chaffeensis- or *E. canis*-infected DH82 cultures (see Basic Protocol 1) or healthy DH82 cultures (from a 25-cm^2 tissue culture flask; see Support Protocol 1)
DH82 culture freezing medium (see recipe)
Liquid nitrogen tank

Biosafety Class II (Type B2) cabinet
15-ml sterile polypropylene centrifuge tubes (Falcon)
Refrigerated tabletop centrifuge
Alcohol-resistant marker pen
2-ml screw-cap cryovials
Nalgene Cryo 1°C freezing container (Fisher Scientific cat. no. 5100-0001)

1. Sterilize a biosafety cabinet by turning on UV light for at least 30 min prior to initiating the culture work.
2. Disinfect the work surface area of the biological safety cabinet with 70% ethanol.
3. Transfer *E. chaffeensis*- or *E. canis*-infected DH82 cultures (see Basic Protocol 1) or 5 ml (∼1×10^7 cells) healthy DH82 cultures (from a 25-cm^2 tissue culture flask; see Support Protocol 1) to a 15-ml sterile centrifuge tube.

   CAUTION: *This step and subsequent handling of cultures must be performed inside the biosafety cabinet.*

4. Centrifuge cultures 10 min at 200 × *g*, 4°C.
5. Discard the supernatant and resuspend the culture pellet in 1 ml DH82 culture freezing medium.
6. Transfer the culture to a properly labeled (with alcohol-resistant marker pen) 2-ml cryovial. Freeze the vials by transferring to a −80°C freezer in a Nalgene Cryo 1°C freezing container.

   The Nalgene Cryo 1°C freezing container is designed to provide a 1°C per min drop of temperature.

7. After ∼24 hr of incubation at −80°C, transfer the vials to a liquid nitrogen tank for long-term storage.

   NOTE: *It is important to avoid defrosting the frozen vials during the transfer from a −80°C freezer to a liquid nitrogen tank. The transfer must be performed very rapidly.*

CULTURING *EHRLICHIA* SPECIES IN A TICK CELL LINE

BASIC PROTOCOL 3

General protocols for growing *Ehrlichia* in tick cell lines (e.g., ISE6, IDE8, or AAE2) are very similar to those for *Ehrlichia* grown in the DH82 cell line (as described in Basic Protocol 1). Major differences include the following: tick cells require a specialized medium and are usually incubated at 34°C in the absence of CO_2 (see Support Protocol 2). *Ehrlichia*-infected tick cells also require a slightly different medium than that used for uninfected tick cells. Both uninfected and infection tick cell media must include several minerals, vitamins, and other supplements. One of the important considerations for growing tick cells and *Ehrlichia* cultured in tick cells is that all the reagents and glassware used for culture work must be free of any endotoxins and should be of the highest quality available (see Support Protocol 3).

Materials

E. chaffeensis and *E. canis* (ATCC or from laboratory stock)
Complete *Ehrlichia*-infected tick cell culture medium (see recipe)

Glassware (see Support Protocol 3)
25-cm^2 tissue culture flasks
34°C humidified incubator

1. Prepare glassware for tick cell culture (see Support Protocol 3).
2. Obtain frozen stocks of *E. chaffeensis* and *E. canis*.
3. Thaw a vial of frozen tick cell stock by following Support Protocol 2, steps 2 to 9, but using complete *Ehrlichia*-infected tick cell culture medium.
4. Inoculate the culture pellet from the frozen stock into a 25-cm^2 tissue culture flask containing tick cells having at least ~80% confluent monolayer (prepared as described in Support Protocol 2).
5. Transfer the flask to a 34°C humidified incubator.
6. Check the infectivity two times a week by making a Cytospin slide and evaluating under a light microscope as described in Basic Protocol 5.
7. If the infectivity is <50%, change half of the medium.

8a. When the infectivity reaches 80% to 90%, subculture by inoculating 10% to 20% of the infected culture into a new 25-cm^2 culture flask having at least 80% confluence of tick cells.

8b. Alternatively, harvest the cultures for nucleic acid or protein preparations (*UNIT 3A.3*).

MAINTAINING UNINFECTED TICK CELL CULTURE

SUPPORT PROTOCOL 2

Two *Ixodes scapularis* embryonic cell lines (ISE6 and IDE8) and an *Amblyomma americanum* embryonic cell line (AAE2) have been used (Munderloh et al., 1994; Singu et al., 2006) to support the growth of *Ehrlichia* and *Anaplasma* species and are useful to serve as in vitro model systems to represent tick hosts (Singu et al., 2006). The tick cell line supports the growth of *E. chaffeensis*, *E. canis*, *Ehrlichia ruminantium*, *A. phagocytophilum*, and *A. marginale* (Munderloh et al., 1996, 1999, 2003; Bell-Sakyi et al., 2000; Singu et al., 2005, 2006). Tick cell lines have also proven to be valuable in understanding host-specific differences in the tick-transmitted rickettsiales, *E. chaffeensis*, *E. canis*, *A. marginale*, and *A. phagocytophilum* (Rurangirwa et al., 1999; Jauron et al., 2001; Lohr et al., 2002; Singu et al., 2005, 2006). The protocols (see Basic Protocol 3 and Support Protocol 2) described are adapted from those described in Kurtti et al. (1988), Munderloh

and Kurtti (1989), and Munderloh et al. (1994). All three tick cell lines are cultured using exactly the same procedures.

Materials

70% ethanol
Complete tick cell culture medium (see recipe)
Frozen uninfected tick cell culture

Glassware for tick cell culture (see Support Protocol 3)
Biosafety Class II (Type B2) cabinet
37°C water bath
15-ml centrifuge tubes
1- and 5-ml sterile serological pipets
25- and 75-cm^2 tissue culture flask
34°C humidified incubator

1. Prepare glassware for tick cell culture (see Support Protocol 3).
2. Sterilize the biosafety cabinet by turning on UV light for at least 30 min prior to initiating the culture work.
3. Disinfect the work surface area of the biological safety cabinet with 70% ethanol.
4. Prepare complete tick cell culture medium (see Reagents and Solutions) inside the biosafety cabinet using the base medium, L15B 300.
5. Obtain a vial of uninfected tick cell culture from a frozen stock and defrost rapidly by incubating for few minutes in a 37°C water bath.
6. When the cell suspension is defrosted completely, wipe the culture vial with a 70% ethanol-soaked paper towel.
7. Carefully transfer the entire contents of culture into a sterile 15-ml centrifuge tube and centrifuge 10 min at 400 × *g*, 4°C.
8. Discard the supernatant and add 1 ml of complete tick cell culture medium to the pellet and gently mix using a 1 ml pipet.
9. Transfer the contents to a 25-cm^2 tissue culture flask containing 5 ml of complete tick cell culture medium and incubate the flask in a 34°C humidified incubator.
10. Replace the entire medium the next day and from then once every week until the desired growth of culture is achieved.
11. Monitor the growth of the culture by following Basic Protocol 5.
12. When the cells reach 90% to 100% confluence, replace the entire medium and detach the cultures by pipetting the medium up and down using a 5-ml sterile pipet to create a stream of medium at the cell layer.
13. Dilute the culture in complete tick cell culture medium (two- to ten-fold depending on the need to expand the cultures) and transfer 5 or 15 ml of the diluted cultures to a new 25- or 75-cm^2 tissue culture flask, respectively.

Splitting tick cell cultures too soon may result in the complete loss of cultures. Enzymatic treatment, such as trypsin, or the glass bead method to separate the cells are not recommended, as these procedures can damage the tick cells.

PREPARING GLASSWARE FOR TICK CELL CULTURE

SUPPORT PROTOCOL 3

All glassware used for tick cell culture must be prepared by following strict standards of a cleaning procedure as outlined here. This protocol is intended for the removal of any endotoxins or other contaminants that stick to glassware.

Materials

Glassware detergent
Glassware for cell culture
Autoclave
Aluminum foil
180°C oven

1. Wash glassware once with a detergent designed for laboratory glassware washing.
2. Rinse several times with tap water and then several times with deionized water.
3. Fill the glassware completely with deionized water and autoclave 15 min at 121°C.
4. Discard water, cover with two layers of aluminum foil, and bake glassware 2 hr in a 180°C oven.

PREPARING UNINFECTED AND *EHRLICHIA*-INFECTED TICK CELL LIQUID NITROGEN STOCKS

BASIC PROTOCOL 4

The procedure of making tick cell stocks is slightly different from that described for preparing DH82 culture stocks. Medium for freezing tick cells (both infected and uninfected) is composed of the complete medium (infected or uninfected), but with an FBS concentration raised to 20% and also by including 10% dimethyl sulfoxide (DMSO).

Materials

Ehrlichia-infected tick cells (Basic Protocol 3)
Freezing medium for uninfected tick cell culture (see recipe)
Freezing medium for infected tick cell culture (see recipe)
Liquid nitrogen tank

2-ml cryovials
Nalgene Cryo 1°C freezing container

1. Check the appearance of healthy cells and infectivity of *Ehrlichia*-infected tick cells by evaluating cultures using the polychromatic staining method (see Basic Protocol 5).

   For preparing infected tick cell culture stocks, select cultures having infectivity ranging from 50% to 70%.

2. Remove the growth medium from the culture flasks.
3. Add 5 ml freezing medium and detach the adhering cells by following Support Protocol 2, step 12.
4. Transfer 1 ml to each properly labeled cryovial.
5. Freeze the vials by transferring to a −80°C freezer in a Nalgene Cryo 1°C freezing container.
6. After ∼24 hr, transfer the frozen vials to a liquid nitrogen tank for long-term storage.

   It is important to avoid defrosting the frozen vials during the transfer from a −80°C freezer to a liquid nitrogen tank. The transfer must be performed very rapidly.

EXAMINATION OF CULTURED HOST CELLS FOR *EHRLICHIA* INFECTION USING POLYCHROMATIC STAINING

It is important to monitor infectivity to ensure that *Ehrlichia* does not outgrow macrophage or tick cell cultures or lyse all of the host cells. Aliquots of infected cultures can be transferred onto glass slides using a Cytospin centrifuge and examined for the presence of morulae (*Ehrlichia* infection in a phagosome) in the cytoplasm of host cells using a polychromatic staining method or an indirect fluorescent antibody (IFA) assay (Fig. 3A.1.1). IFA is a useful tool in identifying *Ehrlichia*-infected in vitro cultures (Ristic et al., 1972) and is particularly useful to monitor infections in host cells having low infection rates.

Materials

Uninfected or *Ehrlichia*-infected host cells
Hema staining kit (Fisher Scientific cat. no. 23-122929, 23-122937, 23-122952) containing:
Hema-3 fixative solution
Hema-3 solution I
Hema-3 solution II

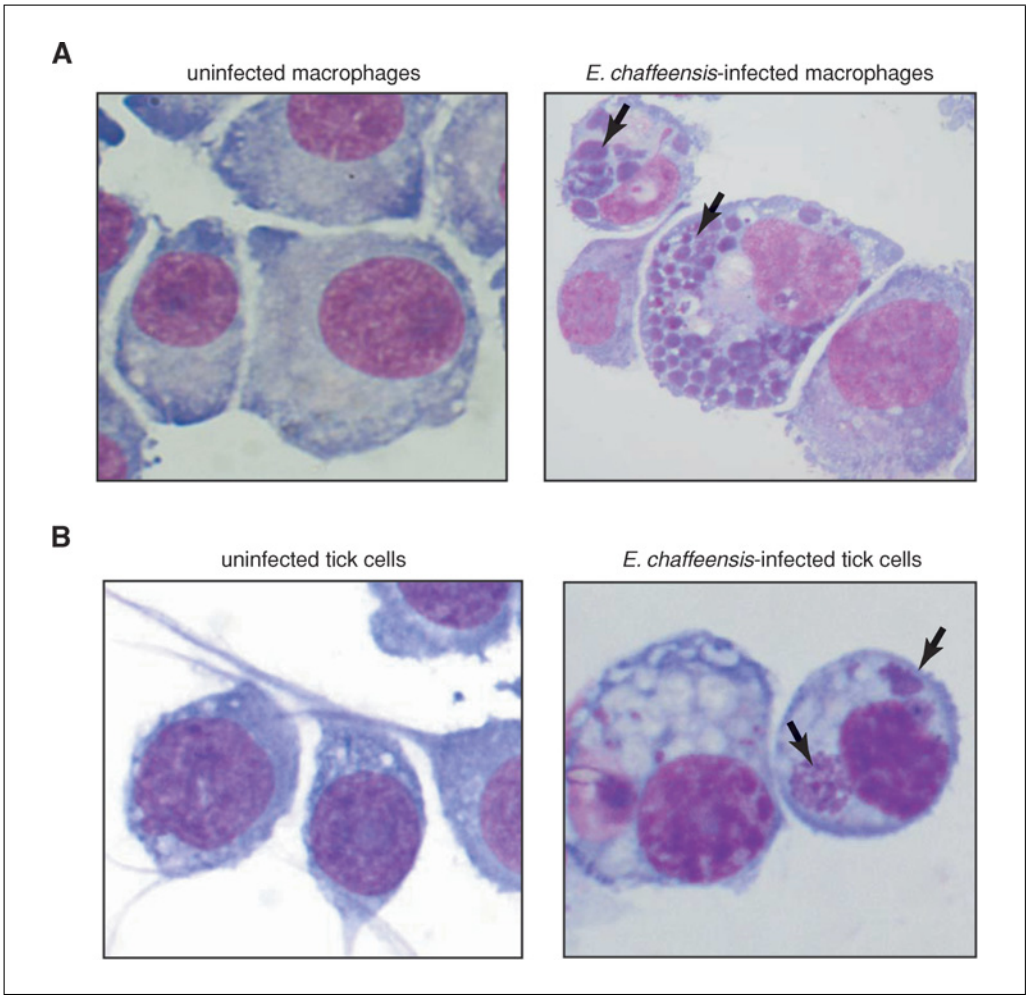

Figure 3A.1.1 Polychromatic staining of healthy and *E. chaffeensis*–infected in vitro cultures of (**A**) macrophage cell line (DH82) and (**B**) tick cells (ISE6). Hema-3 staining kit (Fisher Diagnostics) is used in staining cultures transferred onto a glass slide. *Ehrlichia* organisms are visible in the phagosomes within cytoplasm of infected macrophage and tick cells (identified with arrows). For a color version of this figure, see *http://www.currentprotocols.com*.

Glass microscope slides with frosted ends
Cytospin centrifuge and accessories (Thermo Fisher cat. no. A78300002)
Glass staining jars
Light microscope with 10× and 40× standard objectives and 100× oil immersion objective

1. Label the frosted portion of glass slides with a pencil and assemble for Cytospin analysis.
2. Use 75 to 150 µl of culture suspension (uninfected or *Ehrlichia*-infected host cells) per slide and perform Cytospin as per the manufacturer's instructions by centrifuging culture suspensions 4 min at 400 × *g*.
3. Air dry slides at room temperature until completely dry.
4. Immerse the slides in glass staining jars containing Hema-3 fixative solution for 20 sec, then in Hema-3 solution I for 7 sec, followed by Hema-3 solution II for 4 sec.
5. Rinse the slides in a gentle flow of tap water until excess staining solution is washed off.
6. Air dry slides at room temperature until completely dry.
7. Examine the slides under a light microscope (*UNIT 2A.1*), initially using a 10× or 40× objective to identify the appropriate field containing evenly distributed culture.
8. Use an oil immersion 100× objective to look for the presence of morulae (Fig. 3A.1.1).

INDIRECT FLUORESCENT ANTIBODY ANALYSIS (IFA)

ALTERNATE PROTOCOL

For the most part, polychromatic staining is sufficient for assessing *Ehrlichia* infection. However, IFA may be used to further confirm the presence of *Ehrlichia* in infected cultures. IFA can also be more sensitive in detecting early infections.

Following this procedure, uninfected culture cells will be visible under a microscope with no prominent fluorescence detected, while *Ehrlichia* in morulae will appear as bright green dots (Fig. 3A.1.2).

Materials

Cytospin slides (see Basic Protocol 5)
Acetone
Phosphate-buffered saline (PBS), pH 7.4 (see recipe) containing 1% (v/v) BSA
Polyclonal *E. chaffeensis* or *E. canis* antiserum (obtained from a commercial vendor, e.g., VMRD, cat. no. 211-P-EC, or from infected vertebrate host blood)
1× FA rinse buffer (see recipe)
Fluorescein isothiocyanate (FITC)-conjugated host-specific secondary antibody
Antifade gel mounting medium

Glass staining jars
37°C incubator
Slide box
Paper towels
Aluminum foil
Coverslips
Fluorescence microscope with filters for detecting FITC

1. Prepare Cytospin slides as described for polychromatic staining (see Basic Protocol 5, steps 1 to 3).

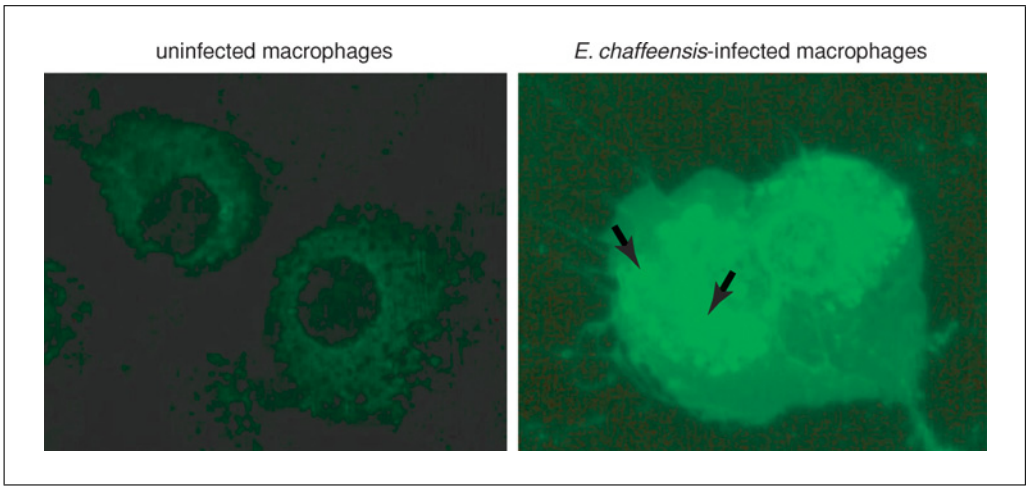

Figure 3A.1.2 Indirect fluorescent antibody (IFA) staining of in vitro cultured *E. chaffeensis*-infected macrophage cell line, DH82. Uninfected DH82 cells are used as a control to demonstrate specific staining only in *E. chaffeensis*–infected cells. *Ehrlichia* organisms in the phagosomes of cytoplasmic compartment of infected macrophages retain strong green fluorescence (identified with arrows). For a color version of this figure, see *http://www.currentprotocols.com.*

2. Immerse slides in a glass jar containing acetone for 1 min.
3. Incubate slides in 1× PBS containing 1% BSA solution for 45 min at 37°C to reduce nonspecific signals.
4. On a flat surface, add 10 μl of prediluted antiserum onto the slides.

   The dilution of antisera can range from 1:64 to 1:4096 depending on the antibody titer.
5. Incubate slides 30 min in a 37°C moist chamber.

   A moist chamber typically consists of a slide box containing water-soaked paper towels.
6. Soak the slides for 10 min with 1× FA rinse buffer in a glass staining jar.
7. Place slides in a moist chamber and add 10 μl of FITC-conjugated host-specific secondary antibodies.
8. Incubate slides 30 min in a dark moist chamber at 37°C (cover slide box with a piece of aluminum foil).
9. Soak slides 10 min with 1× FA rinse buffer in a glass staining jar protected from exposure to light.
10. Add one drop of antifade gel mounting medium and place a coverslip over a slide to allow the medium to spread along the surface of the slide viewing area.
11. Gently press the surface of the coverslip to remove any air bubbles.
12. Examine the slides under a fluorescence microscope initially using a 10× or 40× objective and then using a 100× oil immersion objective.

EHRLICHIA PURIFICATION

BASIC PROTOCOL 6

Recently, methods for purifying host cell-free *Ehrlichia* for use in proteome analysis have been described (Singu et al., 2005, 2006). The protocols have been proven to be useful for isolating different *E. chaffeensis* isolates and *E. canis* cultured organisms. This method can also be used to recover cell-free *Ehrlichia f*or use in antigen analysis or for infection studies using cell-free *Ehrlichia*, or possibly any other molecular biology experiments.

Materials

Uninfected and *Ehrlichia*-infected (Basic Protocol 1) canine macrophage cell line (DH82) or tick cells in a 75-cm^2 confluent flask
SPK buffer (see recipe)

Sonic Dismembrator (Fisher Scientific or equivalent)
3- and 5-μm sterile isopore membrane filters (Millipore)

1. Harvest 15 ml of macrophage or tick cell culture from a 75-cm^2 confluent flask having 80% to 100% *Ehrlichia* infection and centrifuge 15 min at 15,560 × *g*, 4°C, to recover infected host cells and cell-free *Ehrlichia*.

   This protocol can be scaled up or down depending on the volume of purified Ehrlichia needed.

2. Resuspend the resulting culture pellet in 10 ml of SPK buffer and sonicate two times for 30 sec, each time at a setting of 6.5 in a Sonic Dismembrator to release *Ehrlichia* organisms from host cells.

   The sonication setting may vary from instrument to instrument and minor standardization may be required.

3. Centrifuge lysates 5 min at 100 × *g*, 4°C, to pellet host nucleus and other host cell debris.

4. Filter the supernatant sequentially by passing it through 5- and 3-μm sterile isopore membrane filters.

5. Centrifuge *Ehrlichia* organisms from filtrates 15 min at 15,560 × *g*, 4°C and wash pellets two times with 10 ml SPK buffer.

   Avoid disrupting the pellets during this wash protocol. Alternatively, centrifuge cells 5 min at 15,560 × g, 4°C, after each wash.

6. Check the purity of cell-free *Ehrlichia* by preparing a smear on a glass slide stained with a polychromatic stain and examined under a light microscope (see Basic Protocol 5).

PREPARATION OF *EHRLICHIA* PROTEIN EXTRACT

BASIC PROTOCOL 7

The method described here aids in the preparation of total *Ehrlichia* proteins useful for 1-D and 2-D gel analysis.

Materials

Ehrlichia organisms (Basic Protocol 6)
Protein lysis buffer (see recipe)
4:1 (v/v) acetone/trichloroacetic acid, ice cold
Protein sample buffer (see recipe)
RC DC protein assay kit (BioRad)

1. Lyse *Ehrlichia* by adding 0.5 ml of protein lysis buffer to the pelleted organisms obtained in Basic Protocol 6, and incubating 1 hr at room temperature.

2. Precipitate *Ehrlichia* proteins by adding 2 vol of ice-cold 4:1 acetone/trichloroacetic acid and store overnight at −20°C.

3. Centrifuge precipitated protein 15 min at 15,000 × *g*, 4°C, dissolve the final protein pellet in 100 μl sample buffer.

4. Estimate the protein concentration using the RC DC protein assay kit and following the manufacturer's step-by-step protocol.

TWO-DIMENSIONAL (2-D) GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Proteins resolved on a polyacrylamide gel will be useful for identifying pathogen-specific proteins and proteins that are immunogenic. One- and two-dimensional gel electrophoresis–resolved proteins can also be used for establishing the identity of a protein by mass-spectrometry methods (Singu et al., 2005, 2006).

Materials

Ehrlichia proteins (Basic Protocol 7)
Equilibration buffer I (see recipe)
Equilibration buffer II (see recipe)
4% to 20% gradient polyacrylamide gels (BioRad cat. no. 3450104)
Tris-glycine electrophoresis buffer (see recipe)
Silver-staining kit (BioRad)
Blocking solution: 5% nonfat dairy milk in PBS
Primary antibodies (polyclonal sera against *E. chaffeensis* or *E. canis* raised in mouse, dog, or human)
PBS containing 0.1% Tween 20 (PBS-T; see recipe for PBS)
Anti-mouse and anti-dog horseradish peroxidase (HRPO)–conjugated secondary antibodies
ECL western blotting detection reagents (Amersham)

Multiphor II electrophoresis system (Amersham)
Immobiline dry strips (11-cm, pH 3 to 10; Amersham)
Criterion cell apparatus (BioRad cat. no. 165-6001) or equivalent
Hybond-N nitrocellulose membrane (Amersham cat. no. RPN303D)
Protean Trans-blot cell (BioRad) or equivalent
X-ray film (Amersham)

1. Resolve 100 μg *Ehrlichia* proteins in a Multiphor II electrophoresis system using Immobiline 11-cm dry strips, pH 3 to 10.

   The IEF parameters are 300 V, 2 mA, and 5 W for 1 min, followed by 3500 V, 2 mA, and 5 W for 245 min.

2. Equilibrate the strips sequentially for 15 min each in 1 ml of equilibration buffer I and equilibration buffer II.

3. Perform the second dimension (SDS-polyacrylamide gel electrophoresis analysis) on the 1-D resolved strips in a Criterion Cell apparatus using 4% to 20% gradient polyacrylamide gels for 2 hr at 74 V at room temperature using Tris-glycine electrophoresis buffer.

4. Stain the gels using a silver-staining kit per the manufacturer's protocol to identify a pathogen-specific resolved proteome map (Fig. 3A.1.3).

5. Transfer the 2-D-resolved proteins from unstained gel after step 3 (skip step 4) to a nitrocellulose membrane using Protean Trans-blot cell or equivalent as per the manufacturer's instructions.

6. Soak the membrane in 50 ml blocking solution for 1 hr at room temperature.

7. Transfer the membrane to 10 ml of blocking solution containing 1:250 diluted primary antibody (polyclonal sera against *E. chaffeensis* or *E. canis* raised in mouse, dog, or human) and incubate 2 hr at room temperature.

   Polyclonal sera are obtained from wild-type mice (C57BL6 mice) 21 to 24 days after infection with either macrophage-grown or tick cell-grown E. chaffeensis or E. canis

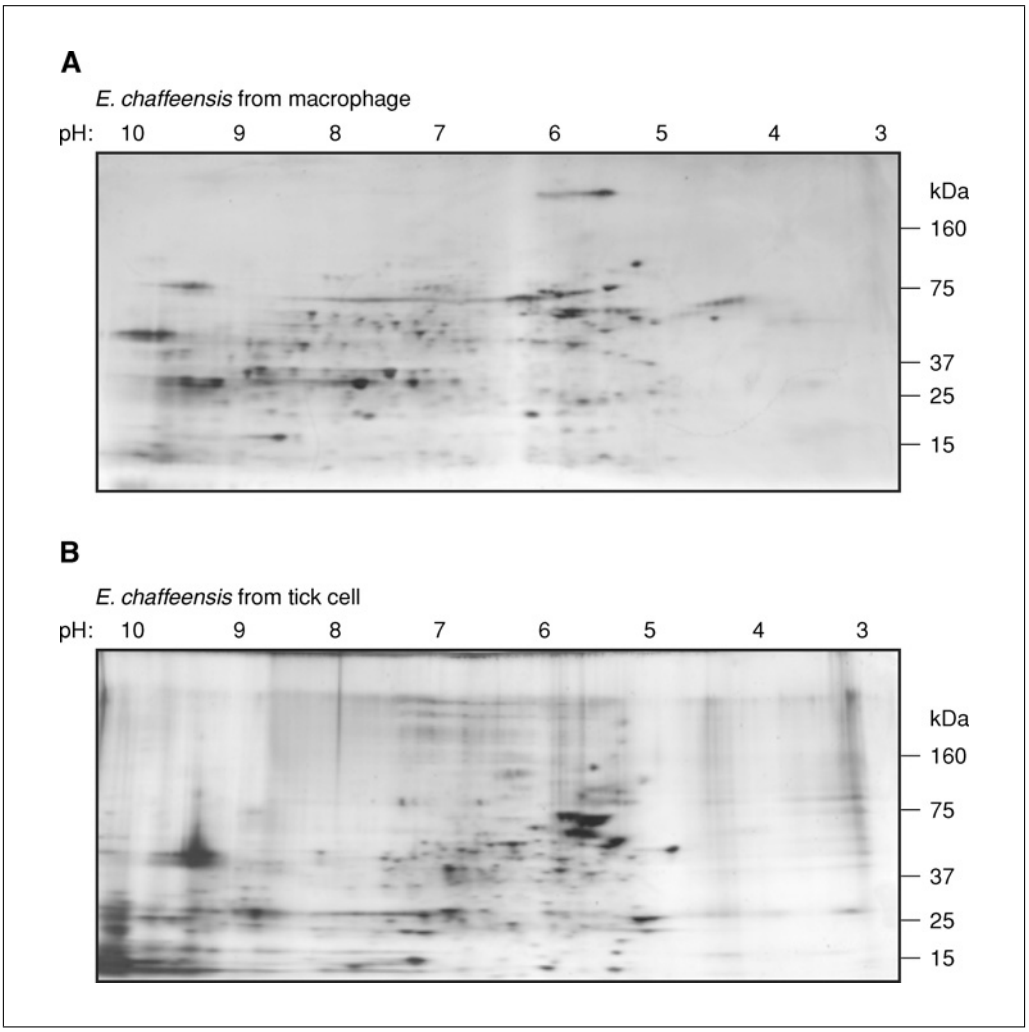

Figure 3A.1.3 Proteome maps of *E. chaffeensis* grown in (**A**) macrophage and (**B**) tick cells (Singu et al., 2005). Scanned images of silver-stained 2-D gels of *E. chaffeensis* proteomes are presented. The approximate location of the pI values of the protein migration and the protein molecular weight standards are shown on the top and right side of the gel images, respectively.

(Ganta et al., 2002, 2004). More details about infection are provided in Ganta et al. (2002, 2004, 2007).

8. Wash the membrane three times, 10 min each time, at room temperature in saturating volumes of PBS-T.
9. Incubate the membrane with appropriately diluted HRPO-conjugated anti-mouse or anti-dog secondary antibody for 1 hr at room temperature.
10. Wash the membrane with PBS-T three times as described in step 8.
11. Detect the antigen-antibody complexes by exposing an X-ray film for 2 to 5 min using the ECL western blotting detection reagents.

Silver-stained gels and X-ray film having western blot data may be digitalized using a scanner and the images may be analyzed with the aid of image analysis software to perform comparisons of 2-D gels.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete Ehrlichia-infected tick cell culture medium

412 ml L15B300 medium (see recipe)
25 ml heat-inactivated FBS (Invitrogen cat. no. 26140079)
25 ml TPB medium (see recipe)
25 ml 5% $NaHCO_3$
12.5 ml 1M HEPES (see recipe)
0.5 ml concentrated lipoprotein (ICN Biomedicals cat. no. 0219147680)

Adjust pH to 7.5 to 7.8 with 1 N NaOH and filter sterilize using a 0.22-μm filter unit. Cover the bottle with aluminum foil to protect from light and store up to 2 months at 4°C.

FBS from Invitrogen works well. FBS is shipped with dry ice, upon arrival, thaw in a 37°C water bath, dispense into 25-ml aliquots, and store at −20°C for future use. Before use, heat-inactivate FBS by incubating 30 min in a 56°C water bath.

Complete MEM medium

500 ml minimum essential medium with Earle's salt (MEM medium: Mediatech cat. no. 15-010-CV)
35 ml heated-inactivated FBS (Invitrogen cat. no. 26140079)
6 ml 100 mM L-glutamine
Mix by inverting bottle several times, filter sterilize using a 0.22-μm filter unit, and store up to 2 months at 4°C.
MEM is used for both uninfected cells and Ehrlichia-infected culture.

Complete tick cell culture medium

449.5 ml L15B300 medium (see recipe)
25 ml heat-inactivated FBS (Invitrogen cat. no. 26140079)
25 ml TPB medium (see recipe)
0.5 ml concentrated lipoprotein (ICN Biomedicals cat. no. 0219147680)

Adjust pH to 7.20 with 1 N NaOH and filter sterilize using a 0.22-μm filter unit. Cover the bottle with aluminum foil to protect from light damage and store up to 2 months at 4°C.

DH82 culture freezing medium

0.5 ml MEM medium (Mediatech cat. no. 15-010-CV)
0.45 ml FBS (Invitrogen cat. no. 26140079)
50 μl DMSO
Prepare fresh

Equilibration buffer I

375 mM Tris·Cl, pH 8.8
6 M urea
2% sodium dodecyl sulfate (SDS)
2% DTT

To prepare 100 ml, mix 37.5 ml of 1 M Tris·Cl, pH 8.8, 36.04 g urea, 2 g SDS, and 2 g DTT. Bring volume up to 100 ml with deionized water, dispense into 2-ml aliquots, and store up to 1 year at −20°C.

This buffer is commercially available from BioRad.

Equilibration buffer II

375 mM Tris·Cl, pH 8.8 (*APPENDIX 2A*)
6 M urea
2% SDS
2.72 mg iodoacetamide/ml
0.01% bromophenol blue

To prepare 100 ml, mix 37.5 ml of 1 M Tris·Cl, pH 8.8, 36.04 g urea, 2 g SDS, 272 mg iodoacetamide, and 10 mg bromophenol blue. Bring volume up to 100 ml with deionized water, dispense into 2-ml aliquots, and store up to 1 year at −20°C.

This buffer is commercially available from BioRad.

FA rinse buffer, 1×

2.85 g Na_2CO_3
8.4 g $NaHCO_3$
2.125 g NaCl
Add distilled deionized water to 1000 ml
Adjust pH to 9 to 9.5 with 1 N NaOH
Store up to 1 year at room temperature

Freezing medium for uninfected tick cell culture

7 ml complete tick cell culture medium (see recipe)
2 ml FBS (Invitrogen cat. no. 26140079)
1 ml DMSO
Prepare fresh

Freezing medium for infected tick cell culture

7 ml complete *Ehrlichia*-infected tick cell culture medium (see recipe)
2 ml FBS (Invitrogen cat. no. 26140079)
1 ml DMSO
Prepare fresh

HEPES, 1 M

23.83 g HEPES (Sigma-Aldrich cat. no. H-3375)
Add cell culture–grade water to 100 ml, filter sterilize using a 0.22-μm filter unit, and store up to 6 months at 4°C.

L15B medium

1 packet of L15B medium powder (Invitrogen cat. no. 41300-039)
0.299 g L-aspartic acid (Sigma-Aldrich cat. no. A-4534)
0.299 g α-ketoglutaric acid (Sigma-Aldrich cat. no. K-1128)
0.292 g L-glutamine (Sigma-Aldrich cat. no. G-6392)
0.3 g proline (Sigma-Aldrich cat. no. P-5607)
0.5 g L-glutamic acid (Sigma-Aldrich cat. no. G-8415)
14.41 g D-glucose (Sigma-Aldrich cat. no. G-7021)
1 ml mineral stock D (see recipe)
1 ml vitamin stock (see recipe)

Add the above ingredients to a 1000-ml volumetric flask, make up the volume to 1000 ml with cell culture–grade water, adjust pH to 6.4 to 6.6 with 10 N NaOH, filter sterilize using a 0.22-μm filter unit, and store up to 6 months at 4°C, covering with aluminum foil to protect from light damage.

L15B300 medium

1000 ml L15B medium (see recipe)
333 ml cell culture–grade water
Store up to 2 months at 4°C

Mineral stock solution A

20 mg $CoCl_2 \cdot 6H_2O$ (Sigma-Aldrich cat. no. C-8661)
20 mg $CuSO_4 \cdot 5H_2O$ (Sigma-Aldrich cat. no. C8027)
160 mg $MnSO_4 \cdot H_2O$ (Sigma-Aldrich cat. no. M1144)
200 mg $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich cat. no. Z0251)

Prepare the mineral stock solution by weighing the desired weights in 100 ml cell culture–grade water (Invitrogen cat. no. 15230-162), filter sterilize using a 0.22-μm filter unit, dispense into 1-ml aliquots into sterile microcentrifuge tubes, and store up to 2 years at −20°C for future use.

Mineral stock solution B

Prepare 20 mg $Na_2MoO_4 \cdot 2H_2O$ (Sigma-Aldrich cat. no. M1003) in 100 ml of cell culture–grade water, filter sterilize using a 0.22-μm filter unit, and store as 1-ml aliquots up to 2 years at −20°C for future use.

Mineral stock solution C

Prepare 20 mg Na_2SeO_3 (Sigma-Aldrich cat. no. S5261) in 100 ml of cell culture–grade water, filter sterilize using a 0.22-μm filter unit, and store as 1-ml aliquots up to 2 years at −20°C for future use.

Mineral stock solution D

1 g glutathione (reduced) (Sigma-Aldrich cat. no. G-6013)
1 g ascorbic acid (Sigma-Aldrich cat. no. A-4544)
50 mg $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich cat. no. F-8633)
1 ml mineral stock A (see recipe)
1 ml mineral stock B (see recipe)
1 ml mineral stock C (see recipe)

Add the above ingredients and make up the volume to 100 ml with cell culture–grade water, filter sterilize using a 0.22-μm filter unit, and store as 1-ml aliquots up to 2 years at −20°C.

$NaHCO_3$, 5%

Add 5 g of $NaHCO_3$ in 100 ml of cell culture–grade water, filter sterilize using a 0.22-μm filter unit, and store up to 2 months at 4°C.

NaOH, 1 N

Add 4 g of NaOH in 100 ml culture grade–water, filter sterilize using a 0.22-μm filter unit, and store up to 6 months at 4°C.

PBS buffer, pH 7.4

8 g NaCl
0.2 g KH_2PO_4
0.2 g KCl
Plus one of the following:
2.17 g $Na_2HPO_4 \cdot 7H_2O$

continued

2.9 g $Na_2HPO_4 \cdot 12H_2O$
1.15 g Na_2HPO_4 (anhydrous)
Make up the volume to 1 liter with deionized distilled water.

Protein lysis buffer

9 M urea
2% Triton X-100
3.24 mM dithiothreitol (DTT)
8 mM phenylmethylsulfonyl fluoride (PMSF)
0.2% ampholyte, pH 3 to 10 (Amersham Pharmacia Biotech)

To prepare 100 ml, add 54.05 g urea, 2 g Triton X-100, 50 mg DTT, 139.3 mg PMSF, and 0.2 ml ampholyte and make up the volume to 100 ml with deionized water and store up to 1 year at −20°C.

Protein sample buffer

9 M urea
0.2% ampholyte, pH 3 to 10 (Amersham Pharmacia Biotech)
2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Sigma)
65 mM DTT
0.52% Triton X-100
0.001% bromophenol blue

Mix 54.05 g urea, 0.2 ml ampholyte, 2 g CHAPS, 1.003 g DTT, 0.52 g Triton X-100, and 1 mg bromophenol blue with deionized water to a total volume of 100 ml and store up to 1 year at −20°C.

SDS-PAGE gel transfer buffer, pH 8.3

39 mM glycine
48 mM Tris base
0.037% SDS (electrophoresis grade)
20% methanol

To prepare 1 liter of transfer buffer, weigh 2.9 g glycine, 5.8 g Tris base, 0.37 g SDS, add 200 ml methanol, and make up the volume to 1000 ml with deionized water. Store up to 1 year at room temperature.

SPK buffer, pH 7.4

0.05 M potassium phosphate
0.2 M sucrose

To make 1 liter of SPK buffer, measure 80.2 ml of 0.5 M K_2HPO_4, 19.8 ml of 0.5 M KH_2PO_4, 68.46 g sucrose, dissolve in deionized distilled water, and make up volume to 1000 ml with deionized distilled water. Store up to 1 year at 4°C.

TPB medium

Add 29.59 g tryptose phosphate broth (TPB; Difco cat. no. 260300) to culture grade–water and make up volume to 1000 ml, dispense into 100-ml aliquots, and autoclave with loose caps for 15 min at 121°C. Store up to 1 year at 4°C.

The color should be pale yellow; if it darkens, the TPB should be discarded as it will be toxic to the tick cells.

Tris-glycine electrophoresis buffer

25 mM Tris base
250 mM glycine
0.1% SDS

Weigh out 15.1 g Tris·Cl and 94 g glycine, dissolve compounds in 900 ml deionized water, and add 50 ml of 10% SDS. Make up the final volume to 1000 ml with deionized water (5× stock). Dilute 1:5 to make 1× working buffer. Store up to 1 year at room temperature.

Vitamin stock

100 mg 4-aminobenzoic acid (PABA; Sigma-Aldrich cat. no. A-9878)
50 mg cyanocobalamine (B12; Sigma-Aldrich cat. no. V2876)
10 mg D-biotin (Sigma-Aldrich cat. no. B-4639)

Prepare the vitamin stock by weighing these ingredients and transferring to 100 ml cell culture–grade water, filter sterilize using a 0.22-μm filter unit, and store as 1-ml aliquots up to 1 year at −20°C for use.

COMMENTARY

Background Information

Ehrlichia culture protocols described in this unit require particular care and attention to details. Two important precautions must always be followed: (1) Monitor the availability of uninfected macrophage and tick cell cultures in sufficient quantities to maintain the *Ehrlichia*-infected cultures. This is especially important for tick cell culture since the doubling time of tick cells is longer than macrophage cultures by about two fold. Typically, three to five times more flasks of uninfected tick cultures to one flask of *Ehrlichia*-infected culture are needed. (2) Care must be taken to avoid culture contaminations, as all the cultures must be maintained in the absence of any added antibiotics. During the initial stages of culture establishment, it may be worthwhile to maintain one or two uninfected host cell culture flasks in the presence of antibiotics, such as gentamycin (50 μg/ml final concentration), or a mixture of penicillin and streptomycin (50 to 100 IU/ml and 50 to 100 μg/ml, respectively) and fungizone (0.125 to 0.25 μg/ml) by following the protocols described by a manufacturer (Sigma-Aldrich).

Polychromatic staining and indirect immunofluorescence assay (IFA) are commonly used for detecting *Ehrlichia* infection in in vitro cell culture as well as in infected animal blood or tissue samples. IFA is a more sensitive method and it is useful to assess low level infection in culture or tissue samples.

Cryopreservation of uninfected and *Ehrlichia*-infected cultures at a regular interval (once every 1 to 2 months) is recommended to facilitate rapid recovery in the event of culture contamination. Host cell–free *Ehrlichia* is needed for use in pathogen-specific protein analysis. Host cell–free *Ehrlichia* can be purified by sonication, differential centrifugation followed by fractionation using small pore size isopore membrane filters. This method aids in the preparation of pure *Ehrlichia* useful in protein analysis and molecular biology experiments.

Critical Parameters

Culturing Ehrlichia in canine macrophage cell line, DH82, and tick cell lines

Sterile culture techniques are a very critical step for *Ehrlichia* culture as all the cultures must be maintained in the absence of any antibiotics to insure bacterial growth. Always keep infected cultures separate from uninfected cultures. Also, maintaining duplicate sets of infected and uninfected cultures by independently prepared media and reagents is preferred.

Tick cells seem to be more sensitive to minor changes of pH and cell density. Cell density of tick cell culture should be between 20% and 80% for optimal growth. Tick cell culture can also be affected due to impurities that may be introduced from glassware and other culture supplies. Thus, it is recommended to use the culture supplies from the same vendor when one finds a product(s) useful in growing *Ehrlichia* in tick cell culture.

Preparing cell culture stocks

One must prepare liquid nitrogen culture stocks on a regular basis to prevent the loss of cell lines due to accidental contamination

or other incidents. It is also recommended to store stocks in two separate liquid nitrogen tanks. Both the uninfected and *Ehrlichia*-infected cultures should be stored by following the slow freezing protocol to reach −80°C overnight and then transferred to a liquid nitrogen tank for long-term storage.

Checking the infectivity by polychromatic staining and IFA methods

It is important to maintain a proper cell density (by assessing under an inverted microscope) so that the culture does not overgrow. It is also critical to check the infectivity of *Ehrlichia*-infected DH82 or tick cells by examining a slide using polychromatic staining or the IFA method. When the infectivity of a flask reaches 70% to 90%, the infected cells need to be harvested and 1/10 to 1/5 of the infected cells can be used to infect a new flask having a confluent monolayer of healthy cells for continued maintenance of the cell culture.

Troubleshooting

Table 3A.1.1 presents some of the common problems associated with the protocols in this unit.

Anticipated Results

Growth of Ehrlichia in DH82 and tick cell culture

DH82 cell culture is easier to maintain as compared to tick cell culture. When recovering a frozen healthy DH82 culture stock, the culture growth should be visible within 2 to 3 days. By 5 to 7 days, a 25-cm^2 tissue culture flask should be confluent and ready to be split. The doubling time for tick cells is longer, and it will take 7 to 10 days for a flask to reach confluency.

When an *Ehrlichia* culture has 70% to 90% infectivity, it is subcultured by inoculating 5% to 10% into a flask containing a confluent monolayer. Infections should reach 80% to 90% within 3 to 5 days after inoculation.

Detection of Ehrlichia-infected cells by polychromatic staining and IFA methods

When examining a Cytospin slide stained with a polychromatic stain, the morulae (cytoplasmic inclusions of *Ehrlichia*) stain purple, while the host cell nucleus stains pink. Morulae in tick cells are more loosely packed compared to those in DH82 cells.

Table 3A.1.1 Troubleshooting Guide for Laboratory Maintenance of *Ehrlichia*

Problem	Possible cause	Solution
Medium color changed to yellow	Bacterial or fungal contamination, or pH may be too low	If contamination was confirmed, discard the medium. If pH too low, adjust pH with NaOH.
Poor culture growth	Cultures were split too fast or diluted too much during subculture	Monitor cell density during growth and subculture
Most of the host cells of an infected culture are lysed	Overgrowth of *Ehrlichia*	Add more healthy cells or re-infect a new flask containing confluent monolayer of host cells
Fewer or no cells detected on a stained Cytospin culture slide	Poor fixation of the cell culture prior to staining	Fix slides in the Hema-3 fixative for a longer period of time
All cellular contents are purple on a stained Cytospin culture slide	Slide incubated too long in Hema-3 solution II	Reduce incubation time in Hema-3 solution II
High background fluorescence on IFA slide	Poor blocking	Increase the length of blocking to >1 hr using BSA or non-fat dairy milk
Low yield of host cell–free *Ehrlichia*	Started with low volume of infected culture or infectivity is low or poor lysis of host cells	Check for infectivity and confluence before use and start with at least 15 ml of 80%–90% infected culture. Sonicate for longer time intervals.

Morulae are visualized as fluorescent green clusters in the IFA slides when examined under a fluorescence microscope.

Purifying host cell–free Ehrlichia

The purified *Ehrlichia* pellet is typically much smaller than the original culture pellet and looks creamy white.

Acknowledgements

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Laboratory Maintenance of *Anaplasma phagocytophilum*

Anaplasma phagocytophilum is the etiologic agent of human granulocytic anaplasmosis (HGA; formerly human granulocytic ehrlichiosis). This unique bacterium resides within salivary glands of its *Ixodes* tick vector or within the intracytoplasmic confines of neutrophils in its mammalian hosts. Because of its obligate intracellular nature, *A. phagocytophilum* is maintained in the laboratory in eukaryotic cell culture or within murine hosts. The human-derived promyelocytic leukemia cell line HL-60 is routinely used for in vitro cultivation of *A. phagocytophilum*. The susceptibility of these cells to *A. phagocytophilum* infection, like that of human neutrophils, is attributable to the expression of P-selectin glycoprotein ligand-1 (PSGL-1) on the cell surface. The rapid rate at which HL-60 cells grow and their permissiveness with respect to *A. phagocytophilum* infection allows one to obtain high yields of in vitro–cultured bacteria. However, the ability of *A. phagocytophilum* to successfully establish in vivo infection declines considerably when continually maintained in cell culture. It is therefore important that the bacterium also be maintained in vivo. Laboratory mice are excellent in vivo models, as infection in these mice partially mimics many of the hematological parameters and histopathological lesions associated with HGA. Peripheral blood infection of immunocompetent mice resolves by the third or fourth week post-inoculation. Moreover, *A. phagocytophilum* pathogenicity wanes with repeated passage through immunocompetent mice. SCID mice are unable to clear *A. phagocytophilum* infection, and are therefore used for continual maintenance of the pathogen in vivo. This unit describes the inoculation and maintenance of *A. phagocytophilum* in vivo using a murine model of infection (see Basic Protocol 1) and in vitro using HL-60 cells (see Basic Protocol 2). Methods for identifying *A. phagocytophilum*–infected cells in a peripheral blood sample (see Support Protocol 1) or infected HL-60 cells (see Support Protocol 2) using either cytological staining (see Support Protocol 3) or immunofluorescent microscopy (see Support Protocol 4) are also discussed. Additionally, cryopreservation of *A. phagocytophilum*–infected HL-60 cell lines (see Support Protocol 3) is described. Furthermore, this unit describes the recovery of semipure preparations of viable *A. phagocytophilum* from infected HL-60 cells (see Basic Protocol 2). Techniques for further purifying these bacterial preparations by density gradient centrifugation (see Support Protocol 6) or for fluorescently labeling them to track infection (see Support Protocol 7) are also provided. Figure 3A.2.1 demonstrates how these protocols are interrelated.

CAUTION: *Anaplasma phagocytophilum* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: The protocols described in this unit involve the use of human and/or murine blood that is infected with *A. phagocytophilum.* Syringe lysis of *A. phagocytophilum*–infected HL-60 cells is also involved. Follow all appropriate guidelines and regulations for the use and handling of human-derived materials and blood-borne pathogens, as well as storage and disposal of "sharps." See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: These experiments require Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

Contributed by Jason A. Carlyon
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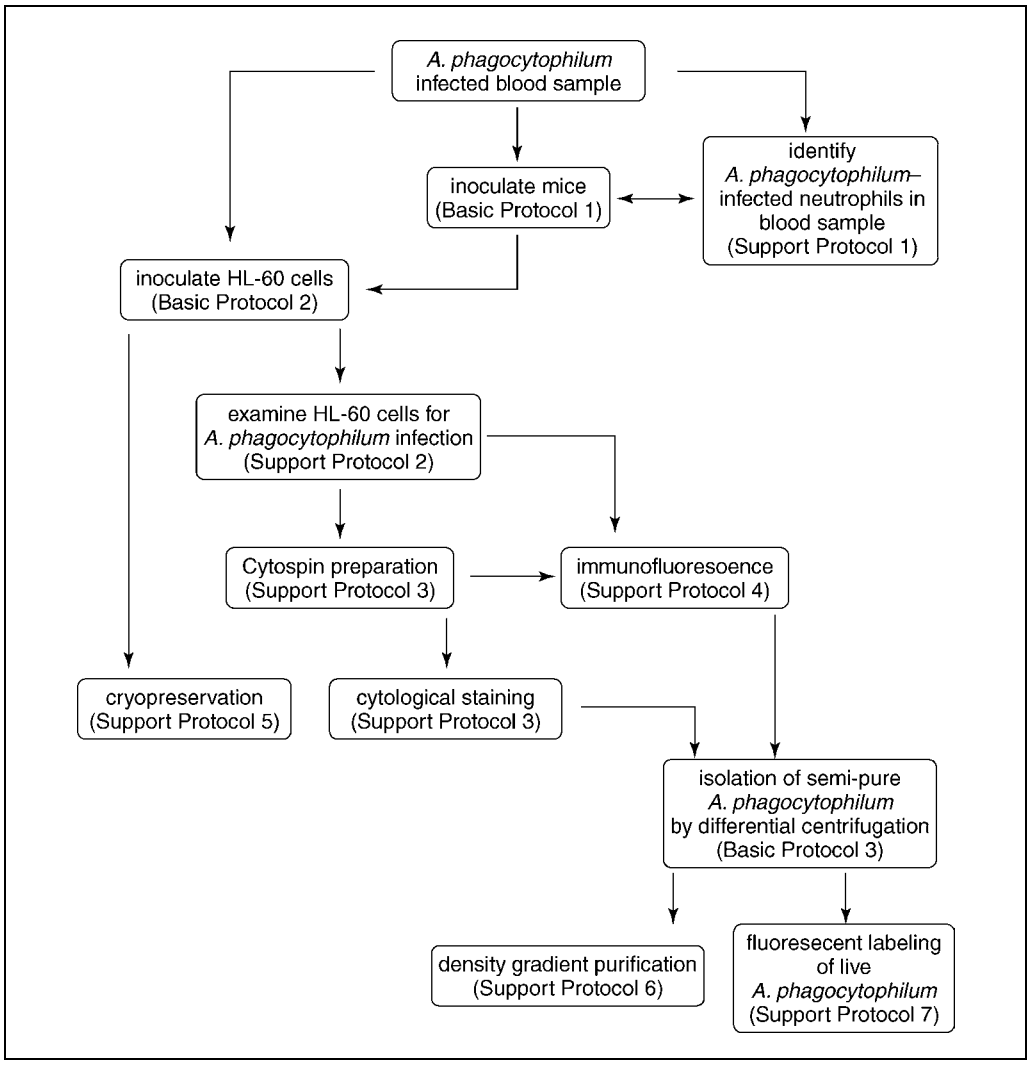

Figure 3A.2.1 Flowchart of protocols described in this unit.

BASIC PROTOCOL 1

INOCULATION OF *A. PHAGOCYTOPHILUM* INTO LABORATORY MICE

Prolonged culture of *A. phagocytophilum* in HL-60 cells results in attenuation of infectivity and pathogenicity of the bacterium. *A. phagocytophilum* infectivity can be maintained by mouse passage. Although it has been demonstrated that blood from infected immunocompetent mice can remain infectious for up to 60 days, the bacterial load in peripheral blood declines significantly by day 30. It is therefore recommended that an immunocompetent mouse remain infected for no longer than 30 days before passing an aliquot of its blood to a naive mouse. Pathogenicity, however, declines with continual passage in immunocompetent mice, but can be restored and successfully maintained by passage in SCID mice. Blood, either from an *A. phagocytophilum*–infected mouse or a patient suspected of having HGA that has yet to be treated with antibiotics, is inoculated into SCID mice. The bacterium is maintained in vivo by passage through SCID mice. *A. phagocytophilum* infection may be maintained in an individual SCID mouse for up to 2 months before being passed to naive SCID mice. For studies of *A. phagocytophilum* in an immunocompetent animal, *A. phagocytophilum*–infected SCID mouse blood is inoculated into C3H/HeN mice. Following inoculation of either SCID or immunocompetent mice, intracytoplasmic colonies of *A. phagocytophilum* are detectable in peripheral blood neutrophils by day 2. Infection is monitored by examination of peripheral blood smears.

While the protocol below calls for C3H/Smn.CicrHsd/*scid* or C3H/HeN mice, C3H/HeJ and their congenic C3H-scid counterparts may also be used. There is no observable difference in bacterial loads between the C3H/ HeN and C3H/ HeJ strains.

Materials

5% (v/v) Lysol
70% (v/v) ethanol
Mouse: C3H/Smn.CicrHsd/*scid* (SCID; 3 to 5 weeks old; Harlan Sprague-Dawley) *or* C3H/HeN (3 to 5 weeks old; National Cancer Institute Animal Production Program, Frederick Cancer Research Center)
Ketamine/xylazine anesthetic cocktail (see recipe) or other anesthetic agent as outlined in approved institutional protocol
Anticoagulant-treated, *A. phagocytophilum*–infected peripheral blood (either from an *A. phagocytophilum*–infected mouse or a HGA patient that has not yet received antibiotic therapy)
Class II, Type B2, vertical flow biological safety cabinet
1-cc syringes with 28-G, $\frac{1}{2}$-in. (12.7-mm) needles
1-cc tuberculin syringes with 26-G, $\frac{1}{2}$-in. (12.7-mm) needles
Gauze
Additional reagents and equipment for anesthesia (Donovan and Brown, 1998) and intraperitoneal injection (Donovan and Brown, 1995a) of mice

NOTE: The total blood volume of a mouse is ~5.5 ml per 100 g body weight. Do not collect more than 20% of total volume at one time. Do not collect more than this volume weekly. There is no limit to the number of times a mouse may be bled, provided that the aforementioned parameters are observed. For repeated blood collection via the retro-orbital route, alternate eyes should be used.

Anesthetize mice

1. Disinfect the work surfaces of a Class II, Type B2, vertical flow biological safety cabinet with 5% Lysol followed by 70% ethanol. Place a paper towel on the work surface of the biological safety cabinet.
2. Anesthetize the mouse (Donovan and Brown, 1998) by injecting 0.1 ml/20 g body weight of ketamine/xylazine anesthetic cocktail intramuscularly using a 1-cc tuberculin syringe with a 28-G, $\frac{1}{2}$-in. needle.

   This dosage should provide effective anesthesia for ~30 to 40 min.

   Once the mouse becomes unresponsive to a toe pinch, the procedure may be continued. If the mouse flinches or otherwise responds to the pinch, wait 5 min and check again. If the mouse still responds, administer an additional dose of 0.1 ml anesthetic cocktail/20 g body weight.
3. Transfer the anesthetized mouse to the biological safety cabinet, placing it on the paper towel covering the flat work surface.

Inoculate mice

4. In the biological safety cabinet, fill a 1-cc tuberculin syringe fitted with a 26-G, $\frac{1}{2}$-in. needle with 100 µl of *A. phagocytophilum*–infected blood.
5. While holding a mouse against the flat surface, slowly inject the 100 µl blood sample into the peritoneal cavity (Donovan and Brown, 1995a).
6. Carefully withdraw the needle and use gauze to apply digital pressure at the injection site to maintain hemostasis.

7. Place the injected mouse in a spare cage and continue injections with additional mice. When injections are finished, check the animals for normal behavior and return them to their normal housing location.

8. Remove unused materials from the biological safety cabinet. Disinfect the interior of the biosafety cabinet with 5% Lysol followed by 70% ethanol.

SUPPORT PROTOCOL 1

PREPARATION OF BLOOD SMEAR FOR IDENTIFYING *A. PHAGOCYTOPHILUM*–INFECTED NEUTROPHILS

Within neutrophils, *A. phagocytophilum* replicates in the confines of intracytoplasmic inclusions to form a cluster of organisms termed a morula. In the following protocol for the identification of *A. phagocytophilum*–infected neutrophils, blood from an infected mouse or HGA patient is smeared onto a glass slide and fixed. Morulae are subsequently identified using either light or immunofluorescent microscopy as discussed in Support Protocols 3 and 4, respectively. The day post-inoculation at which the blood sample is obtained will have bearing on one's ability to detect morulae-positive neutrophils. Following intraperitoneal inoculation of immunocompetent mice with *A. phagocytophilum*, morulae are evident in 2% to 4% of peripheral blood neutrophils on day 2. By days 7 to 8, which typically represent peak infection, morulae are detectable in 7% to 13% of neutrophils. By day 14, the peripheral blood infection wanes considerably, with the percentage of neutrophils with detectable morulae dropping to <2%. Often, morulae are no longer evident in peripheral blood smears by days 28 to 30. The kinetics of infection observed for immunocompetent mice are similar to those observed in human patients with HGA. The kinetics of infection for SCID mice are similar to those observed for immunocompetent mice for the first 7 to 8 days of infection, after which the percentage of infected peripheral blood neutrophils usually remains at 5% to 13% (for up to several weeks) until the mice are euthanized.

Materials

A. phagocytophilum–infected mice (see Basic Protocol 1)
Glass tubes coated with anticoagulant (e.g., EDTA, heparin)
Glass microscope slides with frosted ends
Additional reagents and equipment for blood collection from mice (Donovan and Brown, 1995b)

NOTE: A blood sample from a human patient suspected of having HGA who has yet to receive antibiotic therapy is processed in a manner identical to that described in steps 2 through 6.

1. Collect blood from *A. phagocytophilum*–infected mice (Donovan and Brown, 1995b) via retro-orbital bleed, saphenous vein puncture, or other approved technique and transfer to anticoagulant-treated glass tubes.

2. Place 1 to 2 μl of the anticoagulant-treated blood sample onto one end of a glass slide with frosted end.

3. Holding the slide steady with one hand, touch the end of another slide held at a 45° angle to the drop of blood.

   The blood will spread along the interface of the two slides.

4. Drag the angled slide along the surface of the held slide in a single sweeping motion. Repeat this process to generate at least 3 slides per blood sample.

   The desired result is to achieve a tongue-shaped smear of blood along the slide's surface. The vast majority of neutrophils and other leukocytes are present in the arch-shaped periphery ("tongue") of the smear.

5. Allow slides to air dry for at least 30 min at room temperature.
6. Stain and observe the slides for *A. phagocytophilum*–infected neutrophils as described in Support Protocol 3 or 4.

INOCULATION AND PASSAGING OF *A. PHAGOCYTOPHILUM* INFECTION IN HL-60 CELL CULTURE

BASIC PROTOCOL 2

The human-derived promyelocytic leukemia cell line HL-60 is the predominant choice for in vitro cultivation of *A. phagocytophilum*. Blood from either an *A. phagocytophilum*–infected mouse or a human patient suspected of having HGA that has yet to be treated with antibiotics is inoculated into HL-60 cells to generate a primary in vitro culture of *A. phagocytophilum*. The infected HL-60 cells are subsequently passaged to maintain proper cell density and supplemented with uninfected HL-60 cells to prevent lysis of the entire host cell population.

Materials

5% (v/v) Lysol
70% (v/v) ethanol
HL-60 cells (ATCC #CCL240) growing in culture
IMDM-10: Iscove's modified Dulbecco's medium (Invitrogen 12440-053) supplemented with 10% (v/v) heat-inactivated FBS
A. phagocytophilum–infected blood sample(s) collected in glass tubes coated with anticoagulant (e.g., EDTA, heparin)
Class II, Type B2, vertical flow biological safety cabinet
15-ml polypropylene conical centrifuge tubes with screw caps (BD Falcon)
25-cm^2 tissue culture flasks with vented caps (BD Falcon)
Additional reagents and equipment for counting cells using a hemacytometer (Strober, 1997a) and determining cell viability by trypan blue exclusion (Strober, 1997b)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Inoculate A. phagocytophilum–infected blood into HL-60 cell culture

1. Disinfect the work surfaces of a Class II, Type B2, vertical flow biological safety cabinet with 5% Lysol followed by 70% ethanol. Organize all materials to be used in the biological safety cabinet.

   CAUTION: *Steps 1 to 3 must be performed in the biological safety cabinet. When removing aliquots of uninfected or A. phagocytophilum–infected HL-60 cells from culture flasks in later steps, this must also be done in the biological safety cabinet.*

2. Aliquot 3 ml of HL-60 cells in IMDM-10 at a density of 500,000 per ml into a 15-ml conical polypropylene centrifuge tube. Perform this for each inoculum.

   HL-60 cells are nonadherent.

   RPMI 1640 medium supplemented with 10% FBS (RPMI-10) is equally suitable.

3. Add 100 µl of *A. phagocytophilum*–infected blood, pipet up and down to mix, and transfer the entire volume to a 25-cm^2 culture flask with a vented cap. Perform this for each inoculum.

   At least one flask of HL-60 cells should receive no inoculum, to serve as an uninfected control.

4. Incubate. Bi-weekly, examine the cultures as follows.

   a. Determine the culture densities by examining aliquots using a hemacytometer (Strober, 1997a) and light microscope. Maintain a density of 2–5 $\times$ 10^5 cells/ml by supplementing with fresh medium as needed.

   b. Determine the viability of the cells based on their ability to exclude trypan blue (Strober, 1997b).

      When visualized in the presence of trypan blue under light microscopy, uninfected HL-60 cells are typically round and have smooth borders. The membranes of infected cells, however, become more compromised as the infection progresses. They become permeable to trypan blue, and, as a result, are darker than uninfected cells. Also, their borders are rougher in appearance.

   c. Monitor cultures for infection load to make sure that the bacteria do not outgrow and completely lyse the HL-60 cells, by cytological or immunofluorescence staining as described in Support Protocols 2, 3, and/or 4.

      Morulae, or intracellular inclusions of A. phagocytophilum, should be apparent by ~5 days post-inoculation. The percentage of infected HL-60 cells will depend on the bacterial load of the inoculum.

Maintain A. phagocytophilum–infected HL-60 cells

5. After confirming the presence of *A. phagocytophilum*–infected HL-60 cells, expand the culture to a volume of 10 ml by adding fresh IMDM-10 medium to the flask.

6. Continue incubating. Bi-weekly, determine the cell density by examining aliquots using a hemacytometer. Feed cells by removing 6 to 8 ml of spent medium and supplementing with fresh IMDM-10 to achieve a density of 2 to 5 $\times$ 10^5 cells per ml, and add 0.5 to 1.0 $\times$ 10^6 uninfected HL-60 cells in 1 ml of medium. Determine the percentage of infected cells bi-weekly as described in Support Protocols 2, 3, and/or 4; if percentage of infected cells is <50%, do not add uninfected HL-60 cells.

   For example, if it is determined that the culture density has expanded to 1 $\times$ 10^6 cells/ml, one should remove and discard 9 ml of culture, corresponding to 9 $\times$ 10^6 cells. Remaining in the 25-cm^2 flasks will be 1 ml of A. phagocytophilium–infected culture, corresponding to 1 $\times$ 10^6 cells. It will then be necessary to add 1 $\times$ 10^6 uninfected HL-60 cells (as enumerated using a hemacytometer), suspended in medium, followed by the addition of fresh medium to a final volume of 10 ml, for a final culture density of 2–3 $\times$ 10^5 cells/ml.

SUPPORT PROTOCOL 2 EXAMINATION OF TISSUE CULTURE CELLS FOR *A. PHAGOCYTOPHILUM* INFECTION

Monitoring the percentage of infected HL-60 cells and determining the relative bacterial burden per cell is requisite for ensuring that the bacterial density is neither diluted too greatly by uninfected HL-60 cells nor outgrows and lyses the entire host cell population. Additionally, it is important to gauge the bacterial burden of a given culture prior to lysing it for recovering host cell–free *A. phagocytophilum*. Aliquots of infected HL-60 cells are centrifuged onto glass slides using a Cytospin cytocentrifuge (Thermo Electron). The slides can be examined for the presence of morulae (intracytoplasmic inclusions of *A. phagocytophilum*) using either cytological staining (see Support Protocol 3) or immunofluorescence (see Support Protocol 4).

Peripheral blood, either from an *A. phagocytophilum*–infected mouse or a patient suspected of having HGA that has yet to receive antibiotic therapy may be used in place of *A. phagocytophilum*–infected HL-60 cells in the protocol below. However, examination of peripheral blood using the smear method (Support Protocol 1) is preferable because of the rapidity and ease with which it can be performed.

Materials

5×10^5 cell/ml suspension of *A. phagocytophilum*–infected HL-60 cells in IMDM-10 (see Basic Protocol 2)
5×10^5 cell/ml suspension of uninfected HL-60 cells in IMDM-10 (control)
0.5% (v/v) sodium hypochlorite
Glass microscope slides with frosted ends
Cytocentrifuge setup (Thermo Electron Corp.) including:
Shandon Stainless Steel Cytoclip
Shandon TPX Filter Cards
Shandon TPX Sample Chamber
Shandon Cytospin 4 Cytocentrifuge
Absorbent bench paper with plastic backing

Prepare slides

1. Label the frosted portion of each slide with the appropriate sample designation. Use a Shandon Stainless Steel Cytoclip to secure a TPX Filter Card and TPX Sample Chamber to each slide, situating the filter card between the sample chamber and slide with the holes of the sample chamber and filter card aligned. Place the assemblies in the cytocentrifuge and balance the rotor properly.

   The use of a cytocentrifuge is recommended because the nuclei of HL-60 cells are quite large and can obscure detection of morulae. Centrifugation of HL-60 cells flattens them, which greatly facilitates viewing of their cytoplasmic contents.

2. Pipet 200 µl of a 5×10^5 cell/ml suspension of *A. phagocytophilum*–infected or uninfected (control) HL-60 cells in IMDM-10 into each sample chamber.

   Alternatively, 100 µl of peripheral blood to be examined for A. phagocytophilum may be added to the sample chamber.

3. Centrifuge the suspensions onto the slides for 2 min at $70 \times g$, using a Shandon Cytospin 4 Centrifuge.

4. Unclip the assembly, remove the sample chambers, and soak them in 0.5% sodium hypochlorite for ≥30 min. Afterwards, rinse the sample chambers in deionized water. Dispose of the filter cards into a biohazard-labeled trash receptacle.

   Disposable cytospin funnels are also available from Thermo Electron.

5. Place the slides at a 45° angle on a level surface covered with a sheet of absorbent bench paper with plastic backing. Allow to air dry at room temperature for at least 20 to 30 min, until completely dry.

Stain and examine slides for the presence of A. phagocytophilum

6. Stain and observe the slides for *A. phagocytophilum*–infected HL-60 cells as described in Support Protocol 3 or 4. Perform a minimum of three independent counts of at least 100 cells each, and determine the percentage of infected cells.

CYTOLOGICAL STAINING OF *A. PHAGOCYTOPHILUM*–INFECTED CELLS

SUPPORT PROTOCOL 3

Slides of *A. phagocytophilum*–infected and uninfected control cells are stained using the Protocol Hema-3 staining solutions, which are comparable to Wright and Giemsa stains. Peripheral blood smears (see Support Protocol 1) or cytospins of peripheral blood or HL-60 cells (see Support Protocol 2) may be used. Morulae, or intracytoplasmic colonies of *A. phagocytophilum*, are visualized by light microscopy as stippled vacuolar inclusions that stain more darkly than host cell nuclei. An advantage of this technique is the rapidity with which it can be performed. If a Cytospin cytocentrifuge is unavailable, then immunofluorescent screening (see Support Protocol 4) may be performed.

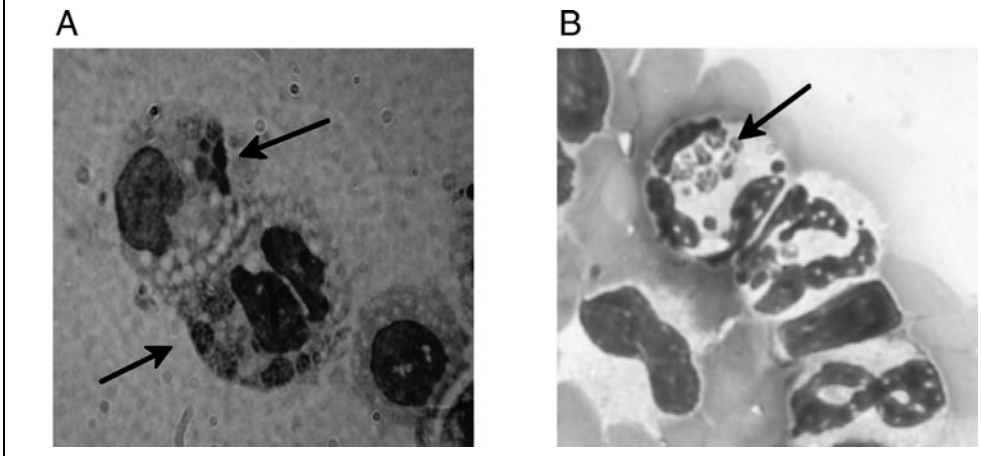

Figure 3A.2.2 Hema-3-stained *A. phagocytophilum*–infected cells visualized by light microscopy. (**A**) Retinoic acid–differentiated HL-60 cells. (**B**) Murine neutrophils. Morulae are denoted by arrows. Photo courtesy of Dr. Venetta Thomas, Yale University; modified from Carlyon and Fikrig (2004), with permission. This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*.

Materials

Protocol Hema-3 staining solutions (Fisher):
  Protocol Hema-3 fixative
  Protocol Hema-3 solution I
  Protocol Hema-3 solution II
Microscope slides with blood smear (see Support Protocol 1 or 2) or HL-60 cells (see Support Protocol 2)
Four glass staining dishes with removable racks (Wheaton model 900200)
Absorbent bench paper with plastic backing
Light microscope (*UNIT 2A.1*)

Stain slides

1. Fill three staining dishes, respectively, with Protocol Hema-3 fixative, staining solution I, and staining solution II, to a depth that will completely immerse the slides when placed in the dishes. Similarly fill a fourth staining dish with deionized water.
2. Place the slides in the staining rack. Immerse the staining rack in the fixative for 60 sec. Remove and allow for drainage of residual fixative.
3. Immerse the staining rack in solution I for 45 sec. Remove and allow for drainage of residual stain. Immerse briefly in deionized water, remove, and allow for drainage of residual water.
4. Immerse the staining rack in Solution II for 45 sec. Remove and allow for drainage of residual stain. Immerse briefly in deionized water, remove, and allow for drainage of residual water.
5. Place the slides on a sheet of absorbent bench paper placed on a level surface at a 45° angle to facilitate drainage of residual water. Allow the slides to air dry for 2 hr to overnight.

Examine slides

6. Observe the slides by light microscopy under oil immersion using the 100× objective. Look for neutrophils with the presence of morulae.

   Morulae are stippled in appearance and stain more darkly than the host cell's nucleus (Fig. 3A.2.2).

IMMUNOFLUORESCENT DETECTION OF *A. PHAGOCYTOPHILUM* IN INFECTED CELLS

SUPPORT PROTOCOL 4

This method is useful if one's laboratory does not possess a Cytospin cytocentrifuge, and therefore serves as an alternative to Support Protocol 3. Also, the increased sensitivity of immunofluorescence microscopy allows one to visualize individual organisms. This is an advantage over cytological staining (see Support Protocol 3), which is better suited for detecting clusters of *A. phagocytophilum*. Slides of *A. phagocytophilum*–infected cells are prepared by aliquotting the cells into wells of Teflon-coated immunofluorescence slides. Alternatively, if one so chooses, slides prepared using the Cytospin procedure (see Support Protocol 2) may be used. The slides are incubated with polyclonal antiserum against *A. phagocytophilum*, followed by incubation with fluorescein isothiocyanate (FITC)–conjugated secondary antibody. The slides are subsequently observed using a fluorescence microscope. Prepare antibodies during the blocking step.

Materials

5% (v/v) Lysol
70% (v/v) ethanol
A. phagocytophilum–infected HL-60 cells (see Basic Protocol 2)
Uninfected HL-60 cells (control)
Phosphate-buffered saline, pH 7.4 (PBS; *APPENDIX 2A*)
Acetone
PBS/1% and 5% BSA: phosphate-buffered saline, pH 7.4 (PBS; *APPENDIX 2A*) containing 1% or 5% (v/v) bovine serum albumin (BSA)
Polyclonal *A. phagocytophilum* antiserum (rabbit or murine; see Cooper and Patterson, 1995)
Fluorescein isothiocyanate (FITC)–conjugated secondary antibody: either goat anti-rabbit IgG (Sigma cat. no. F-6005) or goat anti-mouse IgG (Sigma cat. no. F-8264)
PBS-T: phosphate-buffered saline, pH 7.4 (PBS; *APPENDIX 2A*) containing 0.05% (v/v) Tween 20
20 μg/ml propidium iodide (light-sensitive; store up to 6 months at 4°C; optional)
Antifade gel-mounting medium (Biomeda Gelmount BMM-01)
Clear nail polish
12-well (5-mm diameter) Teflon-coated immunofluorescence slides (Carlson Scientific, cat. no. 101205) with coverslips
Class II, Type B2, vertical flow biological safety cabinet
Plastic, light-impermeable container large enough to accommodate slides
1-ml serological pipets
Forceps
Fluorescence microscope with filters for detecting FITC and rhodamine emission
Additional reagents and equipment for counting cells using a hemacytometer (Strober, 1997a)

NOTE: Propidium iodide staining (steps 30 to 31) is optional. Propidium iodide binds DNA, which results in the host cell nuclei staining red. This can facilitate easier identification of host cells though it is not necessary.

Prepare slides and safety cabinet

1. Label frosted ends of 12-well immunofluorescence slides with sample designations.
2. Disinfect the work surfaces of a Class II, Type B2, vertical flow biological safety cabinet with 5% Lysol followed by 70% ethanol.
3. In the biological safety cabinet, remove 100-µl aliquots from the *A. phagocytophilum*–infected HL-60 cultures to be examined and determine the cell density using a hemacytometer (Strober, 1997a).

Pellet and wash cells

4. Transfer 5×10^5 *A. phagocytophilum*–infected cells to a 1.5-ml microcentrifuge tube. Prepare another tube with the same number of uninfected control HL-60 cells.
5. Microcentrifuge cells 5 min at $210 \times g$, room temperature. Remove the supernatant. Gently resuspend the cells using 1 ml PBS. Microcentrifuge again as before, then remove the supernatant and gently resuspend the cells in 250 µl PBS for a final concentration of $\sim 2 \times 10^3$ cells/µl.

Transfer cell suspensions to immunofluorescence slides

6. Pipet 5 µl of the infected HL-60 cell suspension into the leftmost well of the top row (designated as well no. 1) of the immunofluorescence slide. Use the pipet tip to gently spread the suspension over the entire surface of the well.

   This will yield $\sim 1 \times 10^4$ cells in well no. 1.

7. Pipet 5 µl of PBS to the two wells adjacent to well no. 1 (i.e., wells no. 2 and 3).
8. Pipet 5 µl of infected HL-60 cells into well no. 2. Mix by pipetting to yield a two-fold dilution ($\sim 5 \times 10^3$ cells).
9. Transfer 5 µl of the two-fold dilution from well no. 2 to well no. 3 containing PBS. Mix by pipetting to yield a four-fold dilution ($\sim 2.5 \times 10^3$ cells).
10. Repeat steps 7 to 9 for wells nos. 4 to 6 and nos. 7 to 9 (see Fig. 3A.2.3).
11. Repeat steps 6 to 10 to prepare a control slide using uninfected HL-60 cells.
12. Allow the slides to air dry at room temperature for at least 30 min.

Prepare the humidified chamber

13. Place a paper towel in the bottom of a light-impermeable plastic container. Wet the towel with water.
14. Using a razor blade, section 1-ml serological pipet in lengths that will span the bottom of the container.
15. Place three to four pipet sections ~2 cm apart on top of the wetted paper towel in the bottom of the chamber.

    This chamber will be used in steps 20 to 31.

Fix and permeabilize the cells

16. Pour acetone into the staining jar to a depth that will immerse the slides.
17. Place the slides in the staining rack and immerse the rack in the acetone for 8 min at room temperature.

    If cytospin preparations are used, these must also be fixed with acetone.

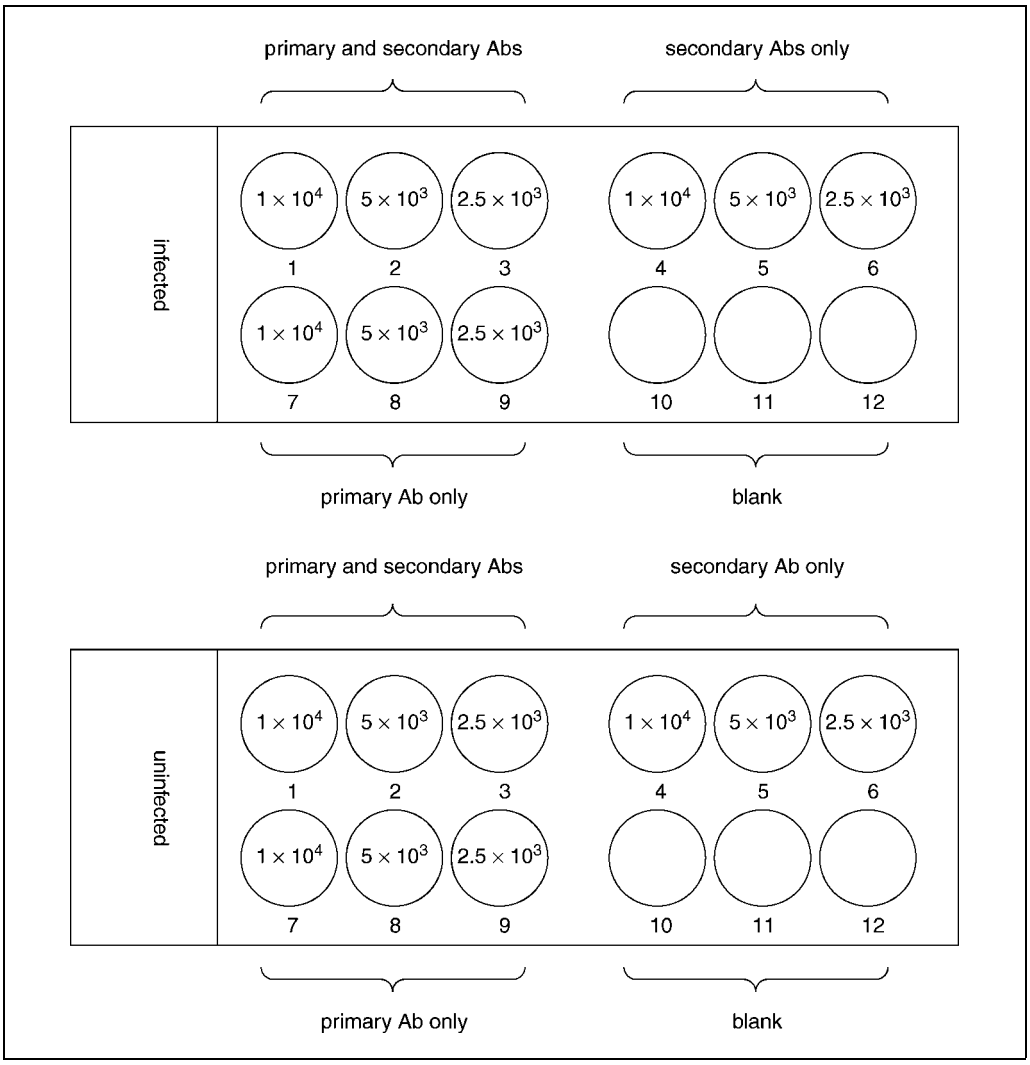

Figure 3A.2.3 Preparation of slides for immunofluorescent detection of *A. phagocytophilum*–infected HL-60 cells.

18. Using forceps, remove the slides and allow excess acetone to drain into the staining jar. Allow residual acetone to evaporate from the slides.

    This will take only a few seconds.

19a. *If slides are not to be screened immediately:* Place pairs of slides back-to-back and wrap in a Kimwipe. Wrap once more using aluminum foil. Label the foil with the sample designations and store up to 6 months (or possibly longer) at –20°C.

19b. *If slides are to be screened immediately:* Proceed to step 20.

Block nonspecific binding sites

20. If the slides were stored at –20°C, unwrap and allow them to sit at room temperature for 10 min. Place the slides on top of the rack of pipet sections in the humidified chamber (see step 15).

21. Add 5 µl PBS to each cell-containing well. Allow the cells to rehydrate for 5 min at room temperature.

    If screening Cytospin preparations, draw a circle around the border of the fixed cell sample using a Pap Pen (e.g., Electron Microscopy Sciences). This will create a barrier that will keep any solution pipetted onto the cells confined to that sample area, in a manner similar to the Teflon-coated wells of the immunofluorescence slides.

22. Remove PBS by aspiration. Add 5 µl PBS/5% BSA to each sample-containing well. Close the humidified chamber and incubate 1 hr at room temperature.

    This blocking step will reduce nonspecific binding of antibodies.

Prepare working stocks of antibodies

Because of the presence of BSA in the antibody solution, the antibodies should be diluted freshly at the time of the experiment and used the same day. However, if sodium azide is added to the solution to a final concentration of 0.1% (w/v), the antibodies can be stored up to 6 months at 4°C.

23. Dilute the polyclonal *A. phagocytophilum* antiserum to an appropriate working concentration in PBS/1% BSA.

    Usually, a 1:500 dilution will suffice. The titer of the antiserum will need to be determined beforehand.

24. Dilute the FITC-conjugated secondary antibody to 1:1000 in PBS/1% BSA. Store at 4°C protected from light until use.

Incubate slides with antibodies

25. Remove the PBS/5% BSA from each well (see step 22) by aspiration.

26. Add 5 µl of the working stock of polyclonal *A. phagocytophilum* antiserum (see step 23) to each sample-containing well. Close the humidified chamber and incubate at room temperature for ≥30 min.

27. Remove the primary antiserum by aspiration. Wash each well by adding 20 µl of PBS-T, then incubating 2 min at room temperature and removing the PBS-T by aspiration. Repeat this wash using PBS-T two more times.

28. Add 5 µl of the working stock of FITC-conjugated secondary antibody (see step 24) to each sample-containing well. Close the humidified chamber and incubate at room temperature for ≥30 min.

29. Wash each well three times as described in step 27.

Counterstain host-cell nuclei (optional)

30. Add 20 µl propidium iodide solution to each sample-containing well. Close the humidified chamber and incubate at room temperature for 3 min.

    CAUTION: *Propidium iodide is hazardous.*

31. Wash each well three times as described in step 27.

Mount and seal slides

32. Add three small drops of antifade gel-mounting medium along the length of the slide between the rows of wells. Place a coverslip on the slide and allow the medium to spread along the surface of the slide until all wells are covered. Gently press the surface of the coverslip to direct any air bubbles until they are released at the edge.

    Be sure to wear clean gloves for this step to avoid leaving any residue or smudges on the coverslip.

33. Allow the slide to dry at room temperature in the dark for 2 hr to overnight. Once the mounting medium has dried, seal the edges with clear nail polish.

Examine slides using fluorescence microscopy

34. Examine the slides using a fluorescent microscope equipped with a filter for detecting FITC emission and either the 40× or 100× objective to visualize *A. phagocytophilum*.

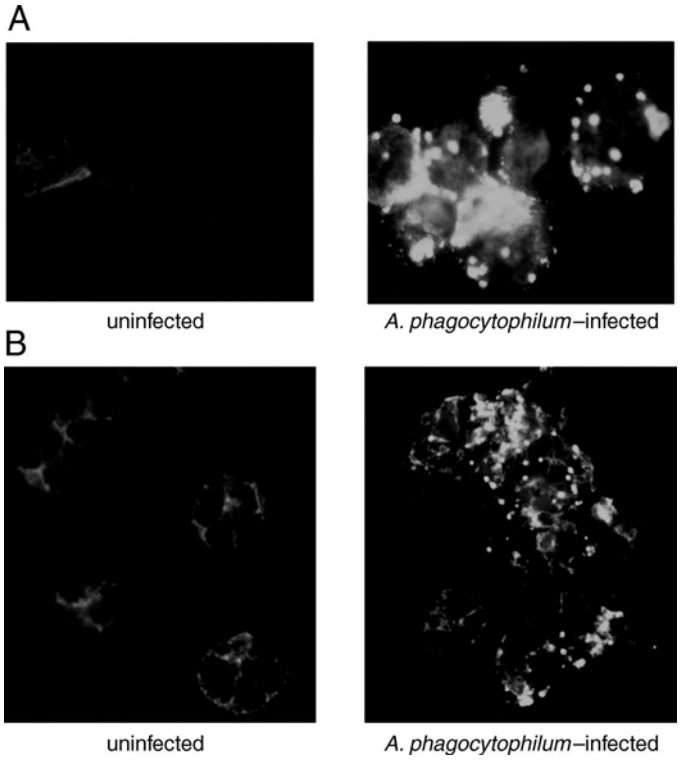

Figure 3A.2.4 *A. phagocytophilum*–infected cells visualized by immunofluorescent microscopy. All samples were incubated with polyclonal rabbit anti–*A. phagocytophilum* serum followed by FITC-conjugated goat anti-rabbit IgG. (**A**) Uninfected and *A. phagocytophilum*–infected HL-60 cells. (**B**) Uninfected and *A. phagocytophilum*–infected human neutrophils. Nuclei were counterstained using propidium iodide. This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*.

35. Visualize propidium iodide–stained HL-60 nuclei by fluorescence microscopy using a filter for detecting rhodamine emission. Representative slides are presented in Figure 3A.2.4.

CRYOPRESERVATION OF *A. PHAGOCYTOPHILUM*–INFECTED HL-60 CELLS

SUPPORT PROTOCOL 5

It is good practice to maintain frozen stocks of the cell lines. To protect against complete loss of cell lines due to accidental contamination or other incidents, stocks of uninfected and *A. phagocytophilum*–infected HL-60 cells should be stored. The cells are pelleted, resuspended in medium containing DMSO, frozen in an isopropanol bath at −80°C, and transferred to liquid nitrogen for long-term storage.

Materials

5% (v/v) Lysol
70% (v/v) ethanol
A. phagocytophilum–infected HL-60 cells growing in culture (see Basic Protocol 2)
100% isopropanol
IMDM-10 with and without 12% (v/v) dimethylsulfoxide (DMSO)
0.5% (v/v) sodium hypochlorite

Class II, Type B2, vertical flow biological safety cabinet
10- and 1-ml serological pipets

15-ml polypropylene conical centrifuge tubes with screw caps (BD Falcon)
Refrigerated tabletop centrifuge
Alcohol-resistant pen (VWR)
250-ml beaker for waste
2-ml screw-cap cryovials
Nalgene Cryo 1°C freezing container
−80°C freezer
Cryobiological storage vessel containing liquid nitrogen
25-cm^2 tissue culture flasks with vented caps (BD Falcon)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Pellet cells

1. Disinfect the work surfaces of a Class II, Type B2, vertical flow biological safety cabinet with 5% Lysol followed by 70% ethanol.
2. Using a serological pipet, transfer 1×10^7 of *A. phagocytophilum*–infected HL-60 cells to a 15-ml conical centrifuge tube.

   Uninfected HL-60 cells are processed in an identical manner.

3. Place the tube in a refrigerated tabletop centrifuge. Centrifuge cells 10 min at $210 \times g$, 4°C. During the centrifugation, label a screw-cap cryovial with the sample designation using an alcohol-resistant pen. Discard supernatant in a 250-ml beaker designated for waste.

   Be sure that the centrifuge is properly balanced.

Prepare the Cryo 1°C freezing container

4. Remove the polyethylene vial holder and foam insert from the freezing container.

   Do not discard the foam insert.

5. Add 100% isopropanol to the fill line of the container. Replace the foam insert and vial holder.

   This will require a volume of ∼250 ml. Do not overfill.

Freeze cells

6. Using a 1-ml serological pipet, gently resuspend the cells from step 3 in 1 ml IMDM-10 containing 12% DMSO. Immediately transfer the suspension to the corresponding labeled, 2-ml screw-cap cryovial. Close the cap.

   DMSO serves as a cryopreservative, which enables the HL-60 cells to survive the freezing process.

7. Immediately transfer the tube to a hole in the vial holder of a Nalgene the Cryo 1°C freezing container. Place the Cryo 1°C freezing container in a –80°C freezer. Leave undisturbed for a minimum of 4 hr.

   This achieves a controlled cooling rate of −1°C per minute, which is recommended for eukaryotic cell lines.

   Cells should be stored for no more than 1 week at –80°C before transferring to liquid nitrogen. Longer storage at the lower temperature will compromise the viability of the culture upon thawing. A temperature of –130°C or below is necessary for long-term storage.

8. After a period of at least 4 hr, remove the frozen vial from the freezing container and place in a cryobiological storage vessel containing liquid nitrogen for long-term storage.

   The long-term storage vessel should have a temperature of –130°C or below. Cells may be stored indefinitely.

Thaw cells

9. Remove the frozen sample from storage and place on ice to thaw slowly.

10. Using a 1-ml serological pipet, gently resuspend and transfer the tube's contents to a 15-ml conical centrifuge tube.

Wash cells

11. Using a 10-ml serological pipet, immediately add 10 ml of IMDM-10.

    DMSO will cause terminal differentiation of HL-60 cells. It is therefore important to thoroughly wash the cells immediately upon thawing to remove all traces of DMSO.

12. Centrifuge the tube as in step 3, then remove and discard the supernatant in the waste beaker.

13. Repeat steps 11 to 12 two additional times.

14. Resuspend cells in 10 ml IMDM-10 and transfer to a 25-cm^2 tissue culture flask with a vented cap.

15. Place newly resuspended culture in incubator and continue culturing as described in Basic Protocol 2.

Decontaminate and clean up work area

16. Add 10 ml of 0.5% sodium hypochlorite to the beaker containing the pooled supernatants. Wait 10 min, then pour the disinfected solution into a sink and flush with running water for 30 sec.

17. Remove unused materials from the biological safety cabinet. Disinfect the interior of the biosafety cabinet with 5% Lysol followed by 70% ethanol.

OBTAINING HOST CELL–FREE *A. PHAGOCYTOPHILUM* BY DIFFERENTIAL CENTRIFUGATION

BASIC PROTOCOL 3

Preparations of *A. phagocytophilum* that are relatively free from contaminating HL-60 cell components are used for a variety of in vitro applications. *A. phagocytophilum*–infected HL-60 cells are lysed via syringe passage, and a semipure preparation of viable bacteria is recovered by differential centrifugation. These preparations can be used in subsequent in vitro infection assays, inoculated into mice (see Basic Protocol 1), subjected to further purification by density gradient centrifugation (see Support Protocol 6), or fluorescently labeled for tracking infection (Support Protocol 7).

Materials

5% (v/v) Lysol
70% (v/v) ethanol
A. phagocytophilum–infected HL-60 cells growing in culture (see Basic Protocol 2)
Uninfected HL-60 cells (control)
Phosphate-buffered saline, pH 7.4 (PBS; *APPENDIX 2A*), prechilled
0.5% (v/v) sodium hypochlorite

Class II, Type B2, vertical flow biological safety cabinet
25-, 10-, and 5-ml serological pipets

15- and 50-ml polypropylene conical centrifuge tubes with screw caps (BD Falcon)
Refrigerated tabletop centrifuge
500-ml beaker for waste
5-cc tuberculin syringes
27-G, $\frac{1}{2}$-in. (12.7-mm) needles
Additional reagents and equipment for counting cells (Strober, 1997a) and cytological (see Support Protocol 3) or immunofluorescence staining (see Support Protocol 4) of *A. phagocytophilum* cultures

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

Determine the cell density and percentage of A. phagocytophilum–infected HL-60 cells

1. Disinfect the work surfaces of a Class II, Type B2, vertical flow biological safety cabinet with 5% Lysol followed by 70% ethanol. Organize all materials to be used in the cabinet.

   With the exception of the centrifugation steps, all work is performed in the hood.

2. Determine cell density of cultures of interest using a hemacytometer (Strober, 1997a).

3. Determine the percentage of infected cells in the cultures of interest using either cytological staining or immunofluorescence microscopy as described in Support Protocol 3 or Support Protocol 4, respectively.

   The percentage of infected cells should be ≥70%. If the percentage of infected cells is ≤70%, return the culture to the incubator and allow the infection to proceed for an additional 1 to 2 days before reassessing it for infection. If necessary, adjust the density of the culture to 2–5 × 10^5 cells per ml by supplementing with fresh medium as described in Basic Protocol 2, step 6.

Pellet cells

4. Using a 10- or 25-ml serological pipet, transfer the appropriate number of *A. phagocytophilum*–infected cells in culture medium to a 50-ml conical centrifuge tube. Prepare a separate tube in a similar manner using uninfected HL-60 cells and carry through the following steps in parallel as a control.

   The number of infected cells can be scaled according to the purpose of the experiment. For instance, if the resulting semipure preparation of A. phagocytophilum is to be further purified by density gradient centrifugation (see Support Protocol 6), pellet 2.5 × 10^8 cells. If using the A. phagocytophilum preparation in an in vitro infection assay, pellet a number of infected cells equal to 1 to 5 times the number of cells to be infected. If host cell–free A. phagocytophilum recovered from lysed HL-60 cells are to be used in subsequent infection assays, it is imperative that uninfected HL-60 cells be processed in an identical manner and used as a control in the subsequent infection assay. This is essential for determining whether results of the infection assay are influenced by the presence of contaminating HL-60 lysate components present in the A. phagocytophilum preparation.

5. Place the 50-ml tubes in a refrigerated tabletop centrifuge. Centrifuge the cells 10 min at 2300 × *g*, 4°C. Discard supernatant in a 500-ml beaker designated for waste.

   Be sure the tubes are properly balanced.

6. Resuspend the pellet in 4 ml chilled PBS.

7. Place a 50-ml conical centrifuge tube upright in a rack. Remove and place the cap to the side.

Lyse cells

8. Remove the plungers from five 5-cc syringes (designated nos. 1 to 5). Attach a 27-G, $\frac{1}{2}$-in needle to each.

   A set of five syringes should also be prepared for the uninfected HL-60 cells.

9. Using a 5-ml serological pipet, transfer the cell suspension to syringe no. 1.

10. Uncap the needle of syringe no. 1 and, using constant pressure, expel the suspension through the needle into the 50-ml tube (see step 7).

    For maximum recovery of the suspension, hold the syringe at a 45^o angle and place the end of the needle as close to the inner wall of the tube as possible without touching. This will allow the suspension to run down the inner wall to the tube bottom and will minimize the chance of producing an aerosol.

11. Discard syringe no. 1 into a sharps container. Repeat steps 9 and 10 four times.

    This process lyses the HL-60 cells without lysing the bacteria.

Recover semipure preparation of host cell–free A. phagocytophilum

12. Cap the 50-ml tubes containing the lysates of *A. phagocytophilum*–infected and uninfected cells. Centrifuge 5 min at 750 × *g*, 4°C.

    This will pellet the majority of host cellular debris.

13. Transfer the supernatants to new 15-ml centrifuge tubes. Discard the tubes containing the pellets of cellular debris into a biohazard-labeled trash receptacle.

14. Centrifuge the tubes containing the supernatants from step 13 for 5 min at 1000 × *g*, 4°C.

    A pellet of considerably smaller size than that generated in step 12 should be visible.

15. Transfer the supernatants to new 15-ml tubes. Discard the tubes containing the pellets of cellular debris into a biohazard-labeled trash receptacle.

16. Centrifuge the tubes containing the supernatants from step 15 for 10 min at 2300 × *g*, 4°C. Remove and discard the supernatant into the beaker designated for waste.

    A small pellet should be visible. The majority of the pellet is composed of host-cell-free A. phagocytophilum. A minor portion of the pellet consists of HL-60 debris and cellular components that were not removed in steps 12 and 14.

Decontaminate and clean up work area

17. Add 10 ml of 0.5% sodium hypochlorite to the beaker containing the pooled supernatants. Wait 10 min, then pour the disinfected solution into a sink and flush with running water for 30 sec.

18. Remove unused materials from the biological safety cabinet. Disinfect the interior of the biosafety cabinet with 5% Lysol followed by 70% ethanol.

DENSITY GRADIENT PURIFICATION OF HOST CELL–FREE *A. PHAGOCYTOPHILUM*

SUPPORT PROTOCOL 6

This method is useful for generating *A. phagocytophilum* preparations that are more highly purified from contaminating host cell components than those prepared by differential centrifugation. Such preparations are used for inoculating animals with the purpose of obtaining anti–*A. phagocytophilum* antiserum that exhibits little to no recognition of HL-60 cells. Alternatively, such preparations are used for subsequent isolation of *A. phagocytophilum* nucleic acids, proteins, or outer membranes. It is best that contaminating host cellular components be minimized for such procedures. An *A. phagocytophilum*

preparation obtained via differential centrifugation (see Basic Protocol 3) is layered onto a discontinuous Renografin gradient and subjected to ultracentrifugation. This results in the resolution of a band of *A. phagocytophilum* that is free from host cell components, with the exception of host cell organelles of a similar density, such as mitochondria.

Materials

5% (v/v) Lysol
70% (v/v) ethanol
A. phagocytophilum–infected HL-60 cells growing in culture (see Basic Protocol 2)
PBS/glucose (see recipe), ice cold
Complete Protease Inhibitor Cocktail Tablets (Roche, cat. no. 1 697 498)
DNase I (from bovine pancreas; Roche cat. no. 776785)
RNase (from bovine pancreas; Roche cat. no. 1 119 915)
30% Renografin (see recipe)
42% Renografin (see recipe)
SPGN buffer (see recipe)
0.5% (v/v) sodium hypochlorite

Class II, Type B2, vertical flow biological safety cabinet
50-ml polypropylene conical centrifuge tubes with screw caps (BD Falcon)
Refrigerated tabletop centrifuge
1-liter beaker for waste
10-cc tuberculin syringe
Beckman L7-55 ultracentrifuge and Beckman SW 28 swinging-bucket rotor (or equivalent)
38.5-ml ultracentrifuge tubes (Beckman Ultra-Clear)
Harvard trip balance
2.0-ml polypropylene microcentrifuge tubes

Additional reagents and equipment for counting cells (Strober, 1997a), cytological (see Support Protocol 3) or immunofluorescence staining (see Support Protocol 4) of *A. phagocytophilum* cultures, preparing a semipure preparation of *A. phagocytophilum* (see Basic Protocol 3), and protein assay (*APPENDIX 3A*)

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

Determine the cell density and percentage of A. phagocytophilum–infected HL-60 cells.

1. Disinfect the work surfaces of a Class II, Type B2, vertical flow biological safety cabinet with 5% Lysol followed by 70% ethanol.

   With the exception of the steps involving centrifugation and water bath incubation, this work is to be performed in the biological safety cabinet. This is especially important if the purified A. phagocytophilum is to be injected into animals.

2. Determine cell density of cultures of interest by examining aliquots using a hemacytometer (Strober, 1997a).

3. Determine the percentage of infected cells in the cultures of interest as described in Support Protocol 3 or Support Protocol 4.

   The percentage of infected cells should be ≥70%.

4. For each Renografin prep to be prepared, place 2.5×10^8 infected HL-60 cells in a (depending on the culture density) of 50-ml conical centrifuge tube.

5. Centrifuge the tubes in a refrigerated tabletop centrifuge 10 min at 2300 × *g*, 4°C.

   The number of processed cells can be scaled according to the purpose of the experiment. As a general rule, process 2.5 × 10^8 cells per Renografin prep.

Lyse the cells

6. While the centrifugation in step 5 is proceeding, transfer 25 ml ice-cold PBS-glucose to a 50-ml conical centrifuge tube. Add 1 Complete Protease Inhibitor Cocktail tablet and vortex to mix. Place the solution on ice.

   Other protease inhibitors may be substituted. However, the author has achieved excellent results with the Complete tablets from Roche.

7. Discard supernatant from step 4 into a 1-liter beaker designated for waste.

8. Mechanically lyse the infected cells and recover a semipure preparation of *A. phagocytophilum* as described in Basic Protocol 3, steps 8 to 16, except use 10-cc syringes in the lysis steps.

Digest host cell nucleic acids

9. Resuspend the pellet in 10 ml ice-cold PBS-glucose with added protease inhibitors (prepared at step 6).

10. Add DNase I and RNase to final concentrations of 50 μg/ml each.

11. Place the tube containing the suspension in a water bath set at 37°C. Incubate 45 min.

    This digests host cell nucleic acids, which reduces the viscosity of the solution.

Prechill rotor and ultracentrifuge

12. Chill the SW 28 rotor and swinging buckets at 4°C. Turn the power switch of the ultracentrifuge to ON. Close the rotor chamber door and turn the vacuum switch to ON. Set the temperature to 4°C.

    Any refrigerated ultracentrifuge and swinging-bucket rotor capable of achieving speeds of at least 87,300 × g may be used.

Prepare discontinuous Renografin gradient

13. Place 10 ml of 42% Renografin in a 38.5-ml ultracentrifuge tube.

14. Gently pipet 10 ml of 30% Renografin over the 42% Renografin layer. If not processing an even number of samples, prepare an identical gradient to use as a balance during centrifugation.

    To avoid mixing the Renografin layers, hold the pipet at a 45° angle to the ultracentrifuge tube and position the tip of the pipet at the inner wall of the tube. This allows the 30% Renografin to settle on top of the 42% layer.

15. Following the nucleic acid digestion in step 11, chill the lysate on ice for 10 min.

16. Using a serological pipet, overlay the lysate on the Renografin gradient. If processing an odd number of samples, overlay the gradient in the balance tube with deionized water.

Recover host cell–free A. phagocytophilum

17. Place the experimental and balance tubes in the SW 28 buckets. Make sure that the combined weight of the balance tube and swinging bucket is equal to the combined weight of the swinging bucket and experimental tube using a Harvard trip balance. If not, adjust the amount in the balance tube.

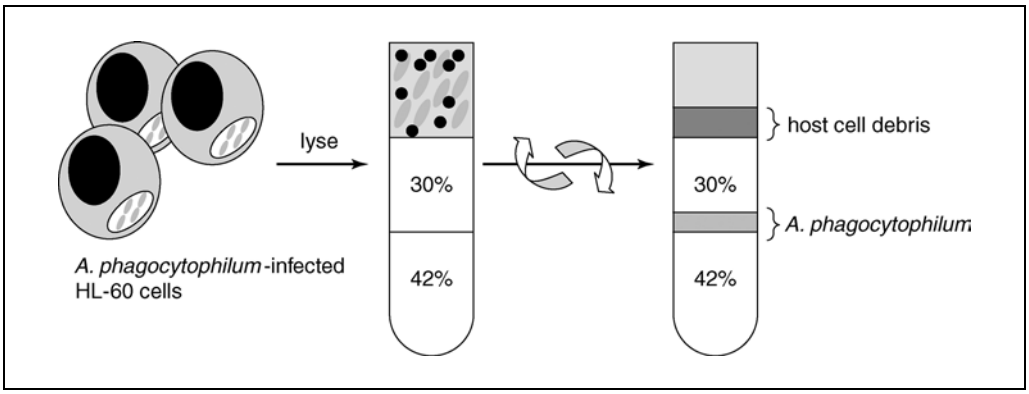

Figure 3A.2.5 Renografin density gradient purification of host cell–free *A. phagocytophilum*.

18. Screw the caps onto all six swinging buckets. Hang each of them at its matched position on the SW 28 rotor.

    CAUTION: *Because of the high speeds at which they are used, ultracentrifuge rotors must be precisely balanced. The SW 28 buckets are numbered 1 through 6. When hanging on the rotor, 1 must lie opposite 4, 2 must lie opposite 5, and 3 must lie opposite 6. Keep this in mind when choosing the swinging buckets.*

19. Turn the vacuum switch to OFF and wait for the rotor chamber to equilibrate. Open the rotor chamber door and place the SW 28 rotor onto the drive spindle. Close the rotor chamber door and turn the vacuum switch to ON. Enter a speed of 22,000 rpm (87,300 × *g*). Enter a run time of 75 min. Leave the temperature setting at 4°C. Set the brake at 800 rpm (115 × *g*).

    The brake switch of the Beckman L7-55 allows either for full dynamic braking until 0 rpm or for full dynamic braking until 800 rpm followed by no braking to a gentle stop. Allowing the rotor to come to a gentle stop allows for minimal disruption of the resulting centrifugation layers. Newer ultracentrifuge models have the option of no brake. Choose this option if using a newer model. However, keep in mind that it will take considerably longer for the rotor to come to a full stop without braking.

20. Press START and allow the centrifugation to proceed.

21. Once the rotor has stopped, turn the vacuum switch to OFF and allow the rotor chamber to equilibrate.

22. Open the chamber door, remove the rotor, and return it to its support stand. Remove the swinging buckets and return them to their rack.

Recover A. phagocytophilum–enriched fraction

23. Unscrew the swinging-bucket caps and remove the ultracentrifuge tubes containing the samples.

    When observing the tubes, three distinct layers of ~10 ml each separated by two distinct bands should be readily apparent (Fig. 3A.2.5). A pellet will also be present at the bottom of the tube.

    The band atop the 30% Renografin layer consists primarily of host cellular debris. The pellet primarily consists of unbroken HL-60 cells. The band between the 30% and 42% Renografin layers is highly enriched for A. phagocytophilum. Likely, host cellular components of similar size and density to that of A. phagocytophilum, such as mitochondria, are present. These, however, constitute a very minor portion.

24. Using a serological pipet, carefully remove the top 18 to 19 ml of the Renografin gradient and discard into the beaker designated for waste. Be sure to leave ~1.5 cm of Renografin above the enriched *A. phagocytophilum* layer.

Wash, pool, and store the A. phagocytophilum–enriched fraction

25. Place several opened 2.0-ml microcentrifuge tubes on ice. Using a serological pipet, remove the entire *A. phagocytophilum*–enriched layer and distribute among the 2.0-ml tubes by pipetting 1 ml into each.

26. Add 1 ml SPGN buffer to each tube. Close the caps. Invert the tubes to mix and to dilute the Renografin.

27. Place the tubes in a prechilled refrigerated microcentrifuge. Microcentrifuge 20 min at 13,000 × *g*, 4°C. Remove and discard the supernatants into the waste beaker. Combine the pellets in a final volume of 500 to 600 μl SPGN buffer.

28. Measure the protein concentration of the enriched *A. phagocytophilum* fraction using the method of choice (*APPENDIX 3A*). Adjust the final concentration to 2 mg/ml by adding SPGN buffer. Store indefinitely at –80°C.

Decontaminate and clean up work area

29. Add 10 ml of 0.5% sodium hypochlorite to the beaker containing the pooled supernatants. Wait 10 min, then pour the disinfected solution into a sink and flush with running water for 30 sec.

30. Remove unused materials from the biological safety cabinet. Disinfect the interior of the biosafety cabinet with 5% Lysol followed by 70% ethanol.

FLUORESCENT LABELING OF LIVE *A. PHAGOCYTOPHILUM*

SUPPORT PROTOCOL 7

This technique enables one to infect and subsequently identify *A. phagocytophilum*–infected cells based on detection of fluorescent bacteria. The difference between this technique and immunofluorescence (see Support Protocol 4), which detects bacteria in fixed host cells, is that this technique utilizes live bacteria and enables one to trace an ongoing infection in live cells. Labeled bacteria will remain fluorescent for up to 4 days. Host cell–free *A. phagocytophilum* preparations obtained via differential centrifugation (see Basic Protocol 3) are incubated with CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA), a vital dye available from Molecular Probes. CellTracker Green CMFDA will not label dead cells or host cell debris.

Materials

Semipure preparation of host cell–free *A. phagocytophilum* (see Basic Protocol 3)
Phosphate-buffered saline, pH, pH 7.4 (PBS; *APPENDIX 2A*), chilled
10 μM CellTracker Green CMFDA (see recipe)
250-ml beaker for waste
Fluorescence microscope with filters for detecting FITC emission

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic technique should be used accordingly.

Wash host cell–free A. phagocytophilum

1. Resuspend pellet of semipure host cell–free *A. phagocytophilum* in 2 ml chilled PBS.

2. Pellet the bacteria in a refrigerated microcentrifuge 10 min at 2300 × *g*, 4°C.

3. Remove and dispose of the supernatant into a 250-ml beaker designated for waste.

4. Repeat steps 1 to 3.

Thorough washing of the A. phagocytophilum is pertinent because any residual serum remaining from the culture medium will inactivate CellTracker Green CMFDA.

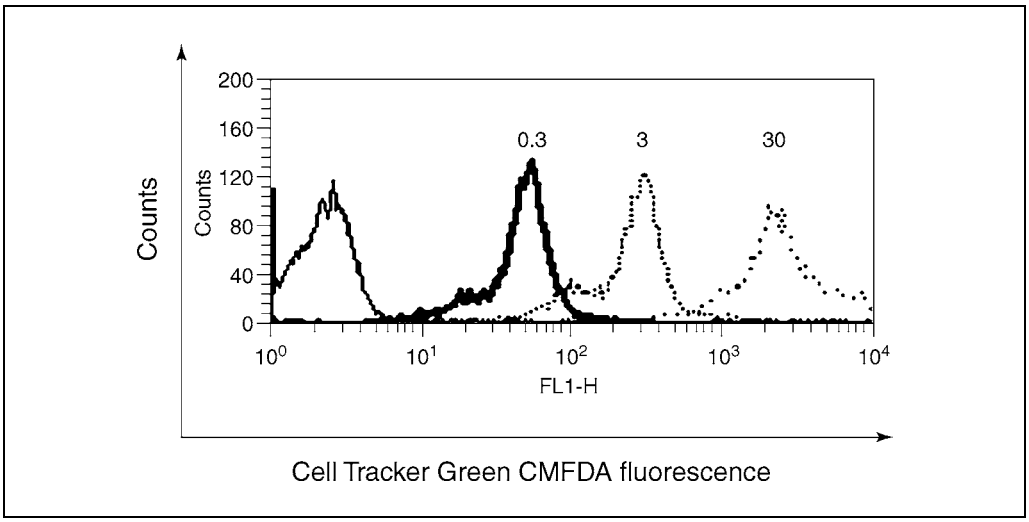

Figure 3A.2.6 Flow cytometric analysis of human neutrophils incubated with CellTracker Green CMFDA–labeled *A. phagocytophilum*. Bacteria were incubated with neutrophils for 60 min at ratios of 0.3 (solid, thick histogram), 3 (dashed histogram), and 30 (dotted histogram) *A. phagocytophilum* organisms per cell. Uninfected neutrophils (solid, thin histogram) served as a negative control.

Label A. phagocytophilum

5. Prewarm the 10 μM working solution of CellTracker Green CMDFA in a 37°C water bath for at least 10 min.

   CellTracker Green CMFDA is light-sensitive; protect the solution from light.

6. Resuspend the *A. phagocytophilum* pellet from step 4 using 1 ml of the prewarmed 10 μM CellTracker Green CMFDA.

7. Close the lid of the tube and seal with Parafilm. Place in the 37°C water bath and incubate 15 min.

Wash labeled A. phagocytophilum

8. Remove the Parafilm. Add 1 ml PBS. Mix by inversion.

9. Pellet the labeled bacteria in a refrigerated microcentrifuge at 2300 × *g* for 10 min at 4°C. Remove and discard the supernatant into the waste beaker.

10. Add 2 ml of PBS and wash the bacteria once more as in steps 8 to 9.

    It is very important to remove any unincorporated CellTracker Green CMDFA, as any remaining dye will label eukaryotic cells when incubated with the A. phagocytophilum preparation.

11. Dilute the labeled bacteria to the desired concentration and incubate with host cells of choice. Infection can be monitored either by flow cytometry (Fig. 3A.2.6) or fluorescent microscopy using a filter equipped for observing FITC fluorescence.

    Labeled bacteria should be incubated with recipient host cells for ≥30 min.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CellTracker Green CMFDA, 10 μM

Prepare a 10 mM stock of CellTracker Green by resuspending 50 μg lyophilized CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Molecular

Probes cat. no. C7025) in 10.8 μl DMSO. Prepare a 10 μM working stock of CellTracker Green CMFDA by adding 1 μl of 10 mM CellTracker Green CMFDA to 1 ml PBS, pH 7.4 (*APPENDIX 2A*), in a sterile microcentrifuge tube.

NOTE: *CellTracker Green CMFDA is sold as a set of twenty individual tubes of 50 μg. Thus, resuspend the entire contents of one tube with 10.8 μl DMSO. CellTracker Green CMFDA is light-sensitive and should therefore be protected from light.*

Ketamine/xylazine anesthetic cocktail

10 ml 100 mg/ml ketamine in sterile 0.9% saline (see *APPENDIX 2A* for saline)
1.5 ml 100 mg/ml xylazine in sterile 0.9% saline (see *APPENDIX 2A* for saline)
After the ketamine and xylazine are combined in a final volume of 11.5 ml, dilute 1:4 in sterile saline (*APPENDIX 2A*) and inject according to body weight as directed in the protocol.

PBS/glucose

0.5 g glucose
250 ml phosphate-buffered saline, pH 7.4 (PBS; *APPENDIX 2A*)
Dissolve glucose in PBS and sterilize using a 0.2-μm filter.
Store up to 2 months at 4°C

Renografin, 30%

125 ml 60% Renografin (Renografin-60; Bracco Diagnostics)
125 ml Renografin buffer (see recipe)
Filter sterilize through a 0.2-μm filter
Store up to 6 months at 4°C

Renografin, 42%

175 ml of 60% Renografin
75 ml Renografin buffer (see recipe)
Filter sterilize through a 0.2-μm filter
Store up to 6 months at 4°C

Renografin buffer

Dissolve the following in 450 ml H_2O
0.163 g KH_2PO_4
0.58 g Na_2HPO_4
3.39 g NaCl
12.5 ml of 200 mM L-glutamine
Adjust pH to 7 using 0.1 N HCl
Bring final volume to 500 ml with H_2O
Filter sterilize through a 0.2-μm filter
Store up to 6 months at 4°C

SPGN buffer

Dissolve the following in 450 ml H_2O
37.3 g sucrose
0.25 g KH_2PO_4
0.80 g K_2HPO_4
12.5 ml of 200 mM L-glutamine
Adjust pH to 7 using 0.1 N HCl
Bring final volume to 500 ml with H_2O
filter sterilize through a 0.2-μm filter
Store for up to 2 months at 4°C

COMMENTARY

Background Information

A. phagocytophilum is the etiologic agent of human granulocytic anaplasmosis (HGA), a potentially fatal disease that is emerging in the United States, Europe, and Asia (for recent reviews see Dumler and Bakken, 1998; Carlyon and Fikrig, 2003; Rikihisa, 2003; Carlyon and Fikrig, 2004). In the U.S., cases of HGA occur primarily in the Northeast, the Upper Midwest, and the West Coast. The incidence of HGA in these areas ranges from ~2 to ≥70 cases per 100,000 persons. *A. phagocytophilum* is a highly unique intracellular bacterium in that it preferentially colonizes neutrophils in mammalian hosts. Within neutrophils, the obligate intracellular bacterium replicates as clusters of organisms, forming intracytoplasmic inclusions termed morulae. In nature, *A. phagocytophilum* cycles between ticks of the *Ixodes persulcatus* complex and its primary mammalian reservoir, *Peromyscus leucopus*, the white-footed mouse. Humans are accidental hosts. Following inoculation via tick feeding, nonspecific symptoms arise, including fever, chills, headache, and myalgia. More distinguishing manifestations follow, including neutropenia, thrombocytopenia, and elevated hepatic transaminases. If left untreated, HGA can be fatal as a result of the patient's increased susceptibility to opportunistic bacterial and fungal pathogens. Chen et al. (1994a) documented the first case of HGA in 1994. Until recently, *A. phagocytophilum* was generically referred to as the agent of human granulocytic ehrlichiosis. Phylogenetic reclassification grouped the HGE agent with *Ehrlichia equi* and *Erhlichia phagocytophila*, which infect horses and sheep, respectively, into the collective designation, *A. phagocytophilum* (Dumler et al., 2001).

Goodman et al. (1996) were the first to demonstrate that *A. phagocytophilum* could infect and be maintained in the human-derived promyelocytic leukemia cell line HL-60. HL-60 cells are highly permissive to *A. phagocytophilum* invasion and intracellular replication, making them an ideal cell line for in vitro propagation. Susceptibility of HL-60 cells to *A. phagocytophilum* infection, like that of human neutrophils, is linked to their surface expression of P-selectin glycoprotein ligand-1 (Goodman et al., 1999; Herron et al., 2000; Carlyon et al., 2003; Yago et al., 2003). Inside HL-60 cells, *A. phagocytophilum* replicates within morulae that are morphologically indistinguishable from those seen within infected neutrophils (Goodman et al., 1996). Successful propagation of *A. phagocytophilum* in the tick embryo cell line IDE8 (ATCC #CRL 11973), isolated from *I. scapularis*, has also been reported (Munderloh et al., 1996, 1999).

Both infectivity and pathogenicity of the bacterium wane considerably with in vitro passage (Hodzic et al., 1998). Thus, *A. phagocytophilum* must also be maintained by passage through inbred mice. As first demonstrated by Barthold, Hodzic, and colleagues, laboratory mice are excellent models, as infection in them partially mimics many of the hematological parameters and histopathological lesions associated with HGA (reviewed in Borjesson and Barthold, 2002). The majority of studies to date have utilized either the C3H/HeN or C3H/HeJ strains and their congenic C3H-*scid* counterparts. However, laboratory propagation of *A. phagocytophilum* in C57BL/6, CD-1, DBA/2, C.B.-17-*scid*, and *Peromyscous leucopus* mice, as well as LVG hamsters, has also been reported.

Unfortunately, peripheral blood infection of immunocompetent mice resolves by the third or fourth week post-inoculation. Additionally, pathogenicity attenuates with successive passage in immunocompetent mice. SCID mice, however, are unable to clear *A. phagocytophilum* infection, and are therefore used for restoring pathogenicity of this organism, as well as for its continual maintenance in vivo (Borjesson and Barthold, 2002; Hodzic et al., 1998).

A. phagocytophilum infection can be assayed using a number of methods (Dumler and Bakken, 1998; Hodzic et al., 1998; Carlyon and Fikrig, 2003, 2004; Dumler and Brouqui, 2004). Direct observation of morulae within neutrophils of a peripheral blood smear or infected HL-60 cells using Romanowsky stains (e.g., Protocol Hema-3) is included here (see Support Protocol 3) because the protocol is relatively simple to perform and requires few materials. It also allows for a determination of the percentage of infected cells. Immunofluorescence microscopy is a more sensitive method in that it can additionally detect individual organisms. This method is quite useful for monitoring *A. phagocytophilum* infection kinetics in an in vitro time course assay. A second means for tracing in vitro infection is the use of CellTracker Green CMFDA to fluorescently label viable *A. phagocytophilum* (see

Support Protocol 7). Herron et al. (2000) first described this technique. Cryopreservation of infected HL-60 cells using standard methods is also presented (see Support Protocol 5). Host cell–free *A. phagocytophilum* is easily recovered from mechanically lysed HL-60 cells by differential centrifugation (see Basic Protocol 3). These preparations can be further purified by Renografin density gradient centrifugation (see Support Protocol 6), which has been utilized for purifying an array of intracellular organisms (McCaul and Williams, 1981; Plano and Winkler, 1989; Weiss et al., 1989; Chen et al., 1994b).

Critical Parameters

Preparing slides and culturing A. phagocytophilum in HL-60 cells

When preparing slides of *A. phagocytophilum*–infected blood to be subsequently screened for morulae-positive neutrophils, it is essential to achieve a good smear. The area of the smear should nearly cover the slide's entire surface and end in a tongue-shaped arc. The vast majority of leukocytes will be present in the periphery of the arc. Confining one's microscopic examination to this region will provide the best opportunity to visualize neutrophils. Once an HL-60 culture is inoculated with *A. phagocytophilum*, it is important to maintain a proper HL-60 cell density such that the culture does not overgrow. Therefore, split the culture bi-weekly to maintain a density of 2–5 $\times$ 10^5 cells/ml. Additionally, it is crucial to determine the percentage of infected cells and add uninfected HL-60 cells as needed to prevent *A. phagocytophilum* from outgrowing and lysing the entire host cell population. For example, if 90% of the cells in a 10-ml culture of HL-60 cells at a density of 1 $\times$ 10^6 cells/ml are infected with *A. phagocytophilum*, discard 8 ml (8 $\times$ 10^6 cells) and add 2 $\times$ 10^6 uninfected HL-60 cells. Bring the final volume to 10 ml with fresh IMDM-10. By day 3, the culture will again contain $\geq$90% infected cells and will need to be split again. Using this example as a guide, cultures can be supplemented while still retaining a high percentage of infected cells.

Lysing infected HL-60 cells

When lysing *A. phagocytophilum*–infected HL-60 cells in preparation for obtaining bacterial preparations that are relatively free from host cellular components, it is crucial to pass the cells through a 27-G, $\frac{1}{2}$-in. (12.7-mm) needle five times to maximize host cell lysis. It is equally important to thoroughly remove the host debris by differential centrifugation. Failure to do so will greatly interfere with density gradient purification of the bacteria. Instead of two distinct bands of host debris and purified *A. phagocytophilum* at the top of the 30% Renografin layer and at the 30% to 42% interface, respectively, excess host cell debris will be distributed throughout the entire gradient.

Bleeding and infecting mice

When obtaining blood from *A. phagocytophilum*–infected mice, do not collect more than 20% of total blood volume at one time. Keep in mind that the total blood volume of a mouse is 5.5 ml per 100 g body weight. For repeated blood collection via the retro-orbital route, alternate eyes should be used. In the event that one eye is damaged, the second eye can be bled. If the second eye becomes damaged, bleeding via the retro-orbital route can no longer be performed. Also, be sure to rapidly transfer collected blood to the anticoagulant-treated tube, as it will otherwise clot. Lastly, do not successively pass *A. phagocytophilum* more than four times through immunocompetent mice, as the bacterium's pathogenicity will become increasingly attenuated with each passage. For in vivo maintenance, it is best to pass the bacterium through SCID mice.

Immunofluorescent microscopy

A critical parameter to keep in mind while preparing slides for examination by immunofluorescence microscopy is that the slides should be left in acetone for 8 min to ensure proper fixation and permeabilization. Also, allow at least 1 hr for the blocking step to prevent nonspecific antibody binding. Additionally, thoroughly resuspend the cells by gently pipetting up and down when transferring cell suspensions into the slide wells. This will minimize clumping and facilitate easier viewing of the cells. When preparing samples for cytological staining, centrifuging the infected HL-60 cells onto the slides results in the host-cell cytoplasm spreading out. This greatly aids in identification of morulae, as they are otherwise often obscured by the large HL-60 cell nuclei. In addition, the Hema-3 solutions can become diluted with repeated use and may require longer incubations for thorough cell staining. If this occurs, it may be necessary to leave the slides in each stain for up to 1 min. If the stains become so dilute that incubations longer than 1 min are required, then discard the old solutions and use fresh stains.

Fluorescent labeling using CellTracker Green CMFDA

When preparing host cell–free *A. phagocytophilum* for fluorescent labeling using CellTracker Green CMFDA, it is crucial to thoroughly wash the bacteria to remove all traces of FBS, as serum components will inactivate the dye. It is equally important to thoroughly wash the labeled bacteria to remove unincorporated label. Otherwise, the entire host cell population will be labeled in the subsequent infection assay, thereby making it impossible to distinguish infected from uninfected cells.

Troubleshooting

Table 3A.2.1 presents some of the common problems associated with the protocols in this unit, as well as their causes and solutions.

Anticipated Results

Inoculation and continual passage of A. phagocytophilum in HL-60 cell culture

Following inoculation of *A. phagocytophilum*–infected blood into HL-60 cell culture, morulae are typically not detectable until day 5 post-inoculation. However, *A. phagocytophilum* has a slow doubling time. Thus, even on day 5, the percentage of HL-60 cells with morulae remains quite low. By days 12 to 14, morulae should be detectable within $\geq$80% of cells.

When viewing HL-60 cells incubated in the presence of trypan blue under light microscopy, uninfected HL-60 cells are typically round and have smooth borders. The membranes of infected cells, however, are rougher in appearance. Moreover, the integrity of the membranes becomes compromised as the infection progresses, and they become permeable with respect to trypan blue. As a result, heavily infected cells and cells that have been lysed upon bacterial exit appear darker than uninfected cells.

Obtaining host cell–free A. phagocytophilum

Following the initial post-lysis spin, a large host cellular debris pellet will be visible at the bottom of the microcentrifuge tube. The pellet obtained after the subsequent centrifugation step is considerably smaller than that obtained after the first spin step. Upon centrifugation at 2300 $\times$ *g*, a small pellet will be visible. This represents *A. phagocytophilum* with minor host cell contaminants.

Inoculation of A. phagocytophilum into laboratory mice

Following intraperitoneal inoculation of immunocompetent mice with *A. phagocytophilum*, morulae are evident in 2% to 4% of peripheral blood neutrophils on day 2. By days 7 to 8, which typically represent peak infection, morulae are detectable in 7% to 13% of neutrophils. By days 14, the peripheral blood infection wanes considerably, and the percentage of neutrophils with detectable morulae drops to <2%. Often, morulae are no longer evident in peripheral blood smears by day 28. With successive passage of *A. phagocytophilum*–infected C3H mouse blood into naive C3H mice, the pathogenicity of the bacterium will rapidly wane. By the sixth or seventh passage, morulae-positive neutrophils will drop to nearly undetectable levels. However, it has been demonstrated that blood from infected C3H mice remains infectious for up to 60 days (Hodzic et al., 1998). Pathogenicity of the bacteria can be restored by inoculation of infected C3H mouse blood into SCID mice. By the third or fourth passage in SCID mice, the percentage of peripheral blood neutrophils with *A. phagocytophilum* infection should rise considerably. The initial kinetics of infection in SCID mice is similar to that observed for immunocompetent mice. However, the maximal level of bacteremia does not subside after day 8, and may be sustained for several weeks. *A. phagocytophilum* should be passaged from infected to naive SCID mice at least every 8 weeks.

Cytological staining of A. phagocytophilum–infected cells

Morula is a Latin term meaning "mulberry." The morphology of the intracytoplasmic inclusions formed by *A. phagocytophilum* makes it an appropriate metaphor. Morulae stain more darkly than the host cell's nucleus and are stippled in appearance (Fig. 3A.2.2). Morulae are most readily distinguishable when present in the cytoplasm and not obstructed by the large HL-60 cell nucleus. For this reason, centrifugation of the cells onto slides using a Thermoshandon Cytospin 4 Cytocentrifuge, which causes the cytoplasm to spread out away from the nucleus, is strongly recommended.

Immunofluorescent detection of A. phagocytophilum in infected HL-60 cells.

Morulae are visualized as fluorescent green clusters of organisms (Fig. 3A.2.4). Because of the enhanced sensitivity of immunofluorescence relative to cytological staining,

Table 3A.2.1 Troubleshooting Guide for Laboratory Maintenance and Staining of *Anaplasma phagocytophilum*

Problem	Possible cause	Solution
Cultivation of A. phagocytophilum in HL-60 cells		
Entire HL-60 cell population lysed	Overgrowth of *A. phagocytophilum*	Add 1×10^6 uninfected HL-60 cells per 2×10^6 infected cells
Obtaining A. phagocytophilum from infected HL-60 cells		
Low yield of host cell–free *A. phagocytophilum*	Low bacterial burden of starting pool of infected HL-60 cells	Determine bacterial load of donor cell population prior to lysing
	Poor lysis of HL-60 cells	Pass infected HL-60 cells through 27-G, 1/2-in. (12.7-mm) needle five times
Inoculation of A. phagocytophilum into mice		
Blood from *A. phagocytophilum*–infected C3H mice has low percentage of morulae-positive neutrophils after third or fourth passage	Pathogenicity of *A. phagocytophilum* has waned with continual passage in immunocompetent mice	Restore pathogenicity by transferring *A. phagocytophilum* to SCID mice
Cytological staining		
Few to no cells present on slide post-staining	Poor fixation; cells washed off	Fix slides in Hema-3 fixative for 1 min
All cellular contents stained purple	Slides incubated too long in Hema-3 Solution II	Reduce incubation time in Hema-3 Solution II
Cellular contents stained too faintly	Staining solutions are too old, diluted	Increase incubation time in each solution or use fresh staining solutions
Immunofluorescence		
Few to no cells present on slide post-staining	Poor fixation; cells washed off	Fix slides in acetone for 8 min
Clumping of cells	Samples not thoroughly resuspended when transferring to slide wells	Pipet samples up and down when transferring to slide wells
High background fluorescence	Poor blocking	Block nonspecific sites by incubating slides with 5% BSA in PBS for $\geq$1 hr
Fluorescence of host cell nuclei is too intense	Propidium iodide staining allowed to proceed for too long	Reduce incubation time to $\leq$3 min and wash slides thoroughly with PBS to remove excess propidium iodide

continued

Table 3A.2.1 Troubleshooting Guide for Laboratory Maintenance and Staining of *Anaplasma phagocytophilum*, *continued*

Problem	Possible cause	Solution
Density gradient purification		
Low yield of host cell–free *A. phagocytophilum*	Low bacterial burden in starting pool of infected HL-60 cells	Determine bacterial load of donor cell population prior to lysis
Host cellular debris present throughout gradient following centrifugation	Ineffective removal of majority of host cellular debris by differential centrifugation	Following syringe lysis, pellet host cell debris by centrifugation as described in Basic Protocol 2
		Dilute host cell–contaminated gradient 4-fold with PBS-glucose and repeat density gradient purification
Fluorescent labeling of A. phagocytophilum		
Poor labeling of *A. phagocytophilum*	Ineffective removal of FBS following lysis	Thoroughly wash host cell–free *A. phagocytophilum* to remove all traces of serum
Fluorescent labeling of entire host cell population in subsequent infection assay	Ineffective removal of unincorporated CellTracker Green CMFDA	Thoroughly wash labeled *A. phagocytophilum* to remove unincorporated dye

individual bacteria can also be discerned. These will be evident in *A. phagocytophilum*–infected HL-60 cells, but will not be detectable in uninfected cells or in cells incubated with only primary or secondary antibody. Host cell nuclei are easily distinguished by their red color when counterstained with propidium iodide.

Cryopreservation of A. phagocytophilum–infected HL-60 cells

The majority of HL-60 cells should survive the cryopreservation procedure. After inoculating a frozen stock culture into fresh medium, the cell density and number of infected cells should begin to increase within 2 to 3 days.

Density gradient purification of host cell–free A. phagocytophilum

Centrifugation of a semipure preparation of *A. phagocytophilum* through the discontinuous gradient (see Basic Protocol 3) will result in three distinct layers of ~10 ml each, separated by two distinct bands (Fig. 3A.2.5). A pellet will also be present at the bottom of the tube. The band on top of the 30% Renografin layer consists primarily of host cellular debris. The pellet primarily consists of unbroken HL-60 cells. The band present at the interface of the 30% and 42% Renografin layers is highly enriched for *A. phagocytophilum*. It is likely that a minor portion will consist of host cellular components that are of similar size and density to that of *A. phagocytophilum*, such as mitochondria.

Fluorescent labeling of live A. phagocytophilum

When cells infected with CellTracker Green CMFDA–labeled *A. phagocytophilum* are viewed using a fluorescent microscope, individual bacteria within or bound to the surfaces of host cells are discernible as bright green dots. When analyzed using flow cytometry, infected cells exhibit a strong rightward shift relative to uninfected cells (Fig. 3A.2.6).

Time Considerations

With the exception of the protocols for establishing *A. phagocytophilum* infection in HL-60 cell culture (see Basic Protocol 2) or in mice (see Basic Protocol 1), each protocol described in this unit can be performed in a single day. Inoculation of HL-60 cells or mice should take <1 hr, depending on the number of inoculations to be performed. Following inoculation of infected blood into HL-60 cell culture, it takes ≥5 days before morulae are evident in even a minor percentage of cells. By days 12 to 14, however, ≥90% of the cells should

be infected. Following inoculation into laboratory mice, *A. phagocytophilum* inclusions are evident in ~2% to 4% of peripheral blood neutrophils by day 2. Maximal bacterial burden, which is represented by infection of 7% to 13% of neutrophils, is achieved by days 7 to 8.

Obtaining host cell–free *A. phagocytophilum* by differential centrifugation will require ~2 hr. Cryopreservation of infected HL-60 cells will require <1 hr to complete, as will the cytological staining procedure. Immunofluorescent staining of samples will take ~4 hr if performed without interruption. However, the protocol can be stopped after acetone fixation and the slides stored indefinitely at –20°C.

In addition, slides can be stored indefinitely at room temperature in the dark after being mounted. Density gradient purification of *A. phagocytophilum* requires ~6 hr and should be performed without interruption. Labeling of host cell–free *A. phagocytophilum* with CellTracker Green CMFDA will require ~3.5 hr and should also be performed without interruption.

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Key References

Borjesson and Barthold 2002. See above.

An excellent review of the use of laboratory mice for in vivo maintenance and studies of A. phagocytophilum.

Carlyon and Fikrig 2004. See above.

This reference provides a comprehensive review of the epidemiology, and clinical symptoms of HGA, as well as of the strategies used by A. phagocytophilum for invasion and intracellular survival.

Goodman et al., 1996. See above.

Presents the first demonstration of in vitro cultivation of A. phagocytophilum.

Herron et al., 2000. See above.

This seminal paper identified PSGL-1 as a ligand required for A. phagocytophilum binding and invasion of human neutrophils and HL-60 cells, and at least partially explains the bacterium's tropism for neutrophils. It also includes the first documentation of the use of CellTracker Green CMFDA for labeling A. phagocytophilum and tracing the bacterium's interactions with host cells.

Hodzic et al., 1998. See above.

This presents the first demonstration of the use of the laboratory mouse as an in vivo model for maintaining and studying A. phagocytophilum.

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Isolation and Molecular Detection of *Ehrlichia* from Vertebrate Animals

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ABSTRACT

Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*, was first recognized in 1986. Infection with this pathogen can be fatal in immune compromised and elderly humans. *E. chaffeensis* can also infect dogs and several wild animals. The clinical symptoms of HME include fever, headache, malaise, myalgia, confusion, rash, lymphadenopathy, and nausea. White-tailed deer serve as the major reservoir host for the natural maintenance of *E. chaffeensis*. *E. canis* is primarily responsible for the canine monocytic ehrlichiosis and is endemic throughout the world. It has a significant impact on the health of dogs. The isolation and growth of *Ehrlichia* species from vertebrate host samples is difficult and time consuming. In this unit, methods to recover *E. chaffeensis* and *E. canis* from infected blood samples collected from dogs, deer, and human patients are described. PCR and RT-PCR methods for sensitive detection of *Ehrlichia* infection are also discussed. *Curr. Protoc. Microbiol.* 9:3A.3.1-3A.3.16. © 2008 by John Wiley & Sons, Inc.

Keywords: *Ehrlichia* isolation • animal blood • PCR • RT-PCR • real-time RT-PCR • DNA isolation • RNA isolation

INTRODUCTION

Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*, was first recognized in 1986 (Dawson et al., 1991a*). E. chaffeensis* can also infect dogs and several wild animals (Dawson et al., 1996; Magnarelli et al., 1997). The clinical symptoms of HME include fever, headache, malaise, myalgia, confusion, rash, lymphadenopathy, and nausea (Rikihisa, 1999). HME can be fatal to the immune compromised and elderly. White-tailed deer are presumed to be the reservoir hosts of *E. chaffeensis* (Dawson et al., 1994; Lockhart et al., 1997). *E. canis* causes canine monocytic ehrlichiosis and was first recognized in Algeria in 1935 (Buhles et al., 1974). Wild and domestic dogs with chronic infection serve as reservoir hosts. During the acute phase of infection, the clinical signs include fever, anorexia, and lymphadenopathy, and, in the chronic phase of infection, the dogs may show emaciation, hemorrhage, and peripheral edema (Buhles et al., 1974).

The first successful culture of *E. canis* was obtained from peripheral blood monocytes obtained from experimentally infected dogs during the acute phase of the disease (Lovering et al., 1980). Similarly, the first isolation and cultivation of *E. chaffeensis* in canine macrophage cell line was reported by Dawson et al. (1991a). The isolation and growth of *Ehrlichia* species is difficult and time consuming. Several laboratories described the isolation of *Ehrlichia* species from vertebrate animals (Dawson et al., 1991a,b; Lockhart et al., 1997; Paddock et al., 1997; Breitschwerdt et al., 1998). Protocols for isolating *E. chaffeensis* from several human blood samples suspected to be positive for *Ehrlichia* are also described (Paddock et al., 1997). Methods of in vitro isolation of *Ehrlichia* from naturally infected deer blood are described in Dawson et al. (1994) and Lockhart et al. (1997). Mice serve as a good model system to understand the host response against *E. chaffeensis* (Winslow et al., 1998; Ganta et al., 2002, 2004). In this unit, methods are

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described to recover *E. chaffeensis* and *E. canis* from blood samples collected from clinically suspected dogs, deer, and human patients. Methods are also described to recover *Ehrlichia* organisms from tissue samples collected from experimentally infected mice.

Molecular detection methods, such as PCR, RT-PCR, and real-time RT-PCR, are useful in detecting *Ehrlichia* infection, as they are very sensitive and the assays can be performed rapidly. Molecular methods have been described by several laboratories and most of them are targeted to either 16S ribosomal DNA or ribosomal RNA (rRNA). 16S rRNA–based tests are ~100 times more sensitive than PCR because the target is available in multiple copies per bacterium (Felek et al., 2001; Sirigireddy and Ganta, 2005). Single or co-infections with three *Ehrlichia* species, *E. chaffeensis*, *E. canis*, and *E. ewingii*, can occur in animals or humans (Breitschwerdt et al., 1998; Murphy et al., 1998; Paddock and Childs, 2003; Dumler et al., 2007). 16S rRNA-based multiplex real-time RT-PCR is most efficient in detecting the aforementioned three *Ehrlichia* species (Sirigireddy and Ganta, 2005). DNA and RNA isolation and their use in detecting *Ehrlichia* by molecular methods further aid in pathogen diagnosis and are complementary to culture recovery methods.

CAUTION: E. chaffeensis and *E. canis* are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of these pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Some of the methods described in this unit also require the use of Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

CAUTION: The protocols described in this unit involve the use of human and/or other animal blood or tissue samples that may be infected with *Ehrlichia* species and/or other potential pathogens. Follow all appropriate guidelines and regulations for the use and handling of human- and animal-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Follow strict sterile culture procedures in all steps involving sample processing and during cultivation to minimize contamination.

NOTE: When preparing solutions, use deionized or double distilled water (ddH_2O) and use reagents of the highest quality. Sterilization by filtration through a 0.22-μm filter or by autoclaving is recommended for most solutions; this is critical for all reagents used for cell culture work. Discard any reagent that shows evidence of contamination, precipitation, or discoloration.

BASIC PROTOCOL 1

ISOLATION AND CULTIVATION OF *E. CHAFFEENSIS* FROM HUMAN BLOOD SAMPLES

This protocol outlines step-by-step instructions for the isolation and cultivation of *Ehrlichia* from infected human blood samples. This method is adapted from the protocol described by Paddock et al. (1997).

Materials

Human blood sample in sterile blood collection tube containing EDTA
Hanks' balanced salt solution (HBSS; without $CaCl_2$, $MgCl_2$, $MgSO_4$, or phenol red; *APPENDIX 2A*)
Histopaque-1083 (Sigma-Aldrich)

Complete MEM medium (see recipe)
DH82 monolayer (~60% confluent) in a 25-cm^2 tissue culture flask (*UNIT 3A.1*)

15- and 50-ml centrifuge tubes
Pasteur pipets
37°C, 5% CO_2 incubator

Additional reagents and equipment for DH82 monolayer culture and Cytospin slide preparation (*UNIT 3A.1*)

1. Collect ~5 to 30 ml of blood in a sterile blood collection tube containing EDTA and use immediately or store up to 1 week at 4°C before use.

   It is best to use freshly obtained blood samples to increase the success of culture work for recovering of Ehrlichia organisms.

2. Dilute the blood sample with 2 vol sterile HBSS.
3. Layer the diluted blood onto 3 ml (for every 5 ml of blood) of Histopaque-1083 in a 15-ml centrifuge tube.
4. Centrifuge the blood/Histopaque tube 20 min at 800 × *g*, room temperature.
5. After centrifugation, carefully aspirate and discard the upper plasma layer using a Pasteur pipet (see Fig. 3A.3.1).
6. Carefully transfer the opaque interface containing mononuclear cells to a 50-ml centrifuge tube containing 10 ml of HBSS with a clean Pasteur pipet, gently mix by inverting tube four to five times, and then centrifuge 10 min at 400 × *g*, room temperature.
7. Discard supernatant and resuspend cell pellet in 5 ml of complete MEM medium.
8. Transfer suspension to a 25-cm^2 tissue culture flask containing a DH82 monolayer that is ~60% confluent (described in *UNIT 3A.1*, Support Protocol 1).

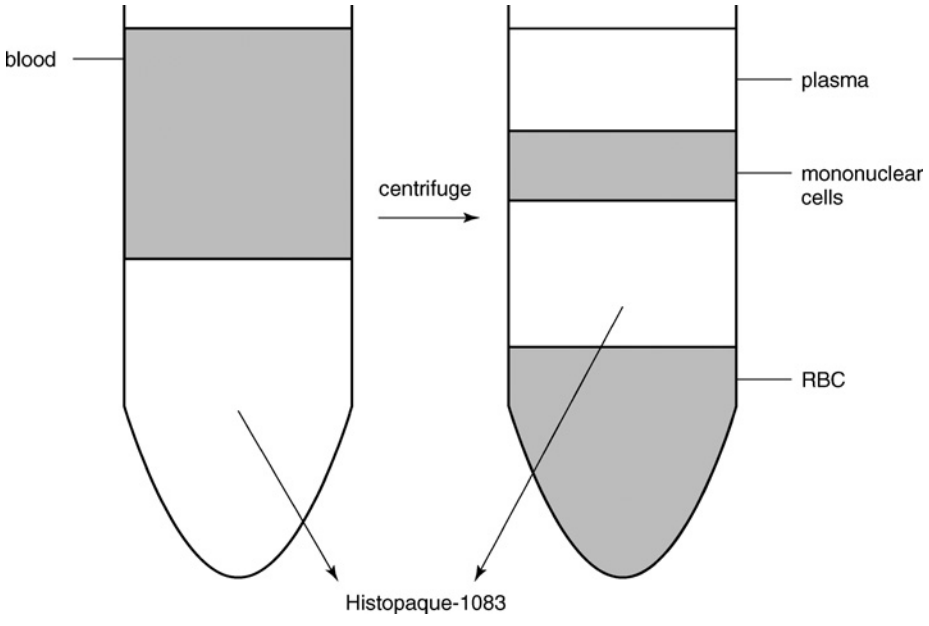


Figure 3A.3.1 Schematic drawing of the different layers of blood sample before and after centrifugation with Histopaque-1083.

9. Transfer flask to a 37°C, 5% CO_2 incubator.

10. Check the infectivity two times a week by preparing a Cytospin slide and evaluating under a light microscope as described in *UNIT 3A.1*, Basic Protocol 5.

    The growth and monitoring should be continued until infection is detected or for up to 10 weeks by changing ~50% of the medium once every 3 to 4 days. If the cells reach 90% to 100% confluence during this incubation period, replace the entire volume of medium, detach the cells by gently rocking the culture flask by hand after adding ~15 4-mm sterile glass beads, discard 50% of the culture using a sterile serological pipet, and replace with an equal volume of fresh culture medium.

BASIC PROTOCOL 2

ISOLATION AND CULTIVATION OF *E. CHAFFEENSIS* FROM DEER BLOOD SAMPLES

This protocol describes a method for the isolation and cultivation of *Ehrlichia* organisms from blood samples collected from naturally or experimentally infected deer. This method is described in two other publications with minor differences to the protocol (Dawson et al., 1994; Lockhart et al., 1997). The protocol described here is adapted from those two publications.

Materials

Heparinized deer blood samples infected with *Ehrlichia chaffeensis*
ACE lysing solution (see recipe)
Complete MEM medium (see recipe)
DH82 cells (~60% confluent) in 25-cm^2 tissue culture flask (*UNIT 3A.1*)

50-ml tubes
37°C, 5% CO_2 incubator

Additional reagents and equipment for DH82 monolayer culture and Cytospin slide preparation (*UNIT 3A.1*)

1. Collect 5 to 7 ml of heparinized deer blood infected with *E. chaffeensis*, transfer to a sterile 50-ml tube containing 25 ml of ACE lysing solution, and mix gently by inverting tube several times to form homogeneous solution.

2. Incubate 5 min at room temperature, centrifuge suspension 5 min at 160 × *g*, room temperature, and discard supernatant.

3. Add 25 ml ACE lysing solution to pellet, mix gently, centrifuge 5 min at 160 × *g*, room temperature, and then discard the supernatant.

4. Repeat step 3 one additional time.

5. Resuspend final pellet in 5 ml complete MEM medium and transfer contents to a 25-cm^2 tissue culture flask containing a ~60% confluent monolayer of DH82 cells.

6. Transfer flask to a 37°C, 5% CO_2 incubator.

7. Check the infectivity two times a week by preparing a Cytospin slide and evaluating under a light microscope as described in *UNIT 3A.1*, Basic Protocol 5. Change ~50% of the medium once every 3 to 4 days.

    Ehrlichia may be first detected in cell culture at ~4 to 5 weeks of evaluation. However, it is advisable to monitor the growth for up to 10 weeks to check for Ehrlichia positives. If the cells reach 90% to 100% confluence during this incubation period, replace the entire volume of medium, detach the cells by gently rocking the culture flask by hand after adding ~15 4-mm sterile glass beads, discard 50% of the culture using a sterile serological pipet, and replace with an equal volume of fresh culture medium.

CULTURE ISOLATION OF *E. CHAFFEENSIS* OR *E. CANIS* FROM CANINE BLOOD

BASIC PROTOCOL 3

This protocol describes a method for isolating *E. chaffeensis* or *E. canis* from the blood of a naturally or experimentally infected dog. This protocol is adapted from Dawson et al. (1991a) with minor modifications. This revised protocol uses a culture medium containing 6.5% FBS rather than 12.5% FBS and the cultures are grown in the presence of 5% CO_2.

Materials

Ehrlichia-infected dog blood in a sterile blood collection tube containing EDTA
Serofuge centrifuge (Becton Dickinson)
37°C, 5% CO_2 incubator

Additional reagents and equipment for DH82 monolayer culture and Cytospin slide preparation (*UNIT 3A.1*)

1. Collect ~6 ml *Ehrlichia*-infected blood from a dog in a sterile blood collection tube containing EDTA (purple tube) and use immediately.

   Blood samples may be stored up to 1 week at 4°C prior to culture isolation. However, it is best to use fresh blood to increase the likelihood of recovering Ehrlichia organisms.

2. Centrifuge blood samples 5 min at 3000 rpm, room temperature, in a Serofuge centrifuge.

3. Transfer 200 µl of the buffy coat to a 25-cm^2 tissue culture flask containing 5 ml (~60% confluent) DH82 culture (see *UNIT 3A.1*, Support Protocol 1 for detailed instructions for DH82 culture growth).

   The buffy coat is a thin fraction in EDTA blood (or in any anticoagulated blood) that is settled between plasma and erythrocyte layers following low-speed centrifugation. It is rich in white blood cells but also contains red and other blood cells.

4. Transfer flask to a 37°C, 5% CO_2 incubator.

5. Check the infectivity two times a week by preparing Cytospin slides of cultures and evaluating under a light microscope as described in *UNIT 3A.1*, Basic Protocol 5.

   If Ehrlichia is not identified within the first week, continue growing until the infection is noted or for up to 8 to 10 weeks. If the cells reach 90% to 100% confluence during this incubation period, replace the entire volume of medium, detach the cells by gently rocking the culture flask by hand after adding ~15 4-mm sterile glass beads, discard 50% of the culture using a sterile serological pipet, and replace with an equal volume of fresh culture medium.

RECOVERY OF *EHRLICHIA* FROM EXPERIMENTALLY INFECTED MOUSE PERITONEAL CELLS

BASIC PROTOCOL 4

This protocol outlines a simple method useful for *Ehrlichia* isolation from experimentally infected mice. The mouse model has been used extensively to understand the pathogenicity and to assess host immune response against *E. chaffeensis* infection (Winslow et al., 1998; Ganta et al., 2002, 2004, 2007).

Materials

E. chaffeensis-infected mice
Sterile PBS (see recipe), ice cold

24-well sterile culture plates
37°C, 5% CO_2 incubator

Additional reagents and equipment for DH82 monolayer culture and Cytospin slide preparation (*UNIT 3A.1*)

1. Aseptically collect peritoneal fluid containing macrophages from an experimentally infected mouse by washing peritoneal cavity, i.e., by injecting 20 ml of ice-cold, sterile PBS using a needle and syringe into the intact mouse.
2. Harvest cells from 5 ml of peritoneal wash solution by centrifuging 10 min at 500 × *g*, 4°C.

   The remaining peritoneal wash solution may be used for other experiments or discarded.
3. Resuspend cell pellet in 1/5 volume of DH82 cells harvested from a 25-cm^2 tissue culture flask having a complete monolayer of cells.
4. Transfer cell suspension to a well of a 24-well sterile culture plate and incubate in a 37°C, 5% CO_2 incubator.
5. Check the infectivity two times a week by preparing a Cytospin slide and evaluating under a light microscope as described in *UNIT 3A.1*, Basic Protocol 5. Change ~50% of the medium once every 3 to 4 days until infection is detected.

   The culture may be monitored for up to 10 weeks if the infection is not observed prior to that. If the cells reach 90% to 100% confluence during this incubation period, replace the entire volume of medium, detach the cells by gently rocking the culture flask by hand after adding ~15 4-mm sterile glass beads, discard 50% of the culture using a sterile serological pipet, and replace with an equal volume of fresh culture medium.

BASIC PROTOCOL 5

DETECT FOR THE PRESENCE OF *EHRLICHIA* BY PCR

The PCR assay described here is useful to confirm infection in host cells. However, it is not necessary to routinely use this method for infection monitoring. In the event that multiple isolates of *Ehrlichia* species are being cultured, it is important to perform a PCR assay to validate the isolate in use. Isolate-specific PCR assays may also be performed using methods described in Cheng et al. (2003).

Materials

PCR kit (Invitrogen cat. no. 10966-034) containing:
  Platinum *Taq* DNA polymerase
  10× PCR buffer
  50 mM $MgCl_2$
PCR/RT-PCR primers for *E. chaffeensis* and *E. canis* (Table 3A.3.1)
10 mM dNTPs
Nuclease-free water
DNA template
1% agarose gel containing 0.1 μg/ml ethidium bromide
1-kb plus DNA ladder (or any other comparable molecular weight DNA standards)

0.2-ml thin-walled PCR/RT-PCR tubes
GenAmp9700 PCR thermal cycler (Applied Biosystems)

1. Prepare PCR master mix by adding the following per reaction (multiply by the number of samples plus one or two extra reactions to compensate for pipetting error):

   2.5 μl 10× PCR buffer
   0.5 μl each of 10 μM forward and reverse primers
   0.5 μl 10 mM dNTPs
   1.5 μl 25 mM $MgCl_2$
   0.2 μl *Taq* DNA polymerase (5 U/μl)
   17.3 μl nuclease-free water
   Mix gently by pipetting up and down.

Table 3A.3.1 Primers and Probes Used in PCR/RT-PCR and Multiplex *Ehrlichia* Real-Time RT-PCR

Primers and probes	Sequence
Primers for PCR/RT-PCR	
E. chaffeensis-forward primer	5′gcatacttggttataaata
E. chaffeensis-reverse primer	5′gtattaccgcggctgctggcac
E. canis-forward primer	5′cctctggctataggaaattg
E. canis-reverse primer	5′gtattaccgcggctgctggcac
Primers and TaqMan probes	
Ehrlichia-common forward primer	5′ctcagaacgaacgctgg
Ehrlichia-common reverse primer	5′catttctaatggctattcc
E. chaffeensis TaqMan probe	5′TET/cttataacctttggttataaataatgttag/TAMRA
E. canis TaqMan probe	5′FAM/tatagcctctggctataggaaattgttag/TAMRA
E. ewingii TaqMan probe	5′ROX/ctaaatagtctctgactatttagatagttgttag/BQH2

For example, if preparing a master mix for ten reactions, add twelve times the suggested volumes of each reagent and then transfer 23-μl aliquots of the homogeneous mixture to each reaction tube.

2. Add 2 μl DNA template or water (to serve as a negative control) to appropriately labeled reaction tubes containing 23 μl of PCR master mix.

   The following precautions must be taken when performing PCR analysis. (1) It is best to set up PCR reactions in a PCR work station (e.g., available from Coy Laboratory Products) to minimize aerosol contamination to PCR reagents and templates. (2) A dedicated set of pipet aids and equipment must be used in setting up PCRs and always use aerosol-barrier tips. (3) All of the PCR reagents should be maintained at 4°C by submerging in a cold chamber or in an ice bucket when setting up PCR reactions (Pollard et al., 2000).

3. Transfer the reaction tubes to a GeneAmp9700 PCR thermal cycler or similar instrument and initiate the PCRs by setting up the machine for the following program:

1 cycle:	4 min	94°C (denaturation)
35 cycles:	30 sec	94°C (denaturation)
	30 sec	55°C (annealing)
	1 min	72°C (extension)
1 cycle:	5 min	72°C (extension).

4. Cool samples to 4°C, either in the PCR machine or by transferring to a refrigerator.

5. Resolve 5 μl of PCR products (along with a 1-kb plus DNA ladder) on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide (Voytas, 2000).

   Molecular weight markers should also be resolved on agarose gels to aid in the identification of the predicted ~0.4-kb DNA amplicon (Fig. 3A.3.2).

   To prevent contamination with previously made amplicons, DNA isolation and PCR assembly should be performed in one laboratory, while the executing PCRs and product analysis should be done in another laboratory. A positive control (with Ehrlichia-specific DNA as a template) and a negative control (reaction mix lacking a template) should also be included in every PCR experiment.

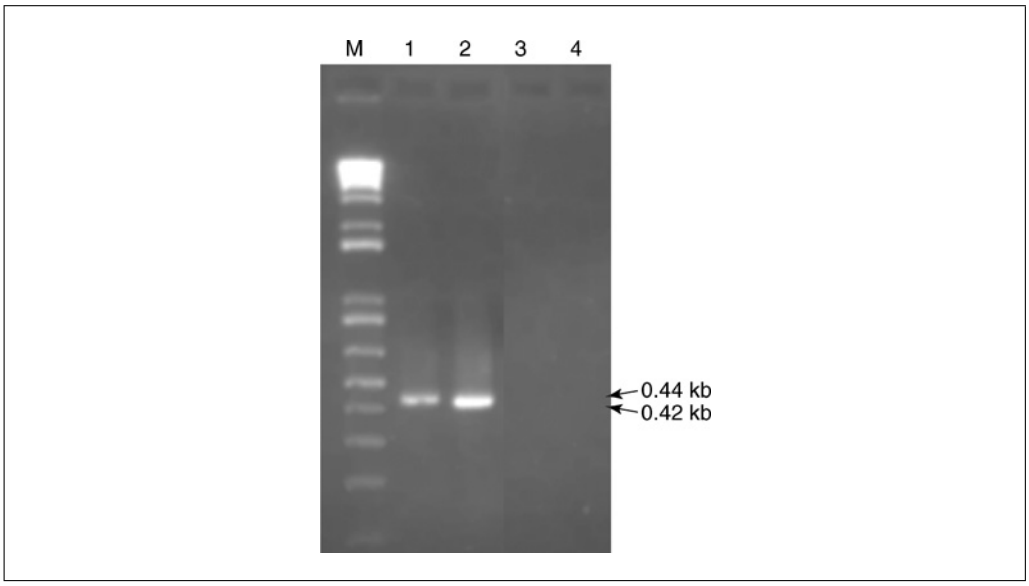


Figure 3A.3.2 PCR product analysis of *E. chaffeensis* and *E. canis.* Lanes 1 and 3 have the PCR products in the reactions containing *E. chaffeensis*-specific PCR primers with and without the templates, respectively. Similarly, lanes 2 and 4 included PCR products with *E. canis* primers with and without the DNA template, respectively. A molecular weight marker (1-kb plus DNA ladder from Promega) is included in lane M. Predicted molecular sizes for the PCR products of *E. chaffeensis* and *E. canis* are 0.44 and 0.42 kb, respectively.

SUPPORT PROTOCOL 1

EHRLICHIA DNA ISOLATION BY SDS/PROTEINASE K METHOD

The DNA isolation method described here is adapted from a previously reported SDS/proteinase K method (Sambrook and Russell, 2001). However, there are also many commercially available kit protocols.

Materials

ACE lysing solution (see recipe)
Whole blood
DNA extraction buffer (see recipe)
20 mg/ml proteinase K in 50 mM Tris·Cl, pH 7.5
Phenol, pH 8.0
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol
24:1 (v/v) chloroform/isoamyl alcohol
70% and 100% ethanol, ice cold
TE buffer (see recipe)

1.7-ml microcentrifuge tubes
Table top high-speed microcentrifuge
65°C water bath

1. Add 1 ml ACE lysing solution to 0.2 ml whole blood in a 1.7-ml microcentrifuge tube, shake gently by inverting tube several times to create a homogenous mixture, and incubate 5 min at room temperature.
2. Centrifuge lysate 5 min at 160 × *g*, room temperature, and discard supernatant.
3. Add 1 ml ACE lysing solution to carefully rinse the white blood cell pellet.

   This procedure aids in the lysis of red blood cells. Steps 1 to 3 are not needed when using this protocol for isolating genomic DNA from a cell culture pellet.

4. Add 0.5 ml DNA extraction buffer containing 12.5 µl proteinase K (20 mg/ml) to the cell pellet, mix gently by pipetting up and down, and incubate 2 hr in a 65°C water bath.

   Incubation may be extended for up to 20 hr.

5. Add 0.5 ml phenol and gently vortex or mix the contents by inverting the tube several times.

   CAUTION: *Phenol is extremely toxic, highly corrosive, and can cause severe burns. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood. Rinse any areas of skin that come in contact with phenol with a large volume of water and wash with soap and water; do not use ethanol. Also see* UNIT 1A.3.

6. Centrifuge 10 min at 10,000 × *g*, 4°C and carefully transfer the aqueous layer to a new tube using a pipet.

7. Add 0.5 ml of 25:24:1 phenol/chloroform/isoamyl alcohol and gently vortex or mix the contents by inverting the tube several times.

8. Repeat step 6.

9. Repeat steps 7 and 8 but using 0.5 ml of 24:1 chloroform/isoamyl alcohol.

10. Add 2.5 vol of 100% ice-cold ethanol and gently vortex or mix the contents by inverting the tube several times.

11. Incubate tube at least 15 min at 4°C, centrifuge 10 min at 10,000 × *g*, 4°C, and discard supernatant.

12. Add 1 ml of 70% ethanol to the tube containing DNA pellet and then carefully discard the supernatant.

13. Air dry the pellet until ethanol is completely evaporated (~10 min) and dissolve DNA in 100 µl TE buffer.

RT-PCR ASSAY FOR DETECTION OF *EHRLICHIA*

BASIC PROTOCOL 6

The RT-PCR assay described here is very similar to the PCR assay outlined above in Basic Protocol 5 with the exception of introducing a reverse transcriptase step. The RT-PCR assay is ~100 times more sensitive than PCR for detecting *Ehrlichia* species (Sirigireddy and Ganta, 2005).

Materials

SuperScript III, one-step RT-PCR system with Platinum *Taq* DNA polymerase and 2× buffer (Invitrogen cat. no. 11732-020)
PCR/RT-PCR primers for *E. chaffeensis* and *E. canis* (Table 3A.3.1)
RNAsin (RNase inhibitor; Promega cat. no. N2515)
Nuclease-free water
RNA template
1.5% agarose gel containing 0.1 µg/ml ethidium bromide (Voytas, 2000)
1-kb plus DNA ladder (or any other comparable molecular weight DNA standards)

0.2-ml thin-walled PCR/RT-PCR tubes
GenAmp9700 PCR thermal cycler (Applied Biosystems)

1. Prepare an RT-PCR master mix by adding the following for a total volume of 23 µl per reaction (multiply each volume with the number of samples plus one or two reactions for pipetting error):

   12.5 µl 2× SuperScript III/*Taq* polymerase buffer
   0.5 µl each of 10 µM forward and reverse primers

1 μl SuperScript III/*Taq* enzyme mixture
0.1 μl RNAsin (40 U/μl)
8.4 μl nuclease-free water.

For example, if preparing a master mix for ten reactions, add 12 times the suggested volumes of each reagent and then transfer 23-μl aliquots of the homogeneous mixture to each reaction tube.

2. Add 2 μl RNA template or water (for negative control) to each reaction tube containing 23 μl of master mix.

3. Transfer reaction tubes to a thermal cycler and initiate RT-PCR by performing the parameters described in Basic Protocol 5, step 3, except add a 1-hr incubation cycle at 48°C at the start of the PCR cycles to aid in synthesizing cDNA.

4. Resolve 5 μl PCR products (along with DNA markers) on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide (Voytas, 2000).

Molecular weight markers should also be resolved on agarose gels to aid in the identification of the predicted ~0.4-kb DNA amplicon (Fig. 3A.3.2).

BASIC PROTOCOL 7

TRIPLEX REAL-TIME RT-PCR FOR QUANTITATION OF *EHRLICHIA*

Triplex real-time RT-PCR is useful to differentiate *Ehrlichia* species. It is also valuable in quantifying the *Ehrlichia* organisms in culture or in a test sample (Sirigireddy and Ganta, 2005).

Materials

SuperScript III, one step RT-PCR system with Platinum *Taq* DNA polymerase and 2× buffer (Invitrogen cat. no. 11732-020)
10 mM dNTPs
Real-time RT-PCR primers and *Taq*Man probes for *E. chaffeensis*, *E. canis*, and *E. ewingii* (Table 3A.3.1)
Nuclease-free water
RNA (see Support Protocol 2)

1.7-ml microcentrifuge tubes
PCR tubes for Smart Cycler (Cepheid cat. no. 11-400-3)
Smart Cycler real-time PRC machine (Cepheid)

1. Prepare a master mix in a 1.7-ml microcentrifuge tube by adding the following reagents for a total volume of 23 μl per reaction (multiply each volume by the number of samples plus one or two reactions for pipetting errors):

12.5 μl 2× SuperScript III/*Taq* polymerase buffer
0.5 μl 10 mM dNTPs
1.0 μl each of 10 μM forward and reverse primers (Table 3A.3.1)
1.7 μl 50 mM $MgSO_4$
0.5, 1.0, and 1.2 μl of 7.5 μM *Taq*Man probes for *E. canis*, *E. chaffeensis*, and *E. ewingii*, respectively
1 μl SuperScript-III/*Taq* enzyme mix along with 3 units of platinum *Taq* DNA polymerase
Bring up to final volume of 23 μl (per reaction) with nuclease-free water.

For example, if preparing a master mix for ten reactions, add twelve times suggested volume of each reagent and then transfer 23-μl aliquots of the homogeneous mix to each reaction tube.

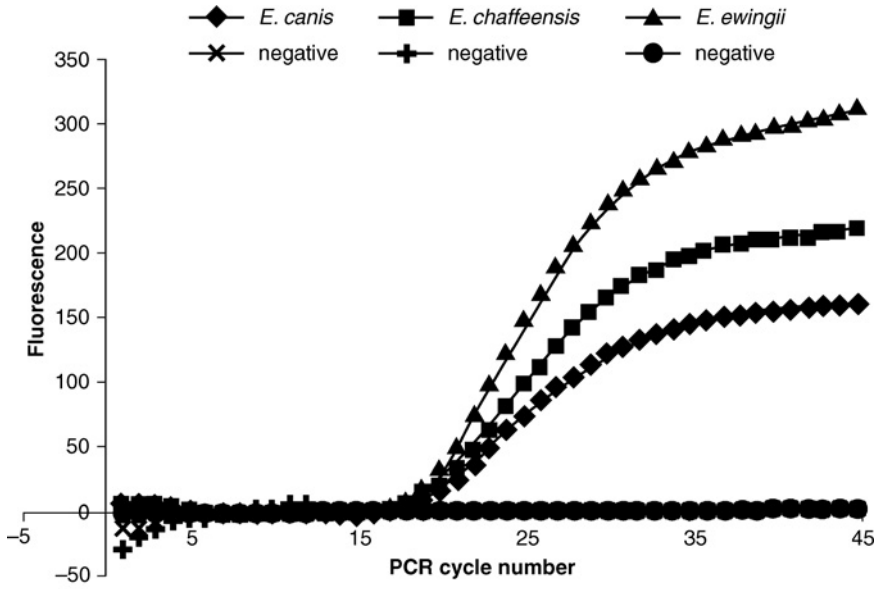
2. Dispense 23 µl of master mix into real-time RT-PCR tubes and add 2 µl of RNA or water (negative control) as templates.

   The experiment should include a positive control (DNA or RNA template from known positives) and a negative control that contains all the reagents except a DNA or RNA template.

3. Perform real-time RT-PCR in a Smart Cycler system (or similar instrument that is designed for use in performing *Taq*Man probe-based real-time RT-PCR assays) using the following parameters:

1 cycle:	30 min	48°C (reverse transcription)
1 cycle:	2 min	94°C (initial denaturation)
45 cycles:	15 sec	94°C (denaturation)
	30 sec	50°C (annealing)
	1 min	60°C (extension)

   The optics of Smart Cycler should be set to turn on during the extension phase of each amplification cycle and, similarly, the threshold fluorescence intensity should be set to 10 U. The dye set should be selected as FTTR (FAM, TET, TAMRA, and ROX). At the completion of the setup cycles, the results are displayed on a computer attached to the machine. If a sample tests positive, a Ct-value (the cycle at which the fluorescence crossed the threshold of 10 U for a specific pathogen probe) will be displayed for the positives (Fig. 3A.3.3). A reaction for which the Ct-value is displayed as ≤39 is considered as positive. If a blood sample has more than one pathogen, real time RT-PCR analysis is expected to yield positive results for more than one pathogen.



Sample	*E. chaffeensis* (TET) Ct (results)	*E. canis* (FAM) Ct (results)	*E. ewingii* (ROX) Ct (results)
DNA	18.4 (positive)	19.12 (positive)	18.00 (positive)
water	0.00 (negative)	0.00 (negative)	0.00 (negative)

Figure 3A.3.3 Real-time data generated for *Ehrlichia* triplex real-time RT-PCR using a Smart Cycler system is presented. Equal molar concentrations of in vitro–synthesized templates of *E. chaffeensis*, *E. canis*, and *E. ewingii* are used to demonstrate the detection by real-time assay (Sirigireddy and Ganta, 2005). Negative controls contained all components of a real-time RT-PCR mixture with the exception of the templates (template volume replaced with water). The Ct values generated by the Smart Cycler are presented as a table format generated by the machine.

EHRLICHIA RNA ISOLATION

The RNA isolation method described here is adapted from the Tri-reagent method kit protocol (Sigma-Aldrich).

Materials

Ehrlichia-infected blood, tissue samples, or culture organisms
Tri-reagent and Tri-reagent BD (Sigma-Aldrich) or similar reagents (available from other manufacturers)
TE buffer (see recipe) or nuclease-free water
RNAsin (RNase inhibitor; Promega cat. no. N2515)

1.7-ml microcentrifuge tubes
Table-top high-speed microcentrifuge

1. Isolate total RNA from *Ehrlichia*-infected blood, tissue samples, or culture organisms using the Tri-reagent or Tri-reagent BD (from blood) method by following the manufacturer's instructions.
2. Dissolve RNA pellet in 100 µl TE buffer or nuclease-free water.
3. Add 40 U RNAsin to purified RNA solution and store up to 2 years at –80°C until use.
4. Use 2 µl of each DNA or RNA in PCR or RT-PCR assays, respectively, to determine the presence of *Ehrlichia*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ACE lysing solution

150 mM NH_4Cl
0.7 mM KH_2PO_4
3 mM Na_4-EDTA

Weigh 8.012 g NH_4Cl, 0.0952 g KH_2PO4, 1.248 g Na_4-EDTA, and dissolve the compounds in a total volume of 1000 ml water and filter sterilize using a 0.22-µm filter unit. Store up to 6 months at 4°C.

Complete MEM medium

500 ml minimum essential medium with Earle salt (MEM medium; Mediatech cat. no. 150-10-CV)
35 ml heat-inactivated FBS
6 ml 100 mM L-glutamine

Mix by inverting the bottle several times and filter sterilize using a 0.22-µm filter unit and store up to 2 months at 4°C.

DNA extraction buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 mM NaCl
50 mM EDTA
0.25% SDS

Add 2.922 g NaCl, 5 g SDS, 50 ml EDTA (0.5 M, pH 8.0), and 50 ml Tris·Cl (0.5 M, pH 7.5), and dissolve compounds in a volume of 500 ml water. Sterilize by passing through a 0.22-µm filter or autoclave. Store up to 1 year at room temperature.

PBS buffer, pH 7.4

8 g NaCl
0.2 g KH_2PO_4
0.2 g KCl
Plus one of the following:
2.17 g $Na_2HPO_4 \cdot 7H_2O$
2.9 g $Na_2HPO_4 \cdot 12H_2O$
1.15g Na_2HPO_4 (anhydrous)
Dissolve the above ingredients in 1 liter water
Sterilize by passing through a 0.22-μm filter or autoclave
Store up to 6 months at 4°C.

TE buffer

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EDTA

Measure 10 ml of 1 M Tris·Cl, pH 7.5 and 2 ml of 500 mM EDTA, pH 8.0, and bring volume up to 1 liter with water. Sterilize by passing through a 0.22-μm filter or by autoclaving. Store up to 1 year at room temperature.

COMMENTARY

Background Information

Several emerging tick-borne rickettsial infections have been reported in recent years and have shown to significantly impact the health of humans and other vertebrates (Paddock et al., 1993; De Silva et al., 1996; Dumler et al., 2001). These include infections by *E. chaffeensis*, a human monocytic agent, *E. ewingii*, a human ewingii ehrlichiosis agent, and *Anaplasma phagocytophilum*, a human granulocytic anaplasmosis agent (Dawson et al., 1991a; Chen et al., 1994; De Silva et al., 1996; Caspersen et al., 2002; Dumler et al., 2007). *E. canis* has been identified as the major cause of tick-borne infections in dogs in nearly all parts of the world for several decades (Haas and Meyer, 1986).

Culture methods that serve as the gold standard for pathogen diagnosis are also described for recovering *Ehrlichia* species from human, deer, and dog blood and mouse tissue samples (Dawson et al., 1991a,b; Lockhart et al., 1997; Paddock et al., 1997; Breitschwerdt et al., 1998; Ganta et al., 2002, 2004, 2007). Simple step-by-step protocols are described in this unit to aid in isolating *Ehrlichia* species from clinically suspected patient samples.

Accurate diagnosis aids considerably in defining treatment regimes for infections with *Ehrlichia* species in humans or animals. In this unit, various protocols for DNA and RNA isolation and molecular methods for rapid diagnosis are presented. Various laboratories have described similar methods (Felek et al., 2001; Sirigireddy and Ganta, 2005) and the methods most suitable for pathogen diagnosis are adapted and described in this section.

Critical Parameters

Isolation and cultivation of Ehrlichia from infected animal samples

Isolating *Ehrlichia* from human and/or animal blood or tissue samples can be very challenging; thus one should carefully follow all the steps for culture isolation as outlined in this unit.

The volume of blood used is important for culture isolation. Consider using a large volume of blood sample (up to 30 ml) for culture recovery experiments, particularly when a blood sample is obtained from an animal suspected to be having a chronic phase of infection.

If an animal is under antibiotic treatment, the probability of culture recovery of *Ehrlichia* significantly decreases.

Culture must be maintained by following strict sterile culture techniques to avoid bacterial and fungal contamination. Cultures should be monitored for up to 10 weeks before considering a sample to be negative.

Nucleic acid isolation and molecular detection methods

It is important to prevent the introduction of previously amplified products into PCR/RT-PCR reactions to avoid false positives.

It is critical to have two independent laboratories to physically separate the pre-PCR assembly (which includes nucleic acid

Table 3A.3.2 Troubleshooting Guide for *Ehrlichia* Isolation and Its Molecular Application[a]

Problem	Possible cause	Solution
No *Ehrlichia*-positive cells observed despite the identification of morulae-like structure in a blood smear	Blood or tissue samples stored too long before use or a sample may contain a significantly different strain, isolate, or species	Use fresh blood samples or confirm by molecular methods
Color of culture flask changed to yellow	Bacterial or fungal contamination	Discard flask, strictly follow sterile culture procedures when conducting culture experiments
Most of the host cells of an infected culture are lysed	Overgrowth of *Ehrlichia*	Add more healthy cells or re-infect a new flask containing a confluent monolayer of DH82 cells
Positive control DNA/RNA did not show positive	One or more ingredients of PCR/RT-PCR reaction mix are missing	Repeat the experiment and carefully check for proper assembly of reaction setup
Negative control gave a positive signal	One or more of the ingredients of master mix are contaminated with previously amplified products or genomic DNA or RNA templates	Carefully follow the protocols for PCR assembly and analysis as described

[a]For additional troubleshooting information related to culture methods, see Table 3A.1.1.

isolation and PCR reaction setup) from post-amplification analysis. Always include positive and negative controls for nucleic acid isolation and PCR setup. The same precautions should be followed when analyzing samples by RT-PCR methods.

Troubleshooting

Table 3A.3.2 presents some of the common problems associated with the protocols in this unit.

Anticipated Results

Isolating Ehrlichia from infected vertebrate host

If starting with a sufficient volume of fresh blood (which may range from 5 to 30 ml), then the *Ehrlichia* inclusions in macrophage cell culture are expected to be seen within ~7 days to 3 weeks. If the bacterial load is very low, detection of culture positives may take up to 6 to 10 weeks. After 10 weeks of culture monitoring, a sample may be considered negative for *Ehrlichia*.

Molecular methods

Both PCR and RT-PCR methods are extremely sensitive in detecting low levels of infection; however, RT-PCR is ~100 times more sensitive as the 16S rRNA target is present in hundreds of copies per cell. If a sample is positive for *Ehrlichia*, a predicted size amplicon is seen following resolution of PCR/RT-PCR products on an agarose gel. Similarly, positive signal is anticipated in the real-time RT-PCR assay. The results should be carefully interpreted in the content of the outcome for the reaction positive and negative controls. The reaction positive control must yield a positive result and the reaction negative control must yield a negative result before the outcomes of the test samples are judged. A test must be rejected if a negative control yields positive or a positive control yields negative.

Acknowledgments

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Transfection of *Wolbachia pipientis* into *Drosophila* Embryos

Interest in *Wolbachia* has grown exponentially in recent years as increasing numbers of biologists discover symbionts in their experimental systems. Considering the major impact that *Wolbachia* can have on infected hosts, it can be expected that research interest will continue to grow. Recent surveys estimate *Wolbachia* infection rates in insects to range between 16% to 76% (Werren et al., 1995; Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000). Given that there are currently >750,000 known insect species globally (Wilson, 1993), extrapolation using a conservative estimate of 10% infected host species gives 75,000 *Wolbachia*-infected insect species, making *Wolbachia* one of the most abundant and widespread groups of parasitic bacteria (Werren et al., 1995). In addition to infections in insects, *Wolbachia* have also been observed in mites (Johanowicz and Hoy, 1995; Breeuwer, 1997), isopods (Rousset et al., 1992), nematodes (Bandi et al., 1998), and a species of spider (Oh et al., 2000).

Wolbachia exhibit multiple mechanisms for manipulating host reproduction to increase the vertical transmission of infections. Reproductive manipulations include cytoplasmic incompatibility (CI; O'Neill et al., 1992), parthenogenesis (*Wolbachia* induction of asexual reproduction; Stouthamer et al., 1993), the reversal of genetic sex-determination (Rousset et al., 1992), and male killing (Hurst et al., 1999). *Wolbachia* are inherited exclusively through females (i.e., maternal inheritance). Thus, the reproductive manipulations serve to bias females in the host population toward infected individuals (Dobson, 2003). The mechanisms responsible for reproductive manipulations act to alter early embryonic events and development and are therefore of interest as tools for studying these processes (Stouthamer and Kazmer, 1994; Reed and Werren, 1995; Dobson and Tanouye, 1996; Lassy and Karr, 1996; Callaini et al., 1997; Dobson and Tanouye, 1998; Presgraves, 2000). *Wolbachia* reproductive manipulations are also of interest for their role in genetic conflict, host reproductive isolation, and speciation (Laven, 1967a; Breeuwer and Werren, 1990; Werren, 1998; Shoemaker et al., 1999). There is additional applied interest in the potential use of *Wolbachia* to manipulate field populations of medically and economically important invertebrates (Laven, 1967b; Curtis and Sinkins, 1998; Sinkins and O'Neill, 2000; Taylor et al., 2000).

The transfer of *Wolbachia* infection (transfection) is used both for basic research examining the *Wolbachia*-host interaction and for applied strategies that use *Wolbachia* infections to affect harmful insect populations. For example, the interspecific transfer of *Wolbachia* between *Drosophila simulans* and *Drosophila melanogaster* has been used to demonstrate host effects on *Wolbachia* infection density and CI (Boyle et al., 1993; Riegler et al., 2004). Applied population replacement and population suppression strategies (Dobson et al., 2002; Dobson, 2003b) require the ability to generate novel *Wolbachia*-host associations. This unit describes a method for *Wolbachia* transfection via the microinjection of *Drosophila* embryos. As demonstrated by recent work, the methods described here for *Drosophila* can be modified and adapted for transfection of additional insect species (Fuji et al., 2001; Kang et al., 2003; Xi et al., 2005, 2006; Zabalou et al., 2004).

CAUTION: Wolbachia pipientis is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

Contributed by Stephen L. Dobson
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MICROINJECTION OF *DROSOPHILA* EMBRYOS

The technique for *Wolbachia* microinjection is based upon that used to introduce DNA into developing *Drosophila melanogaster* embryos (Karess, 1985). The Drosophila embryo is bounded by two membranes at oviposition: an outer chorion and inner vitelline membrane. The chorion may be removed mechanically by gently rolling the embryo on double-sided tape or by submerging briefly in a bleach solution. Once the chorion is removed, embryos are aligned on a tape-covered glass slide and desiccated to minimize yolk leakage from the embryo upon puncturing the vitelline membrane with the microinjection needle. Following desiccation, embryos are covered with oil for injection. Injection is accomplished using a micromanipulator that holds a finely drawn glass needle attached to a microinjector. Although the injection process can be accomplished by a single person, it can be much less frustrating and more efficient when done by a team of two people: one collecting and preparing embryos and another microinjecting. Following injection, *Drosophila* embryos are allowed to develop on the slide. Hatching larvae are immediately transferred to *Drosophila* media. Since the *Wolbachia* infection status of injected individuals is unknown, *Drosophila* females are isolated as virgins and mated to *Wolbachia* uninfected males to assure that CI does not occur. *Wolbachia* is transmitted exclusively through females. Thus, males resulting from microinjection are not useful for establishing transfected lines and may be discarded.

Materials

Wolbachia-infected and -uninfected *Drosophila* embryos (Support Protocol 1)
Apple juice agar plates (Support Protocol 2; Fig. 3A.4.1)
50% bleach solution (Clorox bleach recommended)
Halocarbon oil 700 (Sigma)
Uninfected *Drosophila* males

Camel hair paint brush (Fig. 3A.4.1) or alternate preferred tool for manipulating embryos
Fine mesh for embryo collection and washing
Filter paper, damp
Double-sided tape (e.g., Scotch, #666)
Glass slides
Microinjector, microinjection needles (Support Protocol 3; Fig. 3A.4.2D,E) and micromanipulator
Dissecting microscope
Compound microscope with movable stage

Prepare Drosophila embryos

1. After female flies oviposit in the yeast paste coating of apple juice agar plates (Fig. 3A.4.1), add 2 µl water and agitate using a soft paint brush to suspend embryos.

   Embryos that are inserted into the agar are relatively difficult to remove and may be ignored since their extraction is not an efficient use of time.

   A fine camel hair brush works well, but brush preference varies between users. Thus, several brush types may be purchased and tested. Metal probes are also useful tools for manipulating embryos.

2. Filter embryos suspended in the yeast through mesh and rinse thoroughly with water to remove yeast.

   Figure 3A.4.1 shows an inexpensive design for embryo collection.

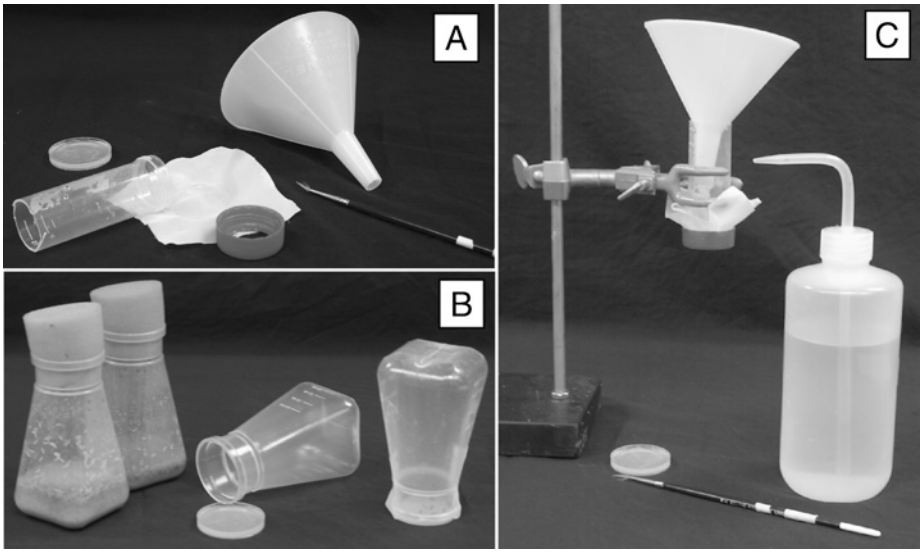

Figure 3A.4.1 Tools for embryo collection. (**A**) Brush for egg manipulation, apple juice plate, funnel, and disassembled mesh sieve. (**B**) *Drosophila* media bottles, a disassembled egg collection chamber, and an assembled egg collection chamber. (**C**) Assembled mesh sieve with water bottle and brush for suspending embryos off the apple juice plates.

3. Dechorionate embryos by submerging briefly in a 50% bleach solution.

   For the authors, 2 min in ~100 ml of 50% bleach is sufficient for dechorionating >90% of embryos. However, results can vary depending upon the bleach used and water quality. Thus, the correct amount of time for dechorionation may need to be determined empirically.

   Care should be taken in the selection of bleach. Many modern brands include fragrances or acids that can significantly reduce embryo survivorship. The label should be examined carefully.

4. Rinse embryos thoroughly with copious amount of distilled water.

5. Use a clean wet brush (or alternate preferred tool) to transfer embryos from the mesh (Fig. 3A.4.2A) onto damp filter paper.

   The filter paper should be saturated but without pools of water. A useful technique is to place the filter paper on a wet sponge.

6. Align embryos on a piece of apple juice agar (Fig. 3A.4.2B) using a wet brush or another preferred tool.

   The anterior of embryos may be recognized by its tapering end and the projecting point of the micropyle (site of sperm entry). Embryos should be oriented in the same anterior/posterior direction, touching laterally and flush along the posterior axis (Fig. 3A.4.2C). Groups of ~30 embryos work well.

7. Place a piece of double-sided tape along the edge of a clean glass slide and invert over the row of embryos on agar. Slowly, lower the tape into contact with the embryos, such that the embryos adhere to the tape.

   Note that the tape may be toxic to embryos. The suggested brand was observed to work well with Drosophila embryos. If it cannot be obtained, alternate brands should be tested empirically for toxicity effects.

8. Lift the tape carefully from the slide so as not to damage the embryos and replace onto the center of the same slide, such that the tape no longer overhangs the slide.

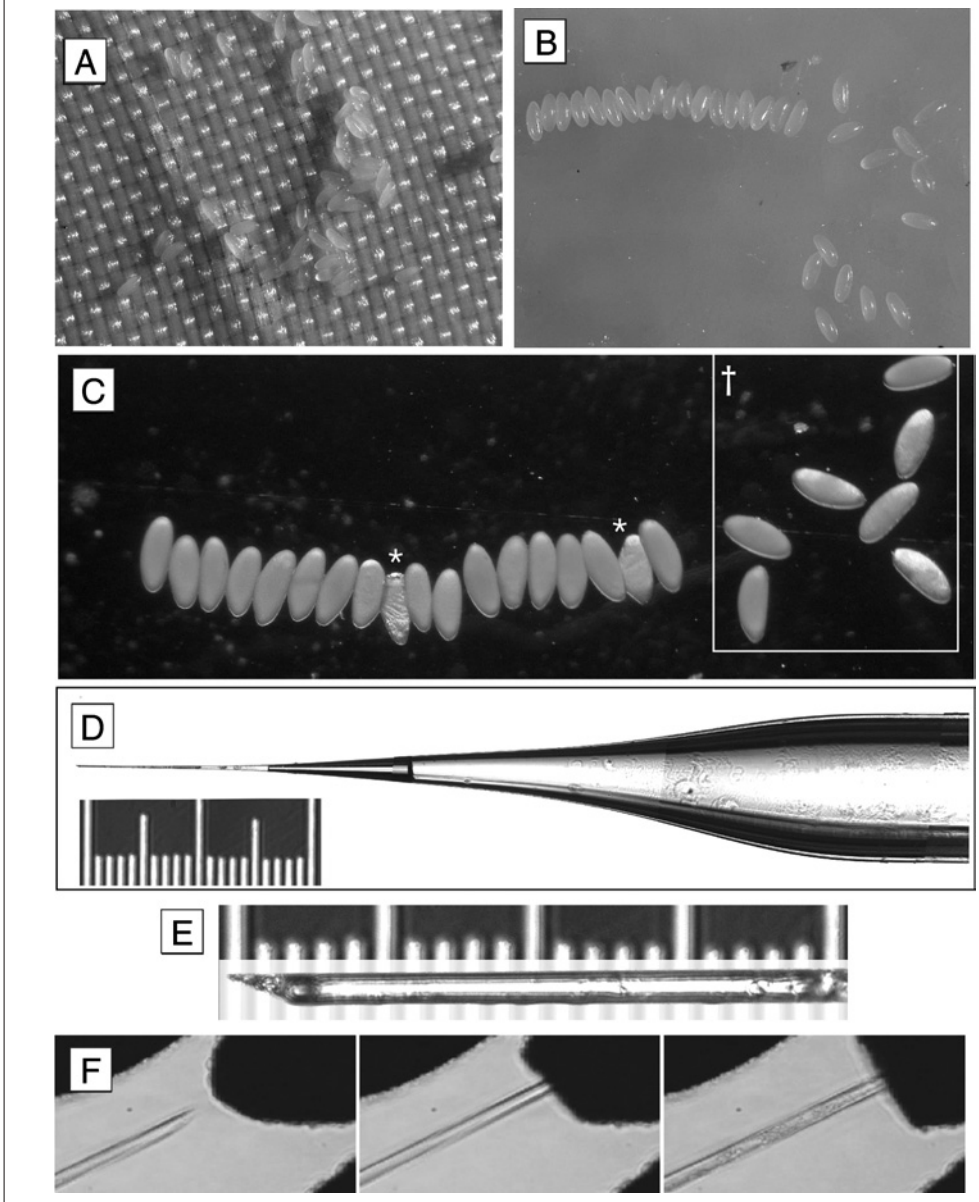

Figure 3A.4.2 Dechorionated embryos on mesh (**A**) are transferred and aligned on agar (**B**). (**C**) Aligned embryos on tape under oil. Note that some embryos are too old (marked with asterisk) or are oriented in the incorrect direction (within box marked with a cross). The latter would be destroyed prior to microinjection. (**D**) Microinjection needle with (**E**) detail of the needle tip. (**F**) A sequence of penetrating and injecting an embryo. The smallest increment of the scale shown in (D) and (E) is 10 μm.

9. Prepare uninfected embryos for injection by desiccating. Do not dessicate donor embryos, which will be used as a source of *Wolbachia*-infected cytoplasm.

   Desiccation is an important step: over-desiccation will reduce survivorship while under-desiccation will result in embryo leaking upon microinjection. As each laboratory differs in its conditions, the desiccation time must be determined empirically for each location. Slides with embryo groups can be desiccated for varying lengths of time and then microinjected. The appropriate time will be based upon observations during injection (e.g., leaking) and observed survivorship. While embryos may be desiccated in ambient room conditions, room conditions may change daily in some locations. Thus, a desiccator may be used to promote uniformity (see http://en.wikipedia.org/wiki/Desiccator for additional information).

10. Cover the embryos with water-saturated halocarbon oil (Fig. 3A.4.2C) and store at 18° to 22°C.

Transfer of cytoplasm from donor to recipient embryos

Temperature during injections is important. The ideal range is 18° to 22°C and should not exceed 25°C. Low temperature slows embryo development and improves survivorship by increasing cytoplasm viscosity.

11. Under a compound microscope, orient the needle such that it is submerged in the oil and parallel with the embryo, with the needle tip pointing at the center of the embryo posterior (Fig. 3A.4.2F).

    To determine the height of the needle relative to the embryo, the needle may be gently pressed against the embryo posterior.

12. Insert the needle into the posterior of a donor embryo and withdraw ~10% of the yolk. Withdraw needle and repeat with an adjacent embryo. Repeat with ten or more embryos to obtain sufficient *Wolbachia*-infected cytoplasm for injections.

    In this step and subsequently, it is important not to expose the needle to air for extended periods, which will allow yolk at the needle tip to dry and clog the needle. Instead, keep the needle tip submerged in oil.

13. Exchange slides, replacing infected donor embryos with uninfected recipient embryos. Orient the needle as described in step 11. Prepare for embryo injection.

    Embryo injection (step 14) should be an abrupt event (Fig. 3A.4.2F). The needle should be quickly plunged into the embryo. If properly desiccated, the embryo should dimple only slightly during injection. Effort should be made to prevent the needle tip from going too deep (>20% of the embryo length).

    If the embryo has not been sufficiently desiccated, cytoplasm can leak from the embryo. Often the leaking cytoplasm can be observed flowing into the needle. If the latter occurs, the needle should be withdrawn from the embryo, and the recipient embryo cytoplasm should be expelled from the needle before proceeding. The cytoplasm may be expelled into the oil away from embryos.

14. Use the microinjector to pump cytoplasm from the needle into the embryo. Withdraw the needle slowly as the embryo is filled.

    The embryo should visibly swell during the injection.

    The pump technique depends upon the type of microinjector. Different needles will also vary in the amount of pressure required. Even using the same needle, the pressure required will change depending upon the amount of cytoplasm remaining in the needle and periodic clogging. Thus, the pressure must be constantly readjusted during injection. A useful strategy permitted by some microinjectors that allow the user to set both the pressure and time of injections is to set the time for a period that is shorter than needed. In this way, the user can adjust for embryo variation by simply adjusting the number of pumps into the recipient embryo.

    Needle clogging is a frequent problem during injections. Many microinjectors include a clearing function that allows for a brief pulse of high pressure to clear clogs. Clearing of clogs should be done away from embryos in the oil.

15. Once finished injecting embryos on the slide, transfer to a dissecting microscope and use a probe to crush or tease away and remove uninjected embryos to simplify the downstream screening of injected lines.

    Killing of embryos should not be done with the injection needle due to the risk of damaging the needle. Instead, while injecting, mentally keep track of the embryos to be killed by their position in the line.

'Problem embryos' occur commonly for several reasons including: (1) embryo overdeveloped, attempting to inject an embryo after cell formation increases the risk of damaging the needle; (2) embryos not attached firmly to tape and move away from the needle when try to inject.

Post-injection care of recipient embryos

16. Maintain glass slides with injected embryos at ~25°C and >90% relative humidity.

    This may be accomplished by placing embryos in an incubator within a sealed Tupperware container with wet paper towels.

17. Observe slides frequently (multiple times per day) for larvae. When observed, gently remove larvae from the oil and transfer onto *Drosophila* media (e.g., Jazz Mix; Applied Scientific).

    If allowed sufficient time, larvae will crawl out of the oil to become lost and die.

    The surface of fly media will dry and develop a skin that is difficult for a single larva to penetrate, resulting in mortality of transferred larvae. Thus, media quality should be examined. The surface of media can be scratched prior to adding larvae and the larvae placed into the resulting groove. Larvae may be gently manipulated on the media to reduce the amount of oil on the larvae.

18. Once pupae are observed (~6 days), monitor frequently to obtain virgin females.

    Injected individuals are often not observed again until pupation, when they migrate to the surface and commonly attach to the container wall.

    Females will not mate for ~8 hr post-eclosion.

    Females and males should be removed when observed and transferred individually into vials with media. The rationale for preventing injected females and males from mating is that their Wolbachia-infection status is unknown.

19. Mate injected females (G_0) with males known to be *Wolbachia* uninfected and then place individually in vials to oviposit. Once sufficient embryos (G_1) are obtained, sacrifice the female and perform PCR to detect *Wolbachia* infection.

    Based upon prior work (Xi and Dobson, 2005), G_0 females that are negative via PCR assay are unlikely to result in transfected lines and may be discarded with their progeny. G_0 females that are positive via PCR assay do not necessarily result in transfected lines. Thus, PCR assays of the G_1 progeny are required for the latter. Isofemale lines with G_1 PCR positive individuals were observed to be stably infected.

    Appropriate PCR primers will depend upon the Wolbachia infection. However, PCR with the general wsp primers (Zhou et al., 1998) have been used by the authors for the general detection of the presence/absence of infections.

SUPPORT PROTOCOL 1

COLLECTION OF *DROSOPHILA* EMBRYOS

An important factor in microinjection is obtaining a sufficient quantity of *Drosophila* embryos that are <1 hr post-oviposition. The requirement of early embryos necessitates a repeated cycle of embryo collection followed by injection. The need for repeated collections of large numbers of young embryos requires access to groups of *Drosophila* females that can oviposit quickly and frequently upon being introduced to the oviposition medium (apple juice plates; Support Protocol 2).

The status of *Drosophila* females (e.g., age, nutrition) is important for obtaining sufficient quantities of early embryos. Protocols for general *Drosophila* care have been previously described in detail (Roberts, 1998). *Drosophila* females begin producing embryos two days after eclosion and can produce up to 100 embryos/female/day for two weeks if treated properly. Females oviposit best in the dark and produce the greatest number of

embryos in the late afternoon or evening. Thus, it may be advantageous to maintain the flies on a shifted light-dark cycle.

To maximize embryo production, it is advantageous to know the age of flies used to generate embryos. Therefore, cohorts of similarly aged flies can be generated by emptying adults from rearing bottles that contain many immatures and recording the date. Adults that are subsequently removed from the same bottles fall within a known time period.

A variety of designs for embryo collecting apparati have been described (Wieschaus and Nüsslein-Volhard, 1986; Santamaria, 1987). An inexpensive version consists of polypropylene *Drosophila* media bottles (AS-355, Fisher Scientific) inverted over apple juice plates with yeast paste (Fig. 3A.4.1B).

Approximately 2 days prior to embryo collection, adults are transferred from culture bottles with fly food media such as Jazz Mix from Applied Scientific ("media bottles"; Fig. 3A.4.1B) into embryo collection chambers to become accustomed to the environment. The flies may remain in the collection chambers for more than a week for continued collections, but the apple juice plates must be exchanged at least once per day. Therefore, if embryo collections are not to be repeated for several days, it may be convenient to return the flies into media bottles when finished with embryo collection.

When collecting embryos, disturbing the flies can reduce oviposition. Therefore, to reduce disturbances, they can be covered with a black cloth. A notation system may be used to identify unproductive collection chambers, which can be replaced with groups of females that are more productive. Ten productive collection chambers should yield a sufficient number of embryos for a two person team. However, if collection is to continue for several hours, additional chambers should be made ready to substitute for exhausted females.

PREPARATION OF APPLE JUICE PLATES

SUPPORT PROTOCOL 2

Apple juice plates are used for collecting embryos from infected and uninfected females (see Support Protocol 1). A thin layer of yeast paste over the agar acts as an oviposition stimulant to the females. If the plates are prepared correctly, a majority of the eggs will be inserted into the yeast paste, not the agar.

Materials

Agar
Sucrose
Apple juice
10% Tegosept (optional, Fisher Scientific)
Live baker's yeast
35 × 10–mm petri plates (BD Falcon)

1. Dissolve 12 g agar and 12.5 g sucrose in 375 ml distilled water by stirring with heat. Stir fast enough to maintain the suspension but do not create bubbles and do not allow to boil over.
2. Once dissolved, remove from heat and continue to stir until the temperature drops to 80°C.
3. While stirring, add 125 ml apple juice followed by 3.5 ml of 10% Tegosept.

   Tegosept (an antifungal agent) is not required, but facilitates long-term storage of plates.

4. Immediately divide into aliquots in small petri plates (35 × 10–mm; BD Falcon; see Fig. 3A.4.1). Add at least enough to cover the bottom of the plate.

   Plating should be done quickly, before the solution solidifies.

   The petri plates work well with the embryo collection chamber that is described above.

5. After solidifying, store the plates at 4°C in a sealed container to prevent desiccation.

6. Immediately prior to use, lightly coat the agar with a yeast paste made by mixing live yeast with distilled water. Spread the paste using a finger or desired tool.

   The consistency of the paste should be similar to that of peanut butter. If plates have been stored at 4°C, they should be allowed to warm prior to use. The yeast acts as an oviposition stimulant for female Drosophila.

SUPPORT PROTOCOL 3

PREPARATION OF MICROINJECTION NEEDLES

The quality of microinjection needles represents a critical consideration for successful transfection. The needle bore must have a diameter large enough to allow the uptake and ejection of the cytoplasm without clogging, however, not so large as to be lethal to the embryos. The parameters below have been used to generate needles appropriate for *Drosophila* transfection.

Materials

Micropipet puller (Sutter Instrument Co., P-87)
Borosilicate glass capillaries (World Precision Instruments, TW100F-4)
Micropipet beveller (Sutter Instrument Co., BV-10)

NOTE: Microinjection needles may be purchased commercially (e.g., Eppendorf). The author does not have experience with purchased needles. Alternatively, needles may be prepared if appropriate equipment is available. The program described here should serve as a starting point only. The parameters will require adjustment depending upon the Puller model, ambient conditions, and the age of the heating element.

1. Using an automated micropipet puller, pull needles to be used for microinjection (Fig. 3A.4.2D,E) from capillaries using the two-step program in Table 3A.4.1.

	Heat	Pull	Velocity	Time	Pressure
Step 1	405	0	10	150	0
Step 2	370	150	40	50	300

2. Bevel needle tips to a 20° angle using the micropipet beveller (Fig. 3A.4.2E).

COMMENTARY

Background Information

Wolbachia is a genus of obligate intracellular alpha proteobacteria represented by the type species *Wolbachia pipientis* (Dumler et al., 2001). *Wolbachia* commonly reside within cytoplasmic vacuoles of arthropods and helminths (Casiraghi et al., 2004; Werren and Windsor, 2000) and are maternally transmitted from mothers to offspring through the embryonic cytoplasm. *Wolbachia* are able to induce a diverse range of phenotypes in their invertebrate hosts, ranging from classical mutualism to reproductive parasitism. Examples of the latter include male killing, host feminization, parthenogenesis, and cytoplasmic incompatibility (reviewed in Dobson, 2003a). Current *Wolbachia* research foci include examining the impact of *Wolbachia* infection on host evolution, characterizing the mechanisms by which *Wolbachia* manipulate invertebrate hosts, and developing applied strategies that employ *Wolbachia* for pest and disease control. Here, a

protocol for the transfer of *Wolbachia* infections (transfection) via embryonic microinjection is described.

Critical Parameters and Troubleshooting

An ability to collect sufficient quantity and appropriate quality of *Drosophila* embryos should be a primary consideration. It is advisable to practice embryo collection, dechorionation, and alignment on tape without the additional steps. Once an acceptable level of survivorship is attained, the additional steps of desiccation and injection can be introduced.

The authors have found the use of a video camera to be helpful in learning to microinject. Embryo injections can be recorded, noting the video time position relative to the slide number. This allows the person injecting to review the injection process once survivorship is known. Comparing lethal and nonlethal injections will help the person injecting to improve his or her technique.

Anticipated Results

Once appropriate technique is developed, >30% hatch rate can be obtained, with ~50% of larvae reaching adulthood (Xi and Dobson, 2005). Transferring from *Wolbachia*-infected *Drosophila simulans* to aposymbiotic *Drosophila simulans*, >60% infection can be detected by PCR of injected individuals surviving to adult (G_0). However, a subset of the G_0 females stably transmit the infection to offspring (Xi and Dobson, 2005).

Time Considerations

With a team of two people and assuming that the flies are laying sufficient eggs, >500 eggs may be injected in a day. Screening slides for survivorship and transferring survivors requires only a few minutes and should be done multiple times per day until the slides are discarded. Once larvae are transferred to media bottles, the development of injected flies may be significantly reduced relative to uninjected flies. Thus, media bottles should be observed for >2 weeks at 27°C.

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Laboratory Maintenance of *Brucella abortus*

This unit presents simple methods for growth and maintenance of *Brucella abortus*. *B. abortus* grows more slowly than *E. coli* K-12; therefore, experiments with this organism require advance planning to have it ready and in the correct growth phase. Since brucellosis is one of the most frequent laboratory-acquired infections, avoidance of infection requires careful microbiological technique. All work with *B. abortus* must be performed at Biosafety Level 3 (BSL-3), and experiments with *B. abortus* cultures are performed in a Class II biosafety cabinet (BSC; see *UNIT 1A.1*). In the U.S., laboratories working with this bacterium are required to seek prior approval from the Select Agent Program of either the Department of Agriculture (USDA) or the Center for Disease Control (CDC; see Internet Resources), and individual laboratory personnel must receive security clearance from the CDC's Select Agent Program to access cultures of *B. abortus*. Other countries may have similar regulations. Be sure to check with the appropriate authorities before starting work with *Brucella* (e.g., see *APPENDIX 1B*). Receipt of *Brucella* cultures from interstate sources is also controlled and requires a separate permit from the USDA for transfer of organisms that may be harmful to livestock or poultry.

Basic Protocol 1 describes how to grow *B. abortus* in liquid culture. Many *Brucella* genes have been shown to be regulated by growth phase; therefore, particular attention is given to growth phases to allow reproducibility between experiments. Basic Protocol 2 describes how to generate laboratory stocks of *B. abortus* for long-term frozen storage and discusses regulations pertaining to maintaining a *Brucella* strain collection. The Alternate Protocol describes how to generate stocks for long-term storage by lyophilization. Basic Protocol 3 gives a method for detecting undesirable rough colonies, which result from spontaneous variants appearing after extended in vitro passages without selection in an animal host.

CAUTION: B. abortus is a BSL-3 pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: B. abortus has been classified as a Select Agent by the United States Government. Refer to the CDC's Select Agent Program for more information (*http://www.cdc.gov/od/sap*). Also refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*).

GROWTH OF *B. ABORTUS* IN LIQUID CULTURE

BASIC PROTOCOL 1

Under the growth conditions specified in this protocol, *B. abortus* 2308 will be in logarithmic growth phase between 8 and 20 hr, in late log/early stationary phase between 20 and 24 hr, and in stationary phase after 24 hr (see Fig. 3B.1.1). If a different *B. abortus* strain is used, then a growth curve should be prepared for each individual strain used.

Materials

B. abortus strain
Agar plates:
- Tryptic soy agar (TSA) plates (see recipe)
- TSA blood agar plates (see recipe)
- *Brucella* agar plates (e.g., BBL, Oxoid, EMD), prepare according to manufacturer's instructions
- Potato infusion agar plates (PIA; see recipe)

Tryptic soy broth (TSB; see recipe)
70% (v/v) ethanol in a spray bottle

Contributed by Yao-Hui Sun, Andreas B. den Hartigh, Renée M. Tsolis, and Thomas A. Ficht
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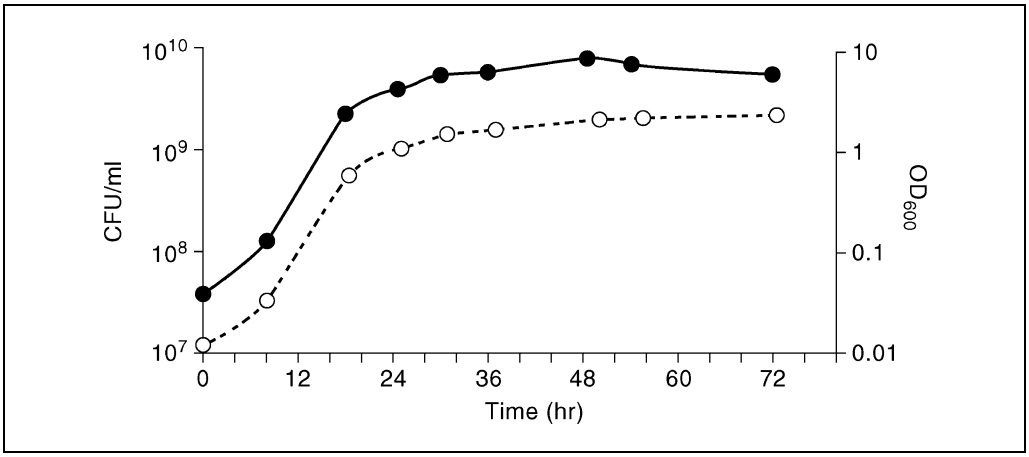

Figure 3B.1.1 Growth of *Brucella abortus* 2308 in tryptic soy broth. Solid circles, CFU/ml; empty circles OD_{600}.

10% (v/v) bleach or 1% (w/v) Virkon S (Dupont) in a spray bottle
Peptone saline maintenance medium (see recipe)
15- and 50-ml disposable polypropylene centrifuge tubes (e.g., Falcon or Sarstedt), sterile
1-µl inoculating loops
10-mm, screw-topped, glass tubes
Closed container (e.g., large plastic box with lid)
Spectrophotometer (e.g., Spectronic 20)
1-liter polypropylene straight-sided jar with screw top (e.g., Nalgene, 2118-0032)
Styrofoam tube racks from 50-ml conical tube package
Shaking incubator

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. In a Class II BSC, streak an agar plate with the *B. abortus* strain of interest and incubate 3 to 4 days.

   Colonies will be colorless and dombed (convex), and will reach 1 to 2 mm in diameter. B. abortus is nonhemolytic on TSA blood agar plates. Potato infusion agar (PIA), Brucella agar, or tryptic soy agar (TSA) can also be used, but B. abortus will grow more slowly on TSA.

   Most field isolates of Brucella require CO_2 for growth, while some laboratory strains are CO_2-independent; therefore, the growth requirements for each individual strain should be tested.

2. Aliquot 5 ml sterile tryptic soy broth (TSB) to a sterile, disposable, 15-ml polypropylene centrifuge tube

3. Using a 1-µl inoculating loop, inoculate the tube with a loopful of bacteria from the plate. Close the tube securely, and vortex to suspend the bacteria.

4. To measure the optical density, add 4 ml TSB into a 10-mm, screw-capped, glass tube that will fit into a Spectronic 20 spectrophotometer. Add 5 ml of TSB to an empty tube as a blank for the spectrophotometer. Pipet 1 ml of the *B. abortus* suspension into the tube with 4 ml TSB (1:5 dilution), close the cap securely, and vortex to mix.

   CAUTION: *All work with open B. abortus cultures must be performed within a Class II BSC (see UNIT 1A.1).*

The authors recommend use of a Spectronic 20 spectrophotometer with holder for 10-mm screw-capped glass cuvettes, or a Klett colorimeter (fitted with a 660-nm filter), which will also hold the screw-capped glass tubes. The advantage of these instruments is that the cuvettes can be tightly closed, allowing the instrument to be located outside the BSC. However, any other spectrophotometer that uses plastic cuvettes without lids can also be used within the BSC. This protocol specifies the Spectronic 20, but can be modified to fit another spectrophotometer by scaling the volumes up or down to fit the capacity of the cuvettes used. The Klett colorimeter uses arbitrary units (Klett units) to measure culture density, so a growth curve similar to the one shown in Figure 3B.1.1 can be established for this instrument using the Klett meter.

5. Place the dilution tubes into a rack. Spray the outside of the tubes and the rack with 70% ethanol and place them into a second, closed container (e.g., a large plastic box with lid) for transport to the spectrophotometer.

   CAUTION: *If there has been any leakage or spillage of bacteria on the outside of the tubes, spray them first with 10% bleach or 1% Virkon S, then with 70% ethanol, to decontaminate them.*

6. Wipe the 70% ethanol off the surface of the tubes and measure the optical density at 600 nm (OD_{600}) against the blank tube containing 5 ml TSB. To be accurate, the OD_{600} reading must be between 0.1 and 0.4; if the OD_{600} is outside this range, make a second dilution of the original suspension from step 3 and repeat the OD_{600} measurement.

   While the screw-capped tubes require a large (5-ml) sample volume, compared to plastic cuvettes, they have the advantage that the sample is contained in a closed tube, which allows the spectrophotometer to be located outside the BSC, saving valuable workspace.

7. Calculate the OD_{600} by multiplying the measured OD_{600} by the dilution factor (e.g., measured OD_{600} of 0.2 × 1:5 dilution = OD_{600} of 1.0 in the original suspension).

   An OD_{600} of 1.0 is equivalent to 2 to 4 × 10^9 CFU/ml of B. abortus.

8. Calculate the dilution factor of the original culture needed to generate a culture with an OD_{600} of 0.01, and dilute accordingly into a sterile, disposable 50-ml polypropylene centrifuge tube containing 5 ml TSB.

9. Place the tubes in a 1-liter polypropylene straight-sided jar that has been fitted with styrofoam tube racks (see Fig. 3B.1.2) and close. Place the jar in a shaking incubator and incubate overnight (16 hr) with shaking at 180 rpm.

10. Remove the jar from the shaker and open it in the BSC.

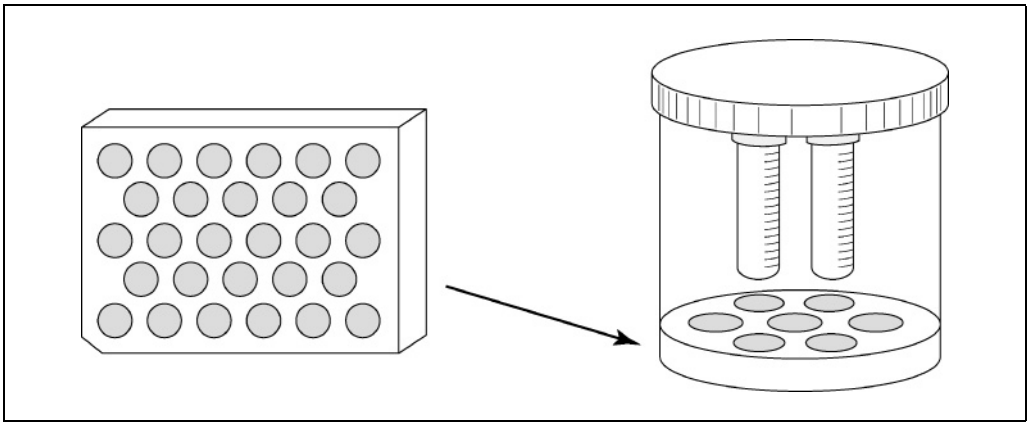

Figure 3B.1.2 Double containment jar for incubating *Brucella* cultures. To construct, cut the styrofoam package insert from the 50-ml conical tubes to fit a 1000-ml straight-sided jar (e.g., Nalgene) and insert. This will secure the 50-ml conical tubes during incubation.

11a. *For immediate use:* Dilute the culture to the required concentration for the application.

11b. *For short-term storage:* Place the culture on ice for 1 to 2 hr.

Liquid cultures of B. abortus will rapidly lose viability if stored at 4°C. Viability can be improved by centrifuging the cells 20 min at 5000 × g, room temperature, and resuspending the pellet in peptone saline maintenance medium before storage.

11c. *For long-term storage:* Proceed to Basic Protocol 2 or the Alternate Protocol.

BASIC PROTOCOL 2

MAINTENANCE OF LABORATORY STOCKS OF *BRUCELLA* BY FREEZING

A consideration for strain collections of *Brucella* maintained in U.S. laboratories is that the Select Agent regulations require close control of strain inventories. Access to the strain collection should be restricted to personnel who have been approved to work with *Brucella*. This can be achieved by locking the freezer, keeping the freezer in a secure location, or a combination of these measures. Note that other countries may have similar regulations. Be sure to check with the proper authorities (e.g., see *APPENDIX 1B*).

Each time a stock is removed from the strain collection, it should be documented in the inventory whether the strain has been destroyed or returned to the freezer. The strain inventory should be checked at regular intervals to ensure that all stocks in the collection are accounted for. The results of the inventory check should be recorded in the inventory documents.

The procedure for long-term storage of *Brucella* cultures at −80°C is similar to that used for many bacterial strains. Stocks prepared in this manner should retain viability for many years. For laboratories without access to a −80°C freezer, a different protocol for preserving stocks by lyophilization is given (see Alternate Protocol).

Materials

B. abortus strain to be stored
TSA blood agar plates (see recipe)
TSB/50% (v/v) glycerol (see recipe)
15-ml disposable polypropylene centrifuge tubes, sterile
1.5-ml freezer vials

1. In a Class II BSC, streak the *Brucella* strain to be stored onto a TSA blood agar plate and incubate 3 to 4 days at 37°C, 5% CO_2, until colonies are visible.

   Colonies will be colorless and domed (convex), and will reach 1 to 2 mm in diameter. B. abortus is nonhemolytic on TSA-blood agar plates. PIA, Brucella agar, or TSA can also be used, but B. abortus will grow more slowly on TSA.

   Not all Brucella strains require CO_2 for growth, so the growth requirements for each individual strain should be tested.

2. Using an inoculating loop, scrape up as many individual colonies as possible from the plate and suspend in 1 ml TSB in a sterile, disposable, 15-ml polypropylene centrifuge tube. Close the tube securely and vortex to suspend the bacteria.

3. Add 1 ml of TSB/50% glycerol to the tube, close the tube securely, and vortex to mix.

4. Aliquot 1 ml of the bacterial suspension to each of two freezer vials. Close the vials securely, spray the outside of the vials with 70% ethanol, and wipe off the ethanol with a paper towel before storing at −80°C.

In the absence of repeated freeze-thaw cycles, the freezer stocks should maintain viability indefinitely. For frequently used strains, it is a good policy to use one of the freezer vials as a working stock to inoculate cultures, as this vial may lose viability over time with repeated thawing and freezing due to removal from the freezer. The second vial should be held in reserve in case the working stock suffers said loss of viability.

Other methods of long-term storage, such as storage in liquid nitrogen or lyophilization (see Alternate Protocol), will also maintain viability of Brucella stocks; however, liquid nitrogen storage of vials has the disadvantage that the vials may explode on removal, creating infectious aerosols.

PRESERVATION OF *BRUCELLA* STOCKS BY LYOPHILIZATION

ALTERNATE PROTOCOL

Lyophilization is used for storage of *Brucella* vaccine stocks. Lyophilization in glass ampules has the disadvantage that it necessitates breaking the ampule, which is a risk to lab workers handling the lyophilized cultures. The handling of sharps, especially glass, should be minimized when working with Brucella, as sharp objects carry the risks of injury and infection. For laboratories without access to a −80°C freezer, a protocol for lyophilization of stocks in glass vials is described below. Lyophilized samples may be maintained at 4°C for up to 2 years without reduction in viability.

CAUTION: This protocol is not the best choice for preservation of virulent *Brucella* stocks, as it requires transport of loosely stoppered vials between the BSC and the lyophilizer. Extra respiratory protection should be used (e.g., a HEPA-filtered respirator) for this step to prevent infection in case of a spill.

Additional Materials *(also see Basic Protocol 2)*

Peptone saline maintenance medium (see recipe)
Bleach
Stabilizing medium (see recipe)

50-ml disposable, polypropylene centrifuge tubes, sterile
Centrifuge with an aerosol-proof rotor
Sterile 10-dram glass vials
Sterile rubber stoppers (autoclaved separately from vials)
Tray
Lyophilizer equipped with an internal stoppering device
Metal crimp closures

1. Grow confluent plates of the strain to be stored by spreading a few colonies from an agar plate or 10 to 50 μl from a frozen stock or liquid culture onto an agar plate in a Class II BSC. Incubate the plates up to 72 hr at 37°C, 5% CO_2, until colonies are visible.

   To avoid accumulation of rough mutants in the stock (see Basic Protocol 3), it is preferable to inoculate from a plate or from frozen stock.

2. Aliquot 10 ml peptone saline maintenance medium into 50-ml sterile, disposable polypropylene centrifuge tubes (one tube per plate).

3. Using an inoculating loop, scrape the confluent lawn gently from the surface of the agar plate and add to the peptone saline maintenance medium by wiping the bacteria onto the inner surface of the tube. Close the tube securely and suspend the material by vortexing.

4. Using an aerosol-proof rotor, pellet the cells 20 min at 5000 × *g*, room temperature. Once the centrifugation is complete, remove the rotor or buckets from the centrifuge and open inside the BSC to remove the tubes (in case of leakage during centrifugation).

CAUTION: *Be sure the tubes are balanced properly, as imbalance can lead to centrifuge accidents, resulting in aerosolization of Brucella.*

5. Carefully pipet off the supernatant and discard into a beaker containing 50 ml undiluted bleach. Resuspend each pellet in 10 ml peptone saline maintenance medium by vortexing.

6. Pellet the cells and resuspend in peptone saline maintenance medium a total of three times, using the same centrifugation conditions as in step 4.

7. After the third wash, resuspend cells in stabilizing medium at a concentration of 10^{10} CFU/ml.

   The concentration can be estimated by measuring in a spectrophotometer (see Basic Protocol, steps 4 to 8).

   The cell suspension may be stored up to 3 days at 4°C before lyophilizing.

8. Aliquot 1 ml of the bacterial suspension aseptically into sterile 10-dram glass vials. Set autoclaved stoppers loosely on top of the vials and place them onto a tray that will fit on a shelf inside the lyophilizer.

   CAUTION: *Use special care when transporting the loosely stoppered vials to the lyophilizer, as potential exists for spilling viable Brucella outside the BSC.*

9. Run the lyophilizer according to the manufacturer's instructions so that the final temperature does not exceed room temperature and the residual moisture content is 1% to 2%.

   The lyophilizer should be located inside the BSL-3 lab.

   The authors routinely employ a temperature range of between −20° and 0°C in the evaporating chamber and −50° to −60°C in the condensing chamber.

10. Use the freeze dryer's internal stoppering device to seal all of the vials at the end of the run and prior to returning the evaporation chamber to atmospheric pressure.

    Failure to properly seal the vials will result in condensation, resuspension of the bacteria, and reduction in viability.

11. Place metal crimp closures on the vials to prevent the rubber stoppers from being removed inadvertently.

12. Check viability of one sample vial by resuspending the contents in 1 ml peptone saline maintenance medium and performing ten-fold serial dilutions. Spread 0.1 ml of each dilution on a TSA plate and incubate for 3 to 4 days at 37°C, 5% CO_2, until individual colonies can be counted.

    Better than 99.9% recovery is expected. Vials may be stored at 4°C for up to 2 years without loss of viability.

BASIC PROTOCOL 3

DETECTION OF ROUGH *BRUCELLA* COLONIES BY CRYSTAL VIOLET STAINING

Brucella may be grown in liquid or on solid medium, but the preferred method for stock culture is growth on solid medium. Choice of media includes PIA, TSA, *Brucella* agar, or TSA blood agar plates. During growth in liquid medium *Brucella* mutants (rough mutants), lacking a complete lipopolysaccharide, may appear spontaneously and can outgrow the smooth wild-type bacteria. Stock cultures can have a large percentage of rough organisms if they have been passaged serially in vitro without selection in an animal host. This is of consequence because rough *Brucella* are attenuated for virulence in animal models of infection. The content of rough *Brucella* may be evaluated by staining

individual colonies on the surface of solid medium with crystal violet, which will stain rough but not smooth colonies.

Materials

B. abortus growing on solid medium
Crystal violet stock solution (see recipe)
Agar plate containing well-spaced individual colonies of *Brucella*
10% (v/v) bleach solution in a beaker

1. Dilute the crystal violet stock solution 1:40 with sterile distilled, deionized water.
2. In a Class II BSC, flood the surface of each plate with 1 ml of diluted crystal violet solution. Allow the plates to stand for 30 sec to 1 min.

   Allowing the liquid to stand too long on the plates may cause the colonies to float off the surface of the agar.
3. Carefully pipet off the crystal violet solution into a beaker of 10% bleach solution.
4. Examine the colonies on the plate. Restreak individual colonies for isolation to make a stock culture.

   Rough colonies are stained dark blue while smooth colonies resist staining and remain white.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Crystal violet stock solution

Dissolve 0.8 g ammonium oxalate in 90 ml water. Dissolve 2 g crystal violet in 20 ml absolute ethanol. Add the ammonium oxalate solution to the crystal violet solution and pass through a sterile filter. Store indefinitely at room temperature.

Peptone saline maintenance medium

1% (w/v) Bacto-Peptone
0.5% (w/v) NaCl
Autoclave and store indefinitely at room temperature

Potato infusion agar (PIA)

Dissolve 49 g dehydrated Bacto Potato Infusion Agar (PIA) powder (Difco) in 1 liter of 2% (v/v) glycerol in a 2-liter Erlenmeyer flask and boil with stirring to dissolve completely. Autoclave 20 min at 121°C with a magnetic stirring bar to sterilize. While the agar is in the autoclave, wipe down the surfaces of a clean bench or tissue culture hood with 70% ethanol. Cool the agar with stirring or by placing in a 50°C water bath. Add any required antibiotics and pour the plates on a clean bench or in a tissue culture hood. Pour 35 to 40 ml agar into each Petri dish to give a thicker plate, which will prevent it from drying out during incubation. Allow the plates to solidify 6 to 8 hr at room temperature, then incubate at 37°C overnight to identify contaminated plates. Remove the plates from the incubator, discard any contaminated plates containing colonies growing on the surface, and allow to cool to room temperature on the bench before storing at 4°C. Store plates up to 1 month at 4°C.

If plates are moved directly from the incubator to the cold room, condensation will occur and the resulting liquid will increase the likelihood of contamination.

Each liter of agar will yield 25 to 30 plates.

Plates are normally opaque at room temperature.

Stabilizing medium

2.5% (w/v) Bacto Casitone (Difco)
5% (w/v) sucrose
1% (w/v) sodium glutamate
Sterilize by passing through a 0.2-μm filter unit
Store indefinitely at 4°C

Tryptic soy agar (TSA)

Dissolve the following in a 2-liter Erlenmeyer flask containing 1 liter H_2O:
15 g pancreatic digest of casein
5 g Bacto Soytone (Difco)
5 g NaCl
15 g agar

Leave the stir bar in the flask and autoclave 20 min at 121°C to sterilize. Prepare and preincubate the plates as described above for PIA (see recipe). Store up to 1 month at 4°C.

TSA is widely used in microbiology, and it may prove more economical to purchase the powdered prepared TSA from Difco or Oxoid than to prepare this medium from scratch.

Tryptic soy broth (TSB)

Dissolve the following in 1 liter H_2O:
17.0 g pancreatic digest of casein
3.0 g Bacto Soytone (Difco)
5.0 g NaCl
2.5 g K_2HPO_4
2.5 g glucose

Aliquot into bottles that can withstand autoclaving. Autoclave for 20 min to sterilize. Store up to 1 month at 4°C.

It is easier and relatively inexpensive to order powdered TSA from a commercial supplier such as Difco or Oxoid.

TSA blood agar plates

Prepare TSA (see recipe), cooling the agar to 50°C after autoclaving. Add 50 ml defibrinated sheep blood (e.g., BBL, Difco) per liter agar and stir to distribute. Add antibiotics as needed and prepare the plates as described above for PIA (see recipe). Store plates up to 1 month at 4°C.

TSA/5% (v/v) blood plates can be ordered relatively inexpensively from microbiology suppliers such as Becton Dickinson. However these plates contain less medium and are prone to drying out during the 3 to 4 day incubation period required to grow Brucella; therefore, it is advisable to incubate them in a humidified incubator.

TSB/50% (v/v) glycerol

Add 50 ml glycerol to 50 ml sterile TSB (see recipe) and stir to dissolve. Filter sterilize by passing through a 0.2-μm filter unit, and store indefinitely at room temperature.

COMMENTARY

Background Information

Brucella abortus is a small, gram-negative coccobacillus. The bacteria are nonmotile and do not form spores. Although many *Brucella* strains can be cultivated aerobically in a bacteriological incubator, some strains require 5% to 10% carbon dioxide for growth. For primary isolation of *Brucella* from clinical specimens, media should be incubated in a 5% to 10% carbon dioxide atmosphere; under these conditions bacteria may take up to 30 days to grow. However, laboratory strains of *B. abortus* will form visible colonies on solid media within 3 to 4 days. *B. abortus* colonies are colorless, 1 to 2 mm in diameter, and have a convex surface. They are nonhemolytic on blood agar. The six species of *Brucella* are designated *B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*. In addition, two new species, *B. pinnepediae* and *B. cetaceae*, have been proposed, based on molecular characterization of *Brucella* isolates from dolphins, porpoises, whales, and seals. While it has been proposed that the different *Brucella* species should be considered as biovars of a single species, *Brucella melitensis*, adoption of this proposal has met with resistance from the *Brucella* research community, which is seeking to maintain the species designations. Therefore, in the scientific literature, both the biovar and the species designations are currently in use. For a discussion of the controversy see Moreno et al. (2002).

B. abortus is a pathogen of cattle, in which it causes abortion late during gestation. In addition, *B. abortus* has been found in wildlife species in the U.S., including bison and elk. This has presented a challenge for wildlife management, particularly in areas of the United States such as Wyoming, as the seropositivity rates have been found to be close to 50% for bison and 28% for elk (for more information on this problem, see *http://wyagric.state.wy.us/relatedinfo/wybrucellosisinfo.htm*). Based on these surveillance results, there is some concern about transmission between these wildlife species and cattle, or transmission to hunters handling infected carcasses.

B. suis, which is closely related to *B. abortus*, is a pathogen of swine, while *B. melitensis* infects goats and sheep. *B. melitensis*, *B. suis*, and *B. abortus* can be transmitted to humans via contact with infected animals or their tissues, or by consumption of unpasteurized dairy products. Brucellosis is rare in the U.S. as a result of its eradication in cattle and pasteurization of dairy products, with fewer than 200 (mostly imported) cases reported per year to CDC. Worldwide, however, over 500,000 cases of human brucellosis are reported to the World Health Organization, and the disease is widespread, especially in Africa, South America, the Middle East, and Russia. Humans generally are considered a "dead end" host and do not transmit the infection. During the 1950's, both the U.S. and the former U.S.S.R. developed technology for use of *Brucella* species as a bioweapon, and, for this reason, *B. abortus*, *B. suis*, and *B. melitensis* are regulated as Select Agents in the U.S.

Some individuals infected with *Brucella* may initially be asymptomatic. Within 8 weeks of exposure, acute onset of symptoms including fever, weakness, headache, malaise, fatigue, night sweats, and muscle and back pain may be reported. Other patients may experience a more gradual development of these symptoms over months. If the illness goes untreated, chronic brucellosis, which is characterized by recurring (undulant) fevers, can develop. Since these symptoms are nonspecific, history of possible exposure will aid in the diagnosis. Blood cultures often fail to yield *Brucella*, hence a diagnosis is made by determining serum agglutination titers with *Brucella* antigen. The recommended treatment for brucellosis is a 6-week course of doxycycline, in combination with rifampin. Treatment with a single antibiotic has been associated with a high rate of relapse of brucellosis.

Critical Parameters and Troubleshooting

Although *B. abortus* grows relatively slowly, with an in vitro doubling time of 3 to 3.5 hr, the techniques for its cultivation are straightforward. An important consideration when working with slow-growing bacteria such as *Brucella* is that any contaminants introduced into cultures will rapidly outgrow *Brucella*, so good aseptic technique is essential.

The protocols described here use TSB and TSA as media for growing *B. abortus*. Many laboratories use *Brucella* agar and *Brucella* broth. Since these are considered specialty media, they are generally more expensive than TSA and TSB, which are widely used in microbiology labs. The authors' *B. abortus* laboratory strain 2308 grows at a similar rate in both *Brucella* broth and TSB, so the less

expensive TSB is generally used. One consideration for the choice of medium is the intended application. For experiments such as enumeration of CFU obtained from tissue culture or animal experiments, where the plates will be discarded after counting, TSA is a good choice because of the cost and the large number of plates used. However, serial passage of *Brucella* on this medium can result in attenuation. In fact, serial passage in vitro was originally used to derive the live, attenuated animal vaccines currently in use, and it has been shown that for vaccine production, use of more nutrient-rich media, such as TSA blood agar or PIA, reduces the appearance of spontaneous rough mutants lacking a complete lipopolysaccharide (Alton et al., 1975). Therefore, for construction of strains to be used for virulence studies, or for strains that will be added to the laboratory stock, it is advisable to keep the number of in vitro passages to a minimum and to maintain stocks on the more nutrient-rich PIA or TSA blood agar. Other media, such as chocolate agar and modified Thayer-Martin medium, will also support growth of *B. abortus* and are routinely used in clinical microbiology laboratories.

Brucellosis is the most frequently reported laboratory-acquired infection, so it is important to follow established laboratory protocols for avoidance of contamination and infection. Disinfectants such as 70% ethanol, 1% Virkon S, or 10% bleach should always be within reach while working in the BSC. Aerosols, cuts, needle sticks, or splashes in the eye have been implicated as causes of laboratory-acquired infections. Use of sharps and glass items should be minimized to reduce the risk of infected wounds. In particular, procedures that can generate aerosols, such as homogenization of tissues, vortexing, electroporation, and centrifugation require special care to ensure containment of the aerosols. Many centrifuge manufacturers now offer special rotors that will contain aerosols in case of a spill: these rotors are highly recommended for centrifugation of samples containing *Brucella*, since centrifuge accidents are a frequent cause of laboratory infection (e.g., see Fiori et al., 2000).

When working in the BSL-3 lab, it is a good idea to plan in advance and, before starting the experiment, make a checklist to ensure that all required materials and equipment are present. A frequent cause of lost time is having to change out of the personnel protective equipment, leave the lab, and fetch whatever is missing in the middle of an experiment. An experiment that takes 1 hr at the bench in a BSL-2 lab will take at least 1.5 hr in the BSL-3 lab because of the extra safety precautions involved, so it is important to plan enough time for experiments. A final consideration is that, in equipping a BSL-3 lab, it may be useful to duplicate existing laboratory items, especially instruments such as pipet fillers or electronic equipment that cannot be readily disinfected or autoclaved.

Anticipated Results

B. abortus grows in TSB with a doubling time of ~3 to 3.5 hr. Under these conditions, a culture of *B. abortus* 2308 inoculated as described in Basic Protocol 1 will be in logarithmic growth phase between 8 and 20 hr, at late log/early stationary phase between 20 and 24 hr, and in stationary phase after 24 hr (Fig. 3B.1.1). If a different *B. abortus* strain is used, then a growth curve should be prepared for each individual strain. Unlike *E. coli* or *Salmonella*, which grow to a density of $\sim 5 \times 10^9$ CFU/ml, *B. abortus* can grow to culture densities exceeding 1×10^{10} CFU/ml. For *B. abortus* cultures, an OD_{600} of 1.0 is log phase and is equivalent to $2–4 \times 10^9$ CFU/ml, which is two to four times higher than an *E. coli* culture with the same optical density. This is likely due to the small size of *B. abortus*, which is ~0.5 μm in diameter and 0.6 to 1.5 μm in length.

Time Considerations

Three days in advance of the planned experiment, the *B. abortus* strain should be streaked out from the frozen stock culture. Cultures on blood agar plates, if sealed with Parafilm, can be maintained at 4°C for up to 4 weeks and still maintain viability; however, the bacteria from these plates will grow more slowly initially on inoculation into a liquid culture, and this should be taken into consideration when the bacterial culture is to be harvested at a particular growth phase. Alternatively, one can use a plate that has been stored at 4°C for up to 4 weeks to inoculate an overnight culture (thickly), then use this saturated overnight culture to reinoculate a new starting culture to an OD_{600} of 0.01.

For work in the BSL-3 lab, always plan extra time (at least 30 min.) for disinfection, and BSL-3 entry and exit procedures. It is wise to wipe the outer and inner surfaces of the BSC with 70% ethanol before starting work, and after completing work, all surfaces in the BSC and the lab (e.g., door handles, incubator

handles) should be wiped down with 70% ethanol. In addition, it saves time and increases safety to keep the blower of the BSC in constant operation, as it takes ~20 min after switching on the fan to establish laminar flow in the hood.

Literature Cited

Alton, G.G., Jones, L.M., and Pietz, D.E. 1975. Laboratory Techniques in Brucellosis, 2nd ed. World Health Organization, Geneva.

Fiori, P.L., Mastrandrea, S., Rappelli, P., and Cappuccinelli, P. 2000. *Brucella abortus* infection acquired in microbiology laboratories. *J. Clin. Microbiol.* 38:2005-2006.

Moreno, E., Clockaert, A., and Moriyon, I. 2002. *Brucella* evolution and taxonomy. *Vet. Microbiol*. 90:209-227.

Key References

Alton et al., 1975. See above.

This manual contains useful information on culturing different Brucella species. It is out of print, but may be obtained through library collections.

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

The CDC's Biosafety in Microbiological and Biomedical Laboratories (BMBL), should be consulted to obtain information on precautions and regulations for work at biosafety level 3.

Internet Resources

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

This site contains the CDC's Biosafety in Microbiological and Biomedical Laboratories (BMBL).

http://serine.urbm.fundp.ac.be/~seqbruce/GENOMES

The University of Namur (Belgium) Bioinformatics group's site contains information on the B. melitensis genome.

http://bbp.vbi.vt.edu/index.php

The Brucella Bioinformatics Portal at Virginia Tech contains updates, links to the sequenced Brucella gemomes and to sites of interest to researchers on brucellosis.

http://www.moag.gov.il/brunet

The BruNet contains epidemiological information about brucellosis and its control

http://www.cdc.gov/od/sap

The CDC site contains information about its Select-Agent Program

http://www.aphis.usda.gov/vs/cvb/selectagent.htm

This site contains information about the USDA's Select Agent Registration Program

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Laboratory Maintenance of *Bartonella quintana*

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ABSTRACT

Trench fever is the common name for the acute febrile syndrome associated with a *Bartonella quintana* bacterial infection. The focus of this unit is to describe reliable methods for cultivation and cryopreservation of *B. quintana* and can be applied to cultivation and preservation of all *Bartonella.* Detailed recipes for preparation of three types of semisolid media are also included. *Curr. Protoc. Microbiol.* 10:3C.1.1-3C.1.13.

Keywords: *Bartonella,* trench fever • culture • HIB-B • BB-H • chocolate agar • cryopreservation

INTRODUCTION

B. quintana infection has affected millions of people during war and is currently reemerging in inner cities throughout the world and in AIDS patients (Maurin and Raoult, 1996; Ohl and Spach, 2000). Of all bacteria, *B. quintana* has the greatest known requirement for exogenous heme, a large cyclic organic molecule (protoporphyrin) containing an iron atom (Vinson, 1966; Myers et al., 1969, 1972). It is generally accepted that this extraordinary supplement requirement is similar for all *Bartonella* species, as erythrocytes, hemoglobin, or hemin are essential for cultivation (Birtles et al., 1995).

The overall function of this unit is to describe reliable methods for cultivation and preservation of *B. quintana* (previously known as *Rochalimaea quintana*). *Bartonella* species are relatively slow growing and require nutrient-rich media; therefore there is a high risk of contamination. Thus, this unit includes a biosafety cabinet preparation protocol (Support Protocol) that should be followed for all procedures. Procedures for inoculation of semisolid media from three sources are described: glycerol stock (Basic Protocol 1), liquid suspension (Basic Protocol 2), and solid plate culture (Basic Protocol 3). A unique harvesting method (Basic Protocol 4) is used to collect bacteria from the surface of a plate, and long-term storage of *B. quintana* is accomplished by cryopreservation with glycerol (Basic Protocol 5).

CAUTION: B. quintana is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

Culture Media

Although several liquid media have been shown to facilitate replication of *B. quintana* (Huang, 1967; Mason, 1970; Maggi et al., 2005), medium solidified with 1.5% agar

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Table 3C.1.1 Semisolid Media Commonly Used for Culture of *Bartonella*

Medium	Comments
HIB-B	Heart infusion broth with 4% blood (v/v), 2% serum (v/v), and 1.5% agar. A good general purpose medium for culturing *Bartonella*.
CHOCO	GC agar with 1% hemoglobin (w/v) and IsoVitaleX medium enrichment. Recommended by ATCC for cultivation of *B. quintana.*
BB-H	Brucella broth supplemented with 1.5% agar and a variable amount of hemin chloride. Facilitates analysis of hemin-associated physiology of *B. quintana* by simulation of natural environments.

Table 3C.1.2 Hemin Supplementation of *Brucella* Agar for Growth of *B. quintana*[a]

H_2O[b]	Hemin chloride[c]	Final concentration	Simulated niche
496.8	3.2	0.05 mM	Human bloodstream (low)
490.3	9.7	0.15 mM	In vitro control[d] (normal)
337.7	162.3	2.5 mM	Louse gut (high)

[a]See Background Information.
[b]Milliliters of H_2O used to initially dissolve *Brucella* broth base.
[c]Milliliters of 5% hemin chloride solution to achieve final concentration listed.
[d]Approximate concentration of heme in a standard HIB-B plate.

provides the most reliable growth substrate. Three separate semisolid formulations containing alternate heme sources are provided in this unit (see Table 3C.1.1): (1) heart infusion broth with 4% (v/v) sheep blood (HIB-B), (2) chocolate agar containing 1% (w/v) hemoglobin (Koehler et al., 1992), and (3) *Brucella* broth supplemented with a variable concentration of hemin (BB-H). Both HIB-B and chocolate agar plates can be used for routine passage of *B. quintana.* Alternatively, BB-H plates are used to study the effect of heme concentration on gene expression and physiology of *B. quintana* (see Table 3C.1.2 and Background Information).

Culture Conditions

B. quintana is considered a microaerophile (only a small amount of oxygen is required for growth) and a capnophile (growth is enhanced in the presence of increased CO_2 tension). This atmosphere is typically achieved using a candle extinction jar. Alternatively, a 5% CO_2 incubator (at atmospheric O_2) can be used. The standard incubation temperature for *B. quintana* is 35° to 37°C in a humidified CO_2-rich environment. Alternatively, incubation can be performed at 30°C to simulate conditions experienced by *B. quintana* in the body louse vector (see Background Information).

Isolation of *B. quintana*

Isolation of *B. quintana* from nature is challenging even for an experienced researcher, and a detailed explanation of the methods employed is beyond the scope of this unit. Although cell coculture systems are considered superior, chocolate agar and HIB-B can be used to isolate *B. quintana* from human samples (Koehler et al., 1992; La Scola et al., 1999), as well as decontaminated body lice (La Scola et al., 2001).

INOCULATION OF *B. QUINTANA* FROM A FROZEN STOCK ONTO SEMISOLID MEDIA

There are three procedures depending on the source of inoculum; (1) glycerol stock, (2) liquid suspension, and (3) agar plate culture. The type of media used is based on the particular experiment (see Strategic Planning). Be sure to properly label the plates with a fine-tipped permanent marker on the agar-side of the petri plate.

Materials

B. quintana glycerol stock (Basic Protocol 5)
Petri plates with solidified medium of choice (see Strategic Planning and Reagents and Solutions)
250 ml glass beaker containing ~100 ml 95% ethanol

Ice bucket
Kimwipes, moistened with 70% (v/v) ethanol
Aerosol-barrier pipet tip
Glass culture spreader, sterilized with ethanol
5% CO_2 incubator with a pan of H_2O to maintain humidity

Additional reagents and equipment for preparing a biological safety cabinet (Support Protocol) and using the spread technique (*APPENDIX 4A*)

NOTE: Cultivation of *B. quintana* should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol and *APPENDIX 4D*).

1. Place glycerol freezer stock in ice bucket in a prepared biological safety cabinet (see Support Protocol) and allow contents to thaw completely.

   Thawing can be expedited by warming the tube between the palms. The tube should be gently inverted to resuspend bacteria and ensure a consistent inoculum.

   Keep the frozen stock tube in an ice bucket when not in use.

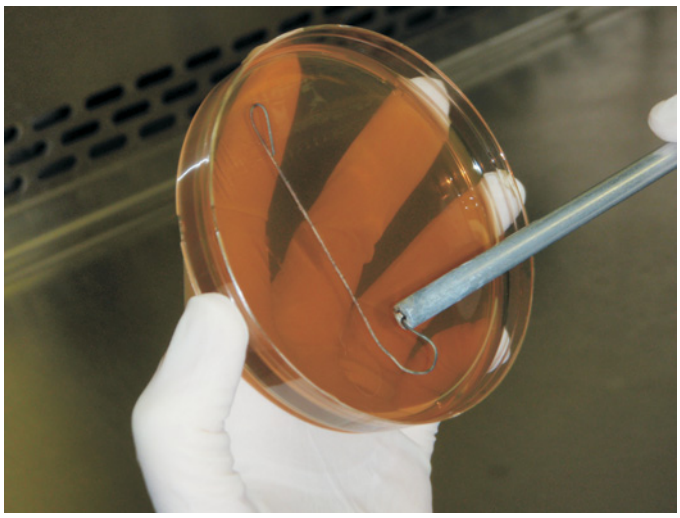

Figure 3C.1.1 Bent inoculation wire. This tool is useful for inoculation of plates from a confluent lawn of a clonal population. A *Brucella* agar plate containing 0.15 mM hemin chloride is shown. For color version of this figure see *http:www/currentprotocols.com.*

2. Sterilize the outside of the screw-capped tube by gently wiping with a 70% (v/v) ethanol-moistened Kimwipe.

   This is to ensure that the freezer stock does not become contaminated. The moistened Kimwipe can also be used to partially unscrew the lid. Do not rub too hard as you are likely to erase the label.

3. Using aseptic technique, remove 10 to 150 µl of thawed freezer stock using an aerosol-barrier pipet tip. Use the spread technique (see *APPENDIX 4A*) to evenly distribute liquid on the surface of the agar-solidified medium using an ethanol-sterilized glass culture spreader.

   Volume used for inoculation is based on empirical determination of the viable density by plating. However, if the volume exceeds 150 µl the plates should be dried agar-side down in the cabinet. Return the glass culture spreader to the ethanol and re-flame prior to spreading each separate plate.

4. Return thawed stock to the freezer.

   The number of freeze-thaw cycles does not seem to have a significant effect on viability.

5. Incubate plates agar-side up in a humidified 5% CO_2 incubator or candle jar at 37°C.

BASIC PROTOCOL 2

INOCULATION OF *B. QUINTANA* FROM A LIQUID SUSPENSION ONTO SEMISOLID MEDIA

The volume used for inoculation is based on the particular experiment. For culturing mammalian blood, larger volumes are often concentrated by centrifugation prior to plating (Koehler et al., 1992; La Scola et al., 1999). For isolation of *B. quintana* from decontaminated body lice (La Scola et al., 2001) a ~1:500 dilution is recommended to allow colony formation in the presence of faster growing bacterial species present. When using a liquid suspension obtained from a harvested plate(s) (see Basic Protocol 4), fragments of solidified media can plug the micropipet tip, resulting in erroneous volume measurements.

Materials

Liquid suspension of *B. quintana* (Basic Protocol 4)
Petri plates with solidified medium of choice (see Strategic Planning and Reagents and solutions)

Aerosol-barrier pipet tip
Glass culture spreader, sterilized with ethanol
5% CO_2 incubator with a pan of H_2O to maintain humidity

Additional reagents and equipment for using the spread technique (*APPENDIX 4A*) and preparing a biological safety cabinet (Support Protocol)

NOTE: Cultivation of *B. quintana* should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol and *APPENDIX 4D*).

1. Using aseptic technique, obtain 10 to 150 µl of the liquid suspension using an aerosol-barrier pipet tip.

2. Use the spread technique (see *APPENDIX 4A*) to evenly distribute the liquid on the surface of the solidified medium using an ethanol-sterilized glass culture spreader.

   Volume used for inoculation is based on empirical determination of the viable density by plating. However, if the volume exceeds 150 µl, the plates should be dried agar-side down in the cabinet. Return the glass culture spreader to the ethanol and re-flame prior to spreading each separate plate.

3. Incubate plates agar-side up in a humidified 5% CO_2 incubator or candle jar at 37°C.

INOCULATION OF *B. QUINTANA* FROM A SOLID PLATE CULTURE ONTO SEMISOLID MEDIA

BASIC PROTOCOL 3

A standard inoculation loop is used to subculture individual colonies of *B. quintana*. A bent inoculation wire (see Fig. 3C.1.1) is a useful tool to inoculate a large number of plates from a confluent lawn of a clonal population.

Materials

B. quintana colony or confluent lawn (from Basic Protocol 1 or 2)
Standard inoculation loop *or* bent inoculation wire (see Fig. 3C.1.1)
Kimwipe, moistened with 70% (v/v) ethanol
Petri plates with solidified medium of choice (see Strategic Planning and Reagents and solutions)
5% CO_2 incubator with a pan of H_2O to maintain humidity

Additional reagents and equipment for sterilizing the inoculation loop or bent wire using a flame (*APPENDIX 4A*)

NOTE: Cultivation of *B. quintana* should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol and *APPENDIX 4D*).

1. Sterilize the handle of an inoculation loop or bent wire with a Kimwipe moistened with 70% (v/v) ethanol.
2. Sterilize the inoculation loop or bent wire using a flame (see *APPENDIX 4A*) and cool it by touching the agar surface of the un-inoculated plate near the outer perimeter.
3. Touch the colony (with a loop) or confluent lawn (with bent wire) and gently streak across the surface of a new agar plate.

   Avoid using too much pressure when streaking as gouges in the surface result in inefficient harvesting (Basic Protocol 4).

4. Incubate plates agar-side up in a humidified 5% CO_2 incubator or candle jar at 37°C.

   The incubation conditions are based on the particular experiment and source of inoculum (see Strategic Planning and Commentary).

HARVESTING *B. QUINTANA* FROM SEMISOLID MEDIA

BASIC PROTOCOL 4

B. quintana harvesting should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol) and inoculation of semisolid media (see Basic Protocols 1 to 3). Harvesting is the collection of large numbers of a clonal population of bacteria from the surface of a plate using a single-edged razor blade and liquid medium.

Materials

Plate(s) containing *B. quintana* clonal populations (Basic Protocols 1, 2, or 3)
Heart infusion broth (HIB; see recipe)

Single-edged razor blades with grooved handle (Smith Brand, cat. no. 67-0238)
Test tube holder (see Fig. 3C.1.2)
250-ml glass beaker containing ~100 ml 95% ethanol
1000-µl micropipettor
Sterile collection tube

Additional reagents and equipment for sterilizing harvesting tool using a flame (*APPENDIX 4A*)

NOTE: Cultivation of *B. quintana* should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol and *APPENDIX 4D*).

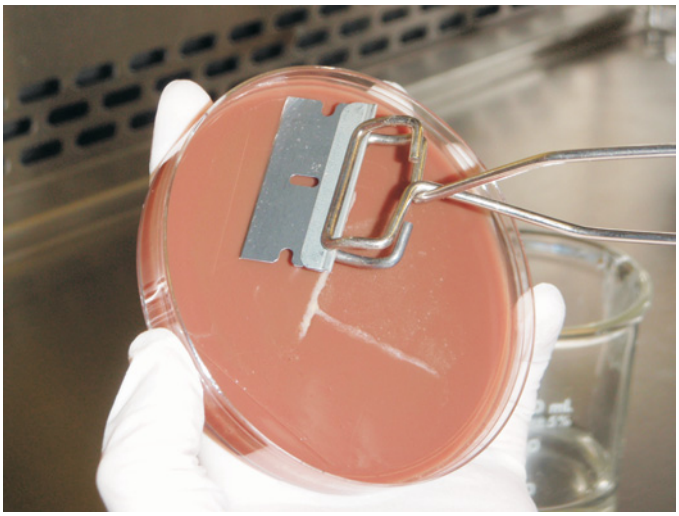

Figure 3C.1.2 Harvesting *B. quintana* from a chocolate agar plate. The harvesting tool, consisting of a single-edged razor blade and a test tube holder, is used to scrape the bacterial lawn toward the outer perimeter of the plate.

1. Carefully analyze plate(s) for contamination in the prepared cabinet.
2. Assemble harvesting tool as shown (Fig. 3C.1.2*)* by clamping the test tube holder along the groove of the razor blade handle.

   Single-edged razor blades with a groove on both sides of the handle provide stability during use. The wire of the test tube holder should firmly clamp the blade.
3. Soak the blade portion of the harvesting tool in a beaker containing 95% ethanol and briefly pass through a flame to sterilize as described for a glass culture spreader (see *APPENDIX 4A*).
4. Hold the plate agar-side-up with one hand, and the sterilized harvesting tool with the other. With the blade slightly angled toward the surface of the agar, gently scrape the bacteria into a single clump close to the outer perimeter of the plate. Return the harvesting tool to the beaker of 95% ethanol.

   CAUTION: *Dispose of the contaminated blade(s) in a sharps biohazard container.*

   Avoid movements of the blade that gouge the surface or create fragments of agar.

   The bacteria have a tendency to stick to each other and the blade. A single blade can be used to harvest >10 plates.
5. Hold the plate agar-side up with one hand, and a 1000-µl micropipettor containing ~300 µl sterile HIB with the other. Slightly tilt the plate such that the clump is facing you and is toward the bottom.
6. Moisten the clump by slowly ejecting this ~300 µl HIB liquid medium directly onto it.

   The liquid decreases the stickiness of the bacteria. The HIB should flow over the clump of bacteria and form a pool in the space between the agar surface and the inner wall of the plate.
7. Holding the pipet tip nearly horizontal to the surface of the plate, moisten the pipet tip in the pool of HIB and push clump of bacteria into this pool.

The goal is not to resuspend the clump, but rather collect it from the surface of the plate such that it can be aspirated and transferred. Avoid movements of the pipet tip that gouge the surface or create fragments of agar.

8. Aspirate and transfer the bacteria to a sterile collection tube.

   The tube size and type is based on the particular experiment. For 1 to 3 plates, use a 1-ml screw-capped vial or 1.5-ml microcentrifuge tube. For >4 plates, 30 ml Oak Ridge-type tubes are useful.

9. Resuspend the bacterial clump by gentle up-and-down pipetting or gentle swirling by hand.

CRYOPRESERVATION OF *B. QUINTANA*

BASIC PROTOCOL 5

Cryopreservation of *B. quintana* should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol). Long-term storage is achieved by freezing at −80°C in a final concentration of 12.5% (v/v) glycerol. Prepare at least two vials of each strain and if possible, store in separate freezers.

Materials

Cryopreservation solution (see recipe)
1-ml screw-capped cryovials (Nunc, cat. no. 375353)
Fine-tipped permanent marker (to label the cryovials)
−80°C freezer

Additional reagents and equipment for growing and harvesting a clonal population of *B. quintana* (Basic Protocol 4)

NOTE: Cultivation of *B. quintana* should be performed in a biosafety cabinet after preparation of workspace and tools (see Support Protocol and *APPENDIX 4D*).

1. Grow a clonal population of *B. quintana* and harvest (see Basic Protocol 4) into liquid HIB.
2. Combine equal volumes of the cryopreservation solution and the liquid suspension in a properly labeled 1-ml screw-capped cryovial.
3. Mix by gentle inversion and store >10 years in −80°C freezer.

PREPARATION OF WORKSPACE AND TOOLS

SUPPORT PROTOCOL

The combination of slow-growing *B. quintana* and nutrient-rich media often leads to contamination. Contamination of cultures is very common but can be reduced when using aseptic technique in a biosafety cabinet. The following procedures are performed to ensure safety for the researcher as well as decrease contamination of bacterial cultures.

Materials

Laminar-flow type biosafety cabinet equipped with germicidal UV bulb
Spray bottle with 70% ethanol (v/v)
Kimwipes
Latex, vinyl, or nitrile protective gloves
Laboratory coat
Touch-O-Matic (Hanau) bunsen burner
10-µl, 100-µl, and 1000-µl micropipettors
Aerosol-barrier micropipet tips, sterile
Biohazard bag

1. Using the spray bottle of 70% ethanol, lightly mist the bench surface of the biosafety cabinet and wipe with a Kimwipe.
2. With the exception of live bacterial cultures, place all items required for a particular procedure in the cabinet, and lightly mist with 70% ethanol paying special attention to the barrels of the micropipettors.
3. Turn on the UV bulb for 10 min to allow ethanol to evaporate and germicidal action to occur.

   Do not sit in front of the cabinet when the UV light is on as damage to skin and eyes can occur.

4. Put on a long-sleeved laboratory coat and protective gloves.

   Ideally, the laboratory coat should have elastic cuffs to reduce exposure of skin.

5. Outside the cabinet, gently mist from the fingertips of gloved hands to elbows with 70% ethanol.

   Use caution when using ethanol spray bottle around open flame and avoid direct contact with eyes or skin. Do not place spray bottle in the cabinet.

6. Turn off the UV germicidal bulb.
7. Begin inoculation/culture (Basic Protocols 1 to 3), harvesting (Basic Protocol 4), or cryopreservation (Basic Protocol 5) procedures.

   To reduce heat generation within the cabinet, bunsen burners equipped with a pilot light (Touch-O-Matic), should be used and ignited only when in use. Outside of the cabinet, periodically mist hands with 70% ethanol throughout the procedures.

8. Remove live bacteria and ensure that gas to the burner is off.

   Live bacterial cultures should be immediately placed in an incubator. Used culture media, micropipet tips, and any other or potentially contaminated material should be discarded in a biohazard bag and autoclaved.

9. Lightly mist micropipet barrels and the surface of the cabinet bench with 70% ethanol.
10. As in step 5 (above), lightly mist hands and arms. Dispose of gloves in biohazard bag.
11. Finally, expose the cabinet to the germicidal bulb for 10 min as in step 3 (above).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

B. quintana cryopreservation solution

Make a 25% glycerol solution (v/v) with water. Sterilize with a 0.22-μm filter or autoclave for 15 min at 121°C. Store up to 1 year at room temperature.

Brucella broth supplemented with a variable concentration of hemin (BB-hemin) agar plates

Dissolve 14 g BBL Brucella Broth (Becton Dickinson, cat. no. 211088) with 496.8 down to 337.7 ml H_2O (see Table 3C.1.2) in a 1000-ml Pyrex flask. Add 7.5 g Difco Agar (Becton Dickinson, cat. no. 214530) and autoclave for 15 min at 121°C. Cool for ~45 min in a 56°C H_2O bath. Lightly mist outside of flask with 70% ethanol

continued

and place in prepared biosafety cabinet. Add 3.2 to 162.3 ml 5% hemin solution (see recipe) to concentration appropriate for specific simulated environment (see Table 3C.1.2). Pour into sterile petri plates in the cabinet, then pass flame over the surface of agar using a standard bunsen burner to remove bubbles. Replace lid. Allow plates to dry overnight (agar-side down) in the cabinet. Re-sleeve, invert, and store up to 1 month at 4°C.

The approximate formula per liter is 10.0 g pancreatic digest of casein, 10.0 g peptic digest of animal tissue, 1.0 g dextrose, 2.0 g yeast extract, 5.0 g sodium chloride, 0.1 g sodium bisulfite, and 15 g agar.

Also see Strategic Planning.

Heart infusion broth with 4% sheep blood (HIB-B) agar plates

Dissolve 12.5 g of Bacto Heart Infusion Broth (Becton Dickinson, cat. no. 238400) with 470 ml H_2O in a 1000-ml Pyrex flask. Add 7.5 g Difco Agar (Becton Dickinson, cat. no. 214530) and autoclave for 15 min at 121°C. Cool for 45 min in a 56°C H_2O bath. Lightly mist outside of flask with 70% ethanol and place in prepared biosafety cabinet, then add 20 ml defibrinated sheep blood (Quad Five, cat. no. 610), 10 ml sheep serum (Quad Five, cat. no. 662), and swirl to mix. Pour into sterile petri plates in the cabinet then pass flame over the surface of agar using a standard bunsen burner to reduce bubbles and replace the lid. Allow plates to dry overnight (agar-side down) in the cabinet. Re-sleeve, invert, and store up to 1 month at 4°C.

Prepare 500 ml batches rather than 1 liter batches as they are easier to pour in the biosafety cabinet. Also see Strategic Planning.

The approximate formula per liter is 10.0 g beef heart infusion, 10.0 g tryptose, 5.0 g sodium chloride, 15.0 g agar (w/v), 40 ml defibrinated sheep blood (v/v), 20 ml sheep serum (v/v).

Heart infusion broth (HIB)

Dissolve 25 g of Bacto Heart Infusion Broth (Becton Dickinson, cat. no. 238400) with 1000 ml H_2O in a 2000-ml beaker. Dispense 100 ml into ten screw-capped 125-ml Pyrex bottles and autoclave for 15 min at 121°C. Store up to 1 year at room temperature.

The approximate formula per liter is 10.0 g beef heart infusion, 10.0 g tryptose, and 5.0 g sodium chloride.

Hemin solution (5 mg/ml)

Dissolve 0.08 g sodium hydroxide with 100 ml H_2O in a 250-ml beaker. Add 0.5 g hemin chloride and stir to mix. Place in a prepared biosafety cabinet and sterilize with a 0.22-μm filter. Store <2 months at 4°C.

Make 100 ml batches rather than 1 liter batches to maintain freshness. Different volumes of this hemin solution are added to Brucella broth (above) to simulate natural hemin concentrations encountered by B. quintana (see Table 3C.1.2 and Strategic Planning).

The approximate formula per liter is 0.8 g sodium hydroxide and 5.0 g hemin chloride.

Koehler's Bartonella chocolate agar plates

Dissolve 7.2 g GC Agar (Acumedia, cat. no. 7104B) with 100 ml H_2O in a 1000-ml Pyrex flask. Dissolve 2.0 g hemoglobin (Acumedia, cat. no. 7195A) with 100 ml H_2O in a 1000-ml Pyrex flask. Autoclave both flasks for 15 min at 121°C, and then cool for ~45 min in a 56°C H_2O bath. In a prepared biosafety cabinet aseptically reconstitute lyophilized IsoVitaleX medium enrichment with 2 ml diluent per manufacturer's instructions (Becton Dickinson, cat. no. 211875). Lightly mist outside

continued

of flasks with 70% ethanol and place in cabinet. Add hemoglobin and IsoVitaleX enrichment to GC Agar. Swirl to mix, and dispense ~28 ml into petri plates with a sterile graduated pipet. Allow plates to dry overnight in the cabinet. Re-sleeve, invert, and store up to 1 month at 4°C.

Prepare 202 ml batches rather than 1010 ml batches as they are easier to dispense in petri plates in the cabinet. Also see Strategic Planning.

The approximate formula per liter is 7.5 g enzymatic digest of casein, 7.5 g enzymatic digest of animal tissue, 1.0 g corn starch, 4.0 g dipotassium phosphate, 1.0 g monopotassium phosphate, 5.0 g sodium chloride, 10.0 g agar, 10.0 g hemoglobin, 0.01 g Vitamin B_{12}, 10.0 g L-glutamine, 1.0 g adenine, 0.03 g guanine hydrochloride, 0.013 g p-aminobenzoic acid, 0.25 g nicotinamide adenine dinucleotide, 0.1 g thiamine pyrophosphate, 0.02 g ferric nitrate, 0.003 g thiamine hydrochloride, 25.9 g L-cysteine hydrochloride, 1.1 g L-cystine, and 100.0 g dextrose.

COMMENTARY

Background Information

The *Bartonellaceae* are members of the order Rhizobiales, a group of Alpha Proteobacteria responsible for a variety of mammalian (e.g., bartonellosis, brucellosis) and plant (e.g., crown gall) diseases. There are currently twenty species in the genus *Bartonella*, nine of which have been associated with human disease (Rolain et al., 2004; Eremeeva et al., 2007). Although the majority of human *Bartonella* infections are considered zoonoses (Breitschwerdt and Kordick, 2000), maintenance of *B. quintana* in nature is thought to be restricted to humans and body lice *(Pediculus humanus corporis*). Two disparate environmental cues experienced by *B. quintana* in these niches are heme concentration (low in human and high in louse; Battisti et al., 2006) and temperature (35°C to 37°C in human and 30°C in louse; Mellanby, 1932; Marsh and Buxton, 1937).

Trench fever is the common name for the acute febrile syndrome associated with *B. quintana* infection. Chronic manifestations of persistent infection include protracted bacteremia, endocarditis, bacillary angiomatosis, and bacillary peliosis (Maurin and Raoult, 1996), which are coincident with its facultative intracellular lifestyle. Transmission to humans occurs when a crushed louse or louse fecal matter containing the bacterium is introduced into the bloodstream by a breach in the skin caused by itching concurrent with louse infestation. PCR is considered the most sensitive method for diagnosis (La Scola and Raoult, 1999), and infections are treated with different antibiotic regimens depending on the acute or chronic nature of the disease (Rolain et al., 2004).

The overall goal of this unit is to convey basic laboratory maintenance procedures for *B. quintana*, which, in general, can be applied to cultivation and preservation of all *Bartonella.* Recent advancements in genetic manipulation (Fournier et al., 2001; Minnick et al., 2003; Battisti et al., 2006), gene regulation (Battisti et al., 2007), proteome analysis (Boonjakuakul et al., 2007), and genome sequencing (Alsmark et al., 2004) have provided a foundation to better understand the molecular aspects of *B. quintana* pathogenesis. Thus, application of the methods provided in this unit can further explain the virulence mechanisms utilized by *Bartonella,* which will certainly lead to more efficient methods of diagnosis, treatment, and prevention of these emerging human pathogens.

Critical Parameters and Troubleshooting

The most critical issue involved in successful completion of the procedures in this unit is proper aseptic technique. For this reason, detailed methods for preparation of workspace and tools are provided (see Support Protocol). In addition to contamination resulting from hands-on manipulation of cultures in the cabinet, improperly prepared medium is another source of unwanted growth. Incubation of an un-inoculated plate is often included to test the sterility of the medium. If the medium is suspect, re-prepare and ensure that all components have been properly autoclaved or filter-sterilized. Finally, condensation often forms within the lid of the plate during extended incubation, and is a vehicle for contamination. The purpose of the overnight drying period (see Reagents and Solutions*)* is to decrease this occurrence. If a considerable amount of moisture is noticed when inspecting a culture, it should be aseptically decanted.

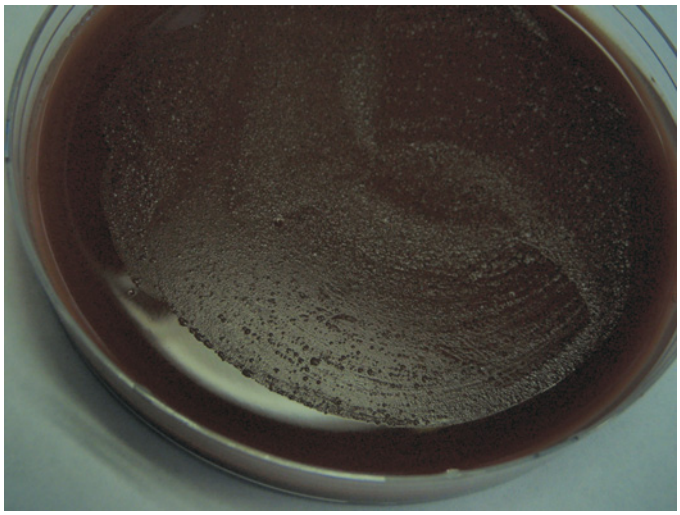

Figure 3C.1.3 Confluent lawn of *B. quintana.* A chocolate agar plate inoculated from a liquid suspension by the spread plate method and incubated in a candle jar for 7 days is shown. Formation of colonies of various sizes within a grainy background sheen is typical. For color version of this figure see *http:www/currentprotocols.com*.

Differentiation of *B. quintana* from other species of bacteria can be achieved at several levels of confidence. First, the colony morphology is described below (see Anticipated Results) and an image is provided (Fig. 3C.1.3). Second, the cellular morphology is best viewed with oil immersion and a phase-contrast microscope at 1000×. The cells are Gram-negative cocco-bacilli, ~0.5-μm wide and 1.5-μm long. Last, PCR and sequence analysis (Koehler et al., 1992; Norman et al., 1995; Minnick and Barbian, 1997) can be employed for genetic identification of the species.

Another challenge in laboratory maintenance of *B. quintana* is variable growth rate, primarily associated with inoculation source and inoculation density. The growth rate of an equal volume of bacteria from freezer stocks can fluctuate, and is often much slower than anticipated. For this reason, it is recommended to inoculate 2 to 3 plates directly from freezer stock, monitor them for growth, and then reinoculate these bacteria onto fresh plates. The second passage of plates is used for the actual experiment, as a more consistent growth rate can be expected. Standard CFU determination and Petroff-Hausser counting chambers (see *APPENDIX 4A*) are routinely used to enumerate cells from a liquid suspension. The BacLight kit (Invitrogen, cat. no., L-7007) can also be used to assist in enumeration and monitor bacterial viability.

Anticipated Results

The growth rate of *B. quintana* can vary greatly depending on the strain, passage number, inoculation source, inoculation density, and culture media. Initial isolation from mammalian blood or decontaminated lice usually requires several weeks of incubation on chocolate agar or HIB-B. Alternatively, colony formation is usually evident in 5 to 7 days from strains that have been grown at least once on HIB-B or chocolate agar.

The colony morphology of *B. quintana* is small (0.2 to 1 mm) and circular, with a convex elevation, even margin, and a translucent-tan color. The color of the colonies can vary with different media. A confluent lawn typically exhibits an opaque sheen with a variety of colony sizes and contains 1×10^7 to 1×10^{10} bacteria (see Fig. 3C.1.3).

Time Considerations

The major portion of hands-on time to complete the basic protocols in this unit is devoted to preparation of growth media. Dissolving the media components (~0.5 hr), autoclaving (~1.0 hr), cooling (~0.75 hr), and dispensing media into plates (~0.25 hr) requires ~2.5 hr. The cooling time can be extended (to ~3 hr) and the overnight drying time can be reduced (to ~3 hr). The major portion of total time for completion of the basic protocols is dedicated to incubation (5 to 21 days; see Anticipated Results). Following preparation of the

workspace and tools (~15 min), the remaining basic protocols listed in this unit (inoculation, harvesting, and cryopreservation) can be completed in <1 hr.

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An extensive and comprehensive review of the epidemiology, clinical manifestations, diagnosis and treatment of B. quintana infections.

Laboratory Maintenance of *Neisseria gonorrhoeae*

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ABSTRACT

Neisseria gonorrhoeae is a human pathogen of mucosal surfaces, thus laboratory manipulations must include appropriate safety measures. The growth requirements and behavior of the gonococcus are significantly different from many bacteria, necessitating modifications of common laboratory techniques. A fastidious organism, *N. gonorrhoeae* requires enriched media in a CO_2 atmosphere at 35° to 37°C for growth. In addition, *N. gonorrhoeae* expresses potent autolysins whose activity increases following glucose depletion during stationary phase, leading to cell death. Long believed to be an obligate aerobe, the gonococcus is capable of anaerobic growth when provided with a suitable electron acceptor. This unit provides information for both aerobic and anaerobic growth, basic long-term and daily maintenance of gonococcal cultures, as well as safety considerations for laboratory studies. *Curr. Protoc. Microbiol.* 8:4A.1.1-4A.1.26. © 2008 by John Wiley & Sons, Inc.

Keywords: *Neisseria gonorrhoeae* • bacterial growth • *Neisseria* media • anaerobic bacterial growth • frozen stock

INTRODUCTION

Neisseria gonorrhoeae ("gonococcus") is an obligate human pathogen, thus all strains are derived from human infections. Best known as the agent of the sexually transmitted disease gonorrhea, this organism readily infects other mucosal surfaces, including the pharynx and eyes, and may cause invasive disease if inoculated into the bloodstream. Thus, appropriate laboratory precautions are needed to prevent laboratory acquired infections following accidental contact with broken skin or procedures that may promote splashes or produce aerosols from gonococcal suspensions. Safety issues specifically related to common laboratory manipulations of gonococci are discussed, including CDC recommendations for *N. gonorrhoeae*.

While *N. gonorrhoeae* can be readily grown in the laboratory, it is a fastidious organism that requires enriched media along with a 3% to 10% CO_2 atmosphere at 37°C for growth, conditions commonly used for clinical isolates. The organism is sensitive to desiccation and cannot survive temperatures <25°C for extended periods of time. In addition, the viability of *N. gonorrhoeae* decreases rapidly in stationary phase, due to enhanced autolysis following the depletion of glucose from the medium. Thus, many techniques used for both long- and short-term growth and storage of most microbes are unsuitable for *N. gonorrhoeae*, such as overnight broth cultures or refrigeration of plate-grown colonies. Detailed protocols are presented for the laboratory growth of *N. gonorrhoeae* under both aerobic and anaerobic conditions. A protocol for a chemically defined medium is also provided for those whose work requires the rigor of exact medium composition.

Neisseria gonorrhoeae is a well-adapted human pathogen, which is demonstrated by the high degree of phase and antigenic variation observed for many surface features of the organism. See *UNIT 4A.3* for detailed information on *N. gonorrhoeae* pilin and Opa variations and their effects on colony morphology.

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CAUTION: Neisseria gonorrhoeae is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Also see Strategic Planning, Biosafety Considerations.

NOTE: N. gonorrhoeae is very fastidious, so it is essential that high-quality distilled and deionized water be used in all recipes and protocol steps.

STRATEGIC PLANNING

Biosafety Considerations

Laboratory associated infections with *N. gonorrhoeae* from contaminated clinical material have been reported in the United States. The primary risk factor centers on the direct or indirect contact of mucous membranes with infectious materials, though the importance of aerosols has not yet been determined. Therefore, the most probable sites of laboratory acquired infections include the pharynx (throat), the conjunctiva (eye surface), and invasive disease acquired through contact with broken skin. *Neisserial* pharyngitis is often asymptomatic, while infection of the conjunctiva rapidly produces a severe, purulent response that can lead to scarring and permanent damage. Infection of the bloodstream, called disseminated gonococcal infection (DGI), initially gives rise to fevers, migratory polyarthralgia (aching joints), and petechial, maculopapular, or pustular rash (skin rash). Without treatment, joints may become infected, leading to septic arthritis often of the elbow or knee. Thus, it is important to seek medical treatment for known accidental exposures or symptoms that may indicate exposure, as gonococcal infections respond readily to standard antibiotic therapy.

In addition, since the risk of aerosols is not yet known, the CDC recommends that production quantities of *N. gonorrhoeae* be handled under BSL-3 containment. The CDC does not qualify what constitutes production level quantities, but does make reference to vaccine production. Thus, the determination for any given set of experiments must be determined through review of the proposed study by the individual Institutional Biosafety Committee.

NOTE: Examples of common laboratory precautions and emergency laboratory procedures for posting in the laboratory are given in Figures 4A.1.1 through 4A.1.3.

Overview of Important Considerations for Laboratory Growth of *N. gonorrhoeae*

Neisseria gonorrhoeae is an obligate human pathogen of mucosal membranes, and is optimized for growth in that moist, nutritionally rich, homogeneous environment with minor temperature and pH variations. Transmission of infection is by direct contact and the gonococcus displays limited ability to survive outside the human host. Thus, the challenge in the laboratory for in vitro growth is to match the conditions of the native environment.

Neisseria gonorrhoeae is characterized as a fastidious organism, requiring enriched media and 3% to 10% CO_2 for initial growth. The CO_2 requirement is not related to anaerobiosis; rather, it is believed to be related to the need for heterotrophs to fix CO_2 to replenish the C_4 dicarboxylic acids for the anaplerotic requirements of the cell.

Many gonococcal clinical isolates are amino acid and/or nucleic acid precursor auxotrophs, traits that are used as epidemiological markers. Due to the known needs of many common laboratory strains of *N. gonorrhoeae*, and the unknown needs of clinical isolates, a rich medium is used for the isolation and growth of gonococci.

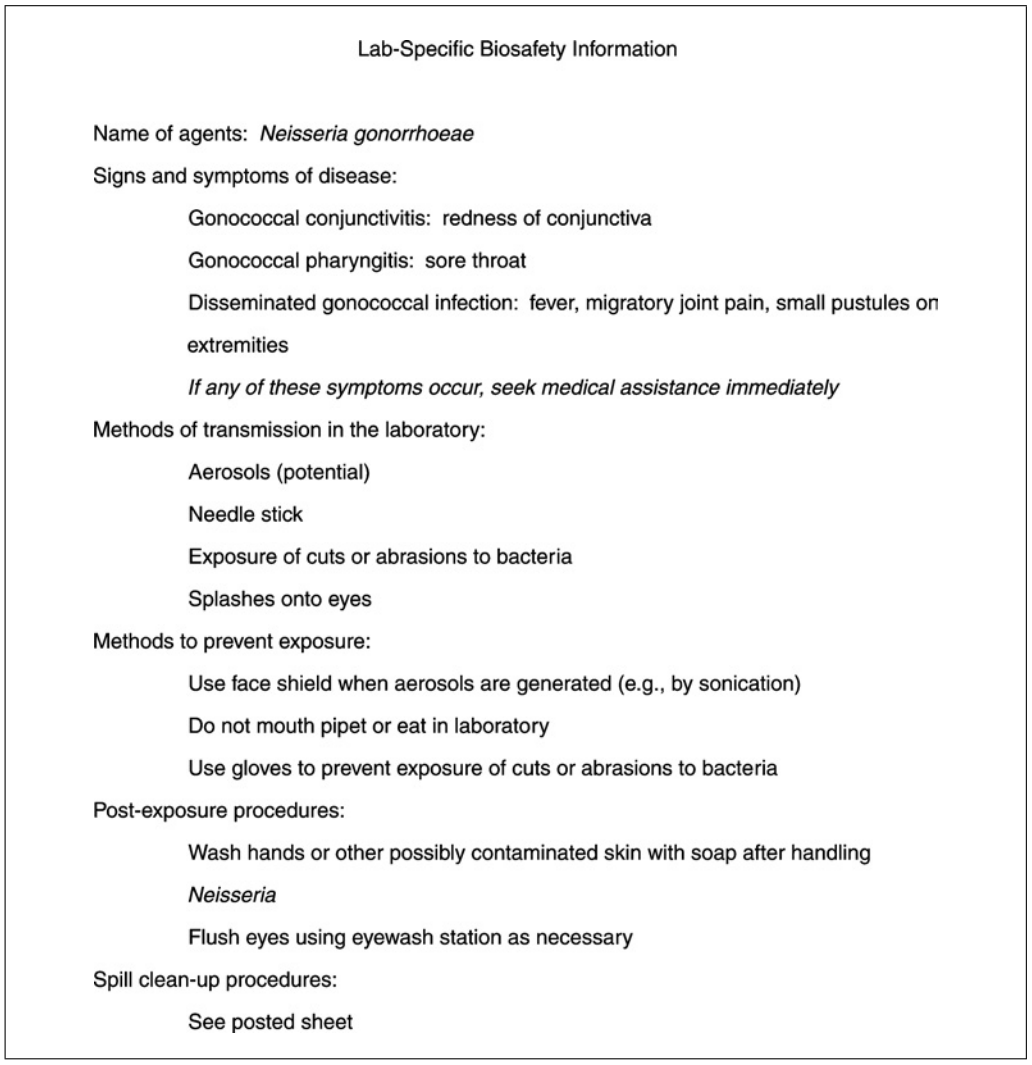
Additional special practices for work with *Neisseria gonorrhoeae* include:

1. A biohazard sign must be posted on the entrance to the laboratory when etiologic agents are in use. Appropriate information to be posted includes the agent(s) in use, the biosafety level, and the investigator's name and telephone number.
2. Biosafety procedures are incorporated into standard operating procedures or in a biosafety manual adopted or prepared specifically for the laboratory by the laboratory director. Personnel are advised of special hazards and are required to read and follow instructions on practices and procedures.
3. The laboratory director ensures that laboratory and support personnel receive appropriate training on the potential hazards associated with the work involved, the necessary precautions to prevent exposures and the exposure evaluation procedures. Personnel receive annual updates or additional training as necessary for procedural or policy changes.
4. A high degree of precaution must always be taken with any contaminated sharp items, including needles and syringes, slides, pipets, capillary tubes, and scalpels. Plasticware should be substituted for glassware whenever possible.
5. The use of needles and syringes or other sharp instruments should be restricted for use only when there is no alternative, such as parenteral injection, phlebotomy, or aspiration of fluids from laboratory animals and diaphragm bottles. Used needle and syringes must be placed in a hard-walled container for transport to a processing area for decontamination, preferably by autoclaving. Needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal.
6. Broken glassware must not be handled directly by hand, but must be removed by mechanical means such as a brush and dustpan, tongs, or forceps. Containers of contaminated needles, sharp equipment, and broken glass must be decontaminated according to any local, state, or federal regulations before disposal.
7. Spills and accidents that result in overt exposures to infectious materials are immediately reported to the laboratory director. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Figure 4A.1.1 An example of a specific biosafety information form required to be posted in the laboratory to comply with the safety requirements of the Institutional Biosafety Committee at the University of Rochester for additional special practices for work with *Neisseria gonorrhoeae*.

CO_2 conditions

The need for enhanced CO_2 to initiate growth of many microbes is well known, especially with the mucosal pathogens. It has been suggested that this mimics the host environment, and is only required initially until the number of microbes in the colony grow sufficiently for their metabolism to generate the needed CO_2. There are several ways to generate the needed $CO_2$to initiate gonococcal growth. Since most liquid cultures are grown in benchtop shaking incubators under room air, a sodium bicarbonate supplement is added to the culture broth to provide the needed CO_2. Pre-incubating the medium in a CO_2 incubator can also provide the needed CO_2; however, this results in more variation than supplementing the medium with sodium bicarbonate. For growth on a solid medium, CO_2 can be provided by the use of a CO_2 incubator, a CO_2-generating GasPak (BBL), or a candle jar. Wheaton Science (*http://www.wheatonsci.com/*) offers a large range of


Lab-Specific Biosafety Information

Name of agents: *Neisseria gonorrhoeae*

Signs and symptoms of disease:

Gonococcal conjunctivitis: redness of conjunctiva

Gonococcal pharyngitis: sore throat

Disseminated gonococcal infection: fever, migratory joint pain, small pustules on extremities

If any of these symptoms occur, seek medical assistance immediately

Methods of transmission in the laboratory:

Aerosols (potential)

Needle stick

Exposure of cuts or abrasions to bacteria

Splashes onto eyes

Methods to prevent exposure:

Use face shield when aerosols are generated (e.g., by sonication)

Do not mouth pipet or eat in laboratory

Use gloves to prevent exposure of cuts or abrasions to bacteria

Post-exposure procedures:

Wash hands or other possibly contaminated skin with soap after handling *Neisseria*

Flush eyes using eyewash station as necessary

Spill clean-up procedures:

See posted sheet

Figure 4A.1.2 An example of a specific biosafety information form required to be posted in the laboratory to comply with the safety requirements of the Institutional Biosafety Committee at the University of Rochester for laboratory specific biosafety information.

suitable, wide-mouth glass jars for use as candle jars. The GasPak system (BBL) is commercially available from BD (*http://www.bd.com/*). It involves the generation of CO_2 from the mixing of sodium bicarbonate with citric acid in a foil-package that is activated by the addition of water. The plates and the CO_2-generating package are placed into an air-tight container (GasPak container). The closed containers are placed into a 37°C incubator overnight to allow gonococcal growth. The candle jar is the precursor to the GasPak. It involves placing the plates into a large, fire-resistant container, traditionally a large glass jar. A small, white, votive candle (unscented) is placed on top of the plates. The candle is lit and the lid tightly closed. The flame will burn until the oxygen in the jar is consumed. At this time, the closed container is placed in a 37°C incubator. The burning candle generates ~3% CO_2, which is sufficient to initiate gonococcal growth.

Starting from frozen stock

If starting the plate culture from frozen stock, it is essential that the stock vial remain frozen. Remove the vial from the freezer and place in an ice-bucket containing crushed dry ice. Use a sterile wooden applicator stick to scrape-up a sample of the frozen stock and place onto a room-temperature GCMB plate. Alternatively, a sterile toothpick can

Emergency Procedures for Spills Involving *Neisseria gonorrhoeae*:
Requires Biosafety Level 2 Containment

1. Alert people in immediate area of spill.
2. Anyone exposed to *N. gonorrhoeae* must clean the affected areas as soon as possible:

   Skin contact/cuts/puncture wounds: wash with soap and water

   Mouth and eyes: flush with water
3. Inform supervisor of exposure.
4. Contact appropriate health service provider for medical attention (name and phone).
5. A properly trained employee must proceed with the clean-up and decontamination of the spill area. Environmental Health and Safety assistance is available by calling Security at (x-xxxx).
6. This person must put on protective equipment (full face shield or mask and safety glasses/goggles, latex gloves, lab coat).
7. Pick out any sharps using tongs or other mechanical means and cover spill with absorbent material such as paper towels.
8. Carefully pour a freshly prepared disinfectant (e.g., 1:5 dilution of bleach in water) around the edges of the spill and then into the spill. Avoid splashing.
9. Allow a 20-min contact period.
10. Use paper towels to wipe up the spill, working from the edges into the center.
11. Clean spill area with fresh paper towels soaked in disinfectant.
12. Place towels into a biohazard bag for disposal.
13. Remove protective equipment and wash hands thoroughly.

Figure 4A.1.3 An example of a specific biosafety information form required to be posted in the laboratory to comply with the safety requirements of the Institutional Biosafety Committee at the University of Rochester for emergency procedures for spills involving *Neisseria gonorrhoeae*.

be used to scrape the frozen stock. Experience has shown that the larger and sturdier applicator stick (i.e., the opposite end of the sterile cotton-tipped swab) does not break as often as toothpicks. The longer applicator stick also appears to minimize contamination of the GCMB plate with *Staphylococci* sp. Therefore, if using toothpicks, wear gloves. Minimize the time the stock vial is held to limit the warming of the vial. The expected viability of gonococcal frozen stocks is not as high as with most other organisms, and the viability decreases with freeze-thaw cycles. This lower viability often necessitates the use of a larger inoculum.

Maintaining anaerobic chamber

Keeping it dry

It is critical to keep the interior of the anaerobe chamber dry. Anaerobic conditions are usually generated by using a palladium catalyst to enhance the reduction of oxygen by hydrogen to form water. The water must be removed from the surface of the catalyst to allow for continued reduction of oxygen. This is often achieved through the use of desiccants. It is very important to maintain the desiccant and catalyst according to manufacturer's directions. Most desiccants have a color indicator that can be used to judge the degree of saturation. Regenerating (usually by baking in an oven or vacuum oven)

the desiccant as soon as the color indicator shows any sign of change is recommended, while the palladium catalyst is dried in a similar manner, at least weekly.

This need for dry conditions means spills in the anaerobe chamber must be cleaned immediately. Spills of media can be as damaging to the functioning of the chamber as spills of cultures. Thus, all chambers should contain disinfectant, either 10% bleach or 70% ethanol in a squeeze bottle, along with towels for blotting and biohazard bags to facilitate removal of contaminated materials through the entry portal.

Measuring function

It is recommended to measure proper functioning of the anaerobe chamber. This can be done by the use of a combined hydrogen and oxygen monitor or anaerobic indicator strips. The use of a dual monitor allows for the assessment of chamber function (oxygen level) as well as monitoring the reducing agent (hydrogen). If sufficient levels of hydrogen are present, the oxygen will be reduced if the palladium catalyst is active. This information aids in troubleshooting of the system if anaerobic conditions are not achieved. Anaerobic indicator strips, which contain methylene blue, are available from BD as part of the GasPak systems. The anaerobic strips become decolorized after 4 to 6 hr in anaerobic conditions.

GCMB Solid Medium

Plate media used for clinical isolation of *N. gonorrhoeae*, including Thayer-Martin, modified Thayer-Martin, Lewis-Martin, and GC-Lect, are chocolate-based media that contain antibiotics to inhibit growth of yeast and bacteria other than gonococci. Most research laboratories use a commercially prepared base media (Difco GC medium–based or BBL GC- or BBL GCII agar–based) for their studies. These recipes are all very similar, using an enzymatic digest of meat proteins or a combination of milk and meat proteins as sources of nitrogen and amino acids. They also include corn starch to bind free fatty acids released by growing gonococci, as these tend to inhibit gonococcal growth at high concentrations. Di- and mono-potassium phosphates are used to buffer the pH of the medium with sodium chloride to maintain osmotic balance. Agar is included in these preparations at a final concentration of 1%, which is a lower concentration than used for most plate media, but empirically determined to enhance the isolation of *N. gonorrhoeae*. See the BD Website (*http://www.bd.com*), maintained by the manufacturer of both Difco and BBL products, for further information on these products.

General considerations

The following is a list of useful tips for preparing GCMB solid medium for culture of *N. gonorrhoeae*.

1. It is important to completely mix GC base media with water prior to autoclaving, as clumps of media stuck to the container tend to burn during the autoclave cycle. Likewise, it is important to remove GCMB from the autoclave at the end of the cycle. Leaving it in the hot autoclave for a prolonged time tends to damage critical components in the media, and the resulting plates will not support gonococcal growth. The soluble corn starch in the media tends to form small flecks. This is not unusual and may be mistaken for contamination. Check by incubating a plate at 37°C to see if the flecks increase in size, as would be expected for microbial contamination.
2. The GC base medium is relatively viscous, so bubbles formed by shaking or vigorous stirring will persist. These bubbles are often the size of the gonococcal colonies and can make it difficult to evaluate growth, especially if performing colony counts. Adding a stir bar to the medium prior to sterilization allows for thorough but gentle

mixing of GC base components prior to pouring plates, as well as mixing supplements added after autoclaving.

3. Supplements are aseptically added after the autoclaved medium has cooled to 55°C. This serves to prevent thermal inactivation of components, as in the case of Kellogg's and certain antibiotics. The Kellogg's supplement, which is stored at 4°C, also needs to be prewarmed so the 4°C solution will not cause localized gelling of the agar.
4. Taping or holding a large stir bar to the outside of the medium container during pouring of the plates will prevent the stir bar in the medium from sliding around in the container, which tends to generate bubbles.

Kellogg's supplement

Douglas S. Kellogg, among others, examined the correlation of colony morphology with virulence. Since colony morphology is dependent on growth media, these studies often included comparisons of media and the effects of different supplementation on *N. gonorrhoeae* growth. One supplement formulation was found to enhance the growth of all tested strains and is now identified as Kellogg's supplement (Kellogg et al., 1963). Often, 500 ml of Kellogg's supplement is made and dispensed into aliquots of multiple bottles during filter sterilization. Kellogg's supplement can be easily contaminated, thus preparing smaller aliquots limits loss due to inadvertent exposure. This 100× solution can be stored at least 6 months at 4°C without loss of activity. The solution is close to saturation for glucose, which makes it somewhat difficult to prepare. It is important to use a large (1-liter) beaker and stir bar to quickly help the glucose into solution and to add the glucose slowly, allowing it to go into solution before adding more. If too much is added at once, the glucose will form a rock-like clump on the bottom of the beaker, which will take a long time to go into solution. Patience is required.

Chemically Defined Medium (CDM)

General considerations

In some cases, it is desirable to determine the exact nutrient requirements of a given strain or to manipulate the type of iron source that can be utilized by a particular strain. The chemically defined medium (CDM) is made from a mixture of several solutions that are prepared and sterilized independently (Morse and Bartenstein, 1980). The CDM stock is often made at 2× concentration, which allows it to be diluted 1:1 with sterile, distilled, deionized water for broth, or 1:1 with sterile 2% to 4% agar for plates. It is recommended to use a high-quality agar to preserve the known concentration of the various components.

While this is a chemically defined medium, it is not a minimal medium and will support the growth of many microbes. Thus, it is advisable to either prepare the exact amount needed, plus allowance for errors, or dispense the 2× CDM into commonly needed volume aliquots during the filter sterilization process.

Iron

Chemically defined medium is often used to test the effect of individual components on bacterial growth. Iron is often a limiting factor in bacterial growth. Thus, testing various types of iron sources addresses the ability of pathogens to utilize iron from the human host.

There are many potential sources of iron that can be used to supplement CDM. Two commonly used iron sources are ferric chloride ($FeCl_3 \cdot 6H_2O$) and ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$). Regardless of the source, a 10 mM stock solution, used at a final concentration of 0.01 mM can be tested with 1× CDM. See Reagents and Solutions for recipes.

Solid medium

It is important to use a high-quality agar when preparing solid medium, such as low-EEO agar for IEF immunological assays, or Noble agar, to prevent the introduction of trace chemicals often found in less-purified preparations.

CDM plates may be soft. It is acceptable to increase the final agar concentration to 2% (w/v) and to chill the plates after pouring to enhance gelling. Warm the hardened plate to room temperature before using to plate *N. gonorrhoeae*.

BASIC PROTOCOL 1

AEROBIC GROWTH OF *N. GONORRHOEAE* ON STREAK PLATES

Most laboratory strains of *Neisseria gonorrhoeae* will form colonies between 0.5 and 2.0 mm in diameter on GCMB plates following overnight incubation with 3% to 10% CO_2 at 37°C. However, a high proportion of cells that have reached stationary phase will be dead due to autolysis, which occurs in gonococci at an accelerated rate following the exhaustion of glucose. Thus, there are some factors that aid in the selection of a high proportion of viable cells for additional studies.

Since the viability of the initial inoculum is often low, it usually is not necessary to heat sterilize the loop between spread-quadrants to achieve isolated colonies. It is also very likely that cells in well-isolated colonies that are 1 to 2 mm in diameter are primarily dead. Experience has shown that the highest viability of cells is found in the colonies that are very close together but not confluent (see Fig. 4A.1.4). These cells were not

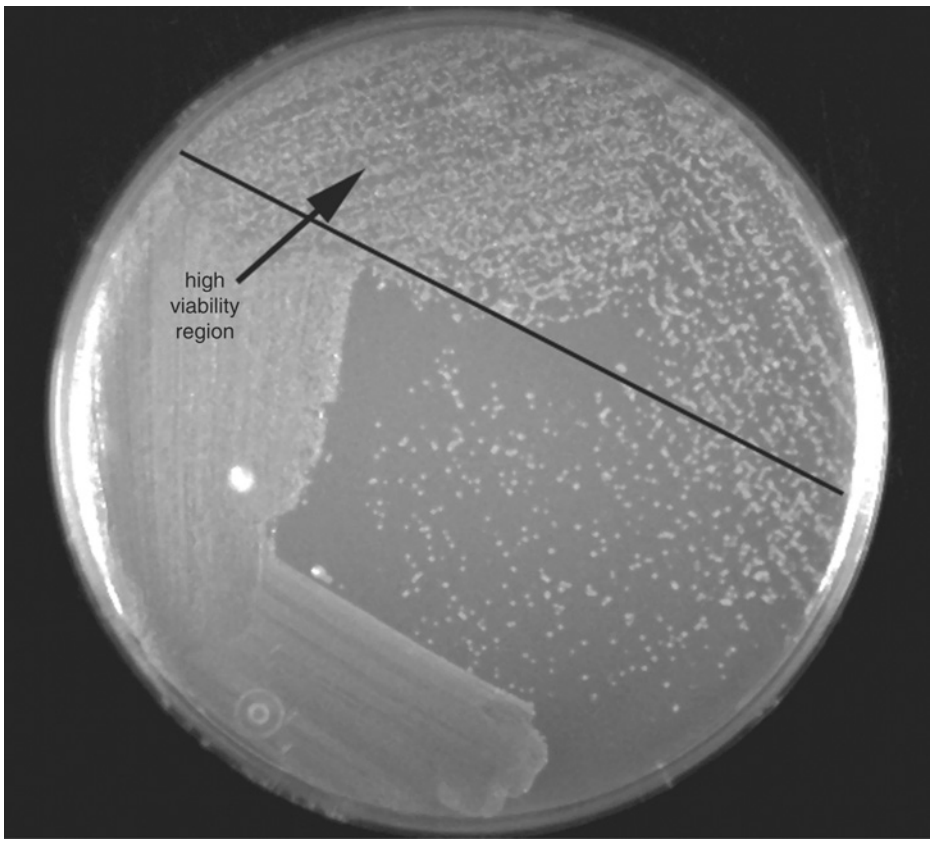


Figure 4A.1.4 Overnight growth of *N.gonorrhoeae* strain F62 on GCMB plate. The ~16-hr culture of F62 shows regions of colony growth that are considered very closely spaced but not confluent. Cells in these colonies tend to have high viability and thus are preferred for use in subsequent experiments.

able to grow as fast as those that produced well-isolated colonies, due to competition for nutrients. Thus, the majority of cells in these small but not confluent colonies have not yet reached stationary phase, and maintain viability. The ability to grow large numbers of viable colonies on plates is critical, as broth cultures of gonococci will not survive an overnight incubation. Thus, scraping cells off plate cultures is the only method to generate large numbers of gonococci for study.

Materials

Gonococci inoculum
GCMB plates (see recipe for GCMB solid medium), at room temperature

Microbiological loop or sterile cotton-tipped applicator
37°C incubator with 3% to 10% CO_2 flow or CO_2-generating GasPak or candle jar (Wheaton cat. no. W216938) with candle
Sterile wooden applicators (opposite end of cotton-tipped swab) or flat tooth-picks, sterilized

1. Using a sterile loop or cotton-tipped applicator, place a small inoculum of gonococci on the edge of the medium. Spread to cover no more than 1/6 of the plate, keeping it near the edge.

2. Without heating the loop, start the second quadrant streak, entering the initial inoculum multiple times. Turn the plate and repeat two times to streak the entire plate. See Figure 4A.1.5.

   Oftentimes, it is not necessary to perform the standard microbiological four-quadrant streak method (see APPENDIX 4A), as there are few colonies found in the fourth quadrant. Thus, depending on the style of the individual, streaking in thirds often generates more viable gonococcal colonies per plate.

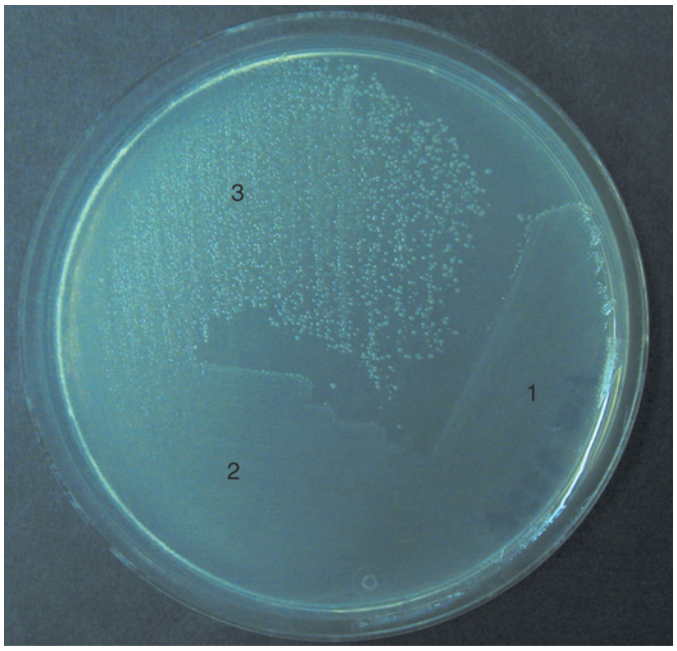


Figure 4A.1.5 Optimal streaking pattern for *N. gonorrhoeae* studies. Standard microbiological plating instructions are designed to provide well-isolated colonies. Since *N. gonorrhoeae* rapidly die following localized depletion of glucose due to rapid growth, streaking patterns are modified to increase the regions of growth constrained colonies to provide a large number of colonies with high viability.

3. If using the cotton-tipped swab, turn the applicator to use a fresh portion of the tip to streak out each of the remaining quadrants.

4. Invert the plates and incubate overnight at 37°C with 3% to 10% CO_2.

   The gonococcal growth will be viable the next day, but the viability will decline rapidly over the next 12 to 24 hr, depending on the strain. It is best to use fresh gonococcal plate cultures for each day.

   A technique to generate large amounts of small but not confluent colonies is to sub-culture late in the afternoon. Spread the initial inoculum, followed by filling the rest of the plate with tightly spaced streaking, each entering the initial inoculum. The majority of these colonies will be small but not confluent if used within 15 to 18 hr of growth. The relatively short incubation time coupled with the close spacing of the cells, to generate competition, will result in a high proportion of the growth with good viability.

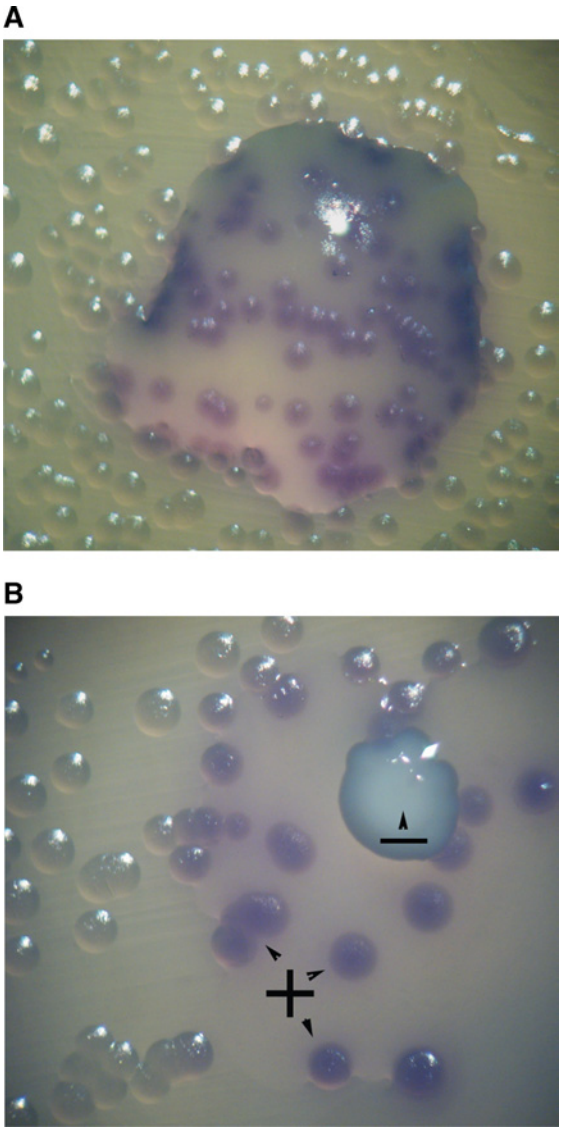


Figure 4A.1.6 Oxidase assay used for presumptive identification of *Neisserial* colonies. **A** shows a GCMB plate of *N. gonorrhoeae* with a small drop of tetramethyl-*p*-phenylenediamine solution. The oxidase-positive colonies rapidly turn pink-purple. **B** shows both oxidase-positive (+) and oxidase-negative (–) colony types. This presumptive assay is used to identify oxidase-positive colony types. Note that tested colonies cannot be used for subculturing or other assays but serve to confirm that a particular colony morphology type has a high probability of being *N. gonorrhoeae*. For a color version of this figure, see *http://www.currentprotocols.com*

OXIDASE TEST

SUPPORT PROTOCOL 1

Colony morphology can vary significantly in *N. gonorrhoeae*, and can be mistaken for contamination by other organisms. A quick method to presumptively identify a colony as *N. gonorrhoeae* is the oxidase test. A freshly prepared 1% di- or tetramethyl-*p*-phenylenediamine solution will react with *Neisseriae*, and turn the colony color pink to purple (see Fig. 4A.1.6). This rapid assay can be performed in a variety of ways. A colony can be picked up onto a swab and then a drop of reagent is applied directly onto the colony on the swab and monitored for color change. Alternatively, a small amount of the reagent can be added directly to the questioned colony type on the culture plate, being careful to limit the spread of the reagent on the plate. Once an oxidase-positive colony type has been identified, the other identical colonies on the plate from non-tested regions can be used with high level of assurance that they are *N. gonorrhoeae*. While some protocols call for a freshly prepared 1% solution, a toothpick full of di- or tetramethyl-*p*-phenylenediamine in 0.5 ml deionized water in a microcentrifuge tube has been found to work well. In addition, some methods require DMSO to solubilize di- or tetramethyl-*p*-phenylenediamine and 2 to 3 days bacterial growth; these protocols are not specific for GC, which are strongly oxidase positive.

CATALASE TEST

SUPPORT PROTOCOL 2

A common contaminant that gives rise to colonies very similar in appearance to GC is *Streptococcal* sp. A rapid method to distinguish these contaminants from GC is the catalase test, as GC are strongly catalase positive while *Streptococcal* sp. are catalase negative. Catalase is a protective enzyme that converts hydrogen peroxide into water and oxygen. In this very simple test, a drop of hydrogen peroxide (3% to 30%) is placed onto a slide and a sample of colony is dragged through this drop, and the immediate formation of bubbles indicates the presence of catalase (see Fig. 4A.1.7). Care must be taken if the colony is obtained from blood or chocolate plates as erythrocytes contain catalase and carryover from medium can give rise to a false-positive test.

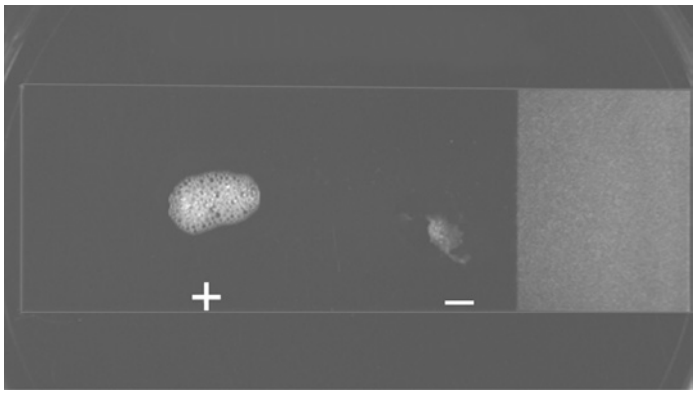


Figure 4A.1.7 Slide catalase assay. Samples of two different colonies were spread on a glass microscope slide. A drop of hydrogen peroxide was placed on each streak. Note the extensive bubbling from the *N. gonorrhoeae* (+) sample.

ESTABLISHING BROTH CULTURES FOR THE AEROBIC GROWTH OF *N. GONORRHOEAE*

BASIC PROTOCOL 2

The growth of *N. gonorrhoeae* in broth requires special consideration. The medium (GCP) is equivalent to GCMB but without agar. Since most shaking incubators are in ambient air, the CO_2 requirement is provided by adding sodium bicarbonate to the broth. *N. gonorrhoeae* may not be well-suited for growth in liquid, as it is a pathogen of mucosal surfaces. This is reflected as poor growth in broth cultures relative to most organisms.

Broth cultures require a relatively high initial inoculum, which may be related to the need for CO_2, as the time in lag-phase is dependent on CO_2 concentration (see Strategic Planning for a discussion of CO_2 conditions). In addition, the growth pattern of different gonococcal strains varies, depending on the expression of pili and other surface adherence factors. Thus, some strains will generate smooth suspensions while others tend to form grainy cultures.

Materials

Supplemented GCP (see recipe)
Overnight plate culture of *N. gonorrhoeae* (see Basic Protocol 1)

3- to 10-ml sterile tubes
Sterile culture flask (volume five times that of broth culture)
Sterile cotton-tipped swab
Spectrophotometer capable of reading at 600 nm and cuvettes
37°C shaking incubator

1. Transfer 1 to 3 ml of supplemented GCP into a 3- to 10-ml sterile tube, and the desired amount into the culture flask.
2. Use a sterile cotton-tipped swab to remove gonococci from the overnight plate(s).

   Be sure to use the small but not confluent colonies from the overnight plate culture to ensure high viability in the inoculum. Some prefer to pre-moisten the swab in the GCP before scraping up the gonococci, as it may make it easier to resuspend the cells from the swab.

3. Vigorously agitate the swab in the tube containing the 1 to 3 ml GCP to create an inoculating suspension of gonococci.

   Do not use a tube that is filled more than 1/3 total height by the GCP, as head-space is needed to allow for vortexing without splashing the gonococcal suspension out of the tube. A capped tube is highly preferred for extra protection.

4. Vortex the suspension at high speed to make a smooth mixture of the gonococci, taking precautions to avoid splashing.
5. Add the inoculating suspension, in small additions, to the GCP in the culture flask until the desired density is reached.

   Many strains of gonococci will not grow if the initial concentration of gonococci, as measured by OD_{600} of the culture, is <0.1, and some may require much higher initial inoculums. Keep the volume of culture to no more than 20% of the flask volume to ensure appropriate aeration of the culture. Successful exponential growth of F62 with initial starting cultures at $OD_{600} = 0.07$ to 0.09 has been obtained, reaching mid-log ($OD_{600} = 0.5$) within 2.5 hr growth.

6. Place the flask on a 37°C shaking incubator at relatively high speed (200 to 250 rpm).
7. Allow the culture to grow until the desired density is reached. Acquire intermediate OD_{600} readings (every hour) to ensure that the culture is growing appropriately, i.e., starting exponential phase within 3 hr of growth.

   The culture will start to lyse if allowed to go into stationary phase for any length of time (often within 1 hr). For the laboratory strain F62, OD_{600} readings of 1 to 1.2 are typical of early stationary phase. The length of time in which it takes a broth culture to reach this level of growth depends both on the strain and on the viability and size of the initial inoculum. Quite often, stationary phase is obtained within 8 to 10 hr of growth from an initial culture OD_{600} of ~0.1. Failure to enter exponential growth may indicate that the initial inoculum was too small, or that its viability was low.

8. Subculture growing broth cultures into fresh complete GCP, however, avoid over-diluting new cultures. Keep the density over $OD_{600} > 0.05$, usually no more than a 1:20 dilution. Grow with shaking at 37°C.

ANAEROBIC GROWTH OF *N. GONORRHOEAE* ON SOLID MEDIUM

BASIC PROTOCOL 3

N. gonorrhoeae was long believed to be an obligate aerobe until it was discovered that nitrite could serve as a terminal electron acceptor, allowing growth under anaerobic conditions (Knapp and Clark, 1984). A technical problem was how to effectively deliver a sub-lethal dose of nitrite. The solution was to place the nitrite on a disc and allow it to diffuse into the medium. This generates a gradient of nitrite in the medium and allows the gonococci to 'select' the preferred concentration for growth. This technique is detailed here.

An effective method to grow *N. gonorrhoeae* anaerobically in broth has not yet been discovered. There have been studies using microaerobic broth, established by either completely filling the culture container with broth (to minimize air space) or capping the culture or by initiating the culture aerobically, then stopping all agitation of the culture (Knapp and Clark, 1984). This loss of motion coupled with the poor solubility of oxygen in liquids will establish an oxygen gradient in the culture. Neither system has been particularly reproducible, therefore, they are not provided in this unit. Contact the authors for more information concerning these techniques (ginny-clark@urmc.rochester.edu).

Materials

GCMB solid medium (see recipe)
Fresh (1-day-old) *N. gonorrhoeae* plate culture
GCP broth (see recipe; non-supplemented GCP is suitable for the temporary resuspension of GC)
70% ethanol
20% sodium nitrite solution, sterilized (see recipe)
100-mm plates
3- to 10-ml sterile tubes with caps
Sterile cotton-tipped swabs
Sterilized glass spreaders (*APPENDIX 4A*)
Tweezers or forceps
Bunsen burner
Paper discs, $\frac{1}{2}$-in. diameter, sterilized (Schleicher and Schuell 740-E)
Anaerobe chamber containing 37°C incubator (highly preferred) or 37°C incubator with H_2/CO_2-generating GasPak (BBL) (BD cat. no. 260678)

1. Pour thick GCMB plates using ~30 ml GCMB solid medium per 100-mm diameter plate (~30 to 36 plates per liter).

   It is important to use fresh (1-day-old), room-temperature, thickly poured GCMB plates, as the anaerobic environments tend to be dehydrating, and normal GCMB plates dry out too much to support gonococcal growth. Allow plates to sit on the benchtop overnight, do not bag or refrigerate.

2. Start a subculture of the gonococci, grow overnight aerobically on GCMB (see Basic Protocol 1).

   Results are more reproducible if the gonococci have been subcultured at least once from frozen stock.

3. Place 1 to 3 ml GCP into a 3- to 10-ml sterile tube, preferably one with a cap.

4. Use a sterile cotton-tipped swab to remove the small but not confluent colonies from the overnight aerobic plate culture of gonococci.

   If the swab is pre-moistened in the GCP before scraping up the gonococci, it may be easier to resuspend the cells from the swab.

5. Vigorously agitate the cotton-tipped swab with gonococci colony in the tube containing the 1 to 3 ml GCP to produce an inoculating suspension of gonococci.

6. Vortex the suspension at high speed to create a smooth mixture of the gonococci, taking precautions to avoid splashing.

   It is desirable to have the concentration at an OD_{600} of ~1.0 to 1.5.

7. Add 100 µl of the gonococci suspension to each plate. Evenly distribute the inoculum by spreading with a sterile glass spreader.

   This method is preferred because spreading the gonococcal suspension with a swab often scratches the medium. These scratches make recovery of the anaerobically prepared gonococci more difficult, as the organisms appear to stick in the scratches.

   Alternatively, heavily inoculate the plates by dipping a fresh, sterile swab into the gonococcal suspension and use it to spread a heavy inoculum evenly across the fresh GCMB plate.

   Spread the inoculum over the plate by streaking the wet swab completely over the plate in one direction, turn the plate 120° and spread again, then turn and spread a third time. This will provide an even suspension of gonococci on the plate. A wet swab is used to limit scratches in the medium and provide a suitable number of gonococci. Allow the plates to dry until the gonococcal suspension is completely absorbed.

8. Dip the tweezer tips into 70% ethanol and flame to sterilize.

9. Use the tweezers to place a sterile $^1/_2$-in. diameter paper disc onto the center of the inoculated GCMB plate.

   Gently tap the disc onto the surface with the tweezers tip to achieve good contact with the medium. This is beneficial if the medium surface is very wet and the disc is sliding on the inoculum. Alternatively, tap the disc onto the plate with a pipet tip used to dispense the nitrite solution.

10. Slowly place 40 µl of 20% sodium nitrite solution onto the paper disc.

    Care must be taken to avoid adding the fluid faster than the disc can absorb it, or the disc will float on the surface of the liquid and shift position on the plate. It is also important to add the nitrite to the disc before it absorbs too much water from the medium, which then limits nitrite absorption. Setting discs onto no more than five to ten plates before adding nitrite is recommended. Allow the nitrite to fully absorb into the disc prior to inverting the plates to prevent the discs from moving.

11. Place all the prepared plates (inverted) into the container for incubation in the anaerobe chamber and load into the entry portal. Alternatively, place the inverted plates into the GasPak container, and activate the H_2/CO_2-generating GasPak.

    In some cases, the gas flow into the entry portal is fast and can move the items in the portal. If the plates are in a container, they are less likely to be tipped. However, make sure the lid is placed on the container in an offset manner, as the lid can become vacuum sealed to the container during the evacuation portion of the cycle. A permanent solution to the vacuum-lock problem is to place a small hole in the lid of the container to allow for gas exchange.

12. Cycle entry portal to switch the atmosphere to 85% N_2, 10% H_2, and 5% CO_2, according to manufacturer's instructions.

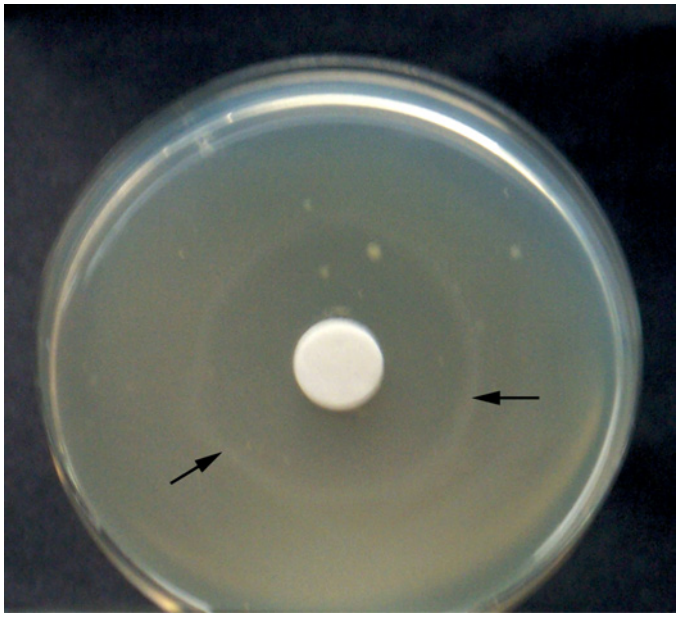


Figure 4A.1.8 Plate of anaerobically grown *N. gonorrhoeae*. A GCMB plate was inoculated with *N. gonorrhoeae* and incubated anaerobically as described in Basic Protocol 3. Arrows indicate the ring of strong *N. gonorrhoeae* growth at the optimal nitrite concentration surrounding the paper disc. Note how the region between the disc and the denoted growth ring is clear and devoid of growth. *N. gonorrhoeae* growth typically extends outward beyond the ring of optimal growth for ~0.5 to 1 in. This is best observed by tipping the plate to find the optimal lighting to show the differences in the medium surface reflectivity due to bacterial growth.

13. Open the internal portal door, remove the plates from the entry portal and place in the 37°C incubator.

    Placing the plates into a container allows easier group handling in the glove box. The container also helps to limit dehydration of the plates during incubation.

14. Check for growth in 24 to 42 hr, depending on the gonococcal strain.

    The anaerobic growth of gonococci does not take the form of colonies, but rather appears as a hazy ring of growth somewhere between the nitrite disc and the outside edge of the plate (Fig. 4A.1.8). This growth is very light and is often discernable as a change in the reflective nature of the medium surface. The nitrite diffuses from the disc and creates a radial concentration gradient in the medium. The area nearest the disc has a lethal concentration of nitrite, so there will be no gonococcal growth near the disc. The gonococci will grow at the optimum nitrite concentration, below toxic levels but yet sufficient to adequately serve as the electron acceptor; see Figure 4A.1.8.

SUBCULTURING ANAEROBIC CULTURES

BASIC PROTOCOL 4

In some cases, it is desirable to regrow the gonococci under anaerobic conditions. Experience has shown that certain measurements of gonococcal physiology are much more consistent when performed on gonococci subcultured anaerobically three times. Thus, it may be important to determine the consistency of the gonococcal behavior as related to anaerobic growth. While it may be preferred to keep gonococci under anaerobic conditions at all times once grown anaerobically, it may be necessary to perform the actual subculture on the benchtop, as would be the case if using the GasPak anaerobic system. Aerobic subculturing of anaerobically grown gonococci often shows no detrimental effect, although this should be tested for any given procedure. It is important to perform the subculture quickly and return the plates to anaerobic conditions. If many plates are involved, the workload should be divided into small units so any given plate is rapidly returned to anaerobic conditions.

Set up the anaerobe chamber so that the instruments and reagents are easily reached. The working space in the anaerobe chamber is often limited, so it is important to maintain a clutter-free area for viewing and manipulating the cultures.

Materials

24- to 42-hr anaerobic gonococcal culture
GCP stock (see recipe)
GCMB plates (see recipe for GCMB solid medium)
20% sodium nitrite solution, sterilized (see recipe)
Squeeze bottle with 70% ethanol or 10% bleach

Anaerobe chamber containing 37°C incubator stocked with:
Pipets and sterile tips
Waste container lined with biohazard bag
Sterile swabs
Paper discs, ½-in. diameter, sterilized (Schleicher and Schuell 740-E)
Tweezers or forceps
Rack to hold tubes, tweezers, marker
Pipet aide
Container for holding inoculated plates
Lab-wipes or paper towels
3- to 5-ml sterile tubes
Mechanical vortexer
Individually wrapped sterile glass spreaders or disposal sterile plastic
Bacteriological loops, optional

1. Remove 24- to 42-hr anaerobic gonococcal plate cultures from the incubator, and identify the ring of gonococcal growth on each plate.
2. Place 1 to 1.5 ml GCP stock broth into a 3- to 5-ml sterile tube.
3. Use a sterile swab to remove the growth ring and resuspend gonococci in the tube containing GCP stock. Agitate swab vigorously and vortex to create a smooth suspension of gonococci, being careful to avoid splashing.
4. Add 100 µl of this GC suspension to a fresh thickly poured GCMB plate.
5. Gently spread with a fresh sterile swab, evenly spreading the suspension in three directions as described in Basic Protocol 3, step 7.

   Alternatively, use autoclaved, individually wrapped glass spreaders or pre-sterilized plastic spreaders. Glass spreaders cannot be sterilized by dipping in ethanol and burning off the alcohol, as the anaerobic environment will not support a flame, therefore, place a sufficient number of sterilized glass spreaders in the chamber for the procedure. One spreader can be used to spread multiple plates inoculated with the same gonococcal suspension if care is taken to avoid contamination.

   An alternative is to use sterile disposable bacteriological loops. These plastic loops can be bent into spreaders by gently pressing the loop end onto the inside of the plate top. This will cause the thinner, ~2 to 3 in. of the loop end to bend at an angle to the handle, which can then be used to evenly spread the inoculum.

6. Prepare the desired number of plates.

   Approximately eight to ten new plates are obtained from every overnight anaerobic culture plate.

   The rate of anaerobic growth is much slower than aerobic growth. To approximate the number of anaerobic plates needed for a given protocol, assume that under anaerobic conditions, 5% to 10% of the gonococci will be obtained as compared to aerobic growth.

7. Use the tweezers to place a sterile paper disc onto the center of the inoculated GCMB plate. Tap to set the disc.

   The tweezers must be sterilized to avoid contaminating the plates. Options include the use of a ceramic incinerator to heat-sterilize the tweezer tips in the anaerobe chamber or to sterilize the tweezers prior to entering the anaerobe chamber, and maintaining sterility by placing them into sterile tube.

   Do not add the discs to too many plates at once, as the paper will absorb water from the inoculated plate. Once the paper is saturated, it cannot absorb the nitrite solution, which will then run out onto the plate.

8. Slowly add 40 μl of 20% sodium nitrite solution onto the paper disc.
9. Let the 20% sodium nitrite solution absorb into the disc before inverting plates so that the disc does not slip from the center of the plate.
10. Invert plates and place into the incubator for 24 to 42 hr.

    A quick and easy way to place inverted plates into the container is to place the container lid on the floor of the anaerobe chamber, top down. Place the plates on the lid, right side (tops) up. Place the bottom of the container over the plates and onto the lid. Slide a hand under the lid and carefully turn the container right-side up. This works best if the plates fill the container. If there are not enough to fill the container, add empty plates to the stack.

LONG-TERM STORAGE OF *N. GONORRHOEAE*

BASIC PROTOCOL 5

In this respect, the maintenance of *N. gonorrhoeae* differs the most from other microbes, as long-term in the *Neisserial* world refers to anything over 2 days. Laboratory stocks of *N. gonorrhoeae* are frozen (−70° to −80°C) in a 25% glycerol solution. Preparing *N. gonorrhoeae* strains for long-term storage is a very easy process, which is advantageous since it needs to be performed quite often. Since *N. gonorrhoeae* modulates its surface features quite frequently through both phase and antigenic variation, it is important to freeze the representative mixture of gonococcal colony types for that particular strain, and to return to this original stock for fresh working material. As the original frozen stock vial is removed and returned to the freezer on a weekly basis, it is important to keep both the original stock vial frozen at all times, and to prepare multiple stock vials from the original stock, taken from an early passage with a representative mixture of colony types.

Variations in piliation and expression of opa proteins give rise to significant differences in colony morphology (see Fig. 4A.1.9). It is often desirable to maintain the complete range of colony types in stock cultures. However, the efficiency of transformation is substantially higher in piliated strains, thus their inclusion in stock cultures is essential if the strain is to be used for genetic experiments. While it is possible to select for non-piliated variants from a predominantly piliated stock, in many strains, the reverse is not feasible, as loss of pili is often due to loss of the pilin expression site (P_E) in the chromosome. See *UNIT 4A.3* for information on discriminating piliated from non-piliated *N. gonorrhoeae*.

Never allow the frozen stock sample to thaw, as the viability of the gonococci will rapidly decline. Always place the frozen stock vial in dry ice when taken out of the freezer, and minimize the time that the vial is held in hand to limit thawing while removing samples to maintain a suitable viability of the stock culture. When the viability appears to be significantly diminished, either switch to a second stock vial or prepare additional stocks from a fresh culture prepared from the original stock.

Materials

GCP stock (see recipe)
18- to 24-hr culture of *N. gonorrhoeae*
50% glycerol (see recipe), autoclaved
Dry ice

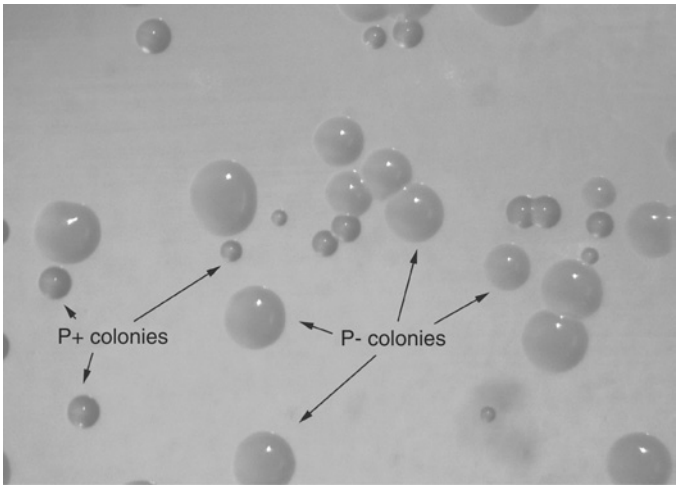


Figure 4A.1.9 Photograph of piliated and non-piliated *N. gonorrhoeae*. Pilin expression can undergo phase variation and is reflected in variation in colony morphology. Piliated (P+) cells form colonies that tend to be smaller, taller, and darker (gumdrop-like) when viewed under a stereoscope while non-piliated (P–) cells form colonies that take a flatter, more wafer-like appearance (Kellogg et al., 1968).

3- to 5-ml sterile tubes
Sterile swab
Spectrophotometer and cuvettes
2-ml cryovials, sterilized
Ice bucket or other suitable container for holding dry ice

1. Place ~2.5 ml of GCP stock broth into a 3- to 5-ml sterile tube.
2. Dip a sterile swab into the GCP stock and rim the swab on the inside edge of the tube to wring out excess broth.
3. Use the wet swab to collect the gonococci colonies from the 18- to 24-hr *N. gonorrhoeae* culture plate, collecting the small but not confluent colonies from the culture.
4. Resuspend the gonococci into the GCP stock broth. Repeat if necessary until the approximate density of the gonococci suspension is equivalent to an $OD_{600} = 1$.
5. Label a cryovial tube using an indelible marking pen.

   It is very difficult to write on frozen tubes, so label first.
6. Add 0.75 ml of sterile 50% glycerol to the cryovial.
7. Add 0.75 ml of gonococcal suspension to the cryovial, cap, and vortex to mix.

   This generates a 25% glycerol suspension of gonococci at an ~OD_{600} of 0.5.
8. Place the cryovial into pulverized dry ice to snap-freeze the sample.

   An ethanol- or acetone-dry-ice mixture can also be used to snap freeze the sample, but the organics tend to remove the ink labeling from the cryovial.
9. Place the frozen sample at –70° to –80°C.

   How long any given stock remains viable varies significantly with the quality of the initial frozen culture, the number of times the frozen stock is accessed, and the degree of thawing that occurred with each handling. Viable N. gonorrhoeae from frozen stocks that were well over 10 years old have been retrieved.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acid-soluble amino acid solution

Dissolve 540 mg L-cystine in 50 ml 1.0 N HCl. Store up to 6 months at −80°C. Discard if precipitates form upon thawing.

Base-soluble essential component solution

To 100 ml of 1.0 N NaOH, add:
750 mg L-hypoxanthine
1065 mg L-tyrosine
1200 mg L-tryptophan
750 mg uracil
Mix until dissolved
Store up to 6 months at −80°C

Calcium chloride, 250 mM

Add 367 mg $CaCl{\cdot}2H_2O$ to 100 ml ddH_2O. Mix to dissolve. Filter sterilize (0.22-μm filter) and store up to 1 year at room temperature.

CDM, 2×

For 1 liter, mix the following:
50 ml CDM stock solution 1 (see recipe)
360 ml CDM stock solution 2 (see recipe)
20 ml CDM stock solution 3 (see recipe)
40 ml CDM stock solution 4 (see recipe)
50 ml 20% (w/v) glucose solution (see recipe)
Add ddH_2O to 800 ml total volume
Adjust pH to 7.4 with 10 N NaOH
Add 2 ml 10 mM ferric chloride ($FeCl_3{\cdot}6H_2O$) or ferric nitrate ($Fe(NO_3)_3{\cdot}9H_2O$) (see recipes)
Add ddH_2O to a final volume of 1 liter
Add 2 ml 250 mM $CaCl_2$ (see recipe; final concentration 0.25 mM)
Sterilize by passing through a 0.2-μm filter sterilization system with sterile storage container
Transfer into appropriate aliquots and store up to 1 week at 4°C

Refer to Table 4A.1.1 for a brief explanation of the component stock solutions.

It is important to add the $CaCl_2$ to diluted CDM as it has a tendency to precipitate. Adding 2 ml to 1000 ml does not significantly alter the concentrations of the final medium.

There are many potential sources of iron that can be used to supplement CDM. Two commonly used iron sources are ferric chloride ($FeCl_3{\cdot}6H_2O$) and ferric nitrate ($Fe(NO_3)_3{\cdot}9H_2O$); see Strategic Planning. Regardless of the source, a 10 mM stock solution, used at a final concentration of 0.01 mM can be tested with 1× CDM.

Table 4A.1.1 CDM Stock Solutions and their Purpose

Stock solution	Purpose
CDM stock solution 1	Salts solution
CDM stock solution 2	Amino acid solution
CDM stock solution 3	Vitamins/biotin solution
CDM stock solution 4	Phosphate solution

CDM, liquid

Dilute 2× CDM 1:1 with sterile ddH_2O and add 1/100 volume 4.2% $NaHCO_3$. Prepare fresh.

CDM, solid

For plates, prepare 3% agar (3 g agar to 100 ml H_2O), autoclave, and cool to 55°C. Mix the agar 1:1 with 2× CDM stock (see recipe; warmed to 55°C), then pour plates.

CDM stock solution 1

To 150 ml ddH_2O, add:
58.45 g NaCl
10 g K_2SO_4
2.18 g $MgCl_2 \cdot 6H_2O$
2.2 g NH_4Cl
30 mg EDTA·Na_2
Stir until dissolved and transfer to 250-ml graduated cylinder
Add ddH_2O to 250 ml total volume
Sterilize by autoclaving at standard conditions
Store up to 6 months at room temperature

CDM stock solution 2

Prepare three intermediate amino acids solutions (acid, base, and neutral soluble essential component/amino acid solutions; see recipes) and mix together to make CDM stock solution 2 to accommodate the different conditions necessary to dissolve the various amino acids and other essential components. This is summarized in Table 4A.1.2.

To combine the acid-, base-, and neutral-soluble amino acid solutions: To 2000 ml neutral-soluble amino acid solution, add 50 ml acid-soluble amino acid solution (L-cystine), and 100 ml base-soluble essential component solution. Add ddH_2O to yield 2700 ml total volume. Filter sterilize. Aliquot into 50-ml tubes. Store up to 6 months frozen at –80°C.

A volume of 40 ml per tube is convenient, as 360 ml is needed per liter of 2× CDM.

Avoid repeat freeze-thaw cycles, as the amino acids tend to precipitate and CDM made with precipitated amino acid solution does not support the growth of gonococci.

CDM stock solution 3

Dissolve the following reagents in 300 ml 95% ethanol:
120 mg thiamine·Cl
28.8 mg Co-carboxylase
180 mg biotin
114 mg D-pantothenic acid
Add ddH_2O to yield 600 ml final volume
Filter sterilize using a 0.22-μm filter
Store up to 1 year at –20°C

Refer to Table 4A.1.3 for component concentrations.

CDM stock solution 4

To 100 ml ddH_2O, dissolve:
13.6 g KH_2PO_4 (1 M)
17.4 g K_2HPO_4 (1 M)
Sterilize by autoclaving and store up to 1 year at room temperature

Table 4A.1.2 Components of CDM Stock Solution 2

Component	Amount	Solvent	Stock concentration (mM)	Final concentration (mM)
Acid-soluble amino acid solution				
L-cystine	540 mg	50 ml 1.0 N HCl	240.3	0.15
Base-soluble essential component solution: add following to 100 ml of 1.0 N NaOH				
L- hypoxanthine	750 mg	100 ml 1.0 N NaOH	136.1	0.37
L-tyrosine	1065 mg	100 ml 1.0 N NaOH	181.2	0.39
L-tryptophan	200 mg	100 ml 1.0 N NaOH	204.3	0.39
Uracil	750 mg	100 ml 1.0 N NaOH	112.1	0.45
Neutral-soluble amino acid solution: add all listed amino acids to 2000 ml ddH_2O				
L-alanine	1500 mg	2000 ml ddH_2O	89.9	1.1
L-arginine·Cl	250 mg	2000 ml ddH_2O	210.8	0.71
L-asparagine·H_2O	385 mg	2000 ml ddH_2O	150.1	0.17
L-aspartic acid (K salt)	9.65 g	2000 ml ddH_2O	171.2	3.8
L-cysteine·Cl	825 mg	2000 ml ddH_2O	157.6	0.35
L-glutamic acid (Na salt)	23.3 g	2000 ml ddH_2O	169.1	8.8
L-glutamine	750 mg	2000 ml ddH_2O	146.1	0.34
Glutathione (reduced)	690 mg	2000 ml ddH_2O	307.3	0.15
Glycine	375 mg	2000 ml ddH_2O	75.1	0.33
DL-histidine·Cl·H_2O	375 mg	2000 ml ddH_2O	209.6	0.12
DL-isoleucine	450 mg	2000 ml ddH_2O	131.2	0.23
DL-leucine	1365 mg	2000 ml ddH_2O	131.2	0.69
DL-lysine·Cl	750 mg	2000 ml ddH_2O	182.0	0.27
DL-methionine	225 mg	2000 ml ddH_2O	149.2	0.1
DL-phenylalanine	375 mg	2000 ml ddH_2O	165.2	0.15
L-proline	750 mg	2000 ml ddH_2O	115.1	0.43
DL-serine	750 mg	2000 ml ddH_2O	105.1	0.48
DL-threonine	750 mg	2000 ml ddH_2O	119.1	0.42
L-valine	900 mg	2000 ml ddH_2O	117.2	0.51

Table 4A.1.3 Concentrations of CDM Stock Solution 3

Component	Stock concentration (mM)	Final concentration (mM)
Thiamine·Cl	0.593	0.59
Co-carboxylase	0.104	0.01
Biotin	1.2	0.12
mg D-Pantothenic acid	0.40	0.004

Ferric chloride, 10 mM

Add 27 to 40 mg $FeCl_3 \cdot 6H_2O$ to a 15-ml conical
Divide the weighed amount by the molecular weight (270.3)
Multiply the result by 100, and add that volume of ddH_2O in milliliters
Mix and filter sterilize (using a 0.2-μm filter)
Store in 1-ml aliquots up to 1 year at 4°C

This iron solution is clear yellow. Discard the solution if the yellow color intensifies or if precipitates form, as that indicates that the iron is oxidized and no longer available for gonococcal growth.

Since the amount of ferric chloride needed to make a 10 mM solution is small, weighing out the reagent first and then adding the appropriate amount of ddH_2O to the reagent is recommended.

Ferric nitrate, 10 mM

Add 40 to 60 mg $Fe(NO_3)_3 \cdot 9H_2O$ to a 15-ml conical tube
Divide the weighed amount by the molecular weight (404.0)
Multiply the result by 100, and add that volume of ddH_2O in milliliters
Mix and filter sterilize (using a 0.2-μm filter)
Store in 1-ml aliquots up to 1 year at 4°C

This iron solution is clear yellow. Discard the solution if the yellow color intensifies or if precipitates form, as that indicates that the iron is oxidized and no longer available for gonococcal growth.

Since the amount of ferric nitrate needed to make a 10 mM solution is small, weighing out the reagent first and then adding the appropriate amount of ddH_2O to the reagent is recommended.

GCMB solid medium

Place 18 g GC base medium (Difco), 500 ml distilled, deionized water, and a stir bar into a 1-liter flask or bottle. Place the flask or bottle onto a stir plate to mix. If necessary, swirl to dislodge the medium components from the bottom edges of the container, as they have a tendency to burn during the autoclave cycle. Loosely cap the bottle or cover the flask opening with foil. Autoclave on liquid cycle according to manufacturer's recommendations. Remove the sterile medium from the autoclave. Once it has stopped bubbling, place into a preheated 55°C water bath. At this time, remove the Kellogg's supplement to the benchtop to allow it to warm to room temperature.

After the medium has cooled to 55°C (~30 min, depending on the number of flasks and the size of the water bath), transfer the cooled medium to a stir plate set on low and add 5 ml (i.e., 1/100 volume of the medium) Kellogg's supplement (see recipe). If needed, add antibiotics at this time. Pour plates using ~20 to 25 ml per 100 × 15–mm diameter plate (20 to 22 plates per 500 ml medium). After the plates have completely set and cooled, invert on the benchtop. For long-term storage (>3 days), place the plates into bags and store, inverted, at 4°C. It is convenient to store poured plates in the bags used to package the Petri plates, as they are sterile and the perfect diameter for the plates. However, it is critical to let the poured plates dry before bagging, as the moisture will condense in the cold and can lead to contaminated plates due to the growth of mold, which gets transferred into the plates via water droplets. As a rule, allow poured media to sit at least overnight, and preferably closer to 48 hr before packaging for long-term storage. If plates must be stored early, it may be beneficial to put small vent holes into the Petri dish bags to allow for air flow.

Refer to Strategic Planning for additional details.

GCP stock

To 1 liter of ddH_2O, add:
15 g proteose peptone no. 3 (Difco)
1 g soluble starch
4 g K_2HPO_4
1 g KH_2PO_4
5 g NaCl

Stir until well mixed, ~5 to 10 min. Note that the solution may not be clear, as the starch may not go fully into solution. Distribute the well-mixed medium into 200-ml bottles, filling no more that half-full. Loosely attach the bottle lids and autoclave. When the medium has cooled to room temperature, tighten the lids and store in 100-ml aliquots up to 1 year at room temperature.

Glucose solution, 20% (w/v)

Add 20 g glucose (dextrose) to 100 ml ddH_2O
Stir to dissolve
Sterilize by filtration using a 0.22-μm filter
Store up to 1 year at room temperature

Glycerol, 50% (v/v)

Glycerol stock is very viscous and difficult to measure. Add 50 ml ddH_2O to a 100-ml graduated cylinder. Carefully pour glycerol into the cylinder until the water level rises to the 100-ml mark. Cover the top of the cylinder with Parafilm and invert several times to start mixing. Add a stir bar and continue stirring to fully mix the water and glycerol. Autoclave solution to sterilize. Store up to 1 year at room temperature.

Kellogg's supplement

To 200 ml ddH_2O, slowly add 200 g dextrose (glucose) in portions, and allow each portion to dissolve before adding more. Mix until all the glucose is in solution. Add 5.0 g glutamine, 250 mg ferric nitrate, and ~10 mg Co-carboxylase (Sigma-Aldrich cat. no. C-8754). Continue stirring until all solids have gone into solution. Transfer to a 500-ml graduated cylinder and add ddH_2O to make 500 ml total volume. Return to beaker and stir for additional 5 to 10 min. Filter sterilize using a 0.22-μm filter into sterile bottles. Store up to 6 months at 4°C.

Refer to Strategic Planning for additional details.

Neutral-soluble amino acid solution

Dissolve the following amino acids in 2000 ml ddH_2O:
1500 mg L-alanine
250 mg L-arginine·Cl
385 mg L-asparagine·H_2O
9.65 g L-aspartic acid (K salt)
825 mg L-cysteine·Cl
23.3 g L-glutamic acid (Na salt)
750 mg L-glutamine
690 mg glutathione (reduced)
375 mg glycine
375 mg DL-histidine·Cl·H_2O
450 mg DL-isoleucine
1365 mg DL-leucine

continued

750 mg DL-lysine·Cl
225 mg DL-methionine
375 mg DL-phenylalanine
750 mg L-proline
750 mg DL-serine
750 mg DL-threonine
900 mg L-valine
Prepare fresh and use immediately for CDM stock solutions

Sodium bicarbonate, 4.2% (w/v)

Add 4.2 g $NaHCO_3$ to 100 ml ddH_2O
Stir to dissolve
Sterilize by filtration using a 0.22-μm filter
Store up to 1 year at room temperature

Sodium nitrite solution, 20% (w/v)

Add 20 g of $NaNO_2$ to 100 ml ddH_2O
Stir to dissolve
Sterilize by filtration using a 0.22-μm filter
Store up to 1 year at room temperature

Supplemented GCP

To GCP stock, add:
1/100 volume 4.2% sodium bicarbonate (see recipe)
1/100 volume Kellogg's supplement (see recipe) or IsoVitaleX (BBL; BD cat. no. 211875-211876)
Prepare only the amount needed for the day

COMMENTARY

Background Information

N. gonorrhoeae is an obligate human pathogen that is considered fastidious in its growth requirements. Many of the studies of gonococcal physiology were performed in the mid 1970s, during the peak years of the U.S. epidemic. Gonococci can grow in a glucose-containing medium over a pH range of 6.0 to 8.0, with an optimum pH of 7.0 to 7.5. Their temperature requirements are narrow, with growth through a range of 25° to 41°C, with an optimum of 36° to 39°C, dependent on the variant (Morse et al., 1979). It is recommended that gonococci be incubated with CO_2 at 3% to 10% as CO_2 enhances the development of exponential growth. Thus, CO_2 is necessary to initiate culture growth, but may not be necessary once exponential growth is established. It has long been reported that all gonococci require cysteine (cystine), however, recent studies found that some gonococci can utilize thiosulfate, suggesting that the cysteine supplementation may, in some cases, also serve as a sulfur source. Gonococci can utilize many forms of inorganic iron, including ferric nitrite, ferric chloride, and ferric citrate. They can also use transferrin- and lactoferrin-bound iron in the human host, as well as heme and hemoglobin (reviewed in Schryvers and Stojiljkovic, 1999). *N. gonorrhoeae* can use three carbon sources: glucose, pyruvate, and lactate (Morse and Bartenstein, 1974). Aerobically grown gonococci primarily utilize glucose via the Entner-Douderoff pathway, with minor use of the pentose phosphate pathway (Hebeler and Morse, 1976). When grown on pyruvate, the use of the pentose phosphate pathway accounts for one-third of carbon metabolism.

For many years, *N. gonorrhoeae* was considered an obligate aerobe, even when isolated from mixed infections with other known obligate anaerobic bacteria. However, the gonococcus can grow anaerobically when provided with nitrite as a terminal electron acceptor (Knapp and Clark, 1984). Under anaerobic conditions, the Entner-Douderoff pathway is used exclusively, forming lactate and acetate in equivalent amounts (Morse et al., 1974).

Many clinical gonococcal isolates show some degree of auxotrophy, which necessitates the use of rich media for isolation

and growth. These traits are often used as epidemiological markers. Some auxotroph types are correlated with antibiotic resistance. In addition, an arginine, hypoxanthine, and uracil auxotroph variant (AHU) was associated with higher rates of asymptomatic gonococcal disease. Subsequent work found that recovery of this auxotroph on standard selective media was low, due to its enhanced susceptibility to vancomycin, used as a selective agent in the medium (Morse and Bartenstein, 1980). This growth inhibition delayed diagnosis and early treatment, factors known to contribute to development of complicated gonococcal disease.

N. gonorrhoeae does not survive long at stationary phase, but undergoes autolysis following exhaustion of glucose from the medium (Morse and Bartenstein, 1974). Some of the genes responsible for this process have been identified, and appear to be related both to virulence and the natural competency of *N. gonorrhoeae*. It has been proposed that limited autolysis could play a role in altering the local integrity of the cell envelope to facilitate the uptake of DNA in the transformation process. Autolysis could also generate pools of available DNA for use by neighboring gonococci, as has been experimentally shown for a lytic peptidoglycan transglycoslase, *AtlAI* (Dillard and Seifert, 2001).

Autolysis is an enzymatic process, following first-order kinetics with a pH optimum of 8.5 and temperature maximum of 40°C. Ions have a marked effect on whole-cell lysis, with potassium enhancing and divalent cations, especially magnesium and calcium, inhibiting lysis. Magnesium and calcium limit visible whole-cell autolysis by stabilizing the outer membrane, with no effect on peptidoglycan hydrolysis (Wegener et al., 1977). It is proposed that autolysis is related to the rapid turnover of peptidoglycan of ~20% to 50% (strain dependent) per generation. Gonococci are somewhat unique in that they secrete peptidoglycan fragments into the surrounding medium. These fragments have cytotoxic properties, and are responsible for the selective killing of ciliated cells in the fallopian tube organ culture system, a primary factor in the development of gonococcal pelvic inflammatory disease (Melly et al., 1984).

Critical Parameters and Troubleshooting

While *N. gonorrhoeae* is a fastidious organism, it is not that difficult to grow in the laboratory as long as care is taken to adhere to its basic minimal requirements. These can be divided into media, appropriate inoculum, and incubation conditions.

Media

It is not unusual for *N. gonorrhoeae* strains to have complex media requirements. The GC base medium used for plate cultures is designed for clinical diagnostic studies, and has been formulated to support virtually all gonococcal strains. Most problems related to media are due to the following:

1. Impurities in the water used to make the medium or supplements. It is important to use high-quality distilled deionized (18 Ω) water.

2. Prolonged or overheating of the medium during the autoclaving process, which inactivates critical components in the medium. The medium should be yellow-gold in color. If it appears more amber in color, it may have been overheated at some point in the process.

3. Contamination of the containers in which the medium was prepared. Flasks used for the preparation of GC media must be carefully washed to remove residual starch, and thoroughly rinsed to remove all traces of detergent.

Inoculum

N. gonorrhoeae viability in frozen stock, or from large/older colonies on plates may not be sufficient to initiate strong growth in subsequent cultures. To limit problems of insufficient inoculum, consider the following:

1. Minimize thawing of frozen stock. This can be done by keeping the vial in dry ice, limiting the amount of time the vial is held in the hand, and using a suitably large scraping from the frozen stock.

2. Choose the most appropriate colonies. Use small but not confluent colonies from an overnight culture. As cultures age, the cell viability declines dramatically.

3. Chilling of the culture may decrease viability. Keep gonococcal cultures at 37°C when not in use, as benchtop conditions are below their survivable temperature range.

Incubation conditions

Incubation conditions refer primarily to the environmental conditions surrounding the culture.

1. Sufficient CO_2 for initial growth. Gonococci require CO_2 to initiate growth in both broth and plate cultures. This must be provided in the atmosphere for plate cultures, and by adding sodium bicarbonate to broth cultures.

2. Appropriate temperature range. While *N. gonorrhoeae* can grow over a temperature

range of 25° to 42°C, they strongly prefer body temperatures of 35° to 38°C.

3. Adequate aeration conditions. Unless purposely growing gonococci anaerobically, they prefer a reasonable amount of oxygen for aerobic growth. Broth cultures can be shaken at moderate speeds (200 to 250 rpm), and it is important to limit the culture volume to 20% of flask volume.

Time Considerations

Gonococcal autolysis significantly impacts the laboratory growth of *N. gonorrhoeae*, as cells do not retain viability on culture medium after reaching stationary phase. Thus, planning for any experiment with gonococci must include 1 day to obtain growth from frozen stock along with a second overnight incubation to obtain suitable numbers of viable organisms for experimental purposes. Clones from genetic studies must be recultured for continued viability every 1 to 2 days, thus being able to rapidly analyze clones is necessary for procedures that may require screening of large numbers of colonies for low-frequency events.

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Genetic Manipulation of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a Gram-negative coccus and the causative agent of the sexually transmitted disease, gonorrhea. The disease generally manifests as localized urethritis in men and cervicitis in women. In women, the infection is often asymptomatic initially, but may spread to the uterus and fallopian tubes, causing pelvic inflammatory disease, chronic pelvic pain, and infertility. Untreated infections in men or women may also cause systemic disease (disseminated gonococcal infection), which may be manifested as tenosynovitis, meningitis, or endocarditis. For laboratory workers, it is most important to remember that *N. gonorrhoeae* causes eye infections. Normally gonococcal eye infections occur in newborns infected during passage through the birth canal of an infected mother; these infections often lead to blindness if left untreated. Laboratory-acquired *N. gonorrhoeae* eye infections have been reported; therefore, it is important to wear safety glasses when performing procedures that may cause gonococci to be propelled into the air and to seek medical treatment if the eyes or other mucosal surfaces are exposed to *N. gonorrhoeae*.

Gonococci are naturally transformable and this property has greatly facilitated its study. Gonococci readily take up DNA containing the 10-bp *Neisseria* DNA uptake sequence (DUS) GCCGTCTGAA (Goodman and Scocca, 1988). The DUS is found frequently in the chromosome of *N. gonorrhoeae* and other *Neisseria* species, often in two copies as an inverted repeat. Plasmids used for transformation of *N. gonorrhoeae* must contain the DUS to be taken up efficiently. Unlike many transformable bacteria, *N. gonorrhoeae* does not regulate transformation: gonococci are transformable at all stages of growth (Sparling, 1966). However, only piliated gonococci are naturally transformable, and piliation is a phase-variable phenotype. Therefore, care must be taken to choose piliated colonies for transformation.

This unit describes methods for genetic manipulation of *N. gonorrhoeae* including various methods of mutagenesis and complementation, as well as methods for growing and purifying DNA from these bacteria.

CAUTION: *Neisseria gonorrhoeae* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. In particular, wear safety glasses as exposure of the eyes to *N. gonorrhoeae* will cause conjunctivitis (see Critical Parameters and Troubleshooting). See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

IMPORTANT NOTE: Unless otherwise indicated, all incubations are to be performed at 37°C, 5% CO_2.

STRATEGIC PLANNING

Growth on Agar or in Liquid Medium

Gonococci are fastidious. They have multiple nutritional requirements and are generally grown in rich medium, either gonococcal base medium (GCB) or chocolate agar. They require CO_2 in the atmosphere for growth on agar plates or sodium bicarbonate in liquid medium (Morse and Bartenstein, 1974). Medium must be supplemented with Kellogg's supplements (Kellogg et al., 1963) or IsoVitalex (Becton Diconson). Recipes for gonococcal base medium and supplements are found in the Reagents and Solution section below. Gonococci should be inoculated from an agar plate into GCBL at a density

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Table 4A.2.1 Antibiotic Concentrations Used for a Common High- and Low-Level Overall Resistance Strains of *N. gonorhoeae*[a]

Antibiotic	MS11 (μg/ml)	FA1090 (μg/ml)
Chloramphenicol	10	2
Erythromycin	10	2
Kanamycin	80	ND
Nalidixic Acid	1.5	1.5
Penicillin G	0.3	ND
Spectinomycin	75	75
Streptomycin	100	100
Tetracycline	2	0.2

[a] Abbreviation: ND, not determined.

no less than 10^7 CFU/ml to reliably obtain a viable liquid culture. The medium must be prewarmed to 37°C. In general, inoculating to a density of 5×10^7 to 1×10^8 (OD_{540} of 0.2) is the most reliable method. Transfer may be done using a sterile Dacron swab. Do not use cotton, as these swabs may contain fatty acids which are inhibitory to some strains of *N. gonorrhoeae*. Optimal numbers of live gonococci for inoculation are obtained from agar plates 18- to 20-hr old. The doubling time for laboratory strains in liquid medium is ~60 min.

Antibiotic Concentrations for Selection in *N. gonorrhoeae* Strains

Some gonococcal strains have much higher levels of antibiotic resistance than others. It is therefore advisable to search the available literature for concentrations of antibiotic used with particular strains. Most strains, however, exhibit either high-level overall resistance levels (e.g., strain MS11) or low-level overall resistance (e.g., strain FA1090); therefore, useful antibiotic concentrations are given for these two commonly used strains (Table 4A.2.1) and may serve as a starting point for the determination of concentrations appropriate for uncharacterized strains.

BASIC PROTOCOL 1

SPOT TRANSFORMATION OF *NEISSERIA GONORRHOEAE* ON AGAR PLATES

N. gonorrhoeae can be transformed at high efficiency by growing the bacteria on agar plates in the presence of high concentrations of DNA. DNA is soaked into the plate at a few spots and the bacteria to be transformed (recipients) are streaked through the spots. Colonies arising on the spots have a high likelihood of containing transformed gonococci, often as high as 20% to 50% of total colony forming units. This method is often used for transformation with plasmids containing mutation constructs created in *E. coli*. Gonococcal DNA may be cloned into an *E. coli* plasmid and then interrupted with the gene for a selectable marker. Gonococcal DNA flanking the marker on both sides facilitates double-crossover homologous recombination and insertion of the marker into the gonococcal chromosome. To prevent the recovery of bacteria that have undergone a single-crossover recombination event and integrated the entire plasmid into the chromosome, the plasmid should be linearized before transformation. Commonly used restriction enzymes and buffers do not inhibit transformation. Therefore, the restriction digest mixture can be used directly without further purification. If the plasmid is 8 kb or larger, it is not necessary to linearize the plasmid, since *N. gonorrhoeae* processes large DNA fragments prior to DNA uptake during natural transformation (reviewed in Hamilton, 2006).

Alternatively, the ability of *N. gonorrhoeae* to incorporate plasmids into the chromosome can be used for making mutations (Hamilton et al., 2001). A single fragment of gonococcal DNA can be cloned into a plasmid in *E. coli*. Transformation with the circular plasmid, followed by selection for the antibiotic resistance marker on the plasmid will result in the isolation of transformants that have integrated the plasmid by single-crossover recombination. Since this method results in duplication of the cloned sequence, the method is often referred to as insertion-duplication mutagenesis.

Due to the high efficiency of transformation observed with this method, it is possible to introduce mutations that do not contain an antibiotic resistance gene or other selectable marker. Plasmids or PCR products containing deletions, insertions, or point mutations can be used for transformation. If plasmids are used, they should be linearized to prevent single-crossover recombination. Potential transformants can be screened by PCR for an increase or decrease in the size of the PCR product amplified from the gene of interest. If small mutations are introduced (e.g., point mutations, epitope tag insertions), it is helpful to also introduce or eliminate a restriction site, allowing potential transformants to be identified by PCR and restriction digest.

Materials

Recipient *N. gonorrhoeae* strain, frozen
GCB plates (see recipe), room temperature and 37°C
≥10 ng/μl plasmid DNA
Appropriate restriction enzyme and buffer (for double-crossover only)
GCB plate containing appropriate antibiotic (Table 4A.2.1; for fragments containing selectible markers only)
Dacron swabs (Fisher Scientific), sterile
Additional reagents and equipment for PCR (see Support Protocol)

1. Streak recipient *N. gonorrohoeae* strain from frozen stock onto a GCB plate. Incubate overnight (<24 hr).

Prepare DNA

2a. *For inserting a construct into the chromosome by double-crossover event:* Linearize the plasmid unless it is large (>8 kb). Be sure not to cut the transforming DNA away from the gonococcal DNA uptake sequence.

2b. *For performing insertion-duplication mutagenesis:* Do not linearize the plasmid.

3. Draw two 1-cm circles on the back of a 37°C GCB agar plate, where, upon streaking, the colony density will be medium and thin, respectively.

   Appropriate places would be one-third and two-thirds of the way down the middle of the plate.

4. Spot ~10 to 20 μl DNA solution (step 2a or 2b) on the agar inside the circles. Maximize the DNA concentration but do not exceed 10 μg. Allow DNA to soak into the plate.

   Amounts of DNA in the hundreds of nanograms are appropriate.

   If the spots are not dry within 10 min, it may be necessary to dry the plate in a biosafety hood with the lid of the plate removed, 20 min or less.

5. Pick a piliated colony of the recipient from the plate prepared in step 1. Streak through the DNA spots and incubate overnight.

   See Background Information for a discussion of appraising the piliation state of N. gonorrhoeae.

6a. *For transformations using fragments containing selectable markers:* Using a sterile Dacron swab, transfer and uniformly spread the colonies that grew on the spots onto a GCB plate containing the appropriate antibiotic.

6b. *For transformations using fragments without selectable markers:* Streak individual colonies to be screened onto GCB plates using an inoculating loop.

CAUTION: *See Critical Parameters and Troubleshooting for important safety considerations.*

7. Incubate transformants overnight.

If using no selection, colonies will be ready for screening in 24 hr or less. Antibiotic selection will significantly slow the appearance of transformants. Transformants selected with chloramphenicol or erythromycin may not be apparent until 36 to 48 hr.

8. Streak individual transformants onto GCB plates and screen for nonselectable mutations by PCR (see Support Protocol).

Due to transformation occurring within only some members of a colony, nonselected mutants identified by this method should be regarded as likely mixed populations and should be restreaked; the presence of the mutation in individual colonies should be verified.

BASIC PROTOCOL 2

TRANSFORMATION OF *NEISSERIA GONORRHOEAE* IN LIQUID CULTURE

Transformation of *N. gonorrhoeae* has traditionally been done in liquid culture. This method is not as efficient as transformation on agar plates as described in Basic Protocol 1; however, it can be quicker, particularly if frozen cultures of piliated bacteria are prepared ahead of time. Also, this method is less likely to give colonies representing mixed populations.

Materials

Recipient *N. gonorrhoeae* strain, frozen
GCB plate (see recipe)
≥10 ng/μl plasmid DNA
GCBL medium (see recipe) containing 5 mM $MgSO_4$, room temperature and 37°C
GCBL medium containing Kellogg's supplements (see recipe)
60% (v/v) glycerol

Dacron swabs, sterile
T-25 tissue culture flask, 37°C

1. Streak recipient *N. gonorrhoeae* strain from frozen stock onto a GCB plate. Incubate overnight.

2. Pick a piliated colony. Streak onto a GCB plate and incubate overnight (18 to 20 hr).

See Background Information for a discussion of appraising the piliation state of N. gonorrhoeae.

3. Add 20 μl plasmid DNA to 200 μl GCBL medium containing 5 mM $MgSO_4$ in a 1.5-ml microcentrifuge tube. Warm mixture to 37°C in a water bath. Maximize DNA amount (not to exceed 10 μg) and minimize DNA volume (not to exceed 20 μl).

Typical DNA amounts are in the hundreds of nanograms (~200 ng to 1 μg). Plasmids smaller than 8 kb should be linearized by restriction enzyme digestion prior to transformation unless insertion of the entire plasmid into the chromosome is desired.

4. Using a Dacron swab, prepare a recipient cell suspension by transfering 1/4 to 1/2 of all of the colonies resulting from step 2 into a 1.5-ml microcentrifuge tube containing 1 ml GCBL medium/5 mM $MgSO_4$, 37°C. Swirl the swab to suspend bacteria.

5. Prepare transformation mix by adding 20 μl recipient cells to the DNA solution (step 3). Incubate 5 to 15 min at 37°C.

6. If it is possible this strain will again be transformed, add 60% glycerol to a final concentration of 15% (v/v) to the remaining recipient cell suspension (without DNA). Flash freeze using liquid nitrogen or a dry ice/ethanol bath.

7. Transfer entire transformation mix to 2 ml GCBL containing Kellogg's supplements in a T-25 tissue culture flask (already at 37°C). Incubate flask with the lid loosened 2 to 6 hr.

Plate transformants

8a. *If selecting for antibiotic resistance:* Prepare 10-fold serial dilutions in GCBL over a range of 10^0 to 10^{-4}. Plate 100 μl of each dilution onto an individual GCB plate with the appropriate antibiotic. Incubate until colonies are observed (24 to 48 hr).

8b. *If screening without selection*: Prepare 10-fold serial dilutions in GCBL over a range of 10^{-3} to 10^{-6}. Plate 100 μl of each dilution onto individual GCB plates without antibiotics. Incubate until colonies are observed (18 to 24 hr).

9. Screen for desired mutation by PCR (Support Protocol).

ELECTROPORATION OF *NEISSERIA GONORRHOEAE*

BASIC PROTOCOL 3

Since the efficiency of electroporation is low and that of natural transformation is high, gonococci are almost never transformed by electroporation. However, there are cases where electroporation is the only reasonable option. For example, transformation of a *recA* mutant with a replicating plasmid, transformation of a nonpiliated, nonreverting strain, or transformation with DNA lacking a gonococcal DNA uptake sequence (DUS) may only be possible by electroporation. Gonococci will begin to undergo death and lysis under the washing conditions required to remove salts prior to electroporation. Different methods have been described for this purpose (Genco et al., 1991). However, it is most important to perform the washing steps and electroporation quickly in order to get the gonococci back into growth medium before too many of the recipients have died.

Materials

Recipient *N. gonorrhoeae* strain, frozen
GCB plates (see recipe) with and without antibiotic (Table 4A.2.1)
0.3 M sucrose: pass through a 0.22-μm filter to sterilize; store up to 1 year at room temperature
10 mg/μl DNA solution
GCBL medium with Kellogg's supplements (see recipe)
GCBL medium (see recipe)
Dacron swabs (Fisher Scientific), sterile
Electroporation cuvette, 2-mm gap length
Electroporatator

1. Streak recipient *N. gonorrhoeae* strain from frozen stock onto a GCB plate. Incubate overnight.

2. Using a sterile Dacron swab, transfer 50% to 100% of the colonies on the plate into 1 ml of 0.3 M sucrose at room temperature.

   Sucrose provides osmotic stabilization. Suspension of N. gonorrhoeae in low osmotic strength buffer results in autolysis and cell death.

3. Microcentrifuge 30 sec at 12,000 rpm, room temperature. Resuspend cells in 1 ml of 0.3 M sucrose and microcentrifuge again. Repeat suspension and microcentrifugation a third time. Suspend cells in 100 μl of 0.3 M sucrose.
4. Add 1 μl DNA solution.

   Plasmid DNA prepared by standard miniprep method and suspended in TE or water works well for electroporation. If arcing occurs during electroporation or if the addition of >3 μl of DNA is desired, the DNA should be concentrated by ethanol precipitation and washed twice with 70% ethanol to remove salts.
5. Transfer cell/DNA mixture into an electroporation cuvette. Electroporate with the pulse controller set at 2.5 kV, 200 Ω, and 25 μF.
6. Resuspend cells in 1 ml GCBL medium with Kellogg's supplements. Transfer culture to a GCB plate without antibiotics.
7. Incubate the plate right-side up 4 to 6 hr.
8. Wash the bacteria off the plate with 1 ml GCBL medium. Plate 50 μl onto a GCB plate containing appropriate antibiotic. Repeat for a second plate if desired. Incubate until colonies are observed (~ 24 to 48 hr).

BASIC PROTOCOL 4

SHUTTLE MUTAGENESIS OF *NEISSERIA GONORRHOEAE* DNA CLONED INTO PHSS6 PLASMIDS USING MTN*CMNS*

One of the most common methods of mutagenesis in *N. gonorrhoeae* is insertional mutagenesis using an antibiotic resistance marker to interrupt a gene or replace part or all of its coding sequence. The number of selectable markers for *N. gonorrhoeae* is limited. Researchers have reported using *cat* (chloramphenicol resistance), *ermC* (erythromycin resistance), *tetM* (tetracycline resistance), *aph*3 (kanamycin resistance), *bla* TEM-type (penicillin resistance), and the omega cassette (spectinomycin and streptomycin resistance). These markers are often introduced directly into the gene of interest cloned on a plasmid in *E. coli* and introduced into the gonococcal chromosome by natural transformation and allelic exchange. However, mutations may also be produced using plasmid or transposon insertions in the chromosome as described below.

Transposons generally do not transpose in *N. gonorrhoeae*. However, researchers wishing to make transposon mutants of gonococci have circumvented this problem in two ways. In the first method, gonococcal DNA is cloned into plasmids and mutagenized with transposons in *E. coli*, then introduced back into gonococci by natural transformation, a technique known as shuttle mutagenesis. Derivatives of Tn3 and Tn1721 have been adapted for use in gonococci, including derivatives containing reporters *lacZ*, or *phoA* (Seifert et al., 1990; Boyle-Vavra and Seifert, 1993, 1994; Haas et al., 1993). A second method is to purify gonococcal DNA and mutagenize it with transposons in vitro. Both Tn5 and Mariner derivatives have been used for this purpose in *N. gonorrhoeae* and *N. meningitidis* (Pelicic et al., 2000; Sechman et al., 2005).

Materials

E. coli strains RDP146(pTCA), RDP146(pOX38::mTnCmNS), and NS2114Sm (Boyle-Vavra and Seifert, 1993)
LB medium (*APPENDIX 4A*)
1 mM HEPES, pH 7 (optional): sterilize by autoclaving; store up to 1 year at room temperature
10 mg/μl target plasmid
LB plates (*APPENDIX 4A*) containing 40 μg/ml kanamycin
LB plates containing 40 μg/ml kanamycin and 12 μg/ml tetracycline

LB plates containing 40 μg/ml kanamycin and 25 μg/ml chloramphenicol

LB plates containing 100 μg/ml streptomycin, 40 μg/ml kanamycin, and 25 μg/ml chloramphenicol

GCB plates (see recipe) containing chloramphenicol (Table 4A.2.1)

Electoporation cuvette, 2-mm gap length

Electroporator

Additional reagents and solutions for PCR (Support Protocol; optional) and spot transformation with *N. gonorrohoeae* (Basic Protocol 1)

1. Incubate *E. coli* strain RDP146(pTCA) in LB medium several hours to overnight at 37°C.

   pTCA encodes the transposase for Tn3.

2. Microcentrifuge desired volume of cells (generally ~1.5 ml) for 30 sec at 12,000 rpm. Resuspend cells in 1 ml low ionic strength buffer, such as sterile 1 mM HEPES, pH 7. Repeat wash two more times.

3. Resuspend cells in 40 μl 1mM HEPES per planned electroporation.

4. Add target plasmid to cell suspension.

5. Transfer cell/DNA mixture into an electroporation cuvette. Electroporate with the pulse controller set at 2.5 kV, 200 Ω, and 25 μF.

6. Plate 10 and 100 μl electrotransformation mix onto LB plates containing 40 μg/ml kanamycin. Incubate overnight at 37°C.

7. Use sterile toothpicks to transfer several potential transformants onto LB plates containing 40 μg/ml kanamycin and 12 μg/ml tetracycline.

   Transformants should be resistant to both antibiotics and show two plasmids when examined by whole-cell lysis (cracking) gel (Kado and Lin, 1981; Dillard and Yother, 1994). Such transformants will serve as the primary recipient.

8. Grow *E. coli* strain RDP146(pOX38::mTn*CmNS*) and primary recipient (step 7) to mid-log phase (OD_{600} of 0.5) without antibiotics. Mate at a ratio of 1:1 for 1 hr at 37°C without shaking.

   This step brings the transposon into the strain carrying the transposase and the target plasmid.

9. Plate 100, 10, and 1 μl onto individual LB plates containing 40 μg/ml kanamycin and 25 μ/ml chloramphenicol to select for Kn^RCm^R transformants. Incubate ~16 hr at 37°C.

   Any RDP146 cells not carrying all three plasmids will be killed in this step.

10. Grow NS2114Sm to mid-log phase (OD_{600} of 0.5) in LB medium without antibiotics (~4 hr).

    This step can be started towards the end of the incubation described in step 9.

11. Wash or scrape transconjugates from plates into LB medium and mix 1:1 with mid-log phase NS2114Sm.

12. Mate 15 min in a 37°C water bath. Incubate 30 min in rotator at 37°C.

13. Plate 100, 10, and 1 μl onto individual LB plates containing 100 μg/ml streptomycin, 40 μg/ml kanamycin, and 25 μg/ml chloramphenicol. Incubate ~16 hr at 37°C.

14. If desired, screen for transposon location and orientation by PCR (see Support Protocol).

15. Transform plasmid DNA into gonococci by spot transformation (Basic Protocol 1). Select for transformants on GCB plates containing 25μg/ml chloramphenicol.

BASIC PROTOCOL 5

CHEMICAL MUTAGENESIS

Chemical mutagenesis has been used in *N. gonorrhoeae*. Its use is infrequent since directed mutagenesis is highly efficient. Chemical mutagens that directly act on the DNA are effective (Campbell and Yasbin, 1984). However, chemical mutagenesis methods that rely on error-prone repair are not, since *N. gonorrhoeae* has no SOS-type system (Black et al., 1998). The procedure below describes use of ethylmethane sulphonate (EMS). *N*-Methyl-*N′*-nitro-*N*-nitrosoguanidine (MMNG) could be used instead, at 5 μg per 10^8 cfu.

Materials

N. gonorrhoeae strain, frozen
GCB plates (see recipe)
GCBL medium with Kellogg's supplements and sodium bicarbonate (see recipe), 37°C
Ethyl methanesulfonate (EMS)
GCBL medium
GCBL medium containing 15% glycerol
GCB plates containing nalidixic acid (Table 4A.2.1)
Dacron Swab

1. Streak *N. gonorrhoeae* strain from frozen stock onto a GCB plate. Incubate overnight.
2. Using a sterile Dacron swab, transfer cells from 1/4 to 1/2 of the plate into 3 ml GCBL medium with Kellogg's supplements and sodium bicarbonate, 37°C, to an OD_{540} of 0.2. Retain a 0.5-ml aliquot.
3. Add EMS to a final concentration of 1% (v/v), e.g., 20 μl to a 2-ml culture.
4. Incubate with aeration and remove 0.5-ml aliquots at 10, 20, 30, and 40 min. For each time point, immediately microcentrifuge aliquot 30 sec at 12,000 rpm and remove supernatant. Resuspend cells with 0.5 ml GCBL.

   Do not forget to include the untreated culture (0-min time point) taken in step 2.

5. Prepare serial dilutions over a range of 10^0 to 10^{-6}. To determine killing frequency, plate 100 μl of each dilution onto an individual GCB plate for quantification of colony forming units. Plate 100 μl of each dilution onto an individual GCB plate containing nalidixic acid to determine mutation frequency.

   Point mutations in the gyrB gene result in nalidixic acid resistance (Nal^R). Thus, measuring the frequency of mutations conferring nalidixic acid resistance gives a measure of the effectiveness of the chemical mutagenesis procedure.

6. Microcentrifuge the remainder of each undiluted aliquot 30 sec at 12,000 rpm. Resuspend cells in GCBL containing 15% glycerol and freeze at –70°C.
7. After overnight growth, identify time-points exhibiting 99% to 99.9% killing and those exhibiting the highest frequency of Nal^R colonies.

   Typically 30- and 40-min time points are optimal.

8. Streak bacteria onto GCB plates from the frozen, mutagenized stock from step 6 that gave the highest mutation frequency or 99% to 99.9% killing frequency, then screen for phenotype.

COMPLEMENTATION ON THE *NEISSERIA GONORRHOEAE* CHROMOSOME

Complementation in *N. gonorrhoeae* is usually done by insertion of the gene of interest onto the gonococcal chromosome. Because a majority of DNA that enters the cell during natural transformation is single-stranded, it is not restricted, and is therefore much more efficiently incorporated than replicating plasmids. Sites that have been used for complementation include the *iga* gene encoding IgA1 protease, and an intergenic region between *lctP* and *aspC* (Mehr et al., 2000). IgA protease is not essential for growth of *N. gonorrhoeae*; therefore, it can be interrupted without affecting in vitro phenotypes (Koomey et al., 1982). However, intergenic insertions are preferred since they are not known to affect any phenotypes. In theory, most any location that does not interrupt a gene or operon will work for this process. However, significant work has gone into making the *lctP-aspC* constructs useful for gonococcal complementation. A series of plasmids was created containing more than 3 kb of the *lctP-aspC* region, interrupted by a polylinker, an antibiotic resistance marker, and in some cases, a regulatable promoter. Transformation of gonococci followed by selection for the antibiotic resistance marker leads to selection of bacteria that have incorporated the construct by double cross-over recombination. Genes cloned into the construct are thus introduced into the chromosome with the antibiotic resistance marker between *lctP* and *aspC*. Plasmid pGCC6 contains two copies of the *lac* promoter-operator, $lacI^{Q}$, allowing for inducible expression of the gene of interest, and *cat*, conferring chloramphenicol resistance (Mehr et al., 2000). Plasmid pKH35 is a smaller pGCC6 derivative with a more extensive polylinker (Hamilton et al., 2005). Plasmid pGCC4 is similar to pGCC6 but carries an *ermC* marker instead of *cat*, and pGCC2 carries only the *ermC* marker (Stohl et al., 2003).

Materials

Gene of interest
pKH35 (Hamiltan et al., 2005) or similar plasmid
E. coli
N. gonorrhoeae mutant of interest
GCB plate (see recipe) containing chloramphenicol (Table 4A.2.1)
1 M IPTG solution
Dacron swab, sterile (Fisher Scientific)
Additional reagents and equipment for preparing a miniprep (Engebrecht et al., 1991), spot transformation (Basic Protocol 1), and PCR (Support Protocol)

1. Clone gene of interest into pKH35 or similar plasmid and transform into *E. coli*.
2. Purify plasmid using standard miniprep procedure (Engebrecht et al., 1991).
3. Use 200 to 500 ng plasmid and the *N. gonorrhoeae* mutant of interest to perform spot tranformation (Basic Protocol 1) on a GCB plate.
4. Use a sterile Dacron swab to transfer all of the resulting colonies from a DNA spot onto a GCB plate containing chloramphenicol. Incubate 24 to 48 hr.
5. Pick individual colonies and screen by PCR for presence of gene of interest adjacent to *lctP* (see Support Protocol).
6. Induce expression of the gene of interest by adding of 1 M IPTG to a final concentration of 1 mM either in liquid culture or on solid medium.

ALTERNATE PROTOCOL 1

COMPLEMENTATION WITH REPLICATING PLASMIDS

Complementation with replicating plasmids is rarely performed with *N. gonorrhoeae*. During transformation, plasmid DNA is cut by a nuclease prior to entry into the cell. Unless the incoming DNA is homologous to the gonococcal chromosome or a resident plasmid, and the cut occurs in the homologous segment, the incoming plasmid cannot reform a circle. A circle can be reformed if the cell brings in more than one copy of the plasmid. Thus, transformation with replicating plasmids follows "two-hit" kinetics. Additionally, the introduced plasmid is subject to restriction, further reducing the transformation frequency. The introduced plasmid is subject to restriction, further reducing the transformation frequency. Use of replicating plasmids would be necessary for complementation of certain recombination- or transformation-deficient mutants that would be unable to take up or incorporate DNA by natural transformation. The complementing plasmid could be introduced by electroporation. Also, higher copy numbers for some replicating plasmids would facilitate increased expression of introduced genes. Plasmids have been developed for complementation in gonococci and commonly rely on the replication origin of the gonococcal β-lactamase plasmids or the cryptic plasmid. Two such plasmids are pLES2 (Stein et al., 1983) and pFP10 (Pagotto et al., 2000). In addition, broad-host range plasmids of the IncP and IncQ groups will replicate in gonococci and can be used for complementation. A complementation method based on the conjugative plasmid p*tetM*25.2 from *N. gonorrhoeae* has been described (Kupsch et al., 1996).

BASIC PROTOCOL 7

PREPARATION OF CHROMOSOMAL DNA FROM *NEISSERIA GONORRHOEAE* GROWN ON SOLID MEDIUM

Purified chromosomal DNA is required for Southern analysis of mutants and complemented strains. For PCR amplification of gonococcal genes for cloning, use of purified chromosomal DNA as template gives cleaner results with fewer false-priming products. Gonococci are less likely to autolyse when grown on agar plates as compared to growth in liquid medium. Therefore, preparation from gonococci grown on agar plates is the most reliable method for chromosomal DNA purification.

Materials

N. gonorrhoeae, overnight culture
GCB plates (see recipe)
TES buffer (see recipe)
10 mg/ml RNase A
10% SDS
Tris-buffered phenol (*APPENDIX 2A*)
Chloroform
3 M sodium acetate
95% and 70% ethanol

Dacron swab, sterile
13-ml snap-cap polypropylene tubes
1-ml pipette tips with wide bore

1. Using an inoculating loop, streak three to five colonies of *N. gonorrhoeae* onto each of eight to ten GCB plates. Incubate overnight.
2. Using one sterile Dacron swab per plate, tranfer and suspend cells in 5 ml TES buffer in a 13-ml snap-cap polypropylene tube.
3. Add 1 μl of 10 mg/ml RNase A (10 mg/ml) and 50 μl of 10% SDS. Incubate 10 min at 37°C to digest RNA.

   Lysis should occur instantly.

4. Extract with an equal volume of tris-buffered phenol. Mix by gentle inversion twenty times. Centrifuge 10 min at 6000 × *g* (7000 rpm), 4°C.
5. Transfer aqueous (top) phase to a new tube using a micropipette equipped with a wide-bore 1-ml pipette tip and repeat extraction.
6. Extract once with chloroform. Invert and centrifuge as in step 4.
7. Transfer aqueous (top) phase to a new tube. Add 1/10 vol 3 M sodium acetate and 2 vol of 95% ethanol. Mix and incubate 30 min at −20°C.
8. Centrifuge 10 min at 12,000 × *g*, 4°C.
9. Wash pellet twice with 70% ethanol.
10. Invert tube and allow pellet to dry until white or clear.
11. Suspend DNA in ∼200 to 500 µl water.

PREPARATION OF CHROMOSOMAL DNA FROM *NEISSERIA GONORRHOEAE* GROWN IN BROTH

ALTERNATE PROTOCOL 2

Growth of *N. gonorrhoeae* in liquid culture makes the preparation steps for DNA purification easier than those for preparing DNA from bacteria grown on plates. The bacteria can be harvested by centrifugation (instead of swabbing) and the recovery of bacteria is more efficient. The drawback to this method is that the cultures must be monitored closely to ensure that the bacteria are still in the growth phase and have not begun to autolyse before chromosome purification begins. Autolysed gonococcal cultures still exhibit significant optical density (OD_{540} of ∼1.2), but purification of DNA from these cultures gives low yields and mostly degraded DNA. However, if growth is monitored to ensure growing cells are used, preparation of DNA from 200 ml of late-log phase gonococci (in this protocol) provides significantly more DNA than the preparation from ten agar plates. This method does not have an RNase step. Rather, the RNA functions as carrier nucleic acid, allowing the DNA and large RNA to be easily recovered with a glass hook.

Materials

N. gonorrhoeae, frozen stock
GCB plate (see recipe)
GCBL medium with Kellog's supplements and sodium bicarbonate (see recipe), 37°C
TE buffer (*APPENDIX 2A*)
10% SDS
5 M potassium acetate
95% and 70% ethanol
Dacron swab
13-ml polypropylene tube
Glass rod
65°C water bath

1. Streak *N. gonorrhoeae* from frozen stock onto a GCB plate and incubate overnight.
2. Using a sterile Dacron swab, transfer 1/4 to 1/2 of colonies on the plate to 3 ml GCBL medium, 37°C, with Kellogg's supplements and sodium bicarbonate.
3. Incubate until culture reaches late log phase (OD_{540} of 1.0 – 1.4; ∼4 to 6 hr). Transfer to 200 ml GCBL, 37°C, with supplements and sodium bicarbonate.

4. Incubate until culture reaches late log phase (OD_{540} of 1.4; <20 hr).
5. Centrifuge culture 10 min at 3,000 × *g*, 4°C. Resuspend in 2.5 ml TE buffer on ice.
6. Transfer to a 13-ml polypropylene tube. Add 10% SDS to 1% (~300 µl of 10%) and lyse the cells 15 min at 65°C.
7. Add 1/5 vol of 5 M potassium acetate (~700 µl) and incubate an additional 15 min in a 65°C water bath. Transfer to ice and incubate 60 min.
8. Remove cell debris by centrifuging 15 min at 12,000 × *g*, 4°C. Add supernatant to 2 vol of 95% ethanol. Hook out the DNA with a glass rod.

   Addition of the cleared lysate to ethanol will cause the DNA and RNA to come out of solution and appear as long white strands or gobs floating in the liquid. A glass hook can easily be fashioned from a Pasteur pipette using a Bunsen burner. The hook should be sealed and contain at least a slight bend. If the DNA cannot be removed with a hook it can still be recovered by centrifugation.
9. Wash DNA once with 70% ethanol, allow it to dry, and resuspend in 200 to 500 µl TE.

SUPPORT PROTOCOL

PREPARING PCR TEMPLATES FROM *NEISSERIA GONORRHOEAE* COLONIES

It is often necessary to screen gonococcal transformants for the presence of the introduced mutation or construct. This process can often be done by PCR or PCR followed by restriction digest. One gonococcal colony contains sufficient DNA for this process, although it will work better with three to five colonies from the isolate. The resulting PCR product can then be examined for its size, size following digestion, or simply its presence using agarose gel electrophoresis.

Materials

N. gonorrhoeae growing on solid medium
Colony lysis solution (see recipe)
Appropriate restriction enzyme(s) and buffer(s) (optional)
Thermocycler and appropriate PCR tubes
Additional reagents and equipement for PCR (Kramer and Coen, 2001)

1. Scrape an amount of *N. gonorrhoeae* equal to one to five colonies and transfer to 50 to 100 µl colony lysis in a PCR tube.
2. Lyse cells 15 min at 94°C in a thermocycler followed by incubation for 5 min at 25°C.
3. Use 2.5 µl lysate as template to a 25-µl PCR reaction (Kramer and Coen, 2001).
4. Use 5 µl PCR product in 30 µl restriction digest, if identification of a restriction site is necessary.

   See Bloch (1995) for more information about restriction mapping.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Colony lysis solution

40 µl 500 mM EDTA
200 µl Tris·Cl, pH 8.5 (*APPENDIX 2A*)
100 µl Triton X-100

9.66 ml H_2O
Store up to 1 year at room temperature

Scale as needed.

GC medium base

42% (w/w) Proteose peptone #3 (15.10 g)
3% (w/w) cornstarch (1.01 g)
11% (w/w) K_2HPO_4 (4.03 g)
3% (w/w) KH_2PO_4 (1.01 g)
14% (w/w) NaCl (5.03 g)
28% (w/w) agar (10.07 g)

Weight amounts given in parentheses indicate appropriate mix for preparing GCB plates (see below).

GCB plates

36.25 g GC Medium Base (Difco; also see recipe)
1.25 g agar
1 liter H_2O
Autoclave with stir bar
Cool to 50° to 60°C
Add 10 ml filter-sterilized Kellogg's supplements I (see recipe)
Add 1 ml filter-sterilized Kellogg's supplement II (see recipe)
Pour into 100-mm plates using 20 ml per plate
Store plates sealed in bags up to 6 months at 4°C

GCBL medium

Dissolve the following in 1 liter H_2O:
15 g proteose peptone #3
4 g K_2HPO_4
1 g KH_2PO_4
1 g NaCl
Adjust pH to 7.2 with HCl if necessary
Autoclave and store indefinitely at room temperature
Add Kellogg's Supplements I & II to 1× each from individual or a combined stock (see recipe)
Add sodium bicarbonate to 0.042% (final) from a 100× stock (0.42 g in 10 ml H_2O; filter sterilize)

Unless the protocol step specifcally indicates that Kellogg's supplements or sodium bicarbonate are to be included in the medium, these reagents should be omitted.

It is only necessary to add Kellogg's supplements when the bacteria are to be grown in the medium. It is not necessary to add Kellogg's supplements when the bacteria are diluted or transformed in GCBL, i.e., steps when the bacteria are only in the medium for a short period of time. Sodium bicarbonate is added only when the bacteria are to be grown in liquid culture with aeration (in sealed tubes or flasks) and not when they are grown in static culture in a CO_2 incubator. Unless the protocol step specifically indicates that Kellogg's supplements or sodium bicarbonate are to be included in the medium, these reagents can be omitted.

Kellogg's supplements

Supplements I and II may be mixed (10 ml supplement I + 1 ml supplement II) and frozen in 11-ml aliquots. One such aliquot is sufficient to supplement 1 liter of GCB or GCBL medium (see recipe). Filter sterilize before use.

Supplement I, 100×
40 g glucose
1 g glutamine
2 mg cocarboxylase (thiamine pyrophosphate)
Adjust volume to 100 ml with H_2O
Sterilize by passing through a 0.2-μm filter
Store indefinitely at –20°C
Supplement II, 1000×
50 mg $Fe(NO_3)_3 \cdot 9H_2O$
Adjust volume to 100 ml with H_2O
Sterilize by passing through a 0.2-μm filter
Store indefinitely at –20°C

An alternative to preparing supplements is to purchase them lyophilized. Supplement IsoVitalex (Becton Dickinson) can be used in place of Supplements I & II.

Do not store Kellogg's supplements at 4°C or room temperature.

TES

250 μl 1 M Tris·Cl, pH 8 (*APPENDIX 2A*)
200 μl 0.5 M EDTA
50 μl 5 M NaCl
4.5 ml H_2O
Store up to 1 year at room temperature

COMMENTARY

Background Information

Natural transformation of *Neisseria gonorrhoeae* was first reported by Sparling (1966). Over the years it has been found that the high degree of gonococcal transformation has led to quick spread of antibiotic resistance markers throughout the gonococcal population and has created a problem for the treatment of gonococcal infections. Transformation also leads to variation in genes coding for gonococcal antigens, allowing the bacteria to avoid the host immune response (Cooke et al., 1998), although significant variation of some antigen-encoding genes occurs by intracellular recombination (Stern et al., 1986; Seifert, 1996). As is the case for many naturally transformable bacteria, the process of transformation involves the components of a type IV pilus. Multiple mutations affecting piliation or pilus function in *N. gonorrhoeae* have been shown to block natural transformation (Chen and Dubnau 2004). The gene for PilE, the pilus subunit, undergoes high frequency recombination. This recombination results in altered pilin protein sequence allowing the bacteria to escape the host immune response. However, some of the recombination events eliminate pilin expression or result in pilin proteins that cannot be assembled into pili. The resulting nonpiliated bacteria are not transformable. Therefore, care should be taken to choose colonies of piliated *N. gonorrhoeae* for transformation procedures. See Figure 4A.2.1.

The gonococcal genome sequence was completed for strain FA1090 and is accessible in GenBank (accession number AE004969). A useful annotation is available at the STD genomes website of the Los Alamos National Laboratories (*http://www.stdgen.lanl.gov*). Although there are some significant differences in the genomes of *N. gonorrhoeae* strains (Gibbs and Meyer, 1996; Hamilton et al., 2005), the sequence information obtained for strain FA1090 is generally useful for searching for genes of interest and for designing primers for cloning gonococcal genes.

Critical Parameters and Troubleshooting

Safety considerations

N. gonorrhoeae is a human pathogen. It should be used under biosafety level 2 (BSL-2) conditions (*UNIT 1A.1*). Most importantly, wear safety glasses. This is vital when working with cultures that might splatter or other conditions that might propel gonococci into the eyes of

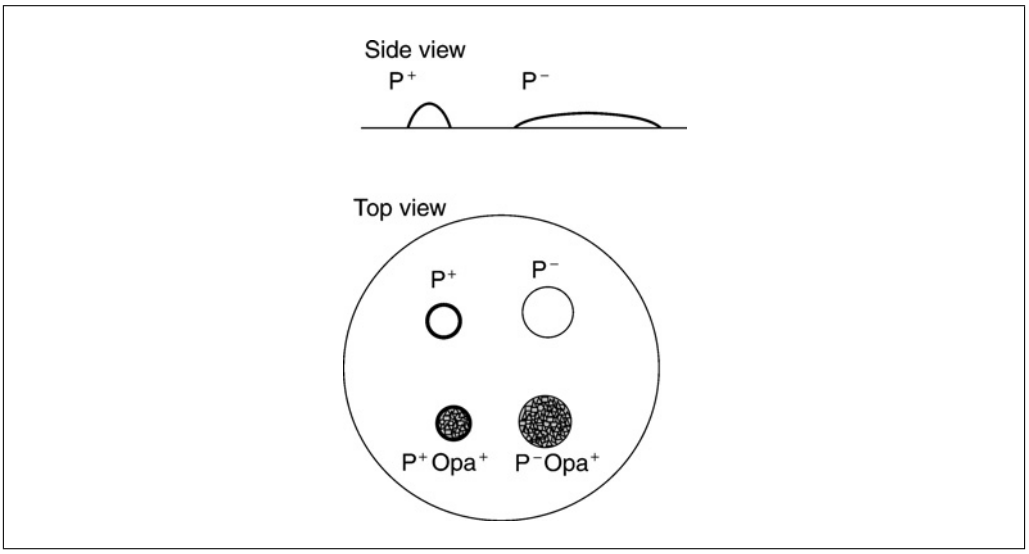

Figure 4A.2.1 Gonococcal colony morphologies. Gonococci expressing pili (P^+) form smaller colonies that appear to have a dark ring around the edge. This effect is due to the cells aggregating to form a mound. Nonpiliated cells form a pancake-shaped colony that has a greater diameter and a fuzzier edge. In addition, when the colonies are viewed with oblique substage lighting, a range of colony opacities will be detected. Colony opacity is due to presence of outer membrane opacity (Opa) proteins. Translucent (Opa^-) colonies appear clear or somewhat blue, whereas opaque colonies may appear yellow and refract the light to give an almost crystalline or granular appearance.

the researcher, such as sonication or streaking colonies with an inoculating loop. Exposure of the eyes will cause conjunctivitis. Exposure of any mucosal surface may result in infection. Gonococcal infections should be considered serious as they can result in septicemia and death. Treatment by a physician should be sought following any mucosal exposure to *N. gonorrhoeae*. Antibiotics are effective in clearing the infection.

Troubleshooting growth, death, and autolysis

One of the most difficult aspects of working with *N. gonorrhoeae* is its tendency to die in culture or to not grow when inoculated into a culture. To avoid these problems, it is best to keep gonococci under growth conditions at all times or to shorten the length of time they are in nonoptimal conditions (such as centrifugations or washes) as much as possible. It is important to prewarm media to 37°C. Streak bacteria from frozen stock onto prewarmed agar plates that contain no antibiotic. Although the bacteria may exhibit a significant level of resistance to an antibiotic while growing, they appear incapable of surviving the selection when restarting from frozen stocks. Finally it is advisable to inoculate at a density of $\sim 10^7$ to 10^8 CFU/ml when growing the bacteria in liquid culture. Although the bacteria may grow in liquid culture at lower densities, they often exhibit a growth lag lasting for a considerable period, and the lag is often followed by autolysis.

Gonococci are fastidious, requiring rich medium for growth. Complex media work best, but defined media have also been devised that will support the growth of gonococci (LaScolea and Young, 1974; Morse and Bartenstein, 1980). Furthermore, *N. gonorrhoeae* will grow in some media designed for tissue culture, particularly if supplemented with a few additional nutrients (Hamilton et al., 2001). The growth rate in the defined media is significantly reduced compared to that in rich media, and doubling time is generally ~120 min.

Gonococci are prone to die under any condition not favorable for growth. Furthermore they appear to have no true stationary phase, and are likely to be actively dying if they are not actively growing (Morse and Bartenstein, 1974; Hebeler and Young, 1975). They cannot be maintained alive on ice. Autolysis generally accompanies death, but there are conditions that can be used prevent autolysis (although the bacteria will still die). Incubation in low pH buffer (usually pH 5.5, 50 mM sodium phosphate or Tris·Cl) and/or on ice will limit autolysis. EDTA and other chelators of divalent cations greatly stimulate autolysis and should be avoided if lysis is not desired (e.g., cell fractionation procedures; Wegener et al., 1977).

Those first starting to work with *N. gonorrhoeae* often experience contamination of their liquid cultures. With a doubling time of 60 min, gonococci grow significantly slower than bacteria that commonly contaminate laboratory cultures. A useful method for overcoming this difficulty is to use streptomycin (100 μg/ml) in the medium. Many gonococcal strains, including the common laboratory strains FA1090 and MS11, carry a mutation in the *rpsL* gene conferring streptomycin resistance. The mutation is not universal; some common strains such as F62 do not carry the *rpsL* mutation.

Mapping insertions or deletions by Southern blotting

Transformants carrying mutations are generally first screened by PCR, however it is often desirable to confirm the mutation by Southern blotting. For this purpose it should be noted that gonococci have extensively methylated DNA, and many restriction enzymes do not cut gonococcal DNA efficiently.

Colony variants

Gonococci undergo spontaneous high-frequency variation of surface molecules by well described mechanisms (Seifert, 1996; Stern et al., 1986). This variation is seen in colony variants that appear on agar plates. For the purposes of growing gonococci, it should be noted that expression of pili and/or opacity proteins results in clumping during growth in liquid culture. The clumps are difficult to disperse and make it difficult to follow culture growth using OD measurements or plating. Therefore it is advisable to use non-piliated (P^-), transparent (Opa^-) variants unless these molecules are required for other purposes (e.g., transformation or adhesion and invasion assays). Piliated variants form colonies that are small and convex and thus appear to have a dark ring around them (Kellogg et al., 1963). Nonpiliated variants form larger, flatter colonies. Opacity protein expression is judged using a stereo dissecting microscope and oblique light. Opa^- variants may appear transparent or slightly blue. Opa^+ variants are opaque, somewhat yellow, and refractory (Swanson et al., 1971). Nonpiliated variants will come to predominate in liquid culture due to a slightly faster growth rate. Nearly all colonies will appear opaque on an old (>24-hr) plate due to variation occurring in a proportion of the cells in a colony at an appreciable rate.

Constructs for controlling pilin antigenic variation

Gonococci are notoriously variable, spontaneously altering pilin sequence as well as expression of genes for opacity proteins, lipooligosaccharide types, and many others. Much of the variation occurs by slipped-strand mispairing and cannot be controlled (Belland, 1991). However, pilin variation can be controlled, thereby preventing variation that might affect adherence and transformation. Two useful genetic alterations have been described that prevent pilin variation. The first, and most widely used constructs, are mutations in *recA*. Since pilin variation requires homologous recombination, mutations in *recA* or eliminating *recA* transcription eliminates pilin variation (Koomey et al. 1987). Both *recA* knockouts and *recA*-inducible constructs have been created. The *recA6* allele carries a *lac* promoter-operator upstream of *recA*, allowing recombination to occur in the presence of IPTG with virtually no recombination (and pilin variation) in the absence of induction (Seifert, 1997). It was recently shown that mutations upstream of *pilE* (the gene for the pilin subunit) will also prevent pilin variation. These insertions allow pilin expression but prevent variation (Sechman et al., 2005). Such strains are more useful since they allow normal homologous recombination required for natural transformation and genetic manipulation.

Troubleshooting transformation

If no transformants are obtained, the problem is often with the donor DNA. Impurities introduced in the preparation of plasmids from *E. coli*, such as salt or ethanol, will kill the potential *N. gonorrhoeae* recipients. In the spot transformation procedure, the bacteria will either not grow on the spot where the DNA was added to the plate or will only form very tiny colonies. Reprecipitation of the DNA followed by two washes with 70% ethanol is usually sufficient to remove the impurities. Adding more DNA to the transformation can also be helpful. Increasing the usual 100- to 200-ng amount of DNA to 1 μg may significantly increase the transformation frequency without damaging viability of the gonococcal recipients. Since DNA must contain the gonococcal DNA uptake sequence to act efficiently in transformation, it is important to make sure the DNA uptake sequence is present in the plasmid and was not cut away from the DNA of interest in preparation for transformation. Plasmids used

Table 4A.2.2 Time Considerations for Select Protocols

Protocol	Description	Time required
Basic Protocol 1 or 2	Gonococcal transformation	4-5 days
Basic Protocol 3	Electroporation	4 days
Basic Protocol 4	Shuttle mutagenesis	12-14 days
Basic Protocol 5	Chemical mutagenesis	2 days
Basic Protocol 6 or Alternate Protocol 2	Complementation	4-5 days
Basic Protocol 7	Chromosome preparation	2 days

for transformation should be linearized prior to transformation if they are smaller than ~8 kb (unless plasmid insertion in the chromosome is desired). Care should be taken to ensure that the enzyme chosen does not separate the uptake sequence from the DNA of interest. As a control for transformation a plasmid carrying a point mutation conferring antibiotic resistance is frequently used. The plasmid pSY6 conferring nalidixic acid resistance is useful for this purpose (Stein, 1991).

Anticipated Results

Transformation of *N. gonorrhoeae* is highly efficient. If using the spot transformation method to introduce mutations into the gonococcal chromosome (Basic Protocol 1), frequencies of 10% to 50% transformant/total CFUs or greater may be expected (Gunn and Stein, 1996). Screening of ten potential transformants is generally sufficient to identify the mutant of interest, even without selection. Using liquid transformation (Basic Protocol 2), frequencies ~1% may be expected. In general, the frequency will depend on the length of the homologous region targeting the mutation and the length of any heterologous sequence being inserted. Lengths of homologous DNA of ~500 to 1 kb on each side of the mutation or insertion to be introduced are sufficient to give efficient transformation. Long lengths of heterologous sequence to be inserted will greatly reduce the transformation frequency, although insertions >6 kb have been obtained (Boyle-Vavra and Seifert, 1993). The insertion of plasmids into the chromosome (Basic Protocol 1) also depends on the length of the homologous sequence with homologous regions of 300 to 500 bp resulting in transformation frequencies in the 10^{-7} to 10^{-6} range (Hamilton et al., 2001).

The insertion of plasmids into the chromosome also depends on the length of the homologous sequence with homologous regions of 300 to 500 bp resulting in transformation frequencies in the 10^{-7} to 10^{-6} range (Hamilton et al. 2001). The frequency of transformation by electroporation (Basic Protocol 3) is very low ($\sim 10^{-9}$) and will only produce a few transformants per electroporation. Shuttle mutagenesis (Basic Protocol 4) works well and has been used to produce multiple mutations in single genes or random mutations in the gonococcal chromosome (Haas et al., 1993; Boyle-Vavra and Seifert, 1993, 1994; Mehr and Seifert, 1997). The constructs for complementation on the chromosome by double-crossover recombination (Basic Protocol 6) are effective for complementation; however, as these contain significant stretches of heterologous DNA, the transformation frequencies are significantly lower than those of simple insertion mutations. The *lac* promoter used in some of the constructs gives relatively high levels of transcription of introduced genes, approaching those of strong gonococcal promoters (Seifert, 1997).

Time Considerations

Although the transformation step can take as little as 5 min, the entire procedure from streaking the bacteria to freezing down the transformed strain may take five days. Most of the time is spent waiting for the bacteria to grow. In particular, selection with chloramphenicol or erythromycin—two of the most useful antibiotics for gonococcal manipulation—significantly slows growth of the bacteria such that transformants will take one and a half to two days to form a colony.

Table 4A.2.2 provides time requirements for many of the protocols presented in this unit.

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Key Reference

Gunn, J.S. and Stein, D.C. 1996. See above.

This article describes the spot transformation method that has greatly increased the frequency of obtaining nonselected mutations and also reviews the data on the many restriction systems of N. gonorrhoeae.

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Interaction of Enterohemorrhagic *Escherichia coli* (EHEC) with Mammalian Cells: Cell Adhesion, Type III Secretion, and Actin Pedestal Formation

The interaction of enterohemorrhagic *Escherichia coli* (EHEC) with host cells is critical for colonization of the mammalian intestine. A likely first step is bacterial adhesion to enterocytes, and Basic Protocol 1 describes the assessment of bacterial binding to cultured mammalian cells. After initial adhesion, the pathogen alters the host cell by injection of effectors via a type III secretion apparatus. Basic Protocol 2 describes methods to detect type III secreted proteins in culture medium, with a method for quantitating the actin pedestal phenotype described in the Support Protocol. Finally, one of the most notable alterations induced by EHEC is the induction of localized actin assembly directly beneath bound bacteria, resulting in the formation of actin pedestals that raise them above the plane of the membrane; Basic Protocol 3 describes detection and quantitation of actin pedestals in vitro.

CAUTION: Enterohemorrhagic *Escherichia coli* (EHEC) is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

ADHESION OF EHEC TO MAMMALIAN CELLS

BASIC PROTOCOL 1

To establish intestinal infection, EHEC must attach to the host mucosa, an event that can be approximated in vitro using cultured cell lines. EHEC are cultured under conditions that have been found to induce the expression of bacterial factors that promote host cell interactions, and cell monolayers are infected with defined numbers of bacteria. Nonadherent bacteria are removed by washing and the monolayers are fixed and stained to visualize the bound bacteria. Microscopic quantitation of stained bacteria provides a measure of bacterial adherence. Commonly, this assay has been performed on Hep-2 or HeLa cells, but other cell lines, such as Caco-2 cells, have been used as well.

Materials

HeLa cells (ATCC #CCL-2)
HeLa cell growth medium (see recipe)
LB broth (*APPENDIX 4A*) containing appropriate antibiotic (Table A.4A.2)
Enterohemorrhagic *Escherichia coli* (EHEC) strain of interest
DMEM/HEPES (see recipe) containing appropriate antibiotic
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
HeLa cell infection medium (see recipe)
2.5% paraformaldehyde (see recipe)
PBS (*APPENDIX 2A*) containing 0.1% (v/v) Triton X-100 (store at room temperature)

Contributed by Pamela J. Savage, Kenneth G. Campellone, and John M. Leong
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PBS (*APPENDIX 2A*) containing 1% (w/v) BSA (add 10 ml 10% w/v BSA to 90 ml PBS)
Anti-0157 rabbit polyclonal antibody (Difco): resuspend lyophilized powder in 3 ml PBS (*APPENDIX 2A*), then dilute 1:500 (recommended) in PBS containing 1% BSA
200 U/ml Alexa568-phalloidin stock in methanol (Invitrogen)
1 mg/ml 4′,6-diamidino-2-phenyindole dilactate (DAPI) stock
2 mg/ml goat anti-rabbit-Alexa488 (Invitrogen)
Mounting medium: 1:1 (v/v) PBS:glycerol or ProLong Gold Antifade Reagent (Invitrogen)
Clear nail polish

Sterile glass coverslips, 12 mm, circular
24-well tissue culture plates
Sterile forceps
14-ml snap-cap culture tubes (e.g., Falcon)
Platform rocker
Centrifuge with plate carrier (e.g., Allegra 6 Benchtop Centrifuge; Beckman Coulter)
Glass microscope slides
Opaque slide box

Additional reagents and equipment for epifluorescence microscopy (*UNIT 2A.1*)

Day 1: Culture HeLa cell monolayers and prepare bacterial cultures

1. Place sterile coverslips into a 24-well tissue culture plate (one coverslip/well) using sterile forceps. Use three coverslips per strain to be analyzed in order to obtain triplicate samples.

2. Seed coverslips with $\sim 10^4$ HeLa cells per ml of HeLa cell growth medium. Incubate at least 16 hr.

   After overnight incubation, the HeLa cell monolayer should be ∼90% confluent. If a different cell line is used, with different growth rate or degree of spreading, initial seeding density should be adjusted to give this level of confluence.

3. For each bacterial strain, inoculate 1.5 ml LB broth (plus appropriate antibiotics; Table A.4A.2) in a 14-ml snap-cap culture tube with a single colony from freshly streaked plates. Incubate with agitation for 8 hr at 37°C until the OD_{600} is between 0.4 to 0.5.

   The antibiotic is selected based on the bacterial antibiotic resistance marker (see APPENDIX 4A).

   It is best to inoculate the bacteria early on the same day that the mammalian cells are seeded.

4. Dilute 10 µl of the bacterial culture into 5 ml DMEM/HEPES, plus the appropriate antibiotic (Table A.4A.2), in a 14-ml snap-cap culture tube or equivalent.

5. Incubate 12 to 14 hr without agitation in a 5% CO_2 atmosphere at 37°C with cap loosely fastened.

   These culture conditions result in EHEC that are capable of efficient interaction with mammalian cells and translocation of protein "effectors," including those required for actin pedestal formation, directly into these cells via a type III secretion system. For review of type III secretion by EHEC, see Garmendia et al. (2005).

Day 2: Infect HeLa cell monolayers with bacteria (Day 2)

6. Briefly mix the bacterial culture from step 5 by inversion.

   This step and the remainder of the assay can be performed on the benchtop.

7. After mixing, dilute the culture in prewarmed (37°C) HeLa infection medium such that the final concentration is 8×10^6 bacteria/ml medium.

   Calculate the concentration of bacteria in the overnight culture based on an OD_{600} reading. Previous titration has revealed that an $OD_{600} = 1.0$ of wild-type EHEC strain EDL933 reflects ~3.5×10^8 bacteria/ml. Pilot titrations of other strains, performed by plating cultures of different OD_{600} for cfu, may be required to determine the precise correlation between OD_{600} and culture density for these strains.

8. Aspirate the mammalian culture medium from each well of the 24-well dish (from step 2) into a waste vacuum flask using a Pasteur pipet. Immediately add 1 ml PBS to each well using a pipet. Aspirate immediately and repeat wash once.

   When adding PBS, the tip of the pipet should be placed against the wall of each well so as not to disrupt the cell monolayers.

9. Add 1 ml bacterial stock (from step 6) per well.

10. Cover and gently rock the tissue culture plate 5 min at room temperature.

11. Centrifuge the plate 5 min at $1462 \times g$ (1000 rpm in a Beckman Coulter Allegra 6 Benchtop Centrifuge), room temperature.

12. Incubate the plate 60 to 90 min at 37°C in a 5% CO_2 atmosphere.

13. Remove the plate from the incubator, aspirate the medium, and immediately wash the coverslips with 1 ml PBS per well. Repeat this PBS wash four times.

    These washes must be done quickly yet gently, to avoid washing the monolayer off the coverslips.

Fix cells

14. Add 0.5 ml room temperature 2.5% paraformaldehyde to each well and incubate, without agitation, at room temperature for 15 min to fix the infected monolayers. Incubate an additional 15 min with gentle rocking.

15. Aspirate paraformaldehyde and add 1 ml PBS per well. Rock the plate gently for 2 min. Aspirate PBS.

16. Add 0.5 ml PBS/0.1% Triton X-100 per well to permeabilize the monolayers. Rock the plate gently for 5 min. Aspirate.

17. Immediately add 1 ml PBS per well to wash. Rock the plate gently for 2 min. Aspirate and repeat the wash two more times.

Stain monolayers and bacteria

18. Add 275 µl of anti-0157 polyclonal antibody (recommended dilution, 1:500 in PBS/1% BSA) per well. Incubate 30 min at room temperature with gentle rocking.

    Alternatively, if using DAPI to visualize the bacteria, skip this step and proceed to step 19. Refer to Critical Parameters for further discussion pertaining to choosing DAPI or specific antibodies.

19. Aspirate medium and wash wells with 1 ml PBS per well, each time rocking gently for 5 min at room temperature. Remove wash medium and repeat two more times.

20. Using a total volume of 275 µl in PBS/1% BSA, treat monolayers with the following:

    2 U/ml (final concentration) Alexa568-phalloidin (add from 200 U/ml stock)
    1 µg/ml (final concentration; add from 1 mg/ml stock) of DAPI (if anti-0157 polyclonal antibody was added at step 18, then add sufficient 2 mg/ml goat anti-rabbit-Alexa 488 for a final concentration of 10 µg/ml, instead of DAPI).

21. Wrap plate in aluminum foil and incubate 30 min at room temperature, with gentle rocking.

22. Aspirate medium and wash with 1 ml PBS per well, each time with gentle rocking for 5 min. Aspirate medium and repeat two more times. Add 0.5 ml PBS to each well.

    Keep the plate wrapped in foil during the washes, because the fluorophores are sensitive to light.

Mount and examine

23. Spot 10 µl mounting medium (1:1 PBS:glycerol or ProLong Gold Antifade Reagent) onto each of a set of glass slides (one slide for each coverslip prepared in this protocol).

24. Remove each coverslip from its well using forceps and mount, inverted, on the its corresponding prepared glass slide. Gently blot off excess mounting medium and seal edges of coverslip with clear nail polish.

25. Allow sealant to dry in the dark and then store slides in an opaque slide box at 4°C until evaluation by fluorescence microscopy.

    The slides can be stored at 4°C for up to 4 weeks.

26. Use epifluorescence microscopy (*UNIT 2A.1*) with a 100× oil-immersion objective (final magnification with oil equal to 1000×) to visualize cells.

    The outline of individual mammalian cells can be visualized using the red channel (λ=560 to 580 nm) to detect Alexa568- phalloidin, which stains filamentous actin (and thus actin stress fibers). If antibody rather than DAPI was used to stain bacteria, visualize the antibody-labeled bacteria using the green channel (λ=490 to 505 nm). See Critical Parameters for further discussion. An excitation wavelength of 395 to 410 nm is then used to visualize DAPI-stained bacteria.

27. For each slide, score the number of bacteria bound per cell for ~50 cells; move the stage in an ordered fashion to avoid counting same field twice.

    The average of triplicate samples will thus encompass data from ~150 cells.

BASIC PROTOCOL 2

FLUORESCENT STAINING OF EHEC-INDUCED ACTIN PEDESTALS

A hallmark of an EHEC infection is the generation of actin pedestals beneath sites of bacterial attachment on the intestinal mucosa. EHEC-induced actin assembly can be reproduced in vitro by infecting cultured cell monolayers, removing unbound bacteria, and staining for F-actin accumulation at sites of bacterial binding using fluorescently labeled phalloidin, a fungal toxin that specifically binds filamentous (F)-actin.

Materials

HeLa cell infection medium (see recipe), prewarmed
Desired primary antibody against host or bacterial protein of interest, diluted appropriately in PBS/1% BSA
2 mg/ml Alexa488-conjugated secondary antibody against species in which primary antibody was raised

Additional reagents and equipment for preparing EHEC-infected HeLa cell monolayers (Basic Protocol 1, steps 1 to 11) and quantitation of actin pedestal phenotype by fluorescence microscopy (Support Protocol)

Prepare EHEC-infected HeLa cell monolayers

1. Perform Basic Protocol 1, steps 1 to 11.
2. Incubate plate at 37°C in a 5% CO_2 atmosphere for 3 hr from initial time of infection.
3. Remove the plate from the incubator, aspirate the medium, and wash monolayer twice with PBS, using 1.0 ml/well.
4. Add 0.5 ml prewarmed HeLa cell infection medium to each well and incubate 2 hr at 37°C in a 5% CO_2 atmosphere.
5. Aspirate the medium and rapidly wash monolayer five times with PBS, each time using 0.5 to 1.0 ml/well.

Fix cells

6. To fix the infected monolayers, add 0.5 ml room temperature 2.5% paraformaldehyde to each well and incubate, without agitation, at room temperature for 15 min. Incubate an additional 15 min with gentle rocking.
7. Aspirate medium and wash monolayer once for 2 min using 0.5 ml PBS/well.
8. Aspirate medium and add 0.5 ml PBS/0.1% Triton X-100 per well to permeabilize the monolayer. Incubate 5 min at room temperature with gentle rocking.
9. Aspirate the medium and wash the monolayer three times, each time for 2 min with 0.5 ml/well PBS, at room temperature with gentle rocking.

Perform staining

10. To localize a host or bacterial protein of interest, add 275 μl of desired primary antibody (diluted appropriately in PBS/1% BSA) and incubate 30 to 45 min at room temperature with gentle rocking.
11. Aspirate medium and wash coverslips three times for 5 min each with 1× PBS (0.5 ml/well) at room temperature with gentle rocking.
12. Using a total volume of 275 μl in PBS/1%BSA, treat monolayers with the following:

    2 U/ml (final concentration) Alexa568-phalloidin (add from 200 U/ml stock) to visualize filamentous actin
    10 μg/ml Alexa488-conjugated secondary antibody (add from 2 mg/ml stock) to visualize the localization of primary antibody added in step 10
    and/or
    1 μg/ml (final concentration) DAPI to visualize bacteria.

    Wrap plate in aluminum foil and incubate 30 to 45 min at room temperature with gentle rocking.

    Researchers may wish to perform their own antibody titrations to establish the optimal concentrations in this step.

13. Aspirate solution, then wash coverslips four times, each time for 5 min with 0.5 ml/well PBS at room temperature with gentle rocking. Leave final wash medium in well.

Mount and examine

14. Spot 10 μl mounting medium or ProLong Gold Antifade reagent on glass slides and mount coverslips, inverted, on the prepared slides. Seal edges of coverslips with clear nail polish and allow to dry in a dark place.

15. Store slides in an opaque slide box at 4°C until ready to perform examination using fluorescent microscopy (Support Protocol).

The slides can be stored at 4°C for up to 4 weeks.

SUPPORT PROTOCOL

QUANTITATION OF ACTIN PEDESTAL PHENOTYPE BY FLUORESCENCE MICROSCOPY

Both EHEC and EPEC-mediated actin assembly activity can be quantitated using epifluorescence microscopy to determine the percentage of cell-associated bacteria generating actin pedestals.

Materials

EHEC grown on HeLa cell monolayers and stained for actin pedestals (Basic Protocol 2)

Additional reagents and equipment for epifluorescence microscopy (*UNIT 2A.1*)

1a. *To identify bound DAPI-stained EHEC:* Visualize cells using epifluorescence microscopy under 100× magnification with an excitation wavelength of 395 to 410 nm (*UNIT 2A.1*).

1b. *To identify associated actin pedestals:* Examine cells using an excitation wavelength of 560 to 580 nm to visualize Alexa568-stained phalloidin (*UNIT 2A.1*), which localizes to filamentous actin.

2. For each slide, score the number of bacteria bound per cell and the number of pedestals as follows:

   a. Score the number of bacteria bound per cell for ~50 cells. Move the stage in an ordered fashion to avoid counting same field twice.

   b. Simultaneously, score the number of pedestals on each of these 50 cells, and thereby derive the percentage of adherent bacteria generating actin pedestals.

   The average of triplicate samples will encompass data from ~150 cells.

BASIC PROTOCOL 3

IDENTIFICATION OF PROTEINS SECRETED BY THE EHEC TYPE III SECRETION SYSTEM

The second step of colonization, intimate attachment, requires the function of the EHEC type III secretion system. When EHEC is grown in LB medium, minimal amounts of protein are secreted by this system. However, under conditions that induce the activity of the type III secretion apparatus, such as growth in DMEM/HEPES or M9 minimal media, proteins secreted via this pathway can be detected in culture supernatants (DeVinney et al., 1999). Nevertheless, because EHEC do not secrete these proteins in large quantities, it is usually necessary to concentrate the supernatant prior to analysis by SDS-PAGE. In addition, although some investigators have utilized variations of this technique to characterize the repertoire of type III secreted proteins by protein staining of the sample, due to the difficulty in obtaining large amounts of these proteins, the authors of this unit routinely use immunoblotting to determine whether a putative type III secreted protein is secreted into culture supernatants in type III secretion–dependent fashion.

Materials

Enterohemorrhagic *Escherichia coli* (EHEC) strain(s) of interest and type III secretion–deficient mutant as control

Luria-Bertani (LB) broth (*APPENDIX 4A*) containing appropriate antibiotics (Table A.4A.2)

DMEM/HEPES (see recipe) *or* M9/glucose/bicarbonate medium (optional; see recipe)

20% glycerol, sterile (see recipe)

1× and 2× SDS sample buffer (*APPENDIX 2A*)

100 mM phenylmethylsulfonyl fluoride (PMSF) stock solution and 1 mg/ml pepstatin, leupeptin, aprotinin stock solutions (see recipe for protease inhibitor stock solutions)

14-ml snap-cap culture tubes (Falcon)

Centrifuge (e.g., Allegra 6 Benchtop Centrifuge; Beckman Coulter)

0.22-μm sterile filters

Centrifugal filter concentrators with MWCO appropriate to the protein of interest (Amicon)

Additional reagents and equipment for SDS-PAGE (Gallagher, 2006), staining of proteins in gels (Sasse and Gallagher, 2003), and immunoblotting (Gallagher et al., 2004)

Grow bacteria

1. For each EHEC strain, inoculate 1.5 ml LB broth (plus appropriate antibiotics; Table A.4A.2) with a single colony from freshly streaked plates in a 14-ml snap-cap culture tube. Incubate 8 hr with agitation at 37°C until the OD_{600} is between 0.4 to 0.5.

   It is important to carry a type III secretion–deficient mutant as a control in this experiment. Refer to Troubleshooting and Critical Parameters for further information.

2. In another 14-ml snap-cap culture tube, dilute bacterial culture 500-fold into 5 ml DMEM/100 mM HEPES (pH 7.4). Incubate 14 hr without agitation at 37°C in a 5% CO_2 atmosphere.

3. Dilute the DMEM culture 1:20 into 5 ml M9/glucose/bicarbonate medium or DMEM/HEPES. Incubate 5 hr at 37°C in a 5% CO_2 atmosphere.

   The use of an M9-based medium compared to DMEM-based medium results in a relative increase in the secretion of translocated effectors (i.e., proteins that are normally injected into mammalian cells) and a reduction in translocators (i.e., secreted proteins that are required for the translocation of effectors into mammalian cells; DeVinney et al., 1999). For more information on this phenomenon, as well as a description of bacterial mutants (deficient in SepL or SepD) that secrete very high levels of effectors, refer to Background Information," identification of proteins secreted by the EHEC type III secretion system."

Prepare bacterial supernatants

4. Collect bacteria by centrifugation at 1462 × *g* (3000 rpm in a Beckman Coulter Allegra 6 tabletop centrifuge), room temperature. Reserve the supernatant(s) on ice.

5. Resuspend pellet in 1 ml of 20% glycerol, transfer to a microcentrifuge tube, and harvest by microcentrifuging 1 min at 2000 × *g*, room temperature. Remove supernatant, repeat addition of glycerol and centrifugation, and again remove the supernatant.

   Washing the bacteria in 20% glycerol is associated with less nonspecific lysis of bacteria than washing in PBS.

6. Resuspend bacterial pellet in 50 μl of 1× SDS sample buffer and reserve for SDS-PAGE (step 11).

7. Sterilize culture supernatant from step 4 by passing through a 0.22-μm filter.

8. To the sterilized supernatant, add PMSF from 100 mM stock to a final concentration of 1 M, and pepstatin, leupeptin, and aprotinin from 1 mg/ml stocks to a final concentration of 10 μg/ml each.

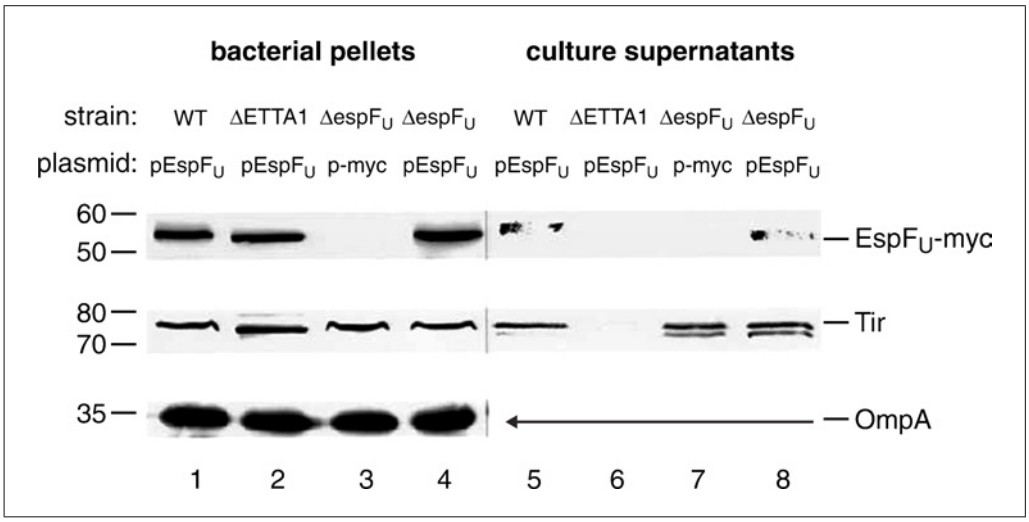

Figure 5A.1.1 Cultured bacterial strains were separated into bacterial and supernatant fractions prior to SDS-PAGE and immunoblotting, as described in Basic Protocol 3. A Type III secretion mutant (ΔETTA1) was included as a negative control and α-OmpA blotting was used as a loading-volume control. Reproduced from Campellone et al. (2004) with permission from Elsevier.

9. Concentrate the supernatants at least 50-fold using centrifugal filter devices.

   The authors have found that the centrifugal filter devices from Amicon work well for this application, but equivalent filters from other sources may be used. Filters with molecular weight cutoffs (MWCOs) of 10 kDa can be used unless very small proteins are of interest. See Critical Parameters section for a further discussion pertaining to MWCO and selection of centrifugal filters.

Analyze supernatants by SDS-PAGE

10. Add an equal volume of 2× SDS-PAGE sample buffer to the supernatant.

11. Visualize secreted protein using SDS-PAGE (Gallagher, 2006) followed by Coomassie Blue or silver staining (Sasse and Gallagher, 2003) or detect specific protein(s) by immunoblotting (Gallagher et al., 2004).

    The authors of this unit have used immunoblotting for detection of several secreted proteins, e.g., Tir or EspF$_U$ were detected by immunoblot after loading sample derived from the equivalent of 1.0 to 1.5 ml of supernatant (Campellone et al., 2004). See Figure 5A.1.1 for an example. Refer to Critical Parameters for suggestions pertaining to selection of controls.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DMEM/HEPES

Dulbecco's Modified Eagle Medium (DMEM), high-glucose formulation with L-glutamine and pyridoxine hydrochloride, without sodium pyruvate (commercially available from Invitrogen), supplemented with 100 mM HEPES (add from 1 M HEPES, pH 7.4, stock solution, also commercially available from Invitrogen). Filter sterilize and store up to 4 weeks at 4°C.

Store both the DMEM medium and the 1 M HEPES stock at 4°C until expiration dates.

Open all reagents, and prepare media, in laminar flow hood. Filter-sterilize and store at 4°C. When dividing complete medium into aliquots for use, perform all procedures in laminar flow hood.

Glycerol, 20%

Add 20 ml of 100% glycerol to 80 ml deionized water. Mix thoroughly and autoclave. Store indefinitely at room temperature.

HeLa cell growth medium

To 178 ml Dulbecco's Modified Eagle Medium (DMEM, high-glucose formulation, containing L-glutamine and pyridoxine hydrochloride, without sodium pyruvate; Invitrogen), add 20 ml heat-inactivated FBS (Invitrogen) and 2.0 ml of 100× penicillin/streptomycin (Invitrogen). Filter-sterilize and store up to 4 weeks at 4°C.

HeLa cell infection medium

To 190 ml Dulbecco's Modified Eagle Medium (DMEM, high-glucose formulation, containing L-glutamine and pyridoxine hydrochloride, without sodium pyruvate; Invitrogen) add 6.0 ml heat-inactivated FBS (Invitrogen) and 5 ml of 1 M HEPES, pH 7.4. Filter sterilize and store up to 4 weeks at 4°C.

M9/glucose/bicarbonate medium

To 750 ml sterile deionized H_2O (cooled to ≤50°C) add:
200 ml 5× M9 salts (see recipe)
2 ml 1 M $MgSO_4$
20 ml 20% (w/v) glucose (filter sterilized)
0.1 ml 1 M $CaCl_2$
44 ml 1 M $NaHCO_3$
Bring volume to 1 liter with deionized H_2O
Autoclave and store indefinitely at room temperature

M9 salts, 5×

Dissolve the following in a total volume of 1 liter deionized H_2O:
30 g $Na_2HPO_4 \cdot 7H_2O$
15 g KH_2PO_4
2.5 g NaCl
5 g NH_4Cl
Divide salt solution into 200-ml aliquots and autoclave
Store indefinitely at room temperature

Paraformaldehyde, 2.5%

Dissolve 0.5 g of paraformaldehyde in 20 ml of PBS (*APPENDIX 2A*). Heat to fully dissolve chemical but be careful not to boil solution. Make solution fresh on day of experiment. Ensure solution is at room temperature prior to use.

Volume can be adjusted depending on needs of experiment.

Protease inhibitor stock solutions

100 mM PMSF: Dissolve 0.174 g phenylmethylsulfonyl fluoride (PMSF) in 10 ml methanol.

1 mg/ml pepstatin: Dissolve pepstatin (Sigma) at 1 mg/ml in 95% ethanol with heating at ~60°C.

1 mg/ml leupeptin: Dissolve leupeptin at 1 mg/ml in water.

1 mg/ml aprotinin: Dissolve aprotinin at 1 mg/ml in water.

Store all protease inhibitor stock solutions up to 6 months at –20°C.

COMMENTARY

Background Information

Pathogenicity of EHEC

Enterohemorrhagic *E. coli* O157:H7 (EHEC) is a highly infectious Gram-negative bacterium that causes an illness often associated with the consumption of fecally contaminated food. During colonization of the large bowel, EHEC generates "attaching and effacing" (AE) lesions, characterized by loss of microvilli on the surface of the enterocyte, intimate bacterial attachment, and formation of an actin pedestal. These AE lesions may both promote colonization and contribute to compromising of the intestinal epithelial barrier. The host cell interaction has been the subject of extensive investigation based on its putative role in colonization, and each protocol in this unit addresses steps required for the production of AE lesions by EHEC. Several other likely important modifications of the host cell are associated with type III secretion of effectors, but a full discussion of this topic is beyond the scope of this protocol (for review, see Garmendia and Frankel, 2005)

In addition to host modification via type III secretion, EHEC produces a Shiga-like toxin (Stx) that can damage the intestinal mucosa and lead to hemorrhagic colitis. The toxins can be absorbed systemically, and severe cases of EHEC infection are associated with the life-threatening complication, hemolytic uremic syndrome (HUS), the triad of anemia, thrombocytopenia, and renal failure. Nevertheless, in most cases, recovery from infection occurs within 5 to 10 days with supportive treatment. The role of antibiotics in the therapy of EHEC infection is somewhat controversial, because Stx is phage-encoded and some antibiotics induce the Stx prophage, resulting in high-level production of toxin (Zhang et al., 2000).

At least two other pathogens also are capable of generating AE lesions. *Citrobacter rodentium* is a natural mouse pathogen that is often used as a model for EHEC. EPEC, mentioned extensively above, is an important cause of infantile diarrhea in the developing world and interacts with mammalian cells in culture with much greater efficiency than EHEC. Hence, as discussed above, it can serve as a useful positive control for each of the three assays described in this unit.

Adhesion of EHEC to mammalian cells

Currently, much is known about type III secretion and the role of some EHEC effectors in host cell modification (see below), but a significant gap exists in our knowledge of the mechanism of initial adherence to mammalian cells. Several potential ligands have been identified (Ebel et al., 1998; Tatsuno et al., 2001; Torres et al., 2002; Badea et al., 2003), but the identity of the adhesin(s) that mediated initial attachment of EHEC to host cell remains unclear. Historically, bacterial adhesion has been assessed using two general methods—the microscopic-based method such as that described above (Basic Protocol 1; Sherman and Soni, 1988; Donnenberg et al., 1993) or one dependent on titration of viable bacterial counts (i.e., colony forming units) of bacteria resistant to washing of the monolayer (Liu et al., 1999; Badea et al., 2003). The initial steps in both methods are identical, and the only difference lies in the mode of detection of bound bacteria. While both methods have been used successfully, in the authors' experience, the viable count assay has sometimes given a high degree of "background" binding, perhaps due to nonspecific attachment of bacteria to tissue culture plastic. Thus, the major advantage to microscopic-based evaluation lies in the ability of the investigator to exclusively count bacteria adherent to mammalian cells.

Fluorescent staining of EHEC-induced actin pedestals

The assay for actin pedestal formation was developed by Knutton et al. (1989) and has been a central assay in virtually all investigations of actin signaling by EPEC and EHEC. It is also commonly referred to as the filamentous actin staining, or FAS, assay. The striking morphology of these lesions and their specificity for pathogenic bacteria has suggested that the ability to form actin pedestals is important in colonization or disease. Thus far, however, experimental evidence directly linking actin signaling to pathogenicity is lacking (Deng et al., 2003). Nevertheless, actin pedestal formation remains a focus of research in EHEC and other attaching and effacing pathogens. Interestingly, whereas Tir is the only effector required for actin pedestal formation by EPEC, a second effector, $EspF_U$ (also known as TccP) is required in addition to Tir for pedestal formation by EHEC (Campellone et al., 2004; Garmendia et al., 2004). For both bacteria, initiation of actin signaling requires clustering of Tir in the plasma membrane, a function provided by the outer membrane protein intimin, which binds Tir and is functionally interchangeable between EPEC and EHEC.

A variety of cell lines have been used to assay actin pedestal formation. One advantage of using HeLa cells is that, not only are pedestals formed efficiently on these cells, but it is often possible to visualize most of the pedestals on a given cell in a single plane of focus because of their relatively flat morphology. Other cell lines have also been used, such as Hep-2 cells (Knutton et al., 1989), Caco-2 cells (which are physiologically more similar to colonic epithelium), and mouse embryonic fibroblasts (MEFs), which can be derived from specific knock-out cells, thereby providing a genetic test of the role of different mammalian signaling molecules in actin pedestal formation (Gruenheid et al., 2001).

Identification of proteins secreted by the EHEC type III secretion system

Type III secretion is critical to the pathogenesis of an EHEC infection. A large pathogenicity island, termed the LEE (for locus of enterocyte effacement) encodes components of a needle-like structure that spans the inner and outer membranes used for the secretion of a specific set of proteins (Jarvis et al., 1995; Abe et al., 1998; Perna et al., 1998), Some of these proteins, such as EspA, EspB, and EspD (where Esp denotes *E. coli* secreted protein), are translocators required for delivery of other secreted "effector" proteins into the host cell. Effector proteins, such as EspF, $EspF_U$, Tir, and Map, modify diverse host cell functions upon translocation (for review, see Garmendia et al., 2005). The ability to perform type III translocation is required for EHEC to establish infection and cause disease (Jarvis and Kaper, 1996). Therefore, the identification and characterization of type III secreted effectors have been essential to understanding of the pathogenesis of EHEC.

The expression of the type III secretion apparatus and effectors depends on the growth conditions of the bacterium. Secretion is very sensitive to temperature, pH, growth phase, and osmolarity (Kenny et al., 1997). Log-phase growth in DMEM, but not LB, gives rise to bacteria that are capable of type III secretion, particularly secretion of those components, such as EspA, EspB, and EspD, that are critical for translocation of effectors into the host cell. Growth in M9 minimal media reduces (but does not eliminate) the secretion of these components, and increases the secretion of effector proteins such as Tir (DeVinney et al., 1999) and $EspF_U$ (also known as TccP; Campellone et al., 2004; Garmendia et al., 2004). The growth conditions presented in the protocol are designed to optimize effector secretion. Recently it has been demonstrated that disruption of the LEE-encoded *sepD* or *sepL* genes results in enhanced secretion of effector proteins (O'Connell et al., 2004; Deng et al., 2005), an observation that has been used to identify a more complete set of effectors. Although beyond the scope of this protocol, *sepD* or *sepL* mutants may allow for more sensitive detection of effectors.

While this technique identifies proteins that are secreted by the type III apparatus, and many of these proteins are likely to be translocated into host cells, other techniques not covered here are required to document translocation. These methods rely on the translocation of hybrid proteins consisting of translocated effectors fused to enzymes (e.g., adenylate cyclase; Wolff et al., 1998) or TEM-1-β lactamase (Charpentier and Oswald, 2004) that exhibit maximal activity only upon entry into in the host cell.

Critical Parameters

Adhesion of EHEC to mammalian cells

It is important to include positive and negative controls when initially developing these assays. A reasonable positive control is enteropathogenic *E. coli* (EPEC, e.g., strain E2348/69; Levine et al., 1985), a close relative of EHEC. Unlike EHEC, EPEC possesses a bundle-forming pilus that promotes efficient bacterial binding to cultured cells. Because EPEC binds more efficiently to cells than EHEC, the authors routinely inoculate monolayers with 5- to 10-fold fewer EPEC. A nonadherent negative control, such as a derivative of *E. coli* K12 (e.g., MC1061), can be used to establish a baseline for "nonspecific" binding. Alternatively, an EHEC mutant defective for type III secretion also is largely defective for binding.

The decision to stain bound bacteria with DAPI or an EHEC-specific antibody is related to the strains being analyzed. For example, EPEC and *E. coli* K12 strains will not stain with anti-O157 antibody, so the authors routinely use DAPI staining in experiments that include either of these control bacteria. The most significant disadvantage to using DAPI is the difficulty in visualizing bacteria that are overlying the mammalian nucleus, which stains intensely with DAPI. However, because scoring of DAPI-stained samples uniformly excludes bacteria in this location, this

limitation should not significantly alter the comparison of bacterial adherence among different strains.

Fluorescent staining of EHEC-induced actin pedestals

Whereas EPEC is highly proficient at generating actin pedestals on cultured monolayers, actin pedestal formation by EHEC is somewhat variable, depending on strain, the particular source of serum, and unknown factors. The authors currently use serum from Gibco (an Invitrogen product line) with success, but other sources of serum may be equivalently efficacious. Because of this potential variability, when initially establishing this assay, EPEC may serve as a useful positive control. Alternatively, a *dam* methylase EHEC mutant is also far more proficient at generating actin pedestals than wild-type EHEC (Campellone et al., 2007) and could similarly function as a positive control.

The temperature of the paraformaldehyde solution used for fixation is critical, because the authors have found that if the paraformaldehyde is not cooled to room temperature, the bacteria-, even those bacteria that are not associated with mammalian cells, are stained nonspecifically with Alexa568-labeled phalloidin.

Identification of proteins secreted by the EHEC type III secretion system

The success of this assay is dependent on the growth conditions that induce the function of the type III secretion apparatus. Thus, bacterial growth conditions are critical. Growth in DMEM/HEPES medium (see Reagents and Solutions) promotes the secretion of translocator proteins, whereas growth in M9 minimal medium (also described in Reagents and Solutions) promotes the secretion of effector proteins while down-regulating the secretion of translocators. Moreover, to rule out the possibility that proteins found in the culture supernatant are the result of nonspecific lysis or secretion via alternative pathways, the parallel analysis of a mutant defective for type III secretion, such as an *ETTA1* deletion mutant, should be performed (Campellone et al., 2004). Similarly, the detection of Tir or other previously characterized secreted effectors in the supernatant of EHEC cultures would provide a positive control. If the putative secreted effector is also expressed by the related pathogen EPEC, this strain, which is more proficient at type III secretion in vitro than EHEC, could provide a more robust signal in this assay.

Finally, the amount of effectors present in the supernatant will obviously vary with the expression level and secretion efficiency of that particular effector. Thus, if one fails to find the putative effector in the supernatant, scaling up the amount of material loaded is a reasonable alternative.

Troubleshooting

Table 5A.1.1 presents some of the problems commonly encountered with adhesion of EHEC to mammalian cells (see Basic Protocols 1 and 2).

Anticipated Results

Adhesion of EHEC to mammalian cells

Using the protocol outlined above, the authors have typically found ~250 wild-type EHEC strain EDL933 bound per 50 mammalian cells. With EPEC strains, one typically finds 2- to 5-fold more bacteria bound per given number of cells than with EHEC (e.g.,

Table 5A.1.1 Troubleshooting Guide for Analyzing Adhesion of EHEC to Mammalian Cells

Problem	Possible cause	Solution
No bacteria bound	MOI too low	Increase number of bacteria initially added
	Washes too harsh	Prior to fixation with PFA, the washes with PBS must be very rapid yet gentle[a]
	Anti-O157 antibody or DAPI concentration too dilute	Titrate the concentration of antibody or DAPI
No mammalian cells left on coverslip	Washes too harsh and/or cells exposed to PBS for too long[b]	Prior to fixation with PFA, the washes with PBS must be very rapid yet gentle[a]
		Consider precoating glass coverslips with gelatin or polylysine in order to facilitate cell attachment

[a] See Basic Protocol 1, step 13.

[b] HeLa cells are very sensitive to serum starvation and can detach from glass coverslips in low serum conditions.

~500 to 1250 bacteria bound per 50 mammalian cells), even with the lower inoculum. At most, one or two of the negative-control *E. coli* K12 are typically bound per 50 cells.

Fluorescent staining of EHEC-induced actin pedestals

Using the protocol outlined above (Basic Protocol 2), one will normally see significant cell-to-cell variation in bound bacteria and pedestals. Many mammalian cells will not be associated with any bacteria, whereas others may harbor ten or more bound bacteria. Normalizing pedestals to bound bacteria, the authors have typically found ~75% of bound wild-type EHEC strain EDL933 are associated with easily detected actin pedestals. For EPEC, this figure is usually closer to 90%.

Identification of proteins secreted by the EHEC type III secretion system

The equivalent of 1.5 ml culture supernatant typically contains enough effector molecule for easy detection by immunoblotting (Campellone et al., 2004), although this may vary significantly depending upon the specific effector.

Time Considerations

Basic Protocols 1 and 2 require multiple days for the culturing of mammalian cell lines, as well as a day to prepare the bacterial cultures. The infection and staining steps can be completed in 1 day. Additionally, Basic Protocol 3 requires 2 days to complete: 1 day for the culturing of the bacteria and an additional day for lysate preparation and examination by immunoblotting.

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Rapid Allelic Exchange in Enterohemorrhagic *Escherichia coli* (EHEC) and Other *E. coli* Using λ Red Recombination

The ability to generate defined mutations in enterohemorrhagic *Escherichia coli* (EHEC) has been essential to the analysis of its pathogenicity. The generation of such mutants has typically been done by integrating a plasmid containing the mutant gene of interest and a selectable marker followed by resolving the co-integrant to remove plasmid backbone sequences. Unfortunately, this procedure does not always transfer the mutation to the chromosome as desired, nor does it occur at a high frequency. Furthermore, the techique is time consuming and requires prior cloning of the gene of interest.

A gene replacement method for *E. coli* that has been gaining widespread adoption over the last five years involves transient expression of the bacteriophage λ Red recombination system (for review, see Court et al., 2002). The practice of using phage recombination systems to promote engineering of bacterial chromosomes is known as "recombineering." The λ Red recombineering technology has simplified allelic exchange in *Escherichia coli* K-12 and *Salmonella enterica* Serovar enteritidis, and has encouraged use of the system in pathogenic species of *E. coli*, including enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *E. coli* (Murphy and Camplellone, 2003).

The regulated expression of the λ bacteriophage genes *bet* (ssDNA annealing function), *exo* (a 5′-3′ dsDNA exonuclease), and *gam* (inactivation of host RecBCD function) in *E. coli* species renders the bacteria hyper-recombinogenic and amenable to gene replacement via electroporated linear DNA substrates. These substrates can be easily generated using PCR, purified using standard DNA purification methods, and introduced by electroporation into electrocompetent bacteria. The methods presented in this unit describe the necessary plasmids and growth conditions needed to render EHEC hyper-recombinogenic, the means via which the authors' laboratory generates linear DNA substrates, and the selection methods employed to obtain potential recombinants (see Basic Protocol).

In addition, a method for the preparation of electrocompetent/hyper-recombinant EHEC cells is presented (Support Protocol), as well as an alternative for the generation of precise unmarked deletions (i.e., deletions containing no exogenous DNA sequences, such as a drug marker) in EHEC using *sacB* counterselection (Alternate Protocol). Variations of these methods and helpful hints for successful gene knockouts are provided in the Commentary section.

CAUTION: Enterohemorrhagic *Escherichia coli* (EHEC) is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

RED-PROMOTED GENE REPLACEMENT IN EHEC

BASIC PROTOCOL

The following protocol is the one used in the authors' laboratory for gene replacement in EHEC. The initial step involves generating a recombination substrate. This is most easily done by generating a PCR product which contains a drug marker flanked by 40 bp of DNA upstream and downstream of the target gene. The PCR product is purified and

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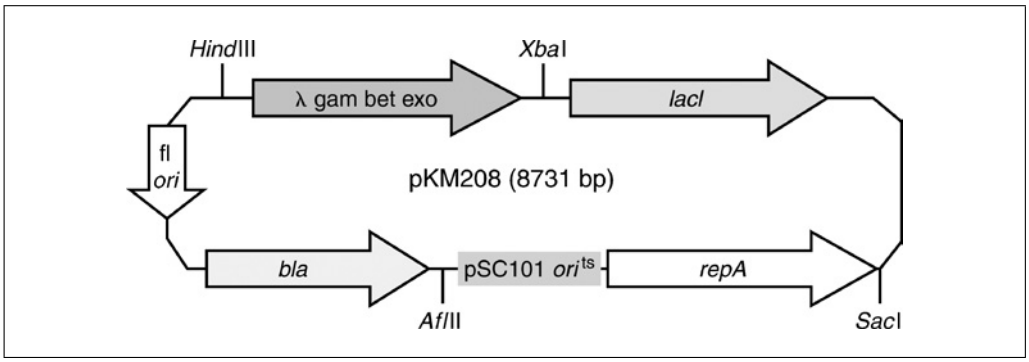

Figure 5A.2.1 Map of pKM208. Positions of key genetic elements in plasmid pKM208 (clockwise from top left): Ptac-*gam-bet-exo* operon, *E. coli lacI* repressor gene, *repA*, pSC101, ampicillin^R-encoding *bla* gene, and the f1 *ori*. A subset of unique restriction sites is shown. The *lacI* gene, contained within a *Not*I fragment (not shown), is absent from plasmid pKM201. The pSC101 ori^{ts} is a low-copy-number replicon which allows growth of the plasmid at 30°C but not at 44°C (Hamilton et al., 1989). While originally described as a tool for generating gene deletions in *E. coli*, it is used here for the Red-producing plasmid to allow for easy curing of the plasmid following Red-promoted gene replacement.

electroporated in EHEC cells containing the Red-producing plasmid pKM208 (Fig. 5A.2.1). Following electroporation, the culture is allowed to proliferate and gene replacements are selected on antibiotic selection plates.

Table 5A.2.1 presents the time required for the major components of this protocol.

Materials

Linear DNA substrate containing drug marker sequence (see Critical Parameters and Troubleshooting)
Polymerase: *Taq* polymerase (New England Biolabs)
dNTPs (*APPENDIX 2A*)
Appropriate PCR buffer (e.g., see *APPENDIX 2A*)
TE buffer (*APPENDIX 2A*)
Electrocompetent EHEC transformed with Red-producing plasmid (Support Protocol)

Table 5A.2.1 Time Considerations for Red-Promoted Gene Replacement in EHEC

Day	Activity	Time required
Day 1	Prepare and purify PCR product	2-3 hr
	Streak and incubate at 30°C to isolate single colonies of EHEC/pKM201 or pKM201 on LB/Amp	Overnight
Day 2	Prepare electrocompetent cells starting with one colony in 20 ml of LB containing antibiotic[a]	6-8 hr
	Electroporate, grow culture, and plate	2 hr
	Incubate to grow visible colonies	Overnight
Day 3	Restreak potential recombinants	15 min
	Incubate to grow visible colonies	Overnight
Day 4	PCR analysis	2-4 hr

[a]As an alternative, electrocompetent cells can be prepared ahead of time and frozen at −80°C.

Primer 1	5′ *GACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGC* AATCTCTGATGTTACATTGC 3′
Primer 2	5′ *AAGAAAGCCTGACTGGCGGTTAAATTGCCAACGCTTATTA* TTCAACTCAGCAAAAGTTCG 3′

Figure 5A.2.2 An example of two 60-oligomer primers used to generate a PCR product for λ Red–promoted gene replacement (in this case, a Δ*lacZ*::*kan* allele). The underlined region in primer 1 contains 5′ to 3′ sequence upstream of the *aph* gene (i.e., top strand reading rightward in the *aph* region when read as a dsDNA sequence file; not shown)—the *aph* gene encodes kanamycin resistance (*kan*). The underlined region in primer 2 contains 5′ to 3′ sequence downstream from the *aph* gene, (i.e., bottom strand reading leftward in the *aph* region when read as a dsDNA sequence file; not shown). These are the sequences that will prime the PCR reaction. The region in italics in primer 1 contains 5′ to 3′ sequence from the N-terminal encoding region of *lacZ* (top strand reading rightward in the sequence file of *lacZ*; not shown), while the region in italics in primer 2 contains 5′ to 3′ sequence from the C-terminal encoding region of *lacZ* (bottom strand reading leftward in the sequence file of *lacZ*; not shown). These are the regions that will define the homology with the target gene, *lacZ*.

SOC medium (*APPENDIX 4A*)

LB plates (*APPENDIX 4A*) containing appropriate antibiotic (see Critical Parameters and Troubleshooting, Drug Markers)

Electroporator and 0.1-cm gap length electroporation cuvettes

Additional reagents and equipment for PCR (Kramer and Coen, 2001), purification of DNA from aqueous solution (Moore and Downhan, 2002), and Southern blotting (*UNIT 14B.1*; optional)

1. Order two 60-oligomer primers with the following characteristics. Try to keep the overall GC content of the primers around 50% ± 5%.

   Primer 1: Should have the first 40 bases (the 5′ end) complementary to the sequence encoding the N-terminal region of the target gene and the final 20 bases (the 3′end) complementary to the sequence on one end of the drug cassette.

   Primer 2: Should have the first 40 bases complementary to the sequence encoding the C-terminal region of the target gene, and the final 20 bases complementary to the other end of the drug cassette.

   The choice of target sequences in these primers will dictate the endpoints of the deletion. See Figure 5A.2.2 for an example.

2. Prepare a PCR reaction by combining linear DNA substrate containing drug marker sequence, primers, polymerase, dNTPs, and appropriate buffer. Perform standard PCR optimized for the sequences involved (Kramer and Coen, 2001).

3. Purify the PCR product using traditional DNA purification methods (Moore and Downhan, 2002) or commercially available PCR product purification kits.

   The purpose of this step is to remove salts from the PCR buffer that would interfere with the electroporation step that follows. In some cases, gel purification of the PCR product is warranted (see Critical Parameters and Troubleshooting, PCR Templates).

   The PCR product contains the drug marker flanked on one end by a 40-bp sequence from the N-terminal encoding region of the target gene, and on the other end by a 40-bp sequence from the C-terminal encoding region of the target gene.

4. Resuspend PCR product in TE buffer.

5. Place electroporation cuvettes in an ice-water bath for 10 min.

   Ensuring the cuvettes are cool is important as electroporation of the sample can generate excessive heat, causing a decrease in cell survival.

6. In a microcentrifuge tube, mix 1 to 5 μl purified PCR product (0.1 to 1 μg) with 50 μl electrocompetent EHEC transformed with Red-producing plasmid (pKM201 or pKM208). Transfer to the precooled cuvette and incubate 1 min on ice.

   PCR product and electrocompetent cells are combined in a microcentrifuge tube rather than directly in the electroporation cuvette to ensure adequate mixing of cells and PCR product.

7. Thoroughly (but quickly) dry the cuvette with Kimwipes. Insert it into the electroporation chamber and quickly pulse at 2200 V or voltage suggested by the electroporator manufacturer for *E. coli*.

   CAUTION*: Maintain the cap on the cuvette during electroporation of EHEC cells to prevent possible aerosol formation.*

8. Immediately add 0.5 ml SOC medium and mix thoroughly with the cells in the electroporation chamber. Transfer 0.5 ml of this mixture to a culture tube containing 1 to 2.5 ml SOC.

   It is not necessary to remove the small amount of mixture contained between the electroporation plates (< 100 μl), as this represents a small fraction of the total sample.

9. Incubate the culture 60 to 90 min at 37°C with rolling to aerate the cells.

10. *Optional*: Save a portion (0.3 ml) of the culture and use to prepare an overnight culture (5 ml total) in LB medium. Plate 0.1 ml of various dilutions of this overnight culture onto LB plates containing appropriate antibiotic.

    This step is required when expression of the drug marker has not reached appropriate levels within the first 90 min of growth following electroporation to confer resistance, as is sometimes the case when tetracycline is used. It is typically unnecessary to perform this step, but it should be tried when cells plated 60 to 90 min following electroporation fail to yield recombinants.

11. Following 60 to 90 min incubation, plate remaining SOC culture from step 9 onto LB plates containing appropriate antibiotic. Incubate overnight at 37°C.

    The appropriate antibiotic is one that selects for the marker contained on the linear DNA substrate (i.e., PCR product; step 1). See Critical Parameters and Troubleshooting, Drug Markers, for important details regarding antibiotic selection.

    The authors typically plate 0.5 ml/plate. To conserve plates, one can concentrate the cells by microcentrifuging 1 min at moderate speed (~2/3 of maximum), 4°C, resuspending in 0.25 ml SOC, and spreading the entire mixture onto one plate.

12. Identify colonies on plates (see Critical Parameters and Troubleshooting). Using an inoculating loop, pick and streak two to four colonies onto fresh LB antibiotic-selection plates. Incubate overnight at 37°C.

    The authors typically streak two colonies per plate.

13. As an alternative (or in addition) to step 12, streak a larger number of colonies (~40 streaks per plate) onto both LB and LB antibiotic-selection plates as follows.

    a. Pick a colony off the transformation plate(s) (step 11) with an inoculating loop.
    b. Transfer using one, small (0.5-cm) streak onto an LB antibiotic selection plate, followed immediately (without flaming) by a streak of similar size onto an LB plate.
    c. Incubate overnight at 37°C.
    d. Identify false positives as those that grow poorly on the LB antibiotic-selection plate relative to their growth on the LB plate.

e. Select two to four candidates that grow well on both plates and streak onto LB-antibiotic-selection plates (one to two colonies per plate) to obtain single colonies.

f. Incubate overnight at 37°C.

The presence and magnitude of false positives varies with bacterial strains, antibiotic used, drug concentrations, and the amount of culture plated in step 11. Many times it does not arise, but can be especially troublesome with the use of chloramphenicol in EHEC (see Critical Parameters and Troubleshooting). With EHEC, false positives are less of a problem if kanamycin is used as the drug marker.

14. Verify true chromosomal gene replacement via PCR (Kramer and Coen, 2001), phenotypic screens, and/or Southern blot analysis (*UNIT 14B.1*).

The authors typically use a series of colony PCR reactions to verify gene replacement (see Critical Parameters and Troubleshooting). Each of the two junctions of marker and chromosomal sequence can be verified by using one primer complementary to the drug marker and the other primer complementary to a nearby region of the chromosome not contained in the original PCR product. Amplification across the drug marker using a primer pair that flanks but does not include the original PCR product should give a product (containing the marker and flanking chromosomal sequence) of the predicted size. Finally, a primer pair specific to the putative deleted sequence should not generate an amplicon from recombinant colonies but should result in the predicted amplicon when the parental wild-type strain is used.

GENERATION OF UNMARKED CHROMOSOMAL DELETIONS IN EHEC USING SACB COUNTERSELECTION

ALTERNATE PROTOCOL

The following method is a variation of the Basic Protocol where instead of using a drug marker in place of the gene, following the protocol first used by Ried and Collmer (1987), a cassette containing the *cat* (positive selection) and *sacB* (negative selection) genes is used. SacB is a levansucrase that transfers fructosyl residues from sucrose to different cellular receptors such as alcohols, sugars, and levans (Dedonder, 1966). Expression of *sacB* kills *E. coli* in the presence of sucrose (Gay et al., 1983). Thus, two rounds of Red recombination are required: the first selects for chloramphenicol resistance (Cam^R/Suc^S), while the second inserts the precise deletion and evicts the cassette (generating Cam^S/Suc^R colonies).

The second electroporation uses a linear DNA species, typically from a previously constructed plasmid, that defines the precise deletion desired. The authors have used a plasmid that contains the upstream region of the target gene fused to the downstream region, constructed in such a way that this linear DNA can be liberated from the plamid by a restiction enzyme digest (Murphy et al., 2000). Using PCR manipulations to construct these plasmids, the authors usually fuse the first three condons of the target gene to the last three codons in order to allow translational read through of the deletion once crossed into the chromosome.

The construction of an in-frame unmarked deletion, one that contains no drug marker or other scar, is useful when one is limited by the number or availability of antibiotic resistance genes used for a particular pathogen. For instance, once a drug marker is removed from the chromosome, it can then be used at a second location within the same bacterium. Also, if one plans to test the mutant bacterial strain in animal studies, it is best to use a strain containing no antibiotic resistant determinants to (1) rule out polar effects of the drug marker (or its expression) on bacterial genes nearby the site of the deletion and (2) prevent the accidental spread of antibiotic resistant determinants in animal populations.

A potential problem with the use of *sacB* is the generation of spontaneous Suc^R colonies due to loss or mutation of the *sacB* gene. The authors have found this to be more of

Table 5A.2.2 Time Considerations for Generation of Unmarked Chromosomal Deletion in EHEC Using SacB Counterselection

Day	Activity	Time required
Day 1	Insert *Not*I fragment from pKM154 containing *cat-sacB* cassette into plasmid containing target site sequences; make minilysate	2 days
Day 3	Prepare and purify fragment containing *cat-sacB* cassette flanked by target sequences by gel purification	4 hr
	Perform first Red-promoted gene replacement (i.e., insert *cat-sacB* in place of target gene)	3 days[a]
Day 5	Perform restriction digestion on plasmid containing target site sequences to liberate deletion-containing fragment	1-2 hr
Day 6	Prepare $Cam^R/Suc^S/Amp^R$ transformant from first electroporation for electrocompetence and hyper-recombinogenicity	6-8 hr
	Perform second Red-promoted gene replacement (insert unmarked deletion in place of *cat-sacB*)	3 days[a]
Day 9	Purify candidates by streaking out for $Suc^R/Cam^S/Amp^S$ transformants; incubate at 37°C	Overnight
Day 10	Test unmarked deletions by colony PCR	2-4 hr

[a]See Table 5A.2.1.

a problem when *sacB* is on a plasmid and much less so when *sacB* is integrated into the chromosome. Starting the electrocompetent culture from a single overnight colony minimizes the presence of spontaneous *sacB* loss and mutation.

Table 5A.2.2 presents time considerations involved in performing this procedure.

Additional Materials *(also see Basic Protocol)*

3.5-kb *Not*I fragment from pKM154 (i.e., the *cat-sacB* cassette; Murphy et al., 2000)
Restriction enzyme(s) and appropriate buffer
LB plates supplemented with chloramphenicol (LB/Cam; for antibiotic concentrations, see Critical Parameters and Troubleshooting, Drug Markers)
LB plates without NaCl, supplemented with 10% sucrose (LB/Suc), room temperature and 30°C
LB plates supplemented with ampicillin (LB/Amp; for antibiotic concentrations, see Critical Parameters and Troubleshooting, Drug Markers)
Linear DNA fragments containing precise (unmarked) deletion of the region of interest (Murphy et al., 2000) suspended in TE buffer
Additional reagents and equipment for constructing plasmids (Murphy et al., 2000), purifying DNA fragments by gel electrophoresis (Moore et al., 2002), and preparing electrocompetent hyper-recombinant cells (Support Protocol, steps 2 to 12)

1. Construct a plasmid containing 0.5 to 1 kb sequence upstream of the target fused to 0.5 to 1 kb sequence downstream of the target (Murphy et al., 2000). Place a *Not*I site between the two.

The plasmid should be constructed in such a manner that a DNA fragment containing the sequence upstream and downstream of the target gene (as well as the cat-sacB cassette if the substrate is to be prepared on a plasmid; see step 2a) can be liberated as an intact fragment by a restriction digest (for details, see Murphy et al., 2000).

2a. *For preparation of recombination substrate on a plasmid:* Construct a second plasmid by placing the 3.5-kb *Not*I fragment from pKM154 (containing *cat-sacB*) into the plasmid constructed above.

While this step involves the construction of an additional plasmid, the recombination substrate will have a greater amount of flanking homology to the target site relative to the PCR-generated substrate described below (0.5 to 1 kb versus 40 bp), thus providing a greater chance of a successful gene replacement.

2b. *For preparation of substrate using PCR:* Prepare a PCR substrate containing the *cat-sacB* cassette flanked by 40 bp of DNA which contains sequence from upstream and downstream of the region of interest (Kramer and Coen, 2001).

This PCR fragment will be larger (~3.6 kb) than the PCR products described in the Basic Protocol, which are typically <1 kb). Thus, PCR-generated sequence errors in sacB that generate a SacB$^-$ phenotype (Suc^R) will have to be screened out of the pool of transformants. Use of high fidelity polymerases will likely alleviate this problem, though this has not yet been tested in the authors' laboratory.

3. If necessary (i.e., for substrates prepared on plasmids), liberate the fragment containing the *cat-sacB* cassette and flanking upstream and downstream sequences using an appropriate restriction enzyme(s). Purify the fragment by agarose gel electrophoresis (Moore et al., 2002).

This DNA fragment must be gel purified prior to electroporation, as simple transformants of uncut plasmid will appear as gene replacement candidates when performing Red-promoted gene replacement.

4. Using either the PCR product or the gel-purified restriction fragment, perform steps 5 to 11 of the Basic Protocol, selecting for Cam^R colonies.

5. Streak isolated Cam^R colonies from the transformation plate onto LB/Cam to verify positive selection, LB/Suc for negative selection, and LB/Amp to verify the presence of pKM201 or pKM208 as follows. Each set of plates can contain ~40 streaks.

a. Pick a colony off the transformation plate(s) with an inoculating loop.
b. Transfer using one, small (0.5-cm) streak onto an LB/Suc plate, followed immediately (without flaming) by a streak of similar size onto an LB/Amp plate, and again onto an LB/Cam plate.
c. Incubate 1 day at 30°C.
d. Identify Cam^R/Suc^S/Amp^R transformants. Streak onto LB/Amp plates to obtain single colonies.
e. Incubate 1 day at 30°C.

Plasmid pKM201 and pKM208 are many times lost spontaneously following electroporation, which is advantageous if one wants to study the gene knockout in an otherwise wild-type background. If it is not lost spontaneously, streaking out the transformation at 42°C will promote loss of the Red plasmid. Here, however, one needs to retain the Red plasmid for the subsequent gene eviction step. Therefore, incubation is performed at 30°C. (Red-producing plasmids contain temperature-sensitive origins of replication; see Fig. 5A.2.1.)

It should be evident here why the recombinant fragment from the second plasmid described in step 1a must be gel purified—i.e., transformation by plasmid would also yield Cam^R/Suc^S/Amp^R transformants. Minilysates can be prepared from the candidates to verify the absence of the substrate plasmid and the presence of Red-producing plasmid.

Also, a PCR test, or a phenotypic test (if available), for the gene knockout can be used at this step to verify the absence of the target gene.

For an unknown reason, selection for Suc^S mutants is more readily achieved using LB plates without sodium chloride.

6. Prepare a $Cam^R/Suc^S/Amp^R$ transformant from this second LB/Amp plate for electrocompetence and hyper-recombinogenicity as described in steps 2 to 12 of the Support Protocol.

7. Using the technique described in steps 5 to 8 of the Basic Protocol, electroporate a restriction digest of the plasmid containing the precise (unmarked) deletion of the target gene (or region of interest) constructed in step 1.

   Note that the recombining fragment need not be gel purified, since the selection in this step (Suc^R) involves loss of a chromosomal marker rather than the acquisition of an antibiotic marker carried by a potentially contaminating plasmid.

8. Incubate the culture at 37°C by rolling at least 5 hr (or overnight).

   Extra incubation time relative to Basic Protocol, step 9, is necessary since $sacB^+$ is dominant to $sacB^-$, and time is needed for recovery from electroporation shock and segregation of multiple chromosomes.

9. If the culture has been grown for 5 hr, plate onto LB/Suc plates at 0.5 ml/plate. Alternatively, if the culture has been grown overnight to saturation, prepare four 10-fold dilutions in LB medium and plate 0.1 ml of each onto LB/Suc.

10. Incubate overnight at 37°C. Select for Suc^R colonies.

11. Streak individual Suc^R colonies onto LB and LB/Cam plates to identify Cam^S transformants.

12. Simultaneously, screen for Amp^S candidates as well to identify cells that have spontaneously lost the Red plasmid. If no Amp^S transformants are found, streak at 42°C to cure the cell of the Red plasmid.

13. Verify deletion by PCR analysis (Kramer and Coen, 2001).

    See Basic Protocol, step 14, and Critical Parameters and Troubleshooting for more information.

SUPPORT PROTOCOL

GENERATION OF ELECTROCOMPETENT HYPER-RECOMBINANT EHEC CARRYING THE λ-RED FUNCTIONS

This protocol describes the growth and preparation of EHEC strains for Red recombineering. Two plasmids have been used successfully in the authors' laboratories for the λ-Red mediated recombination in EHEC: pKM201 and pKM208 (Murphy and Campellone, 2003). Both vectors are low-copy number plasmids, contain temperature sensitive origins of replication, provide ampicillin resistance, and express λ Red functions (*bet*, *exo*, and *gam*) under control of the Ptac promoter. The difference between these plasmids is that pKM208, in addition to Red, expresses the lacI repressor, while pKM201 does not. Thus, while both require IPTG for full induction, the recombination functions from pKM208 are repressed in the absence of IPTG, an advantageous situation given that continued overexpression of Red functions is mutagenic (Murphy and Campellone, 2003). (Under the conditions of transient expression described in this unit for pKM208, Red is not mutagenic.) Both of these plasmids, when induced, will render EHEC hyper-recombinogenic and promote recombination with linear dsDNA substrates.

This technique differs from other methods of electrocompetent cell preparation by the inclusion of a heat shock step. It is thought that the heat shock prepares the cells for the

shock of electroporation, resulting in higher rates of survival, and thus increased numbers of transformants. This effect can vary from 2- to 50-fold; the mechanism of this effect, and its variability, is not understood. In this description, a 20-ml starting culture is used for the prepration of electrocompetent cells, which is enough for two electroporations. The procedure can be scaled up as needed.

Materials

LB plates (*APPENDIX 4A*) containing 100 μg/ml ampicillin (LB/AMP)
EHEC containing pKM201 or pKM208 (Murphy and Campellone, 2003)
SOC medium (*APPENDIX 4A*) containing 100 μg/ml ampicillin, 30°C
100 mM IPTG (see recipe)
20% (w/v) glycerol, 4°C
30° and 42°C shaking water baths

1. Streak EHEC containing pKM201 or pKM208 onto LB plates containing 100 μg/ml ampicillin. Incubate 24 hr at 30°C.

   Lower drug concentrations are sometimes required for selecting plasmid transformants. However, once established, the transformants grow well at higher drug concentrations. The authors have observed this with pKM208 in EHEC. Thus, for transformation of EHEC with pKM208, one should try low levels of ampicillin (50 μg/ml or lower), but once established, the transformant should be maintained at 100 μg/ml. Also see Critical Parameters and Troubleshooting.

2. Inoculate 20 ml warm SOC medium containing 100 μg/ml ampicillin with one to four purified colonies.

   LB medium (APPENDIX 4A) can be used in place of SOC medium.

3. Incubate culture in a 30°C shaking water bath until the OD_{600} reaches 0.3 (cell density $\sim 10^7$ cells/ml).

4. Induce the culture by adding 100 mM IPTG to a final concentration of 1 mM.

   In the case of pKM201, IPTG is used to inactivate endogenous wild-type repressor.

5. Continue 30°C incubation as described until a cell density of $\sim 0.5–0.7 \times 10^8$ (OD_{600} of 0.5 to 0.6) is achieved. Transfer the culture to a 42°C shaking water bath and incubate 15 min.

   The authors have found that survival of EHEC and E. coli K-12 following electroporation is increased if this heat shock step is included. The increase in survivors increases the total yield of recombinants. The heat shock step may also play a role in the spontaneous loss of plasmid pKM201 and pKM208 in some of the recombinants, which is fortuitous, since the gene replacement and curing of the Red-producing plasmid occur simultaneously.

6. Chill the culture in an ice-water bath for 10 min with slow constant swirling.

7. Transfer cells to appropriate bottles and centrifuge 10 min at 5000 × *g*, 4°C. Discard the supernatant.

8. Resuspend the pellet in 1 ml of ice-cold 20% glycerol.

   The purpose of the glycerol is to protect the membranes from the shock of electroporation and to act as a cryoprotectant if the cells are to be frozen for future use.

   The use of 1 mm MOPS has been identified as an optimal component in a survey of electroporation buffers for transformation of P. aeruginosa (Farinha and Kropinski, 1990) and has been adapted for use in some of the authors' experiments, both here and in step 11.

9. Microcentrifuge suspension 30 sec at a moderate speed (e.g., ~2/3 maximum), 4°C. Carefully remove the supernatant.

10. Repeat steps 8 and 9 twice more.

11. Resuspend pellet in 90 μl of cold 20% glycerol.

    This amount of cells can be used for two electroporations (two 50-μl aliquots).

12. Use bacteria within 1 hr or freeze at –80°C for future use (up to 3 to 6 months).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

IPTG, 100 mM

Dissolve 238 mg IPTG powder (Sigma) into 10 ml deionized H_2O
Pass through a 0.2-μm filter (Acrodisc) to sterilize
Store up to 6 months at −20°C

COMMENTARY

Background Information

The mechanism of λ Red-promoted gene replacement with the PCR-generated substrates described in the Basic Protocol surprisingly does not involve many of the proteins normally associated with recombination in *E. coli* (e.g., RecA). It clearly, however, does involve the action of the λ Bet and Exo proteins acting at the ends of a linear dsDNA fragment. Given what is known biochemically about these proteins, a model for the action of Red in promoting gene replacement suggests that λ Exo degrades the 5′ strand of the dsDNA ends, and perhaps loads λ Bet onto the 3′ ssDNA ends that are generated. Bet then likely promotes annealing of the ssDNA ends to ssDNA regions of the lagging strand in the replication fork. How such a structure gets resolved to incorporate the drug marker into the chromosme in place of the target gene is not clear, but multiple possibilities are discussed by Court et al. (2002). Bet can also promote annealing to the leading stand as well, but at a lower frequency. This model is based on the work of Ellis et al. (2001) and their studies with λ Bet–promoted engineering of the *E. coli* chromosome with single-stranded oligonucleotides.

The authors have found that when the regions of homology between the flanking ends of the substrate and the target gene increase from 40 bases to 1 kb (as in the case of the plasmid-generated substrates used in the Alternate Protocol), the recombination events become more dependent upon RecA and thus likely involve host recombination RecF pathway functions. The RedF pathway has been shown to be required when these longer homology substrates are acted upon by the λ Red recombination system in vivo (Poteete and Fenton, 2000).

The use of λ Red for gene replacement in EHEC allows one to quickly make gene deletions to identify virulence factors, transfer in vitro generated mutants to their chromosomal locations, and construct hybrid versions of pathogenicity islands (Campellone et al., 2002, 2004; Murphy and Campellone, 2003). The ease with which gene knockouts can be performed and the simplicity of curing the host of the Red-producing plasmid following genetic manipulation clearly makes this the system of choice in dealing with alterations of the EHEC chromosome. The techniques described here using pKM208 have also been used to make genetic alterations in enteroaggregative *E. coli*, uropathogenic *E. coli*, and *Salmonella typhimurium* (E. Dudley, S. Slechta, and J. Roth, pers. comm. and unpublished results). In all likelihood, the general procedures described here will work for any strain of *E. coli* or related bacteria. Red-promoted gene replacement, using protocols similar to those described here, have also been demonstrated in *Shigella flexneri*, *Salmonella enterica*, and *Yersinia pseudotuberculosis* (Day et al., 2001; Uzzau et al., 2001; Derbise et al., 2003), though with varying degrees of success.

The disadvantage of this system (and virtually all gene replacement techniques) lies in the variability that different loci have with regard to their frequency of recombination. Some loci, like the *lacZ* gene in both *E. coli* K-12 and EHEC, are replaced at higher frequencies relative to other genes tested at the same time. The cause of this

variability is not known, but might result from a context effect on drug marker expression, differences in the presence and number of unknown restriction sites on DNA substrates, differential transcriptional activity, and/or nucleoid partitioning (about which little is known). Another common cause of variability is electrocompetence. The authors find that despite using identical protocols, variability in electrocompetence exist between different batches of cells. One should keep a diluted control plasmid on hand to compare the electrocompetence of cells prepared on different days.

Critical Parameters and Troubleshooting

Drug markers

The authors' laboratory prefers to use either kanamycin or chloramphenicol. However, any antibiotic marker can be used as long as it is different from the marker on the plasmid carrying the λ-Red genes (Amp^R) and is not restricted due to clinical use. (Refer to the local institutional biosafety committee.) Use the lowest drug concentration that just eliminates the background of nontransformants. Many drug markers that work well on plasmids don't do as well at single copy on the chromosome. The authors typically use 15 to 20 μg/ml chloramphenicol and 20 to 25 μg/ml kanamycin.

LB containing 50 μg/ml ampicillin (or lower if necessary) is used for the selection of EHEC cells transformed with pKM201 or pKM208. For growth of liquid cultures containing these plasmids, use LB containing 100 μg/ml ampicillin.

Drug cassette orientation

For the PCR recombination substrate, design the primers to place the drug marker colinear with genes surrounding the target (if possible). If one orientation doesn't work, try the other. The authors have seen context effects on drug expression from the chromosome, i.e., the drug marker works well in one direction, but not at all when turned around.

PCR templates

Don't use intact plasmids for PCR targets. Plasmids (even at low concentration) transform at a high efficiency following electroporation and will yield many false positives. If using a plasmid drug marker as a target, gel purify the PCR product. Better yet, use a gel-purified fragment devoid of an origin of replication as the target, or a target contained within a nonreplicative plasmid. Alternatively, one can also perform colony PCR where the drug marker is contained as a chromosomal element.

The use of long homology substrates

This is a variation of the Basic Protocol described above, where instead of using PCR-generated recombination substrates containing short regions of homology (40 bp), plasmid digests of preconstructed gene deletions containing long regions of homology (0.5 to 1 kb) are used as the source of the recombining substrate. Clearly, the use of PCR-generated substrates is the easiest and quickest route to gene replacement using λ Red. However, there are times when longer homology substrates are warranted. For instance, the overall frequency of recombination is higher with longer homology substrates. Thus, if a particular gene replacement is difficult to generate using short homology (PCR) substrates, success can sometimes be achieved using substrates containing longer regions of homology. Also, when multiple mutant alleles of an already cloned target gene need to be crossed into the chromosome, it saves time to simply clone the mutant allele into a dedicated vector containing the upstream and downstream regions of the target gene, and electroporate a digest of the plasmid (which liberates the recombining substrate) into a cell that contains the *cat-sacB* cassette in place of the target gene on the chromosome.

False positive Cam^R colonies

The authors have found that EHEC exhibits a moderate rate of spontaneous resistance to chloramphenicol. Thus, when using this drug marker, be aware that false positive will sometimes be among the true recombinants. The false positives differ from the true recombinants in both size and color. Generally, larger colonies formed on chloramphenicol plates have a greater chance of being legitimate recombinants than smaller colonies. The true test occurs upon restreaking on LB/Cam plates, where the true recombinants grow well while the false positives show discernibly weaker growth. The authors have found that starting the working culture from a single colony, rather than on overnight culture, reduces the presence of these Cam^R false postives.

Verification of mutants

A traditional method to characterize a mutant would entail Southern blot analysis (e.g., see *UNIT 14B.1*, which, although very informative, is very time consuming. Therefore, the

authors' laboratory uses a PCR-based system which can be used to check whether recombination was successful. It has been found that overnight overexpression of Red induces a 10-fold increase in the rate of spontaneous resistance to rifampicin. Note, however, under the conditions for Red expression used here, no increase in rifampicin resistance was noted. Thus, Red is not likely to be mutagenic for gene replacement purposes. Nonetheless, in *E. coli* K-12, a prudent step following Red-mediated gene replacement is to P1 transduce the mutation into a clean genetic background. Since this is not possible with EHEC, where P1 transduction has not been demonstrated, one is left with two important steps following gene inactivation: (1) cure the strain of pKM208 by growth at elevated temperature (42°C) and (2) prove no ectopic mutation has arisen by complementing the inactivated gene with a plasmid-borne wild type copy. It is always wise to complement individually derived deletion mutants to reconfirm the deletion, as well as determine if the deletion has a polar effect on downstream genes.

No transformants

Follow the guidelines and alternate protocols discussed above. If no transformants are obtained, examine cell survival after electroporation by plating on solid LB medium. In the final culture following electroporation, a survival count of $\geq 10^7$ to 10^8/ml is desired. Cell survivals $< 10^6$ indicate excessive killing. Since frequencies of gene replacement are typically in the range of 10^{-4} to 10^{-5}/survivor, excess killing will prevent successful recovery of transformants. If this happens, search for possible salt contamination in the electrocompetent cell prep or DNA substrate, lower the electroporation voltage and/or decrease or eliminate heat shock exposure.

Anticipated Results

Overall, if performed correctly, this method should yield a fair number of transformants. Typically, the authors obtain between 5 to 50 true transformants per experiment, although in some cases, only one or two transformants have been isolated. Luckily, these few transformants are generally true recombinants as verified by PCR analysis (provided they grow well on post-selection antibiotic-containing plates). The authors have found that the overall frequency of gene replacements in EHEC is not as high as *E. coli* K12. For EHEC, gene replacement frequencies fall in the range of 0 to 20 recombinants per 10^8 survivors and >100 recombinants per 10^8 survivor using long homology substrates. Some sites give higher rates (e.g., *lacZ*) while a minority of sites could not be deleted, perhaps a result of essentiality, though this has not been investigated. However, by making multiple attempts to obtain problematic gene replacments, >90% of the desired EHEC deletions were eventually obtained (B. Skehan, K. Campellone, unpub. obs.).

Time Considerations

Table 5A.2.1 and Table 5A.2.2 present time considerations for the protocols described in this unit.

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Detection, Isolation, and Identification of *Vibrio cholerae* from the Environment

Until the late 1970s and early 1980s, *Vibrio cholerae*, the causative agent of cholera, was viewed as host adapted, i.e., resident in the human gut. Numerous studies conducted during the last three decades, however, have provided clear evidence establishing the microorganism as an autochthonous (i.e., native) member of the aquatic environment (Colwell and Spira, 1992). These organisms can be detected in marine, estuarine, and fresh-water environments throughout the year if appropriately sensitive methods are employed (Colwell et al., 1981; Huq et al., 1990; Louis et al., 2000, 2003). However, *V. cholerae* may not be detected for several reasons—e.g., if it is present in low numbers, if it exists in a state that does not facilitate growth, or if it is simply overlooked. When the bacteria are viable (determined by direct-detection methods) but do not grow on conventional culture media or in broth, they are termed viable but not culturable (VBNC; Xu et al., 1982; Roszak and Colwell, 1987). *V. cholerae*, along with a host of other, mostly Gram-negative bacteria, often occur as VBNC cells in the environment, an indication that this state may be an important strategy for persistence and survival of non-spore-forming microorganisms (Colwell et al., 1985; Roszak and Colwell, 1987).

Currently, many molecular-biological tools are used to study bacteria, including VBNC cells, in clinical, environmental, or industrial samples (Knight, 2000). With the availability of these tools and a renewed interest in *V. cholerae*, the presence of these bacteria in various geographical locations has been reported with increasing frequency. However, standard methods for detection and enumeration have not been universally established. The choice of method for detection and enumeration of *V. cholerae* is largely dependent on the type of sample and the desired end purpose—i.e., simple detection or isolation. Often, combinations of different methods are used for a specific type of sample. Therefore, commonly used bacteriological, molecular, and immunological methods targeted at microbiological analysis of environmental water samples will be discussed in this unit.

Several methods will be described, and investigators will have to determine the most appropriate method consistent with their purpose and needs and with the availability of resources. For the readers' convenience, Table 6A.5.1 provides an overview of the methods provided in this unit.

CAUTION: *V. cholerae* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

There are numerous methods and protocols available in the literature for detecting and isolating *V. cholerae*. A number of them are described in this unit; however, all may not be required in one study. In addition, availability of resources in a particular laboratory will be a critical predetermining factor in making decisions on the choice of methods to include; therefore, stepwise selection cannot be standardized. In other words, it is extremely important to contrive a strategic plan to determine the choice of methods at the beginning of the study. A general method for specimen collection and transportation is provided (Basic Protocol 1). The traditional culture method (Basic Protocol 2) is the so-called "gold standard" in bacteriology, especially when an isolate is needed for the study. In the conventional culture method, selective plates are often used to reduce the

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Table 6A.5.1 Overview of Methods Presented in This Unit for Isolation and Detection of *V. cholerae*

Protocols	Included methodologies
Isolation and identification of V. cholerae using traditional methods	
Basic Protocol 1	Specimen collection and transportation
Basic Protocol 2	Conventional bacteriological culture method for *V. cholerae* and identification of presumptive isolates using standard biochemical tests
Basic Protocol 3	Serogroup determination
Molecular methods for detection and identification of V. cholerae	
Basic Protocol 4	Identification of suspected or presumptive *V. cholerae* by PCR
Basic Protocol 5	Direct PCR
Alternate Protocol 1	Multiplex PCR assay for O1, O139, and *ctxA*
Basic Protocol 6	Colony blot hybridization with labeled DNA or RNA hybridization probes
Alternate Protocol 2	Colony blotting with DIG-labeled probe
Support Protocol	DIG labeling of probes
Direct detection and/or enumeration of V. cholerae using immunological methods	
Basic Protocol 7	Direct fluorescent antibody–direct viable count (DFA-DVC)
Basic Protocol 8	Indirect fluorescent antibody (IFA) method

volume of work and to identify a specific organism or variety of organisms. Once this culture method has been used and an organism isolated, a battery of biochemical tests is employed for characterization and identification. If such colonies are picked from selective plates, the number of biochemical tests needed may decline. Therefore, the number of tests may vary depending on the source of the strain. However, as more tests are performed, more convincing and accurate results will be obtained. Serogroup determination for *V. cholerae* is described in Basic Protocol 3.

Although conventional culture methods, including biochemical tests, are routinely used, they can be time consuming as well as labor intensive. The authors have included two abbreviated alternative methods that use fewer tests for a rapid presumptive identification. Colony blot hybridization (Basic Protocol 6 and Alternate Protocol 2; probe labeling is described in Support Protocol) can be used for rapid detection of *Vibrio cholerae*, and in cases involving large numbers of samples, this method may be preferred. However, it too is dependent on the culturability of the cells.

The viable but nonculturable state represents an important phenomenon, as bacteria present in this state do not grow on conventional culture media. To demonstrate the presence of the organisms in this state, a direct-detection method should be employed, notably fluorescent antibody coupled with direct viable count (DFA-DVC; Basic Protocol 7). No subsequent bacterial isolation is possible using this method, unless a parallel sample is taken. An indirect fluorescent antibody method for detecting *V. cholerae* in environmental samples is described in Basic Protocol 8.

Molecular approaches target specific genes that are unique to the organism in question. Usually, species-specific genes are targeted. This approach is extremely valuable for monitoring the presence of the organism. A single probe, designed carefully, is very specific and will be amenable to screening a larger number of strains at one time. Likewise, a series of such probes can be used for extensive characterization of an organism. These methods can be based on both culturable and nonculturable cells. Community DNA can be used to determine the presence of an organism without culture; however, results will be dependent on the quality and quantity of the extracted nucleic acids.

Strategically, if resources are available, it is appropriate to start with the conventional culture method and the DFA-DVC method. Those samples exhibiting positive results by the DFA-DVC method can be confirmed by PCR (Basic Protocol 4). If one must avoid conventional culture, one may wish to use direct PCR (Basic Protocol 5 or Alternate Protocol 1) and DFA-DVC (Basic Protocol 7), which should provide fairly convincing results. Use of PCR alone for environmental samples may not be reliable, as background noise often poses a significant obstacle in the interpretation of results.

ISOLATION AND IDENTIFICATION OF *V. CHOLERAE* USING TRADITIONAL METHODS

Traditional methods for isolating and identifying *V. cholerae* from environmental water samples were introduced by Colwell et al. (1977), who modified protocols originally intended for clinical samples. Briefly, concentrated water, homogenized plankton samples, or both are added to an enrichment broth and incubated overnight. The alkaline nature of the enrichment broth allows differential multiplication of *Vibrio* species.

Following enrichment, aliquots are subcultured onto selective medium—thiosulfate citrate bile salts sucrose (TCBS) agar, tellurite taurocholate gelatin agar (TTGA), or both (see Reagents and Solutions for compositions)—which offer some selective advantage to *Vibrio* species and aid in the presumptive identification of *V. cholerae*. The identity of presumptive colonies is confirmed by specific biochemical tests designed to differentiate *V. cholerae* from other species and other members of the *Vibrionaceae* and *Enterobacteriaceae*. Confirmed *V. cholerae* strains are then serogrouped as O1 or O139 by simple slide agglutination using specific antisera. Strains of *V. cholerae* that do not agglutinate with specific O1 and O139 antisera are designated as non-O1/non-O139. Stock cultures of confirmed *V. cholerae* are maintained in nutrient agar containing 0.5% NaCl overlaid with oil, or as glycerol stocks at −70°C for future analysis and reference.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

Specimen Collection and Transportation

BASIC PROTOCOL 1

For isolation and detection of *V. cholerae* from the environment, the screening of concentrated water samples and plankton samples is recommended, since a combination of both specimen types provides a higher probability of *V. cholerae* detection (Huq et al., 1990; Binsztein et al., 2004; Huq et al., 2005). Clean, sterile, plastic sampling bottles (for water) and glass jars (for plankton) should be prepared and used. Sufficient sample (depending upon the parameters that need to be analyzed) should be collected in order to perform all examinations. Plankton samples are collected and concentrated using a 64-μm simple plankton net (Fig. 6A.5.1), preferably by towing (just below the surface, to a depth no more than 0.5 m); alternatively, this may be accomplished by manually pouring or mechanically pumping water into the net (Huq et al., 2005).

Processing of samples should begin soon after collection ("on site" or in situ) or within 1 hr of collection. If processing is delayed, the sample should be stored in a cool box at a temperature of 10° to 15°C until processing begins (not to exceed 8 hr; Clesceri et al., 1998). This is a critical parameter if enumeration of *V. cholerae* from the sample will be performed. A recent study suggests that transporting samples at ambient air temperature prior to processing can increase the number of *V. cholerae* strains isolated (Alam et al., 2006). Based on the type of examination, samples may require treatment, e.g., addition of direct viable count (DVC) reagents, before proceeding with further examination and testing. It is recommended that some basic physiochemical parameters—e.g., temperature, salinity, pH, dissolved oxygen, conductivity—of the water source be measured at the time of collection, for later analysis. Results of environmental studies conducted in Bangladesh have revealed a direct correlation of water temperature with clinical outbreaks of cholera. Furthermore, significant correlation of water depth, rainfall, conductivity, and copepod density with cholera outbreaks has been found, with lag periods from 0 to 8 weeks from optimum environmental conditions to cholera outbreaks (Huq et al., 2005). These parameters can be measured on site using portable meters.

Materials

500-ml plastic containers with caps (Nalgene) for water sampling, sterile
Portable meter(s) that measure temperature, dissolved oxygen and pH (dissolved oxygen and pH meter Model 210A from Orion Laboratories), and salinity (Model CO150 conductivity meter from Hach Chemical Company)
Simple plankton net, 64-μm mesh size, or nets of different mesh sizes for size filtration of plankton (Aquatic Research Instruments, *http://www.aquaticresearch.com*; Fig. 6A.5.1)
Net-mounted flow meter (General Oceanics; *http://www.generaloceanics.com/*)
Bucket of known volume (e.g., 5 liters; optional)
240-ml glass containers for plankton collection (Qorpak), sterile

To collect water sample

1a. Measure temperature, dissolved oxygen, pH, and salinity using portable meters.

2a. Uncap presterilized 500-ml plastic bottle and submerge to fill.

3a. Remove sample bottle from water. Cap bottle, leaving enough air inside for agitation and mixing.

4a. Transport samples to the laboratory for processing in a cold box (Styrofoam box with ice, 10° to 15°C), or, preferably, begin processing on site within 1 hr of collection (Basic Protocol 2).

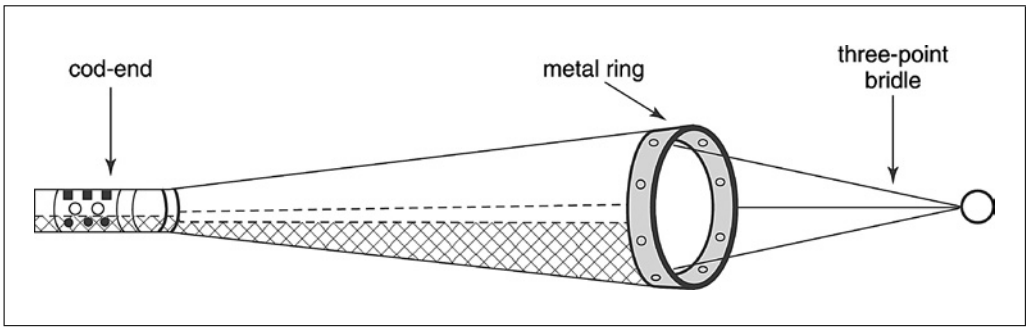

Figure 6A.5.1 Simple plankton net (Aquatic Research Instruments). The net consists of a metal ring and bridle, a heavy-duty nylon net of variable mesh size, and a PVC cod-end or collecting bucket, which can be removed for easy sample collection. A flow meter may be mounted in the mouth of the net on the metal ring to measure volume when the net is towed.

A recent study (Alam et al., 2006) found that transportation or maintenance at ambient air temperature (22° to 25°C) after collection for up to 24 hr may enhance recovery of Vibrio species. This can be a useful alternative to increasing sample volume for geographical areas and/or seasons when the density of V. cholerae is low. This aspect of the protocol may need to be optimized for the water source and environmental conditions.

To collect plankton

1b. Rinse plankton net and cod-end of the net in the body of water to be sampled.

2b. Filter 10 to 40 liters water through the plankton net by towing; use a calibrated, net-mounted flow meter to determine volume. Alternatively, pour known volumes through the net with a small bucket.

3b. Remove cod-end from plankton net and decrease volume to 100 ml by continuing to filter.

4b. Measure (with graduated cylinder) and decant plankton sample into sterile 240-ml glass container.

5b. Transport samples to the laboratory in a cold box (Styrofoam box with ice, 10° to 15°C) for processing or, preferably, begin processing on site within 1 hr of collection (Basic Protocol 2).

See annotation to step 4a for additional considerations.

Conventional Bacteriological Culture Method

BASIC PROTOCOL 2

Conventional culture methods for isolating *V. cholerae* from environmental water samples rely on an enrichment step(s) in broth and plating on selective media, followed by confirmation using a battery of biochemical and serological tests. As stated previously, some knowledge regarding the expected prevalence of *V. cholerae* in the water body to be examined is useful when determining the volume of water to collect for examination. *V. cholerae* is an autochthonous member of the estuarine and aquatic community; however, geographic location and environmental factors such as water temperature play a critical role in determining the number of culturable *V. cholerae* cells present. Most water and plankton samples require concentration, with historical knowledge of the water source helping to determine the concentration factor. Water samples should be concentrated by filtration using 0.2-μm polycarbonate membrane filters (see step 1a, below). A good starting volume is 500 ml.

Overnight enrichment is performed using alkaline peptone water (APW), pH 8.6. Some investigators recommend two successive enrichments. Surface aliquots are streaked onto selective bacteriological media. The two favored selective media used for *V. cholerae* isolation are thiosulfate citrate bile salts sucrose (TCBS) agar and tellurite taurocholate gelatin agar (TTGA), also known as Monsur medium (Monsur, 1961). *V. cholerae* produces translucent, flat, yellow sucrose-fermenting colonies with elevated centers on TCBS (Fig. 6A.5.2A). The organism produces colorless colonies on TTGA (Fig. 6A.5.2B), often with a characteristic dark center after 2 days growth, surrounded by a halo, which appears due to the hydrolysis of gelatin. Both media have advantages as well as disadvantages.

TCBS, or "cholera medium," is highly selective, inhibiting growth of Gram-positive and typical intestinal flora, which cannot grow under alkaline conditions. However, TCBS can inhibit some members of the genus *Vibrio*, including *V. cholerae* itself, as evidenced by comparing simultaneous growth of *V. cholerae* on TCBS versus nonselective media. In addition, TCBS can support the growth of several closely related bacteria, most notably *Aeromonas* and *Proteus*. One of the major advantages of this medium is that it

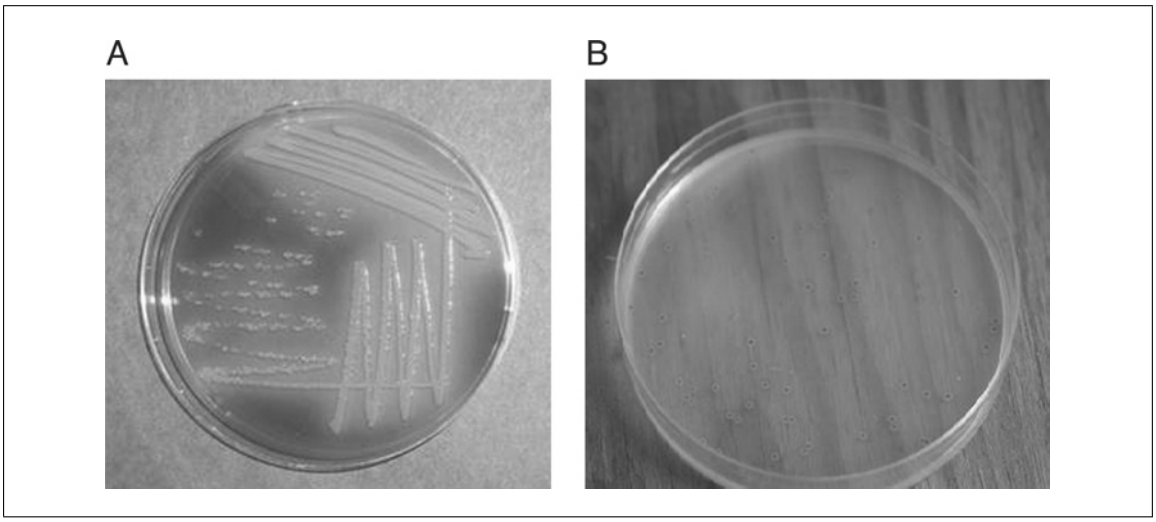

Figure 6A.5.2 Growth of *V. cholerae* O1 on (**A**) TCBS and (**B**) TTGA. Courtesy of Dr. Munir Alam, International Center for Diarrheal Disease Research, Bangladesh. For the color version of this figure go to *http://www.currentprotocols.com*.

is commercially available and does not require an autoclave to prepare plates, thereby making it possible to use the medium under field conditions, without a laboratory or autoclave. TTGA medium is generally considered less selective and less "toxic" to *V. cholerae* than TCBS, so recovery of *V. cholerae* colonies may be enhanced as compared to that obtained with TCBS; however, these plates preferably should be incubated longer (48 hr versus 24 hr for TCBS) to observe the characteristic dark center. Also, the formulation is not commercially available and the medium must be autoclaved after preparation. Once presumptive strains are purified on a nonselective medium such as gelatin agar or modified nutrient agar, they are identified and confirmed by biochemical tests and serogrouped using simple slide agglutination with polyclonal *V. cholerae* O1 and monoclonal O139 antiserum.

Materials

Plankton or water sample (Basic Protocol 1)
Enrichment flask: 150-ml Erlenmeyer flask containing 25 ml sterile alkaline peptone water (APW), pH 8.6 (see recipe); store up to 1 month at 4°C
Thiosulfate citrate bile-salts sucrose (TCBS) agar plates (see recipe)
Tellurite taurocholate gelatin agar (TTGA) plates (see recipe)
Gelatin agar (GA) plates (see recipe)
Modified nutrient agar plates (see recipe)
Filter paper (Whatman 3MM or equivalent) saturated with oxidase reagent (see recipe)
5-ml tubes of medium for biochemical tests (also see Table 6A.5.2):
- Methyl red/Voges-Proskauer (MR-VP) broth (see recipe)
- LB medium (*APPENDIX 4A*) containing 1% (w/v) L-arginine (pH 6.8) and 1 to 2 drops of 10% (w/v) phenol red
- Moeller decarboxylase broth base (see recipe) containing 1% L-lysine
- Moeller decarboxylase broth base (see recipe) containing 1% L-ornithine
- Purple broth base (see recipe) containing 1% (w/v) arabinose
- Purple broth base (see recipe) containing 1% (w/v) mannitol
- Heart infusion agar (BD Biosciences) containing 0.1% (w/v) esculin and 0.05% (w/v) ferric chloride
- Nutrient broth (BD Biosciences) with added NaCl (0%, 6%, and 8%)

Mineral oil, sterile
Kligler iron agar (KIA) slants (see recipe)

Hand-held glass tissue homogenizer, Potter-Elvehjem or Tenbroek style (Kontes), to homogenize plankton
Glass wool
Glass funnel
Filter apparatus with vacuum source
Filter membranes, 47-mm diameter, 0.22-μm pore size
30°C and 37°C incubators
Platinum inoculating loops and needles
Sterile toothpicks

Additional reagents and equipment for serogroup determination (Basic Protocol 3)

Enrich organisms in APW

1a. *For plankton samples:* Homogenize plankton samples using a hand-held glass tissue grinder by moving the pestle up and down in the tube, while rotating, 10 to 20 times. Add 1 ml homogenized plankton to an enrichment flask.

1b. *For water samples:* Filter 500 ml (or 100 to 1000 ml depending on bacterial density) of water first by gravity through glass wool and then under vacuum through a 47-mm, 0.22-μm pore size polycarbonate filter. Add the filter(s) with attached bacteria to an enrichment flask. Shake vigorously to detach bacteria from filter.

Large volumes may require more than one polycarbonate filter, as they will clog.

2. Incubate the flasks statically 16 hr (overnight) at 30°C.

The flasks should not be disturbed or agitated during or after incubation, as Vibrio species tend to migrate to the liquid-air interface.

Selectively plate for Vibrio cholerae

3. After enrichment, collect surface growth (which may be present as a whitish film) from the enrichment flask with a platinum inoculating loop and streak onto TCBS plates, TTGA plates, or both (i.e., selective plates).

4. Incubate the plates at 37°C 16 to 24 hr for TCBS or 48 hr for TTGA.

5. Subculture smooth, flat, sucrose-fermenting (yellow) colonies from TCBS (Fig. 6A.5.2A) and/or translucent, dark-centered colonies with halo zones from TTGA (see Fig. 6A.5.2B) onto GA and/or modified nutrient agar plates (i.e., nonselective plates). Continue incubation for 16 to 24 hr at 37°C.

When subculturing colonies from selective medium onto nonselective medium, touch the top center of the colony using an inoculating loop without touching the agar surface, to avoid carryover of contaminating growth-suppressed bacteria that may be present.

Characterize suspected colonies

Suspected colonies (i.e., those selected for subculture) can be characterized using a traditional battery of biochemical tests (steps 6a to 9a), an alternate KIA-oxidase-antisera protocol (steps 6b and 7b), or a simple two-test protocol (step 6c to 8c).

To characterize by traditional battery of biochemical tests and serogroup determination

6a. Pick suspected colonies from GA and/or modified nutrient agar plates using sterile toothpicks or a platinum loop. Streak onto filter paper saturated with oxidase reagent.

V. cholerae is oxidase-positive, as indicated by the rapid appearance (10 to 30 sec) of a purple color. A delayed color change (60 sec or longer) or no color change is indicative of a negative result.

Table 6A.5.2 Selected Biochemical Tests Used for the Identification of *V. cholerae*

Test	Medium	Temperature	Duration	Reagent to add prior to reading	Positive result indicates
Methyl Red	MR-VP medium	37°C	48 hr	5-6 drops methyl red indicator[a]	Ability to produce stable acid end products from glucose fermentation
Voges-Proskauer	MR-VP medium	37°C	48 hr	3 ml Barritt's reagent A[b] and 1 ml Barritt's reagent B[c]	Production of acetoin, formed from pyruvic acid during glucose fermentation
Arginine dihydrolase	LB medium (*APPENDIX 4A*) containing 1% (w/v) L-arginine (pH 6.8) and 1-2 drops of 10% (w/v) phenol red	37°C	24 hr	None	Presence of the enzyme arginine dihydrolase
Lysine decarboxylase[d]	Moeller decarboxylase broth base (see recipe) containing 1% (w/v) L-lysine	37°C	24 hr	None	Presence of the enzyme lysine decarboxylase
Ornithine decarboxylase[d]	Moeller decarboxylase broth base (see recipe) containing 1% (w/v) L-ornithine	37°C	24 hr	None	Presence of the enzyme ornithine decarboxylase
Arabinose, acid production	Purple broth base (see recipe) containing 1% (w/v) arabinose	37°C	24 hr	None	Utilization of the carbohydrate arabinose by production of acid end products
Mannitol, acid production	Purple broth base (see recipe) containing 1% (w/v) mannitol	37°C	24 hr	None	Utilization of the carbohydrate mannitol by production of acid end products
Oxidase	Oxidase reagent: 1% (w/v) *N,N,N′ N′*-tetramethyl-*p*-phenylenediamine dihydrochloride	37°C	10-30 sec	None	Presence of cytochrome oxidase
Esculin hydrolysis	Heart infusion agar containing 0.1% (w/v) esculin and 0.05% (w/v) ferric chloride	37°C	3 days	None	Hydrolysis of esculin to glucose and esculetin
Growth in nutrient broth with 0% NaCl	Nutrient broth[e] with 0% NaCl	37°C	5 days	None	Organism has minimal salt requirement
Growth in nutrient broth with 6% NaCl	Nutrient broth[e] with 6% (w/v) NaCl	37°C	5 days	None	Organism is tolerant of moderate salt concentration
Growth in nutrient broth with 8% NaCl	Nutrient broth[e] with 8% (w/v) NaCl	37°C	5 days	None	Organism is tolerant of high salt concentration

[a]Dissolve 0.1 g methyl red in 300 ml of 95% ethanol. Adjust volume to 500 ml with water. Store up to 6 months at 2° to 8°C.

[b]Dissolve 5 g α-naphthol in 100 ml absolute ethanol. Store up to 6 months at 2° to 8°C.

[c]Dissolve 40 g potassium hydroxide in 100 ml distilled water. Store up to 6 months at 2° to 8°C.

[d]For lysine and ornithine decarboxylase, it is useful to inoculate a tube of decarboxylase broth base without the addition of either amino acid for comparison as a negative control. All decarboxylase test tubes must be overlaid with mineral oil.

[e]Nutrient broth must not contain any sodium chloride.

Table 6A.5.3 Expected Results of *V. cholerae* for Selected Biochemical Tests[a]

		Results	
Test	% Positive	Positive	Negative
Methyl Red	99	Red	Yellow
Voges-Proskauer	75	Red	Copper
Arginine dihydrolase	0	Pink	Orange
Lysine decarboxylase	99	Dark purple	Yellow
Ornithine decarboxylase	99	Dark purple	Yellow
Arabinose, acid production	0	Yellow	Purple
Mannitol, acid production	99	Yellow	Purple
Oxidase	100	Purple	Clear
Esculin hydrolysis	0	No fluorescence[b]	Fluorescence
Growth in nutrient broth with 0% NaCl	100	Turbid	Clear
Growth in nutrient broth with 6% NaCl	53	Turbid	Clear
Growth in nutrient broth with 8% NaCl	1	Turbid	Clear

[a]Traditional biochemical characterization of presumptive colonies (also see Table 6A.5.2). Adapted from (Lennette et al., 1985).

[b]Loss of fluorescence is used since many *V. cholerae* strains produce melanin, which interferes with the normal indicator (darkening of butt).

7a. If a positive result is achieved using the oxidase reagent test, inoculate one tube of each of the other tests outlined in Table 6A.5.2 (eleven total after oxidase test has been performed) for each suspected colony. Inoculate a second tube of each test with a known positive control. Include a tube of decarboxylase base medium (without the amino acid supplement) as a negative control for the lysine and ornithine decarboxylase tests. Refer to Table 6A.5.3 for interpretation of results.

8a. Overlay arginine, lysine, and ornithine tubes (and decarboxylase broth base used as negative control) with sterile mineral oil. Incubate all tubes at 37°C for the duration indicated in Table 6A.5.2.

9a. Proceed to serogroup determination (Basic Protocol 3) with presumptive *V. cholerae* colonies.

Characterize colonies by simple, rapid biochemical procedure and serogroup determination

6b. Assay suspected colonies from GA and/or modified nutrient agar (from step 5) for arginine dihydrolase and esculin hydrolysis as described in Tables 6A.5.2 and 6A.5.3.

7b. Proceed to serogroup determination (Basic Protocol 3) with presumptive *V. cholerae* colonies (negative for arginine dihydrolase and negative for esculin hydrolysis).

Characterize colonies by KIA-oxidase and serogroup determination

6c. Stab-inoculate suspected colonies from GA and/or modified nutrient agar into KIA slants using an inoculating needle. Incubate at 30°C overnight.

Stab inoculation is accomplished by passing an inoculating needle that has been touched to a suspected colony on the GA or modified nutrient agar plate vertically into the surface of the agar at the center of the slant and down completely into the tube.

See WHO (1974) and Tison (1999) as well as the Commentary in this unit, for more detail.

7c. Perform the oxidase test (step 6a) on colonies giving an alkaline slant over acid butt (purple slant and yellow butt) with no gas production or blackening of the butt due to H_2S production.

8c. Proceed to serogroup determination (Basic Protocol 3) with presumptive *V. cholerae* colonies.

BASIC PROTOCOL 3

Serogroup Determination

Over 210 serogroups of *V. cholerae* have been described to date based on antigenic properties of cell surface polysaccharides, of which serogroup O1 and O139 have been implicated in epidemics of cholera, while serogroup O37 has been held responsible for localized outbreaks of cholera in Czechoslovakia and Sudan. The remaining serogroups collectively and commonly termed "non-O1/non-O139" are predominant among the strains of *V. cholerae* isolated from the aquatic environment (Sack et al., 2003). Although reported mostly in O1 and O139 serogroups, the cholera toxin gene has been reported in non-O1/non-O139 strains, also from the aquatic environment, in India (Chakraborty et al., 2000). However, because of the epidemic potential, the method to determine O1 and O139 serogroup is described below. Strains other than O1 or O139 serogroup need not be serogrouped unless there is a special need, in which case those strains should be sent to a Reference Center for serotyping, since antisera for serogroups other than O1 and O139 are not commercially available.

Materials

Phosphate buffered saline (PBS; *APPENDIX 2A*)
V. cholerae colonies (6- to 16-hr subculture on nonselective modified nutrient agar, e.g., Basic Protocol 2)
Polyvalent antisera for serogroup O1 and O139 *V. cholerae* (BD Biosciences)
Glass microscope slides

1. Add two separate drops of PBS to a microscope slide.
2. Add a loopful of fresh growth from a 6 to 16 hr subculture of *V. cholerae* on nonselective medium to each drop, and mix.
3. Add an equal-sized drop of group O1 polyvalent antiserum to one of the drops.
4. Mix the antiserum-culture suspension by tilting the slide back and forth. Determine if the reaction clumps (i.e., agglutinates) within 0.5 to 1 min, indicating a positive result.

   Autoagglutination, i.e., clumping in the saline solution without antiserum, is indicative of a "rough" morphotype which cannot be typed by antisera. For typing of colonies with rough morphotype, see Alternate Protocol 1.

5. Test non-O1 serogroup colonies using O139 antisera, repeating steps 1 to 4, but substituting anti-O139 antiserum for anti-O1 antiserum.

MOLECULAR METHODS OF DETECTION AND IDENTIFICATION OF *V. CHOLERAE*

Following the introduction of molecular methods into bacteriology, there has been an influx of newer techniques aimed at understanding the mechanism of infection and at the detection of pathogens, or organisms with virulent properties, in clinical or environmental samples. Soon after the *V. cholerae* genome sequence was published in 2000 (Heidelberg et al., 2000), PCR-based simple molecular probes became an important tool for detecting *V. cholerae* and its antigens. Direct-detection methods have proven to be important when conventional culture methods are recognized as ineffective because of the viable but not culturable (VBNC) organisms present in samples collected for analysis.

Identification of Suspected or Presumptive *V. cholerae* by PCR

The polymerase chain reaction (Kramer and Coen, 2001) is a useful alternative to labor-intensive biochemical tests, which are sometimes difficult to interpret and often require replication. Also, many biochemical tests require an additional overnight or longer incubation before results are obtained. It is largely up to the investigator to determine at which step in the traditional, culture-based isolation procedure to switch to this molecular method. Ideally, at least minimal biochemical tests (Basic Protocol 2, step 6b or steps 6c and 7c) should be performed before switching to PCR identification, to decrease the number of strains to be screened to a manageable number. In this method, crude template is prepared by boiling to lyse the cells. This template is then amplified using PCR primers specific to *V. cholerae*, which target the internal transcribed spacer (ITS) region between 16S and 23S rDNA. It is convenient at this stage to characterize a confirmed *V. cholerae* strain by screening for several genes associated with pathogenesis (*ctxA, toxR, tcpA, zot*, and *ompU*) by PCR. PCR products are analyzed by gel electrophoresis and visualized under UV light with ethidium bromide. Positive and negative controls should be run in parallel and should include a eubacterial 16S rDNA PCR reaction on each sample to test template quality. (See Table 6A.5.4 for PCR primers, expected amplicon size and reference).

Materials

Overnight culture of putative *V. cholerae* in liquid medium or on agar plates (Basic Protocol 2)
H_2O, sterile
1× PCR amplification buffer (*APPENDIX 2A*) containing 15 mM $MgCl_2$ and 0.1% (w/v) gelatin
25 mM dNTP stock: dATP, dCTP, dGTP, dTTP (*APPENDIX 2A*)
20 μM PCR primers for ITS (Table 6A.5.4)
Taq DNA polymerase
1.5% agarose gel (Voytas, 2000)
TAE buffer (*APPENDIX 2A*)
Molecular weight ladder (e.g., Hyperladder IV, Bioline)
1 μg/ml ethidium bromide (*APPENDIX 2A*)
2-ml microcentrifuge tubes, sterile
Boiling water bath
PCR tubes
Thermal cycler (MJ Research)
UV transilluminator, handheld UV lamp, or digital gel documentation system
Additional reagents and equipment for agarose gel electrophoresis and ethidium bromide staining of gels (Voytas, 2000)

Prepare crude template

1a. *From liquid medium*: Centrifuge a 1-ml culture (overnight growth at 37°C) and resuspend in 1 ml sterile water.

1b. *From agar plates:* Resuspend a loopful of pure culture (50 to 100 colonies) of suspected or presumptive *V. cholerae* into 300 μl sterile water.

Plates should contain a fresh overnight subculture.

2. In a sterile 2-ml microcentrifuge tube, dilute suspension 1:1000 in sterile water to a final volume of 1 ml.

Alternatively, a single isolated colony can be resuspended in 20 μl sterile water.

Table 6A.5.4 PCR Primers for Genes Associated with Virulence of Toxigenic *V. cholerae* O1/O139 and Expected Amplicon Size

Target	Primer	Sequence (5′-3′)	Amplicon size	Reference
ITS	pVC-F2	TTAAGCSTTTTCRCTGAGAATG	295-310	Chun et al., 1999
	PVCM-R1	AGTCACTTAACCATACAACCCG		
ctxA	PCTA-94F	CGGGCAGATTCTAGACCTCCTG	563	Fields et al., 1992
	PCTA-614R	CGATGATCTTGGAGCATTCCCAC		
toxR	pToxR-101F	CCTTCGATCCCCTAAGCAATAC	778	Rivera et al., 2001
	pToxR-837R	AGGGTTAGCAACCGATGCGTAAG		
tcpA	pTcpA-72F	CACGATAAGAAAACCGGTCAAGAG	452-621	Keasler and Hall, 1993
	pTcpAET-477R	CGAAAGCACCTTCTTTCACGTTG		
	pTcpACL-647R	TTACCAAATGCAACGCCGAATG		
Zot	PZot-225F	TCGCTTAACGATGGCGCGTTTT	946	Rivera et al., 2001
	PZot-1129R	AACCCCGTTTCACTTCTACCCA		
ompU	pOmpU-80F	ACGCTGACGGAATCAACCAAAG	868	Rivera et al., 2001
	pOmpU-906R	GCGGAAGTTTGGCTTGAAGTAG		
ctxA	VCT1	ACAGAGTGAGTACTTTGACC	308	Hoshino et al., 1998
	VCT2	ATACCATCCATATATTTGGGAG		
O1-*rfb*	O1F2-1	GTTTCACTGAACAGATGGG	192	Hoshino et al., 1998
	O1R2-2	GGTCATCTGTAAGTACAAC		
O139-*rfb*	O139F2	AGCCTCTTTATTACGGGTGG	449	Hoshino et al., 1998
	O139R2	GTCAAACCCGATCGTAAAGG		
ompW	ompW-F	CACCAAGAAGGTGACTTTATTGTG	588	Nandi et al., 2000
	ompW-R	GAACTTATAACCACCCGCG		
ctxA	CtxA-F	CTCAGACGGGATTTGTTAGGCACG	302	Shirai et al., 1991; Nandi et al., 2000
	CtxA-R	TCTATCTCTGTAGCCCCTATTACG		
16S rDNA[b]	16S-F	CAGCMGCCGCGGTAATWC	888	Amann et al., 1995
	16S-R	ACGGGCGGTGTGTRC		

[a]Currently, there are no published 23S rDNA PCR primers specific for *V. cholerae*.

3. Place the microcentrifuge tube containing the resuspended culture into a boiling water bath for 10 min.
4. Cool tube to room temperature by allowing tube to sit on bench (~30 min).

Perform Vibrio cholerae–specific PCR (Chun et al., 1999)

5. Set up *V. cholerae*-specific ITS (internal transcribed spacer region) PCR in a PCR tube for a total reaction volume of 25 μl containing the following:

   5 μl template (from step 4)
   1× PCR amplification buffer
   200 μM each dNTP
   800 nM forward and reverse primers for ITS (pVC-F2, pVCM-R1)
   0.625 U *Taq* DNA polymerase.

6. Amplify the *V. cholerae*-specific ITS target with the following cycling conditions:

1 cycle:	1 min	94°C	(initial denaturation)
30 cycles:	1 min	94°C	(denaturation)
	1 min	60°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

Refer to Kraemer and Coen (2001) for information about optimizing PCR.

Perform agarose gel electrophoresis and visualize results

7. Run PCR product out on a 1.5% agarose gel in 1× TAE buffer for 1 to 2 hr at 5 V/cm (Voytas, 2000) along with a molecular weight ladder (e.g., Hyperladder IV).
8. Stain the gel by incubating in 1 μg/ml ethidium bromide staining solution for 15 min at room temperature.
9. Destain the gel by incubating in distilled water for 15 min at room temperature.
10. Visualize the products by viewing the gel using a handheld UV lamp, transilluminator, or digital gel documentation system.

The V. cholerae 16S-23S rDNA intergenic spacer region amplicon is 300 bp in size (Fig 6A.5.3, lane 2).

Screen for toxigenic factors

11. Screen ITS-PCR confirmed *V. cholerae* isolates for the toxigenic factors *toxR*, *tcpA*, c*txA*, *zot*, and *ompU* by PCR according to steps 1 to 10 above, using the PCR primers pToxR, pTcpA, pCTA, pZot, and pOmpU, respectively (see Table 6A.5.4 and Fig. 6A.5.3).

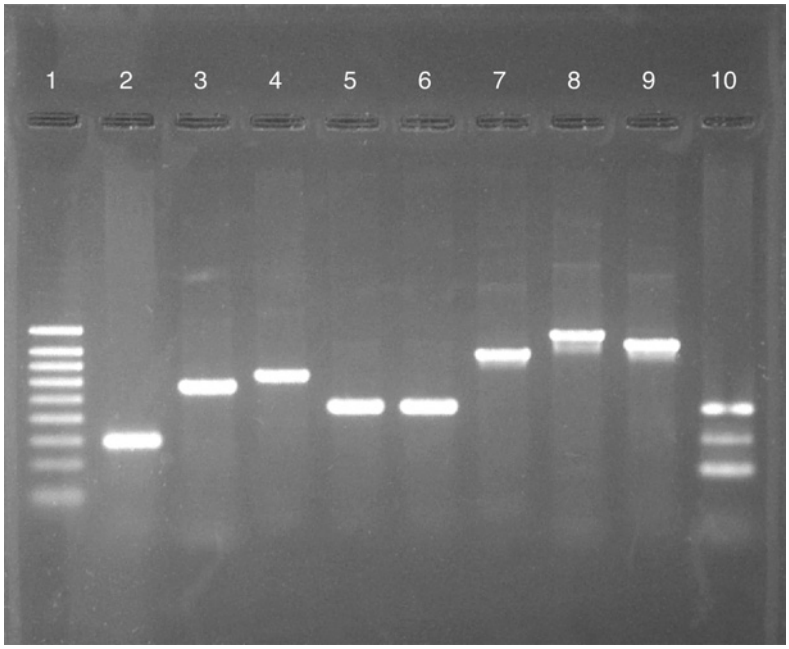

Figure 6A.5.3 Results (gel) of PCR assays used to detect and characterize *V. cholerae*. Lane 1, Hyperladder IV (Bioline); lane 2, *V. cholerae*-specific ITS; lane 3, *ctxA* (pCTA); lane 4, *tcpA* of *V. cholerae* O1 Classical; lane 5, *tcpA* of *V. cholerae* O1 El Tor; lane 6, *tcpA* of *V. cholerae* O139; lane 7, *toxR*; lane 8, *zot*; lane 9, *ompU*; lane 10, O1-O139/*ctxA* multiplex of *V. cholerae* O1 and O139. See Table 6A.5.4 for amplicon sizes.

BASIC PROTOCOL 5

Direct PCR for Environmental Samples

Despite the ubiquitous nature of *V. cholerae*, isolation and detection by traditional methods are difficult since these methods rely on cultivating the organism. These difficulties arise from several possible factors: low density, interspecific competition, cell state (VBNC), and cell health (e.g., starvation). The polymerase chain reaction offers a molecular-based alternative to the traditional culture and immunological methods (discussed later). Three types of targets are used to detect *V. cholerae* in environmental samples by PCR: species-specific genes (16S rDNA, ITS, *ompW*); serogroup-specific genes (O1 and O139 rfb); and toxin and pathogenic factor genes (e.g., *ctx, tcpA*). Briefly, water, plankton, and/or sediment samples are collected and concentrated. DNA is extracted from the samples using a modification of the method of Murray and Thompson (1980) and PCR is performed on the extracted DNA using a multiplex (*ompW* and *ctxA*) primer array. This multiplex PCR can be substituted for the total *V. cholerae* ITS-based PCR identification described in Basic Protocol 4, if desired. Positive and negative controls should be run in parallel and should include a eubacterial 16S rDNA PCR reaction on each sample to test template quality (see Table 6A.5.4 for PCR primers and references).

Materials

Water or plankton sample (Basic Protocol 1) or sediment sample
TE buffer, pH 8.0 (*APPENDIX 2A*)
10% (w/v) SDS (*APPENDIX 2A*)
20 mg/ml (2%) proteinase K
5 M NaCl
CTAB/NaCl solution (see recipe)
25:24:1 phenol/chloroform/isoamyl alcohol (*APPENDIX 2A*)
24:1 chloroform/isoamyl alcohol
Isopropanol
70% ethanol
1× PCR amplification buffer (*APPENDIX 2A*) containing 15 mM $MgCl_2$ and 0.1% (w/v) gelatin
25 mM dNTP stock: dATP, dCTP, dGTP, dTTP (*APPENDIX 2A*)
20 μM forward and reverse PCR primers (Table 6A.5.4) for *ompW* (*ompW*-F, R) and *ctxA* (*ctxA*-F, -R)
Taq DNA polymerase
1.5% agarose gel (Voytas, 2000)
TAE buffer (*APPENDIX 2A*)
Molecular weight ladder (e.g., Hyperladder IV, Bioline)
1 μg/ml ethidium bromide (*APPENDIX 2A*)

37° and 65°C water baths
Vacuum desiccator or lyophilizer
Thermal cycler (MJ Research)
UV transilluminator, handheld UV lamp, or digital gel documentation system

Additional reagents and equipment for sample collection/transportation (Basic Protocol 1) and enrichment (Basic Protocol 2), PCR (Kramer and Coen, 2001), agarose gel electrophoresis and ethidium bromide staining of gels (Voytas, 2000), and isolation of *V. cholerae* by traditional culture method (Basic Protocol 2; optional)

Enrich environmental sample

For water and plankton samples

1a. Transport samples to the laboratory as for the conventional bacterial culture method (Basic Protocol 1).

2a. Filter (water sample) or homogenize (plankton sample), then perform enrichment in APW as in the conventional bacterial culture method (Basic Protocol 2).

For sediment samples

1b. Add sediment to 100 ml distilled water until the final volume reaches 200 ml. Mix well and allow sediment to settle. Remove large particulate matter by centrifuging a 10-ml aliquot of the slurry for 8 min at 1000 × *g*, room temperature.

2b. Enrich 1 ml of the sediment slurry (supernatant from previous step) in an APW enrichment flask, as for water and plankton samples (Basic Protocol 2).

Extract DNA

3. Microcentrifuge a 1-ml aliquot from the upper surface (i.e., top 1 to 2 mm) of the APW enrichment culture for 5 min at 12,000 × *g*, room temperature.

4. Resuspend the cell pellet in 567 µl TE buffer, pH 8.0.

5. Add 30 µl of 10% SDS, then 3 µl of 20 mg/ml proteinase K. Incubate the suspension 1 hr at 37°C.

6. Add 100 µl of 5 M NaCl, then add 80 µl of CTAB/NaCl solution. Incubate mixture 10 min at 65°C.

7. Add 800 µl of 25:24:1 phenol/chloroform/isoamyl alcohol, vortex, and centrifuge 5 min at 12,000 × *g*, room temperature.

8. Transfer aqueous (upper) phase to a new microcentrifuge tube. Add 800 µl of chloroform/isoamyl alcohol (24:1), vortex, and centrifuge 5 min at 12,000 × *g*, room temperature.

9. Transfer aqueous (upper) phase to a new microcentrifuge tube. Precipitate DNA by adding an equal volume of isopropanol.

10. Microcentrifuge 5 min at 12,000 × *g*, room temperature, and remove the supernatant. Wash DNA by adding 1 ml of 70% ethanol and microcentrifuging 5 min at 12,000 × *g*, room temperature.

11. Dry pellet in vacuum desiccator or lyophilizer and resuspend in 100 µl TE buffer, pH 8.0.

Perform multiplex PCR assay for detection of ompW (V. cholerae-specific) and ctxA (toxigenicity) (Nandi et al., 2000)

12. Set up *ompW-ctxA* multiplex PCR in a PCR tube for a total reaction volume of 25 µl containing the following:

    10 to 20 ng extracted genomic DNA (step 11)
    1× PCR amplification buffer
    250 µM each dNTP
    1.2 pmol/µl forward and reverse primers for *ompW* (*ompW*-F, -R)
    0.25 pmol/µl forward and reverse primers for *ctxA* (*ctxA*-F, -R)
    0.625 U *Taq* DNA polymerase.

    This protocol may be modified for the analysis of cultured organisms by using crude template DNA (see Basic Protocol 4, step 4) in this mixture instead of extracted genomic DNA.

13. Amplify the targets with the following cycling conditions:

1 cycle:	5 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	64°C	(annealing)
	30 sec	72°C	(extension)
1 cycle:	7 min	72°C	(final extension).

Perform agarose gel electrophoresis and visualize results

14. Run PCR product out on a 1.5% agarose gel in 1× TAE for 1 to 2 hr at 5 V/cm (Voytas, 2000), along with a molecular weight ladder (e.g., Hyperladder).

15. Stain the gel by incubating in 1 μg/ml ethidium bromide staining solution for 15 min at room temperature.

16. Destain the gel by incubating in distilled water for 15 min at room temperature.

17. Visualize the products by viewing the gel using a handheld UV lamp, transilluminator, or digital gel documentation system.

    The ompW and ctxA amplicons are 588 and 302 bp in length, respectively.

18. Screen samples giving a positive result for isolation of *V. cholerae* using the traditional culture method as described in Basic Protocol 2, if desired.

ALTERNATE PROTOCOL 1

Alternative Multiplex PCR Assay for Detection of O1 and O139 Serogroup *V. cholerae* and *ctxA*

In the following procedure (Hoshino et al., 1998), the multiplex PCR assay is performed to confirm O1 and O139 somatic antigens and for the simultaneous detection of the toxigenic trait gene, *ctxA*. This protocol can be used in place of steps 12 to 17 of the *ompW-ctxA* method (see Basic Protocol 5) for direct detection of *V. cholerae* in environmental samples. In addition, this assay can be used in place of the *V. cholerae*-specific ITS PCR (see Basic Protocol 4) for confirmation of presumptive *V. cholerae* strains isolated by the conventional bacteriological culture method. This protocol is particularly useful for determining the O1 or O139 serogroups of strains that are of the rough morphotype (see Basic Protocol 3, step 4 annotation) in which the serogroup cannot be determined by agglutination with specific antiserum.

Additional Materials *(also see Basic Protocol 4 or 5)*

20 μM forward and reverse PCR primers (Table 6A.5.4) for O1-*rfb* (O1F2-1, O1R2-2) and for O139-*rfb* (O139F2, O139R2)
2.0% agarose gel (Voytas, 2000)

1. Prepare crude template (Basic Protocol 4) or DNA extract (Basic Protocol 5).

2. Set up O1/O139-*rfb*/*ctxA* multiplex PCR in a PCR tube for a total reaction volume of 30 μl containing the following:

   10 to 20 ng of crude DNA template (Basic Protocol 4) or extracted genomic DNA (Basic Protocol 5)
   1× PCR amplification buffer
   210 μM each dNTP
   0.5 μM forward and reverse primers for O1-*rfb* (O1F2-1, O1R2-2)
   0.27 μM forward and reverse primers for O139-*rfb* (O139F2, O139R2)
   0.17 μM forward and reverse primers for *ctxA* (VCT1, VCT2)
   0.75 U *Taq* DNA polymerase.

3. Amplify the targets with the following cycling conditions:

1 cycle:	5 min	94°C	(initial denaturation)
35 cycles:	1 min	94°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	72°C	(extension)
Final extension:	7 min	72°C	(final extension).

4. Run PCR product out on a 2.0% agarose gel in 1× TAE for 1 to 2 hr at 5 V/cm (Voytas, 2000), along with a molecular weight ladder (e.g., Hyperladder).
5. Stain the gel by incubating in 1 μg/ml ethidium bromide staining solution for 15 min at room temperature.
6. Destain the gel by incubating in distilled water for 15 min at room temperature.
7. Visualize the products by viewing the gel using a handheld UV lamp, transilluminator, or digital gel documentation system.

   The O1-rfb, O139-rfb, and ctxA amplicons are 192, 449, and 308-bp in length, respectively (see Fig 6A.5.3).

8. Screen samples giving a positive result for isolation of *V. cholerae* using the traditional culture method as described in Basic Protocol 2, if desired.

Colony Blot Hybridization with Labeled RNA or DNA Probes

BASIC PROTOCOL 6

The colony lift procedure is used to immobilize DNA from bacterial colonies onto nitrocellulose or nylon filters to allow quick screening of a large number of colonies for genetic elements of interest by hybridization. The colony blot hybridization procedure is a culture-based alternative to the conventional culture method (Basic Protocol 2), so it is dependent upon the presence of *V. cholerae* in the sample as viable, culturable cells. Detection by hybridization precludes the necessity of numerous biochemical tests. Its advantage over PCR is that isolation is performed simultaneously with blot preparation, and enumeration can be performed more easily. Briefly, LB or modified nutrient agar spread plates are prepared from water samples and incubated overnight. Other plating media can be used, but the medium should be relatively rich and nonselective to allow for vigorous growth and cells with a high RNA content. Nitrocellulose (or nylon) membranes are overlaid, lifted, and treated to bind RNA (or DNA) to the membrane (Rehnstam et al., 1989). Plates to be lifted should contain 50 to 150 well defined colonies, 2.0 to 3.0-mm in size. Membranes should be handled with sterile forceps only and can be sterilized in an autoclave between two pieces of filter paper for 15 min prior to use. For RNA blots, care should be taken to minimize RNase contamination. Blots are then hybridized with labeled probe specific for *V. cholerae* (and *V. mimicus*), 5′-ACTTTGTGAGATTCGCTCCACCTCG-3′ (Heidelberg, 1997; Heidelberg et al., 2002), or toxigenic *V. cholerae* (*ctxA*). *V. mimicus* is a species closely related to *V. cholerae*, previously described as biochemically atypical *V. cholerae* (nonsucrose fermenting indicated by green colonies instead of yellow colonies on TCBS). *V. mimicus* produces a variety of toxins, including cholera toxin (potential reservoir), and has caused sporadic diarrhea (Ramamurthy et al., 1994). Fluorescently labeled probes are preferred (e.g., see Boyle and Perry-O'Keefe, 1992); however, the DIG system (Roche; see Alternate Protocol 2) offers a good alternative when a variable mode imager (such as Typhoon from GE Healthcare) or equivalent machine for detection of the fluorochrome, such as Dark Reader (Clare Chemical Research, *http://www.clarechemical.com/*), is not available.

Materials

Enrichment flask with *Vibrio cholerae* culture (see Basic Protocol 2) (for detection) or unenriched sample (for enumeration)
Alkaline peptone water (APW; see recipe)
LB plates (*APPENDIX 4A*) or modified nutrient agar plates (see recipe)
10% (w/v) SDS (*APPENDIX 2A*)
3× SSC (*APPENDIX 2A*)
Prewashing solution (see recipe), 60°C
DEPC-treated H_2O (*APPENDIX 2A*)
RNA colony blot hybridization solution (see recipe)
25 ng/μl fluorescein-labeled Vchomim1276 probe (5′-ACTTTGTGAGATTCGCTCCACCTCG-3′, with 5′ fluorescein label; Sigma Genosys)
Washing solution for colony blot hybridization with fluorescein-labeled probes (see recipe), 60°C
85-mm sterile nitrocellulose membranes, 0.22-μm (GE Water and Process Technologies)
65°C incubator
Pyrex dishes
Whatman no. 3 filter paper cut slightly larger than the nitrocellulose membranes
70°C oven
60°C water bath
Hybridization oven
Typhoon Scanner (GE Healthcare) or Dark Reader (Clare Chemical)

NOTE: All solutions should be DNase and RNase-free. For RNA colony blot hybridization, use DEPC-treated water (see *APPENDIX 2A*) to make hybridization solutions.

Prepare spread plates

1a. *For detection:* Prepare spread plates from enrichment flasks using APW as diluent by plating three 10-fold serial dilutions onto LB or modified nutrient agar plates.

1b. *For enumeration (and detection):* Prepare spread plates by plating three 10-fold serial dilutions onto LB or modified nutrient agar plates on-site without APW enrichment.

Alternatively, 100 to 500 ml of water may be filtered through the 0.22-μm nylon membranes and overlaid onto an agar plate. If this method is preferred, incubate the plate containing the membrane overnight at 30°C and then proceed to step 6.

2. Incubate plates overnight at 37°C.

Perform colony lift

3. Using a pencil, mark each membrane on the top side with the blot ID (e.g., recording the medium, sample, dilution) that matches the plate to be lifted. Also place asymmetrical orientation marks on the membrane to facilitate later comparison between membrane and plate.

4. Overlay the corresponding membrane onto each of the incubated spread plates, starting from the center to ensure there are no air bubbles.

5. Allow at least 15 min for transfer.

6. Replica plate the membrane onto a fresh modified nutrient agar plate, transferring the orientation markings. Maintain master and replica plate at 10° to 15°C, sealed with Parafilm.

Perform this step only when subsequent analysis of a positive colony is necessary.

7. For each plate, prepare two Pyrex dishes by placing a piece of Whatman no. 3 filter paper cut slightly larger than the nitrocellulose membrane into each. Prewet the filter paper in one dish with 10% SDS and the other with 3× SSC, pouring or pipetting the liquid onto the filter paper, letting it soak briefly, removing the air bubbles, then pouring off the excess. Ensure that there is no pooled liquid on the filter paper prior to placing membrane.

   It is important to use filter paper prewetted but not saturated with the appropriate solutions, to prevent colonies from over-swelling and losing their circular shape.

8. Preheat 10% SDS, 3× SSC, and the Pyrex dishes with the prewetted filter paper to 65°C.

9. Place membrane colony-side-up on the SDS-prewetted filter paper in the corresponding dish, cover the membrane with Saran Wrap or equivalent plastic wrap to prevent filter paper from drying out, and incubate 5 min at 65°C.

10. Transfer the membrane colony-side-up to the filter paper prewetted with 3× SSC, cover membrane with Saran wrap or equivalent plastic wrap to prevent the filter paper from drying out, and incubate 5 min at 65°C.

11. Transfer membranes to dry filter paper and allow to air dry 10 min at room temperature.

12. Bake membranes 15 min in a 70°C oven.

Perform RNA colony-blot hybridization

13. Wash membranes three times in 150 ml prewashing solution for 15 min at 37°C.

14. Wash membranes in 150 ml prewashing solution at 60°C, with agitation in a shaking water bath.

15. Rinse membranes in DEPC-treated water.

16. Prehybridize the membranes 30 min at 60°C in RNA colony blot hybridization solution at a ratio of 10 ml solution per 100-cm^2 of blot membrane in a hybridization oven.

    85 mm-membranes have a surface area of ~60 cm^2.

    RNA colony blot hybridization solution will form a precipitate when kept at room temperature. If this happens, heat to 40° to 50°C to resuspend.

17. Prepare a sufficient quantity of probe-containing RNA colony blot hybridization solution by adding 32 µl of 25 ng/µl fluorescein-labeled Vchomim1276 probe per 10 ml of RNA colony blot hybridization solution. At the end of the incubation in step 16, pour off the solution from the membrane and add the probe-containing RNA colony blot hybridization solution at a ratio of 10 ml solution per 100-cm^2 blot.

18. Hybridize overnight (ideally for 16 to 20 hr) at 60°C.

19. After hybridization, wash membranes 30 min at 60°C in washing solution for colony blot hybridization with fluorescein-labeled probes.

20. View/image the membrane using Typhoon Scanner or Dark Reader.

21. From replica plates, subculture colonies that were positive by blot hybridization for further analysis, if desired.

ALTERNATE PROTOCOL 2

Colony Blot Hybridization Using DIG-Labeled *ctxA* DNA Probe

The previous colony blot hybridization protocol (Basic Protocol 6) is used to detect *V. cholerae* and closely related *V. mimicus*. This protocol, on the other hand, targets only toxigenic strains of *V. cholerae*. The presence of *ctxA* is confirmed by hybridization using a *ctxA*-specific DNA probe. There may be some cross-reactivity of the probe with the heat-labile toxin (LT) of *E. coli* (Dallas and Falkow, 1980). Colony blots are prepared according to the method of Pal et al. (1992). The hybridization is done according to the DIG protocol. Readers are encouraged to consult the manual accompanying the High Prime kit (Roche); probe-labeling is described in the Support Protocol. The *ctxA* probe can be produced by PCR, using the pCTA primer set (see Table 6A.5.4), or by *Eco*RI digestion of plasmid, pKTN901, which contains a 540-bp *Xba*I-*Cla*I fragment of *ctxA* (Kaper et al., 1988).

Additional Materials *(also see Basic Protocol 6)*

Lysis buffer: 0.5 M NaOH/1.5 M NaCl (prepare fresh)
Neutralization solution 0.5 M Tris·Cl, pH 7.2 (*APPENDIX 2A*)/1.5 M NaCl (store up to 6 months at 2° to 8°C)
1× SSC (*APPENDIX 2A*)
40 μg/ml proteinase K in 1× SSC (store up to 2 months at –20°C)
DIG High Prime DNA Labeling and Detection Starter Kit II: (Roche) including:
  DIG Easy Hybridization Buffer
  10× blocking solution
  Antibody: anti-digoxigenin–AP conjugate
  CSPD
DIG-labeled *ctxA* probe (Support Protocol)
Stringency wash solution I: 2× SSC (*APPENDIX 2A*)/0.1% (w/v) SDS
Stringency wash solution II: 0.5× SSC (*APPENDIX 2A*)/0.1% (w/v) SDS
Washing solution for colony blot hybridization with DIG-labeled probes (see recipe)
Maleic acid buffer (see recipe)
Detection buffer: 0.1 M Tris·Cl, pH 9.5 (*APPENDIX 2A*)/0.1 M NaCl
Sterile nylon (or nitrocellulose) membranes, 85 mm, 0.22-μm (GE Water and Process Technologies)
UV cross-linker or transilluminator
42° and 65°C shaking incubators
Room temperature shaking water bath
Boiling water bath
Hybridization pouches
X-ray film (Kodak or Fuji) and developing facility

Prepare blots

1. Prepare LB agar spread plates as described in Basic Protocol 6, step 1.
2. Incubate plates overnight at 37°C
3. Using a pencil, mark each membrane on the top side with the blot ID (e.g., recording the medium, sample, and dilution) that matches the plate to be lifted. Also place asymmetrical orientation marks on the membrane to facilitation later comparison between membrane and plate.
4. Overlay the corresponding membrane onto each of the incubated spread plates, starting from the center to ensure there are no air bubbles.
5. Allow at least 15 min for transfer.

6. Transfer the blot onto a new LB plate keeping colony side up and incubate 3 hr at 37°C. Wrap the master plate with Parafilm and keep at 10° to 15°C.

   Master plates can be maintained in this manner for up to 2 weeks.

7. Place membranes, colony-side-up, onto Whatman no. 3 filter paper prewetted with lysis buffer in a Pyrex dish (see Basic Protocol 6, step 7, for additional considerations). Incubate 10 min at room temperature.

   It is important to use filter paper prewetted but not saturated with the appropriate solutions, to prevent colonies from over-swelling and losing their circular shape.

8. Remove the membrane from lysis buffer and place onto Whatman no. 3 filter paper prewetted with neutralization solution in a Pyrex dish. Incubate 5 min at room temperature.

9. Transfer membrane to a fresh filter paper prewetted with neutralization solution and incubate 5 min at room temperature.

10. Remove membrane from neutralization solution, place onto dry no. 3 Whatman filter paper and allow to air dry (~15 min) at room temperature, but not to complete dryness.

11. Immobilize colonies onto the membrane using a UV cross-linker or transilluminator.

    Damp membranes should be cross-linked at an output intensity of 120-mJ/cm^2, which corresponds to the optimal or auto-cross-link setting on commercially available machines. Transilluminators or hand-held UV lamps can be used, if calibrated; however, the following times should be sufficient: 1 min for 254-nm lamps or 3 min for 302-nm lamps.

12. Rinse the blot twice in 1× SSC buffer and air dry (~30 min).

13. Treat membranes 30 min at 42°C with 100 ml of 40 μg/ml proteinase K solution using gentle shaking.

14. Rinse filters three times in 1× SSC in a shaking water bath at room temperature, each time for 10 min. Allow to air dry (~30 min) at room temperature.

Prehybridize and hybridize

15. Preheat DIG-Easy Hybridization buffer to 42°C.

16. Prehybridize blots in preheated DIG-Easy Hybridization buffer for 30 min at 42°C with gentle agitation.

17. Denature the DIG-labeled *ctxA* probe by boiling for 5 min and rapidly cooling on ice. Prepare 25 ng/ml denatured probe in DIG Easy Hybridization solution.

18. Pour off prehybridization solution.

19. Add freshly prepared DIG Easy Hybridization Solution plus 25 ng/ml denatured probe and incubate overnight at 42°C.

Perform stringency washes

20. Pour off probe-containing hybridization solution.

    Probe-containing hybridization solution can be reused several times. Store up to 2 months at –20°C.

21. Wash membrane twice in stringency wash solution I for 5 min at room temperature under constant agitation.

22. Wash membrane twice in stringency wash solution II for 15 min at 65°C under constant agitation.

Detect positive colonies

23. Rinse membrane 5 min in washing solution for colony blot hybridization with DIG-labeled probes.
24. Dilute the 10× blocking solution from the kit to 1× in maleic acid buffer. Incubate the membrane 30 min at 25°C in 1× blocking solution (from kit; prepare according to manufacturer's instructions).
25. Prepare antibody solution by diluting the anti-digoxigenin–AP conjugate from the kit 1:10,000 in 1× blocking solution (prepared as in the previous step). Incubate membrane 30 min at 25°C in this diluted antibody solution for 30 min.
26. Wash the membrane twice, each time for 15 min, in solution for colony blot hybridization with DIG-labeled probes, for 15 min each.
27. Incubate membrane in detection buffer for 5 min at room temperature (25°C).
28. Place membrane in hybridization pouch. Add CSPD (from kit) to the membrane, cover and incubate at room temperature for 5 min.
29. Remove excess liquid from pouch, seal, and incubate at 37°C for 10 min.
30. Expose the membrane to X-ray film for 20 min at room temperature and develop.

    If film is under- or overexposed, repeat, varying time of exposure accordingly.

31. From master plates (step 6), subculture colonies that were positive by blot hybridization for further analysis.

SUPPORT PROTOCOL

Digoxigenin Labeling of *ctxA* Probe Using DIG High Prime Kit

PCR- or restriction digestion–generated probes can be labeled using the DIG High Prime kit (Roche), which is based on randomly incorporating digoxigenin-11-dUTP. A 563-bp *ctxA* fragment can be produced by amplifying extracted DNA from a toxigenic laboratory reference strain. The PCR product should be purified by using a PCR clean-up kit or by gel electrophoresis and extraction. Alternatively, a PCR product may be labeled during the PCR amplification using the PCR DIG Probe Synthesis Kit (Roche). A 554-bp *ctxA* probe is also available on a plasmid, pKTN901 (Kaper et al., 1988), which has been widely used (Pal et al., 1992; Islam et al., 2005). The *Xba*I-*Cla*I fragment can be removed from the plasmid by digestion with *Eco*RI, and gel purified. Labeled probes can then be used in the DIG-based hybridization as described in Alternate Protocol 2.

Materials

DNA extracted from toxigenic (*ctxA*$^+$) *V. cholerae* reference strain (ATCC)
Forward and reverse pCTA primers for amplifying *ctxA* (Table 6A.5.4)
Plasmid pKTN901 (available from Dr. James Kaper, *jkaper@umaryland.edu*) or other source of *ctxA* probe
*Eco*RI restriction endonuclease (or other restriction enzyme depending on source of *ctxA* probe) and restriction buffer
5× DIG High Prime labeling mixture (Roche)
0.2 M tetrasodium EDTA, pH 8.0
Boiling water bath
65°C water bath
Additional reagents and equipment for purifying DNA fragments (Moore et al., 2002)

1. *To generate ctxA target fragment by PCR:* Amplify extracted DNA of a toxigenic (*ctxA*$^+$) *V. cholerae* reference strain using the pCTA primer set.
2. *To generate ctxA target fragment by restriction digestion:* Digest plasmid pKTN901 with *Eco*RI for 2 hr at 37°C or digest other source of *ctxA* probe using restriction enzyme and conditions appropriate to construct.
3. Clean up PCR reaction product or digestion product by gel electrophoresis followed by gel extraction (Moore et al., 2002).
4. In a microcentrifuge tube, bring 1 μg purified *ctxA* fragment up to a total volume of 16 μl with sterile distilled water.
5. Denature by placing in a boiling water bath for 10 min, then rapidly transfer to an ice/water bath and chill 5 min.
6. Add 4 μl of 5× DIG High Prime labeling mixture to the DNA solution, mix, then microcentrifuge briefly to collect liquid at the bottom of the tube.
7. Incubate overnight at 37°C.
8. Stop reaction by adding 2 μl of 0.2 M tetrasodium EDTA and/or by heating to 65°C for 10 min.

IMMUNOLOGICAL METHODS FOR DETECTION OF *V. CHOLERAE*

Conventional culture methods are ineffective when bacterial cells have entered into the viable but nonculturable (VBNC) state. Thus, direct or indirect detection of the cells themselves becomes extremely important. The discovery of monoclonal antibodies in the 1980s and, subsequently, the development of a monoclonal antibody against *V. cholerae* O1,s triggered development of direct detection methods for this bacterial species (Xu et al., 1984; Hasan et al., 1992). Using immunological methods, the mystery concerning the inability to culture *V. cholerae* in environmental samples during inter-epidemic periods in Bangladesh was resolved by the discovery of VBNC *V. cholerae* (Roszak and Colwell, 1987; Huq et al., 1990).

Direct Fluorescent Antibody–Direct Viable Count (DFA-DVC) Method

BASIC PROTOCOL 7

The direct fluorescent antibody staining method for rapid detection of *V. cholerae* serogroup O1 and O139 is a very useful, direct, and culture-independent method. Coupled with the direct viable count method of Kogure et al. (1979), it can distinguish culturable, viable cells from viable but nonculturable cells (VBNC) of *V. cholerae* (Chowdhury et al., 1995). It is a two-step method in which samples are incubated with yeast extract in the presence of nalidixic acid, after which actively viable, substrate-responsive cells become enlarged and elongated (Kogure et al., 1979). Next, smears prepared from this suspension on a glass slide are stained with fluorescently labeled monoclonal antibody raised against the "A" factor of *V. cholerae* O1 lipopolysaccharide, which reacts with both serotype Ogawa and serotype Inaba (Colwell et al., 1990; Hasan et al., 1994). Antibodies against *V. cholerae* O139 are also available (Hasan et al., 1995). When observed under an epifluorescent microscope, elongated cells of *V. cholerae* O1 or O139 (based on the type of antibody used) exhibit a bright green–fluorescing periphery (the outer cell wall) with a dark interior (Fig. 6A.5.4). *V. cholerae* O1- and O139 DVC-DFA-positive samples can be confirmed by PCR (Binsztein et al., 2004). DFA-DVC is a rapid method by which one can determine the presence of *V. cholerae* within 8 hr (when the DVC incubation is 6 hr); however, overnight incubation with yeast extract and nalidixic acid is preferred. Kits for *V. cholerae* O1 (Cholera DFA) and O139 (Bengal DFA) DFA tests are commercially available (New Horizon Diagnostics).

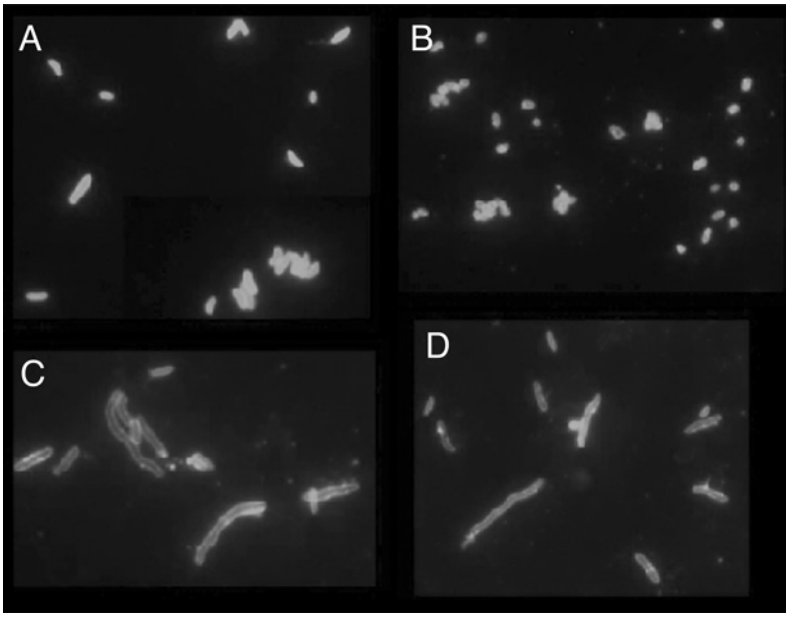

Figure 6A.5.4 DFA staining of *V. cholerae* O1 using the Cholera DFA Kit (New Horizon Diagnostics). (**A**) Fresh culture; (**B**) VBNC cells; (**C**) and (**D**), DVC-incubated cells. For the color version of this figure go to *http://www.currentprotocols.com*.

Materials

Concentrated water or homogenized plankton sample (Basic Protocol 2)
2.5% (w/v) yeast extract in distilled water
0.2% (w/v) nalidixic acid in distilled water
37% to 40% formaldehyde solution or fresh 4% formaldehyde prepared from paraformaldehyde
Absolute methanol
Cholera DFA and/or Bengal DFA kit (New Horizon Diagnostics) containing:
  FITC-conjugated DFA reagent
  Positive and negative control
  Fluorescent mounting medium
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Humidified chamber: 50-ml conical centrifuge tube containing 1 to 2 strips of water-saturated filter paper
Multiwell slides and coverslips
Epifluorescent microscope with FITC filter set

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All solutions should be filter-sterilized through a 0.1-μm filter, as VBNC cells of *V. cholerae* appear as small coccoid cells in a size range of 0.1 to 0.8 μm.

Perform DVC incubation

1. To 1 ml concentrated water or homogenized plankton sample (Basic Protocol 2), add 10 μl of 2.5% yeast extract and 10 μl of 0.2% nalidixic acid.

   The water sample can be concentrated by filtration through a 0.22-μm filter followed by resuspension or by centrifugation for 10 min at 12,000 × g, room temperature, followed by removal of the supernatant and resuspension.

   Freeze a parallel 1-ml sample for PCR confirmation (see Basic Protocol 5).

2. Incubate the mixture at room temperature (25°C) for a minimum of 6 hr to overnight.
3. Fix the sample by adding formaldehyde to a final concentration of 3% (v/v) and incubating 30 min at room temperature in the dark.

   Fixed samples can be stored at 4°C in the dark for up to 6 months.

Perform DFA protocol

4. Place 5 to 10 µl of the fixed sample onto a glass slide and air dry ~15 to 20 min.
5. Fix by adding 5 µl absolute methanol and air dry 1 to 5 min.
6. Add 10-µl of reconstituted FITC-conjugated specific DFA reagent from the Cholera DFA or Bengal DFA kit.

   Use of this kit is discussed in Hasan et al. (1994).

7. Incubate 30 min at 37°C in a humidified chamber. Protect slide from light.
8. Rinse slide with ~50 ml PBS. Air dry slide in the dark ~15 to 20 min.
9. Mount slide with one drop of fluorescent mounting medium and add coverslip.
10. Observe under an epifluorescent microscope (see Fig 6A.5.4).

Indirect Fluorescent Antibody (IFA) Method

BASIC PROTOCOL 8

This immunofluorescent method for detection of *V. cholerae* serogroup O1 in aquatic environmental samples was first introduced by Xu et al. (1984). Antiserum specific for O1 somatic antigen produced in rabbits was used with fluorescein isothiocyanate (FITC)–conjugated goat anti–rabbit immunoglobulin serum, with rhodamine isothiocyanate (RITC)-conjugated bovine serum albumin as the background stain. This indirect staining method was found to be very useful for detecting organisms in samples that gave negative results by culture and was subsequently modified to a direct method (Brayton et al., 1986; Huq et al., 1990). The direct fluorescent antibody method was later optimized (Hasan et al., 1994) and packaged as the Cholera DFA Kit (New Horizon Diagnostics, Inc.). The IFA protocol remains useful for laboratories where commercial DFA kits for *V. cholerae* are not readily available.

Materials

Concentrated water or homogenized plankton sample (Basic Protocol 2)
95% ethanol
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
FA Rhodamine counterstain (Becton Dickinson)
Polyvalent *V. cholerae* O1-specific antiserum (BD Bioscience)
FITC-conjugated anti-rabbit globulin goat serum (Sigma)
Low-fluorescence antiquenching mounting medium: e.g., FA (BD Biosciences) or Citifluor AF1/AF3 (Electron Microscopy Sciences)
Multiwell, Teflon-coated slides
35° and 55°C incubators
Humidified chamber: 50-ml conical centrifuge tube containing 1 to 2 strips of water-saturated filter paper
Glass coverslips
Epifluorescent microscope with FITC bandwidth filter

Prepare and fix samples

1. Add an appropriate amount (dependent on concentration of sample and well size) of each sample to be tested to a Teflon-coated multiwell slide. Air dry 15 to 20 min at room temperature.

2. Fix the sample by adding 95% ethanol to each sample-containing well. Air dry 5 to 10 min at room temperature.
3. Heat slide 10 min in a 55°C incubator.

Slides may be stored up to 1 month at −70°C at this point.

Stain samples

4. Rinse the slide(s) with ~50 ml PBS and air dry 15 to 20 min.
5. While slide is drying, equilibrate humidified chamber in 35°C incubator ~15 min.
6. To each dry sample well, add 1 to 2 drops of a 1:20 dilution of FA Rhodamine in distilled water. Incubate in humidified chamber 30 min at 35°C.

Minimize exposure to light from this step forward.

7. Rinse slide by gently flooding with ~50-ml PBS, then soak in PBS 10 min at room temperature. Remove slide from PBS and rinse again briefly in PBS.
8. Allow slide to air dry 15 to 20 min.
9. Add 5 to 10 µl of *V. cholerae* O1-specific antiserum. Incubate in humid chamber 30 min at 35°C.
10. Repeat steps 7 and 8 using fresh PBS for washing.
11. Add 1 to 2 drops undiluted FITC-conjugated anti-rabbit globulin goat serum and incubate in humidified chamber 30 min at 35°C.
12. Repeat washing steps 7 and 8 using fresh PBS.

Mount and examine slides

13. Mount each slide with a glass coverslip and a low fluorescence, anti-quenching mounting medium, such as Citifluor AF1.
14. Examine samples immediately using a epifluorescent microscope with a FITC band-pass filter.

Slides can be stored in the dark up to 1 to 2 days at 4°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Alkaline peptone water (APW)

10 g peptone
10 g sodium chloride
Add 800 ml H_2O
Adjust pH to 8.6 with NaOH
Adjust volume to 1 liter with water
Autoclave 15 min at 121°C
Store up to 6 months at 2° to 8°C

CTAB/NaCl Solution

Add 4 g NaCl to 80 ml water and dissolve. Slowly add 10 g cetyltrimethylammonium bromide (CTAB) while heating and stirring at 65°C. Adjust volume to 100 ml with water. Store up to 6 months at room temperature.

Gelatin agar

4 g neopeptone
1 g yeast extract
5 g sodium chloride
15 g gelatin
15 g agar
Adjust volume to 1 liter with water
Autoclave to sterilize
Store up to 1 month at 2° to 8°C

Kligler iron agar

Combine the following:
20 g polypeptone
10 g lactose
1 g glucose
5 g sodium chloride
0.5 g ferric ammonium citrate
0.5 g sodium thiosulfate
0.025 g phenol red
15 g agar
Adjust volume to 1 liter with water
Boil to dissolve agar completely

Dispense 5-ml portions of the medium into 16 × 125–mm tubes (other size tubes can be used, adjust dispensed media volume to about 1/3 volume of test tube). Autoclave 15 min at 121°C. Allow tubes to solidify in a slanted position to give a generous butt (underneath a 20° to 40° slant). Store up to 6 months at 2° to 8°C.

This medium is also available commercially as a dehydrated powder from Difco (BD Biosciences).

Maleic acid buffer

0.1 M maleic acid
0.15 M sodium chloride
Adjust pH to 7.5 with solid NaOH
Prepare fresh

Methyl Red/Voges-Proskauer (MR-VP) medium

7 g peptone
5 g glucose
5 g potassium phosphate, dibasic
Adjust pH to 6.9 with HCl
Adjust volume to 1 liter with water
Autoclave to sterilize
Store up to 6 months at 2° to 8°C

This medium is also available commercially as a dehydrated powder from Difco (BD Biosciences).

Modified nutrient agar

3 g beef extract
5 g peptone
10 g sodium chloride
15 g agar

continued

Adjust volume to 1 liter with water
Autoclave
Allow medium to cool to 55°C, then pour plates
Store up to 1 month at 2° to 8°C

Moeller decarboxylase broth base

5 g peptone
5 g beef extract
0.5 g dextrose
0.01 g bromocresol purple
0.005 g Cresol red
0.005 g pyridoxal
Adjust volume to 1 liter with water
Boil 1 min to dissolve
Add 10 g L-amino acid or 20 g DL-amino acid as specified in protocol
If adding ornithine, adjust pH to 6.0 with HCl
Autoclave to sterilize
Store up to 6 months at 2° to 8°C

This medium is also available commercially as a dehydrated powder from Difco (BD Bioscience).

Oxidase reagent

Dissolve 0.05 g *N,N,N′ N′*-Tetramethyl-*p*-phenylenediamine dihydrochloride into 5 ml water. Prepare fresh

Final concentration is 1% (w/v).

Prewashing solution

Prepare the following in DEPC-treated water (*APPENDIX 2A*)

3× SSC (*APPENDIX 2A*), RNase-free
0.1% (w/v) SDS RNase-free
Prepare fresh

Purple broth base

10 g proteose peptone no. 3
1 g beef extract
5 g sodium chloride
0.015 g bromocresol purple
Dissolve in 1 liter cold water
Add carbohydrate (as specified in protocol) to 1% (w/v) final concentration
Autoclave to sterilize
Store up to 6 months at 2° to 8°C

This medium is also available commercially as a dehydrated powder from Difco or BBL (both trademarks of BD Bioscience).

RNA colony blot hybridization solution

Prepare the following in DEPC-treated water (*APPENDIX 2A*)
0.9 M NaCl
50 mM sodium phosphate, pH 8.0 (*APPENDIX 2A*)
5 mM tetrasodium EDTA
0.5% (w/v) SDS
Store up to 1 month at room temperature

Precipitation will occur upon standing at room temperature. Heat to 45° to 50°C to resuspend.

Tellurite taurocholate gelatin agar (TTGA) plates

10 g tryptone
10 g sodium chloride
5 g sodium taurocholate
1 g sodium carbonate
30 g gelatin
15 g agar
Adjust volume to 1 liter with water
Boil to completely dissolve ingredients
Final pH should be 8.5; if not, adjust with HCl
Autoclave and add potassium tellurite to 1% (w/v) final concentration
Allow medium to cool to 55°C, then pour plates
Store up to 1 month at 2° to 8°C

Thiosulfate citrate bile-salts sucrose (TCBS) agar

5 g yeast extract
10 g peptone
10 g sodium thiosulfate
10 g sodium citrate
8 g ox bile
20 g sucrose
10 g sodium chloride
1 g ferric citrate
0.04 g bromthymol blue
0.04 g thymol blue
14 g agar
Adjust volume to 1 liter with H_2O
Boil to completely dissolve; do not autoclave
Allow medium to cool to 55°C, then pour plates

This medium is also available commercially as a dehydrated powder from Oxoid.

IMPORTANT NOTE: TCBS medium is never autoclaved. Sterility is achieved (for practical purposes) by the selective ingredients added to the medium. The high concentrations of thiosulfate and citrate and the strong alkalinity of this medium largely inhibit the growth of Enterobacteriaceae. Ox bile and cholate suppress primarily enterococci. Any coliform bacteria, which may grow, cannot metabolize sucrose. Only a few sucrose-positive Proteus strains can grow to form yellow, Vibrio-like colonies. The mixed indicator thymol blue/bromthymol blue changes its color to yellow, when acid is formed, even in this strongly alkaline medium.

Washing solution for colony blot hybridization with DIG-labeled probes

0.1 M maleic acid
0.15 M sodium chloride
0.3% (v/v) Tween 20
Adjust pH to 7.5 with solid NaOH
Prepare fresh

Washing solution for colony blot hybridization with fluorescein-labeled probes

Prepare the following in DEPC-treated water (*APPENDIX 2A*)
1× SSC (*APPENDIX 2A*)
0.1% SDS
Prepare fresh

COMMENTARY

Background Information

Although microorganisms were isolated and characterized a century ago, the past three decades could be termed as the "golden age of environmental microbiology" (Leadbetter, 1997). Twenty years ago, some microbiologists estimated that perhaps 40% of all prokaryotes were recognized and understood. However, today, it would be overly optimistic to suggest a number even as high as 5%, because new methods employed in environmental microbiology have shown that many microorganisms—perhaps <1% of the microbial species in the world oceans (Venter et al., 2004)—have been cultured. Molecular probes are considered the ultimate tools for detection and characterization of microorganisms (Woese, 1994; Amann et al., 1995) without the need for culture, but probes are designed only on the basis of existing knowledge of molecular signatures of organisms that were obtained originally in pure culture (Leadbetter, 1997). The study of environmental samples is very different from clinical microbiology. The diversity of microorganisms inhabiting highly dissimilar environments is enormous. Therefore, no unique test or set of tests will be appropriate for every environment. Detecting a particular organism will depend on environmental conditions during sample collection. Investigators would be wise first to determine the kind of data or results that will be needed and the appropriateness of available methods, as well as the feasibility of carrying out the procedures to achieve the goal. If a target pathogen is not detected in a sample using a particular method or even by a set of methods, it is risky to conclude that the pathogen is not present, especially if there are other indications that it may be present. Like any bacteriological test, replication and accurate interpretation are critical to achieve reproducible results. Most importantly, it is the responsibility of the investigator to choose appropriate tests and carefully design the study, including controls; otherwise, data interpretation will be very difficult, with misleading conclusions.

Vibrio cholerae is an autochthonous member of estuarine and other aquatic communities (Colwell et al., 1977; Kaper et al., 1979; Colwell et al., 1981; Colwell and Spira, 1992) and the disease with which it is associated, cholera, is largely a waterborne disease. *V. cholerae* also frequently isolated from higher aquatic organisms, such as plankton (Huq et al., 1990; Tamplin et al., 1990; Islam et al., 1993), oysters (Hood et al., 1981; Murphree and Tamplin, 1991), and water hyacinth (Spira et al., 1981). Transmission of cholera occurs predominantly by the fecal-oral route, via ingestion of contaminated water or contaminated seafood. Endemic areas include Southeast Asia, Africa, and Latin and South America, where poverty and poor sanitation are common.

Of the over 200 recognized serogroups of *V. cholerae* (Yamai et al., 1997), only the O1 and O139 serogroups cause epidemic and pandemic cholera outbreaks. Predominantly, non-O1/non-O139 *V. cholerae* strains are isolated from the environment, even in epidemic areas. Of the O1 serogroup strains that are isolated from the environment, most are nontoxigenic; i.e., missing the genes necessary for pathogenicity (*ctxA*$^-$, *tcpA*$^-$; Roberts et al., 1982; Minami et al., 1991).

V. cholerae, along with an ever-increasing number of species of Gram-negative bacteria, is capable of entering a state of dormancy, commonly termed viable but nonculturable (VBNC or sometimes VNC; Roszak and Colwell, 1987; Colwell, 1991; Colwell and Huq, 1994). It is becoming apparent that this state is an important survival strategy and perhaps an essential part of the life cycle of these bacteria. Microcosm experiments using VBNC *V. cholerae* suggest that a large number of environmental isolates exist in this state as compared to culturable cells (Huq et al., 1990; Huq et al., 2000).

Critical Parameters and Troubleshooting

Successful detection and/or isolation of *V. cholerae* may depend upon many environmental variables in addition to the appropriateness of the method used. For example, chances of detection of *V. cholerae* O1 are higher in warmer months when the water temperature is >30°C (Louis et al., 2003; Binsztein et al., 2004). The conventional culture method presented in this unit (Basic Protocol 2) is the most common, and preferred, culture-based protocol used to isolate *V. cholerae* (Morris et al., 1979; Rennels et al., 1980). There have been a number of proposed derivatives of this technique (Basic Protocol 2), based largely on alternative media. For enrichment, alkaline bile peptone water (Spira et al., 1981), Monsur's tellurite taurocholate broth (Monsur, 1961), and sodium-gelatin phosphate broth (Rennels et al., 1980) have been proposed. A second

enrichment step may be used, but this will add an additional 6 to 8 hr to an already lengthy protocol. Equally, a number of differential or selective plating media have been proposed (Tamura et al., 1971; Chatterjee et al., 1977; Morris et al., 1979; Shimada et al., 1990). Spira and Ahmed (1981) reported that using Moore swabs may increase the number of *V. cholerae* isolated over concentration by membrane filtration; however, membrane filtration remains the favored method. The authors of this unit include a subculturing step on to nonselective medium—modified nutrient agar or gelatin agar. This step serves two purposes. First, it is critical that pure colonies be achieved before proceeding with a battery of biochemical tests; mixed cultures will give ambiguous results. Second, growth on TCBS is not satisfactory for the oxidase test or for serotyping. When characterizing by biochemical tests, the authors present eleven biochemical tests to be performed, in addition to the oxidase test (Table 6A.5.2). This list is not definitive; the battery can be increased or decreased, and/or modified.

It is well documented that PCR amplification of environmental samples can be inhibited by dissolved organics, such as humic acid. For that reason, it is suggested the DNA be extracted first, which adds only 3 to 4 hr to the protocol. For all direct PCR examinations, include the eubacterial PCR reaction to confirm template quality on samples that are negative for *V. cholerae* gene targets.

Colony blot hybridization (Basic Protocol 6 and Alternate Protocol 2) is presented here as a culture-based alternative to the conventional culture method described in Basic Protocol 2. At least three serial-fold dilutions should be spread-plated to ensure screening of an ideal number of colonies (50 to 200). As an alternative, the authors suggest filtering water directly through the membrane (0.22-μm) to be used for the colony lift, although some knowledge of the microbial density of the water sample is needed for this procedure. For the *V. cholerae*–specific RNA-colony blot/hybridization, care should be taken to eliminate RNase contamination. Diethylpyrocarbonate (DEPC; *APPENDIX 2A*) is used to eliminate the otherwise stable, ubiquitous enzymes. The *ctxA*-DNA colony blot hybridization (Alternate Protocol 2) is presented for cases in which only toxigenic *V. cholerae* is investigated. This method has two drawbacks: cross-reaction with heat labile enterotoxin (LT) of *E. coli* may occur, and it is relatively expensive if a large number of samples are to be analyzed.

DVC-DFA (Basic Protocol 7) and IFA (Basic Protocol 8) of *V. cholerae* are relatively rapid protocols compared to the other methods presented. One pitfall of these immunofluorescence methods is the confounding observation of autofluorescing constituents found in environmental water samples. All "positive" samples should be documented by digital or film photography; confirmation by PCR of a parallel sample (frozen, but not fixed) is advisable.

Table 6A.5.5 outlines some of the more common problems that may be experienced in performing the basic and alternate protocols from this unit. This is not an exhaustive list; others may be encountered. Consult the troubleshooting sections from the referenced units of *Current Protocols in Molecular Biology* (see Literature Cited) for further advice.

Anticipated Results

V. cholerae can be easily isolated from estuarine environments during the warm summer months—even in nonepidemic areas. The chance of successful isolation decreases during the colder winter months, even from samples that are positive by PCR or DFA. Figure 6A.5.2 shows the typical growth of *V. cholerae* on TCBS and TTGA media. Growth on TCBS by *V. cholerae* appears as small (1- to 3-mm diameter) flat, yellow colonies (Fig. 6A.5.2A). *V. cholerae* appear as medium to large (2- to 5-mm in diameter) flat, translucent colonies on TTGA (Fig. 6A.5.2B). There will be a zone of clearing (or halo) surrounding *V. cholerae* colonies due to hydrolysis of gelatin. A dark center usually develops after 24 hr. All strains of *V. cholerae* can exhibit various colony morphologies, including a rugose form (White, 1940). The strain in Figure 6A.5.2A exhibits two colony morphologies, translucent and opaque. For the sake of comparison, several different positive-control *V. cholerae* strains should be subcultured at the time of subculturing from APW to selective medium. The selective media TCBS and TTGA still allow the growth of other *Vibrio* species and related bacteria. For example, *V. parahaemolyticus* will appear as blue-green colonies, *V. mimicus* will appear as green colonies, and *V. alginolyticus* as larger, yellow colonies on TCBS. Therefore, colonies growing on these media are not necessarily all *V. cholerae* and are labeled "presumptive." The goal of each step in the biochemical identification method is to reduce the number of suspected or presumptive colonies, which will include other *Vibrio* species and related *Vibrionaceae*, until

Table 6A.5.5 Troubleshooting Guide for *V. cholerae* Isolation and Detection

Problem	Solution
Basic Protocol 1	
Little or no plankton in cod-end collecting bucket	Check pore size of plankton net and ensure that it is 64 μm
	Zooplankton should be sampled near dawn or dusk (1-2 hr after sunrise or before sunset) when they are nearer to the surface
	Filter more water through plankton net
Basic Protocol 2	
Failure of positive control to give correct results for biochemical tests	Use multiple positive controls
	Prepare multiple replicates for each positive control
Results of biochemical tests difficult to interpret	Ensure proper incubation temperature and time
	Tests giving variable or weakly positive result should be replicated
	For decarboxylase tests, ensure that a tube without amino acid is used, to demonstrate growth with negative result
	For esculin hydrolysis, use presence/loss of fluorescence as measure of negative/positive
Basic Protocol 3	
Autoagglutination or clumping in saline without antisera	"Rough" morphotypes cannot be serogrouped with anitsera; use O1/O139 *rfb* PCR primers with Basic Protocol 4 to test for toxigenic serogroups
Basic Protocol 4	
No PCR product with positive control	Ensure all components are added to reaction at the proper concentration
	Use fresh dNTPs
	Prepare fresh crude template of positive control as it will degrade over time
	Dilute crude template 1:5000 or more and repeat the control reaction
	Quantify crude template by gel electrophoresis (~10 μl) to ensure sufficient template concentration
PCR product with negative control	Most likely caused by carryover contamination in one of the reaction components; make new components
Basic Protocol 5	
No PCR product with positive control	Ensure all components are added to reaction at the proper concentration
	Use fresh dNTPs
PCR product with negative control	Most likely caused by carryover contamination in one of the reaction components; make new components
Alternate Protocol 1	
No PCR product with positive control	Ensure all components are added to reaction at the proper concentration
	Use fresh dNTPs
PCR product with negative control	Most likely caused by carryover contamination in one of the reaction components; make new components

Continued

Table 6A.5.5 Troubleshooting Guide for *V. cholerae* Isolation and Detection, *Contined*

Problem	Solution
Basic Protocol 6	
Positive control is negative	Ensure solutions used are RNase-free
	Increase incubation time of lysis step, especially if colonies are >3 mm
	Ensure that the correct microscope filter is used for fluorochrome selected (other fluorochromes may be used)
Positive control gives weak signal	Check scanning settings on detection instrument Increase probe concentration and/or hybridization time
Alternate Protocol 2	
Positive control is negative or weak	Ensure that solutions are used in correct order
	Overexposure to UV source will degrade DNA template; consider using positively charged nylon membranes, which do not need cross-linking
	Extend hybridization time
	Extend development time
Positive control is overdeveloped or background is high	Check hybridization temperature
	Do not allow membrane to dry
	Decrease development time
Support Protocol	
Positive control from Alternate Protocol 2 is negative or weak	Check efficiency of probe labeling reaction
	Increase probe concentration (>25 ng/ml)
Basic Protocol 7	
Positive control is negative	Ensure that the proper filter set is used on fluorescent microscope
	Bengal DFA (*V. cholerae* O139) kit positive control is sometimes poor; prepare positive control from laboratory reference strain
Basic Protocol 8	
Positive control signal is weak	Increase amount of *V. cholerae* O1 antiserum and FITC conjugate

eventually only confirmed colonies of *V. cholerae* remain. Table 6A.5.3 lists the expected results of the above prescribed biochemical tests. See Bergey et al. (1994) or Lennette et al. (1985) for more details on each of these tests. Again, it is important to include several positive controls in the biochemical test screening for comparison.

Also, the biochemical confirmation may be shortened as in the alternative "b" and "c" steps of Basic Protocol 2. Figure 6A.5.5 shows the typical reaction of *V. cholerae* on a KIA slant. Most strains of *V. cholerae* isolated from the environment will be non-O1, non-O139, and *ctxA*$^-$, so serotyping usually yields few positive colonies. For O1-positive samples, the O1 serogroup can be further classified into two biotypes, namely Classical (CL) and El Tor (ET). The two biotypes are distinguished from each other on the basis of their differences in several phenotypic properties (WHO, 1987), including hemolysis (sheep red blood cells; CL^-, $ET^{+/-}$), hemagglutination (chicken red blood cell; CL^-, ET^+; Finkelstein and Mukerjee, 1963), Voges-Proskauer reaction (CL^-, ET^+), susceptibility to polymyxin B (CL^+, ET^-; Han and Khie, 1963), and sensitivity to biotype-specific phages (Nair et al., 2002). More recent studies have identified genes unique to the classical and seventh pandemic El Tor strains.

Figure 6A.5.3 shows a gel separation of the expected amplicons for several of the PCR protocols listed in the sections above. Table 6A.5.4 lists the amplicon size for all of the PCR primers mentioned in this chapter. This is not

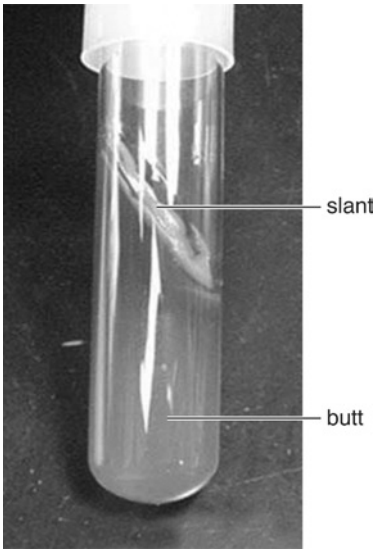

Figure 6A.5.5 Growth of *V. cholerae* in a Kligler iron agar (KIA) slant. Notice the alkaline butt and acid slant, with no gas production or blackening due to hydrogen sulfide production. For the color version of this figure go to *http://www.currentprotocols.com*.

a complete listing of all PCR primers that have been designed for *V. cholerae*-specific targets. Some thought should be given to using PCR identification protocols in lieu of biochemical tests, as PCR screening will provide a definitive answer in a shorter time period.

Figure 6A.5.4 shows the typical results of *V. cholerae* with DFA (Fig. 6A.5.4A,B) and DVC-DFA (Fig. 6A.5.4C,D). DFA-stained cells of *V. cholerae* appear as having a brightly green-fluorescing periphery with a slightly dimmer interior. Fresh, vegetative cells of *V. cholerae* have a distinctive curved-rod, or vibroid shape (Fig 6A.5.4A). VBNC cells of *V. cholerae* appear as rounded, or coccoid cells, which are harder to identify than vegetative cells (Fig 6A.5.4B). The coupling of the DVC procedure (Kogure et al., 1979) with DFA staining can alleviate the difficulty in distinguishing *V. cholerae* cells from autofluorescing objects present in water samples.

Time Considerations

One important parameter to consider in screening for *V. cholerae* is how rapidly an answer is needed. For routine monitoring of environmental water sources, time is usually not as critical as in clinical cases. In clinical settings, a rapid diagnosis is critical prior to initiating antibiotic treatment and oral rehydration. Many clinical laboratories and/or hospitals employ some form of the conventional culture-based method. If the method is followed as prescribed in Basic Protocol 2, the total time before confirmation may be up to 7 days. PCR screening can be performed in a significantly shorter period of time, 6 to 24 hr, depending on the template. DFA can also be completed in a shorter period of time, 4 hr, or 24 hr if coupled with DVC. Confirmation by colony blot hybridization can be performed in 3 days. It is important to balance time considerations with costs, as environmental water screening usually involves numerous sites and frequent sampling.

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Isolation and Classification of *Bdellovibrio* and Like Organisms

Bdellovibrio and like organisms (BALOs) are commonly retrieved from natural and man-made habitats. As they are obligate predators of other Gram-negative bacteria, they should be cultivated with prey cells in two-member cultures. Their growth cycle includes two spatially separated phases: a free-swimming attack phase and an intracellular phase during which the predatory bacterium establishes, elongates, and grows within the periplasm of its host at the expense of the latter's cell content. BALO-infected prey are called bdelloplasts. Isolation from environmental samples requires separation of the small BALOs from the majority of the other bacteria to ensure that plaques are not overgrown by contaminants.

This unit describes ways to isolate BALOs from the environment (see Basic Protocol 1 and Alternate Protocols 1 to 3), store BALOs (see Basic Protocol 2), obtain host-independent derivatives (see Basic Protocol 3), synchronize BALOS (see Basic Protocols 4 and 5), separate the various phases of growth (see Basic Protocol 6), and classify BALOs (see Basic Protocol 7). BALOs are isolated in double-layered agar plates as plaques growing in top agar containing prey cells. When found at very low levels, samples containing BALOs can be enriched by incubation with potential prey prior to isolation. Host-independent (HI) mutants are readily isolated from wild-type BALOs grown in pure and dense cultures, and are grown in rich medium. The different phases of the growth cycle of both wild-type BALOs and HI mutants can be separated to obtain defined fractions. Moreover, wild-type as well as HI-mutant cultures can be synchronized to obtain a majority of cells at the same stage of the growth cycle. Classification is based on the analysis of the 16S rRNA gene with the support of specific primers.

CAUTION: Bdellovibrio and like organisms are Biosafety Level 1 (BSL-1) microbes. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. Prey cells are selected by the experimenter and may therefore vary in biosafety level(s). This should be checked prior to starting work. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

IMPORTANT NOTE: When it is important to remove prey cells from a BALO culture (i.e., DNA, RNA, or protein analysis), lytic suspensions should be passed through a 0.45-μm filter at least once. Also, filtration may have to be performed to remove contaminants carried over during the isolation of BALOs.

IMPORTANT NOTE: When working with bacteria-domain-directed primers, exercise extra care to prevent contamination of PCR reagents.

ISOLATION OF BALOS FROM ENVIRONMENTAL SAMPLES BY DIFFERENTIAL CENTRIFUGATION AND FILTRATION

BASIC PROTOCOL 1

BALO cells are small (0.25 to 0.5 × 0.75 to 1.25 μm); therefore, they can be separated from the major fraction of the bacterial population. This is achieved using differential centrifugation and filtration.

Contributed by Edouard Jurkevitch

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Materials

Soil or water sample suspected of containing BALOs
H_2O, sterile (for soil samples)
HM buffer, 4°C (see recipe)
Potential prey (Support Protocol)
HM top agar, molten (see recipe)
HM plates (see recipe)

1.2-μm Nucleopore filter
28°C incubator
0.45-μm syringe filters (Supor Acrodisc, Pall Corp.; Minisart, Sartorius)

1a. *For soil or rhizosphere samples:* Prepare a slurry containing 10 to 50 g soil using a 1:2 to 1:10 soil/sterile water ratio according to soil texture (i.e., less for heavy clay soils and more for light sandy soils). Shake 30 min at room temperature using a platform shaker at 150 rpm or an orbital shaker at 200 to 250 rpm.

1b. *For aquatic samples:* Collect 50 to 250 ml sample.

2. Centrifuge 5 min at 500 × *g*, 4°C. Discard the pellet.

3. Centrifuge supernatant 20 min at 27,000 × *g*, 4°C. Decant supernatant and dissolve resulting pellet in a few milliliters of cold HM buffer.

HM buffer is used in place of a richer medium to limit the growth of prey or potential environmental contaminants. This is especially important when isolating BALOs from the environment.

4. Pass through a 1.2-μm Nucleopore filter. Maintain at 4°C (up to a few days); process as soon as possible.

If a high level of contaminants (carried over from isolation) grow on the HM top agar, blurring the BALO plaques, an extra filtration through a 0.45-μm filter may be necessary. Such a step may, however, further reduce the yield of BALOs.

To deal with clogged filters, see Alternate Protocol 1 and the Troubleshooting section.

5. Prepare 10-fold serial dilutions of the filtrate in HM buffer, room temperature, at a final volume of 5 ml.

For most environments, useful dilutions range from 10^0 to 10^{-4}.

6. Prepare top-agar infusions in the following manner.

   a. Add 250 to 300 μl (5×10^9 to 10^{10} cfu/ml) suspension of potential prey to test tubes containing 4 ml molten HM top agar.
   b. Vortex quickly.
   c. Add 100 μl filtrate dilution.
   d. Vortex and quickly pour onto an HM plate.
   e. Repeat for each dilution until all dilutions have been plated.

Two to three replicate plates should be made per dilution.

7. Allow top agar to solidify at room temperature (~60 min). Seal each plate with Parafilm.

8. Incubate sealed plates upside-down for 3 to 8 days at 28°C. Check for the growth of regular lytic plaques on the background of the prey lawn.

Plaques may vary in size from one to a few millimeters in diameter.

9. Cut 1-ml pipettor tips with a sterile scalpel. Gently aspirate plaques into the tip. Resuspend individual plaques in 0.5 ml HM buffer, room temperature. Let stand for a few minutes, then vortex.
10. Place a drop of the suspension on a clean glass slide and cover with a cover slip. Check for minute, fast moving cells using phase-contrast microscopy.
11. Pass through a 0.45-μm syringe filter. Maintain at 4°C; process as soon as possible.
12. Repeat steps 5 to 11 twice more to obtain pure cultures.
13. Grow and store as described in Basic Protocol 2.

ISOLATION OF BALOS USING A FICOLL GRADIENT

ALTERNATE PROTOCOL 1

This method more efficiently recovers BALOs while preventing filter plugging with soil colloidal material such as clay. It is, however, more expensive.

Additional Materials *(also see Basic Protocol 1)*

15% (w/v) Ficoll in HM buffer (see recipe): store up to 1 day at 4°C
1.5 × 9.5–cm centrifugation tubes
Gradient maker or peristaltic pump
Tabletop centrifuge with swinging-bucket rotor

1. Prepare sample as in Basic Protocol 1, steps 1 to 3.
2. Prepare 14 ml of a 15% to 1% continuous Ficoll gradient in 1.5 × 9.5−cm centrifugation tubes using a gradient maker or peristaltic pump. Gently pipet the concentrated sample on top of the Ficoll gradient.

   If one does not have access to a gradient maker, it is possible to construct a cushion by layering four 3.25-ml phases (1%, 5%, 10%, and 15%) of Ficoll one on top of the other.
3. Centrifuge in a swinging bucket rotor 20 min at 1,620 × *g*, 4°C, with brakes set at minimum.
4. Retrieve the upper 3 ml.
5. Prepare serial dilutions and plate as in Basic Protocol 1, steps 5 to 13.

ISOLATION OF BALOS FROM THE MARINE ENVIRONMENT

ALTERNATE PROTOCOL 2

Marine BALOs have specific ionic requirements. Consequently, the media used for their isolation should be adapted to this environment. *Vibrio parahaemolyticus* is an effective prey for the recovery of marine BALOs.

Additional Materials *(also see Basic Protocol 1)*

SWYE plates (see recipe)
Vibrio parahaemolyticus P-5 (Sheffield and Williams, 1990)
Sterile ocean water (see recipe)
Polypeptone 20 (Pp) medium (see recipe)
Pp top agar, molten (see recipe)
Pp plates (see recipe)
28° and 25°C incubators

1. To SWYE plates, add 100 μl *V. parahaemolyticus* P-5 and spread evenly to distribute. Incubate 24 to 48 hr at 28°C.
2. Flood the plate with 7 ml sterile ocean water. Resuspend colonies using a sterile cell spreader.

3. Adjust the suspension to $\sim 10^9$ cells/ml (OD_{600} of 0.85).
4. If needed, concentrate by centrifuging 30 min at 10,000 × *g*, 4°C. Resuspend in an appropriate volume of Pp medium.
5. Prepare 10-fold serial dilutions of the sample in ocean water at a final volume of 10 ml in the range of 10^0 to 10^{-4}.
6. Prepare top agar infusions in the following manner.
   a. Add 1 ml prey suspension to 3.3 ml molten Pp top agar.
   b. Vortex and add 5 ml diluted sample.
   c. Vortex and quickly pour onto a Pp plate.
   d. Repeat in triplicate or duplicate for each dilution.
7. Allow top agar to solidify at room temperature (<60 min). Wrap plates in Parafilm.
8. Incubate at 25°C for at least one week.
9. Check for the growth of regular lytic plaques on the background of the prey lawn (up to 8 days). When apparent, treat plaques as in the Basic Protocol 1, steps 9 to 13, substituting Pp medium for HM buffer.

ALTERNATE PROTOCOL 3

ENRICHMENT FOR BALOS IN ENVIRONMENTAL SAMPLES

Whenever quantification is not needed and BALOs are found at very low levels, an enrichment procedure can be applied. See Basic Protocol 1 for materials.

1. Perform step 1a or 1b of Basic Protocol 1. Add potential prey to the soil slurry or aquatic sample to a final concentration of 10^{10} cells/ml. Shake 24 to 48 hr at 28°C on an orbital shaker at 200 rpm.
2. Examine samples daily for 2 to 4 days for reduction in optical density and by phase-contrast microscopy for the presence of small, highly motile cells. If no such cells are seen, prolong the incubation (up to 2 weeks).

   Alternatively, transfer a 1-ml aliquot to 30 to 50 ml of a fresh suspension of 10^8 to 10^9 cells/ml potential prey and incubate an additional 24 to 48 hr.
3. After detection of putative BALOs, treat as described in Basic Protocol 1, steps 2 to 13.

   As enriched suspension contains many BALOs, it is recommended to perform a 0.45-μm filtration step as described. This greatly reduces carried-over contaminants.

BASIC PROTOCOL 2

GROWTH AND STORAGE OF PURE BALO CULTURES

Pure cultures can be cryopreserved; however, BALO strains need to first be cultured in liquid medium.

Materials

Pure culture of BALO in double-layered agar plate (Basic Protocol 1 and Alternate Protocols 1 to 3)
Diluted nutrient broth (DNB; see recipe)
Potential prey (Support Protocol; optional)
80% glycerol, sterile
28°C incubator

1. Cut 1-ml pipettor tips with a sterile scalpel. Gently aspirate a BALO plaque from a double-layered agar plate, including some surrounding agar containing prey. Transfer to a 125-ml Erlenmeyer flask containing 5 ml DNB.

2. *Optional:* Add potential prey to a final concentration of 5×10^9 cfu/ml.

   It is recommended that this step be performed with slower growing prey.

3. Shake on an orbital shaker at 250 rpm 24 to 48 hr, 28°C.

   Suspensions should be clear or almost clear.

4. Check for BALOs by phase-contrast microcopy.

5. Concentrate by centrifuging 10 min at 10,000 × *g*, 4°C. Resuspend in 3 ml fresh DNB. Add 80% glycerol to a final concentration of 20% (v/v).

6. Transfer to appropriate tubes. Quick freeze in liquid nitrogen and store at −80°C, up to 3 to 4 years.

7. If storage of a larger volume is desired, increase culture volume by transferring a fresh BALO inoculum (1:100 to 1:50 of the final volume) in DNB containing prey as above.

ISOLATION AND CULTURE OF HOST-INDEPENDENT MUTANTS

BASIC PROTOCOL 3

Bdellovibrio and like organisms are obligate parasites and as such are retrieved from the environment using prey. However, host-independent (HI) mutants able to grow saprophytically in rich medium appear spontaneously and can be recovered at frequencies of 10^{-5} to 10^{-8}. These HI derivatives exhibit the various stages of wild-type growth, including attack phase, as well as elongating and fragmenting the cell, thereby mimicking the wild-type-cell growth cycle. The absence of prey can be advantageous in molecular analysis.

Note that HI strains lose their predatory behavior when repetitively grown in the absence of prey and also are pleiotropic. HI strains vary in growth rates and other phenotypic characteristics.

Materials

Wild-type, host-dependent BALO strain
Streptomycin-resistant (Sm^r) and -sensitive (Sm^s) prey (Support Protocol)
50 mg/ml streptomycin
Dilute nutrient broth (DNB; see recipe) with and without 50 μg/ml streptomycin
PYE medium (see recipe) containing 50 μg/ml streptomycin
PYE plates with and without host extract (see recipe)
HM buffer (see recipe; optional)
Glycerol, sterile
28°C incubator
0.45-μm syringe filter (Supor Acrodisc, Pall Corp.; Minisart, Sartorius)
Additional reagents and equipment for preparing double-layered HM plates (see Basic Protocol 1; optional)

1. Grow wild-type, host-dependent BALO strain on Sm^r prey (10^9 cells/ml) 24 hr at 28°C. Add 50 mg/ml streptomycin to a final concentration of 50 μg/ml. Incubate an additional 12 hr at 28°C for a total of 36 hr.

   Streptomycin is used to select for Sm^R BALOs.

2. Inoculate 20 ml DNB containing 10^9 cells/ml Sm^r prey and 50 μg/ml streptomycin with the 36-hr culture to a final concentration of 15% (v/v). Incubate at 28°C until the prey is consumed (∼2 hr).

3. Separate the lysate from the antibiotic by centrifuging 5 min at 10,000 × *g*, 4°C. Resuspend pellet containing BALOs in 2 ml DNB and pass through a 0.45-μm syringe filter.

4. Inoculate 50 ml of DNB containing 10^9 to 10^{10} cells/ml Sm^s prey with 1 ml filtrate. Incubate 18 to 24 hr at 28°C.

   The Sm^s prey must be the same strain as the Sm^r prey.

5. Transfer 5 to 10 ml of the resulting lytic culture to PYE medium containing 50 μg/ml streptomycin. Incubate at 28°C until growth of HI cells is apparent (3 to 7 days).

   A large inoculum of a dense BALO suspension assumes that HI mutants will appear. Streptomycin is used to contra select against remaining prey cells.

6. Plate onto PYE plates and incubate up to 7 days at 30°C. If desired, supplement plates with 7% (v/v) host extract to enhance growth.

   HI cultures develop as colonies. Pick larger colonies because tiny ones fail to grow.

7. Streak onto a PYE plate. Incubate at 28°C until colonies develop (up to 3 to 5 days). Repeat this step twice to obtain pure cultures.

8. *Optional:* To retain predatory efficiency in HI mutants, plate HI strains in double-layered HM plates (e.g., Basic Protocol 1, steps 6 to 10) or grow in HM buffer. Include Sm^s prey every second or third transfer.

   Do not subculture HI strains too many times. Start from stock after ∼3 passages.

9. To store, grow an HI colony in 15 ml liquid PYE medium. Centrifuge 5 min at 10,000 × *g*, 4°C. Resuspend in 2 ml PYE. Add glycerol to 20% (v/v) and store at −80°C.

   It is recommended to renew the culture every year.

10. To revive an HI culture, thaw and spread 100 μl on a PYE plate supplemented with 7% (v/v) host extract.

BASIC PROTOCOL 4

SYNCHRONOUS GROWTH OF WILD-TYPE BALOS

In order to study events occurring at the various stages of the growth cycle, it can be advantageous to obtain cultures in which all prey cells are infected simultaneously and the growth cycle of the predator is synchronous. This is achieved by using a surplus of predatory over prey cells, assuring concomitant infection of most prey cells.

Materials

Prey (Support Protocol)
HM buffer (see recipe), 4°C
Pure BALOs (Basic Protocol 1 or Alternate Protocols 1 to 3)
0.45-μm syringe filter (Supor Acrodisc; Pall Corp.; Minisort, Sartorius)
28°C incubator

1. Grow 50 ml prey cells to the end of exponential or early stationary phase.

2. Centrifuge 10 min at 5,000 × *g*, 4°C. Decant the supernatant and add 5 vol cold HM buffer. Repeat centrifugation and resuspend in cold HM buffer such that the final concentration is at least 5 × 10^9 cells/ml.

3. Prepare a fresh 100-ml lytic culture of BALOs on the same prey.

4. Centrifuge the BALO culture 20 min at 10,000 × *g*, 4°C. Decant the supernatant and add 5 vol cold HM buffer. Repeat centrifugation and resuspend in cold HM buffer such that the final concentration is at least 2 × 10^{10} cells/ml.

5. Check the culture by phase-contrast microscopy.

   Bdelloplasts and prey cells should be almost absent from the culture.

6. If needed, pass the BALO culture through a 0.45-μm syringe filter.

   Be aware that this may reduce the yield of attack phase cells.

7. Combine predator (step 6) and prey (step 2) at a ratio of 2:1, at final concentrations of 10^{10} and 5×10^9 cell/ml, respectively.

8. Incubate with strong shaking (~250 rpm) at 28°C.

   Prey cells are penetrated within 30 min and a full cycle completed within 2.5 to 4 hr, depending on the strains and conditions. After the first cycle, the prey culture is essentially consumed and no prey cells remain to start a second cycle.

SYNCHRONOUS GROWTH OF HOST-INDEPENDENT BALOS

BASIC PROTOCOL 5

Asynchronous host-independent (HI) cultures contain a mixture of cells found at the various stages of growth. In synchronous cultures, which are obtained by subculturing a dense suspension of HI cells, most cells are found at the same stage.

Materials

Host-independent (HI) mutant (Basic Protocol 3)
PYE medium with 7% (v/v) host extract (see recipe)
28°C incubator

1. Grow HI mutant in PYE medium with host extract at 28°C overnight.
2. Centrifuge 10 min at 10,000 × *g*, 4°C.
3. Resuspend in fresh PYE supplemented with 7% (v/v) host cell adjusted to 5 mg protein/ml to obtain a final volume 10× larger that of the overnight culture (step 1).
4. Grow at 28°C and check for culture homogeneity according to needs by phase-contrast microscopy.

   Synchrony is maintained until stationary phase (~8 to 12 hr).

   Growth can be tracked by spectrophotometry (OD_{600}).

SEPARATION OF GROWTH PHASES

BASIC PROTOCOL 6

The various growth phases—i.e., free swimming attack cells, bdelloplasts containing intraperiplasmic elongating, replicating predatory cells, and empty prey remains—can be separated to allow stage-specific analysis.

Materials

Synchronous BALO culture (Basic Protocols 4 and 5)
0.05 M potassium phosphate buffer, pH 7.2 (*APPENDIX 2A*)
Percoll
0.25 M sucrose (85.5 g/liter)
14.5 mM NaCl

70-ml ultracentrifuge tube
Sorvall Centricon T-1170 ultracentrifuge and A-641 rotor (or equivalent)

1. Centrifuge a 100-ml synchronous BALO culture 10 min at 20,000 × *g*, 4°C. Wash with 5 to 10 ml of 0.05 M potassium phosphate buffer and resuspend in a few milliliters of the same.

2. Mix five parts Percoll with four parts 0.25 M sucrose. Transfer 66 ml into a 70-ml ultracentrifuge tube.
3. Layer BALOs onto the top of the Percoll-filled tube and ultracentrifuge 30 min at 50,000 × *g*, 4°C.
4. Using gentle pipetting, first remove the floating fluffy white material, then harvest prey cell remains or bdelloplasts (depending on the growth stage) from a ring at the top of the tube.

   The floating fluffy white material is composed of a mixture of growth stages.

5. Harvest the attack phase cells from an opaque band located 1.5 cm from the bottom of the tube, also using gentle pipetting.
6. To remove Percoll, wash the retrieved fractions by diluting in 5 vol of 14.5 mM NaCl. Centrifuge 10 min at 20,000 × *g*, 4°C.
7. Resuspend in a solution appropriate for further work, such as the extraction of cellular constituents.

SUPPORT PROTOCOL

PREY PREPARATION

Prey organisms should be prepared ahead of working with BALOs. In order to accomplish this, first grow potential Gram-negative prey in an appropriate medium (e.g., see *APPENDIX 2C*). Collect cells at the end of exponential to early stationary phase. Centrifuge 10 min at 5,000 × *g*, 4°C. Wash once with HM buffer. Resuspend in HM buffer (see recipe) to a final concentration of $5 \times 10^9$to 10^{10} cfu/ml. For routine growth, prey stock can be used 10 to 14 days if kept at 4°C. The most commonly used prey include *E. coli* ML35 and various strains of *Pseudomonas fluorescens* and *P. putida*.

BASIC PROTOCOL 7

CLASSIFICATION OF BALOS

The classification of BALOs is based on the analysis of the 16S rRNA gene. BALOs belong to two recognized families, Bdellovibrionaceae and Bacteriovoracaceae, forming the order Bdellovibrionales within the δ-Proteobacteria. *Bdellovibrio bacteriovorus* is the only recognized species within the Bdellovibrionaceae, while *Bacteriovorax stolpii, Bacteriovorax marinus, Bacteriovorax littoralis*, and *Peridibacter starrii* belong to the Bacteriovoracaceae. The G+C content of the Bdellovibrionaceae is ∼50%, and that of the Bacteriovoracaceae is between 37% to 44%. Amplified rDNA restriction analysis (ARDRA) of an ∼800-bp fragment enables a first classification of BALOs. Precise phylogenetic affiliation is performed using the (almost) whole 16S rRNA gene sequence length.

Materials

BALO suspension to be analyzed
H_2O, sterile
Dimethyl sulfoxide (DMSO)
3 M $MgCl_2$
2 mM dNTPs (*APPENDIX 2A*): dATP, dCTP, dGTP, and dTTP
1× PCR reaction buffer: 500 mM KCl/100 mM Tris·Cl, pH 9 (*APPENDIX 2A*)/1% (v/v) Triton X-100
Taq DNA polymerase
Primers (Table 7B.1.1)
BSA
1% agarose gel TAE (*APPENDIX 2A*)
Ethidium bromide staining solution (*APPENDIX 2A*)
*Hae*III, *Hin*fI, and *Rsa*I restriction endonucleases and appropriate buffers

Table 7B.1.1 Primers Utilized for Classification of BALOs[a]

Primer	Sequence
BALO-targeted primer 842R	5′-CGWCACTGAAGGGGTCAA- 3′
Bacteria domain targeted primer 63F	5′-CAGGCCTAACACATGCAAGTC-3′
Bacteria domain targeted primer 907R	5′-CCCCGTCAATTCCTTTGAGTTT-3′

[a]Any general Bacteria (i.e., Eubacteria or Archaea) domain targeted primer near the 3′ end can be utilized.

NuSieve GTG agarose (FMC)
*Eco*RI restriction endonuclease and appropriate buffer (for groups C and D only; Table 7B.1.2 and Figure 7B.1.1)
0.45-μm filter (Supor Acrodisc, Pall Corp.; Minisort, Sartorius)
80°C water bath (optional)
Additional reagents and equipment for purification of DNA (Moore and Downhan, 2002) and agarose gel electrophoresis (Voytas, 2000)

Template preparation and PCR amplification

1. Pass the BALO suspension to be analyzed through a 0.45-μm syringe filter. Centrifuge 10 min at 10,000 × *g*, 4°C. Resuspend in sterile water at 1/10 the original volume.
2. Extract DNA according to a standard protocol (e.g., Moore and Downhan, 2002) or immerse in liquid nitrogen then in hot (80°C) water for 3 min, repeating twice.
3. Cool on ice, then add DMSO to a final concentration of 10% (v/v).

   CAUTION: DMSO is hazardous. See UNIT 1A.3 for guidelines on handling, storage, and disposal.
4. Prepare a PCR mix using the following recipe per each 50 μl:

   3 mM $MgCl_2$
   20 μM (each) dNTPs
   1× PCR reaction buffer
   1.25 U *Taq* DNA polymerase
   1 μM each 63F and 842R primer
   12.5 μg BSA
5. Amplify using the following program:

Initial step:	4 min	94°C	(denaturation)
35 cycles:	1 min	94°C	(denaturation)
	1 min	50°C	(annealing)
	1 min	72°C	(extension)
Final step:	5 min	72°C	(extension).

6. Verify the presence of a PCR product by running 5 μl on a 1% agarose gel in TAE (Voytas, 2000). Stain with ethidium bromide staining solution and visualize using shortwave UV light.

   The final product should be ~800 bp.

   If no amplification product is obtained, repeat the procedure (steps 1 to 6), filtering and washing BALO suspensions twice in step 1 and using general primer 907R instead of primer 842R in step 4.

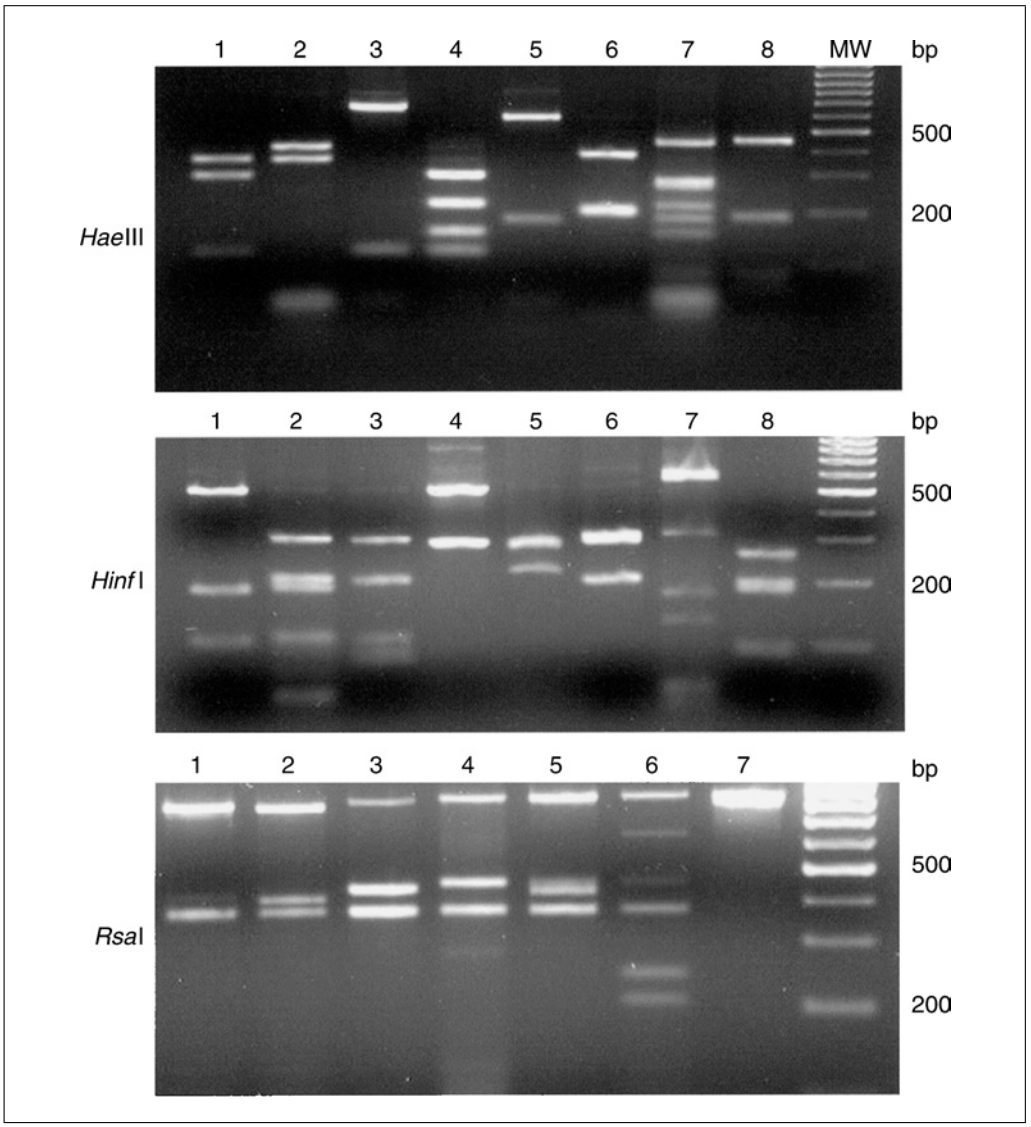

Figure 7B.1.1 Restriction patterns of amplified 16S rRNA gene fragments of *Bdellovibrio* and like organisms after digestion with *Hae*III, *Hin*fI, and *Rsa*I. Lane designated "MW" was loaded with a 100-bp molecular marker. The combination of the patterns defines the different ARDRA groups. (Reproduced with permission from Davidov and Jurkevitch, 2004).

ARDRA

7. Purify amplification products by standard techniques (e.g., Moore and Downhan, 2002).

8. Prepare separate restriction digestions according to manufacturers' protocols utilizing *Hae*III, *Hin*fI, or *Rsa*I, and ~15 µl PCR product.

   See Bloch and Grossman (1995) for general information about performing restriction digestion analysis.

9. Separate for 1 hr at 80 V in a 3% NuSieve GTG agarose gel (or equivalent) using TAE as the running buffer (Voytas, 2000). Stain the gel using 0.5 µg/ml ethidium bromide and make a record of the gel as visualized using short-wavelength UV light.

10. Compare restriction patterns to Figure 7B.1.1, find the corresponding group in Table 7B.1.2, and the phylogenetic affiliation in Figure 7B.1.2.

11. If required (i.e., organism belongs to Group C or D; Table 7B.1.2), prepare a new restriction digestion utilizing *Eco*RI to enhance resolution.

Table 7B.1.2 Patterns Obtained by ARDRA of *Bdellovibrio* and Like Organisms[a]

Group	ARDRA banding pattern[b]		
	*Hae*III	*Hin*fI	*Rsa*I
A	1	1	1
B	1	3	2
C[c]	2	2	1
D[c]	2	2	1
E	10[d]	1	1
F	3	1	1
G	1	2	2
H	1	1	2
I	9[d]	9[d]	1
J	4	4	1
K	5	5	3
L	5	5	7
M	6	6	4
N	6	6	5
O	6	6	6
P[e]	7	7	2
Q[f]	11[d]	10[d]	8[d]
R[f]	12[d]	6	9[d]
S[f]	13[d]	6	10[d]
T[f]	6	6	11[d]
U[e]	8	8	7

[a]Also see Figures 7B.1.1 and 7B.1.2.

[b]Numbers in columns relate to the lanes in Figure 7B.1.1. For example, an organism from group J would be expected to demonstrate the banding pattern shown in lane 4 when digested with *Hae*III (top panel), lane 4 when digested with *Hin*fI (middle panel), and lane 1 when digested with *Rsa*I (bottom panel).

[c]Distinguishable by restriction with *Eco*RI (for patterns, see Jurkevitch *et al*., 2000).

[d]Lanes not shown in Fig. 7B.1.1.

[e]Amplicon obtained using primer 907R instead of 842R. (Davidov and Jurkevitch, 2004, by permission).

[f]Groups Q, R, S, and T are based on in silico digestions with sequences retrieved from the GenBank.

Full gene length amplification

12. Repeat steps 1 to 5 but filtering the BALO suspension twice in step 1 and utilizing primers spanning the (whole) 16S rRNA gene in step 4.

For an example, see Marchesi et al. (1998).

Representatives from each ARDRA group can be sequenced. With this information, more precise taxonomical placement can be achieved.

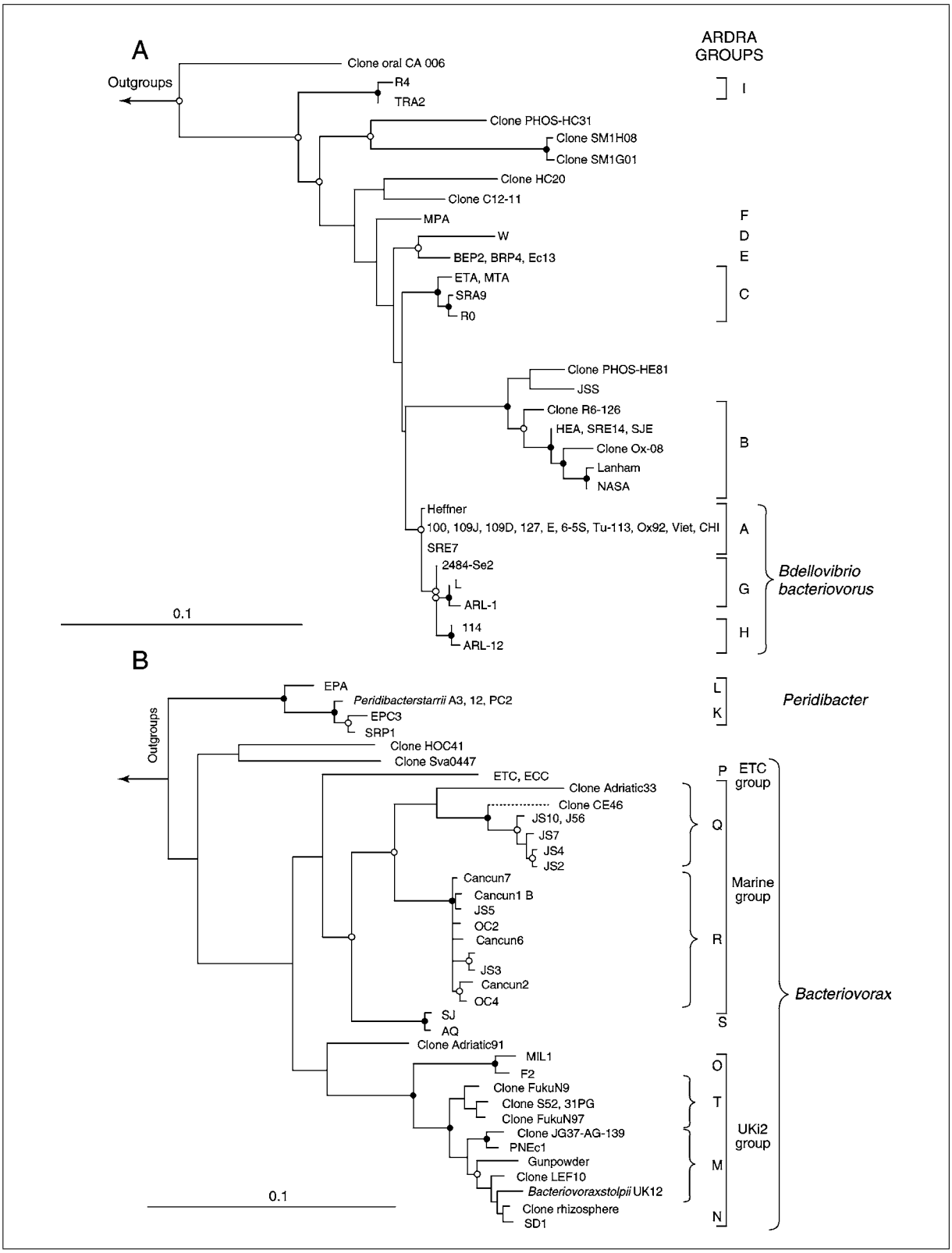

Figure 7B.1.2 Phylogenetic 16S rRNA tree of the (**A**) Bdellovibrionaceae (**B**) Bacteriovoracaceae based on maximum-likelihood analysis. The ARDRA groups shown correspond to the patterns shown in Figure 7B.1.1 and to the key in Table 7B.1.2 (Reproduced with permission from Davidov and Jurkevitch, 2004.)

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Diluted nutrient broth (DNB)

Dilute nutrient broth 1:10 in water and autoclave. Cool to room temperature. Add 6 ml of 0.5 M $CaCl_2 \cdot 2H_2O$ (3 mM final) and 3.33 ml of 0.6 M $MgCl_2.7H_2O$ (2 mM), passing each through a 0.20-μm syringe filter as it is added to the medium. Store up to 3 months at room temperature.

HM buffer

Prepare 25 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES). Adjust to pH 7.4 with 10 N sodium hydroxide. Sterilize by autoclaving and cool to room temperature. Add 6 ml of 0.5 M $CaCl_2 \cdot 2H_2O$ (3 mM final) and 3.33 ml of 0.6 M $MgCl_2.7H_2O$ (2 mM), passing each through a 0.20-μm syringe filter as it is added to the medium.

HM plates

Prepare 25 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES). Add agar to 1.5% final. Adjust to pH 7.4 with 10 N sodium hydroxide. Sterilize by autoclaving and cool to 60°C. Just prior to pouring, add 6 ml of 0.5 M $CaCl_2 \cdot 2H_2O$ (3 mM final) and 3.33 ml of 0.6 M $MgCl_2 \cdot 7H_2O$ (2 mM), passing each through a 0.20-μm syringe filter as it is added to the medium. Pour into standard 100-mm plates using 20 to 25 ml per plate. Let solidify and dry. Seal in bags and store up to 2 months at 4°C.

A concentration of 1.5% can be obtained by adding 15 g agar per liter medium.

HM top agar

Prepare 25 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES). Add agar to 0.7% final. Adjust to pH 7.4 with 10 N sodium hydroxide. Sterilize by autoclaving and cool to 60°C. Add 6 ml of 0.5 M $CaCl_2 \cdot 2H_2O$ (3 mM final) and 3.33 ml of 0.6 M $MgCl_2 \cdot 7H_2O$ (2 mM), passing each solution through a 0.20-μm syringe filter as it is added to the medium. Rapidly prepare 4-ml aliquots in sterile test tubes before the agar solidifies. Prior to use, melt in an 80°C water bath, then maintain at 42°C to keep molten.

A concentration of 0.7% can be obtained by adding 7 g agar per liter medium.

Solidified tubes can be stored for 1 to 2 weeks but should be wrapped to prevent evaporation.

Host extract

Centrifuge one liter of an overnight *E. coli* culture prepared in nutrient broth (e.g., LB or NB; *APPENDIX 4A*). Centrifuge 10 min at 10,000 × *g*, 4°C. Resuspend in 10 ml HM buffer (see recipe), autoclave, and remove debris by centrifuging 10 min at 10,000 × *g*, 4°C. Retain the supernatant. Determine protein concentration using Bradford's reagent (*APPENDIX 3A*) so that the addition of extract to growth medium is standardized.

Ocean water, sterile

Obtain ocean water or prepare from sea salts such as Instant Ocean (Aquarium Systems). Autoclave and pass through a 0.2-μm filter. Store up to 2 months at room temperature.

Polypeptone 20 (Pp) medium

Dissolve 1 g polypeptone 20 (Pp) in one liter ocean water (see recipe for sterile ocean water). If necessary, add HCl to obtain a pH of 7.7 to 7.8. Autoclave and pass through a 0.2-μm filter. Store up to 3 months at room temperature.

Pp plates

Autoclave polypeptone 20 (Pp) medium (see recipe) containing 1.5% (w/v) agar. Cool to 60°C. Pour into 15 × 150–mm plates using ~60 to 65 ml per plate. Let solidify and dry. Seal in bags and store up to 2 months at 4°C.

A concentration of 1.5% can be obtained by adding 15 g agar per liter medium.

Pp top agar

Autoclave polypeptone 20 (Pp) medium (see recipe) containing 1.95% agar. Prepare 3.3-ml aliquots in sterile test tubes. Before use, melt, then maintain at 42°C to keep molten.

A concentration of 1.95% can be obtained by adding 19.5 g agar per liter medium.

PYE medium

Add 3 g yeast extract and 10 g Bacto peptone to 1 liter water. Sterilize by autoclaving. Cool to room temperature and add 6 ml of 0.5 M $CaCl_2 \cdot 2H_2O$ (3 mM final) and 3.33 ml of 0.6 M $MgCl_2 \cdot 7H_2O$ (2 mM), passing each through a 0.20-μm syringe filter as it is added to the medium. Store up to 3 months at room temperature.

PYE plates

Add 3 g yeast extract, 10 g Bacto peptone, and 15 g agar to 1 liter water. Cool to 60°C. If desired, add 70 ml (7% v/v final) host extract (see recipe). Add 6 ml of 0.5 M $CaCl_2 \cdot 2H_2O$ (3 mM final) and 3.33 ml of 0.6 M $MgCl_2 \cdot 7H_2O$ (2 mM), passing each through a 0.20-μm syringe filter as it is added to the medium. Pour into 15-mm plates using ~20 to 25 ml per plate. Seal in bags and store up to 2 months at 4°C.

Salt solution

0.75 g KCl
23.4 g NaCl
6.9 g $MgCl_2 \cdot 7H_2O$
Adjust pH to 7.2 with 1 N NaOH
Store indefinitely at room temperature

SWYE plates

Dissolve 10 g proteose peptone, 3 g yeast extract, and 20 g agar in 1 liter salt solution (see recipe). Sterilize by autoclaving. Cool to 60°C. Pour into standard 1.5 × 90–mm plates using ~20 to 25 ml per plate. Seal in bags and store up to 2 months at 4°C.

COMMENTARY

Background Information

Bdellovibrio and like organisms (BALOs) are small (0.25 to 0.5 × 0.75 to 1.25 μm), rod or vibrio-shaped, highly motile Gram-negative bacteria. The most striking feature of BALOs is their obligate dependence on predation of Gram-negative cells to grow and reproduce. As a result, BALOs exhibit a peculiar life cycle composed of a free-swimming attack phase and, for most BALOs, an intraperiplasmic phase during which the cell grows as a filament that finally fragments to release attack cell progeny from the ghost of the host cell (Martin, 2002). (A few strains are epibiotic and divide by binary fission.) BALOs are ubiquitous in nature and in man-made habitats. They are found in aquatic and terrestrial environments as planktonic cells or associated with biofilms. They are detected in extreme environments as well. Their impact on bacterial populations is still unclear (Jurkevitch and Ramati, 2005).

The discovery of *Bdellovibrio* and like organisms (BALOs) by Heinz Stolp in 1962 (Stolp and Pertzold, 1962) was a serendipitous one: it happened as he pursued the isolation of soil bacteriophages. The basic procedure for isolating BALOs remains similar, i.e., BALOs mixed with a potential host form plaques in double-layered agar plates. However, these plaques differ from phage plaques as they develop more slowly (appearing after 3 to 4 days)

and keep spreading for much longer (a week or more). Although this approach enables the isolation of BALOs, it suffers from a few drawbacks.

1. BALO strains differ in prey range, therefore a particular prey will only enable the growth of the predators able to use it. As most bacteria are not amenable to cultivation, a large proportion of BALOs may go undetected.

2. BALOs are usually found at relatively low levels in nature, therefore there is a risk that colony-developing bacteria, carried over during the isolation procedure, may overgrow plaques on isolation plates.

3. Filtration through 0.45-μm filters reduces the yield of BALOs. The use of a Ficoll gradient may enable a "cleaner" recovery of BALOs, but it is more expensive (Varon and Shilo, 1970).

BALOs belong to the δ-proteobacteria. They form two families, Bdellovibrionaceae and Bacteriovoracaceae. Only one species (*B. bacteriovorus*) is recognized in the former. The latter holds the two genera *Peridibacter* and *Bacteriovorax*, with *P. starrii*, and *B. stolpii*, *B. marinus*, and *B. littoralis* being their recognized species, respectively (Baer et al., 2000, Davidov and Jurkevitch, 2004). The taxonomy of BALOs is mainly based on the phylogenetic affiliation obtained from 16S rDNA analysis (see Basic Protocol 7). No clear connection has been found between BALO prey range and phylogeny. Amplified rDNA restriction analysis (ARDRA) used in conjunction with a BALO-specific primer (Jurkevitch and Ramati, 2000; Davidov and Jurkevitch, 2004) is an efficient tool for an initial determination of the phylogenetic placement of isolates (especially when a large number of strains have to be analyzed).

Host-independent (HI) derivatives of BALOs can be isolated under laboratory conditions (Seidler and Starr, 1969; Barel and Jurkevitch, 2001). These mutants are grown in rich medium and retain the phenotypes of the attack and of the replication-elongation-fragmentation phases (Basic Protocol 3). As such, they can provide information on the growth cycle without the interference of a prey.

In order to perform phase-specific analyses, cultures should be synchronous. This can be achieved with both wild-type and HI strains. Synchrony is realized with the former by using a surplus of predators over prey (Basic Protocol 4), while dilution of dense suspensions induces it in the latter (Basic Protocol 5). Further physical separation of the phases is attained by centrifugation on a Percoll cushion (Basic Protocol 6).

Critical Parameters

Amplification of the 16S rRNA gene (Basic Protocol 7) or part thereof with the polymerase-chain reaction should be performed with great care when using general primers targeting the Bacteria domain. In that case, BALO suspensions should be thoroughly filtered and washed to remove traces of the prey.

Troubleshooting

Table 7B.1.3 presents some common problems encountered when performing the protocols described in this unit, as well as their common causes and potential solutions.

Anticipated Results

Isolation from environmental samples

BALOs plaques should almost always be obtained from environmental samples. The number of plaques may vary greatly between habitats, seasons and the prey chosen, among other parameters. Usually tens to tens of thousands of plaques are formed per milliliter or gram sample.

Isolation of HI mutants

The procedure described has enabled the isolation of HI derivatives from all the strains tested so far (Seidler and Starr, 1969).

Classification

At least the family affiliation (Bdellovibrionaceae, Bacteriovoracaceae, or as yet undescribed groups) of the analyzed isolates should be obtained. Most strains originating from mesophilic, but not extreme terrestrial and aquatic habitats, may belong to one of the described ARDRA groups.

Time Consideration

Isolation from environmental samples

Plaques become apparent 3 to 8 days after inoculation. Pure cultures, obtained by plating the plaques repeatedly, should be obtained within two weeks.

Growth and storage of pure BALO cultures

This procedure should take 2 to 3 days.

Isolation of HI mutants

Isolating host-independent mutants can take up to two weeks.

Table 7B.1.3 Troubleshooting Guide for Isolation and Classification of *Bdellovibrio* and Like Organisms

Problem	Possible Cause	Solution
	Isolation from environmental samples	
No plaques	Prey not adequate	Change prey
	Very low levels of BALOs	Try enrichment protocol (Alternate Protocol 3)
Overgrowth of contaminants	Too many cells pass through 1.2-μm filter	Filter with 0.45-μm membrane
No plaques after filtration through 0.45-μm membrane	Too many BALOs are lost	Try Ficoll protocol (Alternate Protocol 1)
1.2-μm filters clogged	Too much fine material (e.g., clay)	Pass sample through a 5-μm filter step or use a Ficoll cushion prior to passing through 1.2-μm filter
	Culture of host-independent mutants	
Strains grow sluggishly	Uncertain: possibly due to lack of nutritional replacement	Plate on PYE medium supplemented with host extract and start culture from an isolated colony
Classification		
No amplification product	Target sequence in 16S rRNA gene has mismatches with primer 842R	Use primer 907R instead of 842R (see Table 7B.1.1)
DNA sequence not readable: too many multiple peaks	16S DNA from prey or a contaminant is also being amplified	Pass BALO suspension through a 0.45-μm filter twice and be extra careful with PCR reagents

Synchronous growth of BALO cultures

This procedure takes ~1 day.

Separation of growth phase

This method requires 3 to 6 hr.

Classification

Amplified rDNA restriction analysis (ARDRA) patterns can be obtained within a few days.

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Contributed by Edouard Jurkevitch
The Hebrew University of Jerusalem
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Laboratory Maintenance of *Bdellovibrio*

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ABSTRACT

Bdellovibrio bacteriovorus is a Gram-negative bacterium that preys upon other Gram-negative bacteria. It does this by penetrating the outer membrane and peptidoglycan to establish itself in the periplasm where it grows at the expense of the contents of the prey cell before ultimately lysing the prey. Wild-type *Bdellovibrio* are prey-(or host-) dependent and the protocols described in this unit deal with the techniques required to grow this bacterium on its prey cells. Protocols are also presented to generate and culture host-independent mutants as well as novel protocols to evaluate *Bdellovibrio* predation. *Curr. Protoc. Microbiol.* 9:7B.2.1-7B.2.13. © 2008 by John Wiley & Sons, Inc.

Keywords: *Bdellovibrio bacteriovorus* • predator-prey interactions • luminescence assay • plaque assay • host-independent growth • storage of cultures

INTRODUCTION

Bdellovibrio are small, predatory, delta-Proteobacteria. They collide with, attach to, and invade Gram-negative bacterial prey by lysing a small temporary pore into the outer layers of the prey through which they enter and establish growth in the prey periplasm in a structure known as the bdelloplast (Tudor et al., 1990). *Bdellovibrio* growth is achieved by attaching to the cytoplasmic membrane of prey and hydrolyzing and taking up prey biomolecules for *Bdellovibrio* cell synthesis. *Bdellovibrio* grow in a filamentous form within their prey, and when nutrients are exhausted, they septate into individual cells, which synthesize flagella and swim within the bdelloplast. Ultimately, the prey outer membrane is lysed to release fresh, motile progeny *Bdellovibrio*. Their ability to prey upon many disease-causing bacteria raises the interesting possibility that they may be developed for use as biocontrol or therapeutic agents.

Routine laboratory maintenance of *Bdellovibrio* is carried out by growth on suitable Gram-negative prey cells such as *E. coli* by either the double-layer overlay technique (Rittenberg, 1982) or as prey lysates in buffer (Koval and Hynes, 1991). Alternatively, a proportion of the cells of any given population of *Bdellovibrio* can grow axenically in the absence of hosts (Seidler and Starr, 1969a). These are most commonly referred to as host-independent (HI) strains and often have many properties differing from wild-type, such as morphology (Fig. 7B.2.1) and they may or may not retain the ability to form plaques on prey lawns.

Recently, molecular genetic techniques have been developed for the manipulation of *Bdellovibrio*, and since the sequencing of the genome of *B. bacteriovorus* HD100, genes can now be targeted for insertional inactivation or deletion (Lambert et al., 2003). Basic Protocol 1 describes the standard methods of growing *Bdellovibrio* in suspensions of prey *E. coli*. Basic Protocol 2 explains further the standard procedure for rescuing *Bdellovibrio* from long-term storage. Basic Protocol 3 details growth of *Bdellovibrio* as single plaques for enumeration, which is a technique used in many experiments to determine the number of *Bdellovibrio* in a preparation. Basic Protocol 4 describes the differential filtration technique to isolate HI derivatives. Basic Protocol 5 shows how

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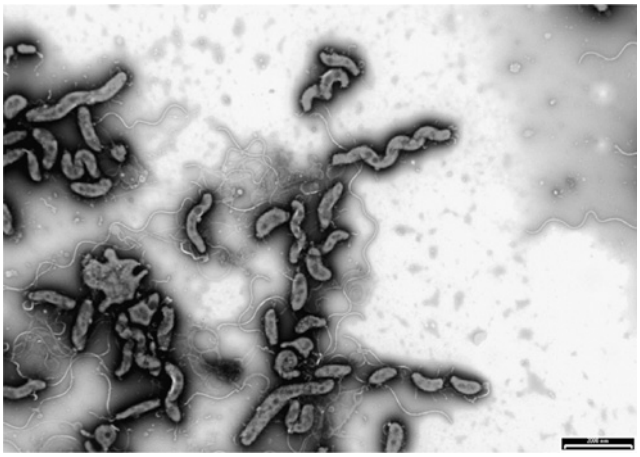

Figure 7B.2.1 Cells of *Bdellovibrio bacteriovorus* HIK3 (a host-independent derivative of strain 109J) grown in PY broth. A wide variety of cell morphology can be observed within one strain; the smaller single cells seen resemble those in predatory cultures.

such strains are grown. Basic Protocol 6 gives a robust protocol for reliable storage of *Bdellovibrio.* Basic Protocol 7 is a novel assay for observing phenotypic differences between strains.

CAUTION: Bdellovibrio bacteriovorus and *E. coli* are Biosafety Level 1 (BSL-1) organisms. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Some prey cells are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

BASIC PROTOCOL 1

ROUTINE CULTURING OF PREY LYSATES

Wild-type *Bdellovibrio* are prey-dependent and therefore need to be grown on suitable Gram-negative prey cells such as *E. coli* K12 (refer to *APPENDIX 4A* for methods of culturing *E. coli* K12). Prey lysates are prey cells suspended in buffer with *Bdellovibrio* so that the prey are the predominant nutrient source for the predator. The HEPES buffer used here is supplemented with $CaCl_2$ (Seidler and Starr, 1969b) to increase the efficiency of prey lysis. A standard prey lysate culture described here can be scaled down to a mini lysate of 2 ml or scaled up to several liters (Koval and Hynes, 1991). Although the experimenter does not need special protection from class I containment organisms such as *E. coli*, it is preferable to conduct culturing of *Bdellovibrio* in a class II containment hood to minimize the risk of contamination by other microorganisms.

Materials

Prey cells (e.g., *E. coli* K12; refer to *APPENDIX 4A* for methods of culturing *E. coli* K12)
1% YT agar plates (see recipe for YT agar)
YT broth (see recipe)
HEPES buffer supplemented with $CaCl_2$ (see recipe)
Overnight culture of prey lysate

37°C static incubator
250-ml conical flasks or sterile universal tubes (Sterilin)
29° and 37°C shaking incubator
Phase contrast microscope with a 100× oil immersion lens

1. Streak prey cells out to produce single colonies on 1% YT agar plates and incubate inverted overnight at 37°C.

   These plates can be stored up to 2 weeks at 4°C.

2. Inoculate an appropriate amount (e.g., 50 ml) of YT broth with a single colony in a vessel at least five times the volume of the broth and incubate overnight in a 37°C incubator with shaking at 200 rpm for aeration.

3. Aseptically add the following to a sterile 250-ml conical flask:

   50 ml sterile HEPES buffer supplemented with $CaCl_2$
   3 ml of overnight prey culture
   1 ml of overnight prey lysate (from previous prey lysate)

   Incubate overnight at 29°C with shaking at 200 rpm.

   The prey should be grown as described here, or rescued from frozen stocks as described in Basic Protocol 2.

4. Check by phase contrast microscopy after 24 hr, preferably with a 100× oil immersion lens, for the presence of bdellovibrios (usually highly motile) and the absence of prey cells and bdelloplasts (indicating successful lysis of prey).

   Prey lysates of this nature retain Bdellovibrio viability for up to 2 weeks if stored at 4°C.

REVIVING *BDELLOVIBRIO* FROM FROZEN STOCKS

BASIC PROTOCOL 2

Bdellovibrio cells can be stored routinely for several years at −80°C (see Basic Protocol 6), but unlike some other bacteria, cannot normally be streaked onto media from such stocks (unless they are HI) as wild-type *Bdellovibrio* are host- (or prey-) dependent. This protocol uses the overlay technique to grow up thawed *Bdellovibrio* on prey cells.

Materials

Prey cells (e.g., *E. coli* K12; refer to *APPENDIX 4A* for methods of culturing *E. coli* K12)
1% YT agar plates (see recipe for YT broth)
YT broth (see recipe)
1% YPSC bottom agar (see recipe for YPSC broth)
25 g/liter $CaCl_2 \cdot 2H_2O$ solution, sterile
0.6% YPSC top agar (see recipe for YPSC broth)
Bdellovibrio frozen stock (made from a previous prey lysate, see Basic Protocol 6)
HEPES buffer supplemented with $CaCl_2$ (see recipe)

29° and 37°C static incubators
37°C shaking incubator
Test tubes
55°C water bath
Phase contrast microscope

Grow E. coli prey cells

1. Streak *E. coli* prey cells onto 1% YT agar plates and incubate inverted overnight at 37°C to produce single colonies.

   These plates can be stored up to 2 weeks at 4°C.

2. Inoculate an appropriate amount (e.g., 50 ml) of YT broth with a single colony in a vessel at least five times the volume of the broth and incubate overnight at 37°C with shaking at 200 rpm for aeration.

   Adjust methods for alternative prey, for example, growth can be in different media such as higher salt media and at different temperatures with many prey preferring 30°C.

Prepare YPSC overlays

3. Pour 1% YPSC agar plates, adding $CaCl_2 \cdot 2H_2O$ to a final concentration of 0.25 g/liter from a 100× stock of 25g/liter after melting the agar.

   The $CaCl_2$ would otherwise precipitate during autoclaving.

4. To a test tube, add 100 µl of the overnight culture of prey cells and 5 ml of 0.6% YPSC top agar incubated at 55°C, with $CaCl_2$ added. Quickly pour this mixture onto the bottom plate and leave undisturbed for 5 min at room temperature to set.

   The agar has to be at 55°C to ensure that it is completely molten so that the prey can be adequately mixed in a room temperature tube before pouring. Therefore, the prey must be able to withstand addition to molten agar at 55°C (as is the case for E. coli prey).

5. Spot 50 µl of a *Bdellovibrio* frozen stock onto the center of this double layer plate and incubate upright overnight at 29°C until a lawn of prey cells is observed.

6. Turn plate upside down and incubate for an additional 1 to 5 days at 29°C until clearing of the prey lawn where the *Bdellovibrio* were spotted is observed.

   Higher concentrations cause quicker clearing of the prey lawn.

7. Pick up some of the area of clearing from the soft agar layer using a 1000-µl pipet set to 250 µl. Aseptically transfer this into a mini lysate containing 2 ml HEPES buffer supplemented with $CaCl_2$ and 150 µl of overnight prey culture.

8. Incubate 1 to 2 days at 29°C with shaking at 200 rpm until lysate appears clear and phase contrast microscopy reveals the presence of bdellovibrios and lysis of prey cells.

   If the Bdellovibrio are pipetted from an area of plate that is thoroughly cleared, then the mini lysate should clear after an overnight (16 hr) incubation. If the plate has only partial clearing, then the lysate may take longer to clear.

   YPSC overlay plates can be stored up to 2 weeks at room temperature and the Bdellovibrio within remain viable, more so than if stored at 4°C.

BASIC PROTOCOL 3

GROWTH OF *BDELLOVIBRIO* AS SINGLE PLAQUES FOR ENUMERATION

For any given *Bdellovibrio*-containing sample, a range of serial dilutions can be used to produce a plate with a countable yet significant number of plaques. This technique is an invaluable way of calculating the number of live *Bdellovibrio* cells being added to an experiment. It acts as an alternative to colony-counting for non-predatory bacteria. Due to the small cell dimensions of *Bdellovibrio*, optical density readings cannot reliably be used to determine the total number of *Bdellovibrio* cells in a sample.

Materials

Prey cells (e.g., *E. coli* K12; refer to *APPENDIX 4A* for methods of culturing *E. coli* K12)
1% YT agar plates (see recipe for YT broth)
YT broth (see recipe)
1% YPSC bottom agar (see recipe for YPSC broth)
25 g/liter $CaCl_2 \cdot 2H_2O$ solution, sterile
HEPES buffer supplemented with $CaCl_2$ (see recipe)

Sample to be enumerated
0.6% YPSC top agar (see recipe for YPSC broth)

29° and 37°C static incubators
37°C shaking incubator
Test tubes
55°C water bath

Grow E. coli prey cells

1. Streak prey cells out onto 1% YT agar plates and incubate inverted overnight at 37°C to produce single colonies.

   These plates can be stored up to 2 weeks at 4°C.

2. Inoculate an appropriate amount (e.g., 50 ml) of YT broth with a single colony in a vessel at least five times the volume of the broth and incubate overnight at 37°C with shaking at 200 rpm for aeration.

Prepare YPSC overlays

3. Pour 1% YPSC agar plates, adding $CaCl_2.2H_2O$ to a final concentration of 0.25 g/liter, after melting the agar, from a 100× stock of 25 g/liter.

   The $CaCl_2$ would otherwise precipitate during autoclaving if added prior to autoclaving.

4. Prepare ten-fold serial dilutions in HEPES buffer supplemented with $CaCl_2$ of the sample to be enumerated.

5. Add 100 µl of these serial dilutions to separate test tubes with 100 µl of an overnight prey culture and then add 5 ml of 0.6% top agar YPSC incubated at 55°C, with $CaCl_2$ added to a final concentration of 0.25 g/liter. Quickly pour this mixture onto the bottom plate and leave undisturbed for 5 min at room temperature to set.

6. Incubate plates inverted 3 to 10 days at 29°C until clear plaques appear in the lawn of prey cells.

   Typically, useful dilutions are 10^{-4} to 10^{-6} for a prey lysate that has completely cleared.

GENERATION OF HOST-INDEPENDENT *BDELLOVIBRIO*

BASIC PROTOCOL 4

Host-dependent *Bdellovibrio* yield host-independent (HI) colonies at a frequency of $\sim 10^{-6}$ to 10^{-8} (pfu/cfu) upon plating on PY agar. While many HI strains retain their capacity for intraperiplasmic growth, it has been noted that continued passage of HI strains in the absence of prey cells tends to lead to a loss of their predatory ability (Seidler and Starr, 1969a). Therefore, excessive passaging of HI strains in the absence of prey should be avoided by returning to frozen stocks of strains frequently. This protocol uses the small size of predatory *Bdellovibrio* to differentially filter them from any remaining larger prey cells; these are then cultured to derive HI colonies. HI cultures exhibit diverse cellular morphologies (Fig. 7B.2.1).

Materials

Overnight prey lysate of host-dependent *Bdellovibrio*
PY agar (see recipe for PY broth)
PY broth (see recipe)

Phase contrast microscope
Petri dishes
0.45-µm syringe filters
10-ml syringe
15-ml tubes (Falcon)

Benchtop centrifuge
Plate spreaders
29°C static incubator
Bijou universal tubes (7-ml screw-top plastic tubes; Sterilin cat. no. 129B)
29°C shaking incubator

1. Grow a 50-ml prey lysate overnight (as in Basic Protocol 1) and check by phase contrast microscopy for the presence of *Bdellovibrio* and complete prey lysis, i.e., the absence of prey cells and bdelloplasts.
2. Pour plates with ~40 ml of 1% PY agar per petri dish and leave to set 10 min at room temperature.
3. Filter 10 ml of prey lysate through a 0.45-μm syringe filter attached to a 10-ml syringe into a 15-ml tube.
4. Balance the tubes and centrifuge 10 min at ~5000 × *g*, room temperature, in a benchtop centrifuge.
5. Pour off the supernatant and resuspend pellet in 100 μl of PY broth.
6. Spread entire 100 μl onto a PY plate and dry briefly by standing at room temperature with lids slightly off (preferably in a laminar flow cabinet). Incubate inverted 3 to 10 days at 29°C.
7. Check on the following day for *E. coli* growth (imperfections in filters can sometimes allow a few cells through).

   Tiny yellow colonies will start to appear, which grow slowly over several days.

8. When colonies are >1 mm, pick into 0.5 ml PY broth in a bijou universal tube and incubate at 29°C with shaking at 200 rpm.

   Growth in broth can be achieved by scraping an area containing many small colonies but such a culture would not be clonal and may have a number of plaquing (and other) phenotypes.

9. In 1 to 3 days, as the culture grows ($OD_{600} > 0.5$), add more PY broth, doubling the volume, then incubate overnight at 29°C with shaking at 200 rpm.

   This process can be continued until the required culture volume has been reached for an experiment. The culture should be incubated in a vessel at least five times the volume of the liquid.

   PY plates of HI cultures can be stored for up to 2 weeks at room temperature.

BASIC PROTOCOL 5

ROUTINE MAINTENANCE OF HI CULTURES

Unlike host-dependent strains, HI strains (Fig. 7B.2.1) can be maintained in rich broth or on plates similar to many other bacteria such as *E. coli,* although growth is relatively slow so incubation times are extended and cultures must gradually be increased in size by sequential addition of growth medium.

Materials

PY agar (see recipe for PY broth)
Frozen stock of HI strain
PY broth (see recipe)

Petri dishes
29°C static incubator
Bijou universal tubes

Conical flasks
29°C shaking incubator

Grow from frozen stocks

1. Pour a petri plate with ~50 ml 1% PY agar and leave to set 10 min at room temperature.
2. Pipet 50 µl of a thawed (5 min at room temperature) stock of HI strain onto the plate.
3. Streak repeatedly across the plate successively to obtain single colonies.
4. Incubate upright overnight at 29°C.
5. Incubate inverted for an additional 2 to 8 days at 29°C.

   A large area of yellow growth will be observed within 2 to 4 days, and later single colonies will be observed.

Grow in broth

6. When colonies are >1 mm, pick and place into 0.5 ml PY broth in a bijou universal tube and incubate at 29°C with shaking at 200 rpm.
7. In 1 to 3 days as the culture grows ($OD_{600} > 0.5$), add more PY medium, doubling the volume, then incubate overnight at 29°C with shaking at 200 rpm.

   This process can be continued until the required volume has been reached for an experiment. The culture should be incubated in a vessel at least five times the volume of the liquid.

8. At any point, add 1 ml of culture sample to a further 1 ml PY broth to increase the number of samples.

PREPARING FROZEN GLYCEROL STOCKS FOR LONG-TERM STORAGE

BASIC PROTOCOL 6

This allows storage for several years.

Materials

Culture of host-dependent *Bdellovibrio*
Culture of host-independent *Bdellovibrio*
Sterile 80% glycerol
Liquid nitrogen in suitable Dewar container

Phase contrast microscope
1.5-ml plastic freezer tubes (Nunc)
Perforated ladle/slotted spoon
−80°C freezer containing freezer box

1. Check culture of host-dependent *Bdellovibrio* by phase contrast microscopy for presence of bdellovibrios and absence of prey cells and bdelloplasts. Check host-independent *Bdellovibrio* culture for presence of bdellovibrios and absence of contaminants.
2. Pipet 700 µl of culture into 1.5-ml freezer tubes.
3. Add 150 µl of 80% glycerol and cap tube. Mix well.
4. Drop into liquid nitrogen and leave until frozen (~1 min). Remove tubes with perforated ladle or slotted spoon.
5. Place in a precooled freezer box in a −80°C freezer.

The stock can withstand several freeze-thaw cycles, but it is advisable to have many stocks and not to access one stock more than three times to ensure growth.

It is important to prepare many frozen stocks of recently isolated HI strains, as upon repeated subculturing they have been demonstrated to lose their ability to infect prey cells.

BASIC PROTOCOL 7

BDELLOVIBRIO PREDATION EFFICIENCY ASSAY ON LUMINESCENT PREY

A relationship was established between light emitted from a population of luminescent *E. coli* and predation of that population (Lambert et al., 2003). This assay was optimized to show predation efficiency of various strains by monitoring the decay in light emission over time (Fig. 7B.2.2).

Materials

Bdellovibrio mutant strains to be tested (usually kanamycin resistant due to insertional inactivation of a gene under study)
109JK control strain
Luminescent *E. coli* S17-1 (pCL100, kanamycin resistant) (Lambert et al., 2003)
E. coli S17-1 (pZMR100) (Rogers et al., 1986)
YT agar (see recipe for YT broth)
YPSC agar (see recipes for both top and bottom agar)
HEPES buffer supplemented with $CaCl_2$ (see recipe)
YT broth (see recipe)
PY broth (see recipe)
50 mg/ml kanamycin sulfate

Petri dishes
55°C and boiling water baths
1.5-ml microcentrifuge tubes
Multichannel pipettor
96-well microtiter plates for luminometer
Photoluminometer capable of reading a kinetic assay of luminescence over time in a 96-well microtiter well and with temperature control and shaking (e.g., Anthos Lucy, Perkin-Elmer Instruments Victor, BMG Labtechnology Fluostar Optima, Tecan Genios)

Grow bacterial strains

1. Grow *Bdellovibrio* mutant strains to be tested and the 109JK control strain for 3 days on luminescent *E. coli* S17-1 (pCL100) (Lambert et al., 2003), subculturing at the same time each day. On the final day, also grow *E. coli* S17-1 (pZMR100) (Rogers et al., 1986) to use to enumerate the *Bdellovibrio.*

Prepare plates and bacteria

2. Pour three 1% YT agar plates and three YPSC bottom agar plates supplemented with 50 μg/ml kanamycin sulfate per *Bdellovibrio* strain tested and melt the appropriate amount (5 ml per plate) of YPSC top agar, maintain melted agar at 55°C.

3. Prepare controls by dispensing 1 ml of each *Bdellovibrio* strain to be tested into 1.5-ml microcentrifuge tubes and boil for 5 min, then leave to cool to room temperature for at least 10 min.

4. Prepare luminescent prey by dispensing 25 ml HEPES supplemented with $CaCl_2$, 25 ml PY broth, and 2 ml of an overnight culture of *E. coli* S17-1 (pCL100) host cells into a petri dish supplemented with 50 μg/ml kanamycin sulfate.

5. Using a multichannel pipettor, dispense 200 μl of this mixture into each well of a 96-well plate suitable for reading in a luminometer.

6. Dispense 1, 2, 4, 8, 16, 32, and 64 μl of *Bdellovibrio* strains to be tested into wells of the plate, avoiding wells at the extremities of the plate as these are prone to evaporation.

7. Make the volume of all the above wells up to 264 μl with heat-killed control *Bdellovibrio* of the appropriate strain, e.g., 32 μl of heat-killed cells added to the well containing 32 μl of live *Bdellovibrio*. Include one well with just 64 μl of heat-killed cells with no live *Bdellovibrio*.

8. Incubate plate at 30°C in a photoluminometer with shaking at 200 rpm, set to read luminescence at 30-min intervals for 24 hr.

Further experiments should also be carried out with a 1:10 dilution of the Bdellovibrio and 1:10 dilution of heat-killed Bdellovibrio.

Enumerate bacteria

9. Immediately after setting the plate for incubation, enumerate samples of each of the *Bdellovibrio* strains used in the assay plate by plating at dilutions between 10^{-4} and 10^{-6} onto YPSC overlays, incubate 3 to 10 days at 30°C and then count plaques.

10. Enumerate the *E. coli* S17-1 (pCL100) in the PY broth and HEPES supplemented with $CaCl_2$ mix by plating dilutions between 10^{-4} and 10^{-6} onto YT plates and incubate overnight at 37°C.

Analyze data

11. Analyze data by plotting $\log_{10}$ luminescence against time.

From this plot, the time taken to reduce to half of the maximum $\log_{10}$ luminescence value for each amount of Bdellovibrio is calculated and this is plotted against the amount of Bdellovibrio initially added as calculated by the plaque overlay enumeration (Fig. 7B.2.3).

If a mutant strain is reduced in its efficiency of predation relative to the 109JK control, then this should be shown on this plot as the line of best fit is shifted (Fig. 7B.2.3).

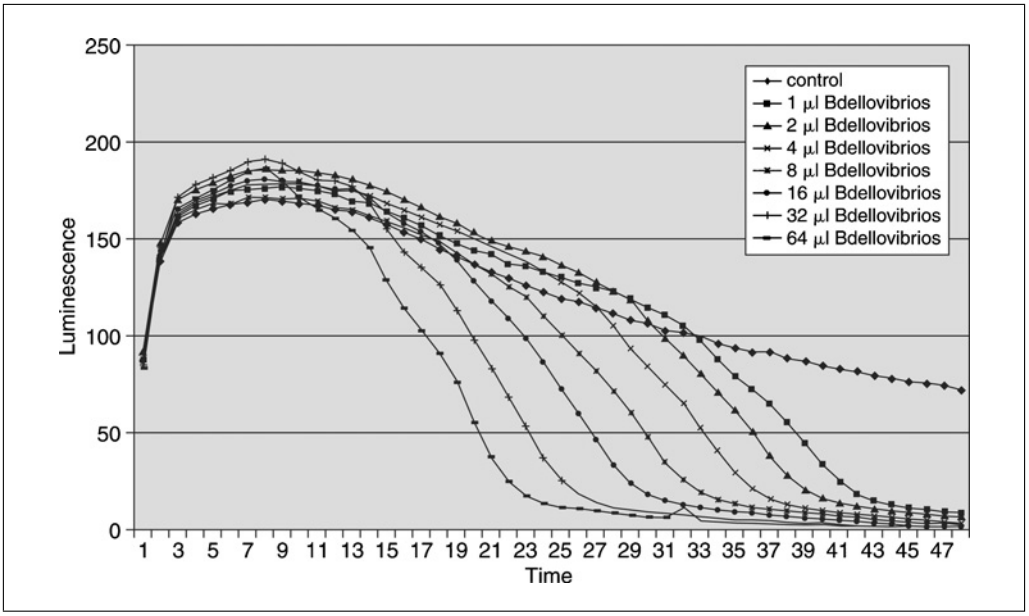

Figure 7B.2.2 Plot showing luminescence (in relative luminescent units) against time (in steps of 30 min; i.e., 1 = 30 min, 2 = 60 min, etc.) for an infection of *Bdellovibrio* on luminescent *E. coli*. After an initial rise in luminescence, more bdellovibrios added results in faster diminution of light. The suspension added contains 1×10^5 viable bdellovibrios as determined by plaque enumeration.

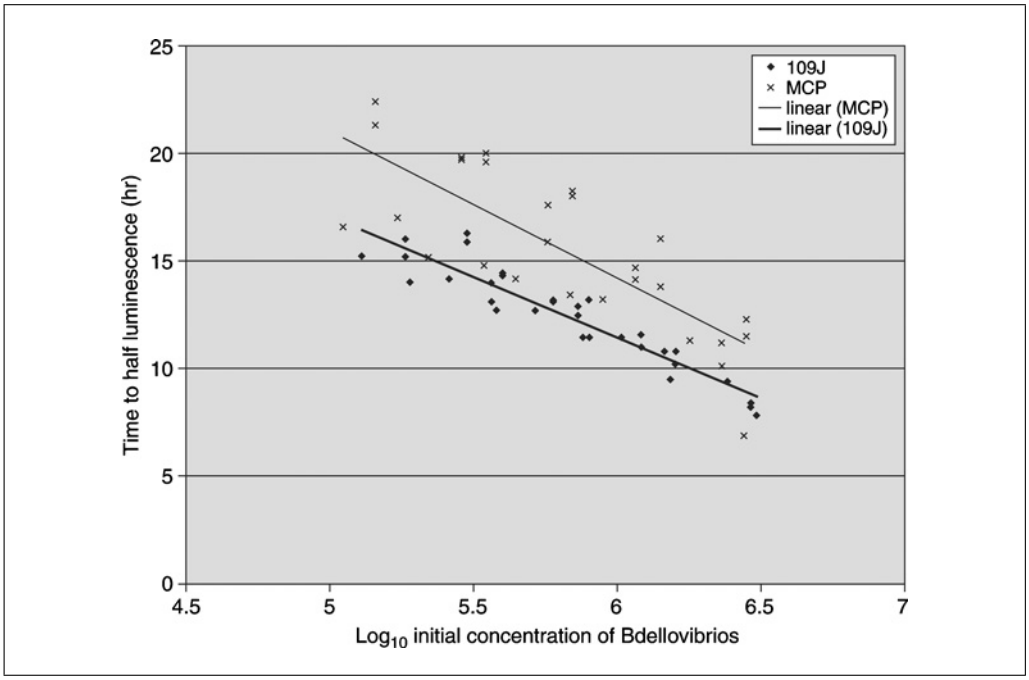

Figure 7B.2.3 Plot showing $\log_{10}$ numbers of bdellovibrios initially added against time to half $\log_{10}$ luminescence values comparing two strains; a wild type 109J and a mutant MCP strain (Lambert et al., 2003).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

HEPES buffer supplemented with $CaCl_2$

5.94 g/liter HEPES free acid
0.284 g/liter calcium chloride ($CaCl_2$) dihydrate
Adjust to pH 7.6 using 2 M NaOH
Sterilize by autoclaving
Store up to 6 months at room temperature

PY broth

10 g/liter peptone
3 g/liter yeast extract
Adjust to pH 6.8 using 2 M NaOH
Sterilize by autoclaving
Store up to 6 months at room temperature
For PY agar, add 10g/liter agar

YPSC broth

0.125 g/liter magnesium sulfate
0.25 g/liter sodium acetate
0.5 g/liter bacto peptone
0.5 g/liter yeast extract
Adjust to pH 7.6 using 2 M NaOH
Sterilize by autoclaving
Adjust to 0.25 g/liter $CaCl_2$ dihydrate using 25 g/liter stock after autoclaving
Store up to 6 months at room temperature
For YPSC bottom agar, add 10 g/liter agar
For YPSC top agar, add 6 g/liter agar

YT broth

5 g/liter sodium chloride
5 g/liter peptone
8 g/liter tryptone
Adjust to pH 7.5 using 2 M NaOH
Sterilize by autoclaving
Store up to 6 months at room temperature
For YT agar, add 10 g/liter agar

COMMENTARY

Background Information

Bdellovibrio bacteriovorus is a highly motile delta-proteobacterium that preys on other Gram-negative bacteria (Stolp and Starr, 1963). *Bdellovibrio* are ubiquitous in nature, having been discovered in a wide variety of environments that include both aquatic and terrestrial habitats as well as mammalian intestines (Varon and Shilo, 1980). Their prey includes plant, animal, and human pathogens (Martin, 2002). Thus, studying the molecular mechanisms of *Bdellovibrio* predation offers cues for the design of antibacterial agents.

Critical Parameters

One important consideration when setting up experiments with *Bdellovibrio* is the physiological state of the prey lysate. As *Bdellovibrio* cycle through attack phase and intraperiplasmic growth, and then release into attack phase again, they undergo many different developmental and metabolic states and this could have a drastic effect on the outcome of any given experiment. Similarly, newly released attack phase cells are very different from cells from older lysates (more rapidly motile and often smaller). To avoid fluctuations between experiments, it is essential to subculture at exactly specified times over a period of several days (e.g., every 24 hr for 3 days). It may also be vital to subculture for several rounds on a new prey, or under new conditions, before using the *Bdellovibrio* progeny for an experiment.

Host-independent *Bdellovibrio* appear to have an as yet unquantified reliance upon cell density, for example, small colonies seem to appear on plates mainly in high density and a relatively high inoculum per volume is necessary for consistent growth.

For culturing, all media and solutions should be sterilized by autoclaving and it is important to work with good aseptic technique. If possible, work should be carried out in a Class II biohazard fume hood to minimize the risk of contamination, although this is not essential.

Troubleshooting

Table 7B.2.1 presents commonly encountered difficulties in maintaining *Bdellovibrio*, as well as the potential causes and possible solutions.

Anticipated Results

For the culture of host-dependent *Bdellovibrio*, overlay plates should develop a large region of clearing where the *Bdellovibrio* has been spotted onto a cloudy lawn of prey cells, or individual plaques where dilutions have been used. These plaques start out as small and cloudy (2 to 4 days) and become clearer and larger over several days (3 to 10 days) incubation.

Culture of *Bdellovibrio* in prey lysates should result in the clearing of the cloudy prey lysate over 1 to 2 days and phase contrast microscopy should reveal prey cells turning into bdelloplasts (rounded prey cells containing *Bdellovibrio* within), then lysing to release small, highly motile attack phase *Bdellovibrio* cells.

Culture and generation of HI *Bdellovibrio* should produce initially (2 to 4 days) very small, yellow-orange colonies on PY plates, which grow slowly over time (3 to 10 days). Similarly, growth in broth produces yellow-orange coloration. Phase contrast microscopy should show, in addition to some small, motile attack phase *Bdellovibrio*, a very heterogeneous mix of cell morphology and size. A much smaller proportion of HI cells tend to be motile and it is not unusual to see no swimming cells at all (Fig. 7B.2.1).

Data from the predation assay on luminescent prey should show light emissions initially increasing as the prey cells grow in the nutrients, followed by the levels slowly lowering in the control with no live *Bdellovibrio*. For each well containing *Bdellovibrio*, the light levels

Table 7B.2.1 Troubleshooting Laboratory Maintenance of *Bdellovibrio*

Problem	Cause	Solution
Contamination of prey lysate	Environmental/skin bacteria	Ensure good sterile technique/work in a Class II containment hood
No clearing of overlay plate after addition of frozen stock and incubation	Frozen stock has been through too many freeze/thaw cycles	Use fresh frozen stocks with fewer than four freeze-thaw cycles
Incomplete lawn formed on YPSC overlay	Not enough viable prey cells added	Use only fresh overnight cultures and ensure correct antibiotic included
Lumpy uneven overlay	Top agar not set properly	Ensure top agar is at 55°C and pour quickly. Do not move the plate for 5 min after pouring
No lawn formed on overlay	Top agar too hot	Incubate top agar at 55°C
No plaques forming/few and cloudy plaques	$CaCl_2$ missing	Remember to add $CaCl_2$
Confluent lysis of overlay	*Bdellovibrio* not diluted enough	Use a range of dilutions to ensure an overlay with discrete plaques
HI starter broth not growing	Cells not in high enough density	Use one large (>2 mm) colony as primary inoculum in a small volume (0.5 ml) of medium, then scale up two fold, five fold, and so on
Prey cells growing on PY HI plates	Filtration of prey lysate has failed (0.45-μm filters are not perfect)	Double filter if necessary
Prey lysate not cleared after overnight incubation	Too few viable *Bdellovibrio* added	Incubate further or only use fresh lysate/large inoculum
Luminescent prey cells not emitting light	Unknown	Revert to frozen stocks

lower more quickly in a manner dependent on the initial number of *Bdellovibrio* added (Fig. 7B.2.2).

For a plot of time to half of maximum luminescence against initial amounts of added *Bdellovibrio*, the line of best fit of different strains can be compared and a mutant with lowered predation efficiency should have a noticeably different line of best fit (Fig. 7B.2.3).

Time Considerations

For each protocol (except those for HI *Bdellovibrio*), advance planning is required to grow prey cells the day before. Time required for all of the protocols is entirely reliant upon the somewhat variable growth rate of the plaques/colonies of *Bdellovibrio*. This tends to be within the range of 4 to 6 days, but can vary from 3 to 10 days.

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Growth and Laboratory Maintenance of *Campylobacter jejuni*

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ABSTRACT

Campylobacter jejuni is a fastidious organism, growing in microaerophilic conditions with a temperature range between 37° and 42°C. Multiple types of media can be used to cultivate it; however, Mueller Hinton broth and agar support the best *C. jejuni* growth. Optimum atmosphere for *C. jejuni* is 85% N_2, 10% CO_2, and 5% O_2. *Curr. Protoc. Microbiol.* 10:8A.1.1-8A.1.7. © 2008 by John Wiley & Sons, Inc.

Keywords: campylobacteriosis • microaerophilic growth • Mueller Hinton

INTRODUCTION

Campylobacter jejuni is one of the major causes of bacterial gastroenteritis worldwide and is primarily acquired through the ingestion of contaminated poultry products. Research on *C. jejuni* has been greatly impaired due to poor culturing techniques and genetic tools. *C. jejuni* was successfully isolated in the early 1970s, utilizing specialty agar and microaerophilic atmospheric conditions. Since its isolation, it has been connected with bacterial gastroenteritis as well as the neurological disorders Guillen-Barré Syndrome and Fisher Syndrome in humans. It is now one of the leading causes of gastroenteritis in both the developed and developing worlds. (Young et al., 2007).

C. jejuni is a fastidious organism, requiring modified atmospheric conditions, a longer growing time, a narrow temperature range, and specialized media when compared to such bacteria as *E. coli*. All these factors must be taken into consideration when working with *C. jejuni.*

STRATEGIC PLANNING

Atmosphere

Campylobacter jejuni requires microaerophilic conditions for growth. Optimum growth is maintained in a tri-gas incubator (e.g., Thermo Forma Series II Water-Jacketed CO_2 Incubator), with 85% N_2, 10% CO_2, and 5% O_2. If a tri-gas incubator is unavailable, specific gas packs (i.e., BBL Campy Pak Plus) or formulated compressed air can be applied with sealable gas chambers or plastic bags, respectively. Gas packs are expensive, and growth in plastic bags is suboptimal compared to that obtained by using a tri-gas incubator. Notes concerning the differences are in the specific protocols outlined. *C. jejuni* is also able to grow in anaerobic conditions.

Temperature

C. jejuni has a narrow temperature range of growth, with optimum growth occurring between 37° and 42°C. The bacteria are able to survive at 7°C and perform vital cellular processes such as protein synthesis; however, growth and recovery at low temperatures

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is difficult (Hazeleger et al., 1998). Significant loss of viable bacteria occurs when the bacteria are left at room temperature and atmosphere for only 10 min. Therefore, when working with *C. jejuni*, the amount of time the bacteria are out of incubators and microaerophilic atmosphere must be limited.

Media

Many types of media have been used to culture *C. jejuni*. Mueller-Hinton medium, blood agar, Columbia blood agar, and BBL medium are commonly used. However, Mueller Hinton (MH) has the highest recovery rate and is recommended in this unit (Ng et al., 1985). *Campylobacter* defined medium was developed by Leach and colleagues (Leach, 1997). Although a minimal medium has not been developed that supports *C. jejuni* growth, the defined medium can be used to adjust components based on experimental needs.

Growth Conditions

C. jejuni can grow in both static and shaking broth cultures. The stringent growth conditions do not lend themselves well to a shaking apparatus. Some tri-gas incubators are equipped to have an internal shaker; however, most are not, and the gas packs in bags or jars do not always allow shaking conditions. *C. jejuni* grown in shaking cultures grow faster than those in static culture. Aggregates form when *C. jejuni* is grown in static broth (Joshua et al., 2006). This aggregation phenotype has been studied extensively, and several genes involved in this have been identified. Based on availability, the protocols suggested in this unit use static growth as the standard.

Strain Selection

Several isolates of *C. jejuni* have been used in previous research (Table 8A.1.1). Strain selection is important, and depends upon the proposed research. Strains have been isolated from chicken, human, and environmental sources. Phenotypic variation among strains from varying sources has been observed for many traits including invasion, cytolethal distending toxin (cdt) production, chick colonization, lipooligosaccharide (LOS)

Table 8A.1.1 Most Commonly Used *C. jejuni* Strains[a]

	Sources	Sequences	Notes
260.94	Human	N	GBS[b]-associated; O:41 serotype
480	Human	N	Electrocompetent; highly invasive
811681116	Human	N	Lab strain
11168	Human	Y	Gaynor (2004)
81-176	Human	Y	Contains pVir, highly virulent (Bacon, 2000)
BTI	Chicken	N	—
CG8245	Human	N	—
F38011	Human	N	—
M1	Environment	N	—
RM1221	Human	Y	—

[a]The most commonly used isolates of *C. jejuni* are listed here, including their source of isolation and whether their genome has been sequenced to date. Strains can be acquired from individual labs working on the particular isolate.
[b]GBS, Guillain-Barré Syndrome.

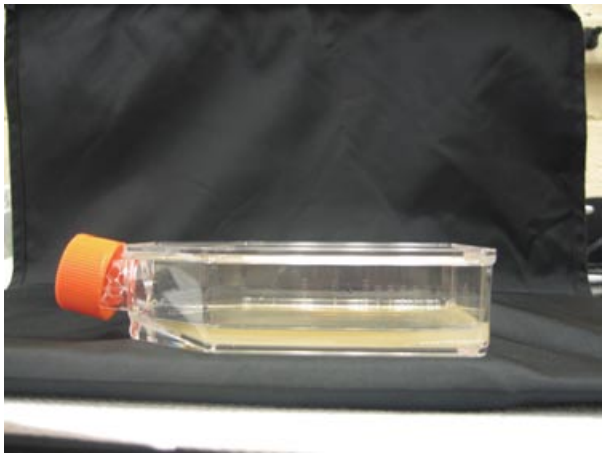

Figure 8A.1.1 Preparation of biphasic medium. In a 75-cm^2 tissue culture flask, 20 ml of MH agar were poured and solidified, followed by 20 ml of MH broth.

production, and natural transformation. The ability of *C. jejuni* to undergo natural transformation has led to a high degree of horizontal gene transfer between *C. jejuni* strains, leading to high genetic diversity among *C. jejuni* strains. *C. jejuni* strains 81-176 and 11168 are well characterized strains, and the most commonly used in pathogenesis studies.

Growth Curves

Mueller Hinton agar is the recommended medium for standard growth curves of *C. jejuni*. Biphasic MH medium, prepared in tissue culture flasks (Fig. 8A.1.1), is the classic way to perform growth curves. The biphasic medium is made using 50% MH agar and 50% MH (poured after the agar solidifies). Note that MH agar should never be microwaved for *C. jejuni* agar plates and biphasic media.

Biphasic medium is recommended for growing conditions outside of a tri-gas incubator, such as in bags or sealed containers. If a tri-gas incubator is used, growth curves can be performed in MH broth alone. However, no volume below 1 ml should be used, due to poor recovery and inconsistent growth.

Each time point should be an individual sample. Static growth of *C. jejuni* results in aggregative bacterial raft formation, and disruption of this growth has led to variable and inconsistent growth. Therefore, each time-point should be separate and undisturbed. Furthermore, each time-point should be done in triplicate to obtain a statistically significant average. Growth curves are normally performed for 36 or 48 hr.

The inoculum for a growth curve is 1×10^6 cfu/ml. From 16- to 18-hr plates, *C. jejuni* should be resuspended to an OD_{600} of 0.4. Dilute this suspension 1:10. Inoculate 80 µl of this dilution into 20 ml of medium. A standard growth curve, measured in cfu/ml over 48 hr, is shown in Figure 8A.1.2.

CAUTION: Campylobacter jejuni is a Biosafety Level 2 (BSL-2) pathogen. Follow appropriate guidelines and regulations for the handling of pathogenic microorganisms. Proper hand washing is essential, as *C. jejuni* has been shown to cause gastroenteritis at a small dose (10 to 100 organisms). See *UNIT 1A.1* and other pertinent resources for more information.

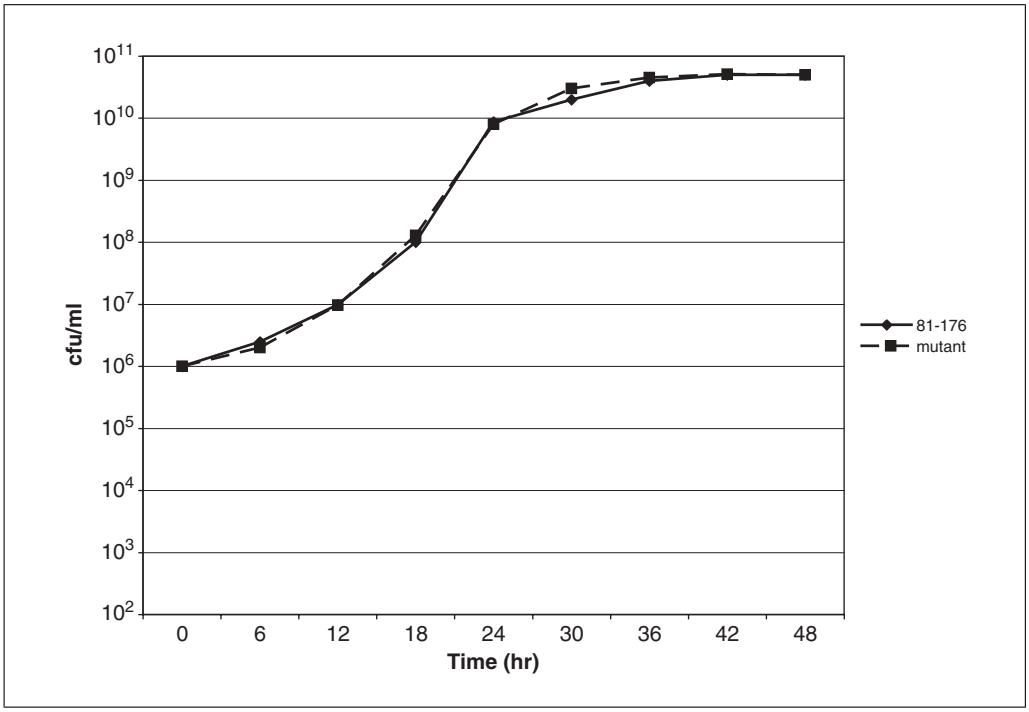

Figure 8A.1.2 Growth curve in biphasic medium of two different isolates of *C. jejuni* 81-176.

BASIC PROTOCOL 1

GROWTH OF *C. JEJUNI* FROM A FROZEN STOCK

C. jejuni is ill equipped for adjusting to changing environments in the lab, especially after being taken out of the freezer. The protocol below supports the best usable *C. jejuni* growth from frozen stocks. Time for growth depends on atmospheric conditions. The protocol outlined below uses Mueller Hinton (MH) broth or MH agar containing 10 μg/ml trimethoprim (TMP) for growing strain 81-176 in a tri-gas incubator. Selective antibiotics can be added to the medium; concentrations commonly used for culturing of and cloning in *C. jejuni* are listed in Table 8A.1.2.

Materials

C. jejuni frozen stock (Basic Protocol 2)
100 × 15–mm Mueller Hinton (MH) agar plates (BD Biosciences, cat. no. 22520; plates are poured in lab) containing 10 μg/ml trimethoprim (antibiotics added in lab)
Mueller Hinton (MH) broth (BD Biosciences, cat. no. 275730) containing 10 μg/ml trimethoprim
Equipment for maintaining *Campylobacter*-specific microaerophilic atmosphere (see Strategic Planning)

Additional reagents and equipment for streaking bacteria (*APPENDIX 4A*)

1. From frozen stock (in MH broth plus 20% glycerol; see Basic Protocol 2), heavily streak out *C. jejuni* strain on 100 × 15–mm Mueller Hinton agar plates containing 10 μg/ml trimethoprim. Grow for 16 to 20 hr in microaerophilic conditions (see Strategic Planning).

   Growth in a tri-gas incubator allows more rapid growth, while bag plating may require up to 48 hr of growth. See Strategic Planning for additional discussion of atmospheric conditions required for growing C. jejuni.

   Streaking of bacteria on agar plates is described in APPENDIX 4A.

Table 8A.1.2 Concentrations of Antibiotics Used in *C. jejuni* Research

Antibiotic	Concentration
Kanamycin	50 μg/ml
Chloramphenicol	15-20 μg/ml
Streptomycin	100 μg/ml to 2 mg/ml
Trimethoprim	10 μg/ml
Cefoperazone	20 μg/ml
Nalidixic acid	30 μg/ml
Tetracycline	12.5 μg/ml

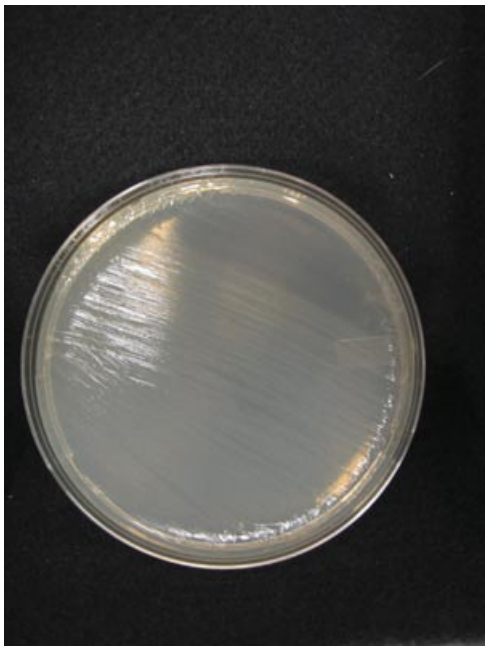

Figure 8A.1.3 Growth of *C. jejuni* 81-176 on an Mueller Hinton agar plate containing 10 μg/ml trimethoprim after 24 hr in a tri-gas incubator.

2. Restreak *C. jejuni* onto a new Mueller Hinton plate containing 10 μg/ml trimethoprim. Grow for 16 to 20 hr in microaerophilic conditions.

   Single colonies will not be observed until culture has been incubated >24 hr. Scrape up a large amount of cells from the lawn (see Figure 8A.1.3).

   After this incubation, C. jejuni is ready to be used.

   For single colonies, streak for isolated colonies on plates and grow for 48 hr or until individual colonies are observed.

3. *Optional:* If large amounts are needed, restreak a heavy inoculum onto several Mueller Hinton plates containing 10 μg/ml trimethoprim or inoculate into Mueller Hinton broth containing 10 μg/ml trimethoprim.

4. *Optional:* Passage *C. jejuni* an additional two to three times.

   Further passages from frozen stock are not recommended.

PRESERVATION OF *C. JEJUNI*

Stocks of *C. jejuni* should be kept in 20% glycerol stocks, stored at −80°C. Incubations can be performed in a tri-gas incubator or in the other recommended conditions at 37°C.

Materials

C. jejuni organisms (cannot be purchased from ATCC or similar sources; must be obtained from individual labs)
100 × 15–mm Mueller Hinton (MH) agar plates (BD Biosciences, cat. no. 22520; plates are poured in lab) containing 10 μg/ml trimethoprim (antibiotics added in lab)
Mueller Hinton (MH) broth (BD Biosciences, cat. no. 275730) containing 20% (v/v) glycerol

Sterile cotton swabs
2-ml cryotubes

1. Heavily streak out *C. jejuni* strain on an MH agar plate containing 10 μg/ml trimethoprim. Grow for 18 to 20 hr.

   After making a mutant (see UNIT 8A.2), streak for single colonies, which takes 36 to 48 hr. Pick a single colony and streak out. Finally, restreak it heavily onto a MH agar plate and carry out the following steps to preserve.

2. Swab bacteria using a sterile cotton swab from plate.

   C. jejuni will appear a pink-peach color (Fig. 8A.1.3).

3. Transfer a sufficient quantity of bacteria to 1.5 ml of Mueller Hinton broth containing 20% (v/v) glycerol in a 2-ml cryotube. Immediately freeze at –80°C.

   The frozen stock should contain a large amount of bacteria; roughly an OD_{600} of 1.5 or more

4. Never thaw frozen stocks. Instead, scrape off an ice chip from the frozen stock using a sterile implement and plate directly onto MH agar plates containing 10 μg/ml trimethoprim.

COMMENTARY

Background Information

Campylobacter jejuni is the causative agent of campylobacteriosis, a self-limiting gastroenteritis. It is one of the major causes of bacterial-associated gastroenteritis in the United States. *C. jejuni* has also been linked to Guillain-Barré Syndrome (GBS), an acute autoimmune neuropathy resulting in flaccid paralysis. Research on *C. jejuni* has been greatly impaired due to poor culturing techniques and genetic tools. *C. jejuni* was successfully isolated in the early 1970s, utilizing specialty agar and microaerophilic atmospheric conditions. Originally thought to be primarily a pathogen of animals, *C. jejuni* is now considered one of the main causes of foodborne bacterial gastroenteritis in humans. The original culturing techniques have expanded to include a number of types of media that *C. jejuni* can use. Although *C. jejuni* is a fastidious organism, its manipulation has become more standardized in recent years. A narrow temperature range, specific media, and specific atmospheric conditions must all be taken into account when growing *C. jejuni.*

Critical Parameters and Troubleshooting

If no growth is seen from the initial streaking of the frozen stock, or from subsequent re-streaks, the inoculum size or selective antibiotics may be the problem. Try to inoculate the plates with a larger amount of bacteria. A usual amount of frozen stock to be struck out is a chunk of frozen sample the size of a grain of rice. Due to the quantity of passages and relatively short bench life of *C. jejuni*, it is recommended that two sets of frozen stocks be kept with highly used strains: a backup stock and a frequently used stock. This will help diminish the possibility of contamination.

Anticipated Results

A large inoculum of frozen stock of *C. jejuni* 81-176 should grow after 18 to 24 hr. The growth will appear as a hazy lawn. The bacteria will have a pinkish-cream color when scraping the cells from the plate. After re-streaking from the overnight plate, growth of *C. jejuni* 81-176 should be observed after 18 to 20 hr. The growth will be hazy. To isolate single colonies, streak for isolation and allow growth for 48 hr.

Time Considerations

Following the protocol, large amounts of viable *C. jejuni* will be available after 36 to 48 hr in a tri-gas incubator. If gas jars are used, growth will be slower, with usable growth appearing after 40 to 48 hr, depending on the amount being streaked.

Freezing a newly constructed *C. jejuni* strain may take 4 to 5 days. Streak from a single colony. Growth will be visible after 48 hr. Heavily restreak the growth onto a new MH agar plate and incubate for another 24 to 36 hr.

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Genetic Manipulation of *Campylobacter jejuni*

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ABSTRACT

Molecular manipulation has been a limiting factor in *C. jejuni* research for many years. Recent advances in molecular techniques adapted for *C. jejuni* have furthered our understanding of the organism. This unit is dedicated to common molecular tools in bacterial research specifically tailored for *C. jejuni*. These include colony PCR, DNA isolation, and RNA isolation. The unit also reviews techniques for genetic manipulation, such as the use of plasmids, natural transformation, electroporation, conjugation, and transposition. In addition, a reporter system, the arylsulfatase assay, can be used to study gene expression. *Curr. Protoc. Microbiol.* 10:8A.2.1-8A.2.17. © 2008 by John Wiley & Sons, Inc.

Keywords: natural transformation • arylsulfatase • genomic DNA • RNA isolation

INTRODUCTION

Molecular biology has been a limiting factor in *C. jejuni* research. Indigenous plasmids are commonly used for cloning and expression vectors. *C. jejuni* is most efficiently transformed by plasmid DNA from its own species, thereby limiting the ease of genetic manipulation. Mutations are produced in *C. jejuni* using allelic replacement via suicide plasmids. Because these methods are described elsewhere (see Hendrixson et al., 2001, for a strategy that works well for *C. jejuni*), we will not discuss them in this unit, which will focus on moving plasmids into *C. jejuni* by conjugation (Basic Protocol 1), electroporation (Basic Protocol 2), and natural transformation (Basic Protocol 3). Numerous plasmids have been developed for *C. jejuni* research and may be used in lieu of specific plasmids described here. However, those discussed (see Table 8A.2.1) are commonly used and have proven successful in many studies. An inducible expression plasmid has yet to be developed for *C. jejuni*.

Table 8A.2.1 Commonly Used Plasmids

	Stable in *C. jejuni*	Source	Notes	Antibiotic resistance	Mobilizable with pRK212.1?	Source
pUC19	N	*E. coli*	Used for cloning	Ampicillin	N (electroporation only)	NEB
pRY108	Y	pILL550	Replicating plasmid	Kanamycin	Y	Yao et al. (1993)
pRY112	Y	pRY110, pWSK29	Replicating plasmid	Chloramphenicol	Y	Yao et al. (1993)
pECO102	Y	pRY112	Replicating plasmid	Chloramphenicol	Y	Wiesner et al. (2003)

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This unit also outlines several common molecular techniques for bacterial studies that have been adapted for *C. jejuni*. Common techniques include transposition (Basic Protocol 4), colony PCR (Basic Protocol 5), isolation of DNA (Basic Protocol 6) and RNA (Basic Protocol 7 and Alternate Protocol), and an arylsulfatase assay used as a reporter for *C. jejuni* gene expression studies (Basic Protocol 8).

The sequencing of genomes of several *C. jejuni* strains, as well as the development of a variety of genetic tools for use in *C. jejuni*, have provided the opportunity for rapid expansion in the knowledge of the biology of this organism. The protocols outlined in this unit, combined with genome sequence data, can be used to make and complement in-frame deletions, create libraries of transposon mutants, and study gene expression using gene fusions and techniques such as microarrays and qRT-PCR.

CAUTION: Campylobacter jejuni is a Biosafety Level 2 (BSL-2) pathogen. Follow appropriate guidelines and regulation for the handling of pathogenic microorganisms. Proper hand washing is essential because *C. jejuni* has been shown to cause gastroenteritis at a small dose (10 to 100 organisms). See *UNIT 1A.1* and other pertinent resources for more information.

NOTE: Incubations are performed at 37°C in a tri-gas incubator, as recommended in *UNIT 8A.1*.

BASIC PROTOCOL 1

CONJUGATION OF *C. JEJUNI* AND *E. COLI*

One method of moving DNA into *C. jejuni* is through conjugation. Conjugation is the transfer of DNA between bacteria though direct cell-to-cell contact. *E. coli* strains carrying the mobilization plasmid pRK212.1 are able to conjugate with *C. jejuni*. This technique is useful for incorporating replicating plasmids, such as pECO101, pECO102, pRY468pRY109, and pRY112 into *C. jejuni* (Yao et al., 1993; Wiesner et al., 2003). This protocol is modified from one used by the laboratory of Dr. Pat Guerry, Uniformed Service University (pers. comm.).

Materials

DNA fragment of interest
C. jejuni plasmid (e.g., pRY108 or pECO101; also see Table 8A.2.1)
Donor strain: *E. coli* DH5α [pRK212.1]
LB agar plates with appropriate selective antibiotics (*APPENDIX 4A*)
Recipient strain: *C. jejuni* (*UNIT 8A.1*)
Mueller Hinton (MH) agar plates, with and without appropriate selective antibiotics (BD Biosciences)
LB liquid medium with appropriate selective antibiotics (*APPENDIX 4A*)
Mueller Hinton (MH) broth without antibiotics (BD Biosciences)

Additional reagents and equipment for cloning of DNA (Struhl, 2000), introduction of plasmid DNA into *E. coli* (Seidman et al., 1997), growth of *E. coli* on solid medium (Elbing and Brent, 2002a) and in liquid medium (Elbing and Brent, 2002b), and growth of *C. jejuni* (*UNIT 8A.1*)

Construct E. coli donor strain

1. Clone fragment to be maintained in *C. jejuni* plasmid (i.e., pRY108, pECO101).

   Struhl (2000) contains protocols for cloning of DNA fragments.

2. Move plasmid into *E. coli* DH5α [pRK212.1].

   Seidman et al. (1997) describe methods for introducing plasmid DNA into E. coli cells.

3. Grow the transformed *E. coli* (Elbing and Brent, 2002a) on LB plates containing selection antibiotics: ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), and plasmid-specific antibiotic (e.g., kanamycin, chloramphenicol).

   Chloramphenicol would be used with pECO102.

Conjugate E. coli donor strain into C. jejuni

4. Grow recipient strain on MH agar with appropriate antibiotics.

   C. jejuni can be selected for resistance to streptomycin, which does not alter its ability to colonize natural host models or to cause disease in the ferret model. The streptomycin resistance phenotype provides a counter-selectable marker for use in these conjugation experiments. Concentrations of antibiotics used in C. jejuni media are listed in Table 8A.1.2. It is also possible to select for C. jejuni using trimethoprim (10 μg/ml) or cefoperazone.

5. Streak *C. jejuni* recipient strain onto MH plate with appropriate antibiotics for 16 to 20 hr at 37°C in microaerophilic conditions (*UNIT 8A.2*). Start an overnight culture of the *E. coli* donor strain in LB liquid medium with appropriate antibiotics.

6. Inoculate 5 ml LB liquid medium containing selective antibiotic for the plasmid with 375 μl of the overnight culture of the donor strain prepared in step 5. Incubate at 37°C while shaking until $OD_{600} = 0.45$ to 0.5.

7. Resuspend recipient *C. jejuni* strain from the 16- to 20-hr plates (step 5) in 1.8 ml MH broth without antibiotics to an OD of ∼1.0.

8. Wash 0.5 ml of the donor strain culture from step 6 once by microcentrifuging 5 min at 10,000 × *g*, 4°C, removing the supernatant, adding 10 ml MH broth without antibiotics, then centrifuging again as before and removing the supernatant to eliminate any antibiotics. Resuspend donor pellet with 1 ml of *C. jejuni* recipient strain suspension.

9. Spin bacterial mixture for 2 min at full speed in microcentrifuge, and remove supernatant.

10. Resuspend cells in 100 μl MH broth without antibiotics. Spot onto an MH plate without any antibiotics.

11. Incubate plate organism-side-up at 37°C in microaerophilic conditions (*UNIT 8A.1*) for 5 hr.

12. Resuspend bacteria from plate in 1.8 ml MH broth. Pellet bacteria by centrifuging 5 min at 10,000 × *g*, 4°C, and removing the supernatant.

13. Spread bacteria on MH agar with 10 μg/ml trimethoprim (TMP), 20 μg/ml cefoperazone, 2 mg/ml streptomycin, and an antibiotic for selection of plasmid.

14. Incubate for 4 to 5 days at 37°C in microaerophilic conditions (*UNIT 8A.1*).

ELECTROPORATION OF *C. JEJUNI*

BASIC PROTOCOL 2

Electroporation of bacteria involves the application of electrical current to the bacterial cells, which permeabilizes the membrane, allowing uptake of DNA from the environment. This technique is primarily used for the incorporation of plasmid DNA. This technique was first described for *C. jejuni* by Miller et al. (1988). Electroporation of *C. jejuni* is primarily done to incorporate DNA from suicide plasmids into the chromosome of *C. jejuni*. Electroporation is useful for this because plasmids derived from *E. coli* will not transform well into *C. jejuni* by natural transformation.

Materials

C. jejuni frozen stock (*UNIT 8A.1*)
Mueller Hinton (MH) agar plates with appropriate selective antibiotics (BD Biosciences)
Mueller Hinton (MH) broth without antibiotics (BD Biosciences)
Wash buffer: 15% (v/v) glycerol/9% (w/v) sucrose
DNA of interest, to be electroporated into *C. jejuni* (15 μg DNA/reaction)
SOC medium (see recipe)

Nitrocellulose membrane (0.025-μm pore size VSWP (Millipore, cat. no. VSWP04700)
Electroporation cuvettes (BioRad Gene Pulser Cuvettes, 0.2-cm electrode; cat. no. 165-2006)
Electroporator (BioRad *E. coli* Pulser)

Additional reagents and equipment for streaking bacteria (*APPENDIX 4A*) and growing *C. jejuni* (*UNIT 8A.1*)

Grow C. jejuni

1. Streak strain (*APPENDIX 4A*) from frozen stock onto one MH agar plate containing 10 μg/ml trimethoprim (TMP). Grow for 24 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

2. Restreak strain onto one MH agar plate containing 10 μg/ml TMP, using a heavy inoculum. Grow for 16 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

Prepare electrocompetent C. jejuni cells

3. Resuspend bacteria in 1.8 ml MH broth (without antibiotics) using an automatic pipettor and 1-ml pipet tip by pipetting 900 μl of broth up and down across the overnight lawn of bacteria.

4. Pellet bacteria by microcentrifugation for 5 min at maximum speed, 4°C.

5. Gently resuspend pellet in 2 ml ice-cold wash buffer.

6. Pellet bacteria by centrifugation for 5 min at full speed in microcentrifuge at 4°C

7. Repeat steps 5 and 6 three times.

8. Resuspend pellet in 900 μl ice-cold wash buffer. Use immediately (keep cold on ice).

Perform electroporation

9. For large volumes (>5 μl) of DNA that have been prepared in TE buffer, dialyze DNA on top of a nitrocellulose membrane floating in 20 ml distilled deionized water for 20 min.

10. Cool electroporation cuvettes on ice.

11. In a microcentrifuge tube, combine 50 μl electrocompetent bacteria (from step 8) and 15 μg DNA. Keep on ice.

12. Transfer bacteria-DNA mix to an electroporation cuvette. Electroporate the sample at 2.5 kV, 200 Ω, and 25 μF.

    The time constant is usually ~5 msec.

13. Flush cuvette with 100 μl SOC medium. Spread bacteria onto MH agar plate containing 10 μg/ml TMP.

14. Incubate plate for 5 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

15. Harvest bacteria by resuspending in 1.8 ml of MH broth without antibiotics. Microcentrifuge bacteria for 2 min at maximum speed and remove supernatant. Resuspend bacteria in 100 μl MH broth. Plate onto MH agar containing the appropriate selective antibiotics.

16. Incubate for 2 to 4 days at 37°C in microaerophilic atmosphere (*UNIT 8A.1*).

NATURAL TRANSFORMATION OF *C. JEJUNI*

BASIC PROTOCOL 3

C. jejuni is naturally transformable, meaning that it readily takes up DNA from the environment. Natural transformation can lead to genetic diversity within a population. It is also a useful tool for molecular biology in bacteria. This technique is useful in *C. jejuni* for the uptake of its own DNA, but not foreign DNA (i.e., *E. coli* derived) from the environment (Wang and Taylor, 1990; Wiesner et al., 2003).

Materials

C. jejuni frozen stock (*UNIT 8A.1*)
Mueller Hinton (MH) agar plates with and without appropriate selective antibiotics (BD Biosciences)
Mueller Hinton (MH) broth without antibiotics (BD Biosciences)
DNA of interest, to be transformed into *C. jejuni* (*C. jejuni* genomic DNA prepared as in Basic Protocol 6)

Additional reagents and equipment for streaking bacteria (*APPENDIX 4A*) and growing *C. jejuni* (*UNIT 8A.1*)

Grow C. jejuni

1. From frozen stock, streak strain (*APPENDIX 4A*) onto MH agar containing 10 μg/ml trimethoprim (TMP). Grow for 16 to 24 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

2. From the 16 to 24 hr growth plate, streak strain onto MH agar containing 10 μg/ml TMP, using a heavy inoculum. Streak plates ~16 hr before the start of the transformation experiment.

3. Make fresh MH agar (without antibiotics) and pipet 1-ml aliquots into plastic tubes.

4. Resuspend the entire growth from the 16-hr plate (from step 2) in 1 ml MH broth (without antibiotics).

Perform transformation

5. Dilute the bacteria to an OD_{600} of 0.5 (~1×10^9 cfu/ml) with MH broth (without antibiotics).

6. Add 0.5 ml resuspended bacteria to each tube (from step 3) containing 1 ml of solidified MH agar. Incubate tubes for 3 hr at 37°C in microaerophilic conditions (*UNIT 8A.1*).

7. Gently mix tubes.

8. Add 5 to 10 μg of the DNA to be transformed to each tube and pipet up and down.

9. Incubate for 4 hr at 37°C in microaerophilic conditions (*UNIT 8A.1*).

10. Transfer bacteria into a microcentrifuge tube. Microcentrifuge for 2 min at maximum speed, room temperature.

11. Resuspend bacteria and plate onto MH agar containing 10 μg/ml TMP and appropriate selection antibiotics.

12. Incubate plates at 37°C in microaerophilic environment (*UNIT 8A.1*) for 2 to 4 days.

TRANSPOSON MUTAGENESIS OF *C. JEJUNI*

Transposons are DNA elements that can insert themselves relatively randomly throughout the genome. Transposon mutagenesis of *C. jejuni* has been a widely accepted tool for genetic manipulation, and this method has identified a number of *C. jejuni* genes needed for colonization and motility (Golden et al., 2000; Hendrixson et al., 2001) A number of transposons have been developed for *C. jejuni*. This section will outline the protocol for in vitro transposition of *C. jejuni* with a mariner-based transposon (Lampe et al., 1999; Hendrixson, 2001). An alternative, pOTHM, is also a mariner-based transposon method, and specifics on that system can be found in Golden et al. (2000). The delivery plasmids and corresponding transposons used in previous studies are listed in Table 8A.2.2.

Materials

5× salt buffer (see recipe)10 mg/ml bovine serum albumin (BSA)
100 mM dithiothreitol (DTT)
Donor DNA (containing transposon)
Recipient DNA (containing target of mutagenesis *or* total *C. jejuni* genomic DNA prepared as in Basic Protocol 6)
Transposase: (available from Dr. David Lampe (Lampe et al., 1999)
TE buffer, pH 8.0 (*APPENDIX 2A*)
dNTP mix: 1.25 mM each dNTP (*APPENDIX 2A*)
T4 DNA polymerase and T4 DNA polymerase buffer (Invitrogen)
T4 ligase and T4 ligase buffer (Invitrogen)
30° and 70°C water baths and 11° and 16°C recirculating water baths (or thermal cycler)
Nitrocellulose membrane (0.025 μm pore size VSWP (Millipore, cat. no. VSWP04700)
Additional reagents and equipment for phenol:chloroform extraction and ethanol precipitation of DNA (Moore and Dowhan, 2002)

Perform transposition reaction

1. Prepare transposition reaction by combining the following:

   16.0 μl 5× salt buffer
   2.0 μl 10 mg/ml BSA
   1.6 μl 100 mM DTT
   1.0 μg donor DNA
   2.0 μg recipient DNA
   500 ng transposase
   Distilled deionized H_2O to 80 μl
   Incubate reactions at 30°C for 4 hr.

Table 8A.2.2 Transposons

Plasmid	Transposon	Transposase	Reference
pFD1	HImar1	Himar1	Rubin et al. (1999); Kakuda and DiRita (2006)
pEnterprise	picard	Himar1	Hendrixson et al. (2001) or Hendrixson and DiRita (2004)
pFalcon	Solo	Himar1	Hendrixson et al. (2001) or Hendrixson and DiRita (2004)
pOTHM	Mariner	Himar1	Golden et al. (2000)

Purify DNA

2. Extract DNA once with phenol:chloroform, then ethanol precipitate DNA (Moore and Dowhan, 2002).
3. Microcentrifuge DNA 30 min at maximum speed, 4°C.
4. Remove supernatant and resuspend pellet in 40 μl TE buffer, pH 8.0.

Perform repair reactions

The repair reactions are necessary to repair small gaps at the transposon-chromosomal junctions. The first reaction (step 5) fills in the gaps, while the ligase reaction (step 8) completes repair.

5. Prepare first repair reaction by combining the following on ice (total volume, 60 μl):

   40 μl DNA from transposition reaction
   6 μl T4 DNA polymerase buffer
   4.8 μl dNTP mix
   7.7 μl distilled H_2O
   1.5 μl 1 U/μl T4 DNA polymerase.

6. Incubate the reaction at 11°C for 20 min in a recirculating water bath or thermal cycler.
7. Inactivate the polymerase by incubating the reaction at 75°C for 15 min.
8. Prepare second repair reaction by combining the following on ice:

   60 μl reaction mixture from first repair reaction (step 5)
   12 μl T4 DNA ligase buffer
   1.5 μl T4 DNA ligase
   46.5 μl dH_2O.

9. Incubate at 16°C overnight in a recirculating water bath or thermal cycler.

Use DNA for transformation

10. Dialyze DNA on top of a nitrocellulose membrane floating on 10 to 20 ml distilled deionized water for 20 min.
11. Use all of the reaction for one transformation of *C. jejuni* (see Basic Protocol 3).

PREPARATION OF *C. JEJUNI* TEMPLATE FOR COLONY PCR

BASIC PROTOCOL 5

Colony PCR is a useful tool to screen for newly constructed mutants. Individual colonies take 3 to 5 days to grow on MH agar containing 10 μg/ml TMP in microaerophilic conditions at 37°C. Once individual colonies are visible, colony PCR can be used to confirm any mutations made. The *C. jejuni* DNA preparation for PCR requires boiling the *C. jejuni* strain in K buffer. This is done by spotting the isolated colony onto a fresh plate, then resuspending the colony in K buffer and incubating the sample at 100°C.

Materials

Proteinase K
K buffer (see recipe)
100°C water bath

Additional reagents and equipment for PCR (Kramer and Coen, 2001)

1. Add proteinase K to a final concentration of 100 μl/ml in K buffer immediately before use.

2. Resuspend one or two isolated colonies in this 100 μl of K buffer containing proteinase K, using a toothpick or a similar implement.

   Spot the individual colonies onto a fresh plate of MH agar containing 10 μg/ml TMP before resuspending in K buffer and keep in microaerophilic conditions at 37°C to preserve the colonies. Individual colonies will take 3 to 5 days to grow on MH agar containing 10 μg/ml TMP in a tri-gas incubator. See UNIT 8A.1 for further information.

3. Place in 100°C water bath for 10 min.

4. Microcentrifuge 5 min at 10,000 rpm to remove cell debris.

5. Use 2 to 5 μl of the supernatant for 50-μl PCR reactions.

BASIC PROTOCOL 6

ISOLATION OF *C. JEJUNI* GENOMIC DNA

Isolated bacterial DNA can be used for a multitude of purposes, such as cloning of specific genes by PCR, sequencing, or transposon mutagenesis. Although kits such as Qiagen's Genomic DNA Isolation Kit can be utilized, higher yields of genomic DNA are achieved by using the protocol outlined here.

Materials

C. jejuni frozen stock (*UNIT 8A.1*)
Mueller Hinton (MH) agar plates (BD Biosciences) with 10 μg/ml trimethoprim (TMP)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.1 mM EDTA
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)/0.1 mM EDTA containing 1 mg/ml lysozyme
Proteinase K
20% (w/v) SDS (*APPENDIX 2A*)
Buffered phenol (*APPENDIX 2A*)
24:1 chloroform:isoamyl alcohol
7.5 M ammonium acetate
Isopropanol
70% (v/v) ethanol
1 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)/0.1 mM EDTA
Filter paper (Whatman)

Additional reagents and equipment for growing *C. jejuni* (*UNIT 8A.1*) and phenol/chloroform extraction of DNA (Moore and Dowhan, 2002)

Days 1 and 2: Grow C. jejuni

1. From a frozen stock, streak strain onto MH with 10 μg/ml trimethoprim (TMP) agar. Grow for 16 to 24 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

2. From the 16- to 24-hr growth plate incubated in step 1, restreak strain onto one plate of MH agar containing 10 μg/ml TMP, using a heavy inoculum. Incubate 18 hr at 37°C under microaerophilic conditions (*UNIT 8A.1*).

Day 3: Isolate and purify DNA from C. jejuni

3. Resuspend *C. jejuni* from 18-hr growth plate in PBS and microcentrifuge 5 min at 10,000 rpm, 4°C.

4. Remove supernatant and resuspend pellet in 200 μl of 10 mM Tris·Cl, pH 8.0/0.1 mM EDTA.

5. Add 400 μl of 10 mM Tris·Cl, pH 8.0/0.1 mM EDTA, containing 1 mg/ml lysozyme.

6. Incubate on ice for 5 min.

7. Add proteinase K to 20 μg/ml and mix gently.

8. Add 50 µl 20% SDS. Mix gently to clear the lysate.
9. Incubate with gentle rocking at 37°C for 30 min.
10. Add 200 µl buffered phenol. Vortex and microcentrifuge 5 min at 12,000 rpm, room temperature.

    Phenol extraction may have to be repeated a couple more times by transferring the aqueous (upper) phase to a fresh tube and adding more phenol. When the phases separate well and quickly, further phenol extractions are unnecessary.

    Additional detail on phenol/chloroform extraction of DNA is found in Moore and Dowhan (2002).
11. Extract twice with 24:1 chloroform:isoamyl alcohol.
12. Add 0.5 vol of 7.5 M ammonium acetate and mix. Fill tube with isopropanol.
13. Mix well. Allow DNA to precipitate at −80°C for at least 1 hr.
14. Collect DNA by microcentrifuging 10 min at 12,000 rpm, 4°C.
15. Carefully decant isopropanol and rinse pellet twice with 70% ethanol. Microcentrifuge 5 min at 12,000 rpm, 4°C.
16. Carefully pour off the last wash, and wick out the remaining ethanol with Whatman filter paper. Dry the pellet under vacuum in a Speedvac evaporator for about 5 min.
17. Resuspend pellet in 300 µl of 1 mM Tris·Cl, pH 7.5/0.1 mM EDTA.

    Resuspension should be allowed to take place overnight. If necessary, pipet with a large-orifice genomic tip.

ISOLATION OF RNA FROM *C. JEJUNI*

BASIC PROTOCOL 7

The protocol outlined below is a variation on the RNeasy protocol. It was modified from a procedure recommended by Drs. Nick Dorrell and Brendan Wren, London School of Hygiene and Tropical Medicine (pers. comm.). As with all RNA work, everything must be RNase-free. Clean the bench and pipets with ethanol and RNase-eliminating solutions, such as Eliminase, before starting. Make sure all solutions are prepared using RNase-free reagents and RNase-free water.

Materials

C. jejuni frozen stock (*UNIT 8A.1*)
Mueller Hinton (MH) agar plates (BD Biosciences) with 10 µg/ml trimethoprim (TMP)
Mueller Hinton (MH) broth (BD Biosciences) with 10 µg/ml trimethoprim (TMP)
RNA Protect Bacteria Reagent (Qiagen)
TE buffer, pH 8.0 (*APPENDIX 2A*) containing 1 mg/ml lysozyme
RNeasy kit (Qiagen) including:
  Buffer RLT
  Buffer RW1
  Buffer RPE
  RNA Mini Spin columns
  2-ml collection tubes
  RNase-free H_2O
2-mercaptoethanol (2-ME)
100% ethanol, ice cold
10× DNase buffer
10 U/µl DNase (Invitrogen)
Buffered phenol (*APPENDIX 2A*)

Chloroform
3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)
70% (v/v) ethanol

75-cm^2 tissue culture flask
15-ml conical centrifuge tubes
Centrifuge
Spectrophotometer

Additional reagents and equipment for growing *C. jejuni* (*UNIT 8A.1*)

Grow C. jejuni

1. Grow a full lawn of *C. jejuni* from frozen stock on one MH agar plate with 10 μg/ml trimethoprim for 16 to 20 hr.
2. Prepare a biphasic 75-cm^2 tissue culture flask (*UNIT 8A.1*; see Fig. 8A.1.1) with 20 ml MH agar and 20 ml MH broth with 10 μg/ml trimethoprim (TMP).
3. Resuspend the bacteria from the prepared overnight plate (step 1) to an OD_{600} of 0.4 in MH broth with 10 μg/ml trimethoprim (TMP).
4. Dilute the bacterial suspension 1:10 with MH broth containing 10 μg/ml trimethoprim (TMP) and inoculate 80 μl into the biphasic flask.
5. Incubate for 48 hr at 37°C in microaerophilic atmosphere (*UNIT 8A.1*).
6. Transfer 4-ml aliquots of the MH broth containing the 48-hr bacterial growth into 15 ml conical tubes.

   Due to the limitations of the RNeasy Spin Column, better yield is achieved when 4 ml of the culture is used per spin column. Multiple samples can be performed and combined prior to DNase treatment, for higher yields.

Lyse bacteria

7. Add 8 ml RNA Protect Bacteria Reagent per 4 ml of the 48 hr culture in the 15-ml conical tube. Vortex for 5 sec and incubate for 5 min at room temperature.
8. Centrifuge tubes at 10 min at 10,000 × *g*, 4°C. Decant supernatant and dab on paper towel.
9. Add 400 μl TE buffer, pH 8.0, with 1 mg/ml lysozyme to each tube and resuspend the bacteria by pipetting.
10. Incubate for 10 min at room temperature, vortexing every 2 min.

Extract and purify RNA

11. Add 1200 μl Buffer RLT and 12 μl 2-mercaptoethanol to each tube, and vortex vigorously.
12. Add 1000 μl of 100% ethanol. Mix by pipetting and transfer all to an RNeasy Mini spin column.
13. Centrifuge for 15 sec at 10,000 rpm and discard the flowthrough.
14. Repeat as required, based on volume.
15. Wash an RNeasy column by adding 350 μl Buffer RW1 onto the column, microcentrifuging 15 sec at 10,000 rpm, and discarding the flowthrough. Repeat this wash a second time.
16. Add 500 μl Buffer RPE onto the RNeasy spin column. Microcentrifuge 2 min at 10,000 rpm and discard the flowthrough.

17. Place column into fresh 2-ml collection tube and microcentrifuge for an additional 1 min at 12,000 rpm, to eliminate any remaining buffer.
18. Transfer RNeasy column into a fresh 1.5-ml microcentrifuge tube.
19. Add 50 μl RNase-free water to the RNeasy membrane in the column and centrifuge for 1 min at 10,000 rpm.
20. Combine any multiple samples done from the original 48 hr overnight culture (see step 6).
21. Dilute sample to 200 μl with RNase-free water and add 20 μl of 10× DNase buffer.
22. Add 1 μl of 10 U/μl DNase to each tube. Incubate at room temperature for 1 hr.
23. Add 220 μl of buffered phenol and vortex. Let sit for 5 min at room temperature.
24. Microcentrifuge 15 min at maximum speed, 4°C.
25. Save top layer and transfer to a new microcentrifuge tube.
26. Add 1 volume chloroform, vortex, and let sit for 5 min.
27. Microcentrifuge 15 min at maximum speed, 4°C.
28. Save top layer and transfer to a new microcentrifuge tube.
29. Precipitate RNA by adding 0.1 vol of 3 M sodium acetate pH 5.2, and 2.5 vol of ice-cold 100% ethanol.
30. Incubate at −20°C overnight (or 2 to 3 hr, minimum).
31. Spin down RNA by microcentrifuging 15 min at maximum speed, 4°C.
32. Remove supernatant, then wash pellet by adding 1 ml of 70% ethanol and microcentrifuging 7 min at maximum speed, 4°C.
33. Remove supernatant and dry pellet completely.
34. Resuspend pellet in 25 to 50 μl RNase-free water.
35. Quantitate RNA by measuring OD_{260} in a spectrophotometer and determine purification of RNA by comparing OD_{260}/OD_{280} (should be >1.8 to 2.0).

    OD_{260} = 1 is equivalent to 40 μg/ml RNA.

TWO-STEP ISOLATION OF RNA FROM *C. JEJUNI* USING TRIZOL

ALTERNATE PROTOCOL

The protocol outlined below, like Basic Protocol 7, utilizes the Qiagen RNeasy kit. However, it has a Trizol extraction preceding the RNeasy columns. The extraction step is for ∼1-4 × 10^9 cfu/ml of *C. jejuni*. The Qiagen columns can easily handle up to 1-2 × 10^{10} cfu/ml of *C. jejuni* RNA as long as it was Trizol-extracted first. This is in contrast to Basic Protocol 7, which dilutes the original 10^9 cfu/ml culture to approximately 10^7 cfu/ml of *C. jejuni*. The protocol outlined below was adapted from one used by the lab of Dr. Erin Gaynor, University of British Columbia (Gaynor et al., 2005).

Additional Materials *(also see Basic Protocol 7)*

10× stop Solution: 5% (v/v) buffered phenol (*APPENDIX 2A*) in 100% ethanol
Liquid N_2
TE buffer, pH 8.0, containing 0.4 mg/ml lysozyme
Trizol reagent (Invitrogen)
Buffer RDD from RNeasy Kit (Qiagen)

Harvest bacteria

1. Prepare a 48-hr liquid culture of *C. jejuni* as described in Basic Protocol 7, steps 1 to 5.
2. Harvest from liquid culture by centrifuging 10 min at 10,000 × *g*, 4°C, removing the supernatant, and resuspending the pellet in 1× stop solution at 1/10 the original volume.
3. Invert several times to mix.
4. Microcentrifuge 5 to 10 min at 11,000 × *g*, 4°C. Aspirate supernatant.
5. *Optional:* Wash by adding 1 ml of 1× stop solution, centrifuging again as in step 4, and aspirating the supernatant.

   This optional wash removes any left-over medium and helps ensure a clean isolation.
6. Freeze in liquid nitrogen in 1ml aliquots in 1.5-ml microcentrifuge tubes and store at −80°C until ready to isolate RNA.

   This is a good stopping point if taking multiple samples over a period of time.

Perform Trizol extraction

7. Remove tubes from freezer and place into ice once thawed.
8. Resuspend bacteria in 50 μl TE buffer containing 0.4 mg/ml lysozyme.
9. Let sample sit at room temperature for ∼5 min.
10. Add 950 μl Trizol reagent and vortex for 1 min to lyse.
11. Add 200 μl chloroform. Shake for 15 sec and let sit at room temperature for 2 to 3 min.
12. Microcentrifuge 15 min at 12,000 rpm, 2° to 8°C.
13. Transfer top (aqueous) phase to a new microcentrifuge tube.
14. Slowly add an equal volume of 70% ethanol to precipitate RNA.

Perform RNeasy purification/DNase treatment

15. Apply RNA solution to an RNeasy Mini spin column in 700-μl aliquots.
16. Add 350 μl RW1 buffer. Microcentrifuge for 15 sec at maximum speed and discard flowthrough.
17. For each column, prepare a mixture of 10 μl of 10 U/μl DNase in 70 μl RDD buffer.
18. Apply the 80 μl of DNase/RDD mix to the middle of the column membrane. Leave at room temperature for 30 min to 1 hr.
19. Add 350 μl RW1 buffer to the column. Microcentrifuge 15 sec at 12,000 rpm, 4°C.
20. Put the column into a new collection tube.
21. Add 500 μl RPE buffer, microcentrifuge 15 sec at 12,000 rpm, 4°C, and discard flowthrough.
22. Add another 500 μl of RPE buffer. Microcentrifuge 15 sec at 12,000 rpm, 4°C, and discard flowthrough.
23. Microcentrifuge 2 min at 12,000 rpm, 4°C, to remove all of the RPE.

24. Transfer column to a 1.5-ml collection tube.

25. Add 50 µl RNase-free water to the membrane. Microcentrifuge 15 sec at 12,000 rpm, 4°C, to collect RNA. Store at −80°C.

ARYLSULFATASE ASSAY

BASIC PROTOCOL 8

Similar to the *lacZ* reporter system in *E. coli*, the arylsulfatase assay, described in Hendrixson and DiRita (2003), quantifies the expression of a gene of interest. Arylsulfatase cleaves sulfates from substrates, and is encoded by the *astA* gene in *C. jejuni*. The assay measures arylsulfatase activity colorimetrically, using nitrophenyl sulfate as a substrate. Arylsulfatase liberates the sulfate from nitrophenyl sulfate (a colorless compound), forming nitrophenol. Nitrophenol is yellow in color, and by monitoring a colorimetric change, this assay can be used to determine the amount of arylsulfatase activity. Briefly, a promoterless *astA* gene is cloned into the gene of interest on the chromosome of *C. jejuni*, creating an *astA* transcriptional fusion. Expression of the gene of interest can then be monitored through the arylsulfatase assay, described below.

Materials

p-Nitrophenol (Sigma, cat. no 104-8)
Arylsulfatase buffer 1 (AB1): 0.1 M Tris·Cl, pH 7.2 (*APPENDIX 2A*)
C. jejuni (*UNIT 8A.1*)
Mueller Hinton (MH) agar plates with antibiotics (BD Biosciences)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Arylsulfatase buffer 2 (AB2): 2 mM tyramine in 0.1 M Tris·Cl, pH 7.2
Arylsulfatase buffer 3 (AB3): 20 mM nitrophenyl sulfate (potassium 4-nitrophenyl sulfate; Sigma, cat. no. N3877) in 0.1 M Tris·Cl, pH 7.2 (*APPENDIX 2A*)
0.2 N NaOH
Spectrophotometer

Additional reagents and equipment for growing *C. jejuni* (*UNIT 8A.1*)

Prepare standard curve

1. Prepare standard curve using *p*-nitrophenol:

   a. Prepare 200 µM nitrophenol in buffer AB1 by dissolving 27.82 mg nitrophenol in 10 ml AB1, then diluting this solution 1:100 with buffer AB1 for a 200 µM solution.

   b. Serially dilute the 200 µM nitrophenol 1:2 with buffer AB1 for six dilutions.

   c. Read OD_{420} for the dilutions of nitrophenol. Create a standard curve of OD_{420} values versus µM nitrophenol.

Prepare bacteria

2. Streak *C. jejuni* onto MH agar containing 10 µg/ml trimethoprim and grow at 37°C for 24 hr under microaerophilic conditions (*UNIT 8A.1*).

3. Restreak heavily onto one MH agar plate. Grow at 37°C for 18 hr.

4. Resuspend the growth in PBS to an OD_{600} of 1.0.

5. Divide each sample into two 1-ml aliquots.

6. Wash one aliquot with AB1 by microcentrifuging 5 min at maximum speed, room temperature, and removing the supernatant. Add 1 ml of buffer AB1, repeat microcentrifugation, and remove supernatant. Resuspend in 1 ml AB1.

7. Wash the second aliquot in AB2 using the technique described in step 6. Resuspend in 1 ml AB2.

Perform assay

8. Add 200 µl of each sample to 200 µl of freshly prepared AB3 in microcentrifuge tubes.
9. Incubate for 1 hr at 37°C.
10. Stop reaction by adding 800 µl of 0.2 N NaOH.
11. Read OD_{420}.

    Samples in AB1 are blanks for AB2 samples.

12. Compare values to standard curve. Report data as nmol or µM nitrophenol.

REAGENTS AND SOLUTION

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

DNase buffer, 10×

200 mM sodium acetate, pH 4.5
100 mM $MgCl_2$
100 mM NaCl
Store up to 1 year at −20°C

K buffer

50 mM KCl
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
2.5 mM $MgCl_2$
0.5% (v/v) Tween 20
Filter sterilize
Store up to 1 year at room temperature

Salt buffer, 5×

6.25 ml 80% (w/v) glycerol (50% final)
1.25 ml 1 M HEPES, pH 7.9 (125 mM final)
1.25 ml 4 M NaCl (500 mM final)
0.25 ml 1 M $MgCl_2$ (25 mM final)
1 ml H_2O
Store up to 1 year at room temperature

SOB medium

2 g Bacto tryptone (BD Biosciences)
0.5 g yeast extract (BD Biosciences)
0.05 g NaCl
10 ml 250 mM KCl
Adjust pH to 7.0 using 1 M HCl
Store up to 1 year at room temperature

SOC medium

1 ml SOB medium (see recipe)
10 µl 1 M $MgSO_4$
10 µl 1 M $MgCl_2$
20 µl 20% (w/v) glucose
Store up to 1 year at 4°C

COMMENTARY

Background Information

Since its discovery, work on *Campylobacter* species has been limited due to challenging culture requirements and limited genetic tools. However, work in recent years has contributed much to our understanding of *Campylobacter jejuni*, as well as the tools used to study it.

Critical Parameters and Troubleshooting

Conjugation

The conjugation of plasmids into *C. jejuni* from *E. coli* containing the conjugation plasmid pRK212.1 (Basic Protocol 1) is a fairly robust protocol. The protocol is quite forgiving of variation in OD_{600} of both the donor and recipient strains, as long as both are collected during exponential growth and mixed at approximately the indicated ratio. The main problem encountered during conjugation is the outgrowth of the donor *E. coli* strain on the selective plates. In general, use of multiple antibiotics to which the *C. jejuni* strain, but not the *E. coli* strain, is resistant solves this problem. In the protocol given here, trimethoprim, cefoperazone, and streptomycin are used together to eliminate growth of *E. coli*. If the recipient strain is resistant to additional antibiotics, they can be used as well. Obviously, streptomycin should only be used with streptomycin-resistant *C. jejuni* (i.e., DRH212).

Electroporation

Electroporations (Basic Protocol 2) may require more troubleshooting and repetitions to achieve introduction of foreign DNA. If the electrocompetent cells are to be used in the future, aliquots can be frozen at −80°C in 20% glycerol until they are needed. In general, if the protocol has been attempted unsuccessfully, new electrocompetent cells and DNA should be prepared before attempting the procedure again.

Electroporation is often used to make insertional mutants, where an antibiotic cassette is inserted into a gene of interest. It can also be used, with the correct intermediate strain, to replace a dual cassette (i.e., chloramphenicol resistance and streptomycin sensitivity) with an in-frame deletion or point mutation. These unmarked mutants can be much more difficult to make, often due to high background (i.e., streptomycin-resistant colonies that retain the chloramphenicol resistance/streptomycin sensitivity cassettes and have not acquired the unmarked mutation). To combat high background in these cases, the following alterations in the protocol can be tried. The electroporation plates can be incubated in a microaerophilic environment (i.e., in zip-lock bags filled with the appropriate gas mix) for the first few days and then transferred to a more stable microaerophilic environment (i.e., Campy-Pak jars or tri-gas incubator; see *UNIT 8A.1*). Additionally, several different concentrations of the selective antibiotic can be tried. If these changes do not lead to successful electroporation, one can test several colonies of the recipient strain for appropriate antibiotic resistance/sensitivity, colony purify the strain, and make a new stock for future electroporation. Alternatively, success can sometimes depend on the location of the insertion in the intermediate strain. Construct a new intermediate strain with the insertion at a different location within the gene of interest.

Natural transformation

With regard to Basic Protocol 3, it should be noted that *C. jejuni* does not efficiently take up DNA from *E. coli*.

In vitro transposition

For the transposon mutagenesis protocol (Basic Protocol 4), recipient DNA here must be *C. jejuni* derived, or it will not be taken up efficiently in the succeeding natural transformation step.

Preparation of template for PCR

The concentration of proteinase K in the K buffer is an important parameter in Basic Protocol 5. If too much proteinase K is added, no PCR product is seen, presumably because the proteinase K degrades the *Taq* polymerase. If multiple PCRs are planned, or must be performed at a later time, the supernatant can be frozen at −20°C, if removed from the cell debris after the spin in step 4.

Genomic DNA isolation

DNA isolated via Basic Protocol 6 is of sufficient quality for cloning, but not for sequencing reactions. If sequence from the chromosome is needed, use of the Qiagen Genomic DNA extraction protocols is recommended.

RNA isolation

Working with RNase-free reagents in an RNase free area is critical to Basic Protocol 7. Additionally, although the Alternate Protocol calls for on-column DNase treatment of the RNA, several labs have indicated that this

Table 8A.2.3 Time Considerations for Protocols

Protocol	Description	Time required
Basic Protocol 1	Conjugation	7-8 days[a]
Basic Protocol 2	Electroporation	5-7 days[a]
Basic Protocol 3	Natural transformation	5-7 days[a]
Basic Protocol 4	In vitro transposition	2 days[b]
Basic Protocol 5	Preparation of template for PCR	<1 day[c]
Basic Protocol 6	Genomic DNA isolation	3-4 days[a]
Basic Protocol 7 or Alternate Protocol	RNA isolation	4-5 days[a]
Basic Protocol 8	Arylsulfatase assay	3 days[a]

[a]Time indicated includes time from streaking *C. jejuni* strain out from freezer stocks to end of protocol as written. For making new strains via introduction of foreign DNA, this includes time until single colonies will appear on selective media, but not the time required to verify the identity of the new strain, isolate single colonies, and freeze down the new strain.

[b]Time does not include purification of transposase or natural transformation of transposition reaction into *C. jejuni*.

[c]Time indicated does not include time required to grow *C. jejuni* strain before beginning the protocol.

procedure does not always efficiently eliminate any contaminating DNA. If DNA contamination is a problem, perform DNase treatment as indicated in Basic Protocol 7. DNase treatment can be repeated multiple times, followed by phenol and chloroform extractions and RNA precipitation. RNA can be tested for contaminating DNA by a PCR reaction (without prior reverse transcription) and DNase treatment can be repeated until there is no PCR product.

Arylsulfatase assay

The substrate for the arylsulfatase assays (Basic Protocol 8), nitrophenyl sulfate in solution, can be unstable and degrade after long-term storage at −20°C, resulting in high background readings. If this occurs, a fresh stock of nitrophenyl sulfate should correct the problem.

Time Considerations

Table 8A.2.3 provides time considerations for the protocols in this unit. It should be noted that for protocols that aim to produce new strains of *C. jejuni*, the time indicated does not include the time to verify the identity of the new strain, colony purify the strain, and freeze the strain down into new glycerol stocks.

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Laboratory Maintenance of *Helicobacter* Species

In vitro, *Helicobacter* species grow quite slowly and only under conditions of reduced (but not absent) oxygen (i.e., microaerophilic conditions) and rich media. Primary cultures may take up to 5 to 7 days to achieve optimal growth, although subsequent passages take only a few days. Identification of *H. pylori* can be made by confirming the presence of urease, catalase, and oxidase enzymes, and by observing microscopic evidence of high motility and a spiral or curved morphology (Windsor and O'Rourke, 2000). Colonies are small and round, and appear translucent against the blood agar background. *H. pylori* is resistant to sulfonamides, vancomycin, trimethoprim, and cephalothin, which allows for the creation of a very effective selective media when isolating bacteria from clinical biopsies. It is sensitive, however, to many common antibiotics including tetracycline, gentamicin, kanamycin, and penicillin. These antibiotics may therefore be used to kill extracellular *H. pylori* when performing co-culture experiments with phagocytes, or as selection markers when introducing gene cassettes into the *H. pylori* genome.

In the present unit, protocols for growing *Helicobacter* organisms on plates (Basic Protocol 1) or in liquid cultures (Basic Protocol 2) are presented first, followed by Basic Protocol 3 for culturing *H. pylori* from clinical biopsy specimens. Three support protocols are presented that describe the preparation of solid media for growth on plates (Support Protocol 1), a method for quantifying numbers of *Helicobacter* organisms in an actively growing culture (Support Protocol 2), and techniques for quantifying *H. felis* in vitro (Support Protocol 3). Additionally, a method for liquid culture of *Helicobacter* has been provided that does not require the use of a tissue culture incubator (see Alternate Protocol). Finally, methods are provided for confirming growth of *H. pylori* (see Support Protocol 4).

CAUTION: *H. pylori* and *H. mustelae* are Biosafety Level 2 (BSL-2) pathogens; *H. felis* is a BSL-1 pathogen. Adult acquisition of *H. pylori* is thought to be relatively rare, and most infected individuals remain asymptomatic and healthy for life. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

Several different *Helicobacter* selective media supplements have been described (see Table 8B.1.1). Skirrow's recipe, available commercially from Difco, BBL, and Oxoid, was originally developed for isolation of *Campylobacter* species. Although Skirrow's supplement (10 μg/ml vancomycin, 5 μg/ml trimethoprim, and 2.5 IU/ml polymyxin B) plus amphotericin B has been used for isolation and culture of *Helicobacter* species, the authors have found the recipes in Table 8B.1.1 to be more effective. In particular, the so-called Glaxo selective supplement A (GSSA) is useful for the isolation of *H. pylori* from mice when attempting to adapt a new *H. pylori* strain to mice (Lee et al., 1997; McColm, 1997; McColm et al., 1995). Note, however, that use of GSSA could induce a bias into the selection/adaptation process, as some *H. pylori* isolates have been shown to be sensitive to nalidixic acid. One or the other of the supplements must be used when attempting to isolate *H. pylori* or one of the other *Helicobacter* species from a human or animal biopsy. This is particularly important for isolating bacteria from mice since they are coprophagic (they consume their own feces). For pure cultures of laboratory strains, selective medium supplementation is less critical, but in order to simplify preparation

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Table 8B.1.1 Supplements for Selective Media

Antibiotic	*H. pylori*	*H. pylori* GSSA[a]	*H. felis*	*H. mustelae*
Amphotericin B	2.5 μg/ml	2.5 μg/ml	2.5 μg/ml	2.5 μg/ml
Bacitracin		20 μg/ml		
Cefsulodin	16 μg/ml			16 μg/ml[b]
Nalidixic acid		10.7 μg/ml		
Polymyxin B		3.3 μg/ml	0.125 μg/ml	
Trimethoprim	20 μg/ml		20 μg/ml	20 μg/ml
Vancomycin	6 μg/ml	10 μg/ml	6 μg/ml	6 μg/ml

[a]GSSA, Glaxo selective supplement A.
[b]For *H. mustelae*, 30 μg/ml cephaloxathin may be substituted for cefsulodin. *H. mustelae* is the only one of these three *Helicobacter* species resistant to cephaloxathin.

of bacterial growth plates, it will do no harm to always use plates prepared with the indicated supplements.

BASIC PROTOCOL 1

CULTURE OF *HELICOBACTER* ORGANISMS ON SOLID MEDIUM

H. pylori is a fastidious organism capable of growing only under a narrow set of conditions. It must be cultured on nutrient-rich agars such as Columbia blood agar base supplemented with whole blood from any one of a variety of large mammals such as horse, ox, or sheep. Reduced oxygen tension is crucial; therefore, a closed-container system capable of generating microaerophilic conditions must be employed. Typically this consists of an anaerobic jar with a microaerophilic system envelope. It is also necessary to incubate for several days at 37°C before colonies can be easily observed. The different gastric *Helicobacter* species such as *H. pylori*, *H. felis*, and *H. mustelae* are distinct in colony morphology. *H. felis* is extremely motile and readily forms confluent lawns which, to the untrained eye, can resemble uninoculated plates. Therefore, the eye must be trained to detect *H. felis* growth as a sheen or haze on the plate. *H. pylori* and *H. mustelae* form small, round, translucent colonies which may first be visible at pin-prick size as early as 3 to 4 days, gradually becoming larger.

Materials

Freshly prepared blood agar plates containing appropriate antibiotic (see Support Protocol 1)
Plates containing viable *Helicobacter* colonies, liquid culture of *Helicobacter* (Basic Protocol 2), or frozen stock of *Helicobacter*
Disposable bacterial spreaders (PGC Scientific), laboratory-made glass spreaders, or inoculating loops
Anaerobic jar(s) with sealable lid(s) (BBL model no. 100 uses one GasPak and holds 12 plates; model 150 uses three GasPak envelopes and holds 36 plates.)
GasPak: BBL CampyPak Plus Microaerophilic System Envelopes with Palladium Catalyst (Becton Dickinson) or equivalent

1. Inoculate freshly prepared blood agar plates with several colonies from a viable plate using an inoculating loop; alternatively inoculate 50 to 100 μl of thawed material from a frozen stock or liquid culture.
2. Use a bacterial spreader or inoculating loop to spread the material back and forth across the entire plate from top to bottom. Turn the plate 90° and repeat.

3. Cover the plates and place them in anaerobic jar(s).

   When culturing H. pylori and H. mustelae on plates, keep the plates inverted during incubation. For Helicobacter felis, however, do not invert the plates. H. felis is very motile, and the extra moisture that drips down onto the plate when cultured in the noninverted fashion helps it to grow (see Critical Parameters and Troubleshooting and APPENDIX 4A).

   Some laboratories include a water-soaked paper towel in the anaerobic jar to provide a humidified atmosphere. However, adding water to the GasPak envelope (see step 4) helps accomplish the same purpose. Because of the high moisture content in the anaerobic jars, it is important to disinfect the jars thoroughly between uses; it may be useful to include an antifungal and/or selective antibiotic supplement in the medium as well (see Table 8B.1.1).

4. Cut the corner off of a GasPak envelope, separate the edges, and place inside the anaerobic jar with the catalyst facing the outside. Add 10 ml water to each envelope, then seal the top of the anaerobic jar. Place the jar in a 37°C incubator or environmental control room.

   There are several different types of gas-generating GasPaks available. Be sure to select the correct one or the Helicobacter organisms may not grow.

   If an incubator is available in which three gases can be controlled, or if custom-blended gas cylinders can be obtained, these may be used in lieu of anaerobic jars and GasPaks. The incubator should be set to deliver 5% to 7% O_2/10% CO_2 with the balance N_2. If the incubator allows control of two gases plus air, set it at 20% to 35% air (which yields 5% to 7% oxygen), 10% CO_2, and the balance N_2. Some trial and error may be required to determine the optimum gas mixture for a particular incubator.

5. After 3 to 4 days open the jar and examine the plates for growth. If no growth or only low growth is observed, plates can be returned to the jar with a fresh GasPak for another 2 to 3 days.

   H. pylori and H. mustelae will form distinct pinhead-sized colonies which gradually become larger and easier to identify. Colonies on densely cultured plates may merge into a confluent lawn that displays a distinctive sheen when the plate is viewed at an angle. Thus, if individual H. pylori or H. mustelae colonies are to be counted, timing is important and it may be necessary to examine the plates at two or three different time points. H. felis tends to form confluent sheets within several days of culture. Early examination may allow for enumeration of individual colonies, but these colonies may be very small and difficult to detect.

PREPARATION OF BLOOD AGAR PLATES FOR GROWTH OF *HELICOBACTER* SPECIES

SUPPORT PROTOCOL 1

Successful culture of *Helicobacter* organisms requires the use of fresh nutrient blood agar plates. Commercially prepared plates may work, but the freshness of these plates cannot be controlled and they can often be too old or too dry. Additionally, they will lack the appropriate selective antibiotics. Unless one has access to a reliable bacteriology laboratory that can prepare fresh plates to specifications, it is better to prepare them in one's own laboratory. Any one of several nutrient agars recommended for growth of fastidious organisms will work, including Columbia Agar Base, Mueller-Hinton Agar Base, and Blood Agar Base (available from BBL, a trademark of Becton Dickinson). The important thing is that the plates be relatively fresh and moist. When preparing the plates, let the agar harden only until it solidifies (not overnight). If the plates are not to be used immediately, store in sealed plastic sleeves at 4°C for no longer than 2 to 3 weeks.

Materials

Nutrient agar base: Columbia Agar Base, Mueller-Hinton Agar Base, or Blood Agar Base (available from BBL; also see *APPENDIX 2C*)
Stock solutions of antibiotics (see Table 8B.1.1)

Defribrinated whole blood (horse, sheep, or ox), sterile (Cleveland Scientific)

1000-ml Erlenmeyer or round-bottom glass flasks or 1000-ml tissue culture bottles
56°C water bath
100-mm sterile polystyrene petri dishes with sleeves

1. Weigh out manufacturer's recommended amount of nutrient agar base for 500 ml medium and add to a flask or bottle containing 500 ml distilled or tap water. Swirl until all the powder is dispersed and freely suspended.

   Settling will occur but it is best not to have any deposits of dry medium when autoclaving.

2. Loosely cover vessels and autoclave under standard conditions for liquid reagents (18 psi/120°C) for 20 min.

3. Remove containers from autoclave and place in a 56°C water bath.

   This allows the medium to cool enough for addition of the blood and antibiotics without denaturing their activity but still being warm enough to prevent the agar from solidifying.

4. Add the appropriate amount of the antibiotics required for the particular *Helicobacter* species (see Table 8B.1.1).

5. Add 35 ml defibrinated whole blood, previously warmed to room temperature.

   Alternatively, "laked" whole blood, which has been frozen and thawed to produce hemolysis, may be used; however, the authors have not found this to be necessary.

6. Remove the flask from the water bath and gently mix until the blood is distributed uniformly. Keep the formation of bubbles to a minimum.

   This is best accomplished by rolling the flask at an angle on the counter top.

7. Immediately dispense the medium into 100-mm petri dishes and remove bubbles by flaming. Stack the warm plates into groups of four or five and allow to cool at room temperature (only until agar has hardened).

   Stacking will allow the agar to cool more slowly and minimize the amount of condensation that forms on the lids (except for the top plate). However, unstacked plates will solidify more quickly.

8. Following solidification of the agar, place plates in a plastic sleeve and store at 4°C until needed.

BASIC PROTOCOL 2

CULTURE OF *HELICOBACTER PYLORI* IN LIQUID MEDIUM

Helicobacter may also be grown in liquid media formulations. Several strategies can be used, depending on the species. Several nutrient-rich media such as Brain Heart Infusion or *Brucella* broth will support growth when supplemented with 10% heat-inactivated fetal bovine serum (FBS). Reduced oxygen tension can be supplied either through the use of a closed container system capable of generating microaerophilic conditions, or, in the case of *H. pylori*, by using a CO_2 incubator. Incubation at 37°C is required for growth. Exponential growth or even saturated cultures can be obtained in as little as 24 hr, depending on the inoculating dose.

Materials

Brucella broth or other suitable liquid medium supplemented with 10% (v/v) heat-inactivated FBS
Agar plate containing a heavy growth of viable *H. pylori* (Basic Protocol 1)

Disposable bacterial spreaders (PGC Scientific) or laboratory-made glass spreaders
25-cm^2 polystyrene tissue culture flasks
37°C, 5% CO_2 incubator

1. Place 1 ml of *Brucella* broth/10% FBS medium on to the center of an 100-mm *H. pylori* plate culture.
2. Using a sterile bacterial spreader, lift *H. pylori* from plate by continuous gentle raking of the surface. Slightly tip plate forward and gather liquid towards the front rim.
3. Transfer suspension of *H. pylori* to a 25-cm^2 tissue culture flask containing 10 ml of *Brucella* broth/FBS media.

   The recovered volume will be <1 ml as some of the liquid is absorbed by the solid medium.
4. Attach the cap of the flask so that it is loose enough to allow gas exchange but not loose enough to fall off.

   Alternatively, flasks with vented caps may by used and screwed on tightly.
5. Place flask in a 37°C, 5% CO_2 incubator standing upright.

   The authors have noticed that keeping the flask upright results in more reliable growth, possibly due to the increased depth of the medium.
6. Allow culture to grow overnight. Check for *Helicobacter* growth by placing the flask horizontally on an inverted microscope at 40× magnification. When the agitation of the culture has stabilized, look for highly motile bacteria by focusing just above the bottom of the flask.
7. Passage culture by transferring a small aliquot to another flask containing 10 ml *Brucella* broth/FBS medium.

   Passage before viability begins to decline (OD of 0.2 to 0.3 or lower).

   The volume of the aliquot used for passage is highly variable and must be based on the density of the culture and how soon one would like the new culture to reach saturation. Generally, 100 to 200 μl of a healthy culture will generate a saturated culture within 24 to 48 hr. At this point, larger cultures can be started by using 60 ml medium in a 75-cm^2 flask or 100 ml in a 150-cm^2 flask.

CULTURE OF *HELICOBACTER* SPECIES OTHER THAN *H. PYLORI* IN LIQUID MEDIUM

ALTERNATE PROTOCOL

Static liquid cultures able to grow under standard tissue culture incubator conditions have only been described for *H. pylori* (see Basic Protocol 2). Other gastric *Helicobacter* species such as *H. felis* require continuous shaking; therefore, alternative methods for growth are required. Unavailability of a CO_2 incubator or unwillingness of colleagues to allow the use of a tissue culture incubator for growth of bacterial cultures (because of concerns about contamination) may also favor alternative strategies. Most gastric pathogens are readily grown in the same liquid culture medium described in Basic Protocol 2 when placed in a suitable flask within an anaerobic jar on a rotary shaker.

Additional Materials *(also see Basic Protocol 2)*

Automatic pipetting device (e.g., Drummond Pipet-Aid or, for volumes ≤1 ml, Gilson Pipetman or equivalent automatic pipettor)
5-ml culture tubes
500-ml Erlenmeyer or tissue culture bottles that fit inside BBL model no. 100 anaerobic jar
Anaerobic jar(s) with sealable lid(s) (BBL model no. 100 or equivalent)
GasPak: BBL CampyPak Plus Microaerophilic System Envelopes with Palladium Catalyst (Becton Dickinson) or equivalent
37°C rotary shaker with 2-liter brackets to accommodate a BBL model no. 100 anaerobic jar

Additional reagents and equipment for quantification of *Helicobacter* (see Basic Protocol 2; see Basic Protocol 3 for *H. felis*) and examining wet mount of *Helicobacter* (Support Protocol 4)

1. Place 1 ml *Brucella* broth/10% FBS medium on to the center of an *H. pylori* plate culture.
2. Using a sterile bacterial spreader, lift *H. pylori* from plate by continuous gentle raking of the surface. Slightly tip plate forward and gather liquid towards the front rim.
3. Transfer harvested bacteria into a 5-ml sterile tube using an automatic pipetting device.
4. Place an aliquot equivalent to the bacteria harvested from one-half of a plate (0.5 ml) into a sterile 500-ml Erlenmeyer flask containing 100 ml Brucella medium/FBS.

   It is also possible to passage a small volume of an existing, actively growing liquid culture. The inoculum will vary between 1 and 10 ml depending on the viability of the culture and the speed at which one wishes new growth to occur.
5. Place the flask containing the inoculated medium within an anaerobic jar.
6. Cut the corner off of the GasPak envelope, separate the edges, and place inside the anaerobic jar with the catalyst facing the outside. Add 10 ml water to each envelope, then seal the top of the anaerobic jar.
7. Seal the jar and place in a 2-liter flask bracket on an orbital shaker at 37°C.
8. Incubate with shaking at 125 rpm for 24 to 48 hr.
9. At the end of this incubation, or earlier, remove an aliquot of the culture and determine optical density (see Support Protocol 2; see Support Protocol 3 for *H. felis*).

   An optical density of 0.1 at 550 nm should yield ~10^7 cfu/ml, but this should be verified by means of one's own growth curve (see Support Protocol 2).
10. Prepare a wet mount (see Support Protocol 4) and examine for motility and absence of contamination with other bacteria.

SUPPORT PROTOCOL 2

QUANTIFICATION OF *HELICOBACTER* ORGANISMS

Both *H. pylori* and *H. mustelae* will form individual colonies when grown on a blood-containing agar base. Thus, serial dilution and counting of colonies can be use to quantify both *H. pylori* and *H. mustelae*. However, for speed and convenience it may be more efficient to prepare a growth curve so that the concentration of bacteria can be determined simply by measuring optical density while growing as a liquid culture.

The following method uses *H. pylori* as an example; however, other *Helicobacter* species can also be quantified using this procedure, with the exception of *H. felis*, which should be treated as described in Support Protocol 3.

Materials

Brucella broth or other suitable liquid medium supplemented with 10% (v/v) heat-inactivated FBS
Actively growing liquid culture of *H. pylori* (see Basic Protocol 2 or Alternate Protocol)
Freshly prepared blood agar plates containing appropriate antibiotic (see Support Protocol 1)
25-cm^2 tissue culture flask
37°C, 5% CO_2 incubator

Spectrophotometer with visible wavelength spectrum, preferably with adapter for microcuvette
Cuvette or microcuvette
Sterile tubes for preparing dilutions
Disposable bacterial spreaders (PGC Scientific) or laboratory-made glass spreaders

Additional reagents and equipment for microaerophilic plate culture of *Helicobacter* (Basic Protocol 1)

1. Place 10 ml *Brucella* broth/5% FBS media in a 25-cm^2 tissue culture flask and inoculate with 100 μl of a log-phase culture of *H. pylori*. Place in a 37°C, 5% CO_2 incubator.
2. Several times each day, resuspend the bacteria uniformly by swirling the flask, remove a 100-μl aliquot, and transfer to a cuvette or microcuvette for the spectrophotometer.

   Depending on the viability of the starting culture, it may take 1 to 3 days, or even longer, to complete the growth curve.

3. Determine the OD_{550} of the culture using *Brucella* broth/10% FBS as a blank.
4. Using sterile tubes, prepare serial 10-fold dilutions of the aliquot from $1:10^2$ to $1:10^5$.
5. Inoculate 10 μl of each dilution onto a blood agar plate supplemented with antibiotics and spread uniformly using a bacterial spreader until no liquid remains on the plate. Mark each plate or fraction of plate used with the time point and dilution.

   To reduce the number of plates required, each plate can be divided into half or even thirds using a marker on the bottom of the plate and distributing each 10-μl inoculum only within the marked boundaries.

6. Cover plates and place in anaerobic jars with GasPaks as described in Basic Protocol 1.
7. Repeat steps 2 to 6 with the liquid cultures until OD_{550} approaches 0.5.
8. Evaluate plates at 4 to 5 days and enumerate colonies, back-calculating for dilution and volume to determine the number of colony forming units (cfu) per milliliter.

   For example, if 10 μl of a 1:1000 dilution yields 25 colonies, then there are $100 \times 1000 \times 25$ cfu $= 2.5 \times 10^6$ cfu per milliliter of the original sample.

9. Plot OD_{550} versus cfu to obtain the growth curve.

   As noted above the authors have found that an OD_{550} of 0.1 generally yields $\sim 10^7$ cfu in their laboratories. However, the specific conditions in another laboratory could be quite different, so it is best to complete one's own growth curve.

10. Repeat this procedure at least twice and average the results in order to obtain a reasonably accurate growth curve.

QUANTIFICATION OF *HELICOBACTER FELIS*

SUPPORT PROTOCOL 3

H. felis will not reliably form individual colonies, and even when individual colonies are observed with *H. felis*, these colonies may under-represent the number of viable organisms present. To attempt an estimation of the number of "cfu" of *H. felis* in stock cultures, the authors have used two approaches. The first is to use an *H. pylori* growth curve as a surrogate for an *H. felis* growth curve. Therefore, if the *H. pylori* growth curve indicates that an OD_{550} of 0.1 equals 10^7 cfu of *H. pylori*, a reasonable extrapolation would be that an OD_{550} for a culture of *H. felis* would also represent $\sim 10^7$ cfu of *H. felis*. The second approach is to prepare a subconfluent culture of *H. felis* and make serial

dilutions of this culture into *Brucella* broth. Pipet an aliquot of appropriately diluted culture into a hemacytometer (*APPENDIX 4A*) and quickly count the number of motile organisms at 400× total magnification. This is difficult, as a high-power microscope objective must be used and *H. felis* bacteria are highly motile and can move into and out of the hemacytometer grid squares. The authors have found that these two methods give numbers which are in reasonable agreement with each other.

BASIC PROTOCOL 3

PREPARING CLINICAL BIOPSIES FOR CULTURE OF *HELICOBACTER PYLORI*

Gastric pinch biopsy material is routinely taken during endoscopy from subjects presenting with symptoms of active gastritis or peptic ulcer disease. Along with identification of *H. pylori* in histologic tissue sections, culture of viable *H. pylori* remains the definitive test for a positive diagnosis of *H. pylori* infection. Additionally, the tremendous genetic and phenotypic diversity between *H. pylori* isolates provides incentives for continuing to isolate novel clinical isolates for further experimental analysis in the laboratory. *H. pylori* can be recovered from the gastric biopsy material of infected subjects by homogenizing the tissue and inoculating nutrient blood agar plates. The use of a urease test broth (see Support Protocol 4) to store or transport the biopsy can provide preliminary evidence of the presence of *H. pylori* if the indicator dye turns bright red within 24 hr of retrieval.

Usually, several pinch biopsies can be obtained from each subject during endoscopy. One or more biopsies are placed into a single tube for culture. In addition to culture, it is generally useful to send additional biopsies to a histology lab for embedding, sectioning, and staining. These biopsies should be collected into 10% buffered formalin. If sufficient biopsies are available, it may also be useful to deposit one or more into a freezing vial and then immerse into liquid nitrogen and store at –80°C or lower, e.g., for RNA isolation.

Materials

Gastric pinch biopsy material
Transport medium (see recipe) in sterile tubes
Brucella broth or other suitable liquid medium supplemented with 10% (v/v) FBS
Freshly prepared blood agar plates containing appropriate antibiotic (see Support Protocol 1)
Sterile forceps
Sterile disposable pellet pestles and 1.5-ml microcentrifuge tubes (Kontes, cat no. 749520)
Disposable bacterial spreaders (PGC Scientific) or laboratory-made glass spreaders
Additional reagents and equipment for microaerophilic plate culture of *Helicobacter* (Basic Protocol 1)

1. Retrieve gastric biopsy material from clinical laboratory in transport medium.

   Biopsies should be obtained for culture as soon as possible after endoscopy, although the authors have had some success in culturing from biopsies stored overnight at room temperature in transport medium.

2. Using sterile forceps, remove biopsy pieces from the transport medium tubes and place in sterile 1.5-ml microcentrifuge tube (provided along with a disposable pellet pestle) with 0.1 ml *Brucella* broth/10% FBS.

3. Grind the tissue using the sterile disposable pellet pestle and add an additional 0.1 ml *Brucella* broth/FBS.

4. Inoculate 10 µl of the homogenate onto a blood agar plate with the appropriate selective antibiotics and spread with inoculating loop or spreader.

5. Cover plates, place in anaerobic jars with GasPaks, and culture as described in Basic Protocol 1, steps 3 to 5.

CONFIRMATION OF *HELICOBACTER PYLORI* GROWTH

SUPPORT PROTOCOL 4

Although the use of selective antibiotics will greatly reduce the growth/isolation of non-*Helicobacter* organisms from animal stomachs, it is still necessary to be sure that the organisms recovered are in fact a *Helicobacter* species. While nucleic acid fingerprinting or PCR procedures can be employed for this, there are also simple morphologic and biochemical tests which can be used to screen suspected *Helicobacter* growth on plates. This is done by using an inoculating loop to pick material from a plate with visible growth and then examining the morphology of the organisms by phase-contrast microscopy of a wet mount and/or of Gram-stained organisms (*APPENDIX 3C*). Additional confirmatory procedures include testing the bacteria for urease, catalase and oxidase activity; these techniques are also described below.

All of the gastric *Helicobacter* species produce large amounts of the enzyme urease, which can cleave urea into carbon dioxide and ammonia. The ammonia is thought to buffer the acidity of the stomach and thus aid the bacteria in its colonization. It is possible, particularly in experimental animals, for other urease-positive micro-organisms (e.g., *Proteus* species) to colonize the stomach. Thus, the combination of colony morphology, urease test, oxidase test, catalase test, and spiral morphology/motility in a wet mount are used to confirm the identity of *H. pylori*. As noted above, nucleic acid fingerprinting and PCR techniques can be used for further verification.

Wet mount/morphology

Viable *Helicobacter* species will be highly motile, and if enough bacteria are placed on the slide they can be easily located by phase-contrast microscopy at 400× to 1000× total magnification. *H. felis* is relatively long and possesses a distinct corkscrew morphology with tight spirals. *H. pylori* and *H. mustelae* are shorter with less distinct spirals. In general, they have a comma- or gull wing–like shape. In a healthy culture, the bacteria will appear to be "swimming" about quite rapidly. An overgrown culture may yield an optical density in the desired range, but observation of low or no mobility will indicate lack of viability. Thus it is important to always check cultures for morphology and motility.

Urease test

Place a loopful of material into 0.3 ml Stuarts urease test broth (see recipe). The broth should turn pink within minutes.

Catalase test

Place a loopful of material into 0.5 ml of 3% H_2O_2. Viable *Helicobacter* will form bubbles.

Oxidase test

Either place a loopful of material into a drop of distilled water on a Bacto Differentiation Disk Oxidase (BD Biosciences, cat. no. 1633) or place a disk over a colony or area of growth on a plate with a drop of distilled water. A pink or maroon color within 10 to 20 min, eventually changing to almost black, indicates a positive test.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

Stuart's urease test broth

0.1 g yeast extract
0.091 g KH_2PO_4
0.095 g Na_2HPO_4
20.0 g urea
0.010 g phenol red
1 liter distilled H_2O
Adjust pH to 6.9 using a small quantity of HCl

Note that this recipe has 100-fold less buffering capacity than some commercially available urease test broths. If the test broth is used with a higher buffer concentration, it will be less sensitive and will give many false negatives. The authors usually make only 500 ml at a time and then use part of that as transport medium (see recipe).

Transport medium for Helicobacter

To Stuart's urease test broth (see recipe), add 20 g glucose or sucrose per 100 ml. Filter sterilize and store up to several months at 4°C.

COMMENTARY

Background Information

Helicobacter pylori is a Gram-negative microaerophilic spiral bacterium which predominantly inhabits the antral region of the human stomach (Marshall and Warren, 1984; Suerbaum and Michetti, 2002). While *H. pylori* can be found in the body or fundus of the stomach or in the gastric metaplasia of the duodenum, it seems well adapted only to gastric tissues. It is not found, for instance, in normal small or large intestine or in the esophagus. The organism resides within the mucus layer of both surface and glandular epithelium. This ecologic niche serves to protect the organism from the harsh environmental conditions of the stomach and from host effector mechanisms. However, despite the fact that host responses are ineffective in clearing the organism, *H. pylori* induces a state of active/chronic gastric inflammation (involving both neutrophils and lymphocytes) in all infected individuals. While there is no overt disease in most infected people, a subset of infected individuals may develop peptic ulcers, gastric cancer, or gastric mucosa–associated lymphatic tissue (MALT) lymphoma. The outcome of infection is determined by a complex interplay between bacterial virulence factors and the host response. Individuals with high gastric acid output who are infected with virulent *H. pylori* strains may be predisposed towards antral-predominant infections and gastritis leading to peptic ulcer disease, while those individuals with lower gastric acid are prone to infection of both the antrum and body of the stomach, and to pangastritis, atrophy of glandular and parietal cells in the body/fundus of the stomach, and gastric adenocarcinoma.

Assessment of the *H. pylori* genome has revealed a great deal of diversity between isolates (Jiang et al., 1996; Tomb et al., 1997; Suerbaum, 2000). Thus, although the complete genomes of two distinct isolates have been published, a hypervariable region of the chromosome has been identified that can house up to 7% of the total number of genes (Alm et al., 1999). Many of these genes are specific to individual isolates. This can make complete characterization of the *H. pylori* phenotype difficult. Additionally, although plasmids have been identified in *H. pylori* strains, few stable *Helicobacter* plasmids suitable for cloning and expression techniques have been described or developed (Kleanthous et al., 1991; Stanley et al., 1992; Bereswill et al., 2005). Introduction of genes directly into the chromosome can be accomplished via natural transformation or electroporation with *E. coli* cloning vectors, but the transformation efficiency of *H. pylori* strains varies dramatically; therefore, specific isolates must be identified that facilitate genetic manipulation (Tsuda et al., 1993).

From a host defense and pathogenesis point of view, animal models for *H. pylori* infection are useful in helping to understand disease processes and vaccine development (Nedrud, 1999). Therefore, a future unit in this series will detail protocols for infecting mice with *Helicobacter felis* and *Helicobacter pylori* and for infecting ferrets with *H. mustelae* and characterizing the bacterial load and gastric pathology.

The procedures in this unit describe how to grow *Helicobacter* organisms using plates or liquid cultures, how to quantify *Helicobacter* species, and how to recover *H. pylori* from clinical biopsies. *Helicobacter* species tend to grow well on media previously established for other enteric fastidious organisms (e.g., see *APPENDIX 2C*), although the addition of animal blood or sera is often required. The growth of pure cultures of *Helicobacter* species has facilitated the study of *Helicobacter* genetics, microbiology, virulence, and immunology. Since *Helicobacter* species are associated with a growing number of gastric and extragastric pathologies, the ability to grow *Helicobacter* species successfully can be an important skill.

Critical Parameters and Troubleshooting

H. pylori and other *Helicobacter* species are considered fastidious organisms which can be difficult to isolate and propagate. Both temperature and oxygen tension are critical parameters for optimal growth. If inoculation of plates from a frozen stock is unsuccessful, the condition of the anaerobic jar and the integrity of the seal when assembled should be tested. However, even if no problems can be identified, it is worthwhile to try again, since establishing the initial culture can be difficult (see Anticipated Results). Similarly, it is not uncommon for established cultures to lose viability and fail to grow when passaged. The jar seal should be checked to make sure that the proper oxygen tension is being obtained. The freshness/moisture of the plates being used to passage the bacteria should also be assessed.

Although bacterial contamination of pure cultures is rare when proper technique is utilized, the presence of mold can be more problematic. Generally, mold contamination occurs under two circumstances. First, plates often get contaminated when they are examined for growth and then placed back in the anaerobic jars for further growth. This is in large part due to removal of the plate lid to get a better look at the colony morphology. The second source of mold contamination is often due to the collection of water on the lids of inverted plates. When the water collects between the edge of the plate and lid, it effectively forms a bridge to the outside of the plate. If mold spores are present inside the jar, they may get inside the plate by this water route. A solution is to maintain the plates right side up, but the collection of water droplets on the agar itself can compromise the ability of the bacteria to grow as isolated colonies, and, if plates are being grown for quantitative counts, reliable numbers may be difficult to obtain. Disinfecting the bell jars between use can also help to reduce mold growth, as can the inclusion of an anti-fungal (amphotericin B) in the growth medium.

If cultures (either plate or liquid) overgrow, the bacteria may die or become coccoid in nature. Although coccoid organisms may not technically be dead, their viability can be difficult to determine. Overgrown, dying, or coccoid cultures will yield a measurable optical density; however, this optical density will not be a true reflection of number of fully viable cfu of *Helicobacter* organisms. Thus, examination of bacterial cultures by wet mount for active motility and expected morphology (see Support Protocol 4) is a critical parameter. When using a growth curve and the optical density to estimate the number of organisms present, it is critical to be on the "up side" of the growth curve rather than on the "down side" or plateau. *Helicobacter* organisms grow much more slowly than many other bacteria, and it is important to check their progress periodically to avoid waiting too long before passaging them or using them for in vitro analysis.

Except for disease conditions characterized by low stomach acidity, where gastric bacterial overgrowth by many species can occur, *Helicobacter* species are usually the only bacteria that can successfully colonize the human stomach. In coprophagic animal species such as mice, however, numerous bacterial species can routinely be cultured from stomach tissues. Thus, recovering clinical or experimental *H. pylori* isolates from gastric biopsies can be associated with contamination by other bacterial species. Normally, few bacteria survive in the gastric mucosa, but when contaminants are present, *Proteus vulgaris* may be suspected. It tends to be resistant to the common *H. pylori* selective media. This is further complicated by the fact that *Proteus* is one of the few enteric bacteria that also produces a urease enzyme, often leading to false-positive urease tests. *Proteus* also tends to grow quickly and forms large sheets, effectively covering any *H. pylori* that might be present as small isolated colonies. Therefore, it is important to plate many dilutions of clinical biopsy homogenates in the hopes that *H. pylori* can be isolated from the background of *Proteus* growth. The combined results of colony morphology observation and the urease, catalase, and oxidase tests, as well as the distinctive morphology (spiral or gull-wing shape) and high motility of *Helicobacter* organisms observed in a wet mount is

usually sufficient to distinguish *Helicobacter* species from *Proteus* species.

Anticipated Results

Establishing viable cultures of *H. pylori* and other *Helicobacter* species may take several attempts, especially when starting from a frozen stock. However, if multiple plates are inoculated or repeated attempts are made, viable cultures can be obtained as long as the anaerobic jars are properly sealed. Performing subsequent passages from a viable culture is generally much more reliable and the setup is easy to maintain. One can expect to observe fairly large and healthy colonies every 3 to 4 days once these cultures are growing. Likewise, once liquid cultures are established, one should expect to passage cultures every 1 to 2 days. Even if microscopic examination reveals dense growth but minimal motile organisms, these cultures may still be viable and suitable for inoculation into fresh media.

Recovering viable *H. pylori* from clinical gastric biopsies can be challenging. It is common for laboratories that perform this function on a regular basis to fail to isolate *H. pylori* even when the rapid urease test is positive. The site of biopsy source, freshness of the biopsy, antibiotic treatment of the subject, and bacterial density within the biopsy can all effect the success of isolating *H. pylori* from human tissue.

Time Considerations

The time necessary to culture *Helicobacter* species on solid media will vary, depending on whether or not blood agar plates have been prepared in advance. It will also vary with the number of plates to be inoculated. In general, plates can be prepared and ready for culturing in several hours. Preparation of the medium and autoclaving takes less than an hour. The two most time-consuming steps are allowing the temperature of the media to cool to 56°C, and allowing the medium to gel adequately following pouring of the plates. The whole procedure can be accomplished in one morning or afternoon. The actual inoculation of plates and preparation of the jars for incubation take only a few minutes.

The number of bacteria in a stock culture can be approximated in a few minutes by measuring the OD_{550} and referring to a growth curve. Rigorous determination of cfu takes longer, but by using the OD and a growth curve, one or two dilutions can be picked for inoculation onto plates, which takes only a few minutes. The plates must then be incubated for several days, but actual counting of colonies after they appear also takes only a few minutes. Homogenization of tissue biopsies and inoculation of the relevant plates will take a few minutes per biopsy. Counting colonies on plates should only require 1 or 2 min per plate unless a very large number of colonies are present. Likewise, testing the cultures for urease, catalase, and oxidase can be performed in a few minutes, provided that the necessary reagents are prepared ahead of time.

Liquid media can be prepared more quickly than solid media because there is no need to pour plates and allow them to gel. It should only take 2 to 3 hr to prepare, sterilize, and cool the medium (to anywhere between 37°C and room temperature) and much of this time is spent waiting, so that other tasks can be performed concurrently. The medium (without serum or antibiotics) can be prepared in advance and stored for at least 1 month. Adding stock solutions of antibiotics and serum and inoculation of the media with *Helicobacter* organisms takes only a few minutes. Preparation of stock antibiotic solutions can be done in 30 min, if necessary.

Construction of a growth curve for liquid cultures requires 1 to 2 weeks, but a large part of the time is spent waiting for the cultures to grow. The actual hands-on time is much shorter, and once the growth curve is prepared under standardized conditions it need not be repeated unless laboratory conditions change dramatically. The first step in a growth curve is to inoculate a liquid culture. This takes only a few minutes. Then, at regular intervals (from every 30 min to several times per day depending on the rate of growth), the optical density must be determined for an aliquot of the liquid culture, which then also needs to be serially diluted and inoculated onto a fresh series of plates. This procedure may take 1 to 2 hr per day. The fresh plates then need to be incubated for several days until individual colonies are visible. If no colonies are yet visible, the plates are returned to the incubator. When colonies become visible, they must be counted. Simply checking the plates takes only a few minutes, but counting colonies can take 1 to 2 hr depending on how many plates were incubated at the same time. Because the whole procedure will have to be repeated to obtain a reasonable average, it can stretch over perhaps 2 weeks, with actual hands-on time consuming 8 to 16 hr; even more time may be required if the culture plates have not been prepared in advance. Since this is a time-consuming and labor-intensive process, it pays to take great

care in doing it and to think twice about altering culture conditions in a way that would require constructing a new growth curve. Note also that, at some time point, the optical density of the growth curve may continue to rise, but that the number of viable cfu will plateau and then decrease.

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Internet Resources

http://www.helico.com

Web site for detailed information about H. pylori history, epidemiology, and pathogenesis.

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Biosafety in Microbiology and Biomedical Laboratories 4th Ed May 1999. Contains details for handling bacteria of differing safety levels and separate sections for individual pathogens such as H. pylori.

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Animal Models of *Listeria* Infection

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ABSTRACT

Listeria monocytogenes is an intracellular foodborne pathogen that causes listeriosis, an infection characterized by gastroenteritis, meningitis, encephalitis, and maternofetal infections in humans. *L. monocytogenes* enters the host via contaminated foods, invades the small intestine, translocates to mesenteric lymph nodes, and spreads to the liver, spleen, brain and, in pregnant women, the fetoplacental unit. Many pathogenicity tests for studying *L. monocytogenes* have been developed, including tests using laboratory animals. A number of small animal species can be experimentally infected with *Listeria*. Mice and guinea pigs can be infected either intragastrically or intravenously, and virulence evaluated either by enumerating bacteria within infected target organs or by evaluating the 50% lethal dose (LD50). Although mice and guinea pigs can be infected with *Listeria* by a variety of routes, the intragastric route is the most relevant to the human foodborne listeriosis. *Curr. Protoc. Microbiol.* 10:9B.1.1-9B.1.17. © 2008 by John Wiley & Sons, Inc.

Keywords: *Listeria monocytogenes* • listeriosis • foodborne pathogen • infection • animal model

INTRODUCTION

Listeria monocytogenes causes listeriosis, a foodborne infection characterized by bacterial dissemination from the intestinal lumen to the central nervous system and the fetoplacental unit. Many pathogenicity tests for studying *L. monocytogenes* have been developed, including tissue culture assays, fertilized hen egg tests, and tests using laboratory animals. A number of small animal species, including mice and guinea pigs, can be experimentally infected with *Listeria*, the mouse species being the most frequently used. Although mice and guinea pigs can be infected with *Listeria* by a variety of routes, the intragastric route is the most relevant to human foodborne listeriosis. Mice can be infected either intragastrically (see Basic Protocol 1) or intravenously (see Basic Protocol 2), and virulence evaluated either by numerating bacteria within infected target organs (see Basic Protocol 3) or by evaluating the 50% lethal dose (LD50; see Basic Protocol 4). The infectious process following intragastric inoculation of *Listeria* can be also assessed in the guinea pig model (see Alternate Protocol).

As a proper cultivation of *L. monocytogenes* is a critical first step for the success of in vivo experiments, a method for preparing *Listeria* inoculum is also described (see Support Protocol).

All strains of *Listeria monocytogenes* are not equally virulent (Barbour et al., 2001; Larsen et al., 2002; Roche et al., 2005). Since the *L. monocytogenes* reference strain *EGDe* genome sequence has been determined (Glaser et al., 2001) and *EGDe* is used by a large number of laboratories, protocols presented here use this strain, but other *Listeria* strains (wild types and mutants) can also be used. The animal model, its genetic

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background, and the route of infection are critical for the outcome of the infection (Cheers et al., 1978; Barbour et al., 1996; Lecuit et al., 2001; Czuprynski et al., 2003; Khelef et al., 2006; Cossart and Toledo-Arana, 2008. Procedures presented here use *iFABP-hEcad* mice and Hartley guinea pigs for intragastric infection, and BALB/c mice for intravenous infection.

CAUTION: Listeria monocytogenes is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for use and handling of pathogenic microorganisms.

CAUTION: These experiments require Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines and regulations for use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

NOTE: The proper handling and restraint of animals needs to be mastered before any inoculations. The experimenter needs to be fully aware of inherent dangers such as accidental needle stick when performing these techniques.

NOTE: The procedure requires familiarity with basic methods for animal care, handling, and restraint (see Donovan and Brown, 2006a).

IMPORTANT NOTE: The success of these protocols is dependent on proper use of aseptic techniques.

BASIC PROTOCOL 1

MOUSE INTRAGASTRIC (IG) INFECTION

In order to increase the permissiveness of the mouse following oral inoculation, a transgenic mouse model expressing human E-cadherin in enterocytes was generated. In this transgenic model, *L. monocytogenes* directly targets enterocytes. This leads to *L. monocytogenes* internalization into these cells and allows the subsequent crossing of the intestinal barrier, followed by bacterial multiplication in the small intestine lamina propria, and dissemination to mesenteric lymph nodes, liver, and spleen (Lecuit et al., 2001).

For this assay, bacteria resuspended in phosphate-buffered saline to the appropriate concentration are inoculated by intragastric gavage into slightly anesthetized mice. This infection route, used at the recommended dose, establishes a sublethal systemic infection that is a suitable model of systemic listeriosis allowing bacterial enumeration in infected organs (see Basic Protocol 3). For LD50 determination, several doses of inoculum must be administered to several groups of mice (see Basic Protocol 4).

Materials

6- to 8-week-old sex-matched *iFABP-hEcad* mice
Bacterial inoculum (Support Protocol)
Phosphate-buffered saline (PBS), sterile
Brain heart infusion (BHI) agar plates
150 mg/ml $CaCO_3$ in PBS, prepare fresh

Housing cages for the mice
Personal protective equipment (e.g., laboratory coat, gloves, and eye protection)
37°C incubator
Sterile 1-ml syringe with a long, bulbous-ended needle (e.g., animal feeding needle 20-G × $^1/_2$ in. with silicon tip)

Additional reagents and equipment for the anesthesia of rodents (Donovan and Brown, 1998)

Prepare animals

1. Acclimate 6- to 8-week-old sex-matched mice to the animal facility environment for at least 1 week prior to infection.

   Animals should be acclimated under standard lighting and temperature conditions with food and water.

2. Starve animals (giving only water) for 12 hr before infection.

   This is done to prevent the delivery of the inoculum into the stomachs of mice that were engorged with mouse chow, which could lead to aspiration of the inoculum in the lung, and also to diminished variability between animals.

3. Just before injection, thaw an aliquot of bacteria on ice. Dilute bacteria appropriately in sterile PBS to achieve 0.2 ml per mouse at 2.5×10^{10} cfu/ml.

   The standard dose recommended for L. monocytogenes EGDe via the intragastric route is 5×10^9 cfu per mouse, delivered in a volume of 0.2 ml. However, lower inocula (10^9, 10^8) also lead to systemic infection, with highly reproducible differences between wild-type (wt) bacteria and isogenic mutants.

4. To confirm the titer of the starting suspension, plate, in duplicate, 0.1 ml of serial dilutions (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}) on sterile BHI agar plates, and incubate the plate 24 to 48 hr at 37°C.

5. Fill the syringe with an inoculum of 0.2 ml containing the bacteria number to be injected, mixed with 0.3 ml of 150 mg/ml $CaCO_3$ suspension in PBS.

   Use a 1-ml syringe with a long, bulbous-ended needle (e.g., animal feeding needle 20-G × 1-1/2 with silicon tip). The amount of material that can be injected orally is ~0.5 ml.

   $CaCO_3$ buffering is used to reduce the gastric acidity and to decrease inter-individual variability which depends upon gastric repletion and pH.

Perform inoculation

6. Remove the mouse from the cage by its tail.

   At this point, mice can be anesthetized (Donovan and Brown, 1998) before gavage. The standard inhalant anesthetics for laboratory animal use are either isoflurane or halothane, delivered to effect in concentrations up to 5% for initial induction.

7. Hold the mouse by the skin on either side of the base of the neck and exert a slight downward and forward pressure under the mandible to tilt the head up slightly, thus aligning the oral cavity and pharynx with the esophagus.

8. Insert the bulbous-ended needle over the tongue into the esophagus and stomach.

9. Slowly inject the inoculum.

   Agents to be administered must be in suspension or solution, and at room to body temperature.

10. After completing the injection, carefully withdraw the needle, place the mouse back into its cage, and continue injections with additional animals. When injections are complete, check the animals for normal behavior and then return them to their housing location with food.

    In case of anesthesia, carefully follow the full recovery of the animal.

11. Discard the needle and syringe and disinfect work instruments and area.

Animals are then either observed every day for determination of mortality (see Basic Protocol 4), or they are euthanized and dissected for determination of bacterial counts from organs (see Basic Protocol 3).

Perform calculations

12. One or two days after incubation at 37°C, count colonies on plates (step 4) and calculate the initial titer of the suspension as follows: cfu/ml = MCC/(VP × DF).

    Where MCC is the mean colony count of replicate countable plates, VP is the volume plated, and DF is the dilution factor of the sample in the plate.

13. Calculate the *Listeria* load delivered by the IG inoculation as: cfu = cfu/ml × DF × VD.

    Where cfu/ml is the initial inoculum titer (step 11), DF is the dilution factor used to prepare the suspension (step 3), and VD is the volume delivered (step 3).

BASIC PROTOCOL 2

MOUSE INTRAVENOUS (IV) INFECTION

In nontransgenic mice, the intravenous route, as opposed to the oral route, can produce a lethal infection, and thus allows bacterial enumeration in infected organs but also the determination of the LD50 for various *Listeria* strains. The infection by intravenous route in the mouse model is widely used for immunological studies.

In this technique, bacteria resuspended in phosphate-buffered saline to an appropriate concentration are inoculated intravenously into lateral veins of the tail.

The IV injection route requires skill in locating one of the two lateral veins of the tail and making sure that the needle is inserted into the vein and not into the surrounding tissue.

This infection route, used at the recommended dose, establishes a sublethal systemic infection that is a suitable model for study of bacterial count in infected organs (see Basic Protocol 3). For LD50 determination, increasing doses of inoculum must be injected (see Basic Protocol 4).

For more details concerning intravenous injection of mice, see Donovan and Brown (2006b).

Materials

6- to 8-week-old female BALB/c mice
Bacterial inoculum (Support Protocol)
Phosphate-buffered saline (PBS), sterile
BHI agar plates

Personal protective equipment (e.g., laboratory coat, gloves, and eye protection)
37°C incubator
Housing cages for the mice
Heat lamp
Restraining device for mice (Harvard Apparatus)
Sterile 1-ml syringe with a small diameter needle (e.g., 26-G × $\frac{3}{8}$ in.)
Sterile gauze

Prepare animals

1. Acclimate 6- to 8-week-old female mice to animal facility environment for ≥1 week prior to infection procedure.

    Animals should be acclimated under standard lighting and temperature conditions with food and water.

2. Just before injection, thaw an aliquot of bacteria on ice. Dilute bacteria appropriately in sterile PBS to achieve 0.3 ml per mouse at 3.3×10^4 cfu/ml.

   The standard dose for L. monocytogenes EGDe via the intravenous route is 10^4 cfu per mouse, delivered in a volume of 0.3 ml.

3. To confirm the titer of the starting suspension, plate, in duplicate, 100 µl of serial dilutions (10^{-3}, 10^{-2}, 10^{-1}, 10^0) on sterile BHI agar plates, and incubate the plate 24 to 48 hr at 37°C.

4. Place the mouse cage under a heat lamp to allow gentle warming in order to promote vasodilation.

   This will increase circulation to the surface blood vessels (as the mouse regulates its body temperature) and expand the diameter of the veins in the tail. Time will depend on the intensity of the lamp. It is essential to observe the mice while they are warming and ensure that they do not become heat stressed while under the lamp. If mice appear to be agitated or heat stressed, remove the cage immediately from beneath the lamp.

5. While the mice are warming, set up a restraining device.

   Numerous devices have been described to facilitate intravenous injections into lateral tail veins, some of which can be obtained commercially (e.g., Harvard Apparatus). Basically, these consist of a cylinder of appropriate diameter, with adjustable length divider, and a slotted end for exteriorizing the tail.

Perform inoculation

6. Fill the syringe with the desired amount of inoculum to be injected. Remove any air bubbles from the syringe and needle.

   Use a 1-ml syringe with a small diameter needle (e.g., 26-G × $\frac{3}{8}$ in.). The amount of material that can be injected IV is ~0.3 ml. Absolutely no air can be accidentally injected, as this may cause a fatal air embolism.

7. When the mice are sufficiently warmed, remove a mouse from the cage by its tail. Holding on by its tail, place the mouse head first into the cylinder of the restraining device.

8. Locate one of the two lateral veins of the tail. Hold the tail with the thumb and first finger, curling the end of the tail slightly over the finger.

   For more details on the injection procedure, see Donovan and Brown, 2006b.

9. Line up the needle exactly in line with the vein and insert the needle into the vein.

   If the needle is in the vein the tip of the needle will be visible inside of the vein. It is desirable to make the first injection attempt as close to the tip of the tail as possible, so that a second attempt, if necessary, can be safely made more proximally or on the opposite vein.

10. Slowly inject the inoculum.

    If the needle is in the vein, the red color of the vein will fade for almost the entire length from the injection site to the tail base near the mouse body as the injected material temporarily flushes the blood. If the needle is not in the vein, a whitening around the injection site will swell up and the plunger will not move easily. Stop injecting and "try again" at a site more proximal (nearer) to the body or use the other lateral vein.

    CAUTION: *NEVER force the syringe if the vein was missed, as this may result in a potential aerosol of the bacterial suspension should the needle hub dislodge from either the syringe or the tail.*

11. After the injection, carefully withdraw the needle and use gauze to apply digital pressure at the injection site for hemostasis.

12. Place the infected mouse in a spare cage and continue injections with the other animals. When injections are complete, check the animals for normal behavior and then return them to their housing location.

13. Discard the needle and syringe and disinfect work instruments and area.

Animals are then either observed every day for determination of mortality (see Basic Protocol 4), or they are euthanized and dissected for determination of bacterial counts from organs (see Basic Protocol 3).

Perform calculations

14. One or two days after incubation of plates (step 3) at 37°C, calculate the initial titer of bacterial stock suspension (se Basic Protocol 1, step 12) and then calculate the *Listeria* load using the dilution factor of the IV suspension (see step 2 above).

ALTERNATE PROTOCOL

GUINEA PIG INTRAGASTRIC (IG) INFECTION

In the guinea pig animal model, *L. monocytogenes* is able to induce a gastroenteritis resembling that observed in humans following intragastric infection. Moreover, *L. monocytogenes* is able to induce a dose-dependent lethality following dissemination to the systemic circulation (Lecuit et al., 2001; Khelef et al., 2006).

In this protocol, bacteria resuspended in phosphate-buffered saline to an appropriate concentration are inoculated by intragastric gavage into anesthetized guinea pigs.

Guinea pigs almost never bite and, although very emotive, can be easily calmed and are very easy to handle. They should always be forewarned before being approached, either by being able to see or hear their would-be handler. They should be lifted by grasping them firmly and gently over their shoulders, with two fingers behind and two in front of the forelimb. The rump should be supported by the other hand when lifting. Essentially the same grip may be used to immobilize the animal, if the hind legs are grasped and extended and the animal is placed on its back on a table top or counter.

The IG infection route, used at the recommended dose, establishes a sublethal systemic infection that is a suitable model for study of bacterial count in infected organs (see Basic Protocol 3). For LD50 determination, increasing doses of inoculum must be injected (see Basic Protocol 4).

Two persons are needed for the infection, one handling the animal, the other one performing the injection.

Materials

300-g, sex-matched Hartley guinea pigs
Bacterial inoculum (Support Protocol)
Phosphate-buffered saline (PBS), saline
BHI agar plates, sterile
15 mg/ml ketamine in sterile PBS, prepare fresh
25 mg/ml $CaCO_3$ in PBS, prepare fresh

Personal protective equipment (e.g., laboratory coat, gloves, and eye protection)
37°C incubator
1-ml syringe with a small diameter needle (e.g., 26-G × $^3/_8$ in.), sterile
20-ml syringe
Sterile suction catheter (1.7-mm × 270-mm; Vygon, ref. no. 530.05)
Animal cages

Additional reagents and equipment for the anesthesia of rodents (Donovan and Brown, 1998)

Prepare animals

1. Acclimate 300-g, sex-matched guinea pigs to animal facility environment for ≥1 week prior to infection procedure.

   Animals should be acclimated under standard lighting and temperature conditions with food and water.

2. Starve animals (giving only water) for 48 hr before infection.

   This is done to prevent the delivery of the inoculum into engorged stomachs and also to diminish variability between animals.

3. Just before injection, thaw an aliquot of bacteria on ice. Dilute bacteria appropriately in sterile PBS to achieve 1 ml per animal at 10^{10} cfu/ml.

   The standard dose for L. monocytogenes EGDe via the intragastric route is 10^{10} cfu per animal, delivered in a volume of 1 ml.

4. To confirm the titer of the starting suspension, plate, in duplicate, 0.1 ml of serial dilutions (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}) on sterile BHI agar plates, and incubate the plate 24 to 48 hr at 37°C.

5. Remove the guinea pig from the cage.

6. For light anesthesia, inject guinea pig intramuscularly with 1 ml of 15 mg/ml ketamine in PBS.

   Use a 1-ml syringe with a small diameter needle (e.g., 26-G × $\frac{3}{8}$ in.). For guinea pigs, ketamine is usually administrated at 50 mg/kg.

7. Wait a few minutes for the anesthesia to take effect.

   The effect of the anesthesia can be determined by toe pinch method.

Perform inoculation

8. Fill a 20-ml syringe with 5 ml of 25 mg/ml $CaCO_3$ in PBS.

9. Fill a 1-ml syringe with 1 ml containing the desired amount of inoculum to be injected.

10. When anesthetized, hold the guinea pig by the skin on either side of the base of the neck and exert a slight downward and forward pressure under the mandible to tilt the head up slightly, thus aligning the oral cavity and pharynx with the esophagus.

11. Insert a suction catheter intragastrically until feeling a light resistance (∼15 cm).

    This procedure is facilitated if carried out by two experimenters. In our experience, home-made suction catheters should be avoided, because of the harmful effect of their distal extremity, which is not smooth enough.

12. Adapt the 20-ml syringe to the catheter and slowly inject intragastrically 5 ml of 25 mg/ml $CaCO_3$ solution.

    $CaCO_3$ is used to reduce the gastric acidity and to diminish variability between animals.

    Agents to be administered must be in suspension or solution, and at room to body temperature.

13. Replace the 20-ml syringe with the 1-ml syringe without removing the catheter, and slowly inject intragastrically 1 ml of inoculum.

14. After completing the injection, carefully withdraw the catheter and place the inoculated guinea pig in a spare cage and continue injections with additional animals.

    While the animal is anesthetized and nonreactive, pay attention not to put the animal's eyes or nose in contact with the sawdust or other bedding material.

15. When injections are complete, check the animals for full recovery and normal behavior after anesthesia, and then return them to their housing location.

16. Discard the needle, syringes, and the catheter. Disinfect work instruments and work area.

Animals are then either observed every day for determination of mortality (see Basic Protocol 4), or they are euthanized and dissected for determination of bacterial counts from organs (see Basic Protocol 3).

Perform calculations

17. One or two days after incubation of plates (step 4) at 37°C, calculate the initial titer of bacterial stock suspension (see Basic Protocol 1, step 12) and then calculate the *Listeria* load using the dilution factor of the IG suspension (see step 3 above).

BASIC PROTOCOL 3

ORGAN RECOVERY AND BACTERIAL ENUMERATION

After ingestion of contaminated food, *Listeria* crosses the intestinal barrier and infects intestinal cells. *Listeria* then disseminates via the lymph and the blood up to the liver and the spleen, and then to the brain and to the placenta after crossing the blood-brain and fetoplacental barriers. Following intravenous or oral inoculation of mice and guinea pigs, the spleen and liver represent the main targets of this pathogen, as the majority of the bacteria are found in these organs. Secondary dissemination to the brain and the placenta may also occur in these animal species, although it does not appear to be as frequent and efficient as it is in humans.

This protocol describes the procedure for harvesting the target mouse organs to be analyzed after infection by *Listeria* (i.e., the small intestine, the mesenteric lymph nodes, the liver, and the spleen), and for bacterial enumeration after organ homogenization. The technique described is applicable to the guinea pig model with minor modifications. Other organs can be harvested if required, for example the brain, the placenta in the case of a pregnant animal model listeriosis, or the lung in the case of pulmonary infection.

CAUTION: All processing of tissues derived from infected animals must be done in an appropriate laboratory equipped with a Class II biohazard hood. All homogenization of infected tissues should be done in the biohazard hood, which protects the user from the significant aerosols generated by tissue grinding and disruption techniques.

Materials

Infected 6- to 8-week-old sex-matched mice (Basic Protocol 1 *or* Basic Protocol 2)
70% ethanol in a spray bottle
Phosphate-buffered saline (PBS), sterile
Dulbecco's minimum essential medium (DMEM)
100 mg/liter gentamicin in sterile PBS, prepare fresh
Sterile Brain Heart Infusion (BHI) medium
BHI agar petri plates, sterile

Personal protective equipment (e.g., laboratory coat, gloves, and eye protection)
15- or 50-ml conical polypropylene centrifuge tubes, sterile
Sterile dissection instruments including:
- Medium dissection scissors
- Tissue forceps
- Small iris scissors
- Iris forceps
- Small-toothed forceps
- Surgical scissors
- Dissecting pins

Styrofoam or cork cutting board and pins
Paper towels
20°C incubator
Rotor-stator homogenizer (IKA UltraTurrax T25, or similar)

Additional reagents and equipment for the euthanasia of rodents (Donovan and Brown, 2006c)

Prepare work area

1. Prepare in advance sterile 15-ml tubes (mouse) or 50-ml tubes (guinea pig) to collect organs. Carefully label the tubes and place on ice.
2. Prepare sterile surgical instruments on a sterile surgical field.

Obtain organs

3. Euthanize the infected mouse by CO_2 inhalation (Donovan and Brown, 2006c). Carefully verify that the mouse is dead.
4. In a Class II biohazard hood, Place the mouse on to its back on Styrofoam or cork board covered with a paper towel, and wet its fur with 70% ethanol to minimize contamination with hair.
5. Pin extremities to Styrofoam or cork board to immobilize animal.
6. With forceps, grab hold of the skin above the urethral opening. Using small iris scissors, cut along the midline from the groin to the chin.

   Be careful to only cut the skin and not the muscle wall underneath.

7. While still grasping the lower peritoneum, make an incision from the start of the first incision to down near the knee on both sides of the animal.

   You should end up with an incision that looks very similar to an upside down Y. Take care not to cut the bladder or the intestines.

8. Peel the skin back from the muscle and peritoneal wall underneath.
9. Open up the abdomen by making an incision in the midline of the peritoneal wall and peeling it back.
10. Remove the spleen, which is behind the stomach and attached to the blood circulation by a single set of blood vessels (splenic artery and vein). Put the spleen into a 15-ml (mouse) or 50-ml (guinea pig) ice-cold empty sterile tube and place on ice.
11. With small-toothed forceps, grasp underneath the right lateral lobe of the liver to a point across the inferior vena cava and the hepatic hilum.
12. Use iris scissors to gently flip these lobes back over the forceps to grasp these lobes as well as the common bile duct.
13. With a firm hold on these tissues, make an incision with the scissors between the liver and the diaphragm and cut the inferior vena cava, freeing the liver.
14. Extricate the liver as one organ with a quick but gentle upward motion. Place the liver in a 15-ml (for mouse) or 50-ml (for guinea pig) ice-cold empty sterile tube and place on ice.
15. Using forceps, lift the intestines and move them to your left side.
16. Identify the bottom end of the stomach (pylorus), cut through the intestine and remove the entire small and large intestines.

17. Remove the whole mesenteric lymph nodes. Put the mesenteric lymph nodes in a 15-ml (for mouse) or 50-ml (for guinea pig) ice-cold empty sterile tube and place on ice.

    The mesenteric lymph nodes can be seen lying along the blood vessels in the fatty tissue that connects the intestine to the back wall of the abdomen.

18. Remove the whole small intestine of the mouse, or the central 20-cm-long portion of the guinea pig small intestine.

19. Open the small intestine longitudinally, remove the intestinal content and put the small intestine in a 15-ml (mouse) or 50-ml (guinea pig) ice-cold tube containing 5 ml (for the 15-ml tubes) or 10 ml (for the 50-ml tubes) of DMEM and place on ice.

20. Eliminate dead animals following appropriate guidelines and regulations for the elimination of infected animals.

21. Rinse the small intestine in sterile DMEM four times to remove all the luminal content.

    DMEM is preferable to PBS because it better preserves the integrity of the tissue, which is critical until the gentamicin treatment ends.

22. Incubate 2 hr at 20°C in DMEM containing 100 mg/liter gentamicin to kill extracellular bacteria from the intestinal lumen.

23. Rinse three times, each time in sterile DMEM. Put the small intestine in a 15-ml (mouse) or 50-ml (guinea pig) ice-cold tube and place on ice.

24. Add sterile PBS to each tube so that the final volume is equal for each organ series (usual final volume is 5 ml for mice organs, and 10 ml for guinea pig organs).

25. In a biohazard hood, grind the tissue and homogenize lysate using a rotor-stator homogenizer (IKA UltraTurrax T25, or similar) until sample is uniformly homogeneous, which usually takes 45 to 60 sec.

    Between samples disinfect the homogenizer tip with 70% ethanol and wash with sterile PBS. Disinfect the homogenizer and the work surfaces of the hood at the end of the homogenization.

26. Determine the number of viable bacteria released from the organs by performing serial dilutions of the organ homogenates in sterile BHI.

    The total number of dilutions depends on the expected bacterial loads within the organs and the time points post-infection.

27. Apply 0.1 ml of each dilution in duplicate to BHI agar petri plates, and incubate 24 to 48 hr at 37°C.

Perform calculations

28. After incubation, determine the bacterial load within the organ using the following formula: cfu/organs = (MCC × VH)/(VP × DFP).

    Where MCC is the mean colony count on duplicate plates, VH is the total volume of the homogenate, VP is the volume plated, and DFP is the dilution factor of sample in the plates.

DETERMINATION OF MORTALITY

BASIC PROTOCOL 4

The classical LD50 test is used to determine the lethal dose of an inoculum that will kill 50% of test animals (LD50). The inoculum is administered at increasing doses, usually a range of 5, to groups of 5 animals. Mortalities are recorded along the time, and the

LD50 is determined by statistical calculations. Although this procedure is very common it consumes a great number of animals, and must thus be thoroughly designed.

Materials

Bacterial inoculum (Support Protocol)
Phosphate-buffered saline (PBS), sterile
BHI agar plates, sterile
37°C incubator

Additional reagents and equipment for the euthanasia of rodents (Donovan and Brown, 2006c)

1. Just before injection, thaw an aliquot of bacteria on ice. Dilute bacteria appropriately in sterile PBS to achieve increasing doses to be administered.

   For BALB/c mice infected intravenously by L. monocytogenes EGDe, typically doses are 10^3, 10^4, 10^5, 10^6, 10^7 cfu per mouse.

2. To confirm the titer of the starting suspension, plate, in duplicate, 100 µl of serial dilutions on sterile BHI agar plates, and incubate the plate 24 to 48 hr at 37°C.

3. Infect animals using one of the inoculation protocols presented in this unit (see Basic Protocols 1 and 2).

4. Each day following infection, record mortality and eliminate dead animals following appropriate guidelines and regulations for the elimination of infected animals.

   The IACUC of some institutions would ask for euthanasia of moribund animals in an LD50 assay. Refer to your institutional guidelines for more information.

5. Two weeks after infection, count remaining live animals.

6. Euthanize the remaining animals by CO_2 inhalation (Donovan and Brown, 2006c). Carefully verify that animals are dead, and eliminate dead animals following appropriate guidelines and regulations for the elimination of infected animals.

7. Determine LD50 with the aid of statistical calculations (e.g., probit analysis; Finney, 1971).

PREPARATION OF BACTERIAL INOCULUM

SUPPORT PROTOCOL

The proper cultivation of *L. monocytogenes* is an essential first step for any experiment utilizing animal models. It is essential that the stock cultures be cultivated with standardized methods and media to ensure that individual strains have been cultivated under similar conditions. Also, to compare data from experiments with different strains of *L. monocytogenes*, it is critical that the strains be grown in the same culture medium and under the same cultural conditions. This protocol outlines the basic cultivation of *Listeria* for use in animal models. Stock cultures are prepared as specific lots with documented titer and preparation conditions.

Materials

Single colony of *Listeria* cells
Sterile Brain Heart Infusion (BHI) medium
Phosphate-buffered saline (PBS), sterile
BHI agar plates

250-ml Erlenmeyer flask
37°C shaking incubator
2-liter flask
50-ml polypropylene conical tubes, prechilled
Microtubes, prechilled

1. Inoculate a single colony of *Listeria* cells into 50 ml sterile BHI medium in a 250-ml Erlenmeyer flask. Grow overnight at 37°C with shaking (200 rpm).

   Alternatively, grow a 5 ml culture overnight in a test tube on a roller drum at 37°C.

2. Inoculate 4 ml of the culture into 400 ml sterile BHI medium in a sterile 2-liter flask. Grow at 37°C, shaking 200 rpm, to an $OD_{600\,nm}$ of 0.7 to 0.8.

3. Aliquot culture into eight 50-ml prechilled, sterile polypropylene tubes and leave the tubes on ice 5 to 10 min.

   Cells should be kept cold for all subsequent steps.

   Larger tubes or bottles can be used to centrifuge cells if volumes of all subsequent solutions are increased in direct proportion.

4. Centrifuge cells 15 min at 4000 × *g*, 4°C. Discard supernatant and resuspend each pellet in 20 ml ice-cold sterile PBS.

   Resuspension should be performed gently and cells kept on ice.

5. Centrifuge cells 15 min at 4000 × *g*, 4°C. Discard supernatant and resuspend each pellet in 20 ml ice-cold sterile PBS.

6. Centrifuge cells 15 min at 4000 × rpm, 4°C. Discard supernatant and resuspend each pellet in 0.5 ml ice-cold sterile PBS.

7. Dispense cells into prechilled, sterile microtubes (0.5-ml aliquots). Freeze immediately at −80°C.

   Bacteria can be stored at −80°C several weeks, but the number of bacteria per milliliter must be determined before each utilization.

8. To determine the actual number of bacteria per milliliter of the frozen inoculum, thaw an aliquot of the bacteria on ice, and plate 100 µl of serial dilutions, in duplicate, on BHI agar plates and incubate overnight at 37°C.

9. One or two days after incubation at 37°C, count colonies on plates and calculate the titer of the suspension as follows: cfu/ml = MCC/(VP × DF).

   Where MCC is the mean colony count of replicate countable plates, VP is the volume plated, and DF is the dilution factor of the sample in the plate.

10. Record the titer of each frozen lot.

COMMENTARY

Background Information

Listeria monocytogenes is an intracellular foodborne pathogen that causes listeriosis, an infection characterized by gastroenteritis, meningitis, encephalitis, and maternofetal infections in humans. *L. monocytogenes* enters the host via contaminated foods, invades the small intestine, translocates to mesenteric lymph nodes and spreads to the liver, spleen, brain and, in pregnant women, the fetoplacental unit. This bacterium has the ability to cross three tight barriers: the intestinal, the blood-brain, and the placental barriers. During infection, it enters, survives, and multiplies inside phagocytic and nonphagocytic cells (Khelef et al., 2004; Hamon et al., 2006; Cossart and Toledo-Arana, 2008).

The existence of animal listeriosis renders possible the use of animal species naturally infected by *L. monocytogenes* as models to study the pathophysiology of human listeriosis. However, this also implies important technical limitations: the animals developing a disease closely resembling human listeriosis are not classical laboratory animals such as the guinea pig or the mouse, but rather farm animals such as ovines, bovines, and caprines. Experimental listerial infections are routinely performed in mouse and guinea pig models. The use of mice and guinea pigs for the study of *L. monocytogenes* infections has been well documented and has a lengthy historical background (Khelef et al., 2004, 2006; Lecuit, 2007).

The mouse model of listeriosis is the most widely used both because of the huge number of immunological reagents available for this species, and because of its cost effectiveness. Mice require a relatively small area for housing as compared to guinea pigs, and a large number of animals can be housed with standard caging and racks. The growth of *L. monocytogenes* following either intragastric or intravenous inoculation is relatively well defined with regard to the distribution of growth in primary target organs, as well as subsequent dissemination into secondary organs (Audurier et al., 1980).

However, whereas the main route of infection by *Listeria* is the oral route, most studies using mice fail to induce a reproducible lethal infection after oral infection with *L. monocytogenes* (Gaillard et al., 1996; Lecuit et al., 2001; Manohar et al., 2001). In contrast to human E-cadherin, mouse E-cadherin is unable to promote entry of *L. monocytogenes* into intestinal cells. This specificity was shown to depend on the nature of a single amino acid of the mature E-cadherin peptidic chain, the sixteenth, which is a proline in humans and a glutamic acid in mice (Lecuit et al., 1999). In order to create a mouse model for orally acquired listeriosis, a transgenic mouse (*iFABP-hEcad)* expressing human E-cadherin was designed (Lecuit et al., 2001). In this transgenic model, *L. monocytogenes* directly targets enterocytes by interacting with enterocytic E-cadherin. The host response to listeriosis can now be studied in the mouse model in depth from its starting point, the intestinal lumen.

In this guinea pig animal model, contrary to what is observed in mice and rats, *L. monocytogenes* is able to induce a gastroenteritis resembling that observed in humans (Aureli et al., 2000). Moreover, *Listeria* is able to cross the intestinal barrier and induce a dose- and internalin-dependent lethality following dissemination to the systemic circulation (Lecuit et al., 2001), making guinea pig a good alternative model for the study of orally acquired listeriosis.

In mice, the intravenous route, as opposed to the oral route, can produce a lethal infection, and thus allows the determination of the LD50, and the comparison of the virulence of various mutants. This route of administration was particularly instrumental in the characterization of most *L. monocytogenes* virulence genes (Dussurget et al., 2004; Cossart and Toledo-Arana, 2008). In addition to the characterization of virulence genes, the infection by intravenous route in the mouse model is widely used in the study of cellular immunity against *Listeria* (Pamer, 2004; Zenewicz and Shen, 2007).

The consequences of foodborne listeriosis being particularly devastating during pregnancy (Mylonakis et al., 2002), and airway infections with *Listeria monocytogenes* being implicated in early onset neonatal listeriosis, the fetoplacental transmission of *Listeria* has been studied in the pregnant mouse or guinea pig (Abram et al., 2003; Bakardjiev et al., 2004). In mice, the reproducible infection of the placenta occurred when mice were intravenously infected at day 14 of pregnancy, and killed on day 1, 2, 3, or 6 post infection which corresponded to days 15, 16, 17, and 20 of pregnancy (Abram et al., 2003; Le Monnier et al., 2006). Alternatively, the pregnant guinea pig animal model can be used to study the fetoplacental transmission of *L. monocytogenes.* Pregnant female Hartley guinea pig of 42 to 52 days gestation are injected intravenously with 10^8 bacteria after subcutaneous injection of 0.05 mg/kg atropine and anesthesia with isoflurane (Bakardjiev et al., 2005). Pregnant guinea pigs can be also used to study the dose response to *L. monocytogenes* after oral exposure (Williams et al., 2007). However, mice and guinea pigs do not appear to be as susceptible as ovines, caprines, bovines, and humans to fetoplacental listeriosis (Lecuit, 2007).

While human listeriosis in most cases starts as an oral infection caused by contaminated food, focal infections are also observed. Especially in early onset neonatal listeriosis, high numbers of *Listeria* are detected in the lung of the newborn, most likely due to uptake of bacteria from contaminated amniotic fluid. Thus, although not observed in adults, the lung represents a potential port of entry for *L. monocytogenes*, at least in newborns contaminated in utero or during parturition. Listeriosis can be studied following intratracheal infection of mice. 10- to 12-week-old female mice were inoculated with 30 µl of bacterial inoculum via view-controlled intratracheal instillation through a catheter (0.7 to 19 mm) towards the bifurcation of the trachea to ensure exclusive delivery to the lungs. After intratracheal application, bacteria were predominantly localized in the peribronchiolar space and invaded alveolar macrophages but rarely lung epithelial cells. Dissemination from the lung into the deep organs started almost immediately after application, although a pulmonary bacterial reservoir remained during the first 4 days (Munder et al., 2005).

Several studies clearly demonstrate that susceptibility to listeriosis depends directly on the bacterial and animal strains used in an experiment. It was demonstrated that the genetic background plays a role in the susceptibility towards *L. monocytogenes*, since the BALB/c or A/J mice are more sensitive to *L. monocytogenes* than C57BL/6 following intravenous or intragastric inoculation (Cheers et al., 1978; Czuprynski et al., 2003). Conversely, the properties of an animal model may vary even among strains from a single bacterial species, as observed for the variation in infectivity of different *L. monocytogenes* strains (Barbour et al., 2001; Kim et al., 2004). These host and microbial differences have to be taken into account when comparing results from one study to the other. Before drawing any conclusion on the relative contribution of one host or bacterial factor in the infection process, one has to make sure that all the other host and microbial variables are equal. In addition, in contrast to strains from other *Listeria* serovars, serotype 4b epidemic strains appear to be able to cause systemic infection in normal mice infected orally. This suggests that there might be serovar specific virulence factors playing a role in mice susceptibility to orally acquired listeriosis (Czuprynski et al., 2003). These observations oblige to take into account carefully all the parameters of the host-pathogen couple for the design of an infection experiment.

Critical Parameters

As age- and sex-dependent factors affect susceptibility to *Listeria* infection (Ohara et al., 1985; Pasche et al., 2005), animals of the same sex and same age/weight must always be used to ensure experiment homogeneity and reproducibility.

The infection of mice or guinea pigs via the intragastric or intravenous route requires as a prerequisite the proper cultivation of stock *Listeria* cultures specifically for use in animal experimentation. It is essential that all bacterial stock cultures be cultivated under similar cultural and physiological conditions. The initial inoculum, temperature, aeration, agitation, and subculture times for different strains need to be standardized. The exact determination of the titer of the bacterial suspension inoculated is critical.

The proper handling, restraint, and injection of animals need to be mastered before any inoculations. The IV injection route requires skill in locating one of the two lateral veins of the tail and making sure that the needle is inserted into the vein and not into the tissue that surrounds the vein. Injection in the surrounding tissue results in a reduced inoculation dose and in an underestimated virulence of the strain used. Conversely, improper intragastric infection can result in small lesions of the esophagus, inducing direct blood contamination by the inoculum, and over-estimation of virulence.

Another common problem associated with the procedure is contamination. It is critical that aseptic techniques are maintained at all times, in particular during the recovery and the homogenization of organs, and the dilution and plating of homogenates.

The outcome of infection varies widely depending on the strain of animals, virulence of the strain of the pathogen, and the route of infection. Thus, these parameters should be carefully considered in the design of experiments that use animal models of *L. monocytogenes*.

Anticipated Results

Bacterial stock suspensions grown on BHI as described reach titers in the range of 10^{11} cfu/ml. Repeated freezing and thawing reduces titers and should be avoided.

After infection of animals, the magnitude of organ titers depends on the bacterial strain used and on the susceptibility of the animal strain. Due to the intrinsic inter-experiment variability of in vivo experiments, it is absolutely critical that adequate controls are included when performing infection experiments (e.g., comparison to wild-type bacteria when analyzing bacterial mutants, comparison to normal or wild-type animals when infecting immunosuppressed or mutant animals).

Mouse intragastric infection

Female *iFABP-hEcad* challenged intragastrically with the laboratory strains *L. monocytogenes EGDe* at a dose of 10^9 cfu develop, as of 24 hr after infection, an actively growing infection in the intestinal tissue ($\sim 10^6$ cfu) that reaches a load of $\sim 10^7$ cfu 72 hr after infection (Fig. 9B.1.1). After invasion of the small intestine from the intestinal lumen, bacteria are subsequently transported via the blood to regional lymph nodes. When they reach the liver and the spleen, most *Listeria* are rapidly killed. Surviving *Listeria* then multiply and grow exponentially in the spleen and liver for the next 48 hr. Maximum counts are observed 72 to 96 hr post-infection ($\sim 10^6$ cfu in the liver and spleen). The protective immune response begins to contain the infection by about hour 96. Bacteria then start to be cleared, leading to their eradication from all organs if the kinetics is followed for additional days.

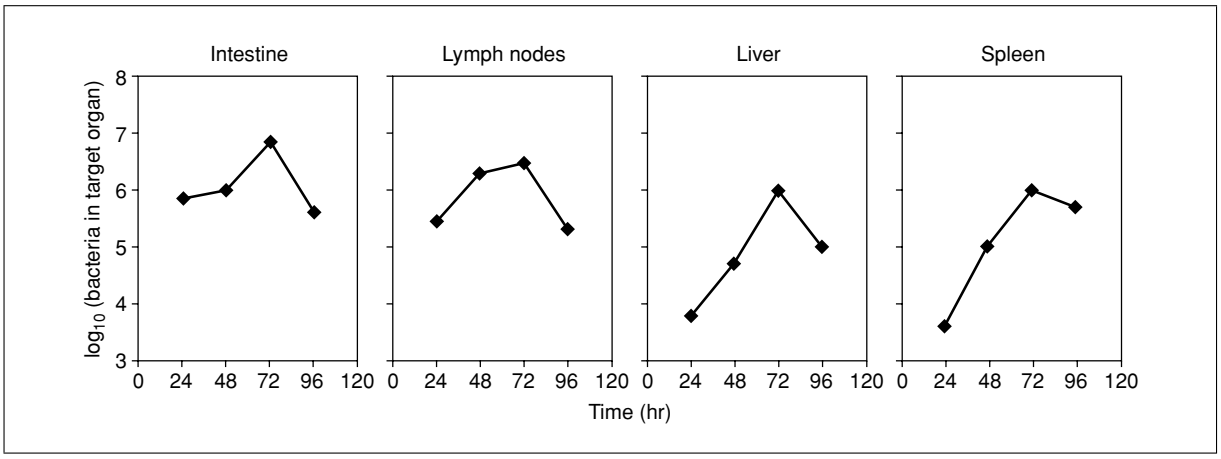

Figure 9B.1.1 Course of infection in *iFABP-hEcad* mice following intragastric inoculation with 10^9 cfu of *L. monocytogenes EGDe.*

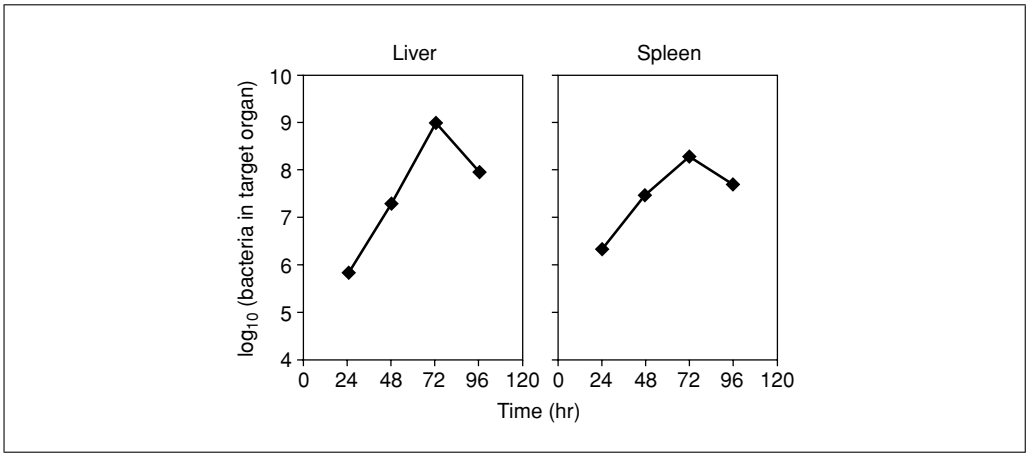

Figure 9B.1.2 Course of infection in BALB/c mice following intravenous inoculation with 10^4 cfu of *L. monocytogenes EGDe.*

Mouse intravenous infection

When female *BALB/c* mice are exposed to a sublethal inoculum (10^4 cfu) of *L. monocytogenes EGDe*, the ensuing infection follows a well-defined course, lasting for ~1 week. Routinely, mice are injected intravenously and bacterial growth kinetics are monitored in the spleen and liver. Within 10 min after intravenous injection, 90% of the inoculum is taken up by the liver and 5% to 10% by the spleen. During the first 6 hr, the number of viable *Listeria* in the liver decreases tenfold, indicating a rapid destruction of most of the bacteria. Surviving *Listeria* then multiply within permissive macrophages and grow exponentially in the spleen and liver for the next 48 hr, peaking at day 2 or 3 post-infection (Fig. 9B.1.2). At ~72 hr after inoculation the infection reaches a load of $\sim10^9$ cfu in the spleen, and $\sim10^8$ cfu in the liver. At a time point 96 hr post-infection, *L. monocytogenes* bacteria start to disappear from mouse organs until their complete clearance as a result of the induction of the immune response.

Guinea pig intragastric infection

The kinetics of infection of guinea pigs inoculated intragastrically with *L. monocytogenes EGDe* at a dose of 10^{10} cfu is similar to that observed with orally infected mice. The intestinal tissue is rapidly infected ($\sim10^6$ cfu at 24 hr post-infection) and load reaches $\sim10^7$ cfu 96 hr after infection (Fig. 9B.1.3). Bacteria translocate to the mesenteric lymph nodes and spread to the liver and the spleen, with maximum counts observed ~96 hr post-infection. Note that the guinea pig spleen is less susceptible than other target organs, and also as compared to mouse spleen ($\sim10^4$ cfu 96 hr after infection). The protective immune response begins to contain the infection by about hour 120. Bacteria then start to be cleared, leading to their total elimination.

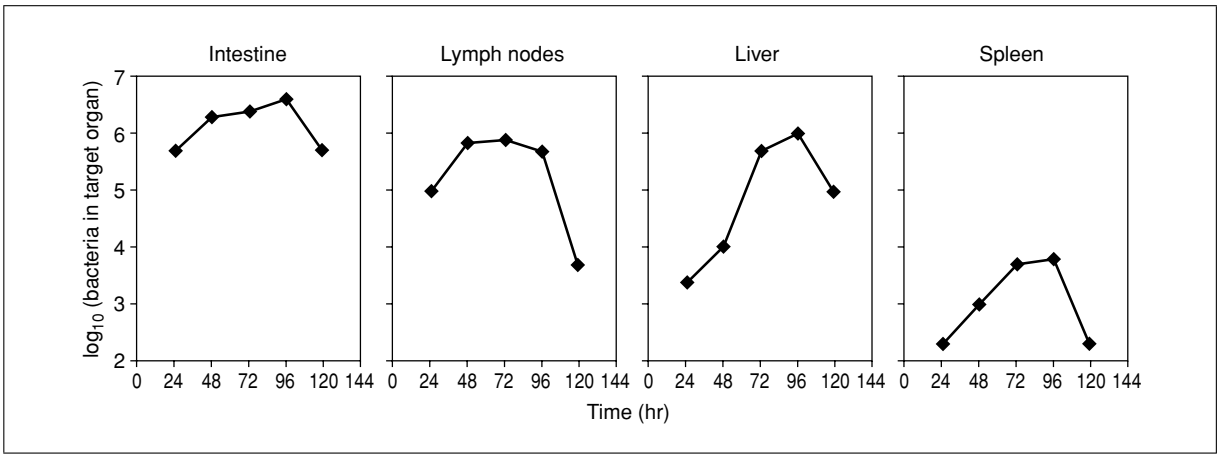

Figure 9B.1.3 Course of infection in Hartley guinea pigs following intragastric inoculation with 10^{10} cfu of *L. monocytogenes EGDe.*

Time Considerations

For in vivo experiments, a carefully planned time schedule is critical. Clearly, the time required for infection and organ recovery depends directly on the skills of the experimenter. Times given here are for experimenter with practice.

Preparation and numeration of bacterial stock solution last 3 days.

Animal should be received on site at least 1 week prior to infection to allow their acclimatization. Dilution and numeration of the bacterial suspension the day of the infection takes 1 hr. Plates are enumerated 2 days after incubation. Light anesthesia and oral inoculation of mice take 5 min per mouse. Intravenous injection of mice takes 5 min per mouse. Intragastric infection of guinea pigs takes 10 min for anesthesia and 5 min for inoculation per guinea pig. Full recovery of animals after anesthesia takes ~1 hr.

For LD50, animal behavior and fate is followed during 2 weeks. Each day following infection, the recording mortality and elimination of dead animals takes 30 min. At the end of the experiment, the euthanasia of the remaining animals by CO_2 inhalation takes 5 min.

For organ recovery and bacterial numeration in infected organs, euthanasia takes 5 min, organ recovery takes 10 min per animal, intestine washing take 2.5 hr, organ homogenization takes 10 min per animal, and dilution and plating of homogenates take 10 min per animal. Plates are enumerated after 2 days incubation.

Contact Information

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Tissue Culture Cell Assays Used to Analyze *Listeria monocytogenes*

Listeria monocytogenes is a facultative intracellular bacterial pathogen that has the ability to invade and multiply in a wide variety of cell types, including professional and nonprofessional phagocytic cells. Many tissue culture cell assays have been developed over the years to evaluate the virulence of *L. monocytogenes* isolates and mutant strains. These assays enable the dissection of steps leading to a successful infection: invasion, escape from primary vacuoles, intracellular growth, and cell-to-cell spread.

Invasion of host cells is a primary and essential step of the infection cycle of intracellular bacterial pathogens. *L. monocytogenes* has the ability to invade nonprofessional phagocytic cells such as epithelial cells, hepatocytes, fibroblasts, and trophoblasts, as well as professional phagocytic cells such as macrophages (Portnoy et al., 1988; Gaillard et al., 1996; Bakardjiev et al., 2004). Invasion of cells by *L. monocytogenes* occurs by the zipper mechanism, requiring interaction of a bacterial ligand with a host cell receptor to cause a cytoskeletal response leading to engulfment of the bacterial cell (Swanson and Baer, 1995). Once inside a professional or nonprofessional phagocytic host cell, *L. monocytogenes* resides in a membrane-bound vacuole for a short period of time. Escape from this vacuole is a prerequisite for the infection cycle as *L. monocytogenes* is incapable of multiplying in a vacuole (Gaillard et al., 1987). Following escape from primary vacuoles, *L. monocytogenes* efficiently multiplies in the cytosol of its host with an initial doubling time of $<$1 hr (Portnoy et al., 1988). Another feature of the *L. monocytogenes* intracellular life cycle is its ability to use host cell actin for motility. Actin polymerization at the bacterial surface begins shortly after bacteria escape from primary vacuoles. Actin filaments initially form a cloud around each bacterium but eventually form a tail at one end of the bacterial cell (Tilney and Portnoy, 1989; Mounier et al., 1990). Polarized actin assembly enables bacteria to move and spread directly from cell to cell by inducing the formation of membrane filopodia, which are taken up by neighboring cells. Spreading bacteria are temporarily located in double membrane vacuoles, also called secondary vacuoles, from which they must escape to access the cytosol of the new host cell and perpetuate the infection cycle.

This unit is comprised of four tissue culture cell assays evaluating in a quantitative and qualitative manner every step of the infectious cycle of *L. monocytogenes*: host cell invasion, escape from vacuoles, intracellular growth, and cell-to-cell spread. Basic Protocol 1 describes an assay measuring invasion by *L. monocytogenes* into Caco-2 cells, nonprofessional phagocytic cells derived from human intestinal epithelia. Basic Protocol 2 describes an assay to quantify the efficacy of *L. monocytogenes* to escape from vacuoles of mouse macrophages; access to a fluorescence microscope is required for this assay. Basic Protocol 3 is used to determine the kinetics of *L. monocytogenes* growth in mouse macrophages. Lastly, Basic Protocol 4 describes a plaquing assay to evaluate bacteria efficacy to spread directly from cell to cell. These assays can be adapted for use with other cell lines or with primary cells.

CAUTION: *Listeria monocytogenes* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

Contributed by Hélène Marquis

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CAUTION: No prophylactic measures, except hand washing, are required for personnel working with *L. monocytogenes*. However, individuals should be informed of the risk of acquiring listeriosis, especially for immunocompromised individuals and pregnant women. If an individual contaminates him- or herself, 70% alcohol or iodine should be used to decontaminate the infected area. Depending on the circumstances, the individual may be advised to see a physician. The following CDC and USDA Websites provide information on listeriosis: *http://www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_g.htm* and *http://www.fsis.usda.gov/Fact_Sheets/Listeriosis_and_Pregnancy_What_is_Your_Risk/index.asp*.

BASIC PROTOCOL 1

HOST CELL INVASION

This protocol describes a tissue culture cell assay to evaluate the ability of *L. monocytogenes* to invade Caco-2 cells (Gaillard et al., 1991; Kim et al., 2004), which are nonprofessional phagocytes derived from human epithelial intestinal cells. This assay is most useful when simultaneously comparing invasion levels of various isolates of *L. monocytogenes*, or invasion levels of a wild-type or reference strain to that of an isogenic mutant strain. Briefly, Caco-2 cells on glass coverslips are infected with *L. monocytogenes*. Infected cells are washed with Ca^{2+}- and Mg^{2+}-free DPBS (CMF-DPBS) at 30 min post-infection to eliminate free bacterial cells. Gentamicin is added at 45 min post-infection to kill extracellular bacteria. Finally, at 75 min post-infection, infected cells on coverslips are lysed and intracellular bacterial counts are determined by plating cell lysates onto solid LB medium. Infected cells on coverslips can also be stained for examination by bright-field microscopy.

Materials

95% ethanol
100 μg/ml type I rat tail collagen (see recipe)
$CaCl_2$ and $MgCl_2$-free Dulbecco's PBS (CMF-DPBS; GIBCO, also see *APPENDIX 2A*), sterile
Caco-2 cells (ATCC #HTB-37), trypsinized
Caco-2 medium (see recipe), 4° and 37°C
BHI medium (see recipe)
H_2O, sterile
Bacterial strain(s) (e.g., *L. monocytogenes*; ATCC, individual researchers working with *L. monocytogenes*, or clinical isolates)
LB plates (*APPENDIX 4A*)
10 mg/ml gentamicin (see recipe)
Diff-Quick stain set (Dade Behring)
Permount (Fisher)

12-mm round, precleaned glass coverslips in 70% ethanol
35-mm tissue culture–treated dishes (BD Falcon)
Pasteur pipets, sterile
15- and 50-ml polypropylene conical tubes, sterile
37°C, 5% CO_2 humidified incubator
37°C water bath
30° and 37°C incubators
1.5-ml microcentrifuge tubes, sterile
15-ml polystyrene conical tubes, sterile

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 4A*)

Prepare coverslips

1. Dip the tip of a pair of forceps in 95% ethanol and flame to sterilize.

   Use caution not to heat the forceps.

2. Pick a single 12-mm round glass coverslip by the edge and dip in 95% ethanol. Blot excess ethanol by touching the edge of the coverslip to a Kimwipe.

   Blot as much ethanol off as possible before flaming.

3. Flame leftover ethanol on coverslip and transfer to a 35-mm tissue culture–treated dish.

   If the coverslip becomes too hot, it will crack.

4. Repeat steps 2 and 3 until there are four coverslips per dish.

5. Cover coverslips with 1.5 to 2 ml of 100 μg/ml type I rat tail collagen. Push coverslips down, as they will tend to float. Incubate for a minimum of 1 hr at room temperature.

6. Aspirate collagen solution with a sterile Pasteur pipet and rinse coverslips once with 2 ml sterile CMF-DPBS. Keep dry and use within 1 hr.

Prepare Caco-2 cells

7. Suspend trypsinized Caco-2 cells in cold Caco-2 medium and count using a hemacytometer (*APPENDIX 4A*).

   A confluent 75-cm^2 flask contains ~3–4 × 10^6 cells.

8. Determine volume of cell suspension needed for the experiment. Calculate 3×10^5 cells per bacterial strain to test.

   The number of Caco-2 cells needed will vary according to cell age and growth rate. Young cells have a slower growth rate. In general, the objective is to obtain a monolayer of cells that is ~70% confluent at time of infection. Fully confluent monolayers do not become well-infected and tend to lift from the coverslips during washes.

9. Transfer volume of cell suspension needed into a 50-ml conical tube and adjust the cell concentration to 1.5×10^5 cells/ml by adding cold Caco-2 medium. Mix well by pipetting up and down.

10. Distribute 2 ml cell suspension (3×10^5 cells) per dish containing four coverslips treated with rat-tail collagen (step 6). Gently swirl the dish to distribute the cells over the coverslips. Incubate overnight in a 37°C, 5% CO_2 humidified incubator.

    Coverslips may need to be pushed down with sterile forceps if they move around, float, or overlap.

11. The next morning, aspirate medium and replace with fresh Caco-2 medium equilibrated to 37°C (i.e., prewarmed in a water bath). Monitor the status of the cells using an inverted microscope.

    Cells should be spread out and ~70% confluent.

    A compacted cell monolayer tends to lift off from glass coverslips during washes.

Prepare bacterial samples

12. Transfer 2 to 3 ml BHI medium into a 15-ml conical tube and inoculate with 1 to 2 colonies of the bacterial strain of interest (e.g., *L. monocytogenes*). Repeat for each bacterial strain to be tested.

13. With the cap on loose, place the tube on its side with a slight slant (~5°) in a 30°C incubator. Incubate overnight (14 to 16 hr).

    L. monocytogenes invades better when grown at 30°C as opposed to 37°C.

    The tubes are slanted to avoid spillage of the culture in the incubator. Fold a paper towel to make a ~2- to 3-cm-wide holder. Rest the top of the tube on the folded paper towel.

14. Following incubation, briefly vortex bacterial culture and transfer 1 ml into a sterile 1.5-ml microcentrifuge tube.

15. Pellet bacteria by microcentrifuging 1 min at maximum speed, room temperature.

16. Aspirate supernatant and suspend bacteria in 1 ml CMF-DPBS. Vigorously vortex to break up bacterial clumps.

17. Repeat steps 15 and 16.

18. Determine the initial inoculum in the following manner.

    a. Prepare a 10^{-6} dilution of the bacterial suspension, making two series of 1/1000 dilutions into sterile water. Be sure to vortex samples very well and change pipet tips between dilutions for accuracy.
    b. Spread 50 µl of this 10^{-6} dilution onto an LB plate until all liquid is absorbed. Prepare in triplicate for accuracy.
    c. Repeat for each bacterial strain tested.

    L. monocytogenes is a Gram-positive bacterial species with a 25- to 50-nm thick and highly cross-linked cell wall, which confers a strong resistance to lysis in water.

    The surface of the LB agar plates should be dry for better absorption. It helps to let the plates dry for 24 to 48 hr at room temperature before use. Alternatively, plates can be cracked open, agar facing down, and dried for 5 to 30 min at 37°C.

Infect Caco-2 cells

19. Infect cells by adding 2 to 20 µl washed bacteria to the dish; swirl gently ($t = 0$).

    Minimal inoculum required to recover detectable numbers of bacteria will vary between strains. If testing strains with various levels of invasion, it is better to use the same number of bacteria for initial infection.

20. Incubate 30 min in a 37°C, 5% CO_2 humidified incubator.

21. During the incubation period, transfer fresh Caco-2 cell medium into a conical tube and incubate in a 37°C water bath. Also prepare 5-ml aliquots of water in 15-ml polystyrene conical tubes and keep at room temperature. Calculate three aliquots per dish.

    The volume of Caco-2 medium needed depends on the number of dishes per assay. Calculate 2 ml per dish.

22. At 30 min post-infection, wash infected cells with CMF-DPBS as follows:

    a. Aspirate medium from the dish using a Pasteur pipet.
    b. Add 2 ml CMF-DPBS equilibrated to room temperature.
    c. Repeat a and b for a total of three successive washes.
    d. After the last wash, replace the CMF-DPBS with 2 ml of Caco-2 cell medium equilibrated to 37°C.
    e. Return dishes to the 37°C, 5% CO_2 incubator.

    Avoid pipetting CMF-DPBS and medium directly onto coverslips to prevent cells from lifting.

23. At 45-min post-infection, add 10 mg/ml gentamicin to a final concentration of 150 μg/ml. Swirl dish gently to equilibrate gentamicin concentration in medium and return dishes to the 37°C, 5% CO_2 incubator.

24. At 75-min post-infection, wash infected cells once by replacing the gentamicin-containing Caco-2 medium with 2 ml CMF-DPBS.

25. Using sterile forceps, pick up a coverslip by the edge, avoid scratching the cell monolayer, and transfer to a 15-ml polystyrene conical tube containing 5 ml water. Repeat for a total of three coverslips, putting a single coverslip in each tube.

26. Vortex tubes containing coverslips at maximum speed for 20 to 30 sec to lyse infected cells. Retain at room temperature and determine invasion levels within 30 min (step 28).

27. Stain the fourth coverslip using a Diff-Quick stain set. After staining, allow coverslip to air dry and mount on glass slide cell-side down on a drop of Permount. Examine stained infected cells by bright-field microscopy using an upright microscope.

    Do not use an oil objective until Permount is solidified (~24 hr).

Determine bacterial counts

28. For determination of invasion levels, vortex each cell lysate prepared in step 26 for 10 to 20 sec. Immediately plate between 5 and 200 μl onto solid LB medium.

    There are three tubes per bacterial strain tested, each tube contains cell lysate from one coverslip, and each cell lysate is plated on one LB plate.

    The volume plated will depend on the level of invasion for each bacterial strain tested.

29. Record numbers of colony forming units (cfu) after incubating the plates for 24 hr at 37°C or 2 days at room temperature.

    Ideally, there should be between 50 and 200 cfu per plate.

    Lysates of infected cells can be generated in 2.5 ml of water rather than 5 ml as for bacterial strains defective in invasion. Alternatively, cells may be infected with more bacteria (see step 19).

30. Calculate the initial inoculum as follows:

$$\frac{\text{average no. CFU per plate} \times \text{vol (ml) inoculum (from step 18)}}{\text{dilution factor} \times \text{vol plated (ml)}}$$

31. Calculate the number of intracellular bacteria per coverslip as follows:

$$\frac{\text{average no. CFU per plate} \times \text{total volume cell lysate (5 ml)}}{\text{volume plated (ml)}}$$

32. Calculate the absolute bacterial invasion levels as follows:

$$\frac{\text{no. intracellular bacteria per coverslip as determined in step 31}}{\text{no. bacteria in initial inoculum as determined in step 30} \times 0.12}$$

    The surface area of one coverslip represents ~12% of the surface area of a 35-mm tissue culture dish. This number needs to be taken into consideration when calculating absolute invasion levels.

33. Calculate the bacterial invasion level of a test strain (or mutant) relative to a reference strain (or wild-type) as follows:

$$\frac{\left(\frac{\text{no. of intracellular bacteria (test) per coverslip}}{\text{no. of bacteria (test) in initial inoculum} \times 0.12}\right)}{\left(\frac{\text{no. of intracellular bacteria (ref.) per coverslip}}{\text{no. of bacteria (ref.) in initial inoculum} \times 0.12}\right)}$$

To simplify this equation:

$$\frac{\text{test strain output/test strain input}}{\text{ref. strain output/ref. strain input}}$$

which is the equivalent of:

$$\frac{\text{test strain output} \times \text{ref. strain input}}{\text{test strain input} \times \text{ref. strain output}}$$

$$\frac{\text{no. intracellular bacteria per coverslip (test strain)} \times \text{no. bacteria in initial inoculum (ref. strain)}}{\text{no. intracellular bacteria per coverslip (ref. strain)} \times \text{no. bacteria in initial inoculum (test strain)}}$$

Use relative percentages or ratios to analyze data from different experiments because of variability in absolute cfu numbers between experiments.

BASIC PROTOCOL 2

ESCAPE FROM VACUOLES

This protocol describes a tissue culture cell assay combined with fluorescence microscopy to evaluate the efficacy of bacterial escape from vacuoles using the mouse macrophage-like cell line J774 (Marquis et al., 1995; Jones et al., 1996). Briefly, J774 cells on square glass coverslips are infected with *L. monocytogenes*. Infected cells are washed with Ca^{2+}- and Mg^{2+}-free DPBS at 30-min post-infection to eliminate free bacterial cells. Gentamicin is added at 60-min post-infection to kill extracellular bacteria. Finally, at 90-min post-infection, infected cells on coverslips are washed and fixed in formalin. Fixed cells are stained for differential visualization of bacteria and actin filaments by fluorescence microscopy. The efficacy of bacterial escape from vacuoles is determined by counting the ratio of intracellular bacteria decorated with actin filaments, as only bacteria that have escaped into the host cytosol become decorated with actin filaments. Primary mouse bone marrow–derived macrophages are also frequently used for this assay. If using nonprofessional phagocytic cells, it becomes important to differentiate extracellular from intracellular bacteria as the efficacy of bacterial invasion is much less than with professional phagocytic cells and nonphagocytosed bacteria may remain attached to the cell despite washes and gentamicin killing.

Materials

95% ethanol
J774 cells (ATCC #TIB-67)
J774 medium with antibiotics (see recipe for J774 and L2 medium), 37°C
$CaCl_2$- and $MgCl_2$-free Dulbecco's PBS (DMF-DPBS, GIBCO; also see *APPENDIX 2A*), sterile, 4°C and room temperature
J774 medium without antibiotics, 4° and 37°C
BHI medium (see recipe)
Bacterial strain(s) (e.g., *L. monocytogenes*; ATCC, individual researchers working with *L. monocytogenes*, or clinical isolates)
10 mg/ml gentamicin (see recipe)
Formalin (see recipe)
TBS-TX (see recipe)

Antibody buffer (see recipe)
Rabbit anti-*Listeria* antibody (Oxoid)
Secondary antibodies (e.g., goat anti-rabbit IgG conjugated to an Alexa Fluor 350 and 568, Invitrogen-Molecular Probes)
Alexa Fluor 488 phalloidin (Invitrogen-Molecular Probes)
TBS (see recipe)
Prolong Antifade (Invitrogen-Molecular Probes)

18-mm square pre-cleaned glass coverslips in 70% ethanol
35-mm tissue culture–treated dishes (BD Falcon)
75-cm^2 non-tissue culture–treated flasks
Pasteur pipets, sterile
15- and 50-ml sterile polypropylene conical tubes
Refrigerated tabletop swinging bucket centrifuge, 4°C
37°C, 5% CO_2 humidified incubator
30°C incubator
1.5-ml microcentrifuge tubes, sterile
37°C water bath
150-mm petri dishes
Fluorescence microscope (filter sets for blue, green, and red fluorophores)

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 4A*)

Prepare coverslips

1. Dip the tip of a pair of forceps in 95% ethanol and flame to sterilize.

   Use caution not to heat the forceps.

2. Pick a single 18-mm square glass coverslip by the edge and dip in 95% ethanol. Blot excess ethanol by touching the edge of the coverslip to a Kimwipe.

   Blot as much ethanol off as possible before flaming.

3. Flame leftover ethanol on coverslip and transfer to a 35-mm tissue culture–treated dish. Prepare one dish per bacterial strain to be tested.

   If the coverslip becomes too hot, it will crack.

   Only one 18-mm square coverslip fits into a 35-mm dish.

Prepare J774 cells

4. Culture J774 cells in a 75-cm^2 non-tissue culture–treated flask in 25 ml of J774 medium with antibiotics (i.e., ampicillin and streptomycin) in a 37°C, 5% CO_2 humidified incubator.

   J774 cells are very adherent cells that are resistant to trypsinization. Harvesting of these cells is facilitated by growing them on non-tissue culture–treated surfaces. Alternatively, J774 cells can be cultured in suspension using a spinner flask. If using a spinner flask, grow cells to a density of 1–2 × 10^6 cells/ml and passage by replacing 60% to 80% of the cell suspension with fresh J774 medium with antibiotics.

5. Wash adherent cells as follows.

   a. Aspirate medium using a Pasteur pipet.
   b. Add 10 to 12 ml of cold CMF-DPBS.
   c. Rinse cell monolayer by rocking the flask sideways.
   d. Aspirate and replace buffer with another 10 to 12 ml cold CMF-DPBS.
   e. Incubate 15 to 20 min on ice or at 4°C.

6. Harvest J774 cells by either of the two following steps:

   a. Detach cells by pipetting CMF-DPBS up and down the monolayer repeatedly; *or*

   b. Alternatively, detach cells by banging the flask (cap side up) three or four times on a hard vertical surface on the side opposite of the monolayer.

7. Transfer detached cells into a 50-ml conical tube and keep on ice. Count cells using a hemacytometer (*APPENDIX 4A*).

   Approximately 10–12 × 10^6 cells can be harvested from a 75-cm^2 flask. J774 cells detach more easily when the monolayer is near confluence.

8. Determine volume of cell suspension needed for the experiment. Calculate 0.8 × 10^6 cells per dish to be prepared.

9. Transfer volume of cell suspension needed for the experiment into a 50-ml conical tube and, using a precooled tabletop centrifuge, centrifuge cells 10 min at 200 × *g*, 4°C.

10. Carefully aspirate CMF-DPBS with a Pasteur pipet by tilting the tube on its side and keeping the tip of the pipet away from the pellet. Work quickly to avoid aspirating cells as the pellet is relatively soft.

11. Suspend cells in cold antibiotic-free J774 medium to a final concentration of 0.4 × 10^6 cells/ml. Pipet cell suspension up and down twelve to fifteen times to break up clumps. Avoid generating foam as much as possible.

    From this point, always use antibiotic-free J774 medium.

12. Immediately transfer 2 ml cell suspension (0.8 × 10^6 cells) into a 35-mm dish containing one square coverslip (step 3). Gently swirl the dish to distribute the cells over the coverslip and incubate dishes in a 37°C, 5%CO_2 humidified incubator overnight.

    Push coverslip down if necessary.

13. Next morning, push coverslips down but avoid scratching the monolayer. Monitor the cell status using an inverted microscope.

    Ideally, cells should be spread out, not clumpy, and 70% to 80% confluent.

Prepare bacterial samples

14. Transfer 2 to 3 ml BHI medium into a 15-ml conical tube, and inoculate with 1 to 2 colonies of the bacterial strain of interest (e.g., *L. monocytogenes*). Repeat for each bacterial strain to be tested.

15. With the cap on loose, place the tube on its side with a slight slant (∼5°) in a 30°C incubator. Incubate overnight (14 to 16 hr).

    L. monocytogenes invades better when grown at 30°C as opposed to 37°C.

    The tubes are slanted to avoid spillage of the culture in the incubator. Fold a paper towel to make a ∼2- to 3-cm wide holder. Rest the top of the tube on the folded paper towel.

16. Following incubation, briefly vortex the bacterial culture and transfer 1 ml into a 1.5-ml microcentrifuge tube.

17. Pellet bacteria by microcentrifuging 1 min at maximum speed, room temperature.

18. Aspirate supernatant and suspend bacteria in 1 ml CMF-DPBS. Vigorously vortex to break up bacterial clumps.

19. Repeat steps 17 and 18.

Infect J774 cells

20. Infect J774 cells (step 13) with 2 to 6 μl washed bacteria. Swirl dish gently to distribute bacteria in medium and transfer dishes to a 37°C, 5% CO_2 humidified incubator ($t = 0$).

21. During the incubation period, transfer cold, antibiotic-free J774 medium into a tube and incubate in a 37°C water bath.

    The volume needed depends on the number of samples tested per assay. Calculate 2 ml per sample (step 22).

22. At 30 min post-infection, wash infected cells with CMF-DPBS as follows:

    a. Aspirate medium from the dish using a Pasteur pipet.
    b. Add 2 ml CMF-DPBS equilibrated to room temperature.
    c. Repeat a and b for a total of three successive washes.
    d. After the last wash, replace the CMF-DPBS with 2 ml J774 medium equilibrated to 37°C.
    e. Return dishes to the 37°C, 5% CO_2 incubator.

    Avoid pipetting CMF-DPBS and medium directly onto coverslips to prevent cells from lifting.

23. At 60-min post-infection, add 10 mg/ml gentamicin to a final concentration of 50 μg/ml. Swirl dishes gently to equilibrate gentamicin concentration in medium. Return dishes to the 37°C, 5% CO_2 humidified incubator.

24. At 90-min post-infection, wash infected cells three times with CMF-DPBS.

    It is important to stop the infection at a time when bacteria have not begun to divide in the cytosol of host cells. The optimal endpoint for this assay will vary in function of the cell type used.

25. Fix the cells by replacing the last CMF-DPBS wash with 2 ml formalin for a minimum of 10 min at 4°C. If the staining is to be done the next day, seal the dish with Parafilm and keep the cells in formalin in the dark at 4°C (up to 24 hr).

Prepare a humid chamber for fluorescence staining

26. Cover the inside bottom of a 150-mm petri dish with a 10 × 10–cm square of Parafilm. Holding the dish by the border, heat very briefly over a flame to soften the Parafilm. Press with finger onto softened Parafilm layer to make it adhere to the bottom of the dish. Avoid trapping air bubbles.

27. To ease identification of the coverslips, mark the outside bottom of the dish with numbers (1 to 9) or letters (A to I). Wrap the dish and its cover separately with aluminum foil to create a dark chamber.

28. Wrap the inside border of the dish with wet (with water) Kimwipes to create a humid chamber.

Stain samples for fluorescence microscopy

29. Wash the fixed samples with CMF-DPBS three times.

    Dispose of the formalin in an appropriate waste container.

30. Transfer coverslips into a humid chamber cell-side up and immediately block by adding 200 to 400 μl of detergent-free antibody buffer to each coverslip. Incubate 10 to 30 min at room temperature.

    It is important that the coverslips never dry during the staining process.

31. Aspirate antibody buffer and replace with 50 µl rabbit anti-*Listeria* antibody diluted 1:500 in detergent-free antibody buffer. Incubate 30 to 60 min at room temperature.

    There are different antisera for different serotypes of L. monocytogenes.

32a. *To wash using the drop/suck method*: Drop 200 to 400 µl of TBS onto each coverslip and gently aspirate the liquid from the side of the coverslip using a pipet tip connected to a vacuum line. Wash with a total of 3 to 6 ml of buffer per coverslip.

32b. *To wash by dipping*: Dip coverslips several times into a 50-ml conical tube containing TBS.

33. Add 50 µl secondary antibody (goat anti-rabbit IgG) conjugated to Alexa Fluor 350 diluted in detergent-free antibody buffer according to the manufacturer's recommendations. Incubate 30 to 60 min at room temperature.

    There are many different conjugated secondary antibodies commercially available. Likewise, secondary antibodies from animal species other than goat can be used.

34. Wash coverslips with TBS as in step 32.

35. Permeabilize infected cells by adding 200 to 400 µl of antibody buffer containing detergent onto each coverslip. Incubate 10 to 30 min at room temperature.

36. Aspirate antibody buffer and replace with 50 µl rabbit anti-*Listeria* antibody diluted 1:500 in antibody buffer containing detergent. Incubate 30 to 60 min at room temperature.

37. Wash with TBS-TX as described in step 32.

38. Add 50 µl secondary antibody (goat anti-rabbit IgG) conjugated to Alexa Fluor 568 diluted in antibody buffer containing detergent according to the manufacturer's recommendations. Incubate 30 to 60 min at room temperature.

    Extracellular bacteria will be stained with both red and blue fluorophores, whereas intracellular bacteria will be stained only with the red fluorophore.

39. Wash with TBS-TX as described in step 32.

40. Add 50 µl Alexa Fluor 488 phalloidin diluted in antibody buffer containing detergent as recommended by the manufacturer. Incubate 10 min at room temperature.

41. Wash with TBS-TX, then with TBS as described in step 32.

    Detergents cause a decrease in fluorescence.

42. Dispose of the wet Kimwipes in the humid chamber and dry the coverslips for 5 to 10 min in the dark at room temperature.

43. Mount each coverslip cell-side down on a drop of Prolong antifade in the middle of a glass slide. Dry overnight in the dark at room temperature. Once dry, seal the edges of coverslips with nail polish.

    Coverslips mounted with Prolong antifade can be stored in a dry slide box for several months at –20°C.

44. Using a fluorescence microscope, count at least 200 intracellular bacteria (stained with red fluorophore only) per sample and, for each counted intracellular bacterium, determine if it is decorated with actin filaments (stained with green fluorophore) by switching to the appropriate filter on the microscope. Do not count extracellular bacteria, which are stained with blue and red fluorophores.

Alexa Fluor 350 is a blue fluorophore that absorbs light at 346 nm and emits at 442 nm. Alexa Fluor 488 is a green fluorophore that absorbs light at 495 nm and emits at 519 nm. Alexa Fluor 568 is a red fluorophore that absorbs light at 568 nm and emits at 603 nm.

KINETICS OF INTRACELLULAR GROWTH

BASIC PROTOCOL 3

This protocol describes a tissue culture cell assay to measure the kinetics of *L. monocytogenes* growth in the mouse macrophage-like cell line J774 (Portnoy et al., 1988). Briefly, J774 cells on glass coverslips are infected with *L. monocytogenes*. Infected cells are washed with Ca^{2+}- and Mg^{2+}-free DPBS (CMF-DPBS) at 30 min post-infection to eliminate free bacterial cells. Gentamicin is added at 60 min post-infection to kill extracellular bacteria. At 2, 5, 8, and 11 hr post-infection, cells on coverslips are lysed and intracellular bacterial counts are determined by plating cell lysates onto solid LB medium. Infected cells on coverslips can also be stained for examination by bright-field microscopy. This assay can easily be adapted for use with other cell lines.

Materials

95% ethanol
J774 cells (ATCC #TIB-67)
J774 medium with antibiotics (see recipe for J774 and L2 medium), 37°C
$CaCl_2$- and $MgCl_2$-free Dulbecco's PBS (CMF-DPBS, GIBCO; also see *APPENDIX 2A*), sterile, 4°C and room temperature
J774 medium without antibiotics, 4° and 37°C
BHI medium (see recipe)
Bacterial strain(s) (e.g., *L. monocytogenes*; ATCC, individual researcher working with *L. monocytogenes*, or clinical isolates)
10 mg/ml gentamicin (see recipe)
LB plates (*APPENDIX 4A*)
Diff-Quick stain set (Dade Behring)
Permount (Fisher)

12-mm round pre-cleaned glass coverslips in 70% ethanol
60-mm petri dishes (Nunc)
75-cm^2 nontissue culture–treated flasks
Pasteur pipets, sterile
15- and 50-ml polypropylene conical tubes, sterile
Refrigerated tabletop swinging-bucket centrifuge, 4°C
37°C, 5% CO_2 incubator
30° and 37°C incubators
1.5-ml microcentrifuge tubes, sterile
37°C water bath
15-ml sterile polystyrene conical tubes
Plotting software (e.g., Prism, Cricket Graph, or Excel)

Additional reagents and equipment for counting cells in a hemacytometer (*APPENDIX 4A*)

Prepare coverslips

1. Dip the tip of a pair of forceps in 95% ethanol and flame to sterilize.

   Use caution not to heat the forceps.

2. Pick a single 12-mm round glass coverslip by the edge and dip in 95% ethanol. Blot excess ethanol by touching the edge of the coverslip to a Kimwipe.

   Blot as much ethanol off as possible before flaming.

3. Flame leftover ethanol on coverslip and transfer to a 60-mm petri dish. Prepare one dish per bacterial strain to be tested.

   If the coverslip becomes too hot, it will crack

   Up to fifteen 12-mm coverslips can fit in a dish.

Prepare J774 cells

4. Culture J774 cells in a 75-cm^2 non-tissue culture–treated flask in 25 ml of J774 medium with antibiotics (i.e., ampicillin and streptomycin) in a 37°C, 5% CO_2 humidified incubator.

   J774 cells are very adherent cells that are resistant to trypsinization. Harvesting of these cells is facilitated by growing them on non-tissue culture–treated surfaces. Alternatively, J774 cells can be cultured in suspension using a spinner flask. If using a spinner flask, grow cells to a density of 1–2 $\times$ 10^6 cells/ml and passage by replacing 60% to 80% of the cell suspension with fresh J774 medium with antibiotics.

5. Wash adherent cells as follows.

   a. Aspirate medium using a Pasteur pipet.
   b. Add 10 to 12 ml of ice-cold CMF-DPBS.
   c. Rinse cell monolayer by rocking the flask sideways.
   d. Aspirate and replace buffer CMF-DPBS with another 10 to 12 ml ice-cold CMF-DPBS.
   e. Incubate 15 to 20 min on ice or at 4°C.

6. Harvest J774 cells by either of the two following steps:

   a. Detach cells by pipetting CMF-DPBS up and down the monolayer repeatedly; *or*
   b. Alternatively, detach cells by banging the flask (cap side up) three or four times on a hard vertical surface.

7. Transfer detached cells into a 50-ml conical tube and keep on ice. Count cells using a hemacytometer (*APPENDIX 4A*).

   Approximately 10–12 $\times$ 10^6 cells can be harvested from a 75-cm^2 flask. J774 cells detach more easily when the monolayer is near confluence.

8. Determine volume of cell suspension needed for the experiment. Calculate 1.5 $\times$ 10^6 cells per dish to prepare.

9. Transfer volume of cell suspension needed for the experiment into a 50-ml conical tube and, using a precooled tabletop centrifuge, centrifuge cells 10 min at 200 $\times$ g, 4°C.

10. Carefully aspirate CMF-DPBS with a Pasteur pipet by tilting the tube on its side and keeping the tip of the pipet away from the pellet. Work quickly to avoid aspirating cells as the pellet is relatively soft.

11. Suspend cells in cold antibiotic-free J774 medium to a final concentration of 0.25 $\times$ 10^6 cells/ml. Pipet cell suspension up and down twelve to fifteen times to break up clumps. Avoid generating foam as much as possible.

    From this point forward, always use antibiotic-free J774 medium.

    If doing a large experiment, it is easier to initially suspend cells and break up the clumps in 10 to 12 ml medium, then adjust the volume of the cell suspension.

12. Immediately transfer 6 ml cell suspension (1.5×10^6 cells) into a 60-mm dish containing coverslips (step 3). Gently swirl the dish to distribute the cells over the coverslips (step 3).

    Coverslips will need to be pushed down with sterile forceps. Make sure coverslips do not overlap.

13. Gently transfer dishes to a 37°C, 5% CO_2 humidified incubator and incubate overnight.

14. On the next morning, push coverslips down but avoid scratching the monolayer. Monitor cell status using an inverted microscope.

    Ideally, cells should be spread out, not clumpy, and cover 60% to 70% of the coverslip surface.

Prepare bacterial samples

15. Transfer 2 to 3 ml BHI medium into a 15-ml conical tube, and inoculate with 1 to 2 colonies of the bacterial strain of interest (e.g., *L. monocytogenes*). Repeat for each bacterial strain to be tested.

16. With the cap on loose, place the tube on its side with a slight slant (~5°C) in a 30°C incubator. Incubate overnight (14 to 16 hr).

    L. monocytogenes invades better when grown at 30°C as opposed to 37°C.

    The tubes are slanted to avoid spillage of the culture in the incubator. Fold a paper towel to make a ~2- to 3-cm wide holder. Rest the top of the tube on the folded paper towel.

17. Following incubation, briefly vortex bacterial culture and transfer 1 ml into a 1.5-ml microcentrifuge tube.

18. Pellet bacteria by microcentrifuging 1 min at maximum speed, room temperature.

19. Aspirate supernatant and suspend bacteria in 1 ml CMF-DPBS. Vigorously vortex to break up bacterial clumps.

20. Repeat steps 18 and 19.

21. Dilute the suspension of washed bacteria 1:20 in CMF-DPBS.

Infect J774 cells

22. Infect cells (step 14) with 6 µl bacterial suspension diluted 1:20 (final dilution of 1:20,000). Swirl dish gently to distribute bacteria in medium and transfer dishes to the 37°C, 5% CO_2 humidified incubator ($t = 0$).

23. During the incubation period, transfer fresh J774 medium into a polypropylene conical tube and incubate in a 37°C water bath. Prepare 5-ml aliquots of water in 15-ml polystyrene conical tubes and keep at room temperature. Calculate three aliquots per sample per time point (step 26).

    The volume of J774 medium needed depends on the number of samples tested per assay. Calculate 6 ml per sample.

    There are enough coverslips for four time points.

24. At 30 min post-infection, wash infected cells with CMF-DPBS as follows:

    a. Aspirate medium from the dish using a Pasteur pipet.
    b. Add 2 ml CMF-DPBS equilibrated to room temperature.
    c. Repeat a and b for a total of three successive washes.
    d. After the last wash, replace the CMF-DPBS with 6 ml J774 medium equilibrated to 37°C.

e. Return dishes to the 37°C, 5% CO_2 incubator.

Avoid pipetting CMF-DPBS and medium directly onto coverslips to prevent cells from lifting.

25. At 60-min post-infection, add 10 mg/ml gentamicin to a final concentration of 50 μg/ml. Swirl dish gently to equilibrate gentamicin solution evenly into medium and return to 37°C, 5% CO_2 humidifed incubator.

26. Determine intracellular bacterial counts at 2, 5, 8, and 11 hr (optional) post-infection in the following manner.

    a. Using sterile forceps, pick up a coverslip by the edge but avoid scratching the cell monolayer.
    b. Dip four to five times in CMF-DPBS to wash off gentamicin.
    c. Transfer the coverslip to a 15-ml polystyrene conical tube containing 5 m water (step 23).
    d. Repeat a to c for a total of three coverslips, putting a single coverslip into each tube.

27. Vortex tubes containing coverslips for 20 to 30 sec at maximum speed to lyse infected cells, and immediately process cell lysates for bacterial count determination. Spread the following volumes of lysates onto LB plates:

    a. $t = 2$ hr post-infection: 25 to 50 μl
    b. $t = 5$ hr post-infection: 2.5 to 5 μl
    c. $t = 8$ hr post-infection: 25 to 50 μl of 1:100 dilution
    d. $t = 11$ hr post-infection: 10 to 25 μl of 1:100 dilution.

    It is important to vortex each cell lysate for 10 to 20 sec just prior to taking an aliquot for plating.

    The volume to be plated may vary according to each bacterial strain tested.

28. Using a Diff-Quick stain set, stain coverslip at 4.5, 7.5, and 10.5 hr post-infection for visual evaluation of the intracellular infection. After staining, allow coverslip to air dry and mount on glass slide cell-side down on a drop of Permount.

29. Examine stained infected cells by bright-field microscopy using an upright microscope.

    Do not use an oil objective until Permount is solidified (~24 hr).

Determine kinetics of intracellular growth

30. Calculate the number of intracellular bacteria per coverslip as follows:

$$\frac{\text{no. CFU per plate} \times 5\,\text{ml}) \times \text{dilution factor}}{\text{volume plated (ml)}}$$

    Average numbers for the three coverslips for a specific time point for each strain tested.

31. Plot the growth curve using programs such as Prism, Cricket Graph, or Excel. Use a log scale to report the number of bacteria per coverslip on the *y* axis, and a linear scale to mark time points on the *x* axis.

32. Calculate the doubling time (τ) between time points using the following equations:

    rate constant (k) $= \{\ln[y(t)/y_0]\}/\Delta t$

    doubling time (τ) $= \ln 2/k$

where, Δt is the time elapsed between two time points, y_0 is the number of cfu per coverslip at the first time point, and $y(t)$ is the number of cfu per coverslip at the second time point.

CELL-TO-CELL SPREAD

BASIC PROTOCOL 4

This protocol describes a tissue culture–cell plaquing assay to evaluate *L. monocytogenes* efficacy at spreading from cell to cell (Sun et al., 1990). The most appropriate cell line to use for this plaquing assay is the mouse fibroblast L2 cell line, which is a variant of the L929 line (Dales, 1962). Mouse L2 cells are relatively round when grown in tissue culture–treated dishes and form nicely demarcated plaques when infected with *L. monocytogenes*. This cell line can generally be obtained from scientists working with Corona viruses or with *L. monocytogenes*. For the assay, mouse L2 cells in six-well tissue culture–treated plates are infected with *L. monocytogenes*. Infected cells are washed with Ca^{2+}- and Mg^{2+}-free DPBS (CMF-DPBS) at 60-min post-infection to eliminate free bacterial cells, then overlaid with tissue culture medium mixed with agarose and gentamicin. After a 4-day incubation period, a second overlay containing neutral red is added to each well. Neutral red diffuses down staining live host cells at the bottom of the wells and revealing clear plaques formed by foci of infection. Plates are scanned and plaques measured using commercially available software.

Materials

L2 mouse fibroblast cell line (individual researchers working with Corona viruses or with *L. monocytogenes*), trypsinized
L2 medium (see recipe for J774 and L2 medium) without antibiotics, 4°C
BHI medium (see recipe)
Bacterial strain(s) (e.g., *L. monocytogenes*; ATCC, individual researchers working with *L. monocytogenes* or clinical isolates, sterile)
Dulbecco's PBS $CaCl_2$- and $MgCl_2$-free (CMF-DPBS, GIBCO; see *APPENDIX 2A*)
2× *L. monocytogenes* plaquing medium (see recipe), 37°C
10 mg/ml gentamicin (see recipe)
1.4% (w/v) agarose: autoclave and allow to harden
1 N HCl
0.5% neutral red solution, pH 5.2 (Sigma)
15- and 50-ml conical polypropylene tubes, sterile
6-well tissue culture–treated plates or 35-mm tissue culture–treated dishes
37°C, 5% CO_2 humidified incubator
30°C incubator
1.5-ml microcentrifuge tubes, sterile
37°C water bath
50° to 55°C water bath
Pasteur pipets, sterile
Scanner
Plaque measuring software: Adobe Photoshop or equivalent
Additional reagents and solutions for counting cells in a hemacytometer (*APPENDIX 4A*)

Prepare L2 cells

1. Suspend trypsinized mouse L2 cells in antibiotic-free L2 medium and count using a hemacytometer (*APPENDIX 4A*).

   A confluent 25-cm^2 flask contains ~8–9 × 10^6 cells.

2. Determine volume of cell suspension needed for the experiment. Calculate 0.7×10^6 cells per bacterial strain to test.

   The number of cells to put down varies according to cell age and growth rate. Young cells have a slower growth rate in general. The objective is to obtain a monolayer of cells that is 80% to 90% confluent at the time of infection.

3. Transfer volume of cell suspension needed into a 50-ml conical tube and adjust the cell concentration to 0.35×10^6 cells/ml with cold, antibiotic-free L2 medium.

4. Distribute 2 ml cell suspension (0.7×10^6 cells) per well of a 6-well tissue culture–treated plate or 35-mm tissue culture–treated dish.

5. Incubate 40 to 48 hr in a 37°C, 5% CO_2 humidified incubator.

   Cell monolayer should be 70% to 90% confluent.

Prepare bacterial samples and infect L2 cells

6. The day before infection, transfer 2 to 3 ml BHI medium into a 15-ml conical tube, and inoculate with 1 to 2 colonies of bacterial strain of interest (e.g., *L. monocytogenes*). Repeat for each bacterial strain to be tested.

7. With the cap on loose, place the tube on its side with a slight slant (~5°) in a 30°C incubator. Incubate overnight (14 to 16 hr).

   L. monocytogenes invades better when grown at 30°C as opposed to 37°C.

   The tubes are slanted to avoid spillage of the culture in the incubator. Fold a paper towel to make a ~2- to 3-cm wide holder. Rest the top of the tube on the folded paper towel.

8. On the next morning, briefly vortex bacterial cultures and make a 1:100 dilution in Ca^{2+}- and Mg^{2+}-free DPBS (CMF-DPBS) in 1.5-ml sterile microcentrifuge tubes.

9. Infect cells (step 5) with 10 to 20 µl of a 1:100 bacterial dilution (1:10,000 to 1:20,000 final dilution), and incubate 1 hr in a 37°C, 5% CO_2 humidified incubator.

   During incubation, pepare L2 overlay (steps 10 to 16).

Prepare L2 cell overlay

10. Transfer a calculated volume of 2× *L. monocytogenes* plaquing medium to a 50-ml conical tube. Calculate 1.5 ml for each well.

11. Add 10 mg/ml gentamicin to a final concentration of 20 µg/ml.

    Final gentamicin concentration will be 10 µg/ml after diluting the 2× plaquing medium with agarose (step 15).

12. Equilibrate medium in a 37°C water bath.

13. Melt 1.4% agarose in the microwave at 30% power.

14. Cool down the melted agarose in a 50° to 55°C water bath.

15. At t = 60 min post-infection, wash infected cells with CMF-DPBS as follows:

    a. Aspirate medium from the dish using a Pasteur pipet.
    b. Add 2 ml CMF-DPBS equilibrated to room temperature.
    c. Repeat a and b for a total of three successive washes.

    Avoid pipetting CMF-DPBS and medium directly onto coverslips to prevent cells from lifting.

16. Mix the 2× plaquing medium containing gentamicin (step 11) with the 1.4% melted agarose in a 1:1 ratio. Transfer 3 ml to each well and return plate to the 37°C, 5% CO_2 humidified incubator for 4 days.

Work quickly to avoid allowing agarose to solidify.

The final composition of L2 overlay is 1× DMEM, 5% FBS, 10 μg/ml gentamicin, and 0.7% agarose.

Stain and measure plaques

17. Prepare the staining overlay as described in steps 10 to 16 (omit step 15) except that the calculated volume should be 2 ml for each well.

18. Add 50 μl of 1 N HCl per 10 ml of overlay.

    Medium should appear red-orange but not yellow (see Troubleshooting).

19. Add 600 μl of 0.5% neutral red solution per 10 ml overlay (final neutral red concentration of 0.03% w/v).

    Neutral red tends to precipitate if solution is too alkaline or if added to 2× plaquing solution.

20. Transfer 2 ml to each well on top of the first overlay and return to the 37°C, 5% CO_2 humidified incubator for 6 to 8 hr.

    Neutral red diffuses in the overlay and stain cells at the bottom of the wells revealing clear plaques.

21. Scan plates with a resolution of 300 to 600 dpi.

22. Zoom in on plaques and measure the diameter of 20 plaques per well using the measuring tool of Adobe Photoshop. Avoid measuring plaques that are located at the edges of the well.

23. Calculate the average surface area (πr^2) of plaques formed by each bacterial strain. The plaque size is usually reported as the percentage or ratio relative to plaques formed by the reference or wild-type strain:

$$\frac{\text{average surface area of plaques (test or mutant strain)}}{\text{average surface area of plaques (ref. or wild-type strain)}}$$

    Use relative percentages or ratios to analyze data from different experiments because of variability in absolute plaque size between experiments.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibody buffer

10 ml TBS or TBS-TX (see recipe)
100 mg BSA (1% w/v final)
Dissolve on rocker at 4°C
Prepare fresh each day and keep on ice

Brain heart infusion (BHI) medium

100 ml H_2O
3.7 g BHI (BD Biosciences)
Stir to dissolve
Autoclave for 15 min
Store up to 2 months at 4°C

Caco-2 medium

500 ml EMEM (GIBCO or CellGrow)
130 ml FBS (20% v/v final)
6.5 ml 200 mM L-glutamine (2 mM final)
6.5 ml 10 mM nonessential amino acids (0.1 mM final)
6.5 ml 100 mM sodium pyruvate (1 mM final)
Store in the dark up to 2 months at 4°C

Verify expiration date on EMEM.

Use only sterile solutions to make this medium.

Formalin

40 ml PBS (*APPENDIX 2A*)
10 ml 16% formaldehyde stock solution, EM-grade (3.2% w/v final)
Wrap tube in aluminum foil
Store up to 1 month at 4°C

Formaldehyde is a known human carcinogen. It is toxic by inhalation, causes eye burn or blindness, is a strong sensitizer, and irritates the skin, eyes, and respiratory tract.

Gentamicin, 10 mg/ml

10 ml H_2O
100 mg gentamicin (10 mg/ml final)
Pass through a 0.22-μm filter to sterilize
Dispense into 1-ml aliquots
Store up to 2 years at –20°C or up to 2 months at 4°C
Always vortex before use

Follow the conversion factor indicated on powder stock container: 100 mg of powder does not equal 100 mg of gentamicin.

J774 and L2 medium

500 ml high-glucose DMEM without sodium pyruvate
41 ml FBS (7.5% v/v final)
5.5 ml 200 mM L-glutamine (2 mM final)
If needed, add penicillin G to 100 U/ml and streptomycin to 100 μg/ml final concentrations just before use
Store in the dark for up to 2 months at 4°C

Verify expiration date on DMEM.

Use sterile solutions to make this medium.

L. monocytogenes plaquing medium, 2×

High glucose DMEM powder with L-glutamine and pyridoxine HCl (for 1 liter mix)
3.7 g sodium bicarbonate
Dissolve in 450 ml H_2O
Measure volume and add H_2O to 500 ml
Pass through a 0.22-μm filter to sterilize
Add 55 ml FBS (10% v/v final)
Store in the dark at 4°C
Verify expiration date on DMEM

TBS

25 ml 1 M Tris·Cl, pH 8.0 (25 mM final; *APPENDIX 2A*)
30 ml 5 M NaCl (150 mM final)
945 ml H_2O
Store up to 1 year at room temperature

TBS-TX

500 ml TBS (see recipe)
0.5 ml Triton X-100 (0.1% v/v final)
In order to dissolve Triton X-100, solution may need to be warmed to ~50° to 55°C
Make fresh daily

NP-40 or saponin can substitute for Triton X-100.

Type I rat tail collagen, 100 μg/ml

10 ml 0.1 N acetic acid (filter-sterilized)
10 mg type I rat-tail collagen (Sigma)
Rock for a minimum of 3 hr at room temperature to dissolve
Freeze aliquots of 1 mg/ml solution indefinitely at –80°C
Just prior to use, dilute 1:10 in 0.02 N acetic acid (100 μg/ml final)
Discard solution after use

Dissolve collagen directly in original vial.

COMMENTARY

Background Information

L. monocytogenes is a saprophytic, Gram-positive bacterial rod found ubiquitously in nature (Fenlon, 1999). It is the etiologic agent of listeriosis, a food-borne disease affecting humans and a variety of vertebrates (Vazquez-Boland et al., 2001). Food-borne infections by *L. monocytogenes* initially cause a febrile gastroenteritis that is self-limiting in healthy individuals. However, in the immunocompromised or elderly, the infection becomes systemic, causing a meningoencephalitis and possibly a septicemia. In pregnant women, *L. monocytogenes* infects the fetus, causing abortion. As a food-borne pathogen, the bacterium has become a significant public health problem and has caused several epidemics in the United States and Europe (CDC, 1999).

The tissue culture cell assays that are described in this unit represent an in vitro approach to evaluate the virulence of *L. monocytogenes* isolates and mutant strains. These assays enable the dissection of steps leading to a successful infection: invasion, escape from primary vacuoles, intracellular growth, and cell-to-cell spread. These assays have been used to screen libraries of transposon-insertion mutants and to examine the phenotype of specific mutants leading to the discovery and characterization of important virulence factors (Portnoy et al., 1992; Dussurget et al., 2004). These assays have also been used to phenotype the intracellular behavior of isolates from clinical and food sources (Gray et al., 2004).

Critical Parameters

For every assay described in this unit, it is always important to repeat them more than once to verify reproducibility and to acquire enough data points for statistical analysis. In addition, as the physiological and biochemical status of tissue culture cells may change with the number of passages, one should try to perform replicate experiments within a short period of time or to use cells that have not been passaged excessively. Another potential source of variability is the FBS. Before buying a new lot of FBS, each assay should be performed with the old and new lots of FBS in parallel to verify reproducibility. It is preferable to buy a large quantity of FBS from a single lot to avoid reproducibility problems.

Stocks of *L. monocytogenes* should be stored at –80°C in 50% glycerol. Cultures of *L. monocytogenes* on BHI agar plates can be stored for up to 4 weeks at 4°C. However, it is preferable to streak a fresh plate every week. Spontaneous rough variants will appear on older cultures (Lenz and Portnoy, 2002). These variants have a defect in cell septation and will show deficiency in many of the assays described in this unit.

Troubleshooting

Table 9B.4.1 presents some commonly encountered problems with the assays discussed in this unit, as well as their possible causes and potential solutions.

Table 9B.4.1 Troubleshooting Guide for Tissue Culture Cell Assays Used to Analyze *Listeria monocytogenes*

Problem	Possible cause	Solution
Coverslips break	Too much heat	Blot as much ethanol as possible before flaming. Hold the coverslip by the edge. Avoid trapping ethanol between the arms of the forceps.
	Forceps with tip bend inward	The tip of fine forceps will bend easily if heated too much and a bent tip tends to cause the coverslip to crack when pressure is applied. Do not overheat the tip of forceps.
Coverslips move around	Air bubbles formed under coverslips *or* liquid movement displaces coverslips	Push coverslips down with sterile forceps. Make sure there is no partial overlap of coverslips. Handle dishes very gently. Wash gently.
Caco-2 cell monolayer peels off coverslip	Cells too dense	Reduce number of cells put down for the experiment.
	Dirty coverslips	Verify 70% and 95% ethanol for presence of precipitate material. Change solutions.
	Collagen treatment not adequate	Treat coverslip with 1× collagen solution freshly made from stock and seed with cells immediately after treatment.
Large variability between bacterial counts for a specific time point from three coverslips coming from the same dish	Unequal cell numbers on coverslips *or* unequal bacterial distribution in dish *or* unequal gentamicin equilibration in dish	When adding cells, bacteria, or gentamicin to a dish, always swirl dish gently to distribute evenly. Just before an infection, examine coverslips individually for cell distribution. Avoid using those that have more or less cells than the average coverslip for bacterial counts.
	Lysis of cells incomplete	Vortex coverslips for at least 20-30 sec at maximum speed to lyse infected cells. Make sure the coverslip is not stuck at the bottom of the tube.
	Uneven distribution of lysate in H_2O	Vortex again just prior to taking an aliquot out as membranes from cell lysates tend to aggregate possibly trapping bacteria.
Poor infection level	Overnight bacterial culture did not grow well or too old	Make fresh BHI.
		Use colonies from freshly streaked plates.
		Start bacterial cultures with a small inoculum as late as possible the day before.

continued

Table 9B.4.1 Troubleshooting Guide for Tissue Culture Cell Assays Used to Analyze *Listeria monocytogenes, continued*

Problem	Possible cause	Solution
	Cells too dense	Confluent monolayers do not infect well. Either decrease cell number or increase infection level.
No bacteria recovered	Penicillin and streptopmycin present in tissue culture medium	Always use antibiotic-free tissue culture medium when putting down cells for an assay and when adding fresh medium after washes.
Streaks of bacterial colonies on LB plates	Plates too wet: did not absorb the liquid.	Do not use agar plates with condensation. Do not use very fresh plates. Dry agar plates before use.
	Liquid not spread properly	Spread inoculum until no visible traces of wetness on agar surface.
Diff-Quick–stained samples look fuzzy under the microscope	Coverslip mounted cell side up	Put a drop of permount on the coverslip and add a clean coverslip on top.
	Coverslip not dry when mounted	Dry stained coverslip completely before mounting.
Difficulty to identify bacteria decorated with actin	High phalloidin background	Use freshly made antibody buffer and block longer. Stain for a shorter period of time or dilute phalloidin further.
		Wash more extensively.
Diffuse staining	Detergent present	Use detergent-free TBS at the end of the last wash.
	Mounting medium incompatible with fluor	Try a different mounting medium.
	Oxidation of fluor	Use mounting medium with antioxidant or a more stable fluorophore.
Clear crescent in monolayer of overlaid L2 cells	Overlay too hot when added onto cells	Cool down overlay by pipetting the solution up and down before adding to the wells.
	PBS pipetted directly onto cells or with too much strength during washes	Wash gently. Avoid pipetting PBS directly onto cells.
Overlay turns yellow before the fourth day of plaquing	Cells were too confluent at time of infection	Seed wells with less cells and infect before cells reach confluence.
	Bacterial growth	Make sure that gentamicin is added or use new stock of gentamicin.
Monolayer lyses after staining with neutral red	Unknown	Scan plates as soon as possible.

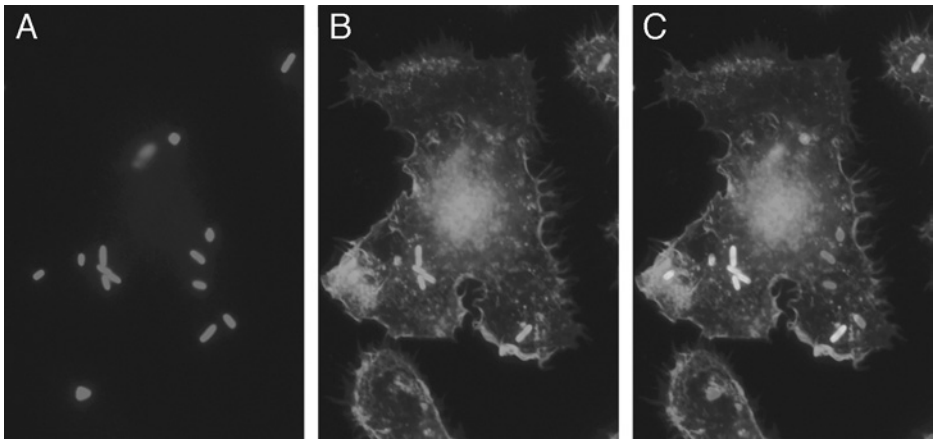

Figure 9B.4.1 Fluorescence micrograph of infected J774 cells showing bacteria decorated with actin filaments. **(A)** Bacteria were labeled with anti-*L. monocytogenes*–specific antibodies and with rhodamine-conjugated goat anti-rabbit IgG. **(B)** Actin was labeled with Alexa Fluor 488 phalloidin. **(C)** Overlay: most of the bacteria are yellow because of co-localizion with a cloud of actin. A few bacteria are red because they have not yet escaped the vacuole. For the color version of this figure go to *http://www.currentprotocols.com*.

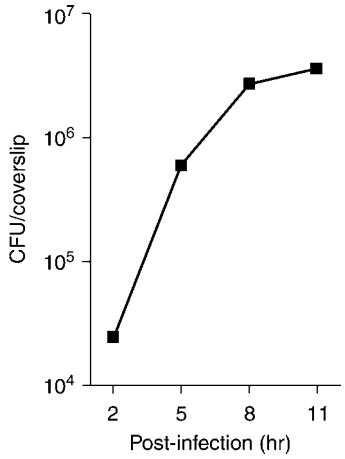

Figure 9B.4.2 Graph showing a typical *L. monocytogenes* growth curve in J774 cells.

Anticipated Results

Anticipated results reported here are based on experiments performed with wild-type strain 10403S, a streptomycin-resistant serotype 1/2a derived from *L. monocytogenes* 10403 (Bishop and Hinrichs, 1987), and with specific tissue culture cell lines. Variations should be anticipated when using other background strains and cell lines. The efficacy of *L. monocytogenes* 10403S to invade Caco-2 cells is <0.1% of the initial inoculum within the time period of the assay. Invasion rates increase with time, but one should be careful not to extend the experimental period beyond the point at which bacteria begin multiplying in the host cell. Efficacy of bacterial escape from vacuoles of mouse macrophages varies from 50% to 70% depending on the cell type used. A fluorescent micrograph representative of cells infected with vacuolar and cytosolic bacteria is shown in Figure 9B.4.1. Similar to the invasion assay, rates of escape from vacuoles increase with time, but one should be careful not to extend the experimental period beyond the point at which bacteria begin multiplying in the host cell. The kinetics of intracellular growth in mouse macrophages is ~45 min between 2 and 5 hr post-infection, ~90

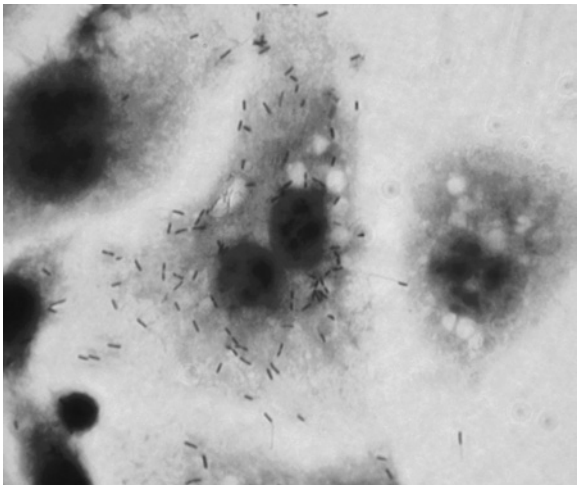

Figure 9B.4.3 Micrograph of J774 cells infected with *L. monocytogenes* and stained with Diff-Quick. Bacteria show as dark small rods. Notice the primary infected cell in the middle with neighboring cells in the process of becoming infected. For the color version of this figure go to *http://www.currentprotocols.com*.

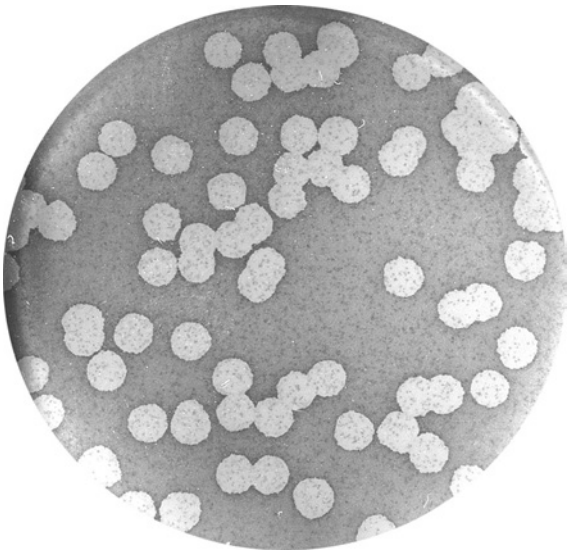

Figure 9B.4.4 L2 cells stained with neutral red 4 days after infection with *L. monocytogenes*. Clear zones in the monolayer represent plaques formed by foci of infection.

min between 5 and 8 hr post-infection, and ~180 min between 8 and 11 hr post-infection. A typical *L. monocytogenes* growth curve in J774 cells is presented in Figure 9B.4.2 and a micrograph of infected J774 cells stained with the Diff-Quick stain set is presented in Figure 9B.4.3. The kinetics of intracellular growth is slightly slower in other cell types. Plaques formed by 10403S in L2 cells have a diameter of ~2 to 2.4 mm (surface area of ≈ 3.0 to 4.5 mm^2) 4 days post-infection. An example of stained plaques formed by *L. monocytogenes* is shown in Figure 9B.4.4.

Time Considerations

The time required for preparing the coverslips depends on the experience of the person. Initially, it may take as long as 30 min for a dish with fifteen coverslips, but eventually it will take 10 min or less. For Basic Protocols 1 to 3, the cells are seeded the day before the infection is to be performed. For

Basic Protocol 4, the cells are seeded 2 days before the infection is to be performed. The invasion assay takes between 2 and 2.5 hr from washing the bacterial cells to plating cell lysates. It takes 24 hr at 37°C or 48 hr at 30°C for *L. monocytogenes* to form a decent size colony on LB agar. The first part of the protocol for measuring bacterial escape from the vacuole takes between 2 and 2.5 hr from washing the bacterial cells to fixing the infected cells. The fluorescence staining part takes between 2.5 and 5 hr depending on the number of samples. It takes between 30 and 60 min to examine each sample by fluorescence microscopy. The kinetics of intracellular growth takes 9 hr for an 8-hr growth curve or 12 hr for an 11-hr growth curve from washing the bacterial cells to plating the last time point. The infection part of the plaquing assay takes 1.5 hr from washing the bacteria to adding the overlay. There is a 4-day incubation period before staining the plaques, and there is a 6- to 8-hr incubation period after adding neutral red. Scanning the plates takes ~10 min per plate. Measuring plaque size takes 10 to 15 min for 20 plaques.

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Tissue Culture Assays Used to Analyze Invasion by *Staphylococcus aureus*

S. aureus is an important human pathogen that has the ability to invade many types of host tissues including skin, lung, cardiac endothelium, bone, and soft tissues, particularly in patients with immunosuppression and those harboring intravascular devices. Despite its maligned character, this bacterium normally does not cause disease and is frequently found as a commensal in the nares of ~25% of healthy individuals. However, in diabetics, i.v. drug abusers, and patients with cystic fibrosis, renal failure, or immunosuppression, the incidence of colonization and disease by this pathogen increases. Although it has long been held that *S. aureus* is an extracellular pathogen, recent experimental evidence suggests that *S. aureus* is capable of invading and surviving in a broad range of host cells including keratinocytes, pulmonary epithelial cells, osteoblasts, and cultured endothelial cells (Cheung et al., 1991; Bayles et al., 1998; Lowy, 1998). These intracellular sites can serve as reservoirs that enable the microorganism to avoid innate immunity and extraneous antimicrobial agents. Additionally, quorum sensing (Section 1C) and virulence gene expression inside professional phagocytes such as neutrophils and macrophages would confer additional survival advantages upon the bacteria, leading to apoptosis or lysis of host cells.

The culture of mammalian cells and tissues is now a widely accepted technique for studying the interactions of bacterial pathogens with host cells. This unit describes some of the cell lines commonly used to study *S. aureus* adherence, internalization, and intracellular trafficking, and the induction of apoptosis in host cells.

CAUTION: Staphylococcus aureus is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: The protocols described in the unit involve the use of human and/or murine tissues that may be infected with *S. aureus*. Follow all appropriate guidelines and regulations for the use and handling of human-derived materials and blood-borne pathogens, as well as for storage and disposal of "sharps." See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STANDARD *S. AUREUS* INVASION OF MAC-T CELLS

BASIC PROTOCOL 1

Bovine mastitis is a classic persistent infection that causes annual losses of billions of U.S. dollars to the dairy industry. It has been hypothesized that *Staphylococcus aureus*, the primary cause of bovine mastitis, avoids the host immune system by invading and surviving inside epithelial cells that line the mammary gland (Fox et al., 2000). Thus, the study of *S. aureus* invasion of cultured mammary epithelial cells via an in vitro assay using an immortalized bovine mammary epithelial cell line, designated MAC-T (Hyunh et al., 1991), is valuable in understanding the processes involved in intracellular survival. In addition, the induction of apoptosis in MAC-T cells by intracellular bacteria can be routinely monitored using a variety of protocols including (a) an agarose gel assessment of DNA laddering and (b) a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Facchinetti et al., 1991; Gavrieli et al., 1992; Wijsman et al., 1993; Piqueras et al., 1996).

Contributed by Ambrose L. Cheung and Kenneth W. Bayles
Current Protocols in Microbiology (2007) 9C.4.1-9C.4.7

Materials

MAC-T cells (contact Kenneth W. Bayles, *kbayles@unmc.edu*)
MAC-T growth medium (see recipe)
0.25% trypsin in HBSS (Sigma)
Invasion medium: MAC-T growth medium (see recipe) without antibiotics or FBS
S. aureus (strain RN6390; Network on Antimicrobial Resistance in *Staphylococcus aureus*; *http://www.narsa.net*)
Tryptic soy broth (TSB; see recipe) as bacterial growth medium
Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), filter sterilized
Gentamicin sulfate (Invitrogen)
0.025% (v/v) Triton X-100 (U.S. Biochemical) in sterile distilled water
Tryptic soy agar plates (see recipe)

24-well tissue culture plates (Costar)
Shaking bacteriological incubator

Additional reagents and equipment for counting cells using a hemacytometer (*APPENDIX 4A*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. Seed MAC-T cells at 6×10^4 cells/well (containing 1 ml of MAC-T growth medium) in 24-well tissue culture plates and grow until the density reaches $\sim 5 \times 10^5$ cells/well ($\sim$3 days). Periodically determine cell density by incubating the cells from a control well with 0.25% trypsin for 5 min at 37°C to release the cells from the surface of the plate, then counting using a hemacytometer (*APPENDIX 4A*).
2. At a time point $\sim$16 hr prior to the invasion assay (and prior to reaching 5×10^5 cells/well), replace the growth medium in each well with 1 ml of invasion medium.
3. Inoculate the *S. aureus* bacteria into 10 ml TSB and incubate overnight at 37°C with vigorous shaking (250 rpm).

   This should give a final bacterial cell density of $\sim 10^{10}$ cfu/ml.
4. On the day of the invasion assay, harvest 1 ml overnight culture of *S. aureus* by centrifuging 10 min at $2500 \times g$, room temperature, and removing the supernatant. Wash the cells three times each time by adding 3 ml sterile HBSS, centrifuging again as before, and removing the supernatant. Resuspend pellet in 1 ml invasion medium.
5. Wash the MAC-T cell monolayers in each well once with 1 ml of invasion medium, then add the bacterial suspension to the cells. Incubate 2 hr.

   In a typical experiment, 100 μl per well of a 1:100 dilution (in invasion medium) of the bacterial cells washed in step 4 works well. The cfu of the bacteria used should be enough to achieve a final multiplicity of infection (MOI) of ~50.
6. At end of 2-hr incubation, replace the supernatants of the cocultures with 1 ml invasion medium containing 100 μg gentamicin sulfate. Incubate the cocultures an additional 3 hr.

   In control experiments in the absence of MAC-T cells, bacterial growth should not be observed with the above gentamicin sulfate treatment.
7. Discard the supernatants of the cocultures and wash the monolayers in each well three times, each time with 1 ml HBSS.

8. Add 100 μl of 0.25% trypsin to each well and incubate 5 min at 37°C. Lyse the MAC-T cells by adding 900 μl of 0.025% (v/v) Triton X-100 and vortexing briefly.
9. Prepare 10-fold serial dilutions of the cell lysates in TSB and plate in triplicate on tryptic soy agar plates to quantify the bacteria.

ASSAY FOR ADHERENCE OF *S. AUREUS* TO CULTURED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVECs)

BASIC PROTOCOL 2

S. aureus bacteremia and dissemination into organ tissues are essential steps in the pathogenesis of staphylococcal disease (Lowy, 1998). One unique aspect of the pathogenesis of *S. aureus* bacteremic infections in humans is the ability of the organism to adhere to and colonize endothelial cells during transient bacteremia. To define this interaction, several investigators have used cultured human umbilical vein endothelial cells (HUVEC) as a model. A consequence of this interaction is the internalization of *S. aureus* by HUVEC, eventuating in apoptosis of the endothelial cells (Kahl et al., 2000). The protocol below describes the use of human umbilical cord endothelial cells in adherence and apoptosis assays (Jaffe et al., 1973; Jaffe, 1980).

Materials

S. aureus (strain RN6390; Network on Antimicrobial Resistance in *Staphylococcus aureus*; *http://www.narsa.net*)
Bacterial growth medium: e.g., tryptic soy broth (TSB; see recipe) or brain-heart infusion medium (e.g., Difco)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
M199 medium (Bio-Whittaker)
Tryptic soy agar for growing *S. aureus* (see recipe)
Human umbilical vein epithelial cells (HUVEC) grown to confluence in gelatin-coated 96-well plates (*APPENDIX 4B*)
0.05% (w/v) collagenase/0.01% (w/v) EDTA
0.25% trypsin/0.1% EDTA (Cellgro) in M199 medium (Bio-Whittaker)
2% (v/v) Triton X-100 in H_2O
10 μg/ml lysostaphin (Ambi Biologicals)

18-mm glass culture tube
Humidified 37°C, 5% CO_2 incubator
Microtip sonicator (e.g., Branson)

Additional reagents and equipment for culture of HUVEC (*APPENDIX 4B*) and trypan blue exclusion test of cell viability (Strober, 1997)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly.

NOTE: Unless otherwise stated, all reagents and incubations are at 37°C.

Prepare and enumerate bacteria

1. Grow *S. aureus* in 10 ml culture medium to $OD_{650} = 1.0$ in an 18-mm glass tube.

   This should correspond to ~1 × 10^9 cfu as measured in a Spectronic 20.

2. Harvest the bacteria by centrifuging 10 min at 3000 × *g*, room temperature, and removing the supernatant. Wash twice, each time by adding 10 ml PBS, centrifuging again as before, and removing the supernatant.
3. Resuspend the bacterial pellet in M199 medium and adjust to $OD_{650} = 1.0$ with M199 medium in a spectrophotometer.

4. Confirm the bacterial density by plating serial dilutions on tryptic soy agar plates and counting colonies to determine the number of cfu.

Enumerate HUVEC

5. Enumerate the number of HUVEC in each of three wells of a confluent 96-well plate by adding 200 µl of 0.05% collagenase/0.01% EDTA, incubating 5 min, then pipetting up and down to mix and counting viable cells by trypan blue exclusion (Strober, 1997).

   A viability of ≥ 95% is anticipated as confirmed by the exclusion of the trypan blue dye.

   In these studies, the authors grow HUVEC to confluence on gelatin-coated 96-well microtiter plates (Corning) in culture medium in the absence of antibiotics (APPENDIX 4B). Similar studies can also be performed in 24-well or 6-well plates (Tables A.4B.1 to A.4B.3).

   Separate sets of triplicate wells of confluent HUVEC will be required for enumeration of total bacteria (adherent plus internalized; steps 6 to 10) and for enumeration of internalized bacteria (steps 11 to 12).

Enumerate total bacteria (adherent plus internalized)

6. Add 100 µl of the bacterial suspension (see step 3) to triplicate wells containing HUVEC and incubate at 37°C in a humidified 5% CO_2 incubator for varying time periods.

   For example, incubate for time periods ranging from 30 to 120 min to ascertain the optimal time period for the adherence assay. This may differ between bacterial strains.

7. After incubation, wash the monolayers gently with 200 µl/well of M199 medium, tilting the microtiter plate slightly and aspirating the wash medium under gentle suction with a Pasteur pipet. Repeat this wash three times.

   Take special care to avoid direct contact of the pipet tip with the cells.

8. Detach the HUVEC and adherent bacteria by adding 200 µl of 0.25% trypsin/0.1% EDTA in M199 medium to each well.

9. Mix well by pipetting up and down with a 1000-µl pipet tip several times. Transfer to a microcentrifuge tube containing 200 µl of 2% Triton X-100 in water.

10. Sonicate for 20 sec with a microtip sonicator set at maximum power to disrupt bacterial clusters. Plate serial dilutions on tryptic soy agar plates and count colonies to determine the number of cfu, reported as the mean from triplicate wells.

    This assay will yield both adherent and internalized S. aureus bacteria.

Enumerate internalized bacteria

Separate sets of triplicate wells are used for determining the internalized bacteria.

11. To ascertain the number of internalized bacteria, add 100 µl of 10 µg/ml lysostaphin per well to the HUVEC and incubate for 20 min to lyse extracellular *S. aureus*.

12. Wash cells three times, detach with trypsin/EDTA, lyse HUVEC using Triton X-100 and sonication, and count colony growth as described in steps 7 to 10, to enumerate the number of internalized bacteria.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

MAC-T growth medium

Dulbecco's modified Eagle's medium (DMEM) containing:
10% (v/v) fetal bovine serum (FBS; heat-inactivated; omit for invasion medium)
5 mg/ml insulin
1 mg/ml hydrocortisone
44 mM $NaHCO_3$
100 U/ml penicillin G (omit for invasion medium)
100 mg/ml streptomycin sulfate (omit for invasion medium)

Tryptic soy agar (TSA)

Dissolve the following in a 2-liter Erlenmeyer flask containing 1 liter H_2O:
15 g pancreatic digest of casein
5 g Bacto Soytone (Difco)
5 g NaCl
15 g agar

Leave the stir bar in the flask and autoclave 20 min at 121°C to sterilize. While the agar is in the autoclave, wipe down the surfaces of a clean bench or tissue culture hood with 70% ethanol. Cool the agar with stirring or by placing in a 50°C water bath. Add any required antibiotics and pour the plates on a clean bench or in a tissue culture hood. Pour 35 to 40 ml agar into each Petri dish to give a thicker plate, which will prevent it from drying out during incubation. Allow the plates to solidify 6 to 8 hr at room temperature, then incubate at 37°C overnight to identify contaminated plates. Remove the plates from the incubator, discard any contaminated plates containing colonies growing on the surface, and allow to cool to room temperature on the bench before storage. Store plates up to 1 month at 4°C.

TSA is widely used in microbiology, and it may prove more economical to purchase the powdered prepared TSA from Difco or Oxoid than to prepare this medium from scratch.

Tryptic soy broth (TSB)

Dissolve the following in 1 liter H_2O:
17.0 g pancreatic digest of casein
3.0 g Bacto Soytone (Difco)
5.0 g NaCl
2.5 g K_2HPO_4
2.5 g glucose

Aliquot into bottles that can withstand autoclaving. Autoclave for 20 min to sterilize. Store up to 1 month at 4°C.

It is easier and relatively inexpensive to order powdered TSA from a commercial supplier such as Difco or Oxoid.

COMMENTARY

Background Information

S. aureus is an opportunistic pathogen that can infect humans and other mammals. In humans, *S. aureus* can cause a variety of superficial infections (e.g., cellulitis or skin abscesses) and deep-seated infections ranging from bacteremia to abscesses in almost any organ, or multiple organs, of the body. A commonly occurring scenario is that the microorganism gains access to the bloodstream via an infected catheter or other intravascular device. Dissemination to distal organs from the bloodstream would require bacterial attachment to endothelial cells followed by invasion

and extravasation. However, the specific mechanism of bacterial entry into endothelial cells and exit to surrounding tissues has not been defined. Studies of *S. aureus* with cultured HUVEC represent an important step in understanding the mechanism of *S. aureus* dissemination.

An important staphylococcal disease in mammals that has human relevance is bovine mastitis. It has been estimated that ~8% of cows are infected. The primary concern is the effect of this disease on cows, resulting in the cessation of milk production with consequent financial impact on the dairy industry. Segregation of infected cattle and cessation of milking have often been used as the control strategy. These techniques for preventing dissemination of *S. aureus* among cows are time consuming, labor intensive, and expensive. A better strategy will be to understand the physical and immunologic defenses in bovine mammary epithelial cells and how *S. aureus* can usurp these defense mechanisms. To enable these studies, cell culture studies of *S. aureus* with bovine mammary epithelial cells (MAC-T cells) have been initiated.

Many successful pathogens have evolved unique mechanisms to evade the host cell killing mechanisms both extracellularly and intracellularly. Although it had been previously thought that *S. aureus* primarily caused diseases outside host cells, recent studies suggest that *S. aureus* can be internalized into a variety of cultured nonphagocytic cells, including human keratinocytes, pulmonary epithelial cells, endothelial cells, osteoblasts, fibroblasts, and bovine mammary epithelial cells (Bayles et al., 1998; Lowy, 1998; Cheung et al., 1991; Cheung et al., 2004; Qazi et al., 2004). In this unit, discussion has been limited to only two cell lines, HUVEC and MAC-T cells, with the hope of expanding the number of cell types in future updates. Individual cell lines discussed here represent a close approximation of the model system that investigators can use to examine a particular infection syndrome (i.e., HUVEC for disseminated sepsis syndrome in humans and MAC-T cells for bovine mastitis) while minimizing the extensive use of animal models. Regardless, these methods have demonstrated that *S. aureus* can be actively internalized by cultured human endothelial cells and bovine mammary epithelial cells, thus making it possible to study the endosomal fate of *S. aureus*-containing vesicles inside these nonprofessional phagocytes. In some cases (Bayles et al., 1998; Kahl et al., 2000; Qazi et al., 2004), the maturation of the endosomes may be altered or delayed (e.g., cystic fibrosis bronchial epithelial cells, CFT-1) or the bacteria may escape into the cytosol (MAC-T cells).

Critical Parameters

In adherence assays with HUVEC, take extreme care in washing between incubation steps to avoid dislodging HUVEC from the gelatin-coated wells. An alternative is to lightly fix the HUVEC with 0.05% glutaraldehyde for 5 min followed by washes; however, a pilot study should be performed to ensure that bacterial adherence to HUVEC is not affected by the fixing procedure.

A recent study by Qazi et al. (2004) indicates that gentamicin can inhibit the growth of the intracellular bacteria and, thus, may impact the normal metabolic processes that occur within the intracellular niche. Although the authors of this unit have not observed these effects on bacterial growth, incubation at 37°C with 50 μg/ml lysostaphin for 10 min can be used in place of gentamicin treatment.

Troubleshooting

If enumeration of cfu leads to significant variations in results of the adherence assays with HUVEC, one can deploy bacteria labeled with methyl-[^{3}H]thymidine. These labeled bacteria, once prepared and stored at 4°C, can be used for several assays and, if heat-killed (by incubating at 55°C for 1 hr), can last for as long as 3 to 4 weeks. After adherence, the HUVEC and adherent bacteria can be lysed with 200 μl of 2.5% SDS and 0.2 M NaOH (if in microtiter wells) and 100 μl of this lysate is neutralized with 400 μl of 0.05 M acetic acid and counted for radioactivity in the presence of scintillation fluid (Readysafe, Beckman Instruments).

Anticipated Results

In the authors' experience, the adherence varies significantly among clinical isolates. With most studies, ~1% of the bacteria showed nonspecific adherence to tissue culture wells. Adherence to HUVEC can be increased by precoating the wells with plasma, probably simulating what happens in vivo (10% to 20% of the inoculum can adhere in this manner).

The percent survival of MAC-T cells is quite variable, ranging from 5% to 40%, depending on the specific bacterial strain used and the calculated MOI (Hyunh et al., 1991; Bayles et al., 1998; Fox et al., 2000). Presumably due to the induction of apoptosis and subsequent permeabilization of the host cell

membrane, the percent survival will drop substantially after ~10 hr post-invasion.

Time Considerations

With a primary culture of HUVEC in a 25-cm^2 flask, the cells can be passaged every 3 to 4 days, depending on the level of confluence. Once confluence is reached, the experiments can typically be accomplished in a single day, beginning with bacterial harvest and enumeration of bacteria by cfu or counting radioactivity from tritium-labeled bacteria.

The procedure, from seeding of the cell culture to counting colonies, should take approximately 4 days. Approximately 3 days of incubation are required to reach a MAC-T cell density of $\sim 5 \times 10^5$ cells/well, and a total of 16 hr is needed to grow the bacteria to a density of $\sim 1 \times 10^{10}$ cfu/ml. On the day of the invasion assay, allow 3 hr for preparing the MAC-T cell/*S. aureus* coculture and 4 hr for incubation with gentamicin and plating of the surviving (intracellular) bacteria. Another 24 hr is required for incubation and enumeration of bacterial colonies.

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Murine Models of *Streptococcus pyogenes* Infection

Because *S. pyogenes* is a strictly human pathogen (with the exception of one strain, see below), there is no really good animal model for studying its virulence. In the absence of such a complete model, methods have been devised to model different aspects of the human disease. The three infection models presented are valuable in assessing different aspects of *S. pyogenes* virulence. The long-term throat colonization model (Basic Protocol 1) uses the single *S. pyogenes* strain isolated as a mouse pathogen by Lancefield's group (Hook et al., 1960). It also causes disease in humans. This strain was chosen in the hope that it would initiate a more natural infection in the mouse. This model is valuable for studies of the early colonization steps of infection and for studies of long-term persistence (at least one month). Basic Protocol 2 (pneumonia model) also uses the mouse-pathogenic strain. Although it bypasses the initial steps of infection by using intratracheal inoculation, it gives rapid, reproducible results about virulence of the strain due to systemic dissemination. It may serve as a more natural alternative than the model employing intraperitoneal inoculation. The third model (Basic Protocol 3) uses a human isolate of *S. pyogenes* and introduction of the bacteria by subcutaneous inoculation into out-bred mice. Many virulent strains of *S. pyogenes* can be used in Basic Protocol 3. This model mimics one major type of human disease: infection through the skin followed by systemic dissemination of the bacteria and possible death. Additional protocols (Basic Protocols 4 to 11) present methods for determining the amount of *S. pyogenes* in the different mouse tissues following infection.

The procedures presented in this unit use healthy mice, and no additives (e.g., antibiotics, culture media, or beads) are introduced during the inoculation. These models are generally useful in testing the virulence of mutants constructed in the laboratory. For example, to determine whether the capsule is important for virulence and which step(s) of infection it affects, a capsule-deficient mutant can be used in these models. In addition, the models provide assays to assess the effectiveness of potential vaccines or therapeutics. It should be remembered that different strains of *S. pyogenes* behave very differently in many assays, including animal models. Because *S. pyogenes* secretes many known and potential virulence factors, experiments performed with washed bacteria (as presented below) will give different results from those performed with unwashed inocula.

CAUTION: Streptococcus pyogenes is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: This experiment requires Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: When performing counts on a hemacytometer (*APPENDIX 4A*) for *S. pyogenes*, each chain should be considered a single colony forming unit (cfu). Be certain that only cocci

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in chains are present in the culture. Cultures should be abandoned if other species are present.

BASIC PROTOCOL 1

THROAT COLONIZATION MODEL OF *STREPTOCOCCUS PYOGENES*

This model was developed to assess the ability of mutants of the mouse-pathogenic strain of *S. pyogenes* (Hook et al., 1960) to persist in the throat for long periods following intranasal inoculation (Husmann et al., 1997). The strain of mouse chosen is critical for the outcome of long-term persistence. The authors' observations suggest that if the bacteria are present on day 3 or 4 in throat swabs from the mice, they will persist for at least one month. Thus, the model assesses the contribution of the strain being tested to long-term throat colonization. It should be valuable as an assay for vaccines or therapies designed to prevent or cure *S. pyogenes* infection. The drawback to this model is that it may not work with other *S. pyogenes* strains.

Materials

Streptococcus pyogenes strain B514-Sm (Hook et al., 1960)
2× brain heart infusion (BHI)/2% (v/v) supplement B (see recipe)
0.9% saline (*APPENDIX 2A*)
7-week-old female (preferred) C57BL10/SnJ mice (Jackson Laboratories): place order to allow for acclimatization one week prior to inoculation at the age indicated (see Critical Parameters and Troubleshooting)
Solution containing 1.5 mg/ml xylazine and 100 mg/ml ketamine
THY plates containing 1000 μg/ml streptomycin (see recipe)
85 μg/ml hyaluronidase (optional)

15-ml screw-cap conical tubes
Klett flasks, screw-cap (Bellco)
Klett colorimeter with red filter
Refrigerated centrifuge (e.g., Beckman with JA-14 rotor) and centrifuge bottles
Rodent ear punch
Balance accurate to 0.01 g
20-μl micropipettor and round-tipped sequencing pipet tips

Additional reagents and equipment for counting cells (*APPENDIX 4A*) and for anesthesia (Donovan and Brown, 1998)

1. Grow a 6-ml overnight culture of *S. pyogenes* strain B514-Sm in 2× BHI/2% supplement B. Incubate at 37°C, static, in a 15-ml screw-cap conical tube.

   IMPORTANT NOTE: *DO NOT use hyaluronidase. Capsule production by the bacteria is essential for induction of throat colonization.*

2. Add the 6-ml overnight culture to 144 ml of 2× BHI/2% supplement B (a 1/25 dilution). Incubate at 37°C, static, in a screw-cap Klett flask until the Klett reading is 85 ± 5 (late log phase; *APPENDIX 4A*).

3. Harvest bacteria by centrifuging the culture 20 min at 8500 × *g* (7500 rpm in a JA-14 rotor), 4°C.

4. Pour off the supernatant carefully.

   IMPORTANT NOTE: *Mucoid strains do not form very tight pellets. Use caution when pouring to retain the pellet.*

5. Place the culture pellet on ice and keep on ice throughout the rest of the inoculation procedure.

6. Resuspend the bacteria in the small amount of liquid remaining in the centrifuge bottle.

7. Determine cfu per milliliter by counting in a hemacytometer (*APPENDIX 4A*).

   Be certain that only cocci in chains are present in the culture.

   Note that each bacterial chain is considered a single cfu (see Critical Parameters and Troubleshooting).

8. Adjust the density of the bacterial suspension with 0.9% saline so that the number of cfu to be delivered to each mouse is contained in a 10-µl volume.

   For example, if each mouse is to be given a dose of 1×10^7 cfu, the bacterial suspension should be at a density of 1×10^9 cfu/ml.

9. Anesthetize each 7-week-old female (preferred) C57BL10/SnJ mouse by intraperitoneal injection with 30 µl of a solution containing 1.5 mg/ml xylazine and 100 mg/ml ketamine (see Donovan and Brown, 1998).

   Female mice are preferred because they are less aggressive.

   For alternate anesthesia, see Basic Protocol 3, steps 11 and 12.

10. Once the animals become unresponsive to having their hind feet pinched, punch their ears for identification using a standard rodent ear punch. If necessary, weigh the animals at this time using a balance accurate to 0.01 g.

    Use the system of no punches, one in the right ear, one in the left ear, two in the right ear, etc.

11. Lay each mouse horizontally on its back.

12. Inoculate each mouse by depositing 10 µl bacterial suspension on the exterior portion of the left nares using a 20-µl micropipettor and a round-tipped sequencing tip. Place all 10 µl on the left nares only.

    If the bacterial suspension is gently dropped onto the exterior of the nares, the mouse will breathe in the liquid as it inhales.

13. Allow the animals to remain on their backs, undisturbed, in their cages until they recover from the anesthesia.

    This will take ~30 min.

14. Plate in quadruplicate 10-fold serial dilutions of the inoculum on THY plates containing 1000 µg/ml streptomycin to perform viable plate counts and confirm the cfu present in the suspensions used for inoculation.

    Countable plates should have only 25 to 50 colonies per plate because the colonies are very mucoid. Alternatively, plate on THY plates containing 85 µg/ml hyaluronidase and 1000 mg/ml streptomycin to allow counting of higher numbers on more plates with lower dilutions.

    THY plates containing 1000 µg/ml streptomycin are used for growing group A Streptococcus (GAS) streptomycin-resistant strains to prevent growth of contaminating Staphylococcus. If using wild-type Streptococcus, isolate a streptomycin-resistant strain by plating $>10^9$ bacteria on THY medium containing 1000 µg/ml streptomycin.

PNEUMONIA MODEL OF *STREPTOCOCCUS PYOGENES* INFECTION (INTRATRACHEAL)

BASIC PROTOCOL 2

This model was developed in an attempt to mimic the normal mode of introduction (i.e., inhalation) of *S. pyogenes* into an organism (Husmann et al., 1996; 1997). The natural mouse-pathogenic bacterial strain identified by Lancefield's group (Hook et al., 1960) is used. Because intranasal inoculation caused infection in a maximum of 60% of the mice regardless of dose, this method could not be used to calculate an infectious or lethal dose (Husmann et al., 1996). Instead, the initial infection steps are bypassed by introducing the

bacteria intratracheally. The model is expected to be useful as a reporter of the bacteria's ability to disseminate from the initial site of infection and cause systemic disease. It is valuable because it represents a natural site of infection and results in rapid spread, allowing for an experiment of short duration (72 hr).

Materials

S. pyogenes strain B514-Sm (Hook et al., 1960)
2× BHI/2% supplement B (see recipe)
0.9% saline (*APPENDIX 2A*)
5-week-old female C3HeB/FeJ mice (Jackson Laboratories; *http://www.jax.org*): place order to allow for acclimatization one week prior to inoculation at the age indicated (see Critical Parameters and Troubleshooting)
Solution containing 1.5 mg/ml xylazine and 100 mg/ml ketamine
THY plates containing 1000 μg/ml streptomycin (see recipe)
85 μg/ml hyaluronidase (optional)
15-ml screw-cap conical tubes
Klett flask, screw-cap (Bellco)
Klett colorimeter with red filter
Refrigerated centrifuge (e.g., Beckman with JA-14 rotor) and centrifuge bottles
Rodent ear punch
Balance accurate to 0.01 g
22-G straight feeding needle (Popper and Sons) and 1-cc tuberculin syringe
Additional reagents and equipment for counting cells (*APPENDIX 4A*) and for anesthesia (Donovan and Brown, 1998)

1. Grow a 6-ml overnight culture of *S. pyogenes* strain B514-Sm in 2× BHI/2% supplement B. Incubate at 37°C, static, in a 15-ml screw-cap conical tube.

   IMPORTANT NOTE: *DO NOT use hyaluronidase (capsule production by the bacteria is essential for induction of pneumonia).*

2. Add the 6-ml overnight culture to 144 ml of 2× BHI/2% supplement B (a 1/25 dilution of the culture). Incubate at 37°C, static, in a screw-cap Klett flask until the Klett reading is 85 ± 5 (late log phase; *APPENDIX 4A*).

3. Centrifuge culture 20 min at 8500 × *g* (7500 rpm in a JA-14 rotor), 4°C to harvest bacteria.

4. Pour off the supernatant carefully.

   IMPORTANT NOTE: *Mucoid strains do not form very tight pellets. Use caution in pouring to retain the pellet.*

5. Place the culture pellet on ice and keep on ice throughout the rest of the inoculation procedure.

6. Resuspend the bacteria in the small amount of liquid remaining in the centrifuge bottle.

7. Determine cfu per milliliter by counting in a hemacytometer (*APPENDIX 4A*).

   Be certain that only cocci in chains are present in the culture.

   Note that each bacterial chain is considered a single cfu (see Critical Parameters and Troubleshooting).

8. Adjust the density of the bacterial suspension with 0.9% saline so that the number of cfu to be delivered to each mouse is contained in a 20-μl volume.

   For example, if each mouse is to be given a dose of 1.0×10^7 cfu, the bacterial suspension should be at a density of 5.0×10^8 cfu/ml.

9. Anesthetize each 5-week-old female C3HeB/FeJ mouse by intraperitoneal injection of 30 µl of a solution containing 1.5 mg/ml xylazine and 100 mg/ml ketamine (see Donovan and Brown, 1998).

   For alternate anesthesia, see Basic Protocol 3.

   Female mice are preferred because they are less aggressive.

10. Once the animals become unresponsive to having their hind feet pinched, punch their ears for identification using a standard rodent ear punch. If necessary, weigh the animals at this time using a balance accurate to 0.01 g.

    Use the system of no punches, then one in the right ear, one in the left ear, two in the right ear, etc.

11. Deposit 20 µl bacterial suspension into the lower trachea by holding the mouse vertically with its head up, inserting a 22-G straight feeding needle attached to a 1-cc tuberculin syringe into the mouth and then into the lower trachea.

    Keep in mind that the trachea has a rough "vacuum cleaner hose" texture while the esophagus is very smooth. Upon placing the needle into the trachea, the rounded tip of the needle should be felt from the outside. When the tip of the needle reaches the bronchi of the mouse, resistance will be felt. Once this happens, do not insert the needle any further; pull the needle up just slightly.

12. Place the mouse on its back and allow it to remain in that position until it recovers from anesthesia.

    This will take ~30 min.

13. Plate in quadruplicate 10-fold serial dilutions of the inoculum on THY plates containing 1000 µg/ml streptomycin to perform viable plate counts and confirm the cfu present in the suspensions used for inoculation.

    Countable plates should have only 25 to 50 colonies per plate because the colonies are very mucoid. Alternatively, plate on THY plates containing 85 µg/ml hyaluronidase and 1000 µg/ml streptomycin to allow counting of higher numbers on more plates with lower dilutions.

    THY plates containing 1000 µg/ml streptomycin are used for growing group A Streptococcus (GAS) streptomycin-resistant strains to prevent growth of contaminating Staphylococcus. If using wild-type Streptococcus, isolate a streptomycin-resistant strain by plating >10^9 bacteria on THY medium containing 1000 µg/ml streptomycin.

SUBCUTANEOUS INOCULATION OF CD1 MICE WITH *STREPTOCOCCUS PYOGENES*

BASIC PROTOCOL 3

One major route of infection in humans is through the skin. Such infections can result in systemic spread and serious, life-threatening disease. This model is modified from one first described by Bunce et al. (1992) and Ashbaugh et al. (2000) and was used as described below by Limbago et al. (2000, 2001). It appears that most or all strains of mice will be infected (immunocompetent hairless mice were used first; out-bred mice are used below), and the outcome depends on the bacterial strain. Many cause only local lesions, the sizes of which can be measured to report degree of virulence. With some strains, the introduction of cytodex beads along with the bacteria has proven necessary for successful infection. More virulent *S. pyogenes* strains, e.g., the one described below, lead to systemic dissemination of the bacteria and death of the mouse without the introduction of foreign irritants in the inoculum. This model is probably closer to a type of human disease than the previously described models, and it may accurately mimic virulence following skin infection in people. It should be noted that lesion size and systemic spread in different hosts might not be influenced equally by the virulence factor being studied (Dalton et al., 2006).

Materials

6-week-old female CD1 (outbred) mice, 21 to 23 g (Charles River Laboratories; *http://www.criver.com*): place order to allow for acclimatization one week prior to inoculation at the age indicated (see Critical Parameters and Troubleshooting)
THY medium (see recipe)
Group A *Streptococcus* (GAS) strain, preferably streptomycin-resistant (e.g., AM3; Stamp and Hendry, 1937)
0.9% saline (*APPENDIX 2A*), 4°C, sterile
2.5% Avertin (see recipe)
Depilatory agent (e.g., Nair)
THY plates containing 1000 μg/ml streptomycin (see recipe)
85 μg/ml hyaluronidase (optional)
Isoflurane

15-ml conical tube
Klett flask (Bellco)
Klett colorimeter and with red filter
Refrigerated centrifuge (e.g., Beckman with JA-20 rotor)
Balance accurate to 0.01 g
Rodent ear punch
Cotton-tipped applicators
Gauze pads
Alcohol wipes
27-G needle and 1-ml insulin/tuberculin syringe
Calipers (optional)
Urogenital swab, single-use (Puritan)
Dissecting tools, sterile
Dounce tissue homogenizer

Additional reagents and equipment for counting cells (*APPENDIX 4A*), removal of spleen (Basic Protocol 6, steps 1 to 3), and anesthesia (Donovan and Brown, 1998)

Prepare bacterial cultures

1. Inoculate 5 ml THY medium with a GAS strain in a 15-ml conical tube. Incubate overnight at 37°C, static.

   The authors use AM3 because a streptomycin-resistant strain is preferable.

2. Inoculate 2.5 ml overnight culture into a Klett flask containing 50 ml THY medium (a 1:20 dilution). Incubate in a 37°C static water bath until the Klett reading is 50 to 55 Klett (mid-log phase).

   This usually takes ~4 to 5 hr.

3. Harvest bacteria by centrifuging 15 min at 6000 × *g* (7,000 rpm in a JA-20 rotor), 4°C.

4. Pour off the supernatant carefully.

   IMPORTANT NOTE: *Mucoid strains do not form a very tight pellet. Use caution when pouring to retain pellet.*

5. Resuspend pellet in 10 ml 0.9% saline and centrifuge 15 min at 6000 × *g* (7,000 rpm in a JA-20 rotor), 4°C.

6. Carefully pour off supernatant.

7. Place the culture on ice and keep on ice throughout the remainder of protocol.

8. Resuspend the cell pellet in 4 ml of 0.9% saline.

9. Determine cfu per milliliter by counting in a hemacytometer (*APPENDIX 4A*).

   Also use this opportunity to ensure that the culture was not contaminated. Each chain is considered one cfu. Cultures should be discarded if other bacterial species are present.

10. Adjust the density of the culture with 0.9% saline so that the desired cfu are delivered in a 100-μl volume.

    For example, if delivering 10^8 cfu, the culture should be at 10^9 cfu/ml.

    The authors use 2×10^7 cfu/mouse for inoculation. This is above the LD_{50} for GAS strain AM3.

Inoculate mice

11. Weigh each mouse using a balance accurate to 0.01 g and multiply the weight in grams by fifteen to determine the number of microliters of 2.5% Avertin to administer.

12. Anesthetize each mouse by intraperitoneal injection of the calculated volume of 2.5% Avertin (see Donovan and Brown, 1998).

13. Once the animals have become unresponsive to having their hind feet pinched, punch their ears for identification using a standard rodent ear punch.

    Use system of zero punch, 1 left, 1 right, 2 left, 2 right.

14. Lay each mouse on its stomach and apply a quarter-sized amount of depilatory agent to the back of the back haunch of the mouse with a cotton-tipped applicator. Wait ~5 min, then remove hair with a gauze pad. Swab depilated spot with alcohol wipe.

15. Inoculate 100 μl bacterial suspension just under the skin surface in the center of the bald spot using a 27-G needle and 1-ml insulin/tuberculin syringe.

    Keep the syringe at an ~45° angle to the skin, bevel side up. Insert the needle just under the skin. If done correctly, this should result in a small "bleb" under the skin with no bleeding.

16. Allow animals to remain on their stomachs in cages until they recover from anesthesia.

    This should take ~30 min.

17. Plate in quadruplicate 10-fold serial dilutions of the inoculum on THY plates containing 1000 μg/ml streptomycin to perform viable plate counts and confirm the cfu present in the suspensions used for inoculation.

    Countable plates should have only 25 to 50 colonies per plate because the colonies are very mucoid. Alternatively, plate on THY plates containing 85 μg/ml hyaluronidase and 1000 μg/ml streptomycin to allow counting of higher numbers on more plates with lower dilutions.

    THY plates containing 1000 μg/ml streptomycin are used for growing group A Streptococcus (GAS) streptomycin-resistant strains to prevent growth of contaminating Staphylococcus. If using wild-type Streptococcus, isolate a streptomycin-resistant strain by plating $>10^9$ bacteria on THY medium containing 1000 μg/ml streptomycin.

Evaluate infected mice

18. Observe mice twice daily for signs of illness (lethargy, weight loss, abscess formation) and chart accordingly. If desired, measure lesion size with calipers.

    Abscess will usually progress from purulent (raised, whitish) to necrotic (sunken, scabbed) within 1 to 2 days postinoculation.

    Mice generally are moribund within 3 to 4 days postinoculation.

19. Euthanize mice by inhalation of isoflurane followed by cervical dislocation.

20. Remove spleens (see Basic Protocol 6, steps 1 to 3) using sterile dissecting tools and transfer aseptically to a Dounce tissue homogenizer.
21. Homogenize in 1 ml of 0.9% saline and plate (undiluted) in duplicate for viable bacteria on THY plates containing 1000 μg/ml streptomycin.
22. Wipe the wound site area with an alcohol wipe. Lift the scab with sterile forceps and swab with a single-use urogenital swab placed in 1 ml THY medium containing 1000 μg/ml streptomycin.
23. Incubate 2 days at 37°C and evaluate by microscopic examination to determine if streptococci are present.

CULTURING OF VARIOUS MOUSE ORGANS AND FLUIDS TO ASSAY FOR *STREPTOCOCCUS PYOGENES* STRAIN B514-SM

Prior to collecting any tissues or doing any culturing (except for throat culture or collection of blood sample by exsanguinations) the mouse should be sacrificed using an approved method (e.g., anesthesia followed by cervical dislocation).

Use proper aseptic technique throughout these procedures to prevent cross contamination of tissues and samples. This means carefully cleaning and flame-sterilizing tweezers and scissors before cutting into any new membrane or tissue. Also spray the entire exterior surface of the mouse with 95% ethanol using a squirt bottle before beginning tissue collection. For any culture which is to be quantitated, keep the specimens on ice prior to plating for viable counts.

When liquid medium is called for, use THY medium containing 1000 μg/ml streptomycin; for solid media, use THY plates containing 1000 μg/ml streptomycin. Use screw-cap or snap-cap disposable plastic tubes for all liquid cultures. Incubate all cultures at 37°C and read the results after 2 days.

BASIC PROTOCOL 4

Preparing Cultures from the Meninges

A mouse that tilts its head to one side and has difficulty maintaining balance very likely has a meningeal infection. Prepare a culture to determine if streptococci have spread to the meninges.

Materials

Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocol 1, 2, or 3), sacrificed
THY medium (see recipe)
Surgical scissors, sterile
Sterile swab, calcium alginate urogenital

1. Cut off the head of the mouse with surgical scissors. Insert the point of the scissors into the skull portion of the spinal column, which is very white in color.
2. Using the spinal column as a guide, cut up into the skull between the two main brain hemispheres.
3. Using fingers, peel back the skull on each side, exposing the brain.
4. Swab the surface of the brain (i.e., the meninges) and the area between the two hemispheres with a sterile swab.
5. Place the swab in 1 ml THY medium. Incubate 2 days at 37°C and evaluate by microscopic examination to determine if streptococci are present.

Preparing Cultures from Trachea

BASIC PROTOCOL 5

To determine the number of bacteria in the trachea, prepare cultures.

Materials

Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocol 1, 2, or 3), sacrificed
THY medium and plates (see recipe)
0.9% saline (*APPENDIX 2A*)
Surgical scissors and forceps, sterile

1. Cut into the throat area of the mouse, using sterile surgical scissors, to expose the trachea.
2. Remove the three tracheal rings immediately below the larynx. Place in 500 μl THY medium.
3. Vortex 10 sec on high speed.
4. Perform serial 10-fold dilutions in 0.9% saline and plate (in duplicate) the undiluted and 1/10 dilutions.
5. Incubate 1 to 2 days at 37°C and count colonies to determine cfu present in the trachea.

Preparing Cultures from Spleen

BASIC PROTOCOL 6

To assess tissue dissemination, cultures are taken from the spleen.

Materials

Mouse infected with *S. pyogenes* strain B514-SM or AM3 (Basic Protocol 1, 2, or 3), sacrificed
THY medium and plates (see recipe)
Surgical scissors and forceps, sterile
Dounce tissue homogenizer, sterile

1. Pull the skin up using forceps and cut through the skin and into the peritoneal cavity using surgical scissors. Take care not to cut into the intestines.
2. Remove a 3 × 3–mm section of the spleen and place in 500 μl THY medium.

   The spleen is located behind the stomach (and is actually attached to the stomach) and is brick red in color.

3. Carefully transfer the entire spleen and medium to a sterile Dounce tissue homogenizer.

   The homogenizer should be thoroughly cleaned (with soap and water), rinsed, and autoclaved before use.

4. Thoroughly homogenize the tissue and plate (undiluted) in duplicate on THY plates.
5. Incubate 1 to 2 days at 37°C and count colonies to determine cfu present in the spleen.

Preparing Cultures from Lung

BASIC PROTOCOL 7

A mouse with pneumonia will have breathing difficulties and a hunched-over appearance, appear lethargic, and may also have watery eyes. Prior to culturing the lungs, be sure to inspect for signs of pneumonia (i.e., white areas, redness, swelling, abscesses, and consolidation). Note that in the mouse, there is only one left lung lobe but there are several lobes on the right.

Materials

Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocols 1 or 2), sacrificed
THY medium (see recipe)
0.9% saline (*APPENDIX 2A*)

Surgical scissors and forceps, sterile
Dounce tissue homogenizer

1. Open the chest cavity of the mouse and peel back the ribs, exposing the heart and lungs.
2. Remove the entire left lung lobe and place in 500 μl THY medium.
3. Carefully transfer the entire lung and medium to a Dounce tissue homogenizer.
4. Thoroughly homogenize the lung in the medium, remove a 0.1 ml portion of the homogenate, and perform 10-fold serial dilutions in 0.9% saline.
5. Plate the undiluted and 1/10 dilutions (in duplicate) on THY plates.
6. Incubate 1 to 2 days at 37°C and count colonies to determine if streptococci were present in the lungs.

BASIC PROTOCOL 8

Preparing Cultures from Blood

The following protocol presents a method for obtaining blood from the axillary artery of a mouse infected with *S. pyogenes*. Blood obtained using this method can also be assayed for the presence of antistreptococcal antibodies, if desired. For more information about blood collection, refer to Donovan and Brown (1995).

Materials

Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocols 1, 2, or 3), sacrificed
THY medium (see recipe)

Surgical scissors, sterile
200-μl micropipettor and appropriate tips
15-ml screw-cap conical tube

1. Cut into the large axillary artery of the mouse using surgical scissors.

   This artery is located in the "arm pit" area under either front leg.

2. Collect as much blood as possible using a 200-μl micropipettor.

   Usually 200 to 400 μl is obtainable.

3. Add the entire blood sample to 10 ml THY medium in a screw-cap conical tube.
4. Incubate 2 days at 37°C and evaluate by microscopic examination to determine if streptococci had entered the bloodstream of the mouse.

BASIC PROTOCOL 9

Preparing Cultures from Nasal Passages

The following protocol presents a method for obtaining *S. pyogenes* from the nasal passages of an infected mouse and transferring it to appropriate medium for culture.

Materials

Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocols 1 or 2), sacrificed
THY medium (see recipe)

200-μl micropipettor and appropriate tips
1.5-ml snap-cap tube

1. Open the jaws of the mouse very wide.
2. Flush 200 μl THY medium through the nasal passages and out through the nares by inserting a yellow pipet tip attached to a 200-μl micropipettor into the soft palette.
3. Collect the medium as it exits the nares into a snap-cap tube.
4. Adjust the volume of medium in the tube to ~500 μl and incubate.
5. Incubate 2 days at 37°C and evaluate by microscopic examination to determine if streptococci are present in the nasal passages.

Collection of Saliva from Mice

BASIC PROTOCOL 10

The following protocol presents a technique by which an *S. pyogenes*–infected mouse is induced to salivate, and the resulting saliva collected and stored in the presence of protease inhibitors. This method can be used to look at antibodies produced by the mouse or to assay for proteins secreted by streptococci while in the mouse oral cavity.

Materials

Carbamyl chloride (carbachol; Sigma-Aldrich)
Soybean trypsin inhibitor (Sigma-Aldrich)
Phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich)
BSA (Sigma-Aldrich)
Sodium azide (NaN_3)
Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocols 1 or 2)
22-G straight feeding needle (Popper and Sons) and 1-cc tuberculin syringe
200-μl micropipettor
−80°C freezer

1. Prepare a fresh solution of 20 μg/ml carbamyl chloride (carbachol) in water
2. Prepare a protease inhibitor solution containing 2 mg/ml of each of the following in water:

   soybean trypsin inhibitor
   PMSF
   BSA
   NaN_3.

   CAUTION: *PMSF and sodium azide are highly toxic (see UNIT 1A.3). Wear gloves and do not mouth pipet. Discard into a properly labeled screw-cap jar or bottle for disposal.*

   If using horseradish-peroxidase detection in an ELISA assay to assay the saliva, omit sodium azide because azide inhibits peroxidase activity.

3. Inject 0.1 ml carbachol solution into the mouse intraperitoneally (see Donovan and Brown, 1998).

   About 5 min after the injection, the mouse will start to salivate (drool).

4. Collect the saliva with a 200-μl micropipettor. Keep the saliva sample on ice.

   Usually, the mouse will continue to salivate for ~15 min and it is possible to collect ~200 μl of saliva.

5. Following collection, add 1 μl protease inhibitor mix to the saliva sample.
6. Clarify the saliva by microcentrifuging at top speed, 4°C.

7. Store the saliva sample up to several months at −80°C.

The mouse is sacrificed after collection of saliva.

BASIC PROTOCOL 11

Assay of Group A *Streptococcus* Colonization by Throat Culture from Mice

The following protocol details a method whereby *S. pyogenes* is obtained from an infected mouse via throat swab and then transferred to appropriate solid and liquid medium. Plates are then examined by eye to determine if the organism is present, and upon a negative result, are also examined in liquid culture via microscopy.

Materials

Mouse infected with *S. pyogenes* strain B514-SM (Basic Protocols 1 or 2)
Calcium alginate swab (urethro-genital applicator; Baxter Healthcare)
THY plates and medium containing 1000 μg/ml streptomycin (see recipe)

1. Hold the conscious mouse firmly by its dorsal neck surface and place its tail between the ring finger and the little finger to aid in immobilizing the mouse.
2. Insert a sterile calcium alginate swab into the mouth far enough so that the back of the throat is reached.

   Resistance can be felt when the swab reaches the back of the throat (that area of the oral cavity is fairly hard). Another good indicator that the swab has been inserted far enough is when all of the white portion of the swab is inside of the mouth and none is visible from the outside.

3. Rotate the swab from one side of the throat to the other at least three times.
4. Rotate the swab over the surface of a THY plate containing 1000 μg/ml streptomycin. Break off the metal end of the swab and place it in a snap-cap tube containing 1 ml THY medium containing 1000 μg/ml streptomycin.
5. Incubate 2 days at 37°C.
6. Examine the plates for the presence of mucoid, streptomycin-resistant colonies.
7. If the plate culture is negative, lightly vortex the medium culture, and place a few drops of the culture on a glass slide and examine using a microscope.

   A positive medium culture is indicated by the presence of many cocci in chains within a single field.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Avertin, 2.5%

Dilute 1 ml Avertin stock solution (see recipe) in 39 ml PBS (*APPENDIX 2A*). Mix thoroughly at room temperature for at least 10 min. Pass through a 0.22-μm filter to sterilize. Store in the dark up to 3 months at room temperature.

Do not place on ice because it will precipitate.

Avertin stock solution

Add 5 g tribromoethanol (Sigma-Aldrich) to 5 ml *tert*-amyl alcohol (Sigma-Aldrich), to produce a 100% (w/v) solution. Store in the dark up to 1 year at 4°C.

Brain heart infusion (BHI) broth (2×)/2% (v/v) supplement B

Prepare BHI broth (Remel) at twice the recommended concentration (2× BHI). Add Supplement B (Difco) to a final concentration of 2% (v/v). Store up to 1 month at room temperature.

THY medium and plates, with and without 1000 μg/ml streptomycin

15 g Todd-Hewett dehydrated broth (Difco)
1 g yeast extract (Difco or Fisher Scientific)
8 g agar (if preparing plates; omit for liquid medium)
500 ml H_2O
Sterilize by autoclaving
Cool to <50°C and add 1000 μg/ml streptomycin (or other appropriate antibiotic) as required (see *APPENDIX 4A*).

If using hyaluronidase, add to a final concentration of 85 μg/ml before pouring plates.

THY medium derives its name from the first letters of Todd-Hewett and yeast.

COMMENTARY

Background Information

Streptococcus pyogenes is an important Gram-positive human pathogen that is responsible for a spectrum of mild to severe infections of throat, skin, and deep tissues. *S. pyogenes* causes pharyngitis (strep throat), which is characterized by a reddened sore throat, difficulty swallowing, fever, and swollen lymph nodes. A "butterfly" facial rash accompanying these symptoms is an indication of scarlet fever, which is caused by certain strains of *S. pyogenes*. Infection of the epidermis by *S. pyogenes* (impetigo) is highly contagious and characterized by inflamed and purulent lesions on the skin. Both pharyngitis and impetigo are mild infections that can be diagnosed in the clinic by culturing the infected site and assaying for the presence of *S. pyogenes*. More severe local infections caused by *S. pyogenes* include erysipelas, which involves dermal layers of the skin, and cellulitis, which can involve subcutaneous tissue. Analysis of serum for anti-streptococcal antibodies is often necessary to diagnose *S. pyogenes* as the causative agent of erysipelas or cellulitis. Localized streptococcal infections can and should be treated with antibiotics (usually penicillin or clindamycin) to limit the potential for development of invasive streptococcal disease or post-streptococcal sequelae (such as rheumatic fever or glomerulonephritis).

In the last twenty years, *S. pyogenes* has gained notoriety due to an increase in the incidence of necrotizing fasciitis and the emergence of streptococcal toxic shock syndrome (reviewed by Bisno and Stevens, 1996). Necrotizing fasciitis is a rapidly spreading infection of deep tissue (fat and fascia) and is characterized by severe pain and swelling and often gross discoloration. A necrotic streptococcal infection often requires surgical removal of the infected area and is usually not amenable to antibiotic therapy. If not treated in time, necrotizing fasciitis can lead to toxic shock and/or organ failure. Toxic shock can also develop from a pharyngeal infection or from *S. pyogenes* that entered the body through the vagina (puerperal sepsis). Due to the prevalence of these invasive streptococcal infections and the difficulty in treating them, investigation of the *S. pyogenes* components that promote invasive disease is crucial.

Although *S. pyogenes* is an obligate human pathogen, animal models that imitate aspects of human disease are useful to identify streptococcal proteins important for various stages of virulence. Several vertebrate models have been developed that utilize mice, rats, rabbits, baboons, and zebrafish, each of which possesses innate and adaptive immunity (Bunce et al., 1992; Hollingshead et al., 1993; Piepmeier et al., 1995; Husmann et al., 1996; Ashbaugh et al., 2000; Neely et al., 2002). Each model has its own advantages and is used to mimic specific phases of human infection. The mouse models presented herein offer the opportunity to look at the infection process from the natural routes of inhalation or skin infection and the opportunity to examine persistent colonization or systemic spread of *S. pyogenes* to distal organs and tissues. Mouse models are useful because mice are well characterized genetically and immunologically, are relatively inexpensive and easy to handle, and have a rapid breeding cycle. The murine pneumonia

and throat-colonization models offer the additional advantage that the strain used is a natural pathogen of mice as well as of humans. The murine models can be used to assess the virulence of different strains of *S. pyogenes* by comparing the ability to colonize and persist in the mouse, the ability to disseminate and cause a systemic infection, and the time to death. These models can also be used in competition experiments where two streptococcal strains are co-inoculated and bacteria are isolated after the infection to determine which strain had a selective advantage in vivo.

Critical Parameters and Troubleshooting

Several technical issues are important to consider when handling *S. pyogenes* strains. Before beginning experiments in mice, it is important to conduct growth curves with all wild-type and mutant streptococcus strains so that doubling times and final cell densities are known. This information will provide an idea of how long the strains will take to grow on the day of the experiment. Grow the wild-type and mutant strains in the same media on the same day for an accurate comparison of growth. The use of complex media to grow *S. pyogenes* can lead to a lack of consistency. Do not use antibiotics when growing the bacteria unless selection is necessary to maintain an unstable plasmid or chromosomal insertion. On the day of the experiment, if the bacteria are not growing with the expected doubling time, they should not be used. Problems growing the bacteria may be due to improperly washed glassware or medium that was not correctly prepared. When performing microscope counts on vortexed bacterial cells that grew to the correct cell density, each chain should be considered one cfu. Be sure that only cocci in chains are present at this time. If other cell types are present, a contaminating bacterium has been introduced, and these cells should not be used. Proper sterile technique should prevent this problem. If working with a strain that has an unstable insertion or replicating plasmid, the bacteria should be isolated at the end of the experiment and plated on medium without antibiotic, then replica plated onto medium with the appropriate antibiotic to determine rates of reversion (loss of the insertional mutation) or rates of plasmid loss.

Before beginning an experiment, the mice should be given one week to acclimatize to their new environment. Therefore, one should order mice that are a week younger than the recommended age for the experiment. Mice that are too old may not respond to *S. pyogenes* as expected. It is important that throughout the experiment the mice are treated with proper husbandry practices that minimize stress. For example, shield them from loud noises and avoid changes in their light-dark cycle or diet. When performing the experiment, it is recommended to only anesthetize and inoculate four to five mice at a time. Do not try to anesthetize all mice at once and then inoculate all at once, as some may begin to wake up before inoculations are complete. One common problem with the protocols in this unit is improper inoculation technique (e.g., in the pneumonia model, bacteria may be deposited in the esophagus rather than the trachea). This will result in a lower incidence of infection than expected. Trial inoculations followed by surgical inspection of the appropriate organs can be performed with a dye (e.g., crystal violet or methylene blue) to assess proper inoculation technique.

Anticipated Results

Anticipated results and signs of illness are discussed to some extent in the protocol section. In the throat colonization model (Basic Protocol 1), it is expected that the wild-type strain (B514-Sm) should be able to establish long-term colonization of the throat in up to 60% of infected mice. Colonization is assayed by throat culture as described in Basic Protocol 11. While this model does not have a linear dose-response relationship, the ability of isogenic strains to colonize the mice can be compared. In the pneumonia model (Basic Protocol 2), it is expected that the wild-type B514-Sm strain will produce pneumonia in 50% of inoculated mice within 72 hr when administered at a dose of 1×10^7 cfu. In the subcutaneous infection model (Basic Protocol 3), it is expected that all mice will develop skin lesions within 1 to 2 days, and 50% will develop a lethal systemic infection within 4 days when wild-type strain AM3 is administered at a dose of 2×10^7 cfu. The time to death induced in mice can be compared between isogenic strains of AM3. Isolation and culturing of the spleen as described in Basic Protocol 6 can be used to determine if the mouse suffered from systemic infection.

Time Considerations

There are several important considerations when planning the timing of experiments. One week must be allowed for the mice to acclimate to their environment before inoculation. On the day of inoculation, it will take several

(usually up to 5) hours for *S. pyogenes* strains to reach the desired phase of growth (late exponential). Depending on the researcher's level of technical skill and the number of mice (usually 20) included in the experiment, between 30 min and 2 hr are needed to inoculate the mice. After inoculation, the time required for the experiment is dependent upon the infection model. In the throat colonization model (Basic Protocol 1), mice should be monitored for at least 4 days. In the pneumonia model (Basic Protocol 2) the mice will need to be monitored for at least 72 hr. In the subcutaneous infection model (Basic Protocol 3), mice should be monitored for one week, or less at a higher dose.

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Laboratory Maintenance of *Mycobacterium tuberculosis*

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ABSTRACT

This unit includes protocols for the laboratory maintenance of *Mycobacterium tuberculosis*, including growth on liquid and solid media as well as recommendations for long-term strain storage. Considerations for working with *M. tuberculosis* at Biosafety Level 3 containment are also discussed. *Curr. Protoc. Microbiol.* 6:10A.1.1-10A.1.8. © 2007 by John Wiley & Sons, Inc.

Keywords: tuberculosis • medium • Middlebrook • 7H9 • 7H10 • 7H11 • Sauton • strain storage

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), infects almost one third of the world's population. Yearly, there are an estimated 10 million new cases of TB, resulting in 2 to 3 million deaths. Over the last fifteen years, genetic tools to manipulate *M. tuberculosis* have proliferated (*UNIT 10A.2*). Furthermore, availability of the complete *M. tuberculosis* DNA genome sequence (as well as genome sequences of other related mycobacteria) has yielded tremendous information allowing a better understanding of the bacterium and the disease it causes.

The protocols in this unit will detail common medium preparation considerations and strain storage.

CAUTION: *Mycobacterium tuberculosis* is a Biosafety Level 3 (BSL-3) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See Strategic Planning, Safety Considerations, for further discussion. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for additional information.

CAUTION: Use disposable plastic inoculation loops and plate spreaders in the BSL-3 setting, since the alternative of sterilizing glass or metal inoculation loops or plate spreaders with ethanol and an open flame generates aerosols.

STRATEGIC PLANNING

Safety Considerations

Standards of BSL-3 containment should be followed for work with *M. tuberculosis*. Investigators should consult with the local safety officer and/or institutional biosafety committee for additional guidance. See *http://www.cdc.gov/OD/ohs/symp5/jyrtext.htm* for a brief description of general BSL-3 laboratory features. Complete inactivation of *M. tuberculosis* cells is necessary to perform any studies outside the BSL-3 laboratory. All methods for inactivation should be validated in individual laboratories, taking into consideration the concentration of cells used. An article by Schwebach et al. (2001) provides some guidance on determining if particular conditions are adequate for inactivation of mycobacteria. For many procedures, the authors of this unit have found the following conditions sufficient for inactivation of *M. tuberculosis*: incubation at 65°C for 12 hr,

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Table 10A.1.1 Antibiotic Supplements for Medium[a]

Antibiotic	Suggested supplier
30 mg/liter apramycin sulfate[b]	Sigma (cat. no. A-2024)
50 mg/liter hygromycin B	Roche Applied Science (cat. no. 843-555)
10 to 20 mg/liter kanamycin monosulfate[b]	Sigma (cat. no. K-4000)

[a] All antibiotics in this table can be stored up to 6 months at –20°C.
[b] H_2O as solvent.

80°C for 2 hr, or 95°C for 15 min; alternatively, incubation for 1 hr in a final concentration of 2.5% glutaraldehyde, 2% paraformaldehyde, or 5% formalin, or in Vesphene IIse. During heat inactivation, care should be taken to ensure that the entire tube is immersed in a water bath preheated to the appropriate temperature. Incubation of *M. tuberculosis* cultures with glutaraldehyde, paraformaldehyde, formalin, or Vesphene IIse should be done in a matter that ensures that the entire interior surface of the tube has been coated by gently rolling and inverting the tube.

Cultivation of *Mycobacterium tuberculosis*

Although the growth requirements of *M. tuberculosis* are not complex, the long doubling time (~18 to 22 hr for wild-type strains, longer for some mutant strains) presents specific challenges. Careful attention should be paid to the use of proper aseptic technique. In addition, hydrophobic mycolic acids (see *UNIT 10A.3*), a hallmark of mycobacteria, cause the bacteria to clump together, and can interfere with the establishment of clonal isolates. This problem can be prevented by the addition of detergent (either Tween 80 or Tyloxapol) to the culture medium. The most widely used medium formulations for laboratory cultivation of *M. tuberculosis* were developed by Middlebrook (Cohn et al., 1954). Middlebrook 7H9 is a liquid medium, while Middlebrook 7H10 and Middlebrook 7H11 are the corresponding formulations for growth on agar plates. Middlebrook 7H11 differs from 7H10 in that casamino acids are added and it is a "richer" medium. Middlebrook 7H9, 7H10, and 7H11 contain bovine albumin, which can lead to technical difficulties in subsequent procedures (e.g., see *UNIT 10A.4*). Sauton medium does not contain albumin, and is an alternative medium for some applications. However, because it is a minimal medium, doubling times can be longer, and some mutant strains grow very poorly in Sauton medium. Antibiotics like kanamycin, hygromycin, and apramycin are used to retain plasmids that bear the corresponding antibiotic-resistance cassette. Refer to Table 10A.1.1 for suggested antibiotic concentrations.

BASIC PROTOCOL 1

GROWTH OF *M. TUBERCULOSIS* IN MIDDLEBROOK LIQUID MEDIUM

Cultivation of *M. tuberculosis* in Middlebrook liquid medium is important for many genetic manipulations including transposon mutagenesis, electroporation, and phage transduction (see *UNIT 10A.2*).

Materials

Middlebrook 7H9 liquid medium (see recipe)
M. tuberculosis

30-ml square medium bottles (Nalgene, cat. no. 2019-0030), sterile
Disposable plastic inoculation loops, sterile
Incubator containing platform shaker or roller-bottle apparatus
490-cm^2 roller bottles (Corning, cat. no. 430195)

Additional reagents and equipment for bacterial culture techniques including inoculation of liquid medium (*APPENDIX 4A*)

For small-volume cultures

1a. Transfer 10 ml Middlebrook 7H9 medium to a sterile, square medium bottle.

2a. Transfer a colony of *M. tuberculosis* to the bottle with a disposable inoculating loop, or add 0.1 to 0.2 ml of a thawed strain stock.

APPENDIX 4A describes basic bacterial inoculation techniques.

3a. Close lids on bottles enough to keep them from coming off, but leave loose enough to allow for exchange of gases.

4a. Incubate the square medium bottle cultures in a plastic box on a platform shaker with mild agitation (100 rpm) in an incubator at 37°C.

For large-volume cultures

1b. Transfer 100 ml of Middlebrook 7H9 medium to a 490-cm^2 roller bottle.

2b. Transfer 0.5 to 1 ml of a smaller culture or a thawed strain stock to the roller bottle.

3b. Close lids on bottles enough to keep them from coming off, but leave loose enough to allow for exchange of gases.

4b. Incubate roller bottles in a roller bottle apparatus at 1.25 rpm in a 37°C incubator.

Liquid cultures will take a few days to a few weeks to grow at 37°C, depending on the amount of inoculum and the strain.

The correlation between colony-forming units (cfu) and optical density (OD_{600}) can vary between strains but a good reference point is that an OD_{600} of 1.0 is roughly equivalent to 3×10^8 cfu/ml. The OD_{600} of a culture in logarithmic growth will double every 18 to 24 hr. OD_{600} readings above 1.5 are not reliable, as the density of the culture makes it more likely to form clumps.

GROWTH OF *M. TUBERCULOSIS* IN SAUTON LIQUID MEDIUM

ALTERNATE PROTOCOL 1

Cultivation of *M. tuberculosis* in Sauton liquid medium is important for preparation of culture filtrates, as the albumin in Middlebrook medium would complicate protein analysis (see *UNIT 10A.4*).

Materials

Sauton medium (see recipe)
M. tuberculosis

30-ml square medium bottles (Nalgene, cat. no. 2019-0030), sterile
Disposable plastic inoculation loops, sterile
Incubator containing platform shaker or roller-bottle apparatus
490-cm^2 roller bottles (Corning, cat. no. 430195)

Additional reagents and equipment for bacterial culture techniques including inoculation of liquid medium (*APPENDIX 4A*)

For small-volume cultures

1a. Transfer 10 ml Sauton medium to a sterile, square medium bottle.

2a. Transfer a colony of *M. tuberculosis* to the bottle with a disposable inoculating loop, or add 0.1 to 0.2 ml of a thawed strain stock.

APPENDIX 4A describes basic bacterial inoculation techniques.

3a. Close lids on bottles enough to keep them from coming off, but leave loose enough to allow for exchange of gases.

4a. Incubate the square medium bottle cultures in a plastic box on a platform shaker with mild agitation (100 rpm) in an incubator at 37°C.

For large-volume cultures

1b. Transfer 100 ml of Sauton medium to a 490-cm^2 roller bottle.

2b. Transfer 0.5 to 1 ml of a smaller culture or a thawed strain stock to the roller bottle.

3b. Close lids on bottles enough to keep them from coming off, but leave loose enough to allow for exchange of gases.

4b. Incubate roller bottles in a roller bottle apparatus at 1.25 rpm in a 37°C incubator.

Liquid cultures will take a few days to a few weeks to grow at 37°C, depending on the amount of inoculum and the strain. Strains grown in Sauton medium may have longer doubling times compared to strains grown in Middlebrook 7H9 medium.

BASIC PROTOCOL 2

GROWTH OF *M. TUBERCULOSIS* ON SOLID MEDIUM

Cultivation of *M. tuberculosis* on Middlebrook solid medium is important for many genetic manipulations including transposon mutagenesis, electroporation, and phage transduction (see *UNIT 10A.2*).

Materials

M. tuberculosis
Middlebrook 7H10 or 7H11 agar plates (see recipes and Strategic Planning)
Vesphene IIse disinfectant (Fisher)

Sterile plastic inoculating loops
Sterile plastic plate spreaders
Heavy-duty aluminum foil or sterile metal-plate canisters

Additional reagents and equipment for bacterial culture techniques, including spreading and streaking plates (*APPENDIX 4A*)

1a. *Usual procedure:* Using sterile, plastic inoculating loops, streak *M. tuberculosis* across Middlebrook 7H10 or 7H11 agar plates (see Strategic Planning) as described in *APPENDIX 4A*.

1b. *For plating larger volumes of bacterial culture (e.g., serial dilutions, transductions, transformations):* Put 100 to 200 µl of the culture in the center of an agar plate. Using a sterile, plastic plate spreader, distribute the liquid using a circular motion, focusing the liquid towards the center of the plate (also see *APPENDIX 4A*). When most of the liquid has been absorbed, expand the circular motion to distribute all the way to the edges of the plate.

If liquid is pushed too early to the edges, a large fraction of cells will grow along the rim, making counting and picking colonies for later applications difficult.

2. Invert a stack consisting of <10 inoculated agar plates and wrap tightly in heavy-duty aluminum foil or place in a sterile metal canister to prevent plates from drying out during prolonged incubation. Moisten a disposable paper towel or shopcloth and lightly coat the inside layer of aluminum foil with a dilute solution of Vesphene IIse, to prevent contamination during incubation.

3. Incubate 3 to 6 weeks at 37°C.

STORAGE OF *M. TUBERCULOSIS*

Typically, mycobacteria can be stored as lyophilized stocks, agar slants, or frozen stocks. Since lyophilization is not a cost-effective product-storage alternative for virulent *M. tuberculosis*, and agar slants can take up considerable BSL-3 storage space, the best alternative for strain storage is frozen stocks. It is recommended that, for frequently used strains, a working stock of a few dozen vials be made. For all strains, save two vials of each strain and, where possible, distribute the strain duplicates between two different −70°C freezers.

Materials

M. tuberculosis culture (Basic Protocol 1 or Alternate Protocol 1)
M. tuberculosis strain storage medium (see recipe)
Sterile 2-ml cryotubes

1. Transfer 1 ml of mycobacteria culture to a sterile microcentrifuge tube and microcentrifuge 10 min at 11,000 rpm, 4°C, to pellet cells.
2. Discard supernatant and resuspend cell pellet in 1 ml *M. tuberculosis* strain storage medium.
3. Transfer to a 2-ml cryotube. Store up to 2 years at –70°C in a freezer in a BSL-3 laboratory.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

M. tuberculosis strain storage medium

0.47 g Middlebrook 7H9 powder (Difco, cat. no. 271310)
90 ml 50% (w/v) glycerol
10 ml OADC (see recipe)
0.25 ml 20% Tween 80 (see recipe) or 20% Tyloxapol (see recipe)
Sterilize by filtration through 0.22-μm membrane
Store up to 6 months at 4°C

Middlebrook 7H9 liquid medium

Dissolve 4.7 g Middlebrook 7H9 powder (Difco, cat. no. 271310) in 900 ml H_2O, then add:
100 ml OADC (see recipe)
10 ml 50% (w/v) glycerol
2.5 ml 20% Tween 80 (see recipe) or 20% Tyloxapol (see recipe)
Sterilize by filtration through 0.22-μm membrane
Store up to 3 months at 4°C

For 7H9 powder, the approximate formulation when dissolved in 900 ml water is 0.5 g ammonium sulfate, 0.5 g L-glutamic acid, 0.1 g sodium citrate, 1.0 mg pyridoxine, 0.5 mg biotin, 2.5 g disodium phosphate, 1.0 g monopotassium phosphate, 0.04 g ferric ammonium citrate, 0.05 g magnesium sulfate, 0.5 mg calcium chloride, 1.0 mg zinc sulfate, and 1.0 mg copper sulfate (taken from http://www.bd.com/ds/technicalCenter/inserts/Middlebrook_7H9_Broths.pdf)

Middlebrook 7H10 solid medium

Dissolve 19 g Middlebrook 7H10 powder (Difco, cat. no. 262710) in 900 ml H_2O
Autoclave 20 min
Cool on bench 30 min with stirring

continued

Add 100 ml OADC (see recipe)
Add 10 ml of 50% (w/v) glycerol

For 7H10 powder, the approximate formula when dissolved in 900 ml water is 0.5 g ammonium sulfate, 1.5 g monopotassium phosphate, 1.5 g disodium phosphate, 0.4 g sodium citrate, 25.0 mg magnesium sulfate, 0.5 g calcium chloride, 1.0 mg zinc sulfate, 1.0 mg copper sulfate, 0.5 g L-glutamic acid (sodium salt), 0.04 g ferric ammonium citrate, 1.0 mg pyridoxine hydrochloride, 0.5 mg biotin, 250.0 μg malachite green, and 15 g agar (taken from http://www.bd.com/ds/technicalCenter/inserts/Middlebrook_7H10_Agar.pdf)

Middlebrook 7H11 solid medium

Dissolve 21 g Middlebrook 7H11 powder (Difco, cat. 283810) in 900 ml H_2O
Autoclave 20 min
Cool on bench 30 min with stirring
Add 100 ml OADC (see recipe)
Add 10 ml of 50% (w/v) glycerol

For 7H11 powder, the approximate formulation when dissolved in 900 ml water is 0.5 g L-glutamic acid, 0.4 g sodium citrate, 1.0 mg pyridoxine, 0.5 mg biotin, 0.04 g ferric ammonium citrate, 0.5 g ammonium sulfate, 1.5 g disodium phosphate, 1.5 g monopotassium phosphate, 0.05 g magnesium sulfate, 15 g agar, and 1.0 mg malachite green (taken from http://www.bd.com/ds/technicalCenter/inserts/Seven_H11_Agars.pdf).

Oleic Acid–Dextrose–Catalase (OADC)

Dissolve 8.5 g NaCl and 50 g BSA fraction V (Roche Applied Science, cat. no. 100-021) in 950 ml water. Add 20 g D-dextrose, 40 mg catalase, and 0.5 g oleic acid. Split into four 250-ml aliquots in centrifuge bottles. Precipitate insoluble material by centrifuging 30 min at 1500 × *g*, 4°C. Sterilize clarified solution by filtering through a 0.22-μm membrane. Incubate bottles at 37°C overnight to detect possible contamination. Store up to 6 months at 4°C

OADC is also available commercially from BD Microbiology Systems (cat. no. 212351).

Sauton liquid medium

0.5 g KH_2PO_4
0.5 g $MgSO_4$
4.0 g L-asparagine
60 ml glycerol
0.05 g ferric ammonium citrate
2.0 g citric acid
0.1 ml 1% (w/v) $ZnSO_4$
H_2O to 900 ml
Adjust pH to 7
Autoclave, cool, and add 2.5 ml 20% Tween 80 (see recipe)
Store up to 6 months at room temperature

Tween 80, 20%

20 ml Tween 80 (Fisher, cat. no. T164-500)
80 ml H_2O
If needed, heat to 56°C to speed Tween going into solution
Sterilize by filtration through 0.22-μm membrane
Store up to 2 months at room temperature

The final concentration of Tween 80 in used in the media in this unit is 2.5% (v/v).

Tyloxapol, 20%

20 ml Tyloxapol (Sigma, cat. no. T-8761)
80 ml H_2O
Sterilize by filtration through 0.22-μm membrane
Store up to 1 year at 4°C

The final concentration of Tyloxapol used in the media in this unit is 0.05% (v/v).

COMMENTARY

Background Information

Tuberculosis (TB) is a disease caused by *M. tuberculosis*. *M. tuberculosis* is spread via the aerosol route and predominately causes disease in lung tissue, although it can spread to other organs. It is estimated that nearly one-third of the world's population is infected with TB; many of those are cases of latent infection. When the immune system wanes as a result of aging or infection with HIV (*UNIT 15J.1*), TB can reactivate from its latent form, even after decades of quiescence. Antibiotic therapy is available for the treatment of TB; however, due to the slow growth of the organism, long

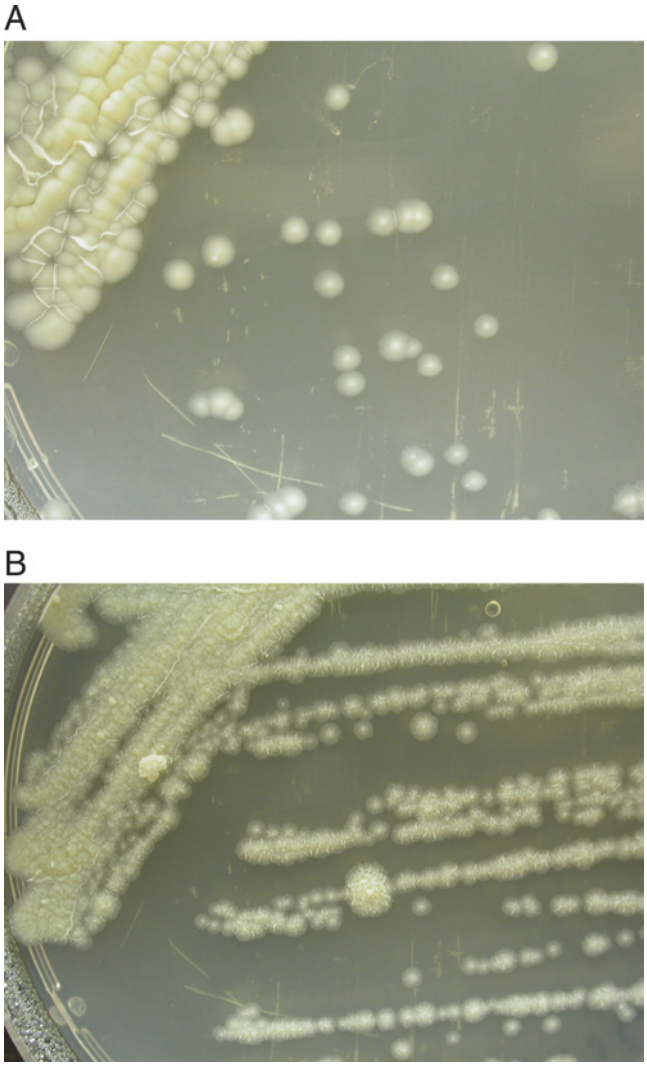

Figure 10A.1.1 Colony morphology. Colonies of *Mycobacterium tuberculosis* appear different when grown on solid medium (**A**) with or (**B**) without Tween-80. In the presence of a detergent, like Tween-80, *M. tuberculosis* colonies appear smooth, round, and have a shiny surface as depicted in panel A. If no detergent is present in the medium, the colonies will appear more rough, wrinkled, and matte, as depicted in panel B.

courses of treatment (6 months to 1 year) are recommended.

Critical Parameters and Troubleshooting

One of the most important aspects of working with *M. tuberculosis* is to minimize any possible aerosol exposure. Second in importance to safety considerations, scrupulous attention to detail in preparing medium and reagents is crucial. Given the long doubling time of the organism (3 weeks for colonies to be visible on solid medium), care should be taken to minimize any possible sources of contamination of the medium.

Strain storage

Care should be taken to save strains when they are fresh. Also, some strains are more stable than other strains (i.e., titers for frozen stocks may remain stable for some strains, while others will show a 10- to 100-fold drop in titer over 6 months to 1 year).

Anticipated Results

Figure 10A.1.1 provides an example of the colonies that arise from growth on Middlebrook 7H10 medium following 4 weeks of incubation at 37°C.

Time Considerations

Medium preparation and strain storage should only take a few hours, with the majority of the time spent on medium preparation.

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Genetic Manipulation of *Mycobacterium tuberculosis*

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ABSTRACT

This unit includes protocols for the genetic manipulation of *Mycobacterium tuberculosis*, including nucleic acid extraction (plasmid DNA, genomic DNA, and mRNA), and methods for electroporation (transformation), transduction (including allelic exchange), and transposon mutagenesis. Considerations for working with *M. tuberculosis* at Biosafety Level 3 containment are also discussed. *Curr. Protoc. Microbiol.* 6:10A.2.1-10A.2.21. © 2007 by John Wiley & Sons, Inc.

Keywords: tuberculosis • plasmid • genomic DNA • mRNA • transformation • transduction • bacteriophage • mutagenesis

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), infects almost one third of the world's population. Yearly, there are an estimated 10 million new cases of TB from which 2 to 3 million people die. Over the last 15 years, the development of genetic tools to manipulate *M. tuberculosis* has proliferated. Furthermore, completion of the *M. tuberculosis* DNA sequence (Cole et al., 1998), as well as other related mycobacteria, has yielded tremendous information for better understanding the bacterium and the disease it causes.

The three biggest challenges in working with *M. tuberculosis* are first, the risk of aerosol infection, second, the long doubling time of the bacterium, and third, the consequences of a cell wall rich in mycolic acids, which leads to cells that clump. Since *M. tuberculosis* is a human pathogen and can infect via the aerosol route, it is important to minimize the production of aerosols (see Strategic Planning, Safety Considerations). The long doubling time (~18 to 22 hr for wild-type strains, longer for some mutant strains) presents specific challenges in aseptic technique (refer to *UNIT 10A.1*). Finally, mycolic acids, a hallmark of mycobacteria, are hydrophobic and cause bacteria to clump together, interfering with the establishment of clonal isolates. (See *UNIT 10A.3* for more information about mycolic acids and their isolation.)

CAUTION: *Mycobacterium tuberculosis* is a Biosafety Level 3 (BSL-3) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See Strategic Planning for further discussion. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

Growth

UNIT 10A.1 discusses laboratory maintenance of *M. tuberculosis* including media preparation, inoculation of solid and liquid media, and strain storage.

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Safety Considerations

Standards of BSL-3 containment should be followed for work with *M. tuberculosis*. Consult with the Safety Officer and/or Institutional Biosafety Committee for additional guidance. See *http://www.cdc.gov/OD/ohs/symp5/jyrtext.htm* for a brief description of general BSL-3 laboratory features. Inactivation of *M. tuberculosis* cells is necessary to continue some studies outside the BSL-3 laboratory. All inactivation methods should be validated in individual laboratories, taking into consideration the concentration of cells used. To test if conditions are adequate for inactivation of *M. tuberculosis*, an article by Schwebach et al. (2001) provides some guidance. For many of the procedures, the authors have found the following conditions sufficient for inactivation of *M. tuberculosis*: incubation for either 12 hr at 65°C, 2 hr at 80°C, or 15 min at 95°C; incubation in a final concentration of either 2.5% gluteraldehyde, 2% paraformaldehyde, 5% formalin, or Vesphene IIse for 1 hr. Care should be taken to ensure that the entire tube is immersed in the water bath. Likewise, incubation of *M. tuberculosis* cultures with gluteraldehyde, paraformaldehyde, formalin, or Vesphene IIse should be done in a matter that ensures that the entire interior surface of the tube has been coated by gently rolling and inverting the tube.

BASIC PROTOCOL 1 EXTRACTION OF GENOMIC DNA USING THE CTAB-LYSOZYME METHOD

Genomic DNA preparations are useful for a number of applications, including PCR and Southern blot analyses. The following protocol for the cetyltrimethylammonium bromide (CTAB) genomic DNA prep is the most reliable method of DNA extraction, although it requires more time than the GTC (guanidine thiocynate) method (see Alternate Protocol 1). The CTAB-lysozyme method of genomic DNA extraction yields good quality DNA. The DNA is suitable for Southern blotting and PCR but not for applications that require larger DNA fragments (>50 kb) such as pulsed-field gel electrophoresis. The CTAB method is also suitable for smaller culture volumes (10 ml) and is useful when screening a number of clones. See also Connell (1994). After completion of step 11, this method can be processed in a BSL-2 laboratory.

Materials

10% (w/v) glycine (see recipe)
Cell culture
GTE solution (see recipe)
Lysozyme solution (see recipe)
10% SDS (see recipe)
10 mg/ml proteinase K (see recipe)
5 M NaCl
CTAB solution (see recipe), 65°C
24:1 (v/v) chloroform/isoamyl alcohol
Isopropanol
70% ethanol
TE buffer (*APPENDIX 2A*)

37°C incubator
15-ml polypropylene conical tubes
Refrigerated tabletop centrifuge
2-ml microcentrifuge tubes
Microcentrifuge

1. (Optional) Twenty-four hr before harvesting cells for genomic DNA preparation, add glycine to a late log culture to a final concentration of 1% using a 10% (w/v) glycine stock. Incubate 24 hr at 37°C.

   The glycine weakens the cell wall and for some strains will lead to a higher yield of DNA.

2. Transfer 10 ml culture to a 15-ml conical tube and centrifuge in a tabletop centrifuge 10 min at 2000 × *g*, room temperature.

3. Discard supernatant, resuspend cell pellet in 450 μl GTE solution, and transfer to a 2-ml microcentrifuge tube containing 50 μl of a 10 mg/ml lysozyme solution.

4. Mix gently and incubate overnight at 37°C.

5. Make a 2:1 solution of 10% SDS solution and 10 mg/ml proteinase K. Add 150 μl of this solution to the cells and mix gently. Incubate 20 to 40 min at 55°C.

6. Add 200 μl of 5 M NaCl and mix gently.

   NaCl blocks the binding of DNA to cetrimide.

7. Preheat CTAB solution to 65°C, add 160 μl, and mix gently. Incubate 10 min at 65°C.

8. Add an equal volume (~1 ml) 24:1 (v/v) chloroform/isoamyl alcohol, shake vigorously to mix, and microcentrifuge for 5 min.

9. Transfer 900 μl aqueous layer to a fresh 2-ml microcentrifuge tube.

10. Repeat extraction with 24:1 (v/v) chloroform/isoamyl alcohol, shake vigorously to mix, and spin in microcentrifuge for 5 min.

11. Transfer 800 μl to fresh 2-ml microcentrifuge tube.

    CAUTION: For BSL3 organisms, dip tube in a disinfectant such as Vesphene IIse to disinfect outer surface. From this point on, the supernatant can be processed in a BSL-2 laboratory (see Strategic Planning).

12. To 800 μl aqueous layer, add 560 μl (0.7 vol) isopropanol, mix gently by inversion until the DNA has precipitated out of solution.

13. Incubate 5 min at room temperature. Microcentrifuge for 10 min, room temperature.

14. Aspirate supernatant and add 1 ml of 70% ethanol to wash DNA pellet. Mix gently by inversion and microcentrifuge 10 min at room temperature.

15. Carefully aspirate supernatant, avoiding the pellet, and air-dry DNA pellet for 15 min. Do not overdry.

16. Add 50 μl TE buffer to dried DNA pellet and store overnight at room temperature or 4°C to allow pellet to dissolve. Store up to 1 year at −20°C.

    RNase A can be added to TE (1 μg/ml) to reduce RNA contamination.

EXTRACTION OF GENOMIC DNA USING THE GTC METHOD

ALTERNATE PROTOCOL 1

The GTC (guanidine thiocynate) method is a relatively faster protocol than the preceeding CTAB genomic DNA prep, but the yield of DNA can be variable. It is recommended for 50- or 100-ml culture volumes of *M. tuberculosis* but does not work as well for 10- or 20-ml cultures. The CTAB procedure (see Basic Protocol 1) has less variable yields, regardless of culture size. After completion of step 7 in this protocol, the GTC genomic DNA prep can be processed in a BSL-2 laboratory.

Materials

M. tuberculosis cell culture
3:1 (v/v) chloroform/methanol (using water-saturated chloroform)
Phenol, Tris-buffered (*APPENDIX 2A*)
GTC solution (see recipe)
2-Mercaptoethanol
Isopropanol
70% ethanol
TE buffer (*APPENDIX 2A*)

Spectrophotometer
50-ml polypropylene conical tubes
Tabletop centrifuge
Vortex

1. Grow cells to late log phase (OD_{600} of ~0.8 to 1.0).
2. Transfer 50 ml cell culture to a 50-ml conical tube and centrifuge 10 min at 2000 × *g*, room temperature.

   Use polypropylene tips and tubes—no polystyrene plastic.

3. (Optional) Microcentrifuge cells for 2 min, then use 10 to 20 µl of the supernatant in a 100-µl PCR reaction (see Alternate Protocol 2).
4. Remove all of the culture supernatant.
5. Add 6 ml freshly prepared 3:1 (v/v) chloroform/methanol mixture to the pellet.
6. Vortex for 30 to 60 sec on high to lyse the cells.

   Lysis is evident when a clear bottom layer is visible after the tube has sat undisturbed for a few minutes.

7. Add 6 ml Tris-buffered phenol and vortex 30 sec.
8. Add 9 ml GTC solution (containing 1% 2-mercaptoethanol, added just before use) and invert tubes several times.

   CAUTION: For BSL-3 organisms, dip tube in Vesphene IIse to disinfect outer surface and take out of hood. From this point on, the suspension can be processed in a BSL-2 laboratory (see Strategic Planning).

9. Centrifuge 10 to 15 min at 2000 × *g*, room temperature. Remove and save upper phase.
10. Precipitate lysate with an equal volume of isopropanol. Centrifuge 5 to 10 min at 2000 × *g*, room temperature, to collect DNA.
11. Wash with 70% ethanol.
12. Air dry pellet and resuspend in 100 µl TE buffer. Store up to 1 year at −20°C.

ALTERNATE PROTOCOL 2

SMALL-SCALE, RAPID EXTRACTION OF GENOMIC *MYCOBACTERIUM TUBERCULOSIS* DNA FOR PCR

The polymerase chain reaction (PCR) is a versatile tool for screening *M. tuberculosis* clones. In this protocol, either *M. tuberculosis* cells from solid media or liquid culture (*UNIT 10A.1*) can be used for PCR template preparation. The main advantage of this protocol is that DNA samples suitable for PCR analysis can be prepared rapidly compared to the CTAB or GTC methods. This is useful when screening a large number of clones; however, the resulting yield and quality of DNA cannot be used for Southern analysis; therefore, eventually a CTAB or GTC genomic DNA prep is necessary.

Materials

Plated colonies or liquid cultures
24:1 (v/v) chloroform/isoamyl alcohol
3 M ammonium acetate
95% ethanol

Plastic loop
200 μl microcentrifuge tubes with screw-caps and O-rings
Vortex
95°C heating blocks
Microcentrifuge

1. For colonies, scrape a clump of cells off a plate using a plastic loop. Transfer the cells to a 200-μl sterile water in screw-cap (O-ring) microcentrifuge tube. For liquid culture, transfer 200 μl of culture to a 200-μl microcentrifuge tube.
2. Briefly vortex to resuspend cells. Incubate 30 min in a 95°C heating block with another heating block placed on top.

   For BSL3 organisms, dip closed tube in a disinfectant such as Vesphene IIse to disinfect the outer surface of the tube. From this point on, the filtrate can be processed in a BSL2 laboratory.

3. Microcentrifuge for 2 min. Add 200 μl of 24:1 (v/v) chloroform/isoamyl alcohol to the supernatant and briefly vortex to extract proteins and lipids.
4. Microcentrifuge 5 min and transfer the upper (aqueous) phase to fresh tube.
5. Add 1/10 vol of 3 M ammonium acetate and 2.5 vol of 95% ethanol.
6. Microcentrifuge for 5 min, pour off supernatant and air-dry pellet until ethanol has evaporated.
7. Resuspend the DNA pellet in 100 μl sterile water and use 10 μl of this DNA prep for each 100-μl PCR reaction.

EXTRACTION OF PLASMID DNA

BASIC PROTOCOL 2

The majority of the work done with vectors in *M. tuberculosis* strains is with plasmids that have been manipulated and characterized first in *E. coli*. Most plasmids or cosmids used for studies in *M. tuberculosis* are shuttle plasmids that have two plasmid origins of replication: one for use in *E. coli* and one for use in Mycobacteria. Cloning and other nucleotide manipulations of plasmids are much more efficient and less time consuming in *E. coli*; however, on occasion, it is necessary to retrieve a plasmid from an *M. tuberculosis* transformant to verify the integrity of an area of interest (promoter, open reading frame, marker, etc.). The following protocol describes modifications made to the Qiagen Midi-prep kit (Qiagen) for extraction of plasmid DNA from *M. tuberculosis*. Other versions of Qiagen plasmid purification methods (e.g., mini-prep) also work effectively for *M. tuberculosis* with one essential modification: for the lysozyme incubation step, incubate 1 hr at 37°C, then 2 hr at 80°C. Adjust volumes according to the manufacturer's directions. The lysis of bacteria for plasmid extraction is based on the *E. coli* method pioneered by Birnboim and Doly (1979).

NOTE: The organic solvents will not harm the column or matrix, but ensure that the plastic pipets and receptacles are made of polypropylene, not polystyrene.

NOTE: The yield of an 8-kb plasmid from a 50-ml culture of *M. tuberculosis* is ~1/10 what is expected from a similar culture in *E. coli*, most likely because of profound

differences in cell wall composition and subsequent cell lysis and DNA recovery, and also because of lower plasmid copy number in *M. tuberculosis*.

Materials

10% (w/v) glycine (see recipe)
Cell culture
Qiagen midi-prep kit containing:
  P1 buffer
10 mg/ml lysozyme
1:1 (v/v) chloroform/methanol

1. Grow a culture of mycobacteria to log phase ($OD_{600} = \geq 1$). Prior to harvest (3 to 24 hr), add glycine to the culture at a final concentration of 1% (w/v) from a 10% stock solution in water. Incubate culture at 37°C.

   The addition of glycine serves to weaken the cell wall, making it easier to lyse the cells with lysozyme.

2. Pellet cells 5 min at room temperature and resuspend in P1 buffer containing lysozyme at 10 mg/ml (freshly prepared). Use the same volume of P1 buffer as for an *E. coli* Qiagen prep. Incubate 1 hr at 37°C, then 2 hr at 80°C.

3. Proceed with the Qiagen midi-prep protocol according to the manufacturer's instructions, with one important modification: after passing the cleared lysate through the column, and before performing the two washes with wash buffer, wash the column with a 1:1 mixture of chloroform/methanol.

   The volume used is the same as that used for the two washes with wash buffer, i.e., if using a Q-100 tip, then use 10 ml of the 1:1 mixture for the washes—use more if using a larger column. After this organic wash, proceed with the two buffer washes and elute.

BASIC PROTOCOL 3

EXTRACTION OF RNA WITH TRIZOL

RNA extraction from *M. tuberculosis* (Mahenthiralingam, 1998; Dietrich et al., 2000) is useful for RT-PCR assays as well as microarray analysis. The RNA-Trizol protocol is useful for recovering RNA for RT-PCR and the following fast prep and GTC methods have been used for preparing RNA pools for microarray analysis.

Materials

M. tuberculosis culture
3:1 (v/v) chloroform/methanol, prepare fresh
Trizol
Chloroform
Isopropanol
70% ethanol (made with DEPC-treated water), cold
Diethylpyrocarbonate (DEPC)-treated water

Spectrophotometer
15-ml polypropylene tubes
Tabletop centrifuge
Vortex
13-ml Sarstedt tube
1.5- and 2-ml RNase-free tubes (Ambion)

1. Grow 50 ml culture of *M. tuberculosis* to an OD_{600} of 0.5 to 1.0.

2. Transfer culture to four 15-ml polypropylene tubes and centrifuge 10 min at 2000 × *g*, 4°C.

3. Discard supernatant and centrifuge tubes 1 min at 2000 × *g* to bring down residual supernatant to be discarded.
4. Add 1 ml freshly prepared 3:1 (v/v) chloroform/methanol mix to each pellet and immediately mix well using a 1-ml disposable pipet tip.
5. Vortex 1 min, then add 5× vol (5 ml) Trizol to each tube.
6. Vortex each sample for 15 sec to mix well
7. Incubate 10 min at room temperature (layers should separate).
8. Centrifuge 15 min at 2000 × *g*, 4°C.
9. Rinse 13-ml Sartstedt tubes with chloroform. Transfer aqueous upper level into the rinsed Sartstedt tube and add an equal volume of isopropanol.

   For BSL3 organisms, dip closed tube in a disinfectant such as Vesphene IIse to disinfect the outer surface of the tube. From this point on, the filtrate can be processed in a BSL2 laboratory.
10. Precipitate overnight at –20°C. Centrifuge 30 min at 2000 × *g*, 4°C, pour off supernatant and wash pellet two times with cold 70% ethanol (made with DEPC-treated water).
11. Resuspend RNA pellet in 100 µl cold DEPC-treated water and store at −20°C.
12. Check sample on a 1% agarose gel. If chromosomal DNA is present, repeat Trizol extraction.

EXTRACTION OF RNA FROM *MYCOBACTERIUM TUBERCULOSIS* USING THE FAST RNA PREP

ALTERNATE PROTOCOL 3

This protocol for RNA preparation uses the Fast RNA prep machine (FastPrep; FP120, Bio101, Thermo Electron Corporation). In brief, using the FastRNA Pro Blue kit in conjunction with the FastPrep machine allows for homogenization of mycobacterial cells that allows for release of RNA into the FastPrep RNApro solution. For mycobacteria, the key point is not to overload the matrix provided in the kit—use 5 ml of a mycobacterial culture with an OD_{600} of 0.5 to 1.0 or 10 ml culture with a lower OD_{600}.

Materials

Mycobacterial culture
Qiagen RNA protect reagent (Qiagen cat. no. 76506)
Qiagen RNeasy kit (Qiagen cat. no. 74104)
Absolute ethanol

Spectrophotometer
2-ml microcentrifuge tubes
Microcentrifuge
Lysing Matrix B tube (MP Bio cat. no. 6911-100)
Fast RNA prep machine (FastPrep; FP120, Bio101, Thermo Electron Corporation)

1. Culture 10 ml mycobacterial culture grown to an OD_{600} of 0.2 and collect by centrifuging for 10 min at 2000 × *g*, room temperature.
2. Decant the supernatant and resuspend the cell pellet in 1 ml of Qiagen RNA protect reagent. Incubate the cell suspension 1 hr at room temperature.

   CAUTION: Although this treatment has been found to sterilize M. tuberculosis and M. bovis cultures such that subsequent steps can be performed in a BSL-2 laboratory, this should be confirmed in each laboratory.

   At this point, the cells can be stored for 6 months at −80°C or RNA extraction can begin.

3. Transfer the cell suspensions to 2-ml microcentrifuge tubes and microcentrifuge cells 1 min at 15,800 × *g*, 4°C.
4. Decant the supernatant and resuspend cells in 1 ml Qiagen buffer RLT (Qiagen RNeasy kit).
5. Transfer the cell suspension to a Lysing Matrix B tube and process in a FastPrep machine for 45 sec at speed 6.
6. Incubate the tubes 1 min on ice, then microcentrifuge 1 min at 15,800 × *g*, 4°C.
7. Remove 750 µl processed liquid to a new 2-ml microcentrifuge tube, taking care not to transfer any of the glass beads. To these tubes, add 525 µl absolute ethanol and apply the contents to Qiagen RNeasy mini columns (two applications of ~650 µl each).
8. Purify RNA according to Qiagen RNeasy kit manufacturer's instructions. Assay the yield and integrity of the RNA by typical methods.

SUPPORT PROTOCOL 1

GTC METHOD FOR STABILIZATION OF THE *MYCOBACTERIUM TUBERCULOSIS* mRNA POOL

This is a protocol to use before RNA extraction to prevent alterations in transcription for experimental methods that study gene expression and require accurate mRNA representation (Stewart et al., 2002; Butcher, 2004). Bacterial mRNA is quite labile and rapid alterations in transcription can occur as a result of environmental changes that take place during most preparations. Some researchers use rapid harvesting by centrifugation or RNA stabilization with commercially available reagents (e.g., RNA Protect; Qiagen). This protocol uses guanidinum thiocyanate (GTC).

Materials

M. tuberculosis culture
GTC solution (for RNA prep; see recipe)

15- or 50-ml conical tubes
Tabletop centrifuge

1. Grow *M. tuberculosis* to desired growth phase.
2. In a 15- or 50-ml conical tube, prepare a volume of GTC solution equal to the cell culture volume ready.
3. Add culture straight into the tube and mix by vortexing or inverting the capped tube.
4. Centrifuge 10 min at 2000 × *g*, room temperature.
5. If processing by Trizol for RNA extraction (see Basic Protocol 3), resuspend well in Trizol (1 ml/25 ml culture), then proceed with RNA preparation in Basic Protocol 3, step 6 or store at –80°C.

   Alternatively pellet can also be processed using the Fast RNA prep (see Alternative Protocol 2, beginning at step 4), taking care not to overload the matrix.

BASIC PROTOCOL 4

ELECTROTRANSFORMATION OF *MYCOBACTERIUM TUBERCULOSIS*

Electrotransformation of mycobacteria was first demonstrated in *M. smegmatis*, a fast-growing non-pathogenic mycobacterium (Snapper et al., 1990). *M. tuberculosis* strains are amenable to electroporation (Wards and Collins, 1996) but not all strains have equal transformation efficiencies.

Materials

M. tuberculosis culture
Middlebrook 7H9 broth
10% glycerol with Tween-80 (see recipe), sterile
DNA
Selective plates

Spectrophotometer
490-cm^2 roller bottle (Corning cat. no. 430195)
37°C incubator
50-ml conical tubes
Tabletop centrifuge
1.5-ml microcentrifuge tubes
0.2-cm electroporation cuvettes
Electroporator (e.g., BioRad Gene Pulser)
15-ml snap-cap plastic tube (Falcon, cat. no. 2059 or equivalent)

1. Grow *M. tuberculosis* to mid-log phase (OD_{600} should be between 0.5 and 1.0) in Middlebrook 7H9 broth in a 490-cm^2 roller bottle at 37°C.

   A culture volume of 100 ml is sufficient for five electroporations if the culture is resuspended in 1/100 volume or 25 electroporations; if resuspended in 1/10 the volume, see step 5. Check the OD_{600} the day before preparing the cells to make sure that the culture is still in log phase (i.e., the OD_{600} has approximately doubled in 1 day).

2. Transfer cells to 50-ml conical tubes and centrifuge 15 min at 2000 × *g*, room temperature.

3. Discard supernatant and wash cells with an equal volume of sterile room temperature 10% glycerol with Tween-80. Pellet cells by centrifugation for 15 min at 2000 × *g*, room temperature.

4. Repeat the wash and centrifugation two additional times.

5. Resuspend the cell pellet in sterile, room temperature, 10% glycerol with Tween-80, using 1/100 the original volume of the culture. Use 200 μl of cells per transformation.

   Alternatively, resuspend the cell pellet in 1/10 of the original volume of the culture and use 400 μl of cells per transformation.

6. Mix DNA and bacteria in a 1.5-ml microcentrifuge tube and incubate for 10 min or longer at room temperature.

7. Transfer bacteria and DNA to 0.2-cm cuvette and electroporate (BioRad Gene Pulser, settings: 2500 mV, 1000 Ω, 25 μF). To avoid overheating the electroporator, allow the electroporator to cool for 1 to 2 min between transformations.

8. Add 1 ml of Middlebrook 7H9 medium to each cuvette and transfer cells to a 15-ml snap-cap plastic tube.

   Alternatively, add 1 ml Middlebrok 7H9 medium to each cuvette and do not transfer to another tube (replace the cuvette cap).

9. Allow for expression of antibiotic resistance by incubating overnight at 37°C.

10. Plate transformation on selective plates and incubate 3 to 4 weeks at 37°C (*UNIT 10A.1*).

BASIC PROTOCOL 5

GENERATING *MYCOBACTERIUM TUBERCULOSIS*–KNOCKOUT MUTANTS WITH SPECIALIZED TRANSDUCTION

The ability to generate null mutants in *M. tuberculosis* is critical for rigorous genetic analysis. In any knockout scheme, an allelic exchange substrate containing the modified gene of interest needs to be delivered to the cells. A means should be available for selecting for recombinants that have the knockout allele. A number of methods have been used to successfully generate knockout strains of *M. tuberculosis* (reviewed in Bardarov et al., 2002) including transformation with linear DNA substrates, suicide plasmids, chemical modification of allelic exchange substrates, and specialized phage transduction (Bardarov et al., 2002). Both suicide plasmids and chemical modifications of plasmids have the advantage that only a plasmid construction and subsequent *M. tuberculosis* strain electroporation are necessary. The disadvantage of using suicide plasmids or chemically modified plasmids is that electroporation can be very inefficient and electroporation efficiencies vary widely between *M. tuberculosis* strains.

Specialized phage transduction has the disadvantage of more preparation steps, but the advantage of higher efficiencies of substrate delivery compared to electroporation as well as less variability in efficiency for many different strains of *M. tuberculosis*.

Generation of knockout mutants with specialized transduction entails five steps: (1) construction of allelic exchange cosmid, (2) phasmid transduction, (3) phage lysate preparation, (4) phage transduction, and (5) generation of double-crossover alleles. Figure 10A.2.1 illustrates steps 2, 3, and 4. For construction of the allelic exchange

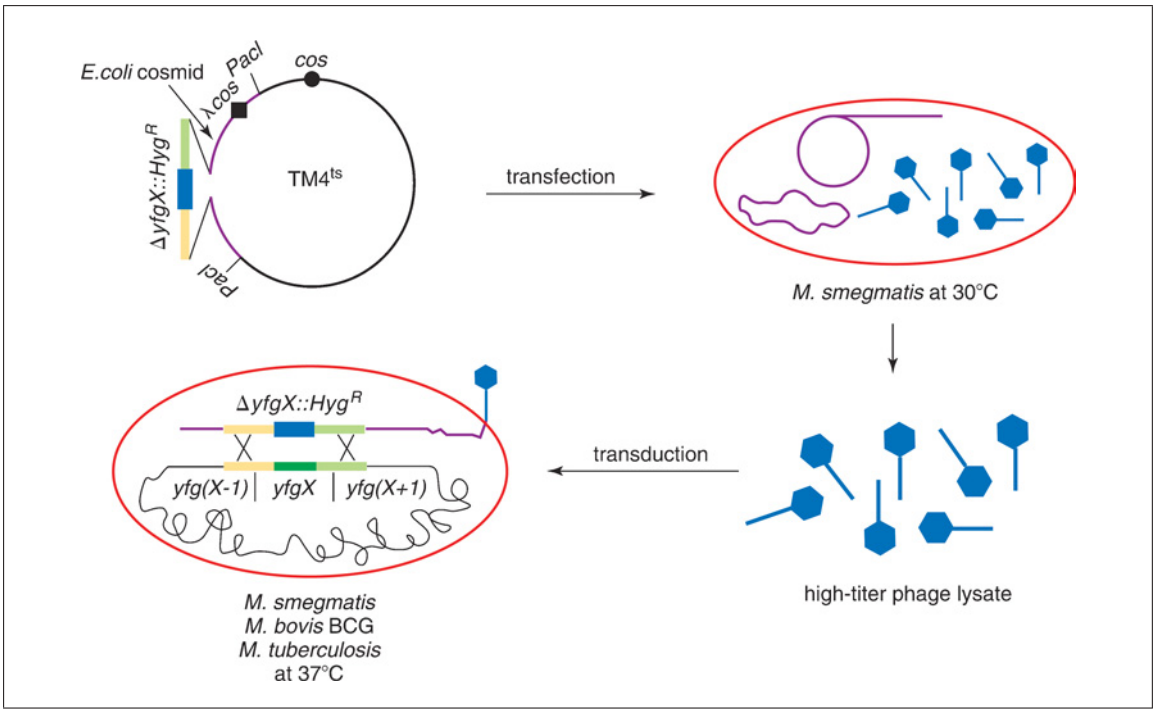

Figure 10A.2.1 Specialized transduction. Regions flanking the upstream and downstream regions of "your favorite gene" (*yfg*) are cloned into a hygromycin-containing cassette on a plasmid containing both lambda *cos* sites and mycobacteriophage *cos* sites. *M. smegmatis* is transfected at 30°C to allow for production of a high-titer phage lysate containing the Δ*yfgX*::*Hyg*^{R} allele. The high-titer phage lysate is then used to transduce *M. tuberculosis* at 37°C (non-permissive temperature for phage lysis) to deliver Δ*yfgX*::*Hyg*^{R} substrate to the cell for subsequent allelic exchange.

substrate (AES) cosmid, Bardarov et al. (2002) reviews the necessary features of an AES cosmid. Briefly, the AES plasmid has multiple cloning sites (MCS) upstream and downstream of an antibiotic-resistance marker (hygromycin), and a counter-selectable marker (*sacB*). Also on the AES plasmid is *oriE*, an *E. coli* plasmid origin of replication, a λ-cos site for lambda packaging, and a unique *Pac*I restriction site for eventual ligation to a modified, temperature-sensitive mycobacteriophage such as TM4 (Bardarov et al., 2002). The lengthy protocol for generating knockout strains of *M. tuberculosis* includes: (1) construction of allelic exchange subsrates (AES) phasmids, (2) phasmid screening, (3) phage preparation and verification, (4) preparation of high-titer phage lysates, and (5) phage transduction. Only the final step of phage transduction that utilizes an *M. tuberculosis* strain is performed at BSL-3 (*M. smegmatis* is a BSL-1 organism; see *UNIT 1A.1* for more information on biosafety levels).

Materials

Upstream and downstream regions (1-kb each) of gene of interest
pYUB854 plasmid or equivalent (jacobsw@hhmi.org; Bardarov et al., 2002)
*Pac*I restriction endonuclease and buffer 1
phAE159 or equivalent (jacobsw@hhmi.org)
Shrimp alkaline phosphotase (SAP)
1% agarose gel
T4 DNA ligase and 10× T4 ligase buffer
Lambda in vitro packaging mix (e.g., MaxPlax, Epicentre cat. no. MP5110)
Mycobacteriophage buffer (MP buffer; see recipe)
E. coli HB101 cells (see Support Protocol 2)
LB broth with and without hygromycin (150 mg/liter)
LB plates with hygromycin (150 mg/liter)
Qiagen miniprep kit (Qiagen cat. no. 27106)
10× BSA
M. smegmatis mc^2155
Middlebrook 7H9 broth without Tween-80
Middlebrook top agar (see recipe)
Middlebrook 7H10 plates without Tween-80
M. tuberculosis
Mycobacteriophage wash medium (see recipe)

37° and 65°C water baths
Agarose gel electrophoresis apparatus
Microcentrifuge
15-ml snap-cap tubes (Falcon cat. no. 2059 or equivalent)
Oak Ridge centrifuge tubes (Nalgene cat. no. 3115 or equivalent)
0.45- and 5-μm syringe filters
Spectrophotometer
37° to 39°C heating block
2-ml screw-cap tubes

Additional reagents and equipment for electroporation (see Support Protocol 3)

Construct allelic exchange substrate (AES) phasmid

1. For the target DNA sequence to be deleted, clone ~0.5 to 1 kb of sequence upstream and downstream of the region into a plasmid such as pYUB854 (described in Bardarov et al., 2002).

   Upstream and downstream regions are typically each amplified by PCR, cloned into a vector such as pTOPO (Invitrogen, cat. no. K4500-40), sequenced to confirm the insert, then sequentially subcloned into pYUB854.

2. For sequence-confirmed AES plasmids, digest 10 μl of construct with *Pac*I enzyme 2 hr at 37°C.
3. Following the same procedure, digest 10 μg phAE159 in a total volume of 50 μl.
4. After digestion, add 0.5 μl SAP and incubate 20 min at 37°C followed by incubation for 20 min at 65°C.
5. Run 1 μl phAE159/*Pac*I and 3 μl prephasmid/*Pac*I on a 1% agarose gel to determine the amount of DNA to be used for the ligation reaction.
6. Set up the ligation reaction overnight (or >2 hr) at room temperature. For example:

   2 to 4 μl phAE159
   4 to 6 μl plasmid
   1 μl 10× T4 ligase buffer
   0.5 μl T4 DNA ligase (400 U/μl)
   Bring up to 10 μl with water

7. Add 5 μl ligation mixture to a MaxPlax in vitro packaging extract on dry ice. Remove and mix gently by tapping lightly with finger as the sample thaws.
8. Incubate for up to, but no more than, 2 hr at room temperature.
9. Stop reaction by adding 400 μl MP buffer.
10. Add 1 ml *E. coli* HB101 host cells directly to the packaging tube, mix, and incubate 1 hr at 37°C without shaking (see Support Protocol 2 for preparation of HB101 host cells).

    Alternatively, remove half of packaging mixture and store for future use (in the case where HB101 cells are not competent).

11. Microcentrifuge 1 min at 15,800 × *g* (13,000 rpm), 4°C.
12. Remove supernatant and resuspend pellet in 1 ml LB broth.
13. Plate cells on LB plates with hygromycin and incubate overnight at 37°C.

Perform phasmid screening

14. Screen for correct phasmids by picking six colonies individually into 5 ml LB broth with hygromycin. Incubate overnight at 37°C, with shaking at 200 rpm.
15. Prepare a plasmid miniprep from the 5-ml culture using a Qiagen miniplasmid preparation kit and elute with 50 μl of 65°C EB buffer (from Qiagen kit).

    Optional: Prepare glycerol stocks of cultures first, e.g., 100 μl of 10% glycerol with 100 μl cells stored at –20°C.

16. Digest the phasmid minipreps with *Pac*I to screen phasmids that have the correct insert. For example, for a total volume of 10 μl, add:

    5 μl DNA
    1 μl 10× NEB buffer 1
    1 μl 10× BSA
    1 μl *Pac*I
    2 μl water.

17. Check the digest pattern against expected results.

    Normally, one to six out of six phasmid colonies screened contain the insert.

Prepare phage

18. Eighteen to 24 hr prior to the electroporation, start a fresh culture of *M. smegmatis* mc^2155 for use in step 21.
19. Electroporate 5 to 10 μl phasmid DNA into 400 μl competent *M. smegmatis* mc^2155 cells (see Support Protocol 3, steps 1 through 7).
20. Add 1 ml Middlebrook 7H9 broth (without Tween-80) and incubate 1 hr at 37°C.
21. Set up two dilutions:

    (a) Add 400 μl transformed mc^2155 to 400 μl actively growing mc^2155.
    (b) Add 110 μl transformed mc^2155 to 1 ml actively growing mc^2155.

22. Add 300 to 600 μl of dilutions a and b, separately, to 3 ml Middlebrook top agar in 15-ml snap-cap tubes, vortex to mix, and plate onto prewarmed 7H10 plates (without Tween-80).
23. Incubate the plates 3 days at 30°C.
24. With a sterile pipet tip, core out one to two plaques each into 200 μl MP buffer to recover phage; incubate at 1.5 hr at room temperature, then store at 4°C.

Prepare high-titer phage lysate

25. Set up two dilutions:

    a. Add 2 to 5 μl phage from step 24 to 300 μl mc^2155 culture.
    b. Dilute original phage from above 1:5 with MP buffer. Add 2 to 5 μl diluted phage to 300 μl mc^2155 culture.

26. Set up two plates for each dilution—mix phage with 3 ml Middlebrook top agar and pour onto 7H10 plates as described in step 22.
27. Incubate plates 2 days at 30°C.
28. Select the plates with the appropriate number of plaques per plate for high titer preparation (~1000 per plate, which will give a lacey appearance).
29. Add 5 ml MP buffer to these plates. Shake for 2 to 4 hr at 4°C, then incubate plates 1 to 2 hr at 37°C.

    Better titers will be obtained if plates are incubated 2 to 4 hr at 4°C and then 1 to 2 hr at 37°C. The top agar first shrinks at 4°C and then expands at 37°C and this "breathing" of the top agar helps more phage to be released in the lysate.

30. Harvest the phage lysate by pipetting up the MP buffer from each plate and pooling. Centrifuge this agar-phage mix in an Oak Ridge centrifuge tube 15 min at 15,800 × *g*, 4°C.
31. Filter the phage lysate through a 5-μm filter and then a 0.45-μm filter.
32. Make serial dilutions of the phage lysate in MP buffer. In duplicate, mix 300 μl phage lysate dilutions with 300 μl mc^2155 cells and incubate 30 min at 30°C.
33. To each phage dilution, add 3 ml Middlebrook top agar and plate on Middlebrook 7H10 plates.
34. For each dilution, incubate one plate at 30°C and one plate at 37°C to confirm temperature-sensitive phenotype and to determine phage titer—i.e., plaque forming units (pfu)/ml.

Perform phage transduction

35. Grow culture of targeted *M. tuberculosis* to OD 0.8 to 1.0.

    A 10-ml volume of culture will be needed for each transduction.

36. Transfer 10 ml culture to a 15-ml conical tube and centrifuge 15 min at 2000 × *g*, 4°C.

37. Decant supernatant and wash pellet once with an equal volume of mycobacteriophage wash medium.

38. Centrifuge 15 min at 2000 × *g*, decant supernatant and resuspend cell pellet in 1 ml MP buffer.

39. Prewarm phage at 37°C to 39°C in a heating block containing water in the wells.

40. In a 2-ml screw-cap tube, mix 1 ml cells in MP buffer and 1 ml high-titer phage. Gently mix the cells and phage using a 1000-µl tip. Incubate overnight in a 37° to 39°C heating block. Include cells only and phage only controls.

41. Microcentrifuge 10 min at 15,800 × *g*, 4°C, to pellet cells. Remove supernatant and resuspend pellet in 0.2 ml Middlebrook 7H9 broth.

42. Plate on one selective plate. Incubate 3 to 4 weeks at 37°C.

SUPPORT PROTOCOL 2

LAMBDA PHAGE TRANSDUCTION OF *E. COLI* HB101

The *E. coli* HB101 strain is ideal for propagation of phasmids containing allelic exchange substrates. Specifically, the HB101 strain does not have a γ-δ resolvase function (γ-δ resolvase sites flank the hygromycin cassette in the allelic exchange substrate plasmid—inclusion of γ-δ resolvase at this juncture would promote the loss (looping out) of the hygromycin cassette).

Materials

E. coli HB101 (ATCC #33694)
LB broth supplemented with 10 $MgSO_4$ and 0.2% maltose
10 mM $MgSO_4$

37°C incubator with shaker
Spectrophotometer
50-ml conical tubes

1. To prepare *E. coli* HB101 for transduction, grow the HB101 strain overnight in LB broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose at 37°C with shaking.

2. Inoculate 25 ml fresh medium with 0.5 ml overnight culture.

3. Shake culture at 200 rpm at 37°C until OD_{600} reaches 0.8 to 1.0.

4. Transfer cells to a 50-ml conical tube and centrifuge 10 min at 2000 × *g* (3000 rpm), 4°C.

5. Decant supernatant and resuspend cell pellet in 12.5 ml of 10 mM $MgSO_4$.

6. Store cells up to 2 days at 4°C until ready for use.

ELECTROPORATION OF *M. SMEGMATIS* mc^{2}155

Preparation of *M. smegmatis* mc^{2}155 cells for electroporation is similar to the electroporation protocol for *M. tuberculosis* (see Basic Protocol 4) with some modifications.

Materials

M. smegmatis mc^{2}155 cells
Middlebrook 7H9 broth or LB broth with 0.05% Tween
10% glycerol with 0.05% Tween, 4°C
Transforming DNA
Medium plates with antibiotic (e.g., 10-20 μg/ml Kanamycin at; at 50 μg/ml Hygromycin B)

Spectrophotometer
50-ml conical tubes, prechilled
0.2-cm electroporation cuvettes (GenePulser, BioRad cat. no. 165-2086)
GenePulser electroporator (BioRad cat. no. 1652076)
15-ml snap-cap plastic tube (Falcon cat. no. 2059 or equivalent)
Tabletop centrifuge

1. Grow fresh 50 ml culture of *M. smegmatis* mc^{2}155 in either Middlebrook 7H9 broth or LB with Tween (0.05%) to mid-log phase (OD_{600} 0.5 to 1.0).
2. Incubate cells 10 min (no longer than 2 hr) on ice.

   For the following steps, keep cells as close to 0°C (ice bath) as possible. The electroporation cuvettes, 50-ml conical tubes, and 10% glycerol should be pre-chilled to 4°C.

3. Transfer chilled cells to a 50-ml prechilled conical tube. Centrifuge cells 10 min at 2000 × *g*, 4°C. Decant supernatant from cell pellet.
4. Wash cells two times with 40 ml ice-cold 10% glycerol.

   For optimal results, 10% glycerol should be prepared fresh weekly with distilled water. Sterilize by filtration through 0.2-μm membrane pore, do not autoclave.

5. Resuspend the washed cell pellet in 5 ml ice-cold 10% glycerol (1/10 culture volume) and store on ice (0.4 ml per sample).
6. Dispense transforming DNA (1 to 10 μl) into a prechilled electroporation cuvette with 0.2-cm electrode gap and incubate on ice. To each aliquot of DNA, add 400 μl of prepared cell suspension and mix by gently pipetting up and down (use prechilled pipet tips for cell transfer).
7. Pulse one time with a GenePulser electroporator set at 2.5 V, resistance 1000 Ω, capacitance 25 μFD.

   The time constant reading should be ~19 to 21 for control cells with no DNA added.

8. Immediately dilute transformed cell mixture with 2 ml medium. Transfer cell suspension to a 15-ml snap-cap plastic tube.
9. Incubate 2 hr at 37°C (with or without shaking) to allow cell recovery and expression of antibiotic resistance.
10. Centrifuge 10 min at 2000 × *g*, 4°C. Aspirate supernatant from cell pellet; resuspend cell pellet in 0.5 ml medium.
11. Plate desired volume of cell resuspension on plates containing the antibiotic of choice (e.g., 10 to 20 μg/ml kanamycin or 50 μg/ml hygromycin B).

12. Incubate plates at 37°C.

Colonies should appear in 3 to 4 days.

BASIC PROTOCOL 6

TRANSPOSON MUTAGENESIS OF *MYCOBACTERIUM TUBERCULOSIS*

Transposon mutagenesis of *M. tuberculosis* strains is important for identifying genes that are involved in a wide range of functions (virulence, cell wall growth, amino acid biosynthesis, etc.) (Kriakov et al., 2003). Successful transposon mutagenesis depends on having (1) a transposon that can insert throughout the chromosome and (2) a way to reliably deliver the transposon to an entire population of cells. The Mariner transposon functions in *M. tuberculosis* with thousands of estimated integration sites throughout the *M. tuberculosis* chromosome. Specialized phage transduction provides an ideal means to deliver a substrate to an entire culture. For the following protocol, a mariner transposon such as Himar1 is cloned onto the temperature-sensitive TM4 phage phAE159. A high titer phage lysate is prepared (as described previously) to deliver the transposon to a culture of *M. tuberculosis* (Rubin et al., 1999).

Materials

M. tuberculosis culture
Mycobacteriophage (MP) buffer (see recipe)
High-titer phage lysate containing transposon
Middlebrook 7H9 broth
Middlebrook 7H10 plates with 50 mg/liter hygromycin

Spectrophotometer
15-ml conical tube
Centrifuge (Sorvall RT 600 tabletop centrifuge or equivalent)
37°C incubator

1. Grow 10 ml *M. tuberculosis* culture to an OD_{600} of 0.8.
2. Transfer to a 15-ml conical tube and centrifuge 10 min at 3450 × *g* (4000 rpm), room temperature.
3. Decant supernatant and resuspend pellet in 10 ml MP buffer.
4. Centrifuge cells 10 min at 3450 × *g* (4000 rpm), room temperature.
5. Decant supernatant and resuspend cell pellet in 200 μl MP buffer.
6. In a sterile 15-ml conical tube, mix 200 μl of cells with 1 ml of high-titer phage lysate (10^{10} to 10^{11} pfu/ml) containing transposon.
7. Incubate 4 hr at 37°C.
8. Add 1 ml of Middlebrook 7H9 broth to the transduction mixture and incubate 24 hr at 37°C.
9. Centrifuge cells 10 min at 3450 × *g* (4000 rpm), room temperature.
10. Decant supernatant and resuspend cell pellet in 1 ml Middlebrook 7H9 broth.
11. Plate the cell suspension on eleven plates of Middlebrook 7H10 with 50 mg/liter hygromycin B: one plate with 10 μl, one plate with 50 μl, and nine plates with 100 μl each of cell suspension.
12. Incubate plates 3 weeks at 37°C
13. After the 3-week incubation, count the colonies to determine the number of transposition events and screen as needed.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CTAB solution

Dissolve 4.1 g sodium chloride in 90 ml water. While stirring on a magnetic stirrer, add 10 g cetrimide (hexadecyltrimethylammonium bromide, Sigma cat. no. H5882). Incubate in 65°C water bath to dissolve cetrimide into solution. Store 1 year at room temperature.

Dextrose, 20%

Dissolve 20 g dextrose in water and bring up to 100 ml with water. Sterilize by autoclaving or by filtration through a 0.22-μm pore membrane. Store 1 year at room temperature.

Glycerol, 10% with Tween

Weigh out 50 g of glycerol in flask. Add 1.25 ml of 20% Tween-80. Bring up to 500 ml with water. Sterilize by filtering through a 0.22-μm pore membrane. Store 1 year at room temperature.

Glycine, 10% (w/v)

Dissolve 10 g glycine in a small volume of water and bring up to 100 ml water. Sterilize by filtering through a 0.22-μm pore membrane. Store 1 year at room temperature.

GTC solution (for DNA prep)

4 M guanidine thiocyanate
0.1 M Tris·Cl, pH 7.5 or 8.0
0.5 % sarcosyl
ddH_2O
Heat to 55° C to dissolve
Filter through no. 1 Whatman paper
Store 1 year at room temperature

GTC solution (for RNA prep)

4 to 5 M guanidinum thiocyanate (or isothiocyanate) (50 to 60 g/100 ml)
0.5% sodium-*N*-lauryl sarcosine (0.5 g/100 ml)
25 mM tri-sodium citrate (citric acid, trisodium salt) (1 g/100 ml)
0.1 M 2-mercaptoethanol (BME) (0.7 ml/100 ml)
Store up to 6 months at room temperature

Note: BME should be added just before using GTC solution.

GTE solution

25 mM Tris·Cl, pH 8.0
10 mM EDTA
50 mM glucose
Autoclave and store 1 year at room temperature

Lysozyme solution

For optimal lysozyme activity, make a fresh 10 mg/ml solution of lysozyme (Sigma cat. no. L6876) in 25 mM Tris·Cl, pH 8.5 buffer.

Middlebrook top agar

0.47 g Middlebrook 7H9 powder (Difco cat. no. 0713-01-7)
0.75 g noble agar (Difco cat. no. 0142-17-0)
ddH_2O to 100 ml
Autoclave; add 1 ml sterile 20% dextrose (see recipe)
Store up to 6 months at room temperature

Mycobacteriophage buffer (MP buffer)

50 mM Tris·Cl, pH 7.6
150 mM NaCl
10 mM $MgCl_2$
2 mM $CaCl_2$
Store up to 1 year at room temperature

Mycobacteriophage wash medium

5% glycerol
10% albumin dextrose saline
Store up to 6 months at 4°C

Proteinase K

10 mg/ml proteinase K (Sigma cat. no. P4914) in water. Store 6 months at –20°C.

SDS, 10%

10 g sodium dodecyl sulfate in 100 ml water
Adjust pH to 7.2 with NaOH or HCl
Store 1 year at room temperature

COMMENTARY

Background Information

Tuberculosis (TB) is a disease caused by *M. tuberculosis*. *M. tuberculosis* is spread via the aerosol route and predominately causes disease in lung tissue, although it can spread to other organs. It is estimated that nearly one-third of the world's population is infected with TB, many of those are cases of latent infection. Upon waning of the immune system through aging or infection with HIV that compromises the immune system, TB can reactivate from its latent form after decades of quiescence. Antibiotic therapy is available for treatment of TB, although due to the slow-growth of the organism, long courses of treatment (6 months to 1 year) are recommended.

Critical Parameters and Troubleshooting

One of the most important aspects of working with *M. tuberculosis* is to minimize any possible aerosol exposure to this pathogen (see Strategic Planning, Safety Considerations). Second to that, scrupulous attention to detail in preparing media and reagents is crucial. Given the long doubling time (3 weeks for colonies to be visible on a solid medium; refer to *UNIT 10A.1*) of the organism, care should be taken to minimize any contamination of media, as such contaminants could "out grow" the *M. tuberculosis* cells.

Extraction of genomic DNA

For most preparations, the glycine incubation step is not needed. However, if yields are unacceptably low, include a glycine incubation.

Extraction of plasmid DNA

If plasmid yield is too low for manipulations, consider scaling up DNA extraction by processing more culture in parallel rather than trying to load more lysate onto the extraction column.

PCR template preparation

Strains vary in how well the PCR protocols work. An alternative to this protocol is to use the Genomic DNA—GTC prep.

Extraction of RNA

Microarray, RT-PCR, and other RNA methodologies are important for characterizing *M. tuberculosis* under a variety of in vitro

and in vivo conditions. Care should be taken to minimize contamination with RNase.

Electrotransformation

Each strain of *M. tuberculosis* has a different efficiency of transformation. If no or very few transformants are obtained, it could be because of the quality or quantity of input DNA or because of the cells. DNA can be checked for its ability to transform fast-growing mycobacteria. For critical transformations, cells should be harvested at the lower range of the OD_{600} suggested. More important than the actual OD_{600} is that the cells are in logarithmic growth and the OD_{600} has doubled from the day before.

Generation of knockouts with specialized transduction

Knocking out genes in *M. tuberculosis* is a complex process. Ultimately, the failure to obtain a recombinant with the gene of interest knocked out could be due to the essentiality of the gene. For such knockouts, a meridiploid strain can be constructed where the wild-type allele is placed at the *att*/*int* site prior to transducing the strain with the deletion substrate. Care should be taken along the way to ensure the sequence of the substrate is correct, the phage carrying the allelic exchange substrate is temperature sensitive, and the phage used for the transduction has a high titer ($\sim 10^{10}$ pfu/ml). Additionally, if the deletion of the allele of interest will result in a potential auxotrophy, appropriate media supplementation must be made.

Figure 10A.2.2 demonstrates what a knockout looks like as confirmed by Southern analysis.

Transposon mutagenesis

This method is a powerful tool for genetic manipulation of *M. tuberculosis*. Preparation of a high-titer phage lysate as well as appropriate media supplementation and selection is key for good results.

Anticipated Results

Extraction of genomic DNA

Yield of DNA from this method is lower than the CTAB method but may be adequate for PCR and some Southern analysis. In contrast, the quality of the DNA from the CTAB method is higher than the GTC prep, but the time investment is much greater, which is a consideration when there are many cultures to screen (see Time Considerations).

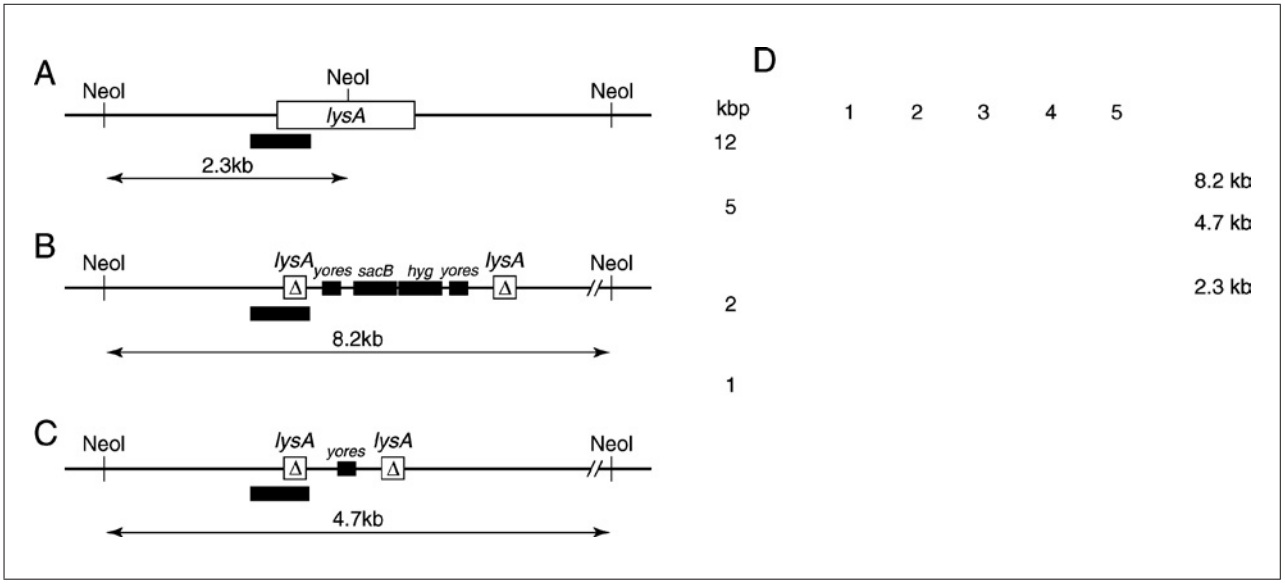

Figure 10A.2.2 Southern analysis. Chromosomal loci for *M. tuberculosis* (**A**) wild type, (**B**) marked Δ*lysA* mutant, and (**C**) unmarked Δ*lysA* mutant. See Basic Protocol 5 for construction of the knock-out phages and phage transduction. The probe is the upstream flanking sequence (UFS) of *lysA* that was used for the knock-out construct and represented as a black box. The arrows identify the fragments that will be detected by Southern analysis. (**D**) Southern blot of *Nco*I-digested genomic DNA (see Basic Protocol 1 for DNA isolation) probed with the UFS of *lysA* that was used for the knock-out construct. Lanes 1 and 2, molecular weight = 3 (unmarked Δ*lysA* mutant); lanes 4 and 5, fragment size indicated (marked Δ*lysA* mutant).

Extraction of plasmid DNA

Using this method, the yield of plasmid is influenced by the size of the plasmid (cosmid versus smaller plasmid) and the strain. Overall, plasmid yield is ≤10% of what would be expected for extraction of plasmid DNA from a similar volume of *E. coli*.

PCR template preparation

The yield from this procedure varies by *M. tuberculosis* strain and some strains do not work well at all. In that case, it is recommended to grow up a 10-ml volume of culture and extract the genomic DNA with the GTC method.

Extraction of RNA

The yield of RNA generated by this method is dependent on strain as well as growth conditions for the experiment.

Electrotransformation

Preparation of the cells for electroporation by repeated washes with glycerol is key for removing salts that might interfere with electroporation. Day 1 of this protocol takes ∼2 hr followed by an overnight incubation. The plating on day 2 takes <30 min. Transformants will not be evident for another 3 to 4 weeks.

Time Considerations

Extraction of genomic DNA

The GTC method takes ∼1 hr in the BSL-3 laboratory and then another 1 hr in the BSL-2 laboratory. In contrast, the CTAB method has at least one to two overnight incubations. If glycine pretreatment is used, then it requires <1 hr on day 1, 30 min on day 2, and 2 hr on day 3. For some strains, better DNA yield is observed if the pellet is allowed to dissolve overnight so further analysis could begin on day 4 (or day 3 without the overnight incubation).

Extraction of plasmid DNA

This protocol is spread over 3 days depending on the length of the incubation with glycine. Day 1 takes <10 min, day 2 requires 30 min, and day 3 takes ∼2 hr.

PCR template preparation

This protocol takes ∼1 hr for less than ten samples.

Extraction of RNA

The main protocol takes ∼1 hr followed by an overnight incubation. If there are more than four to six samples to process, the time required will be longer.

Electrotransformation

Preparation of the cells for electroporation by repeated washes with glycerol is key for removing salts that might interfere with electroporation. Day 1 of this protocol takes ∼2 hr followed by an overnight incubation. The plating on day 2 takes <30 min. Transformants will not be evident for another 3 to 4 weeks.

Generation of knockouts with specialized transduction

To knockout a gene with specialized transduction can take weeks to months. Beginning with target DNA sequence already cloned in pYUB854, construction of the allelic exchange phasmid is spread out over 3 days, with ∼2 hr of work each day. Phasmid screening takes another 2 days. Day 1 of phage preparation takes ∼10 min, day 2 (electroporation of *M. smegmatis*) requires 2 to 3 hr, and day 5 requires ∼2 hr. For preparation of high titer phage lysate, day 1 requires ∼1 hr and day 4 requires ∼10 hr (not all hands-on time, as there are a number of incubation steps). For phage transduction, day 1 requires ∼1 hr, and day 2 requires ∼30 min. Transductants will not be evident for another 3 to 4 weeks following plating.

Transposon mutagenesis

Preparation of the transposon transduction takes ∼2 hr on day 1 with a 4-hr incubation followed by an overnight incubation, day 2 only requires ∼30 min. Colonies will not be evident for 3 to 4 weeks.

Acknowledgments

Special thanks to Kieran Bhatt, Bing Chen, Zhiyan (Annie) Dai, Dee Dao, Robert Glover, Morad Hassani, Jordan Kriakov, Sunhee Lee, Catherine Vilcheze (see *UNIT 10A.3*), Brian Weinrick, and Torin Weisbrod for contributions and helpful discussions.

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Isolation and Analysis of *Mycobacterium tuberculosis* Mycolic Acids

This unit describes the isolation of *Mycobacterium tuberculosis* mycolic acids and their analysis by either thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). The term "mycolic acid" was given by Stodola et al. (1938) to designate the principal ether-soluble fraction of the wax isolated from *M. tuberculosis* (Stodola et al., 1938). Mycolic acids are long-chain α-alkyl β-hydroxy fatty acids (70 to 90 carbons) that are present within the mycobacterial cell wall. *M. tuberculosis* comprises three major mycolic acid classes (Fig. 10A.3.1): the α-mycolic acids (containing cyclopropane rings and/or double bonds with either a *cis* or *trans* configuration), methoxy-mycolic acids (containing methoxy groups and cyclopropane rings), and keto-mycolic acids (containing keto groups and cyclopropane rings).

Mycolic acids are either covalently bound to arabinogalactan or found as free lipids esterified to trehalose or glucose in the mycobacterial cell wall. They have been shown to modulate cell wall fluidity, protect mycobacteria against chemicals such as antibacterials or stress, play a role in pathogenecity and persistence, and have diverse immunological functions (Kremer and Besra, 2005).

Analysis of mycolic acids by TLC has been widely used to study mycolic acid biosynthesis, the formation of unusual mycolic acids in mycobacterial mutant strains, and the effect of antimycobacterial drugs targeting the cell wall. It is mainly a qualitative method, which allows for an easy identification of the three major mycolic acid classes. HPLC analysis is a quantitative method that efficiently separates different structures based on functional groups and chain length. It has been extensively used to classify and identify mycobacterial species. When linked to a mass spectrometer, HPLC is a powerful tool to isolate and characterize mycolic acids.

Both protocols utilize the same initial steps: the mycolic acids are hydrolyzed from the cell wall by saponification and then derivatized. In the first method, the saponified mycolic acids are methylated and then extracted prior to TLC analysis. In the second method, the mycolic acids are extracted as free fatty acids and then derivatized to UV-absorbing esters for HPLC analysis. Both methods will work for the extraction and analysis of mycolic acids from other mycobacteria, such as *M. smegmatis*, *M. avium*, or *M. leprae*, or other bacterium that contain mycolic acids, such as *Corynebacterium*, *Gordonia, Nocardia*, and *Rhodococcus*. However, mycolic acids from other bacteria have different structures

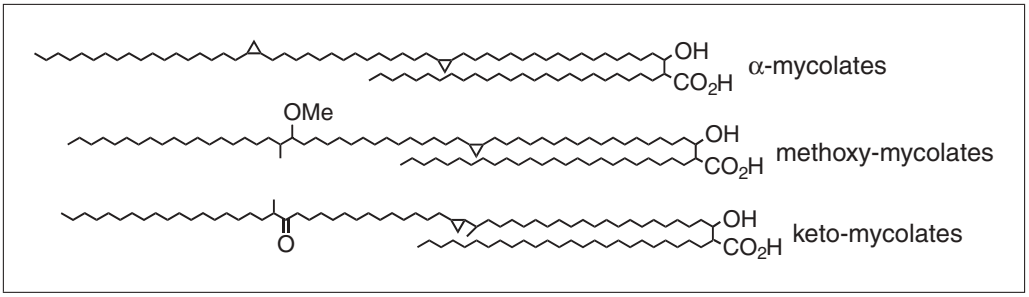

Figure 10A.3.1 Structure of α-, methoxy-, and keto-mycolates from *M. tuberculosis*. Only one type of mycolic acid for each class is represented. Reprinted from Minnikin et al. (2002), with permission from Elsevier.

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Current Protocols in Microbiology (2007) 10A.3.1-10A.3.11

(chain length, number of unsaturations, or presence of oxygenated groups); therefore, the TLC or HPLC pattern for mycolic acids will differ.

CAUTION: *Mycobacterium tuberculosis* is a Biosafety Level 3 (BSL-3) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Chemical extraction and TLC studies should be performed in a chemical fume hood under BSL-2 conditions.

BASIC PROTOCOL 1

ISOLATION AND ANALYSIS OF MYCOLIC ACIDS BY TLC

To visualize the different mycolic acid classes by TLC from a small culture of *M. tuberculosis*, the cells are first labeled with [1-^{14}C]-acetate. Mycolic acids are long-chain fatty acids synthesized by the fatty acid synthases type I and II, which use acetate as a building block. During the labeling period, all molecules that use acetate for their biosynthesis will incorporate ^{14}C. At the end of the labeling period, the cells are centrifuged, washed once to remove any trace of labeled materials from the supernatant, and then saponified with tetrabutylammonium hydroxide to hydrolyze the mycolic acids from the cell wall. The mycolic acids are then methylated with methyl iodide to form mycolic acid methyl esters (MAMEs). This step turns the mycolic acids into less polar molecules that are easier to analyze by normal phase TLC. After washing and drying, the organic phase is evaporated, resuspended in a smaller volume of methylene chloride, and loaded onto a normal-phase TLC plate. Radiolabeled species such as MAMEs are detected by autoradiography.

This protocol is also useful to study shorter-chain fatty acids (C_{16} to C_{26}). Along with the mycolic acids, the shorter-chain fatty acids are saponified, methylated, and extracted during the protocol and the resulting fatty acid methyl esters (FAMEs) elute on TLC above the MAMEs (see Fig. 10A.3.2).

CAUTION: Radioactive materials require special handling. Follow the guidelines provided by the local radiation safety adviser. See *UNIT 1A.4* for more information.

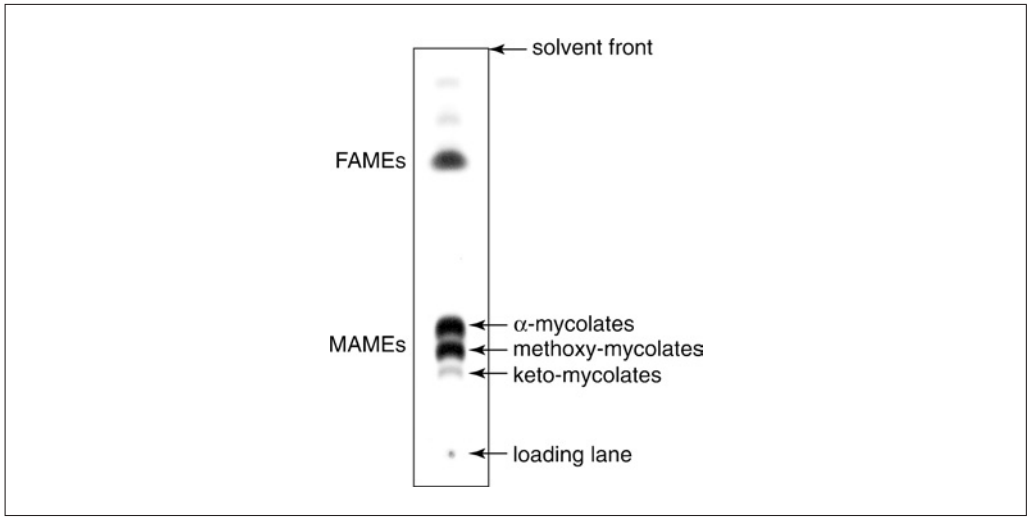

Figure 10A.3.2 Autoradiography TLC of [^{14}C]-labeled fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) isolated from *M. tuberculosis.* FAMEs are a mixture of fatty acids having from 14 to 26 carbons in length, and are usually not separated. Using three elutions in a nonpolar solvent system allows for the separation of the three major classes of mycolic acids: α-, methoxy-, and keto-mycolic acids.

Materials

M. tuberculosis culture (*UNIT 10A.1*)
[1-^{14}C]-acetic acid, sodium salt in ethanol (1.0 mCi/ml)
Vesphene
40% aqueous tetrabutylammonium hydroxide solution
Methylene chloride (optima-grade)
Methyl iodide
3 N aqueous hydrochloric acid solution
Anhydrous sodium sulfate
Nitrogen gas
Liquid scintillation counting solution
Hexanes (HPLC-grade)
Ethyl acetate (HPLC-grade)

30-ml PETG media bottles (Fisher)
37°C incubator with shaker
15-ml conical tubes
Pyrex glass tube (13-mm o.d. × 100-mm length) with Teflon-lined screw caps
50° and 100°C heating block
Tube rotator (e.g., Glas Col)
Cotton-plugged Pasteur pipets (optional)
4-ml vial with screw-thread cap
5-µl capillary pipets
Silica gel 60 F_{254} 250-µm glass plates (5 × 10–cm or 10 × 10–cm)
TLC tank with lid
Autoradiography cassette
X-ray film

Culture and label M. tuberculosis cells

1. Under BSL-3 conditions (*UNIT 1A.1*), grow a 10-ml culture of *M. tuberculosis* (*UNIT 10A.1*) to log-phase (OD_{600} = ~0.6) in a 30-ml sterile PETG media bottle.
2. Label the culture by adding 10 µCi of [1-^{14}C]-acetic acid, sodium salt (1 µCi/ml culture).
3. Incubate 20 hr (one generation time) at 37°C with shaking (~100 rpm).
4. Transfer the culture to a 15-ml conical tube and centrifuge 10 min in a tabletop centrifuge at 2000 × *g*, 20°C. Discard the supernatant in a bottle containing Vesphene and labeled for radioactive waste.

   M. tuberculosis is extremely resistant to chemical germicides due mostly to its cell wall composition. Vesphene has been shown to effectively sterilize M. tuberculosis cultures (Schwebach et al., 2001).

5. Resuspend the cell pellet in 10 ml distilled water and centrifuge 10 min at 2000 × *g*, 20°C. Discard the supernatant in a bottle containing Vesphene and labeled for radioactive waste.

Perform saponification and methylation

6. Resuspend the cell pellet in 1 ml distilled water. Transfer into a Pyrex glass tube with Teflon-lined screw cap.
7. Add 1 ml of a 40% aqueous solution of tetrabutylammonium hydroxide to the tube. Close the tube and heat 20 hr in a 100°C heating block.

8. Remove tube from heat and allow it to cool to room temperature before opening. Add 2 ml methylene chloride and 100 µl methyl iodide. Close the cap tightly.

   At this point, the outside of the tube can be washed with Vesphene and the tube removed from the BSL-3 facility according to standard procedures. Continue the protocol in the fume hood of a BSL-2 laboratory.

9. Place the tube on a rotator and rotate 1 hr at 25 rpm, room temperature.

Extract

10. Centrifuge the tube 1 min at 1400 × *g*, room temperature, to allow for phase separation. Using a Pasteur pipet, discard the upper aqueous phase.

11. Add 1 ml of 3 N aqueous hydrochloric acid solution. Mix the phases well by vortexing the tube for 10 sec.

12. Let the phases separate or centrifuge the tube 1 min at 1400 × *g*, room temperature, to achieve phase separation. Discard the upper aqueous phase.

13. Add 1 ml of distilled water to the organic phase. Mix the phases well by vortexing the tube for 10 sec.

14. Let the phases separate (~5 min) or centrifuge the tube 1 min at 1400 × *g*, room temperature, to achieve phase separation. Discard the upper aqueous phase.

15. Remove any residual water in the organic phase by adding 2 to 4 g anhydrous sodium sulfate. Vortex and let stand in the hood for 5 min.

16. Filter the suspension through fluted filter paper and transfer the organic phase into a 4-ml vial.

    Alternatively, filter the sample using a cotton-plugged Pasteur pipet. Push the cotton to the tapered part of the pipet with another pipet and use it as a disposable filtering funnel.

17. Evaporate the organic phase to dryness under nitrogen by placing the vial in a 50°C heating block and directing a gentle stream of nitrogen gas into the vial.

    The residue, which contains FAMEs and MAMEs, can be stored at −20°C for several months.

Analyze by TLC

18. Resuspend the residue in 200 µl methylene chloride.

19. Measure cpm for the sample (ratio sample/scintillation liquid: 1/100).

    This step gives the cpm/ml of each sample and allows, if required, the loading of equal amounts of counts (cpm) for each sample on the TLC plate. In that case, the volume spotted on the TLC plate might differ for each sample.

20. Using a 5-µl capillary pipet, load a 5-µl aliquot onto a Silica Gel 60 F_{254} plate. Allow the aliquot to dry before placing the TLC plate into the TLC chamber.

    The aliquot should be spotted 1 cm from the bottom of the plate. If more than one sample needs to be spotted, leave at least 1 cm between samples.

21. Add the elution solvent: 75 ml of 95:5 (v/v) hexane/ethyl acetate to the TLC chamber. Ensure that the solvent level in the TLC chamber is below the loading lane.

22. Place the TLC plate into the chamber. Let the elution solvent migrate until it is 0.5 cm from the top of the TLC plate. Remove the plate from the chamber.

23. Let the plate dry (leave it in the fume hood for 5 min), then repeat the elution two additional times (three elutions total).

    The three elutions are necessary for the separation of the major classes of mycolic acids by TLC.

24. In a dark room, place the dry TLC plate into an autoradiography cassette. Place a sheet of X-ray film on top of the TLC plate. Close the lid and place the cassette in a −80°C freezer.

25. Expose for 24 to 36 hr at −80°C to obtain the autoradiogram.

ANALYSIS OF MYCOLIC ACIDS BY TWO-DIMENSIONAL SILVER TLC

ALTERNATE PROTOCOL 1

To improve the separation and visualization by TLC of mycolic acids containing double bonds, argentation single- and two-dimensional TLC (2-D TLC) can be used. The complex between silver and a *cis*-alkene bond is more stable than the complex between silver and a *trans*-alkene bond, therefore, a different retention time on TLC is observed between mycolic acids containing *cis* and/or *trans* double bonds. The advantage of 2-D TLC is a better resolution of mycolic acids. This can be useful when analyzing mycolic acids from strains that make unusual mycolic acids, differing in their level of unsaturation or their chain length. The disadvantages are (1) time (this protocol will require an extra 90 min) and (2) the fact that only one sample can be analyzed per TLC. For single-dimension silver TLC, follow Basic Protocol 1, steps 1 to 18, prepare the silver-impregnated TLC as described below and then follow the TLC analysis protocol from Basic Protocol 1, steps 19 to 25. The protocol for two-dimensional silver TLC is described below.

Materials

0.6 M aqueous silver nitrate solution
Petroleum ether (b.p. 35° to 60°C, ACS-certified)
Anhydrous diethyl ether (ACS-certified)
125°C oven

Additional reagents and equipment for culturing, labeling, saponifying, methylating, and extracting mycolic acids (see Basic Protocol 1)

Culture, label, saponify, methylate, and extract mycolic acids

1. Culture, label, saponify, methylate, and extract mycolic acids as described in Basic Protocol 1 steps 1 to 18.

Prepare silver-impregnated TLC

2. Use a 10 × 10–cm silica gel 60 TLC plate. Place the plate in a TLC tank containing 75 ml of 10% aqueous silver nitrate solution. Allow the solution to migrate until it is 2 cm from the top. Remove the TLC plate, dry in a fume hood for 5 min, and then activate 15 min in a 125°C oven.

    The aqueous silver nitrate solution can be stored up to 1 year at room temperature in the dark and reused until it runs out.

3. Load a 5-µl aliquot of the mycolic acid extract onto the non-silver nitrate–impregnated section of the TLC plate. Allow the aliquot to dry before placing the TLC plate into the TLC chamber.

    Make sure the sample is loaded 1 cm from the bottom of the plate and 1 cm from the left side of the plate, in the non-silver nitrate–impregnated part of the TLC (see Fig. 10A.3.3A).

Perform first dimension elution

4. Add the elution solvent: 75 ml of 95:5 (v/v) hexane/ethyl acetate to the TLC chamber. Place the TLC plate in the chamber. Make sure that the solvent level in the TLC chamber is below the sample. Let the elution solvent migrate until it is 0.5 cm from the top of the TLC plate. Remove the plate from the chamber and air-dry in the fume hood for 5 min. Repeat the elution one additional time (two elutions total; Fig. 10A.3.3B).

Perform second dimension elution

5. Turn the plate 90° counterclockwise. Place in the TLC chamber containing the second dimension elution system: 75 ml of 85:15 (v/v) petroleum ether/diethyl ether. Make sure that the solvent level in the TLC chamber is below the eluted sample. Allow the elution solvent to migrate until it is 0.5 cm from the top of the TLC plate. Remove the plate from the chamber and air-dry in the fume hood for 5 min. Repeat the elution two additional times (three elutions total). Air-dry the TLC plate (Fig. 10A.3.3C).

6. In a dark room, place the dry TLC plate into an autoradiography cassette. Place a sheet of X-ray film on top of the TLC plate. Close the lid and place the cassette in a −80°C freezer. Expose for 24 to 36 hr at −80°C to obtain the autoradiogram.

Figure 10A.3.3D shows the 2-D TLC of mycolic acid methyl esters (MAMEs) from M. tuberculosis.

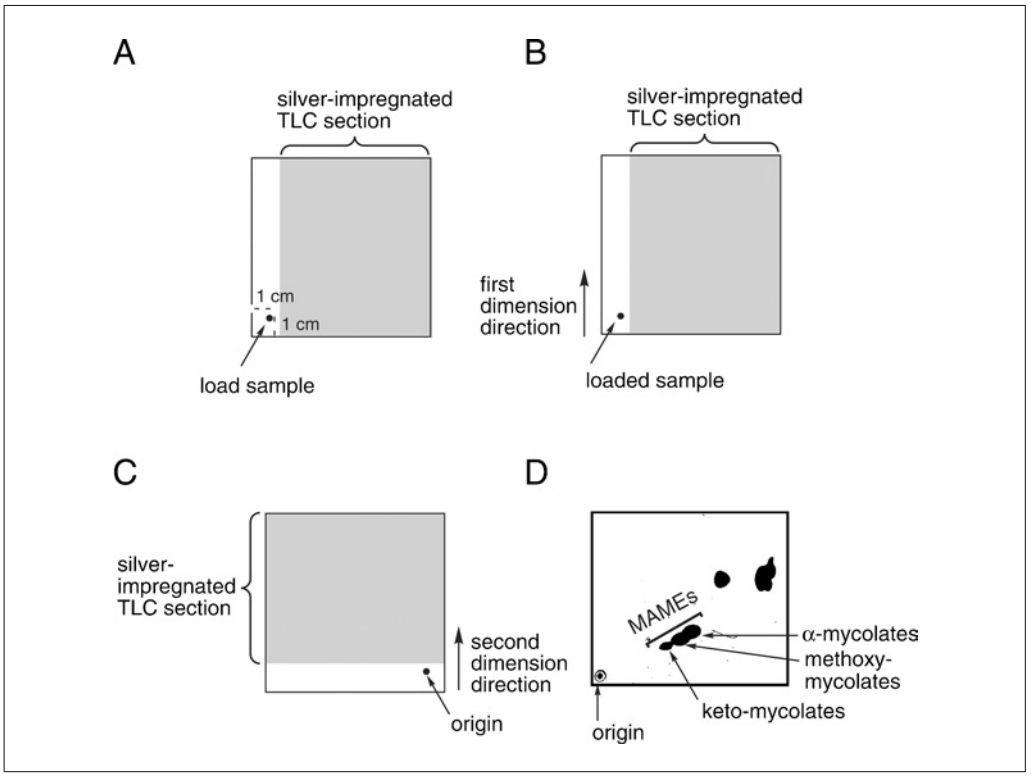

Figure 10A.3.3 (**A-C**) Illustration of the protocol for 2-D TLC. (**D**) Autoradiography 2-D TLC of [^{14}C]-labeled mycolic acid methyl esters (MAMEs) isolated from *M. tuberculosis.* The different mycolic acid classes, α-, methoxy-, and keto-mycolic acids, are indicated.

ISOLATION AND ANALYSIS OF MYCOLIC ACIDS BY HPLC

In this protocol, the mycolic acids are saponified using a methanolic solution of potassium hydroxide. After acidification, the free mycolic acids are extracted with chloroform and then derivatized to UV-absorbing esters to be detected by the UV detector of an HPLC setup. This technique has been used to analyze mycolic acid profiles from numerous *Mycobacterium* species (see Internet Resources).

As in Basic Protocol 1, this protocol also extracts and derivatizes shorter-chain fatty acids (C_{14} to C_{26}), which elute in the first 5 min of the HPLC run. These shorter-chain fatty acids can be separated by HPLC using the same reversed-phase C_{18} column with the following settings: column temperature at 45°C, flow rate at 2 ml/min, and wavelength at 260 nm. The mobile phase is 83:17 (v/v) acetonitrile/water used as an isocratic elution for 20 min, followed by a linear increase to 100% acetonitrile in 2 min, and held at 100% acetonitrile for 18 min (Zimhony et al., 2000).

Materials

M. tuberculosis (*UNIT 10A.3*)
Vesphene
50% (w/v) aqueous potassium hydroxide solution (9 N)
Methanol (HPLC-grade)
6 N aqueous hydrochloric acid solution
Chloroform (HPLC-grade)
Anhydrous sodium sulfate
Nitrogen gas
p-bromophenacyl ester derivatization kit (Alltech Associates)
Potassium bicarbonate
2-Propanol (HPLC-grade)

490-cm^2 sterile roller bottle
Pyrex glass tube (16-mm o.d. × 150-mm length) with Teflon-lined screw caps
50°, 85°, and 95°C heating blocks
Conical funnel
2-ml screw-thread vial with cap
Reversed-phase C_{18} column (4.6 × 150–mm, 3-μm particle size)

Culture M. tuberculosis

1. Grow a 30-ml culture of *M. tuberculosis* (*UNIT 10A.3*) to an optical density of ~1 in a 490-cm^2 sterile roller bottle.
2. Centrifuge 10 min at 2000 × *g*, 20°C. Discard the supernatant in a waste bottle containing Vesphene.
3. Resuspend the cell pellet in 10 ml distilled water. Centrifuge 10 min at 2000 × *g*, room temperature, and discard the supernatant in a waste bottle containing Vesphene.

Perform saponification

4. Resuspend the cell pellet in 2 ml of 50% aqueous potassium hydroxide solution. Transfer into a Pyrex glass tube with Teflon-lined screw cap. Add 2 ml methanol to the tube.
5. Close the cap and heat 16 hr in a 95°C heating block.

Make sure that only the part of the Pyrex glass tube containing the solution is in the heating block and that at least the upper half of the glass tube is outside of the heating block. This allows for cooling and condensation of any vapor coming from the saponification mixture and prevents possible leakage.

6. Let the tube cool to room temperature before opening.

   At this point, the tube can be washed with Vesphene and removed from the BSL-3 facility according to standard procedures. The remainder of the protocol can be done in the chemical fume hood of a BSL-2 laboratory.

Perform extraction

7. Slowly, add 6 N aqueous hydrochloric acid solution until the pH is below 3 (~3 to 4 ml), checking the pH with pH indicator paper.
8. Add 2 ml chloroform and carefully vortex.
9. Allow the phases to separate by gravity. If phase separation does not occur in 5 min, the sample can be centrifuged 5 min at 1000 × *g*, 20°C.
10. Using a Pasteur pipet, transfer the lower organic phase into a new Pyrex glass tube and set aside. Extract the aqueous phase a second time with chloroform, repeating steps 8 and 9. Combine the organic phases.
11. Remove any residual water in the organic phase by adding anhydrous sodium sulfate (~3 g). Vortex and allow it to stand for 5 min.

    The sodium sulfate will clump while being hydrated.

12. Filter the suspension through a piece of fluted filter paper in a conical funnel to remove the sodium sulfate clumps and transfer the organic phase into a new Pyrex Glass tube.

    Alternatively, filter the sample using a cotton-plugged Pasteur pipet. Push the cotton to the tapered part of the pipet with another pipet and use it as a disposable filtering funnel.

13. Evaporate the organic phase to dryness under nitrogen by placing the tube in a 50°C heating block and direct a gentle stream of nitrogen gas into the tube (10 to 15 min is usually necessary to obtain a dry sample).

Derivatize for HPLC analysis

14. Derivatize the mycolic acids to UV-absorbing *p*-bromophenacyl esters using the Alltech derivatization kit as follows:

    a. To the residue, add 0.5 ml of dicyclohexyl-18-crown-6 (Alltech kit's ampule B), 40 mg of potassium bicarbonate, and 0.5 ml of *p*-bromophenacyl bromide (Alltech kit's ampule A).
    b. If needed, add 0.5 ml chloroform to the tube to help dissolve the residue.
    c. Close the cap, vortex, and heat 30 min at 85°C.
    d. Cool the tube to 50°C.

15. Filter the suspension and transfer the solution into a 2-ml screw-thread vial.

    The sample is ready for HPLC analysis.

Analyze by HPLC

16. Using a reversed-phase C_{18} column, set the flow rate at 1.5 ml/min, and the wavelength at 260 nm.
17. Equilibrate the column with 60:40 (v/v) methanol/2-propanol using isocratic elution.
18. Inject the sample.

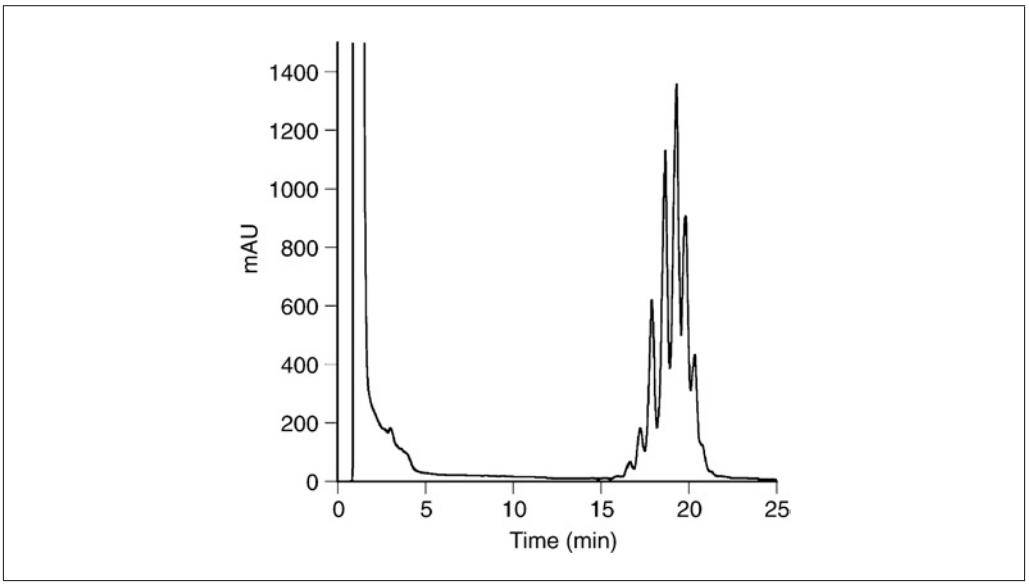

Figure 10A.3.4 HPLC chromatogram of mycolic acids isolated from *M. tuberculosis* H37Rv. The mycolic acid species elute between 15 and 25 min.

19. Elute isocratically in 60:40 (v/v) methanol/2-propanol for 3 min followed by a linear increase to 6:94 (v/v) methanol/2-propanol over 18 min. Elute isocratically with 30:70 (v/v) methanol/2-propanol for 4 min.

    The mycolates will elute between 15 and 22 min. In the first 5 min of the elution, the byproducts of the derivatization reaction and the shorter-chain fatty acids are present in the eluate (see Fig. 10A.3.4).

ANALYSIS OF MYCOLIC ACIDS BY HPLC USING A CHLORINATED SOLVENT ELUTION SYSTEM

ALTERNATE PROTOCOL 2

Butler and Guthertz (2001) have published a general method for isolation and analysis of mycolic acids by HPLC. The advantage of their method is time: their saponification requires only 1 hr (versus overnight) and the HPLC analysis is quicker with mycolic acids eluting between 7 and 9 min (versus 15 to 25 min). The disadvantages are: (1) the HPLC elution system uses a chlorinated solvent (methylene chloride) and, therefore, the seals of the HPLC pump and the HPLC tubing must be resistant to chlorinated solvents, (2) autoclaving tubes containing organic solvents (step 5) may not be authorized in some institutions for safety purposes, and (3) a quicker elution of mycolic acids might result in a poorer resolution of peaks and compromise the HPLC mycolic acid analysis. Follow Basic Protocol 2 with alterations in the following steps.

5. Close the cap and autoclave 1 hr at 121°C.

   Autoclaving glass tubes containing organic solvents may not be authorized in some institutions.

Perform elution

The HPLC elution system uses methanol and methylene chloride instead of methanol and 2-propanol as mobile phase and proceeds as follows.

17. Set the flow rate at 1.5 ml/min and equilibrate the column with 98:2 (v/v) methanol/methylene chloride using isocratic elution.

18. Inject the sample.

19. Elute isocratically by increasing linearly the mobile phase to 80:20 (v/v) methanol/methylene chloride for 1 min followed by a second linear increase to 35:65 (v/v) methanol/methylene chloride for 9 min. Elute isocratically for 2 min using 98:2 (v/v) methanol/methylene chloride.

COMMENTARY

Background Information

Mycobacterial mycolic acids are key constituents of the mycobacterial cell wall. They play an important role in maintaining the structure and permeability of the cell wall and thus preventing the entry of many antibiotics. Changes in mycolic acid structures have been associated with variation in cell wall permeability and attenuation of pathogenic mycobacterial strains. Mycolic acids are synthesized by the combined action of a type I fatty acid synthase (FASI) and a type II fatty acid synthase (FASII). FASII elongates the short-chain fatty acids arising from FASI into meromycolates (C_{56}). Mycolic acids are then formed by a claisen condensation between the meromycolates and the major FASI end-product (C_{26}). Takayama et al. (2005) have recently published a review on the biosynthesis of mycolic acids and their processing in *M. tuberculosis* and presented an assessment of all previously published data.

The protocols in this unit describe the extraction of *M. tuberculosis* mycolic acids from the cell wall and their analysis by TLC or HPLC. These analyses can be used to study mycolic acid biosynthesis and detect unusual mycolic acids accumulating due to mutations or environmental conditions. Isolation and quantification of individual mycolic acid species is readily done by HPLC.

A 10-ml *M. tuberculosis* culture does not form enough mycolic acids to be easily visualized on TLC. Therefore, the first step in the TLC protocol is labeling with [1-^{14}C]-acetate, which allows for the visualization of mycolic acids by autoradiography. After labeling, the mycolic acids are saponified and then methylated using methyl iodide. Other groups have used diazomethane for the methylation reaction but due to the highly explosive character of diazomethane, this is not recommended. The recommended procedure for TLC analysis of MAMEs is to use 95:5 (v/v) hexane/ethyl acetate as the elution solvent. The use of low-boiling-point solvents such as acetone or diethyl ether in chromatographic systems should also be avoided, as these solvents are too volatile to ensure a constant ratio between elution solvents.

Critical Parameters and Troubleshooting

If the autoradiogram or the HPLC chromatogram does not show any mycolic acid signal or very faint signals, several points should be assessed, such as the state of the cells before labeling (for the TLC protocol), the efficiency of the saponification, and the purity of the chemical reagents.

For TLC analysis, the cells should be actively growing (OD_{600} = 0.4 to 0.8) during the labeling procedure. Cells that are in the stationary phase should be avoided, as they may not incorporate acetate as efficiently as actively replicating cells. A culture with an optical density <0.4 will yield small amounts of labeled mycolic acids. Therefore, it will be necessary to reduce the volume of solvent used to resuspend the dry residue after extraction (see Basic Protocol 1, step 13) to ≤100 µl.

The saponification step in both protocols is important as it will determine the amount of mycolic acids that will be extracted. This step is also necessary for the killing of *M. tuberculosis*, which allows the sample to be removed from the BSL-3 facility. The incubation time of this step should not be reduced.

To ensure a good transfer of the derivatized mycolic acids into the organic phase during the extraction, vortex the biphasic solution vigorously.

Anticipated Results

The autoradiogram shown in Figure 10A.3.2 was obtained from a 10-ml *M. tuberculosis* H37Rv culture labeled when the culture had reached an OD_{600} of 0.47. Labeling lasted 20 hr followed by a 20-hr alkaline treatment. MAMEs were resuspended in 200 µl of methylene chloride and 5 µl of the solution (6000 cpm) were spotted onto the TLC plate. The autoradiogram was obtained after exposure for 36 hr at –80°C. Figure 10A.3.4 shows the HPLC chromatograph of the mycolic acids from *M. tuberculosis* H37Rv. Chloroform was added to the derivatization mixture and 95 µl of the final solution was injected onto the HPLC reversed-phase C_{18} column (4.6 × 150–mm, 3-µm particle size).

Time Considerations

Once the culture is ready (3 to 7 days of culturing, depending on the inoculum size), the TLC protocol takes ∼3.5 days: 20 hr for the labeling, 20 hr for the alkaline treatment, and 2 to 3 hr for the methylation and extraction. At this point, the dried sample can be stored in a −20°C freezer. The three elutions of the TLC plate take between 30 and 45 min, and then the film is exposed for 24 to 36 hr. The HPLC protocol takes ∼1 day, the longest step being the saponification. The protocol can be stopped after the drying step (step 13) and the dried sample can be stored in a −20°C freezer. The *p*-bromophenacyl mycolic acid esters in solution in acetonitrile (step 15) can be stored at 4°C.

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Internet Resources

http://www.cdc.gov/nchstp/tb/Laboratory_Services/maps_tagged.pdf

Mycolic acid pattern standards for HPLC identification of mycobacteria.

Contributed by Catherine Vilchèze and William R. Jacobs

Albert Einstein College of Medicine

Bronx, New York

Analyses of *Mycobacterium tuberculosis* Proteins

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ABSTRACT

This unit includes protocols for the isolation of proteins from *Mycobacterium tuberculosis*. Considerations for working with *M. tuberculosis* at Biosafety Level 3 containment are also discussed. *Curr. Protoc. Microbiol.* 6:10A.4.1-10A.4.5. © 2007 by John Wiley & Sons, Inc.

Keywords: tuberculosis • TB • protein • culture filtrate • Sauton

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), infects almost one third of the world's population. Yearly, there are an estimated 10 million new cases of TB resulting in 2 to 3 million deaths. Over the last 15 years, the development of genetic tools to manipulate *M. tuberculosis* has proliferated (*UNIT 10A.2*). Furthermore, availability of the complete *M. tuberculosis* DNA genome sequence (as well as other related mycobacteria) has yielded tremendous information towards a better understanding of the bacterium and the disease it causes.

The protocols in this unit will detail bacterial protein extraction and culture filtrate preparation. These proteins may then be analyzed using standard methods such as SDS-PAGE or 2-D electrophoresis. See Braunstein et al., 2003, and Kriakov et al., 2003, for examples of *M. tuberculosis* protein analyses.

CAUTION: *Mycobacterium tuberculosis* is a Biosafety Level 3 (BSL-3) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See Strategic Planning, Safety Considerations for further discussion. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for additional information.

SAFETY CONSIDERATIONS

Standards of BSL-3 containment should be followed for work with *M. tuberculosis*. Consult with the Safety Officer and/or Institutional Biosafety Committee for additional guidance. See *http://www.cdc.gov/OD/ohs/symp5/jyrtext.htm* and *UNIT 1A.1* for a brief description of general BSL-3 laboratory features. Complete inactivation of *M. tuberculosis* cells is necessary to perform some studies outside the BSL-3 laboratory. All methods for inactivation should be validated in individual laboratories, taking into consideration the concentration of cells used. Schwebach et al. (2001) provides some guidance on determining if particular conditions are adequate for inactivation of mycobacteria. For many of the procedures, the authors have found the following conditions sufficient for inactivation of *M. tuberculosis*: incubation for 12 hr at 65°C, 2 hr at 80°C, or 15 min at 95°C; or incubation for 1 hr in a final concentration of one of the following solutions: 2.5% glutaraldehyde, 2% paraformaldehyde, 5% formalin, or Vesphene IIse. During heat inactivation, care should be taken to ensure that the entire tube is immersed in a water bath preheated to the appropriate temperature. Incubation of *M. tuberculosis* cultures with gluteraldehyde, paraformaldehyde, formalin, or Vesphene IIse should be done in a

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matter that ensures that the entire interior surface of the tube has been coated by gently rolling and inverting the tube.

BASIC PROTOCOL 1

EXTRACTION OF *M. TUBERCULOSIS* PROTEINS USING THE GLASS BEAD METHOD

For preparation of protein extracts, the glass bead method is preferred. Some researchers suggest more than one wash step (step 1) to remove any residual albumin from Middlebrook broth while other researchers prefer to avoid any albumin contamination by growing cultures in Sauton's medium (see *UNIT 10A.1*). Glass beads are necessary to lyse the cells as the large amount of lipids retained by mycobacteria make boiling alone an inefficient method for protein extraction. Protein extracts can then be used in SDS-PAGE or 2-D protein gel electrophoresis for further analysis.

Materials

Mycobacteria (*UNIT 10A.1*)
Phosphate buffered saline (PBS; *APPENDIX 2A*)
Protein extraction buffer (see recipe), ice cold
2× SDS sample buffer (*APPENDIX 2A*)
15-ml screw-cap conical tubes

Refrigerated tabletop centrifuge
Vortex and tube adaptor
2-ml screw-cap tubes (Sarstedt)
Microcentrifuge
Glass beads (106-μm, Sigma cat. no. G4649)
95°C water bath

1. Wash cells two times with an equal volume of PBS to remove BSA that might interfere with resolution of mycobacterial proteins by SDS-PAGE.
2. Centrifuge culture 10 min at 2000 × *g*, 4°C, in 15-ml screw-cap conical tubes.
3. Discard supernatant and add 10 ml PBS to cell pellet. Vortex to resuspend cell pellet then centrifuge 10 min at 2000 × *g*, 4°C.
4. Discard supernatant and resuspend cell pellet in 1.5 ml PBS. Vortex to resuspend cell pellet.
5. Transfer cell suspension to a 2-ml Sarstedt screw-cap tube. Microcentrifuge 5 min at 15,800 × *g*, 4°C.
6. Discard supernatant and resuspend cell pellet in 300 to 400 μl of ice-cold protein extraction buffer.
7. Add 0.4 g of 106-μm glass beads.
8. Vortex 5 min at top speed using an adaptor to hold the tubes.
9. Microcentrifuge 5 min at 15,800 × *g*, 4°C, to pellet glass beads and debris at the bottom of the tube.
10. Transfer supernatant to a fresh 2-ml Sarstedt screw-cap tube.
11. Add an equal volume of 2× SDS sample buffer.
12. Vortex briefly to mix sample, then incubate 5 min in a 95°C water bath.
13. Store sample up to 2 weeks at –70°C; thaw at 2°C (~10 min) just prior to use.
14. Perform SDS-PAGE (CPMB *UNIT 10A.2*).

PREPARATION OF CULTURE FILTRATES

M. tuberculosis secretes a number of proteins of immunological interest. For preparation of culture filtrates, it is essential that cultures be grown in Sauton medium, which does not have the albumin enrichment that is found in Middlebrook medium (refer to *UNIT 10A.1*). Cultures will take longer to grow in Sauton medium (both initial growth and overall doubling time) compared to growth in Middlebrook 7H9 medium. Culture filtrates can then be used in SDS-PAGE or 2-D protein gel electrophoresis for further analysis.

Materials

Bacterial culture in Sauton medium (*UNIT 10A.1*)
50-ml conical tubes
Sterile cell scraper
Refrigerated tabletop centrifuge
Filter units (0.45- and 0.2-μm)
Cell concentrator (Amicon Ultrafiltration unit or equivalent)

1. Grow 100 to 200 ml bacterial culture in Sauton medium.
2. Transfer culture to 50-ml conical tubes.

   Bacteria will be difficult to manipulate because of a lack of albumin and detergent in the medium; the culture will grow as a pellicle on the surface of the medium and will stick to pipets. For most analysis, it is useful to compare the supernatant and pellet; therefore, it is important to collect as much of both components as possible. Decanting the culture directly to 50-ml conical tubes and using a sterile cell scraper will help in increasing the amount of material recovered.

3. Pellet bacteria by centrifuging 20 min at 2000 × *g*, 4°C.
4. Transfer supernatant to a fresh tube and centrifuge again.
5. Filter supernatant through a 0.2-μm filter unit, then through another 0.2-μm filter unit.

   For BSL-3 organisms, dip closed tube in a disinfectant such as Vesphene II to disinfect the outer surface of the tube. From this point on, the filtrate can be processed in a BSL2 laboratory.

6. In the BSL-2 laboratory, concentrate supernatant 100× in a cell concentrator with appropriate cutoff filter.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Protein extraction buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
5 mM EDTA
0.6% SDS
10 mM $NaPO_4$
1.1 mg/liter pepstatin (Roche, cat. no. 10253286)

Add 50× proteinase inhibitor cocktail (PIC) to protein extraction buffer (see recipe) for a final 1× concentration (i.e., 2 μl PIC to 98 μl protein extraction buffer).

The protein extraction buffer without PIC can be stored for 1 year at room temperature. The protein extraction buffer with PIC must be used on the same day and cannot be stored for later use.

Proteinase inhibitor cocktail (PIC), 50×

Dissolve one Complete Proteinase Inhibitor Cocktail tablet (Roche cat. no. 11836145001) in 1 ml dH_2O. Store up to 3 months at –20°C.

COMMENTARY

Background Information

Tuberculosis (TB) is a disease caused by *M. tuberculosis*. *M. tuberculosis* is spread via the aerosol route and predominately causes disease in lung tissue, although it can spread to other organs. It is estimated that nearly one-third of the world's population is infected with TB, many of those are cases of latent infection. Upon waning of the immune system through aging or infection with HIV, TB can reactivate from its latent form, even after decades of quiescence. Antibiotic therapy is available for the treatment of TB, although due to the slow-growth of the organism, long courses of treatment (6 months to 1 year) are recommended.

Critical Parameters and Troubleshooting

One of the most important aspects of working with *M. tuberculosis* is to minimize any possible aerosol exposure. Second to important safety considerations, scrupulous attention to detail in preparing medium and reagents is crucial. Given the long doubling time (18 to 20 hr, longer for some mutant strains) of the organism, care should be taken to minimize any possible sources of contamination of medium.

Extraction of proteins

Contaminating albumin from the medium is one of the major obstacles to recovering protein samples. If albumin contamination is a problem, increase the number of washes with PBS. Furthermore, the time and speed that the samples are processed in step 8 can be adjusted. If extended times are used, a 5-min rest period with incubation on ice is recommended.

Preparation of culture filtrates

Care must be taken to use a non-albumin-containing medium such as Sauton. The bulk of the cells will grow as a sticky lawn on the surface of the medium. For many applications, it is important to compare the proteins in the cell bulk to the proteins in the culture filtrate. For this reason, as much of the cells should be recovered and saved for potential analysis.

Anticipated Results

Extraction of proteins

Yield can be altered by increasing the amount of starting culture and/or adjusting the

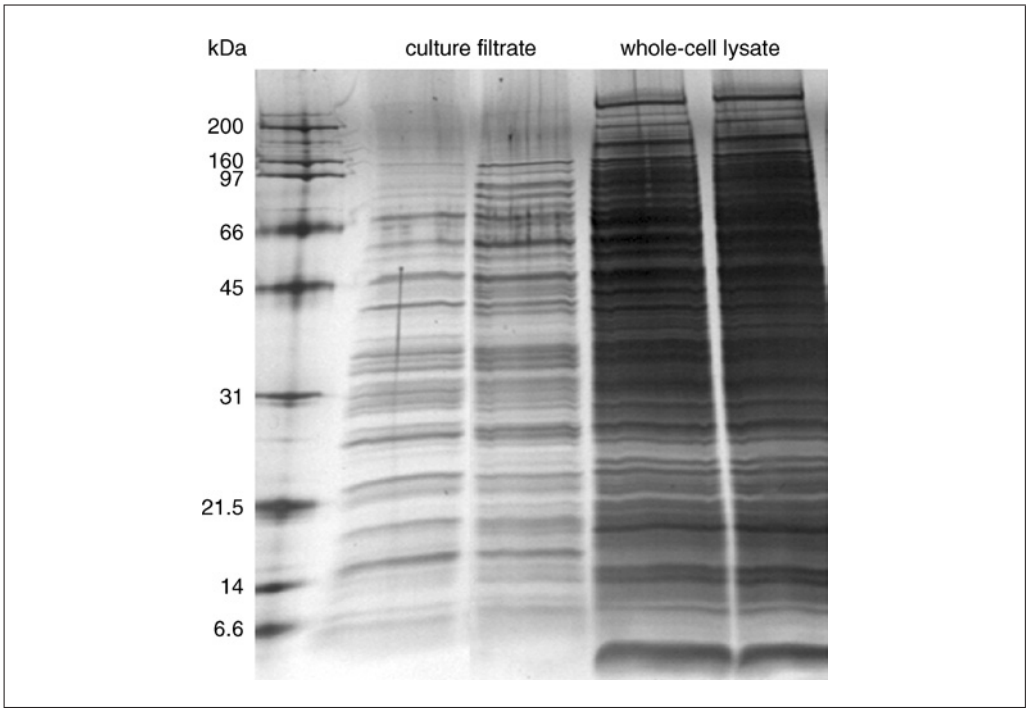

Figure 10A.4.1 SDS-PAGE gel. Typical distribution of *M. tuberculosis* proteins from culture filtrates and whole-cell lysates. Cells were grown to an OD_{600} of 0.4 in Sauton medium without detergent. Proteins were extracted following Basic Protocols 1 and 2.

conditions for cell lysis. A Bradford assay is useful for determining the protein concentration for each sample.

Figure 10A.4.1 shows a protein gel of culture filtrate proteins isolated from *M. tuberculosis* grown on either Middlebrook or Sauton medium.

Time Considerations

Extraction of proteins

The protocol takes ~2 hr.

Preparation of culture filtrates

Centrifugation and filtering of the culture filtrate take <1 hr. Depending on the cutoff filter used, application of the supernatant to the cell concentrator can take 2 to 8 hr.

Acknowledgments

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Animal Models of *M. tuberculosis* Infection

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ABSTRACT

Animal models of tuberculosis infection continue to provide useful information about the nature of the disease process, including specific information about the immune response to the infection and the disease pathology. In addition, standardized animal models are now used extensively to test the capacity of new vaccines to inhibit the course of the infection, as well as test the capacity of new drugs to sterilize the infection. This unit describes some basic protocols illustrating established protocols for infecting animals with tuberculosis, subsequent processes for analysis, and various aspects of biosafety that must be observed. Using these protocols the course of infection, the basic immune response, and the extent of lung pathology can be determined in mouse and guinea pig models of experimental tuberculosis. *Curr. Protoc. Microbiol.* 7:10A.5.1-10A.5.29.

Keywords: mouse • guinea pig • tuberculosis • animal models • immune response • pathology

INTRODUCTION

The past decade has seen an explosion of knowledge regarding the immunological and pathological basis of the host response to *Mycobacterium tuberculosis*, much of it coming from the mouse model and, to a lesser extent, from the guinea pig model. As more and more laboratories have acquired aerosol generation devices, studies have shifted away from intravenous and other routes of infection towards the much more realistic low-dose aerosol infection model.

The mouse remains the chosen model for basic immunological investigations because of the wealth of available reagents and, over the past two decades, much has been learned about the T cell response, including its various subsets, soluble mediators such as cytokines and chemokines, signaling pathways, and basic pathology, as well as a growing appreciation of the layers of innate immunity that comprise the earliest responses in the mouse lung. In contrast, far fewer immunological reagents are available for the guinea pig, although this is now changing. This animal model remains an important step for new vaccine testing, reflecting similarities in the pathology of the disease process to that seen in humans.

Whereas the early literature focused on changes in the bacterial load in the two models after various manipulations, the focus of present day has been pathology of tissues, information about RNA message, or data obtained by flow cytometry, and so forth. As a result, this unit includes some basic protocols describing how to prepare lungs and other tissues for such analyses.

SAFETY CONSIDERATIONS

The basis of safety in a laboratory is facility design, fail-safes, precise training, and an exposure control plan. Biosafety Level III laboratories have become more commonplace,

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driven by the current interest in biodefense. These laboratories vary between laboratories working with small inocula in which *M. tuberculosis* can be safely handled in appropriate Class II biosafety cabinets located within restricted access areas, and laboratories working with large numbers of animals infected with this organism.

Biosafety Level III laboratories by definition have defined air handling systems that remove air under negative pressure many times per minute. These can be solid structures or movable (e.g., biobubbles within a larger room) and operate best as stand-alone buildings. This is not always possible, and where Biosafety Level III laboratories arise from the renovation of regular laboratories, great care must be taken in terms of the design, air flow, access, adjacent traffic, and so forth. At a minimum, tandem HEPA filter systems are required, and these should be checked on a regular basis. Renovating laboratories to become Biosafety Level III laboratories where there are regular laboratories on the floors above is not recommended.

Fail-safes refer to levels of safety. For example, a technician loading a nebulizer of an aerosol chamber should be wearing an OSHA-approved mask with a respirator (e.g., powered air purifying respirator, PAPR) on top. The technician may be in a biobubble surrounding the generator, but should be in a room that has the greatest negative air pressure in relation to the Biosafety Level III facility as a whole. The technician may work with a colleague to check procedures and to make sure that there are no distractions. After completing the aerosol run and decontaminating equipment, the technician should shower out of the facility, leaving all protective clothing (e.g., surgical scrubs, etc.) behind for autoclaving. The technician should undergo extensive training before directly working with *M. tuberculosis*, including knowledge of required actions to take should anything go wrong while in the Biosafety Level III area.

The central figure in any institution is the Biosafety Officer. This individual should review procedures on a regular basis and pre-approve the agents used. Clinical samples of *M. tuberculosis*, which may include drug-resistant isolates, should be listed by the Biosafety Officer, and the laboratory responsible should know their location (usually in a -70°C freezer) and the number of vials present at any time.

Official guidelines for the United States are readily available from the National Institutes of Health, from the Centers for Disease Control (which now regard drug-resistant *M. tuberculosis* as a Class C Select Agent), and from the Occupational Safety and Health Organization. Other national and international agencies have guidelines that cover other countries (refer to *APPENDIX 1B*). These organizations cannot be responsible for individual laboratory conduct, therefore, it is the responsibility of the faculty and staff that work each day in the Biosafety Level III laboratories to establish their own guidelines and strictly adhere to them.

IMPORTANT NOTE: Refer to Section 1A for more information on laboratory safety issues.

BASIC PROTOCOL 1

AEROSOL INFECTION OF MICE USING THE MIDDLEBROOK APPARATUS

To establish an animal model of pulmonary tuberculosis to resemble the normal route and site of infection in humans, it is necessary to use an aerosol generation device. A few are available, but the most widely used is the Middlebrook Airborne Infection apparatus, which has been used for this specific purpose for the past 30 years (Fig. 10A.5.1). This instrument is currently manufactured by Glas-Col and has changed very little in configuration from its original design. The instrument basically consists of a large circular tank (aerosol chamber) that contains a circular basket/cage with five

Figure 10A.5.1 Glas-Col aerosol device. This instrument is based upon earlier models originally developed by Middlebrook. The central gasket contains a large steel cage in which up to 80 to 100 mice can be exposed to aerosol at any one time. The device attached on the right is a heat sterilizer to incinerate any bacteria escaping the internal HEPA-filter systems.

pie-shaped compartments for the animals. Each of the compartments can accommodate as many as 25 mice. The aerosol chamber has a heavy acrylic lid with two locking handles that lock the lid tightly against a heavy-duty rubber gasket. The lid also has two ultraviolet lamps on its underside and these lamps come on during the decontamination cycle of instrument operation. The front of the instrument consists of a control panel with four timers for the various cycles of operation; two air flow meters, two air control knobs, as well as an on/off switch for the exhaust air incinerator. On the front of the instrument, there are three hoses with clamps used to attach the Venturi nebulizer. When the instrument is in operation, compressed air flows through the nebulizer and produces a very fine mist of the bacterial suspension, which is then carried by a larger volume of air flowing into the aerosol chamber. This fine mist is then inhaled by the animals within the basket. It then exits the chamber to flow through two HEPA filters and then a super-heated exhaust stack where any residual particles are incinerated. After the nebulization procedure, the machine runs a "cloud decay" process in which the aerosol chamber is purged with fresh air. This may also help in drying any bacilli attached to the fur of the animal. A final step is an "ultraviolet cycle" in which the UV lamps come on and decontaminate the top surfaces of the basket.

Materials

Suspension of bacteria in 5-ml syringe with 18-G needle
Mice
5% Lysol wash bottle
70% ethanol

Airborne infection apparatus (Glas-Col)
Venturi nebulizer (Glas-Col)
Large and extra-large autoclave bags
Autoclave tape

Prepare apparatus and load animals

1. Ensure that the aerosol basket in the airborne infection apparatus has been cleaned and is ready for use.

2. Place an In Use light switch just outside the aerosol room and turn it on.
3. Fill the nebulizer with the bacterial suspension.
4. Secure nebulizer to airborne infection apparatus by clamping to inlet ports. Ensure that the nebulizer is secure to avoid the possibility of the aerosol being released into the room.
5. Place aerosol basket stand and basket into airborne infection apparatus.
6. Load animals into basket and secure lid. If loading different groups of mice, mark the tail of each mouse with an indelible pen designating group name. Place no more than 25 mice into each chamber of the basket.

Run apparatus

7. Turn Power switch on airborne infection apparatus to On position.
8. When LED screen reads Glas-Col Apparatus Co., turn on UV Light switch and Program switch.
9. When LED screen reads Enter When Ready, press Enter.
10. When LED screen reads Enter Preheat Time. . . 900, press Enter to accept.
11. When LED screen reads Enter NEB Time. . . 1800, change value to 2400 and press Enter.
12. When LED screen reads Enter CD Time. . . 1800, change value to 2400 and press Enter.
13. When LED screen reads Enter DEC Time. . . 900, press Enter to accept, LED screen will read Preheat Cycle In Process.

    The right-hand (compressor) gauge should read 40-60 psi; if it does not, adjust upward with corresponding control beneath.

14. Leave the room while the instrument is running.

End program and clean apparatus

15. After the cycle is finished (alarm will sound and UV lights will go off), re-enter the room. Inspect the nebulizer to make sure the fluid has nebulized correctly.
16. Turn off all switches and open airborne infection apparatus.
17. Remove the nebulizer and place in a metal dish containing 5% Lysol. Seal with autoclave tape and mark as “fragile” (nebulizers are easily damaged and expensive to replace).
18. Wipe down the apparatus with Lysol.
19. Remove animals from the apparatus basket. Place the basket in a large autoclave bag and seal with autoclave tape. Place this in a second autoclave bag for further safety.
20. Sweep up and dispose of any debris from animal cages, both inside the infection apparatus and on the floor. Avoid sweeping any debris into the aerosol port on the bottom of the instrument.
21. Wipe the inside of the instrument with Lysol, followed by 70% ethanol.
22. Turn off In Use light switch.

AEROSOL INFECTION OF GUINEA PIGS USING A MADISON CHAMBER

Because of their size, guinea pigs need a larger device for efficient aerosol infection exposure. An exposure chamber developed and designed by engineers at the University of Wisconsin at Madison has proven to be highly effective, and much of the classical literature on the guinea pig model arose from the use of this device.

Materials

5% Lysol wash bottle
70% ethanol wash bottle
M. tuberculosis H37Rv stock
Guinea pigs

Biosafety Class II cabinet
50-ml conical tubes
1- and 20-ml syringes
18- and 26-G needles
Madison infection chamber
Autoclaved glass nebulizer jars
Guinea pig cages
Stainless-steel container(s)
Absorbent bench-top paper
Disposable surgical gown
Infection basket(s)
5-ml tubes (Falcon)
Powered air purifying respirator (PAPR)
Large/extra-large biohazard bags
Autoclave tape

Prepare work area and run test

1. Sterilize the surfaces of the Biosafety Class II cabinet with paper towels wet with 5% Lysol, followed by wiping the surfaces two times with 70% ethanol.
2. Transport all materials from airlock into the barrier, and take the *M. tuberculosis* stock out of the dry ice–containing ice chest and place into a 50-ml conical tube, and begin thawing the stock inside the biosafety cabinet.
3. Load 15 ml of sterile distilled water into a 20-ml syringe affixed to an 18-G needle (this will be used for the test run).
4. Begin the test run in the Madison infection chamber (15-min cycle) to ensure that the pressure driving the vacuum is adequate.

   a. Dispense the 15 ml of sterile distilled water into the autoclaved glass nebulizer jar by removing the plug screw on the Collison nebulizer (Fig. 10A.5.2), unsheathing the needle, and placing the needle tip in through the orifice to rest against the side of the jar (so as not to create any aerosols when dispensing).
   b. After dispensing the distilled water into the jar, refasten the plug screw on the nebulizer.
   c. *For the test run only*, unlatch the Control Box lid and check that the time delay relay, TD1, is set to 900 sec/On Delay, and that the second time delay, TD2, is set to 300 sec/Interval Delay.
   d. Close/latch the Control Box lid, and supply power to the chamber system, which powers the compressor/vacuum pump unit.

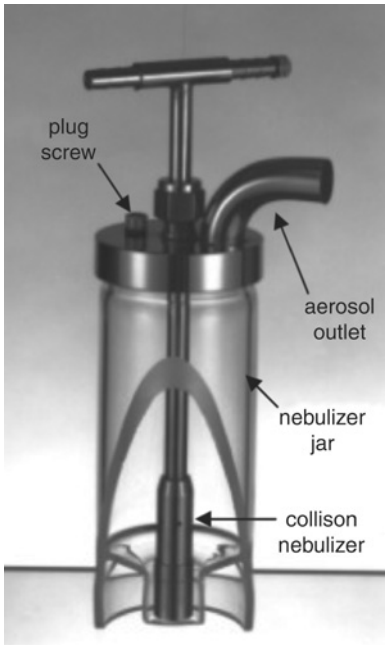

Figure 10A.5.2 Collison nebulizer jar for the Madison guinea pig aerosol chamber.

Figure 10A.5.3 Photohelic meter for the Madison chamber.

e. Check for the proper amount of airflow into the system (secondary air, 40 to 45 liters/min) indicated on the flow meter on the right side of the flow panel. Also, be sure to check the Photohelic unit that the proper amount of vacuum is being maintained (consistently 9 to 10 in. water; Fig. 10A.5.3).

5. Proceed with the test run. Observe the flow pressures on the Control Box (Fig. 10A.5.4).

   a. Turn on the Control Box Power switch by turning clockwise.

   b. Depress the Nebulizer Test button to check for the proper airflow into the nebulizer (~3.5 to 4 liters/min) as indicated on the flow meter at left on flow panel.

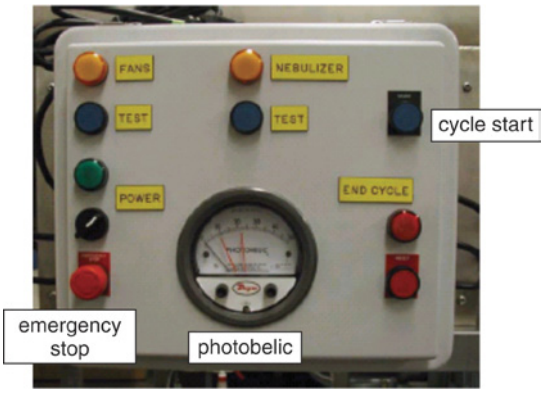

Figure 10A.5.4 Control panel for the Madison chamber.

c. Unlatch the chamber door (eight latches around the perimeter) and open the door to visualize the operation of the mixing fans—two at the rear and two at the front of the chamber.

d. Test the mixing fan(s) operation by depressing the Fan Test button on the Control Box.

e. If everything is operational, reseal the airtight door with all eight latches.

f. Initiate the test run by depressing the Cycle Start button on the Control Box.

g. Log both the reading on the primary airflow meter into nebulizer (left side of flow panel) and the reading on the pressure gauge beneath the regulator into a log book.

h. Also, before leaving the room, be sure that the nebulizer is fully functional by checking for spray coming out of each of the three jets of the nebulizer probe.

i. Illuminate the In Use light.

Prepare guinea pig cages and M. tuberculosis

6. While waiting for the *M. tuberculosis* vial to thaw, label each of the corresponding guinea pig cage cards for the guinea pigs to be aerosolized by writing "aerosolized with ~20 cfu *M. tuberculosis* on date/initials" onto each card.

7. Place the stainless-steel container(s) (depending on how many aerosol runs are required) and fill with a 5% Lysol solution. Place stainless-steel container(s) next to the infection chamber.

8. After the *M. tuberculosis* vial has thawed, place some absorbent bench-top paper inside the biosafety cabinet, put on a disposable surgical gown, double gloves, and fill one 50-ml conical tube with the 5% Lysol solution (~25 ml).

   NOTE: *If two or more staff members are present, while one prepares the inoculum, the other staff member(s) can load the guinea pigs to be challenged into the infection basket. This can be performed for the first two aerosol runs inside the guinea pig housing rooms (since both infection baskets have been autoclaved).*

9. Spray the top of the *M. tuberculosis* vial with 70% ethanol.

Load bacteria

10. Fit a 1-ml syringe with a 26-G needle, insert needle into the *M. tuberculosis* vial, and invert vial.

11. Pull bacteria in and out of syringe at least ten times; do not allow frothing to occur.

12. Pull ~1 ml into the syringe, then, turn the bottle upright and pull ~100 µl of air into the syringe (so that the vial does not expel any liquid when needle is removed), and withdraw needle.
13. Slowly dispense the 1 ml of bacteria into a 5-ml tube so as not to create an aerosol.
14. In another 5-ml tube, perform the necessary dilution(s) from concentrated stock to arrive at the working stock concentration needed to place x ml into 16 ml – x ml of sterile distilled water such that the final concentration is 1×10^6 cfu/ml.
15. Put aside 1 ml of the stock solution for plating on agar to verify the inoculum concentration.
16. Transport the diluted inoculum in a 20-ml syringe to the aerosol chamber.
17. Put on the PAPR.

    When the End Cycle indicator sounds for the test run, press Reset on the Control Box and remove a sterile autoclaved nebulizer.

18. Carefully place the inoculum into the nebulizer and screw into place. Place the syringe into Lysol.

Infect guinea pigs

19. Load the guinea pigs into their individual holders in the chamber by aligning the cart to the opened chamber door, latching the lock-pin, and sliding the basket into the chamber.

    Be sure that the spacer is in place at the posterior (the spacer should be placed between the rear mixing fans and the infection basket of the chamber) before relatching the chamber door.

20. Relatch the door, and be sure that all the eight latches are fastened on the door so as to create the necessary airtight seal.
21. Recheck for the proper amount of airflow being channeled through the chamber (40 to 45 liters/min). Also, be sure to check that the vacuum gauge reads 9 to 10 in. water (after opening/closing the chamber door this may take 1 to 2 min).
22. Initiate the cycle by depressing the Start button, and set a timer for 15 min.
23. Be sure to check the three jets on the nebulizer probe for proper spray/dispersion before leaving the room.

Remove guinea pigs and clean up

24. Once the End Cycle indicator light/alarm sounds, depress the Reset button.
25. Open the chamber door and remove the infection basket.
26. Transfer each of the infected guinea pigs back into their cages.
27. Carefully unscrew the nebulizer jar from the apparatus, liberally wrap in paper towels, and place into the 5% Lysol solution inside the stainless-steel autoclavable container, and place the lid on the container.
28. Thoroughly, but gently, wipe the entire stainless-steel nebulizer probe with paper towels wet with 5% Lysol.

    Do not press the paper towels too hard over the nebulizer probe. There are minute holes that paper towel filaments can plug and, thus, block the nebulizer jets.

29. Gently clean the probe with a paper towel stack wet with 70% ethanol.

30. Pour ~20 ml water (from a sterile 50-ml conical tube) inside an additional autoclaved nebulizer jar and screw into the apparatus.
31. Check for the proper amount of airflow being channeled through the chamber (40 to 45 liters/min). Also, be sure to check that the vacuum gauge reads 9 to 10 in. water.
32. Initialize the Cleaning run by depressing the Start button on the Control box.
33. Following the Cleaning run, turn off all switches.
34. Replace the nebulizer jar with the final autoclaved nebulizer jar.
35. Place the "used" jar into 5% Lysol solution.
36. Close the lid to the container, place into a large biohazard bag, and mark "FRAGILE—glass nebulizer jars"
37. Remove the infection basket(s) from the cart(s), double bag with extra-large biohazard bags, and seal with autoclave tape.
38. Open the chamber door, and wipe inside of chamber apparatus with 5% Lysol; repeat process with 70% ethanol (while always wearing the PAPR).
39. Sweep up and dispose of any debris from animal cages, etc.
40. Autoclave the packed nebulizer/aerosol baskets.

Although these chambers are well made, resist the temptation to run an aerosol infection experiment when you first receive the instrument. Run water alone at least three or four times before you do anything else. Look carefully at the various joints for bubbling when run under pressure; some joints may need tightening. Only when you are sure the instrument is operating well should you then run the instrument containing mycobacteria.

PREPARING *M. TUBERCULOSIS* INOCULUM FOR AEROSOL EXPOSURE

BASIC PROTOCOL 3

To infect mice or guinea pigs by aerosol with *M. tuberculosis*, a preparation of bacteria from a stock of known cfu/ml is required. To avoid clumps of bacteria in the nebulizer, the bacilli are thawed, after which it is important to run the bacteria through a 1-ml tuberculin syringe fitted with a 26-G needle at least ten times. To minimize the possibility of aerosol from the needle, the suspension is slowly dispensed down the inside wall of a plastic tube. Then, dilute the inoculum in sterile distilled water to achieve the required cfu per ml. For low-dose challenge infections, the concentration of bacteria should be $\sim 2 \times 10^6$ cells/ml. If more than one round of aerosol exposures are run, keep the stock solution at 4°C, until the next dilution is made. Run the suspension through the syringe as above each time.

To verify the concentration of the inoculum used in the nebulizer, an aliquot of the mycobacterial suspension is diluted and plated on 7H11 agar (see *UNIT 10A.1*). The dilutions should be ten-fold serial dilutions, and are easily done using 24-well plates (100 μl of the bacterial suspension in 900 μl saline or PBS). A volume of 100 μl of each dilution (10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8) is plated onto quadrants of petri dishes containing 7H11 agar. Colonies are counted 3 weeks later to verify the inoculum size.

NOTE: Take great care when pipetting the serial dilutions to prevent aerosol generation. Any small spills or drops should be disinfected with 5% Lysol. All pipet tips used to serially dilute the mycobacteria are placed in a pipet boat containing 5% Lysol solution.

Materials

M. tuberculosis bacterial stock
5% Lysol solution
70% ethanol

50-ml conical tubes
Tube rack
Pipet boat, optional
Small biohazard bag
3- and 10-ml syringes
18- and 26 1/2–G needles

1. Retrieve bacterial stock from freezer and place into 50-ml conical tube, close tube, and write your initials. Transfer to biosafety cabinet, place tube into rack, and allow stock to thaw.
2. Fill one 50-ml conical tube (or fill pipet boat about half full) with 5% Lysol solution. Place a small biohazard bag inside of hood.
3. Remove aluminum seal from bacterial stock, and spray top of bacteria vial with 70% ethanol.
4. Fit a 3-ml syringe with a 26 1/2–G needle, insert needle into vial, and invert vial.
5. Pull bacteria in and out of syringe at least three times; do not allow frothing to occur.
6. Pull 1 ml of bacteria into syringe, revert tube, and pull 100 µl air into syringe (so that excess liquid does not come out when needle is removed), then withdraw needle.
7. Remove cap from diluent tube, move hand away from tube.
8. Place needle tip just below meniscus of diluent and slowly add bacteria. Avoid touching the side of the needle to the sides of the diluent tube.

   If required, make serial dilutions in additional conical tubes to reach desired dilution for inoculum.

9. Disinfect syringes/needles by drawing 1 ml of 5% Lysol from conical tube (or pipet boat), then place syringe into tube.
10. Using a 10-ml syringe fitted with an 18-G needle, pull 5 ml of inoculum into syringe. Pull 100 µl air into syringe so that inoculum does not drip when needle is removed and withdraw needle (this is the inoculum).
11. Change gloves before removing hands from hood. Gloves should be placed into biohazard bag inside of hood.
12. Upon initiating aerosol cycle, return to biosafety cabinet, place syringe(s) containing Lysol into a sharps container. Clean work area with 5% Lysol, then followed by 70% ethanol.

BASIC PROTOCOL 4

INTRAVENOUS INFECTION OF MICE WITH *M. TUBERCULOSIS*

Intravenous injection of the mouse is not by itself a difficult procedure, but it requires absolute concentration, no distractions, and good eyesight. The inexperienced investigator should perform several practice sessions just using saline before attempting to inject live bacteria. If at any time you do not feel confident to do this procedure, do not attempt it.

Materials

Bacterial suspension at desired concentration (see Basic Protocol 3)
Mice
70% ethanol

1-ml syringe with 25- to 30-G needle
Restraint device (e.g., Braintree Scientific)
Heat lamp
Gauze sponge or swab

IMPORTANT NOTE: Restraint devices vary and are often homemade; they are also available commercially from suppliers such as Braintree Scientific. Most are metal or plastic holders with an opening at one end to expose the tail of the mouse without harming the animal. The procedure must be done in a BSL-3 facility.

1. Fill a 1-ml syringe with the injectate and be careful to remove all air bubbles to avoid risk of air embolism.
2. Gently place the mouse in a restraint device so that the tail can be immobilized without the mouse being able to turn.
3. Warm the tail with a heat lamp by placing the lamp 6 to 9 in. away. Look for tail vein vasodilation, when adequate vasodilation is observed, move the lamp away. Do not expose the animal to any more heat than necessary.
4. Swab the tail with 70% ethanol.
5. Find the lateral vein (one on each side of the tail) and insert the needle parallel to the vein a few millimeters into the vessel. Keep the bevel of the needle facing up.
6. Inject slowly. No bleb should form if needle is properly located in the vein. If a bleb appears indicating failure to properly enter the vein, try again further up the tail.
7. Withdraw the needle and apply digital pressure if necessary to achieve hemostasis.

If you feel any resistance to the injection, stop because the vein was probably missed. Never force the injection, if forced, the mycobacteria will spray back at you. Always wear a plastic face protector in addition to a mask and usual protective clothing.

EUTHANASIA OF TEST ANIMALS

Details regarding euthanasia are described in CP Immunology, *UNIT 1.8*; Care and Handling of Laboratory Animals.

Euthanasia methods must be humane, painless, and quick. Requirements and procedures approved by Institutional Animal Care and Usage Committees can differ quite widely. For many years, cervical dislocation was a common method, but is now more likely to follow some form of anesthesia. Some IACUCs also recommend subsequent decapitation, although this may not be a method of choice for some. The authors recommend CO_2 asphyxiation, which is quick and totally effective. Cervical dislocation is avoided primarily because this introduces large amounts of blood into the upper thorax, potentially confounding studies on the lungs. Check the guidelines provided by your IACUC before proceeding.

BASIC PROTOCOL 5

Euthanasia of Mice by Carbon Dioxide Asphyxiation

Materials

CO_2 source
Euthanasia chamber (e.g., Perspex box)

1. Use an appropriate euthanasia chamber, such as a Perspex box.

   The euthanasia chamber must be at least two times as tall as the rodent (standing up) to be euthanized. CO_2 is heavier than air and will settle to the bottom of the container. Never blow CO_2 directly onto animals in cages.

2. Fill the euthanasia chamber with CO_2.

   Do this slowly, over a few minutes. A fast flow will cause turbulence in the container and will, therefore, create a mixture of air and CO_2. A slow flow will allow the CO_2 to settle to the bottom creating close to 100% saturation. Concentrations of CO_2 >80% will cause unconsciousness of the animal in a matter of seconds, minimizing any sensation of suffocation.

3. Leave animals in the chamber for at least 5 min.

BASIC PROTOCOL 6

Euthanasia of Guinea Pigs

Because guinea pigs are larger animals, pentobarbital overdose is recommended. Consult your IACUC for specific guidelines.

Materials

Sodium pentobarbital (120 mg/kg)
1- to 3-ml syringe with 20- to 25-G needle

1. Weigh the animal and calculate dose of sodium pentobarbital at 120 mg/kg.
2. Gently restrain the animal.
3. Inject sodium pentobarbital via the intraperitoneal route using a 1- to 3-ml syringe and 20- to 25-G neeedle.
4. Verify death by lack of cardiac pulse, respiration, and eye pupil response/dilation.

DETERMINATION OF BACTERIAL LOADS

In studying the course of *M. tuberculosis* infection in animal models, determination of the bacterial load in target organs is fundamental information. The study design depends upon the time course to be studied, which usually involves a relatively long period due to the chronic nature of the infection, and, therefore, time points for analysis need to be determined to avoid the experiment becoming too expensive. Given the extensive literature on the mouse model, this is now easily determined. A second consideration is the number of animals per time point, which can be determined using a statistical power calculation. For a bacterial load curve, a power of >0.8 can be achieved using four to five mice.

The process consists of six procedures:

1. Preparation of agar plates
2. Bacterial count set up
3. Necropsy
4. Homogenization of tissues
5. Plating
6. Counting bacterial colonies

Collection of Samples from *M. tuberculosis*–Infected Animals

It is important to have all necessary items in the safety hood before beginning the experiment. However, for the hood to function correctly and safely, it is essential that the airflow is not restricted, so make sure that the hood vents are not obstructed. Moreover, to reduce the risk of contamination and waste of resources, first practice with uninfected animals so that you are completely familiar with techniques, including dissection and removal of target organs, before attempting these procedures with infected animals.

Mouse organ-sized pestles are normally packaged in five- or ten-packs. Pestle packs are placed plastic side down inside a red autoclave bag and packed in a cooler with dry ice in a bag placed on top of them. The pestle packs must not be in direct contact with the dry ice. Guinea pig pestles are packaged in three- to five-packs. They are autoclaved and packed in the cooler as described for the mouse pestles. It is important to keep the pestles cold so they fit snuggly into the homogenization tubes; if they warm up, they slightly expand and do not fit.

NOTE: All processing of tissues from *M. tuberculosis*–infected animals must be done in the biosafety cabinet and glove box. Ensure that the blower, power, and light switches of the biosafety cabinet are in proper working order before starting any laboratory techniques in this instrument.

Materials

70% ethanol
Mice or guinea pigs
5% Lysol solution

Necropsy board
Absorbent paper
250-ml plastic beakers
Surgical kits containing scissors and forceps
Small biohazard bags
Tube rack
Homogenization tubes (Glas-Col cat. no. 099C S31 for mouse or cat. no. 099C S37 for guinea pig)

Prepare for necropsy

1. Place a necropsy board into the hood in a central location without blocking any of the air vents. Cover the board with absorbent paper.
2. Fill a 250-ml plastic beaker with 70% ethanol and place it inside the hood. Place the scissors and forceps into the beaker.
3. Place a small biohazard bag inside the hood.
4. Place one rack containing labeled homogenization tubes inside the hood.

   Several racks may be required for the entire count but only one rack should be in the hood at any given time.

5. Place any additional supplies inside the hood (PCR tubes, histology cassettes, syringes, etc.).

Collect sample

6. Euthanize animals usually four to five at a time according to Basic Protocol 5 or 6. Using a wash bottle, thoroughly wet the fur with 70% ethanol to sterilize the area.

7. Transfer the animals to the hood and place them on clean, dry, absorbent paper on the necropsy board. Place the animals on their backs and pin them to the board using syringe needles.
8. Make a midline incision(s) using the scissors.
9. Retract the skin above the head and below the thighs by pulling it with gloved fingers to expose the peritoneum.
10. Cut the peritoneum using scissors and retract it to expose the internal cavity.

Remove spleen

The spleen is attached to the greater curvature of the stomach by connective tissue.

11a. *To remove the spleen all at one time:* Take hold of as much of the spleen as possible with forceps. Gently pull the spleen free of the peritoneum, tearing the connective tissue behind the spleen.

11b. *To remove the spleen in sections:* Grab hold of the top and bottom portions of the spleen and tear from the connective tissue one part at a time.

It is usually more efficient to remove the spleen all at once, particularly if large numbers of animals will be used. Avoid cutting into the intestine.

12. Uncap the appropriate tube and place the spleen into the tube. Place the spleen as far down the tube as possible to avoid any tissue touching the cap once it is replaced. Cap the tube.

Remove liver

13. With small forceps, grasp the right lateral lobes of the liver to a point across the inferior vena cava.
14. With scissors, gently flip these lobes back over so that the top portion of the forceps can grasp these lobes as well as the common bile duct.
15. When the forceps have a firm hold, make an incision with the scissors between the liver and the diaphragm and cut the inferior vena cava—this then frees the liver.
16. With a quick but gentle upward motion, extricate the free liver as one organ.
17. Uncap appropriate tube, place the liver in designated tube, and re-cap tube.

Remove lungs

18. Make an incision through the thoracic cavity using scissors and cut back the rib cage (the lung lobes should be readily visible on either side of the heart). With small forceps, take hold of the bronchial tree close to the heart and cut the lung lobes away.
19. Uncap appropriate tube, place the lung in designated tube, and re-cap tube.
20. If collection of tissue for PCR is required, proceed to the glove box to homogenize tissue.

Formalin-fixed tissue may be infused, placed in cassettes, and stored (see below).

Clean up

21. Place mouse carcasses into the biohazard bag situated in the hood.
22. Remove gloves and place them in the biohazard bag. Exit the hood and put on a fresh pair of gloves. Proceed to the next experimental group.

To prevent the possibility of a spill, racks of completed tubes should be placed at the back of the bench to ensure that they cannot be tipped over.

23. At the completion of necropsy, place all mice and trash in the small biohazard bag and tape it closed. Place the entire bag inside another bag and re-tape. Remove the bag from the hood and autoclave as soon as possible (never store). Rinse the necropsy board with 5% Lysol, wipe, and place the board back into its container. Wipe the entire hood with 5% Lysol, followed by 70% ethanol and discard the paper towel in a biohazard bag for disposal.

Organ Homogenization

Once organs have been harvested and placed in homogenization tubes, they are ready to be disrupted. Once this is performed, they can then be serially diluted in dilution tubes to prepare for plating on agar. The homogenization procedure creates a significant aerosol hazard, therefore, using a glove box married to the biosafety cabinet in which the organs were dissected is recommended.

Materials

5% Lysol solution
Organs in homogenization tubes (see Basic Protocol 7)
70% ethanol
7H11 agar plates
Saline, sterile
Glove box in biosafety cabinet
Aluminum boat
Pestles (Glas-Col cat. no. 099C S21 for mice or Biospec Products cat. no. 985370-14 for guinea pigs), sterile and chilled
Homogenizer (e.g., Glas-Col homogenizer system)
Pipet boats
24-well tissue culture plates
Automated pipettor
Multi-channel pipettor

Set up for homogenization

1. Open the outer access door of the airlock entry port for the glove box chamber and place an aluminum boat containing 5% Lysol within the airlock chamber. Close and secure the outer door of the airlock and then place hands in the glove ports and open the inner access door of the airlock entry port.

2. Remove the boat from the airlock and place it within the glove box chamber with the lid off. Close the inner access door.

3. Transfer a tube rack with ten homogenization tubes containing organs through the airlock as described above. Transfer paper towels soaked in 70% ethanol into the glove box.

4. With hands in the glove ports, carefully remove the foam test tube stoppers from the homogenization tubes and place them off to the left side of the hood on a paper towel.

5. Transfer a pack of sterile chilled pestles into the glove box. Carefully open the pestle pack and place the chilled pestles into the homogenization tubes, taking care not to splash or spill the tube contents.

   Pestles can be placed into the tubes one at a time before homogenizing, or in groups of ten (remember that the pestles warm up rapidly in the tubes).

Homogenize organs

6. Remove the homogenization tubes one at a time from the test tube rack and attach the shaft of the pestle into the chuck of the grinder.

   There are various ways to do this, but the authors recommend the Glas-Col homogenizer system—an alternative is electric power drill bits (e.g., Black and Decker), which also fit—which must be secured in the glove box (Fig. 10A.5.5) with an activating power pedal that is placed outside the box. This has an appropriate chuck to attach to the top of the pestle, plus it spins at a high speed needed for thorough homogenization.

Figure 10A.5.5 (**A**) Homogenizing tube for mouse organs fitted tightly with a plastic pestle. (**B**) Homogenizer system with pestle fitted into the chuck.

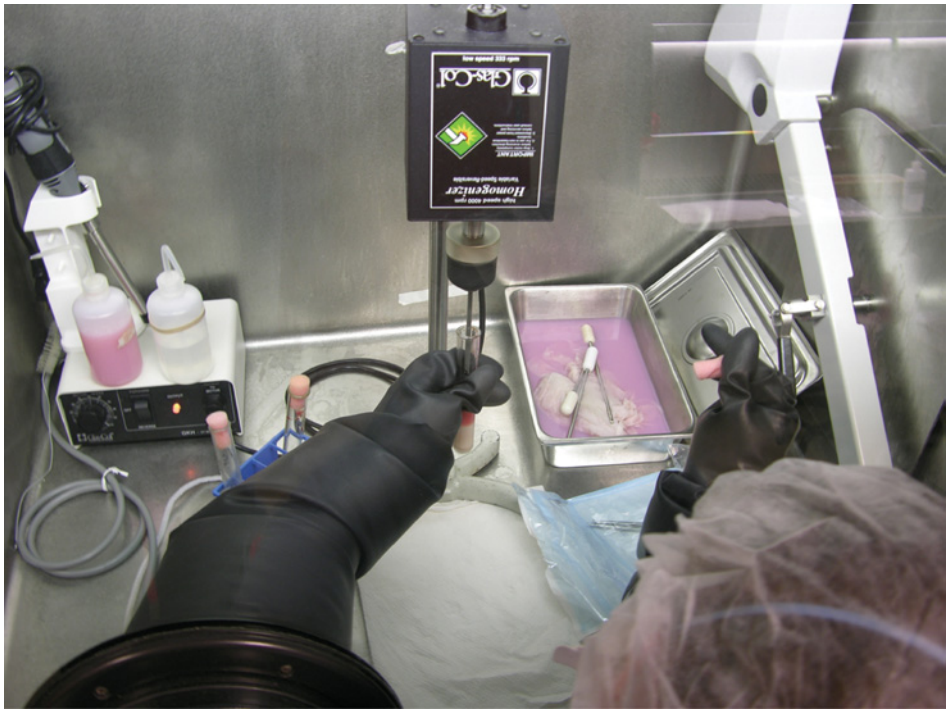

Figure 10A.5.6 In the glove box, the pestle has been fit into the homogenization tube and the unit activated to spin the pestle, disrupting the tissue in the tube. Do not rush this step; a high shearing force is needed to disrupt tissues and reduce the bacteria to a single cell suspension so that subsequent plating and counting is accurate.

7. When secured, firmly hold the homogenization tube with both hands and then actuate the grinder via the foot pedal.

   It is essential that a firm grasp of the homogenization tube is maintained while performing this technique as the pestle will rotate in the tube at a high rpm (Fig. 10A.5.6).

8. While the pestle is rotating, move the tube up and down slowly so that all the tissue is homogenized well.

   Do not rush this step. If the tissue cells are not completely disrupted and the bacterial clumps are not completely disassociated, there may be an undercount of the bacterial load.

Clean up

9. After homogenization is complete, remove the pestle from the chuck (keeping it inside the tube), hold the tube and pestle over the Lysol-filled aluminum pestle boat, and gently pull the pestle out and place it into the boat.

10. Return tube to the test tube rack and repeat until all tubes with organs have been homogenized.

11. After homogenization, recap the tubes with foam plugs and allow the chamber to purge for ~5 min to clear any residual aerosols.

12. When all homogenizations have been completed, pass paper towels and wash bottles containing 5% Lysol and 70% ethanol into the glove box. Wipe the interior surfaces of the glove box with 5% Lysol followed by 70% ethanol.

13. Place all used paper towels and pestle packs into the pestle boat. Cover the pestle boat with a lid, remove from the glove box, and place into a red biohazard bag contained within a pan. Autoclave as soon as possible.

Plate infected tissues

14. Half fill a pipette boat with 5% Lysol and place it in the hood either near the back or at the side. Ensure that airflow is not compromised. Leave the pipet boat lid outside the hood.

15. Fill a rack with eight to ten tubes containing organ homogenates.

16. Place a pack of 7H11 agar plates inside the hood.

    The agar plates are light sensitive, therefore, only place those plates immediately necessary for use inside the hood.

17. Remove the plates from their plastic bag and place them on the hood surface. Make sure the underside is properly labeled for each dilution to be plated.

18. Dispense 800 µl of sterile saline into 24-well tissue culture plates using an automated pipettor, leaving the first row empty.

    Always use some type of mechanical pipet-aid to pipet homogenates.

19. Dispense undiluted homogenate into the first row of a 24-well plate using a 1-ml pipet. Discard the pipet into the Lysol-filled boat.

20. Using a multi-channel pipettor and sterile wide-bore tips (to prevent blockage), carefully make serial 200-µl dilutions across the plate (1:5 dilutions using 200 µl in 1000 total). Change tips with every row. Dispense used tips into the Lysol-filled pipet boat.

21. Using a 1-ml pipet, dispense 100 µl of each homogenate dilution onto the agar plates. Use a separate pipet for every dilution; if this is not done, bacteria will carry over from dilution to dilution and massively over-count the bacterial load.

22. Bag, label, and incubate the agar plates at 37°C as described above (see also *UNIT 10A.1*). Count bacterial colonies ~3 weeks later, when colony formation is clearly visible and easy to count.

    Most laboratories prefer to store incubated plates upside down. This does not influence colony formation, but it does allow water that sometimes condenses out of the agar to accumulate on the lid. If left upright this water can flush the individual colonies onto the sides of the plate making counting difficult. Count colonies on quadrants on which there ~20 to 100 and then calculate back from the dilution on that quadrant. Liver plates may give nothing at zero or –1 dilutions, do not panic, this is simply due to inhibition by liver enzymes at higher concentrations.

SUPPORT PROTOCOL 1

PREPARING FOUR-CHAMBER (QUADRANT) PLATES SUPPLEMENTED WITH OADC

Materials

Agar, autoclaved
Filtered oleic acid albumin dextrose complex (OADC)

Laminar flow hood
5-ml repeating syringe (Wheaton), sterile
Petri dishes
Foam rack to support a 2-liter flask
Indelible colored markers
Plastic tub (large enough to hold poured plates)
Plastic sandwich bags

1. When the autoclaved agar has cooled to 56°C, remove the flask from the water bath and place it inside a laminar flow hood. Carefully loosen the foil and aseptically transfer 100 ml of sterile OADC into 900 ml agar.

2. Replace the foil on the flask. Gently swirl the flask to mix the OADC solution with the agar.
3. Prime the 5-ml repeating syringe by pushing the plunger up and down until the barrel fills with medium and there are minimal air bubbles.
4. Dispense 4.5 ml into each petri dish quadrant, or if using one without divisions, fill with 18 ml of agar (any bubbles that form should be gently shaken off to the edge). Carefully stack the plates as they are filled making sure to work toward the center of the sterile hood.
5. After dispensing the agar, immediately remove the flask with the syringe and tubing still in it from the hood and take to the sink. Set syringe aside for cleaning at a later time.
6. Turn off the hood light. Carefully push the stacks of plates to the center back of the hood. Do not cover with foil. Allow the plates to stand for at least 1 hr at room temperature with the blower of the hood turned on and the light turned off.

   This is sufficient time to allow the agar to solidify. Even though the agar in these plates is light sensitive, leaving them for 1 hr or so has no effect. Always check that the plates have solidified before proceeding to step 7.
7. When the agar has solidified, label plates on one edge of the lid with an indelible colored marker and be sure to record method of labeling.

   For example, place a blue line on the side of the lid on all the plates to make it easy to identify this batch.
8. Re-bag the plates upside down in plastic bags, and seal with tape.
9. Remove bagged plates from the hood and place in plastic tubs. Label the outside of the tub with a piece of tape with the batch no. and the date of the pour and your initials written on it.
10. Place the entire tub for 3 days in a 37°C incubator.
11. After 3 days, check plates for contamination.
12. Remove two plates from the batch and place into a plastic sandwich bag. Write the batch number and date poured on the bag and incubate for an additional 4 to 5 days in the 37°C incubator.

    This is to ensure there is no slow growing contamination on the plates.
13. Keep the remaining plates at room temperature.
14. After 5 days, check the two bagged plates in incubator for contamination.
15. Plates may be stored for ~2 months at room temperature.

    After such time the plates are to be discarded.

PREPARATION OF MIDDLEBROOK OLEIC ACID ALBUMIN DEXTROSE COMPLEX (OADC)

SUPPORT PROTOCOL 2

There are various Middlebrook agar formulations designed for growing colonies of *M. tuberculosis*; the authors recommend 7H11. It is necessary to supplement this agar with OADC to facilitate growth. A method for OADC preparation is provided here, or it may be commercially purchased (VWR).

Materials

6 M sodium hydroxide (NaOH)
Oleic acid ($C_{18}H_{34}O_2$)
Sodium chloride (NaCl)
Bovine serum albumin (BSA fraction V)
Dextrose
Antibiotic(s) (see recipe)

37°, 50°, and 56°C water baths
1-, 2-, and 4-liter Erlenmeyer flasks
500-ml sterile filter units
Sterile OADC bottles (500-ml clear glass bottles)
37°C incubator
115-ml sterile filter unit
Hand-held vacuum pump

Prepare OADC

1. Prepare the sodium oleate solution as shown in Table 10A.5.1. Warm in a 50°C water bath until the solution is clear.
2. While the sodium oleate solution is in the water bath, dissolve the following in 950 ml water:

(a) NaCl:	8.1 g/liter
	16.2 g/2 liters
	32.4 g/4 liters
(b) BSA:	50.0 g/liter
	100.0 g/2 liters
	200.0 g/4 liters
(c) dextrose:	20.0 g/liter
	40.0 g/2 liters
	80.0 g/4 liters

   Mix well until dissolved in solution. Adjust to pH to 7.0 with 4% NaOH.
3. When both solutions are clear, combine them to make the complete OADC enrichment medium. Bring to a final volume of 1000 ml with water.
4. Filter sterilize this medium through a 500-ml sterile filter unit.

   Use two units per liter of medium.
5. Aseptically dispense ~100 ml of sterile medium into sterile 500-ml OADC bottles and place the bottles 1 hr in a 56°C water bath.
6. After 1 hr, remove bottles from the water bath and place the medium overnight in a 37°C incubator.

Table 10A.5.1 Sodium Oleate Solution

	For 1 liter	For 2 liters	For 4 liters
Reagent	Volume of reagent to add (ml)		
H_2O	30	60	120
6 M NaOH	0.6	1.2	2.4
Oleic acid	0.6	1.2	2.4

7. Heat the bottles 1 hr in a 56°C water bath and place back in the 37°C incubator overnight.
8. Check the bottles for contamination (medium remains cloudy); discard medium if this happens.
9. Assign a batch number and store upto 1 month at 4°C until needed.

Filter OADC

10. Place one bottle of OADC into a 37°C water bath for at least 20 min prior to use to allow it to warm up.
11. Open the sterile 115-ml filter units and place in clean hood.
12. Aseptically dispense 100 ml of OADC into the filter unit.
13. Add the appropriate amount of antibiotics to the OADC.
14. Filter each bottle of OADC using a hand-held vacuum. Do not apply more than 15 lbs of pressure at one time.

PREPARATION OF 7H11 AGAR PLATES

SUPPORT PROTOCOL 3

Plates should be labeled with the experiment identifier, time point, group, organ, and date of experiment on the bottom of the base. If there is more than one mouse in each group, the plate should also have an A, B, C, etc. written next to the group identification. Quad plates should also be labeled with the dilution factor. Also see *UNIT 10A.1*.

Materials

70% ethanol
7H11 agar base
Asparagine
Glycerol

2-liter Erlenmeyer flask
Aluminum foil
Autoclave tape
56°C water bath

1. Wearing a clean laboratory coat, face mask, and gloves, rinse gloves with 70% ethanol.
2. Weigh out the following ingredients and place them into a 2-liter Erlenmeyer flask:

   21.0 g 7H11 agar base
   1.0 g asparagine
   5.0 ml glycerol
   900 ml ddH_2O.

3. Dissolve and cap flask with two layers of aluminum foil. Tape the foil to the flask with autoclave tape.
4. Autoclave for 20 min at 121°C on slow exhaust cycle.
5. Remove the flask from the autoclave immediately and check the color of the agar.

   The agar should be an emerald green color. If not, discard.

6. After checking the color of the agar, place the flask into a 56°C water bath and allow the agar to equilibrate for at least 30 min

BASIC PROTOCOL 9

ISOLATION OF SAMPLES FOR DETERMINING *M. TUBERCULOSIS* LOAD BY RT-PCR

Measurement of the expression of multiple genes during infection with *M. tuberculosis* is achieved by isolation of total RNA and then running a PCR assay. This protocol describes how to collect samples of infected tissue to be processed for PCR analysis. Mice are euthanized and samples of spleen, liver, or single lung lobes (upper left lobe for example) are collected. Homogenization of samples for total RNA extraction creates large aerosols and therefore collection of samples should be performed in a biosafety cabinet with an adjacent glove box.

When performing PCR assays, it is important to be aware of potential contamination of the test samples with RNase, as well as extraneous sources of DNA. All pipets used to aliquot cDNA should be considered contaminated; they either should be kept separate from pipets used to handle reagents, or if the same pipets are to be used, they should be cleaned with RNase-Away spray (alternatively, use a dilute solution of HCl: 10 ml of 12 N HCl:1 liter H_2O). Gloves should always be worn and changed frequently throughout the procedure. Handle samples containing RNA or cDNA in separate work areas if possible. For additional information on RT-PCR analyses of bacterial gene expression during mammalian infection, including troubleshooting tips, see *UNIT 1D.3*.

Materials

Ultraspec (Biotecx Laboratories)
95% ethanol
Lysol
Distilled water

Figure 10A.5.7 Commercial tissue tearer that can be used to rapidly disrupt samples followed by processing for PCR techniques. The worker is holding the tissue sample in a homogenizing tube large enough for guinea pig tissues.

5-ml round-bottom tubes (Falcon cat. no. 35-2063)
Nitrocellulose pen
50-ml conical tubes
Tissue tearer (Fig. 10A.5.7)

1. Mark each 5-ml round-bottom tube using a nitrocellulose pen.

   Solutions such as Ultraspec will remove anything but the most tenacious ink.

2. Fill the tubes with 1 ml of Ultraspec (agitated and warmed up to room temperature prior to use).

3. Prepare two 50-ml conical tubes, one with 40 ml water and the second one with 40 ml of 95% ethanol.

4. Collect infected tissue samples and place a small piece of each organ into a 5-ml round-bottom tube containing 1 ml of Ultraspec. Immediately proceed to homogenization in the glove box using a tissue tearer (Fig. 10A.5.8).

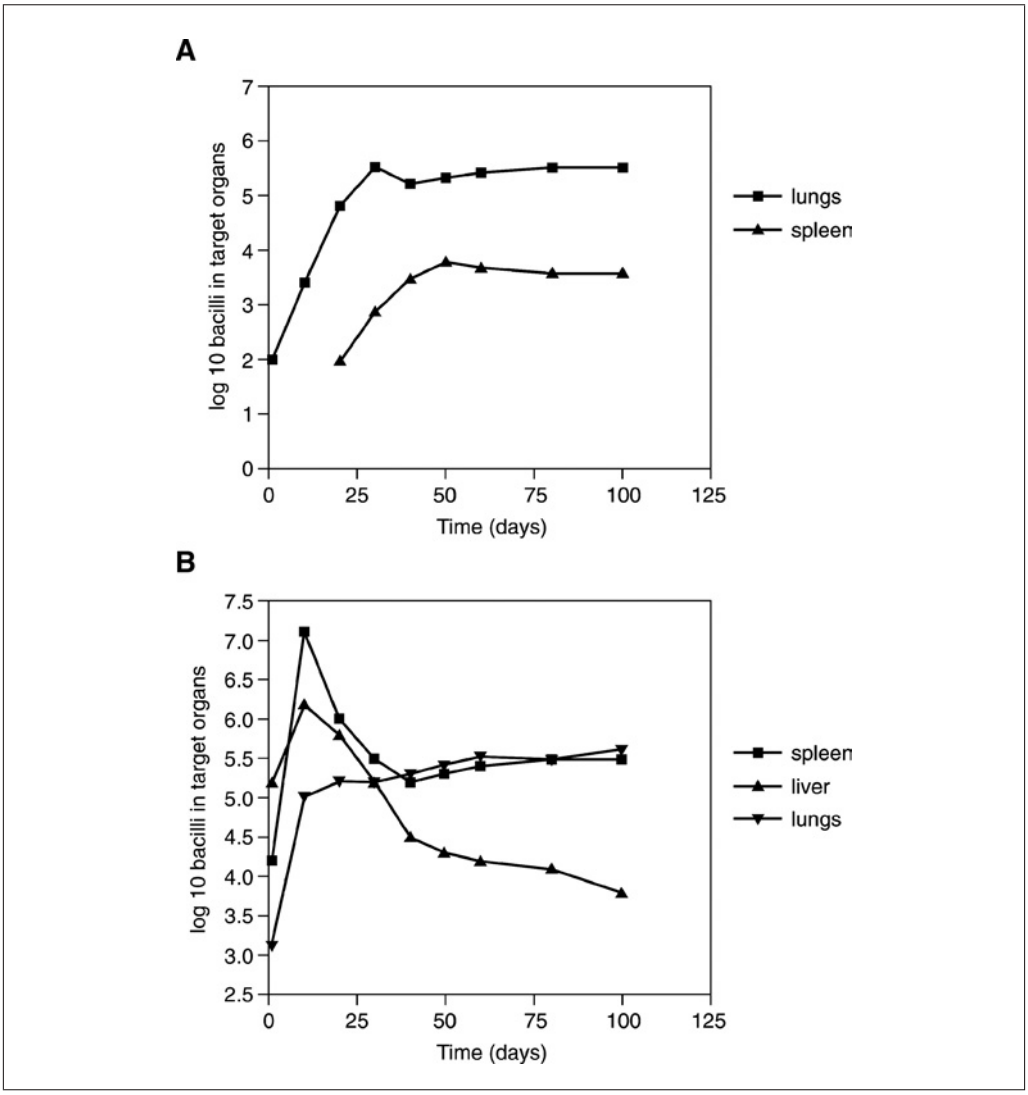

Figure 10A.5.8 Representative results for bacterial load determinations in mice after aerosol (**A**) or intravenous (**B**) infection. Notice the distribution in the target organs in the latter. If higher uptakes (especially in the lungs) are observed, then the inoculum was clumped together.

5. Rinse the tissue tearer tip in 95% ethanol for ~30 sec followed by a second rinse in distilled water. Disrupt the tissues using the tearer at full speed for 10 sec or until the tissue is completely disrupted.
6. Clean the outside of the tubes with Lysol. At this point, disrupted tissue can either be processed for RNA or be frozen on dry ice and kept at −70°C.
7. Clean the tearer in 95% ethanol and then water between each use.

BASIC PROTOCOL 10

PREPARATION OF LUNGS AND OTHER TISSUES FOR HISTOLOGY

The histological analysis of organs from infected mice can provide useful information about the host response. The size of granulomas, their make-up in terms of lymphocytes and macrophages, the degree of lung tissue they are consolidating, the development of necrosis, and so forth, can be invaluable information. New techniques, such as morphometry and stereology are gradually being developed, which will improve the understanding even further.

After fixation and paraffin embedding, sections are usually stained with hematoxylin and eosin, or with more specialized stains such as Masson's trichrome to detect fibrosis and such. Actual bacteria in the sections can be visualized by the Ziehl Neilson acid fast staining method.

The distribution of different cell populations of lymphocytes and macrophages within the lungs of mice infected with *M. tuberculosis* can be studied using immunohistochemistry. Tissue sections are stained using monoclonal or polyclonal antibodies specific for cell surface markers, as well as for cytokine and chemokine expression.

It is very important that specimens are collected and frozen as soon as possible in order to retain the morphology of the tissue and integrity of the antigens. Although samples can be stored for a few weeks at –80°C, it is recommended that they be processed as soon as possible.

Materials

Lung
10% formalin
5% Lysol
Histology cassettes

1. Infuse the lung with 10% formalin by injection down the trachea or bronchus.
2. Place in a histology cassette in 10% formalin.
3. Keep the cassette in the BSL III laboratory for at least 48 hr.
4. Wipe the cassettes with 5% Lysol.

These can now be removed and processed for histology.

BASIC PROTOCOL 11

PREPARATION OF OCT SNAP-FROZEN, *M. TUBERCULOSIS*–INFECTED TISSUE FOR ANALYSIS BY IMMUNOHISTOCHEMISTRY

Although there are newer techniques describing ways to perform immunohistochemistry on plastic embedded tissues, the best way is still to preserve the tissues in fixatives such as optimal cutting temperature compound (OCT) medium. This preserves cell membrane markers (CD4, CD8, etc.) so that they can be stained by specific antibodies. A further advantage of this approach is that the data can be combined with flow cytometric analysis to show both specific cell numbers and the distribution of these cells in a given tissue.

Materials

Mice or guinea pigs
70% ethanol
30% OCT (VWR cat. no. 25608-930) in 1× PBS
Dissection board with pins
Surgical instruments
5- or 10-ml syringes with 18-G needles
Embedding capsules (flat bottom 4 × 4): e.g., Peel-A-Way disposable embedding molds (22 × 30–mm, 20-mm deep; Thermo Scientific cat. no. 1830)
Aluminum foil
Plastic lock baggies
Small biohazard bags

Procedure for mice

1a. Euthanize the animal (see Basic Protocol 5), pin to board, and rinse with 70% ethanol.

2a. Cut back the skin as described above so that the chest cavity is exposed and the lungs can be viewed.

3a. Place a syringe needle barrel under the trachea to elevate it in relation to the chest cavity.

4a. Fill a 5-ml syringe with 30% OCT mixture and cap with an 18-G needle.

It is very important that there are no bubbles in the syringe, this can be accomplished by filling the syringes early and allowing the bubbles to rise to the top so they can be flushed out.

5a. Slide the needle into the trachea and begin gently injecting the mixture into the lungs to inflate them.

6a. While leaving the syringe in place in the trachea, remove the whole lung and place it directly into the labeled embedding capsules, which have been coated with OCT.

7a. When all samples have been extracted, care should be taken to center the samples (to make the sectioning procedure easier). Also, make sure the tissues are covered with the OCT.

8a. Place the capsules directly onto liquid nitrogen (they will float).

In a few minutes the OCT will begin to appear white in color.

9a. Remove the capsules, wrap in aluminum foil, and place into plastic lock bags. Seal, label, and place directly onto dry ice. Transfer to a freezer if you intend to store for any time.

Procedure for guinea pigs

1b. Infuse the left cranial lung lobe until it is fully inflated with OCT medium.

It takes ~5 to 10 ml to infuse the entire lobe using an 18-G needle. The needle should be inserted ~1/8 in. into the hilus to achieve this.

2b. Place the infused lobe in a Peel-A-Way mold that is 1/4 full of OCT. Begin to freeze immediately by dipping the mold into liquid nitrogen.

3b. As soon as the bottom starts to freeze fill the rest of the mold with OCT and continue freezing.

This is done in two steps to prevent the lung from floating to the top of the mold.

4b. Wrap frozen samples individually in aluminum foil and label for storage as needed.

PREPARATION OF LUNG CELL SUSPENSIONS

Cell suspensions from naïve and *M. tuberculosis*–infected mice are now routinely used for studies involving single-cell analysis by flow cytometry or by cell culture. For example, flow cytometric studies have provided a vast amount of information regarding the precise nature of the host response to *M. tuberculosis* infection. In addition, approaches such as the culture of cells obtained from infected animals have provided useful information regarding cell activation, proliferation, and production of multiple cytokines and chemokines.

Materials

Mice
10-ml syringes filled with ice-cold heparin solution fitted with 26-G needles
Tissue culture medium (e.g., RPMI or DMEM), cold
Collegenase/DNase (150/50 U/ml) (Sigma cat. no. C-7657 and D-5025) solution thawed and on ice
Gey's solution: 4.15 g NH_4Cl, 0.5 g $KHCO_3$, 500 ml ddH_2O, bring to pH 7.2

Dissection board with pins
Surgical instruments: long and small scissors, two tissue forceps
Autoclave bags
Small Petri dishes (60 × 15–mm)
Sterile razor blades or long-sharp scissors
37°C water bath with rocking
Nylon strainers (70-μm; VWR cat. no. 21008-952)
Sterile 15-ml centrifuge tubes

1. Euthanize mouse (see Basic Protocol 5). Retract skin and open thoracic cavity as explained above. Hold the heart gently using forceps.
2. Using a 10-ml syringe filled with ice-cold heparin solution, insert the needle into the 'dark spot' on the right ventricle of the heart and inject the heparin until the lungs turn white and inflate.

   A concentration of 50 U/ml is used per lung.

3. Remove the lung and place in 2 ml ice-cold tissue culture medium.
4. Double wrap mouse carcasses in autoclave bags for disposal.
5. Place lungs in a small petri dish. Use a sterile razor blade to cut up the lungs into very small pieces.

   If ethanol is used to sterilize the razor blade, rinse the blade in incomplete-medium or make sure that the ethanol has completely dried before using on tissues.

6. Add 2 ml collagenase/DNase per organ.
7. Incubate 30 min in a 37°C rocking water bath.
8. After incubation, place on ice and neutralize the enzyme mixture by adding 5 ml of medium to each tube.
9. Create single-cell suspensions using a syringe plunger and forcing the lung tissue through 70-μm nylon strainers. Rinse strainer with 3 ml medium. Pipet cell solution back into the 15-ml tube.
10. Centrifuge 5 min at 250 × *g* (1200 rpm), 4°C.
11. Resuspend cell pellet and add 2 ml Gey's solution. Leave for 5 min at room temperature.

12. Add 5 ml tissue culture medium to neutralize the Gey's Solution.

13. Centrifuge 5 min at 250 × *g* (1200 rpm), 4°C.

14. Decant supernatant and resuspend the pellet. Place suspension on ice.

15. Proceed with staining of cells for flow cytometry, or for cell culturing.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibiotics

10 mg cycloheximide
50 mg carbenicillin
2.0 ml ddH_2O

Weigh out each antibiotic, using an analytical balance, into separate 5-ml snap-cap tubes. Add 1.0 ml of ddH_2O to each tube and mix to dissolve. When dissolved, combine antibiotics into one tube.

Filter sterilize the antibiotic mixture prior to adding to a bottle of OADC. Prepare fresh.

COMMENTARY

Background Information

A large number of laboratories have now developed reproducible models of tuberculosis infection in the mouse or guinea pig. This has resulted in major advances in the understanding of the host response to *M. tuberculosis* at both the immunological and pathological levels.

In the mouse, early studies, primarily using the technique of passive cell transfer (Orme and Collins, 1983; Lefford, 1984; Orme, 1987), demonstrated the role of T cells and their subsets, but this approach has now been largely replaced by the use of gene-disrupted mouse models, which have been used to show the importance, or lack of importance, of various T cell subsets (D'Souza et al., 1997; Flynn and Chan, 2001; Turner et al., 2001), the role of key cytokines, such as gamma interferon, IL-12, IL-23, and IL-27 (Cooper et al., 1993; Flynn et al., 1993; Pearl et al., 2004; Holscher et al., 2005), and chemokines, particularly CCR2 (Peters et al., 2001; Kipnis et al., 2003). The pathologic process in the lungs of mice and guinea pigs (Rhoades et al., 1997; Turner et al., 2003) is now also routinely reported.

This model is also very useful for the more practical issue of identifying new drugs and vaccines. Drugs that are active in the mouse invariably give similar efficacy in humans, and thus can be evaluated in intravenously or aerogenically infected mice both as full-term regimens (Nuermberger et al., 2004) and more rapid screening models (Lenaerts et al., 2003). As for vaccines, the mouse model is a convenient and cost-effective way to screen new vaccines (Orme, 2006). The degree of protection given by a vaccine candidate in the mouse, coupled with immunogenicity data such as cytokine secretion and generation of activated T cell phenotypes, can be used to prioritize vaccines for further assessment (Orme, 2003).

Such additional evaluations are usually done in the guinea pig model. It has the advantage of exhibiting a course of infection and developing caseous necrosis that is similar to that in untreated humans with active disease, making it a good model to determine the protective effects of a new vaccine (McMurray et al., 1999). In short-term assays, this model can be used to evaluate protection, and in long-term assays, the capacity of the vaccine to modify the pathological process and engender long-term survival can be assessed. A guide to the various stages of the lung pathology in this model has recently been published (Turner et al., 2003). New techniques that can be applied to this model such as flow cytometry (Ordway et al., 2007) and magnetic resonance imaging (Kraft et al., 2004) continue to appear.

Critical Parameters

A factor critical to the success of any model of mycobacterial infection in mice or guinea pigs is the condition of the inoculum. Large

batches of bacteria should be prepared by laboratories intending to do this on a routine basis, so that the inoculum used is consistent. Having said that, batches do tend to have a certain shelf-life, and should be replaced every few years. New batches should be compared to standardized laboratory strains (H37Rv, Erdman, Erdman K01).

Cultivation methods vary from laboratory to laboratory, and this can affect the virulence of the inoculum. Pellicle growth certainly increases this factor, but in general, most laboratories report peak levels of 10^5 to 5×10^6 in the lungs after aerosol infection with ~100 organisms. With either aerosol or intravenous infection, it is very important to ensure that the inoculum contains a single-cell suspension, and incorporation of a detergent such as Tween-80 (0.05%) helps to eliminate this problem. It should be borne in mind however that this may reduce the virulence of the organism, although usually only marginally.

Anticipated Results

The growth of *M. tuberculosis* in the mouse is influenced by the virulence of the inoculum, the infection route, and the degree of resistance of the mouse strain used. In the case of immunocompromised mice, such as those with specific gene deletions, or those devoid of lymphocytes (SCID, Rag−/−, etc.), the infection may be progressive in the lungs.

Representative growth curves for *M. tuberculosis* H37Rv in 8- to 10-week-old female C57BL/6 inbred mice are shown in Figure 10A.5.8. In the aerogenic model, the infectious load peaks initially ~30 to 40 days, then may drop slightly, before becoming a chronic infection. In this stage, the bacterial load in the lungs appears relatively flat, or may slowly rise with time. In other strains such as the CBA/J, there is apparent regrowth of the infection after ~150 days (Turner et al., 2002), indicating reactivation of the infection. In the intravenous model, immunity is induced far more quickly, peaking initially ~10 to 15 days. Note the target organ uptake values; they should be ~90% in the liver, 10% in the spleen, and 2% to 3% in the lungs. Higher values in the spleen and (especially the) lungs indicates possible clumping of the inoculum.

Although guinea pigs can be productively infected with as few as three to five bacilli, exposure to inocula implanting ~20 to 25 gives reproducible uniform results in this model (adjusting the stock dilutions until detection of this number by plating ~24 hr after running the aerosol is necessary; see also Kraft et al., 2004). The infection grows rapidly for ~30 days, before becoming chronic. After ~100 to 150 days, animals suddenly lose weight and die. Because this results in tissue autolysis and therefore data loss, it is recommended that body weights are monitored during this time and animals euthanized rather than being allowed to die. Vaccination of guinea pigs with 10^3 BCG intradermally protects animals for ~1 year, as shown in Figure 10A.5.9.

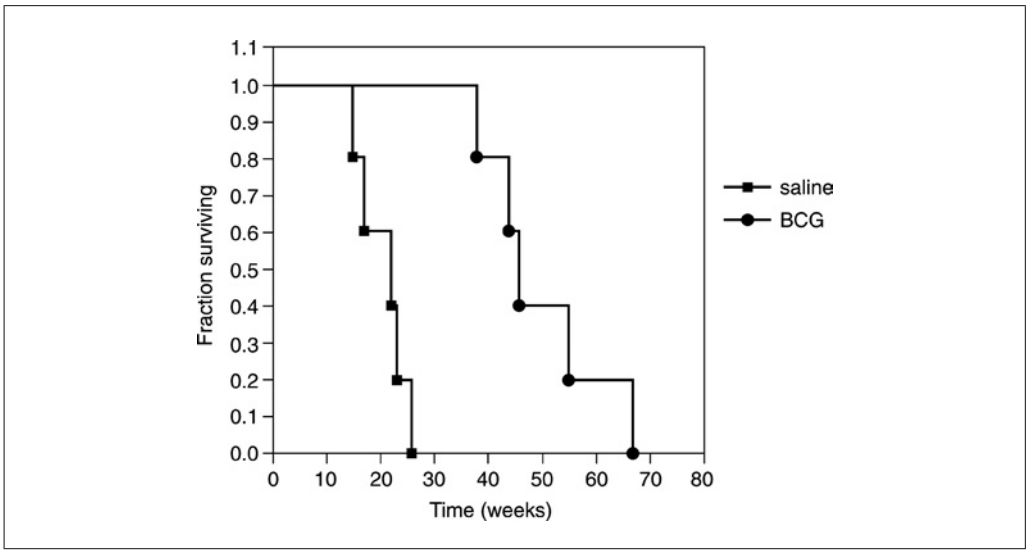

Figure 10A.5.9 Survival times of guinea pigs infected with *M. tuberculosis* H37Rv. Note that if more virulent infections (such as Erdman K01) are used, expect the curves to move to the left. Carefully begin monitoring animals 8 to 10 weeks after exposure so that animals in distress can be euthanized.

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Laboratory Maintenance of *Mycobacterium marinum*

This unit describes methods for laboratory culture, storage, and maintenance of *Mycobacterium marinum*, and for processing this organism for infection assays. *M. marinum* is a natural pathogen for fish and amphibians, and the disease symptoms that it causes in those animals are very similar to human infections by *M. tuberculosis*. *M. marinum* is also closely related to *M. tuberculosis* at the genetic and biochemical levels.

Although the methods for culturing, handling, and maintenance of *M. marinum* have some aspects in common with those for *M. tuberculosis* (*UNIT 10A.1*), there are many others that apply strictly to *M. marinum*. *M. marinum* grows optimally in vitro in bacterial culture medium at 30° to 32°C, with a generation time of 6 to 8 hr at the mid-log phase of growth, and its biosafety requirement is BSL-2 (*UNIT 1A.1*). Hence, *M. marinum* grows significantly faster (~4 to 6 times) and can be handled in the laboratory more easily (BSL-2, meaning that it can be manipulated on the benchtop) than *M. tuberculosis* (BSL-3, meaning that it must be kept strictly in a biosafety hood). For these reasons, *M. marinum* has been increasingly used as a model for studying the pathogenesis of *M. tuberculosis*.

In this unit, methods are described for culturing *M. marinum* in liquid medium (see Basic Protocol 1) and on solid agar medium (see Basic Protocol 2), and for processing the organism to be used in infection assays (see Basic Protocol 3).

CAUTION: *Mycobacterium marinum* is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: It is important to use a refrigerated centrifuge when performing centrifugations at 25°C to prevent the sample from exceeding 32°C.

CULTURING *M. MARINUM* IN LIQUID MEDIUM

BASIC PROTOCOL 1

This protocol describes methods for culturing *M. marinum* in liquid medium. *M. marinum* cultured in liquid medium is used for the following applications: (1) to amplify the bacterial number; (2) to incubate the bacteria for certain assays, such as drug-sensitivity tests; and (3) to analyze the physiologic or biochemical properties of the bacteria, e.g., to examine gene expression or protein secretion. Like *M. tuberculosis*, *M. marinum* is aerobic and requires oxygen for optimal growth. There are two major differences between *M. marinum* and *M. tuberculosis* that distinguish them from one another in laboratory culturing. One is that *M. marinum* requires an optimal growth temperature of 30° to 32°C. The other is that *M. marinum* is a photochromogen and its physiology and biochemistry vary in light versus dark, with the organism growing optimally in the dark. In addition to methods for *M. marinum* culturing, this protocol also describes methods for short- and long-term storage. The methods described in this protocol are essential for every aspect of *M. marinum* research, including genetics, biochemistry, physiology, and pathogenesis.

Materials

7H9 liquid culture medium (see recipe) *or* Sauton's liquid culture medium (see recipe)
M. marinum: ATCC #BAA-535 (human isolate) or ATCC #927 (fish isolate)
M. marinum freezing medium (see recipe)
Liquid nitrogen tank with canes to accommodate cryovials

Contributed by Lian-Yong Gao and Joanna Manoranjan
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Culture containers: sterile 250-ml flask or bottle (glass or plastic)
30° to 32°C incubator with orbital shaker
Spectrophotometer capable of measurements at 600 nm
50-ml conical centrifuge tubes
Cryovials

1. Aliquot 50 ml 7H9 or Sauton's medium into a sterile 250-ml flask or bottle (glass or plastic). Warm up the culture medium and container in an orbital shaking incubator set at 30° to 32°C.

   Use 7H9 medium for regular culture of M. marinum; however, use Sauton's medium under the following two circumstances: (1) when performing bioassays on the culture supernatant, for example, analyzing secreted proteins, or (2) when analyzing M. marinum in a defined medium, for example, examining M. marinum gene expression under phosphate-starvation conditions (see Critical Parameters). For optimal aerobic growth of M. marinum, keep the volume of the culture medium at no more than one-fifth of the total volume of the culture container. See Anticipated Results for doubling times.

2. Thaw a vial of *M. marinum* frozen stock (as obtained from supplier, or see step 5b) on ice for 15 to 20 min, and inoculate 10 μl of the stock into the prewarmed culture container from step 1. Tightly seal the container and culture *M. marinum* in the shaking incubator at 30° to 32°C with continuous shaking at 100 rpm for 4 days in the dark, to reach mid- to late-log phase of growth (OD_{600} ~1.2 to 1.5, corresponding to a density of 1–2 × 10^9 cells/ml).

   To make the M. marinum culture reach mid-to late-log phase faster, use a higher inoculation volume. For example, add 200 μl of the thawed frozen stock to 50 ml of the culture medium to reach mid-to late-log phase in 2 days. The disadvantages of heavier inoculation are: (1) the culture will contain a higher percentage of bacteria originating from the frozen stock, some of which may not be viable, and this may affect subsequent assays; and (2) the frozen stock will be used up more quickly, which may be an issue with certain strains that are not available in large quantities, e.g., low-passage clinical isolates.

   M. marinum grows mostly as clumps/aggregates, and the optical density needs to be measured immediately after addition of the culture to the spectrophotometer cuvette. Evaluation of M. marinum density in relationship to OD_{600} is achieved by measuring OD_{600} of the M. marinum single-cell suspension and enumerating CFU of the single-cell suspension on 7H10 agar medium (see Basic Protocol 2).

3. Transfer 4 ml *M. marinum* confluent culture from step 2 to a new culture container with 46 ml prewarmed fresh liquid culture medium. Culture the bacteria in the shaking incubator with continuous shaking (as in step 2) for 2 days to reach confluence (OD_{600} ~1.5, late-log-phase, corresponding to 2 × 10^9 cells/ml).

   At this point, the M. marinum culture is ready to be processed for various assays. Although M. marinum is an aerobic bacterium, micro-oxygenation under the shaking conditions described here is sufficient to support appropriate growth, and there is no need for intentional aeration of the culture.

4. To maintain *M. marinum* culture for frequent use, subculture as in step 3.

   Reduce the inoculation volume accordingly in order to increase the amount of time before the culture reaches confluence. To maintain M. marinum fully virulent, minimize the time period of the subcultures (less than three to four subculture passages) as mutations may accumulate during the period of the subcultures and may affect the assays.

5a. *For short-term storage:* Seal the culture container and store it at room temperature in a dark place.

   M. marinum can remain viable for 2 months at room temperature in the dark.

5b. *For long-term storage:* Transfer 50 ml of the confluent culture from step 3 to a 50-ml conical tube and centrifuge 10 min at 2700 × *g*, 25°C. Remove and discard the supernatant using a pipet, then resuspend the pellet in 10 ml freezing medium at 1×10^{10} cells/ml. Divide into 1-ml aliquots in cryovials and store at −80°C.

M. marinum remains viable for over 20 years under these conditions. Storing M. marinum in liquid nitrogen can preserve viability for an even longer period of time.

CULTURING *M. MARINUM* ON SOLID MEDIUM

BASIC PROTOCOL 2

This protocol describes methods for culturing *M. marinum* on solid agar medium. Culturing *M. marinum* on agar medium is used for the following applications: (1) to select for antibiotic-resistant *M. marinum* clones; (2) to test for *M. marinum* susceptibility to drugs/antibiotics; (3) to enumerate *M. marinum* colony-forming units; (4) to isolate a single colony of *M. marinum*. This protocol also describes the method for maintaining and storing *M. marinum* culture on agar medium. The methods described in this protocol are essential for many aspects of *M. marinum* research, including studies on genetics, biochemistry, physiology, and pathogenesis.

Materials

M. marinum: ATCC #BAA-535 (human isolate) or ATCC #927 (fish isolate) growing in liquid culture (see Basic Protocol 1)
100-mm 7H10 agar plates (see recipe)
Ethanol

15- and 50-ml conical centrifuge tubes
10-ml syringes with 26-G needles
Tabletop centrifuge
Hemacytometer
Microscope with 40× objective
30° to 32°C incubator
Spreader
Sealable plastic bags to accommodate petri dishes (Fisher)
Inoculating loop

Additional reagents and equipment for counting cells using a hemacytometer (Strober, 2003)

1. Transfer 10 ml of liquid *M. marinum* culture into a 50-ml conical centrifuge tube.
2. Draw the cell suspension into a 10-ml syringe and pass it through a 26-G needle three times to break the bacterial clumps. Transfer the suspension to a 15-ml conical centrifuge tube.
3. Centrifuge 2 min at 1000 × *g*, 25°C, to separate single-bacteria suspension from clumps. Transfer the upper, clearer single-bacteria suspension to a new 15-ml conical tube.
4. Repeat centrifugation as in step 4, then transfer the upper, single-bacteria suspension to a new 15-ml conical tube. Count the density of *M. marinum* using a hemacytometer under a microscope with a 40× objective (Strober, 2003). Make appropriate dilutions for the next step of plating.
5. Transfer 100 µl from each of the above *M. marinum* dilutions of varying densities to the center of an individual 100-mm 7H10 agar plate that has been prewarmed to 30° to 32°C in the incubator.
6. Dip the triangular part of the spreader in ethanol, then flame to sterilize the spreader. Use the spreader to spread the bacterial suspension evenly on the agar surface.

7. Place plates bottom-side-up in a sealed plastic bag and incubate at 30° to 32°C for 5 to 7 days in the dark before visualizing and enumerating the *M. marinum* colonies.

   The M. marinum colonies have a rough morphology, irregularly shaped at the edge and waxy as a whole. The colonies are white when incubated in the dark but turn golden in 12 to 24 hr in the light. M. marinum colonies are easily distinguished from contaminants by the appearance of the colonies and the incubation time.

   For short-term maintenance/storage of M. marinum on the agar medium, keep the sealed plates at room temperature in the dark for up to 2 months.

8. *To isolate M. marinum single colonies:* Transfer a small amount of *M. marinum* from either a bacterial culture or frozen stock to a 7H10 agar plate prewarmed to 30° to 32°C. Streak on the plate with an inoculating loop for single-colony isolation. Incubate the plate as in step 7.

BASIC PROTOCOL 3

PROCESSING *M. MARINUM* FOR INFECTION ASSAYS

This protocol describes methods for processing *M. marinum* for infection assays. To fully develop the advantages of the *M. marinum* model for studying *M. tuberculosis* pathogenesis, cell and animal infection assays are essential. *M. marinum* can infect cells from fish and mammals that are maintained at 32°C, and the infections manifest pathologic features similar to those brought about by *M. tuberculosis*. In addition, the infection of fish or frogs with *M. marinum* results in the development of pathology similar to *M. tuberculosis* infection in humans.

M. marinum cultured in liquid medium is ideal for infection assays, as it maintains superior virulence than when it is cultured on solid medium. Also, because *M. marinum* grows in clumps/aggregates, the culture needs to be processed for isolating single bacteria and quantifying the bacterial number prior to the infection assays. In addition, as *M. marinum* produces a number of molecules that are released into the culture medium, the organisms need to be extensively washed in saline (usually phosphate-buffered saline, PBS) prior to the infection assays.

Materials

M. marinum: ATCC #BAA-535 (human isolate) or ATCC #927 (fish isolate) growing in liquid culture (see Basic Protocol 1)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

15- and 50-ml conical centrifuge tubes
10-ml syringes with 26-G needles
Tabletop centrifuge
Hemacytometer
Microscope with 40× objective

1. Transfer 50 ml of liquid *M. marinum* culture into a 50-ml conical tube. Centrifuge 10 min at 2700 × *g*, 25°C. Remove and discard the supernatant using a pipet, then resuspend the pellet in 30 ml of PBS prewarmed to room temperature.

2. Repeat step 1 twice to wash away the bacterial culture medium, and finally resuspend the pellet in 10 ml PBS.

3. Draw the cell suspension into a 10-ml syringe and pass it through a 26-G needle three times to break the bacterial clumps. Transfer the suspension to a 15-ml conical tube.

   This procedure can be performed on the benchtop. Aerosols are generally not a health/safety issue with this organism; however, safety glasses are strongly recommended to prevent splashing into the eyes.

4. Centrifuge 2 min at 1000 × *g*, 25°C, to separate single-bacteria suspension from clumps. Transfer the upper, clearer single-bacteria suspension to a new 15-ml conical tube.

5. Repeat centrifugation as in step 4, then transfer the upper, single-bacteria suspension to a new 15-ml conical tube. Count the density of *M. marinum* using a hemacytometer under a microscope with a 40× objective (Strober, 2003).

The M. marinum single-cell suspension can be used for animal or cell infection assays (Ramakrishnan et al., 1997; Talaat et al., 1998; Gao et al., 2004). The above procedures ensure infections with fully virulent M. marinum. However, it is not recommended that the frozen stock be used directly for infection assays, as the virulence properties change during freezing and thawing.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ADC supplement

Add the following components to 400 ml water with constant stirring at room temperature and continue stirring for 40 min:
4.25 g NaCl
25 g bovine serum albumin (BSA) fraction V
10 g dextrose (glucose)
15 mg catalase (Sigma; store up to 1 month at 4°C)
Adjust the final volume to 500 ml with H_2O
Adjust pH to 6.8 to 7.0 using 1 N NaOH
Pass through 0.22-μm filter to sterilize
Store up to 1 month at 4°C

Catalase is added to degrade oxygen intermediates such as H_2O_2, produced by mycobacteria during growth.

M. marinum freezing medium

Add the following components to 140 ml water with constant stirring at room temperature and continue stirring for 10 min:
60 ml glycerol
1 ml 10% (v/v) Tween 80
Pass through 0.22-μm filter to sterilize
Store up to 3 months at 4°C

Tween 80 is viscous; it is therefore more convenient to make a 10% Tween 80 stock solution than to add the reagent directly.

7H9 liquid culture medium

Add the following components to 891 ml water with constant stirring at room temperature and continue stirring for 30 min:
4.7 g Middlebrook 7H9 powder (Difco)
4 ml glycerol
5 ml 10% (v/v) Tween 80
100 ml ADC supplement (see recipe)
Pass through a 0.22-μm filter to sterilize. Alternatively, autoclave the above medium without Tween 80 and ADC, then supplement the cooled medium (<50°C) with the abovementioned amounts of these reagents. Store up to 1 month at 4°C.

7H10 agar plates, 100-mm

Add 5 ml glycerol to 895 ml water in a 2-liter flask with constant stirring on a magnetic stirrer. To this, add 19 g dehydrated Middlebrook 7H10 agar medium (Difco) slowly with continual stirring, and heat the medium until it just starts to boil. Autoclave using liquid cycle for 30 min. Cool down to 50°C in a water bath. Move the flask to a warm (~50°C) hot plate. With gentle stirring, add 100 ml OADC supplement (see recipe). Continue stirring and add appropriate antibiotics and/or other heat-sensitive chemicals at this point. Stir to mix well. Place 20 ml of this mixture in each of the 100-mm petri dishes. Keep the plates at room temperature for 2 days to dry up the condensation and agar surface. Place the plates bottom-side-up in sealed plastic bags and store up to 2 months at 4°C.

For plates containing certain antibiotics, e.g., Zeocin, that are light-sensitive and that have a short shelf life, store the plates in the dark and used them within 1 month of storage.

OADC supplement

Add 15 ml oleic acid solution (see recipe) to 485 ml ADC supplement (see recipe) and mix well on a magnetic stirrer. Adjust pH to 7.0 with 1 N NaOH. Pass through a 0.22-μm filter to sterilize. Store up to 1 month at 4°C.

Oleic acid solution

Add 2.4 ml oleic acid and 2.4 ml of 6 M NaOH to 120 ml H_2O. Mix well. Store up to 1 year at 4°C

Sauton's liquid culture medium

Add the following components to 925 ml water with constant stirring on a warm (50°C) hot plate and continue stirring for 40 min:

0.5 g KH_2PO_4
0.5 g $MgSO_4 \cdot 7H_2O$
4.0 g L-asparagine
10 ml 0.5% (w/v) ferric ammonium citrate solution
2.0 g citric acid
0.1 ml 1% (w/v) $ZnSO_4$ solution
60 ml glycerol
5 ml 10% (v/v) Tween 80
2.0 g dextrose (glucose)
Adjust pH to 7.0 with 1 N NaOH
Cool to room temperature

Pass through a 0.22-μm filter to sterilize. Alternatively, autoclave the above medium without Tween 80, then supplement the cooled medium (<50°C) with the above-mentioned amount of Tween 80. Store up to 1 month at 4°C.

COMMENTARY

Background Information

Because of some of the unique properties of *M. marinum*, the methods for its laboratory culture and maintenance are different from those for *M. tuberculosis* in many aspects. For example, *M. marinum* is cultured at 30° to 32°C, due to its lower temperature requirement for optimal growth (Clark and Shepard, 1963). Also, *M. marinum* displays somewhat less clumpy growth than *M. tuberculosis* and can be cultured in liquid medium on an orbital shaker (Gao et al., 2003). The clumps/aggregates are formed because of the lipid (mycolic acids) composition in the cell wall. During mid-log phase of growth of *M. marinum*, >98% of the bacteria are in clumps that contain >100 bacteria in aggregates. However, during the late- or post-log phase of growth, the percentage of single bacteria increases up to 10%. Finally, unlike *M. tuberculosis*, which requires handling under BSL-3 level containment (*UNIT 10A.1*),

M. marinum is handled at the BSL-2 level, meaning that the latter organism can be manipulated on the laboratory benchtop (*UNIT 1A.1*). Finally, unlike *M. tuberculosis*, *M. marinum* is photochromogenic and grows optimally in the dark.

On the other hand, *M. marinum* has much in common with *M. tuberculosis*. For example, *M. marinum* causes diseases in fish with pathological features similar to *M. tuberculosis* in humans, including disseminating infections, formation and development of necrosis in granulomas, and transmission of the infection (Talaat et al., 1998; Gao et al., 2004). At the genetic and biochemical levels, *M. marinum* is very closely related to *M. tuberculosis* (Tonjum et al., 1998; Gao et al., 2003, 2004). *M. marinum* mutants defective for growth in macrophages and for virulence toward fish can be fully complemented by *M. tuberculosis* gene homologs (Gao et al., 2003, 2004). Because of these and other properties of *M. marinum* (fast growth rate and the ability to handle the bacteria on the benchtop), *M. marinum* has been increasingly used as a model for studying the molecular mechanisms of *M. tuberculosis* pathogenesis (Ramakrishnan et al., 1997; Talaat et al., 1998; Gao et al., 2003, 2004).

Critical Parameters and Troubleshooting

Media

The most commonly used mycobacterial liquid culture media are those developed by Middlebrook and Cohn (1958), and Sauton (1912), initially designed for *M. tuberculosis*. Middlebrook's 7H9 liquid culture medium is a complex medium containing bovine serum albumin (BSA). It is most widely used to support large amounts and high-density growth of mycobacteria, including *M. tuberculosis* and *M. marinum*. Sauton's is a defined medium that does not contain proteins. This medium is often used under the following circumstances: (1) when the mycobacterial culture supernatant is to be analyzed for secreted proteins (Andersen et al., 1991; Gao et al., 2004); (2) when analyzing the effect of the nutrient components of the culture medium on *M. marinum*, e.g., under low phosphate concentration or depletion of phosphate in the culture medium (Lefevre et al., 1997).

Aeration conditions

M. marinum requires oxygen to grow. To avoid contamination, the culture container should be kept sealed. Keeping the liquid culture volume at one-fifth the full volume of the container should provide adequate oxygen to allow optimal growth of *M. marinum* (Gao et al., 2003). However, oxygen requirements may differ among strains of *M. marinum*. If the culture does not proceed to mid-log phase, oxygen may be a limiting factor. Try increasing the size of the culture container to achieve optimal growth.

Agitation conditions

M. marinum grows optimally with gentle agitation. Shaking at 100 rpm on an orbital shaker is ideal (Gao et al., 2003). However, as agitation is more intense for larger culture containers (e.g., bottles or flasks >1 liter), use slower shaking speeds (<100 rpm).

Temperature requirements

The optimal growth temperature for *M. marinum* is 30° to 32°C. Below 30°C, *M. marinum* grows at lower rates. *M. marinum* remains static at 35° to 37°C and loses viability at higher temperatures (Clark and Shepard, 1963). Pay attention to the incubating temperature. If the culture is accidentally exposed to 37°C or higher for an extended period of time (>1 day), start a new culture.

Light requirements

M. marinum grows at an optimal rate under dark conditions (Gao et al., 2003). Strong light conditions decrease the *M. marinum* growth rate while inducing production of golden pigments. However, the light effect is reversible. If the growth of *M. marinum* is halted by exposure to strong light, transfer the culture back to dark conditions to resume bacterial growth.

Anticipated Results

For *M. marinum* growth in liquid culture medium, the anticipated doubling time is 6 to 8 hr in 7H9 and 8 to 10 hr in Sauton's medium. For *M. marinum* growth on agar-based culture medium, the anticipated time for colony formation is ~6 days. In the presence of antibiotics or certain chemicals, slower growth rates are anticipated. The human (ATCC #BAA-535) and the fish (ATCC #927) isolates grow at similar rates, and both can infect cells (of fish and mammalian origins) and animals (fish) with similar virulence potential (Talaat et al., 1998).

Time Considerations

Starting with the inoculation of *M. marinum* frozen stock into the liquid culture medium

and ending at the point where one is ready to perform infection assays, the procedures described in this unit generally take ~4 to 6 days. Starting at that same point and ending at the point where one has obtained results on the agar plates for antibiotic selection and drug sensitivity tests, the entire process takes 11 to 13 days.

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Key References

Clark and Shepard, 1963. See above.

Demonstrates growth conditions, including temperature requirements, for M. marinum.

Gao et al., 2003. See above.

Demonstrates various culture methods and genetic manipulations in M. marinum.

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Zebrafish and Frog Models of *Mycobacterium marinum* Infection

Mycobacterium marinum, the causative agent of tuberculosis in poikilothermic species and a close genetic relative of *Mycobacterium tuberculosis*, is gaining popularity as a model for the study of *Mycobacterium* pathogenesis (Pozos and Ramakrishnan, 2004; Ramakrishnan, 2004). Several natural hosts have been used to study experimental mycobacterioses in the laboratory (Ramakrishnan et al., 1997; Talaat et al., 1998; Davis et al., 2002; Gauthier et al., 2003; van der Sar et al., 2004; Swaim et al., 2006). Infection in such hosts can be monitored by conventional methods such as determining bacterial load in tissues or whole animals. It can also be monitored by newer methods that involve differential interference contrast (DIC) and fluorescence microscopy of tissues or of live infected animals in real time. This unit presents protocols for the administration and monitoring of *M. marinum* infections in three animal models: leopard frogs, adult zebrafish, and zebrafish embryos. Also included in this unit is information for the preparation of bacterial inocula and relevant aspects of animal husbandry (see Strategic Planning). Additional information and alternative methods for the culture of *M. marinum* can be found in *UNIT 10B.1.*

CAUTION: Mycobacterium marinum is a Biosafety Level 2 (BSL-2) pathogen (see Strategic Planning). Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: This experiment requires Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

STRATEGIC PLANNING

Safety Considerations

M. marinum is a BSL-2 organism, and an occasional human pathogen, causing a superficial granulomatous infection of the skin (Wolinsky, 1979). Users should comply with their institution's requirements for BSL-2 pathogens. The authors recommend gloves, laboratory coats, and face shields as personal protective equipment. Furthermore, as a fish pathogen, extreme care should be taken to disinfect all *M. marinum* cultures with Amphyl or Lysol IC at a final concentration of 1%, or by sterilization in an autoclave prior to disposal into sewage systems. Water used for housing of fish and frogs should be decontaminated with 10% bleach prior to sewage disposal. Any equipment should be wiped down with 70% ethanol, 0.004% NPD (Steris Corporation), or 1% Lysol after use. Carcasses of infected animals should be treated as biohazardous waste and disposed of according to institutional guidelines. Accidental splashes to the skin or eyes, or accidental inoculations (i.e., needle sticks) should be washed immediately and thoroughly with warm water. Soap should be used on the skin. A qualified physician should be consulted for consideration of preemptive treatment.

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Selecting the Right Animal Model

The choice of model for the study of *M. marinum* pathogenesis largely depends on the aspects of infection to be investigated. The adult frog is a natural host for *M. marinum* and typically sustains a chronic and asymptomatic infection, even in the presence of high tissue burdens (Ramakrishnan, 1997). As it is relatively easy to isolate different tissues aseptically from the frog, this is a useful model for evaluating the ability of different *M. marinum* strains to establish and sustain a chronic infection in a relatively resistant host. This model is also useful for studies that require a large amount of tissue for analysis. However, as these frogs are wild-caught, they represent a heterogeneous, outbred population and infection results may vary widely from animal to animal. The use of relatively large groups of animals is required to obtain statistically significant data. Furthermore, supply may be more limited than for animals raised in captivity. Frogs take up more space than do fish, although their maintenance is relatively simple (see Systems for Housing Frogs and Fish).

Adult zebrafish are more susceptible than frogs to acute infection with *M. marinum* and suffer a progressive deterioration over the course of weeks to months (Swaim et al., 2006). As such, they represent a more sensitive system for the evaluation of virulence of different *M. marinum* strains by survival analysis, determination of bacterial burden, and/or pathology. Furthermore, zebrafish, but not frogs, develop necrotic, caseating granulomas, and are an appropriate model for the study of *Mycobacterium*-induced necrosis. Finally, zebrafish families (the authors have used the AB and WIK lineages) are more inbred than wild-caught frogs, yet less so than mouse strains. As detailed below, maintenance of zebrafish requires a significant commitment of funds and personnel, yet many fish can be housed at one time.

Zebrafish embryos are acutely susceptible to infection by wild-type strains of *M. marinum* and in the vast majority of cases will die within 2 weeks of infection. However, this system provides some unique advantages. Due to its optical transparency, the zebrafish embryo is ideal for visualizing cellular and infectious processes in vivo in real time. Furthermore, the contributions of innate immunity can be studied in isolation due to the absence of an adaptive immune system at this stage of development. A type of oligonucleotide antisense technology called morpholinos is routinely used in studies of zebrafish development, and can similarly be used to examine the role of various host genes in the infection process (H. Clay and L. Ramakrishnan, unpub. observ.). Embryos are abundant and relatively easy to maintain; therefore, a laboratory without a fish facility may be able to obtain embryos for short-term experiments from fellow investigators at the same or nearby institutions, or from a supplier (see Sources of Frogs and Fish).

One further advantage of the zebrafish models is that genomic information and immunological tools for the frog are virtually nonexistent. In contrast, mapping of the zebrafish genome is nearing completion and immunological tools are being developed. See Commentary for further considerations.

Sources of Frogs and Fish

Wild male frogs can be purchased from JM Hazen Frog Company (Alburg, VT) or Nasco (Ft. Atkinson, WI). They are generally available year-round, however, they hibernate from November to March, and supply may be limited during this time. The authors' laboratory has experienced a higher rate of mortality when warming the frogs up to laboratory temperature (22°C) during hibernation months. However, regardless of when received, frogs should be acclimated to their new environment for 2 to 4 weeks, or until 2 weeks pass without mortalities or other signs of illness, prior to experimental manipulation. Frogs are relatively inexpensive (less than $2 USD each), but housing costs can be steep, depending on the researchers' institution, and crickets cost about $60 per week.

Zebrafish embryos, juveniles, and adults can be purchased from the Zebrafish International Resource Center (ZIRC), University of Oregon, Eugene (*http://www.zfin.org*). Adults are $7.00 each while 100 embryos can be purchased for about $40 to $80 (higher prices for commercial organizations). Again the cost of the animals is insignificant relative to the cost of housing (a typical system to house 1000 fish, or 1 rack, costs about $10,000, see Systems for Housing Frogs and Fish), care (fish food costs $100/month for 1000 fish), supplies, and personnel costs.

Systems for Housing Frogs and Fish

In general, installing fish and frog systems requires more time and expertise than most investigators have available, and is thereby best accomplished by hiring an aquaculture technician or having the systems installed by the manufacturer. There are three types of systems for housing frogs and fish: (1) static, (2) recirculating, and (3) flow-through. Static and recirculating systems use reverse osmosis or deionized water to which salts are added (see frog and fish water recipes). The flow-through system uses tap water to which salts are added. The discussion below elaborates on all three types of systems: each is useful for different aspects of frog and fish husbandry as they pertain to mycobacterial infection experiments.

Static systems

Static systems refer to no-flow housing or tanks for rearing larval zebrafish and housing frogs. Fresh water is exchanged manually by transferring the animals to a clean tank, daily for zebrafish larvae and every other day for frogs. The drawback of the static system is the potential for wide fluctuations in water quality (i.e., pH, temperature, conductivity, and ammonia). The static bath is a convenient way to concentrate food for larval fish, which require vision for hunting paramecia. The static bath is also a convenient way to simulate the natural pond-type conditions for frogs. The frog tanks used in the authors' laboratory are polysulfone rat cages purchased from Alternative Design Manufacturing and Supply, Inc. (*http://www.altdesign.com*). They measure 10.25-in. (26-cm) wide × 18.75-in. (18-cm) deep × 8-in. (20-cm) high. The lids are an open style with replaceable filter paper. Within the tank, ~1 in. (2.5 cm) of water is added and an over-turned plastic basket measuring 8-in. (20-cm) wide × 10-in. (25-cm) long × 2-in. (5-cm) deep allows the frogs to rest out of the water.

Recirculating systems

Recirculating systems are used to house uninfected juvenile and adult zebrafish. They are designed to support multiple tanks situated on the shelves of a rack that can stand alone on a laboratory bench or the floor, or can be combined to create a multi-rack system. There are several manufacturers of such systems, namely Aquatic Habitats (*http://www.aquatichabitats.com*); Aquaneering, Inc. (*http://www.aquaneer.com*), Allentown Caging Equipment Company, and Thoren Caging Company. The principle of the recirculating system is that water flowing to the tanks is reused or recirculated continuously. The water is filtered in steps comprised of mechanical, biological, and chemical disinfection. A pad mechanically filters the solids, such as uneaten food and bioflocculants, and then the water flows into a sump. A pump forcibly pushes water first through a 50-μm cartridge filter and then through a biofilter containing a substrate such as sponge pads, or plastic or ceramic balls or beads, which supply a large surface area onto which denitrifying bacteria colonize and metabolize fish waste. For a comprehensive description of biofiltration, see Malone and Rusch (1997). Water then flows into an activated carbon canister, which chemically adsorbs the organic molecules from soluble fish waste (Moe, 1992). An ultraviolet (UV) light sterilizer disinfects the water before returning it to the fish tanks. Recirculating systems are water efficient and exchange rates vary depending on the density of fish (3% to 10% per day).

Flow-through systems

The flow-through system is ideal for housing experimentally infected fish and uses large amounts of water to provide a continuous supply for the animals at precise environmental conditions. Flow-through systems are single pass, meaning the water flows into the tanks only once and is then discharged. Because large amounts of water are required for the flow-through system, tap water is used as a water source. The only filtration required is a two-step pretreatment process involving mechanical removal of small particles via a 50-μm cartridge filter followed by dechlorination using large tanks of carbon (US Filter Corp, *http://www.usfilter.com*). The filtered water is mixed in a large tank by means of a continuous environmental monitor (YSI 5200, YSI Incorporated), which automatically doses sea salt solution to maintain conductivity and sodium bicarbonate solution to maintain pH, before flowing to the fish tanks. The discharged water is UV sterilized or chlorinated to kill the mycobacteria before draining to the sewer.

Frog Care

Materials needed for storing the frogs, proper housing maintenance, and proper handling include: tanks, plastic buckets, a mesh screen, nets and net soak, and bleach. Crickets are needed for feeding the frogs and can be kept in a 50-gallon plastic garbage container with screen-paneled lid.

Maintaining frog environment

House five or fewer frogs per static tank (described above) in 1× frog water (see recipe). Move frogs to fresh water in a clean tank every other day (e.g., Monday/Wednesday/Friday), using nets to catch and transfer them. Latex gloves irritate their skin so minimize handling, and never handle infected frogs or their water without personal protective equipment (see Safety Considerations). Disinfect nets between uses by soaking in Net Soak (Jungle Laboratories Corporation; *http://www.junglelabs.com*).

Change water, tank, and haul-out platform every other day after feeding day. Performing tasks as specified here ensures that frogs are not sitting in excess waste products on the off day (Saturday in the authors' laboratory). However, animals should be monitored daily.

Frog food: crickets

Order crickets weekly through a supplier such as Fluker (*http://www.flukerfarms.com*). Feed two to three crickets per frog, every other day (e.g., Tuesday/Thursday/Sunday). Crickets must be kept in a well-ventilated container. A 50-gallon (189-liter) trash barrel with a screened-panel in the lid can be used. Bulk crickets are shipped in a cardboard egg crate within a larger box. Place the crickets with the egg crate into the trash barrel lined with two heavy-duty plastic bags (crickets chew through plastic). Feed crickets 4 to 7 mouse chow pellets (Lab Diet brand) to prevent frog metabolic disease and water them by shaking water droplets into the barrel. Alternatively, if mouse pellets are not available, Fluker makes a high-calcium diet for crickets.

Fish Care

Synthetic sea salt mix (Instant Ocean, Aquarium Systems., Inc.) should be used to prepare the water in the tank. Other materials necessary to maintain a suitable living environment are: bleach (used for disinfection), sodium thiosulfate (removes chlorine from the water), crushed coral (calcium chloride), and sodium bicarbonate (helps maintain pH at 7.4). Conductivity and pH meters will be needed to monitor the temperature and pH of the tank, respectively. An aquaculture water chemistry kit (model FF-1A, Hach Company; *http://www.hach.com*) will be needed to test for ammonia. Also, a mesh bag will be needed for proper handling of the fish.

Maintaining fish environment

In order to establish a biological filter for the first time, the substrate comprising the filter must be seeded with biofloculant from an existing system or a commercial product like Bacta-Pur N3000 (Aquatic Eco-systems). Add 1.5 g of flake food per 50 gallons of water three times weekly and allow the system to run for several weeks. Test for ammonia using an aquaculture water chemistry test kit (model FF-1A, Hach Company). The concentration should first increase due to the deamination of proteins, and, as the denitrifying bacteria colonize the ammonia concentration will again decrease. Following the decrease, denitrifying bacteria are well enough established to add fish. Adding carbon and fresh water exchange helps to decrease the ammonia concentration. When fish are added to the system and are being fed, the pH will tend to decrease, an indication that the biofilter is working. Add 1 teaspoon (4.9 cm^3) sodium bicarbonate per 75 gallons of system water to maintain pH at 7.4. Always check the accuracy of the pH meter against the chemical test provided in the aquaculture water chemistry kit. Hardness should be in the moderate range (50 to 150 mg per liter) for spawning.

It is important to remember that clean tanks reduce normal flora associated with fish allowing for better re-isolation of mycobacteria during infection experiments. As a general rule, small tanks become soiled more quickly, and should be checked for normal flow every 2 weeks (1- to 3-liter tanks). Check and rotate larger (>9-liters) tanks monthly. Disinfect all tanks, lids, and baffles with bleach, thoroughly rinse three times, and air dry before adding water and fish. It is best to have extra tanks of each size for cleaning rotation. Replace filters as described in Table 10B.2.1.

Always keep a spare pump on hand. Depending on the system, ask the dealer what is the weakest link and be sure a spare is included with the system. Service pumps every 2 years.

Fish Feeding

At the larval stage, the feeding materials needed will be: TetraMin flake food (Drs. Foster and Smith; *http://www.drsfostersmith.com*), *Paramecium Multimicronucleatum* starter culture, larval powder food consisting of 1:1:1 Spirulina (Argent Chemical)/Paenaeid shrimp diet (Argent Chemical)/OSI microfood (Drs. Foster and Smith), and *Artemia* (brine shrimp cysts; Argent Chemical). At the adult stage, the fish will continue to be fed TetraMin flake food and *Artemia* along with Aquatox food (Aquatic Eco-systems; *http://www.aquaticeco.com*). Two brine shrimp hatchers (Aquatic Ecosystems) along with an air pump will be needed during both the larval and adult stages. To culture the paramecia, 5-ml syringes, 500-ml clear, sterile glass bottles, and a 5-liter rectangular plastic container, with secure lid (e.g., Rubbermaid), will be needed.

Table 10B.2.1 Fish Tank Filtration Replacement Schedule

Filter	Change frequency
Mechanical prefilter pads	Every 1-3 days according to system loading or as necessary
Cartridge filter	Every 2 days or whenever pressure drops after filter
Carbon	Every 2 weeks
UV lamps	Every 4-6 months
Biofilter	Every 6 months[a]

[a] Set aside 1/8 of (seed) substrate in fish water (see Reagents and Solutions). Clean remaining substrate and place into filter with seed substrate loaded nearest to incoming flow of water.

Feeding larval fish

Larval fish can be moved to their rearing container on day 5 post-fertilization (pf). For a detailed description of breeding, egg collection, and embryo incubation, see Westerfield (2000). Larval fish are fed paramecium culture (see below) and larval powder food twice daily, morning and evening. Their water is changed between feedings by pouring off the dirty tank water into a spare tank that catches any larvae that may be accidentally poured off with the dirty tank water. Escapees may be recovered with a large-bore pipet such as those described in Basic Protocol 6. On day 10 pf, feed the larval zebrafish live *Artemia nauplii* (brine shrimp, see below). An indication that the larval zebrafish have weaned onto the shrimp is that their bellies turn an orange color. At this point, they can be transferred to a system tank with a small mesh baffle designed specifically for housing larval fish. Continue to feed paramecia and larval powder food for another week while also feeding ample amounts of shrimp. Replace the larval powder with crushed flake food around day 15 pf.

Feeding adult fish

Adult fish are fed twice daily, first with flake food followed by live brine shrimp. If they are fed live food first, they will not eat the dry or flake diet, which fulfills important nutritional requirements. The flake food used is a mix of 1 part TetraMin and 1 part Aquatox.

Fish food: brine shrimp (Artemia nauplii)

Prepare a saturated salt solution in the brine shrimp hatcher. Add 1 tablespoon of *Artemia* cysts per liter. Attach air pump to vinyl tubing to keep the cysts in continuous suspension. Hatched brine shrimp are orange in color compared to the brown cyst. Upon hatching (~48 hr), shut off air supply and allow remaining unhatched cysts to sink. Drain the sunken cysts until pure hatched shrimp flow from the hatcher. Catch the pure shrimp in a net and transfer them to new salt water (Instant Ocean prepared as per package instructions) for storage or fish water for feeding.

Fish food: paramecium

Culturing paramecium is a two-stage process that involves first inoculating 500-ml clear, sterile glass bottles and growing them to a density ~200 cells/ml. These bottles are then used to inoculate rectangular tubs. Both the bottles and tubs require 1 to 2 weeks incubation before use.

To prepare the paramecium culture, the para medium (PM) must first be prepared. This will require the use of protozoan pellets (Carolina Biological Supply), sterile deionized water, and five 500-ml bottles. Bottle culture preparation will require deionized water, paramecia starter culture, Nekton bird vitamins, and wheat berries. The preparation of the bird vitamins will require deionized water, a 30-ml syringe, and a 0.45-μm filter. The final step in the paramecium tub culture preparation will require NPD (1-Stroke Environ germicidal detergent; Steris Corporation, *http://www.steris.com*), large rectangular plastic containers (e.g., Tupperware), paramecium bottle culture, Nekton bird vitamins, and wheat berries.

Preparation of para medium (PM)

Add 10 protozoan pellets plus a pinch of sea salt to 1 liter deionized water in a 2-liter Erlenmeyer flask and bring to a boil, monitoring carefully to prevent boiling over. Add the resulting slurry to five 500-ml bottles, equally distributing the liquid as well as the green sediment among the five bottles (~200 ml per bottle). Top off with sterile deionized water, bringing volume to 500 ml, and store at 4°C.

Paramecium culture step 1: Bottle culture preparation

Bottle cultures are usually prepared five at a time. To each of five sterile 500-ml bottles, add 100 ml PM (described above), 300 ml sterile deionized water, 100 ml paramecia stock from a previously cultured bottle, 5 ml sterile bird vitamins (see below), and five boiled wheat berries. Incubate for a minimum of 1 week at 28°C.

Be sure that the paramecium starter culture used to seed the next generation of cultures is free of contamination by *Coleps*, a ciliated protozoan smaller than the paramecium and recognized by its whirling swim behavior. *Coleps* feed on larval zebrafish younger than 7 days pf.

To prepare bird vitamins, dissolve $^1/_2$ scoop (scoop provided with vitamins) into 25 ml deionized water in a conical centrifuge tube. Filter-sterilize using a 30-ml syringe with a 0.45-μm filter fitted to the end of the syringe. Vitamins should be prepared fresh each time. This vitamin formulation, although manufactured for birds, provides a combination of nutrients necessary for optimal paramecium growth (Westerfield, 2000).

Paramecium culture step 2: Tub culture preparation

Disinfect the tub and lid with NPD by spraying all surfaces. Wait a few minutes and then wipe dry with a large Kimwipe. To the tub, add 250 ml PM (described above), and top off with sterile deionized water. One tub holds ~2 to 2.5 liters. Add 250 ml paramecium bottle culture (prepared in part 1, above). Add 10 ml filter-sterilized Nekton bird vitamins and 15 wheat berries to tub. Incubate for 1 to 2 weeks at 28°C before using. Always check tub cultures for purity and health by sampling 1 ml of the culture and observing with a dissecting microscope prior to feeding to larval fish. There should be ~200 paramecia/ml, which should possess a distinct slipper-shaped body plan (i.e., not skinny or short and stubby).

INFECTION OF ADULT FROGS (*RANA PIPIENS*) AND ENUMERATION OF *M. MARINUM*

BASIC PROTOCOL 1

This protocol describes a method for infecting adult frogs, and subsequent enumeration of bacterial colony forming units (cfu). At the desired times following infection, frogs may be euthanized and tissues harvested for analysis. See Background Information and Anticipated Results and references therein for guidance on the course of infection in frogs. The three-lobed liver is large (Fig. 10B.2.1), and as such, provides abundant material for analysis—that is, cfu determination as well as ample material for sectioning and histology. In addition, liver granulomas are discrete and well formed, while those of the spleen are sometimes more diffuse. The spleen is much smaller and usually serves as an additional source of cfu counts. However, any tissue may be harvested depending on the interests of the investigator. Furthermore, all procedures described here have been developed for *M. marinum* strain M (ATCC # BAA-535) and its derivatives. Other strains may be utilized depending on the interests of the investigator.

Materials

Frogs (see Strategic Planning)
0.2% (w/v) tricaine (ethyl 3-aminobenzoate, methanesulfonate salt), pH 7.0
70% ethanol
7H9 liquid culture medium (see recipe)
PBST: PBS (*APPENDIX 2A*) supplemented with 0.05% (v/v) Tween 80, sterile
Bacteriological plates: usually 7H10 agar plates (see recipe) supplemented with 10 mg/liter amphotericin B and antibiotics (see recipe for supplements to 7H10 agar plates) if appropriate

Plastic bags, ~1-gallon
1-ml syringes with 27-G needles (tuberculin syringes)
Frog tanks (see Strategic Planning)
2-liter beaker
Dissecting board and pins
Dissecting forceps, both with and without teeth
Surgical scissors for dissection
50-ml conical tube, preweighed
Omni homogenizer (model TH-115; Omni International) with sterile Omni tips (disposable generator probes)
33°C incubator
Additional reagents and equipment for culturing *M. marinum* for inoculation (see Support Protocol 1)

Prepare cultures and inject frog

1. Prepare log-phase cultures of desired *M. marinum* strains as described in Support Protocol 1. Load culture into a 1-ml syringe fitted with a 27-G needle.

   Such cultures will typically be ~1 × 10^7 cfu/ml. If a lower dose is desired, culture may be diluted with 1× PBST prior to loading into the syringe.

2. Place frog in a plastic bag and lay it on the bench surface, ventral side up. Place hand over frog, as shown in Figure 10B.2.2, to restrain.

   The plastic bag merely aids in restraint of the frog. To avoid suffocation, leave enough air in the bag and do not restrain frog for more than 1 min. Frogs must be healthy and acclimated before beginning the experiment.

3. Select an intraperitoneal injection site just left or right of midline and above the hind legs, but avoiding organs (see Fig. 10B.2.2).

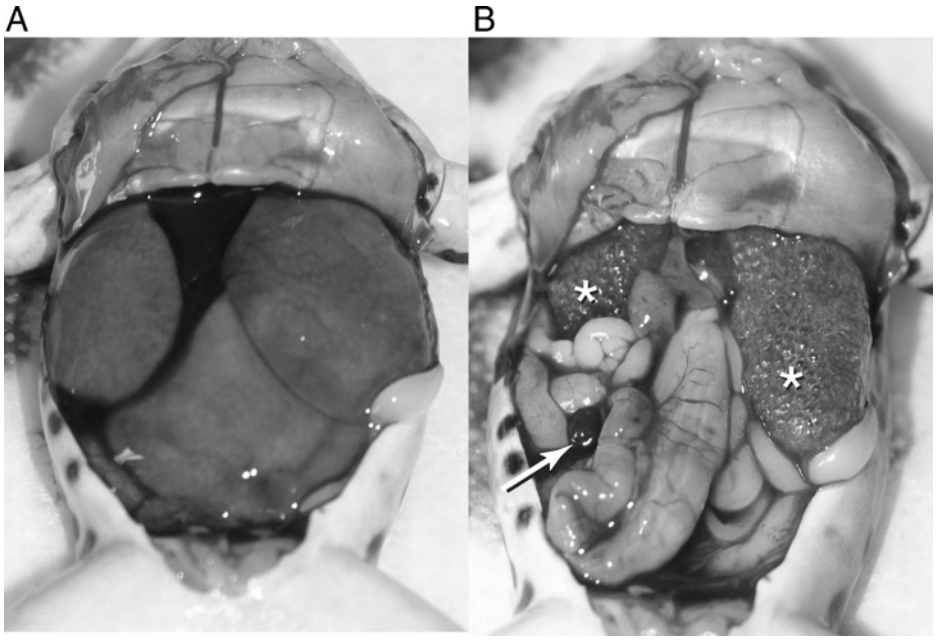

Figure 10B.2.1 Internal organs of *Rana pipiens.* The large tri-lobed liver (**A**) can be removed to reveal lungs (*) and spleen (arrow) shown in **B**. For color version of this figure, see *http://www.currentprotocols.com*

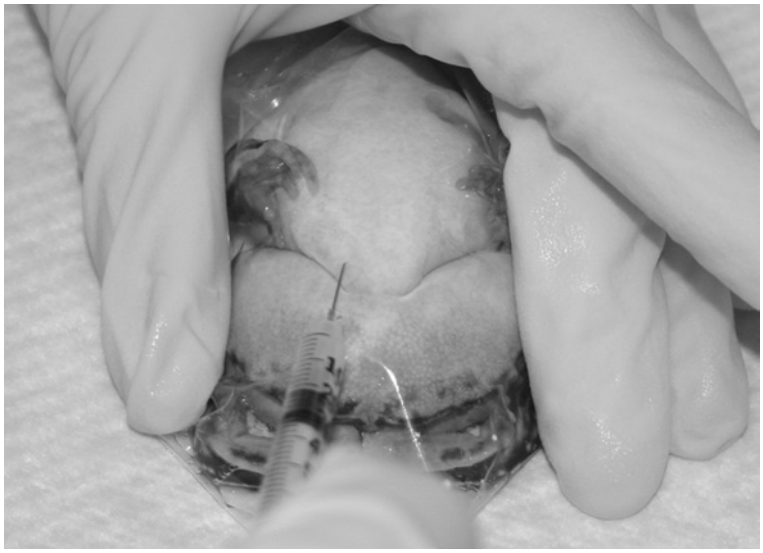

Figure 10B.2.2 To inject frogs, place in a clear plastic bag and restrain as shown. Injection site should be at the base of the abdomen to either the right or left of the midline. Insert needle far enough to penetrate musculature, but not deep enough to pierce organs. For color version of this figure, see *http://www.currentprotocols.com*

4. Using a tuberculin syringe fitted with a 27-G needle, insert needle through the plastic bag and into the frog at the injection site, and dispense 0.1 ml culture into the peritoneal cavity.

5. Quickly return frog to its tank and resume normal care schedule (see Strategic Planning).

Perform dissection

6. At the desired time after infection (see Time Considerations), anesthetize frogs in a 2-liter beaker with ~200 ml of 0.2% tricaine, pH 7.0, or in another suitable container with sufficient tricaine solution to reach a depth of ~0.5 in.

   Tricaine dissolved in water is highly acidic (pH 2 to 3) and must be neutralized (e.g., with NaOH or sodium bicarbonate) prior to placing animals in the solution. Failure to do so will result in undue suffering for the animals, and poor absorption of the tricaine.

7. Remove frog from beaker and pinch legs with considerable pressure. If any responsiveness is observed, return frog to the anesthetic and try again after several minutes.

   Frogs should be completely unresponsive in ~10 min.

8. Pin frog ventral side up and spray with 70% ethanol to disinfect skin.

9. Dissect frog using sterile instruments to remove skin in the following manner.

   a. Using dissecting forceps (with teeth), grasp the skin midline at the base of the abdomen and make a small incision in the skin.

   b. Inserting one prong of a pair of scissors at the incision site, cut in a straight diagonal line from the incision site up to the axilla.

   c. Repeat on the other side.

d. When both cuts have been made, fold the skin up over the head.

Some cutting of connective tissue between the skin and the underlying musculature may be required.

10. Switch to new, sterile instruments and cut open the underlying musculature in a similar manner to expose body cavity (Fig. 10B.2.1). To avoid damaging tissues, use toothless dissecting forceps.

11. First, make a cut to remove the ventricle of the beating heart to ensure death.

Some bleeding may occur, but this does not pose a problem for subsequent manipulations.

Remove desired tissues

12. Aseptically remove the liver, found in three lobes (Fig. 10B.2.1A) and transfer to a preweighed, sterile 50-ml conical tube.

If performing fluorescence microscopy, remove a ~5-mm × 5-mm × 5-mm piece first and place into a glass vial (see Basic Protocol 5, step 1a). Similarly, samples may be removed for histological analysis if desired.

13. If desired, remove underlying organs (Fig. 10B.2.1B), revealed after removal of the liver, in a similar fashion.

Two additional organs often used for bacterial enumeration are the spleen and lungs. The spleen is small and round, typically 2 to 5 mm in diameter. Lungs are unilobular and may be punctured prior to removal. Since these organs are small, they are not typically weighed, and cfu are represented on a per-organ basis. Avoid cutting gastrointestinal organs, doing so will contaminate the body cavity and organs with intestinal flora.

For tissues to be analyzed by microscopy, refer to Basic Protocol 5.

14. Weigh the liver and add 5 ml sterile 7H9 liquid culture medium supplemented with OADC and 0.05% Tween 80.

Use 2 ml for spleen or 3 ml for lungs. When processing many frogs, tissues may sit in closed tubes for up to 2 hr at room temperature before adding medium.

Homogenize and plate tissues

15. Homogenize tissues using an Omni homogenizer with sterile Omni tips at full speed until smooth. Measure the volume of homogenate with a serological pipet.

The homogenate should be free of tissue clumps large enough to block pipetting.

16. Prepare serial dilutions of homogenate in PBST and plate desired dilutions on bacteriological plates.

Typically, a frog infected with 1×10^6 bacteria will have 1×10^4 to 1×10^7 cfu/ml of liver homogenate at 6 weeks post-infection. Refer to Anticipated Results and references therein for more guidance on estimating the bacterial load.

17. Incubate plates for ~5 to 7 days at 33°C, or until colonies can be counted (*APPENDIX 2A*).

Additional colonies may continue to arise for 2 to 3 days after the plates are first counted. M. marinum colonies will continue to increase in size over time, therefore, they should be counted early, while they are still distinct, by marking plates with a pen. Continue incubation for several more days to catch any additional colonies that may arise. Refer to Support Protocol 1 and UNIT 10B.1 for further information on axenic culture of M. marinum.

INFECTION OF ADULT ZEBRAFISH (*DANIO RERIO*) AND ENUMERATION OF *M. MARINIUM*

Infection of Adult Zebrafish with *M. marinum*

BASIC PROTOCOL 2

Similar to frogs, adult zebrafish can be infected via intraperitoneal injection using a $\frac{1}{2}$-in. 30-G needle. As *Mycobacterium* infection of zebrafish appears to be a more progressive disease than that seen for frogs, fish may also be monitored for survival over time to generate a mortality curve. Alternatively, fish may be euthanized at various time points following infection and *Mycobacterium* counts enumerated. This protocol describes the infection of adult zebrafish, while Basic Protocol 3 describes the enumeration of bacterial cfu per fish.

Materials

M. marinium (ATTC #BAA-535 or other strain of interest)
PBS (*APPENDIX 2A*), sterile
Bacteriological plates: usually 7H10 agar plates (see recipe) supplemented with 10 mg/liter amphotericin B and antibiotics (see recipe for supplements to 7H10 agar plates) if appropriate
0.02% (w/v) tricaine in fish water (see recipe for fish water)
Fish (see Strategic Planning)

50-ml vaccine bottles
1-ml syringes with 21-G (or larger) needles
$\frac{1}{2}$ in., 30-G needles
33°C incubator
250-ml beakers
Plastic spoon
Petri dish

Additional reagents and equipment for culture of *M. marinum* for inoculation (see Support Protocol 1)

NOTE: After inoculation, fish must be housed in a flow-through system (see Strategic Planning). For most consistent results, use age-matched fish of the same family, and acclimate fish prior to injection to ensure maximal health. See Strategic Planning for details.

1. Prepare log-phase cultures of desired *M. marinum* strains as described in Support Protocol 1. Prepare serial dilutions in PBS, with the final dilution made in a 50-ml vaccine bottle.

2. To eliminate shearing of the mycobacteria, fill each 1-ml syringe using a 21-G (or larger) needle, and then carefully exchange for a 30-G needle.

   Dosage volume should be 50 μl per fish, thus allowing 20 doses per 1-ml syringe. A single syringe will be sufficient to inject a group of fifteen fish, with enough culture remaining in the syringe for plating in triplicate. Depending upon the virulence of the strain, inoculates of 5 to 500 cfu/fish are appropriate. Make dilutions of the culture accordingly to achieve this number.

   A 30-G needle is used to minimize trauma to the fish. Otherwise, avoid passing bacterial stocks through such small needles to minimize lysis.

3. Plate two 50-μl aliquots from this needle onto bacteriological plates to enumerate the average number of bacteria per injection. Incubate injection plates at 33°C until colonies can be counted.

   Since the needle may become contaminated with intestinal flora, microorganisms in the water, or those on the surface of the skin upon inoculation of the fish, the authors recommend plating two aliquots prior to inoculation of the fish (step 5, below). A final

aliquot may be plated after the last fish has been injected (step 7, below) to help determine whether the dose remains constant for all fish injected with that needle, although this plate may become contaminated. Cells should be spread on the plate using a spreader (see APPENDIX 4A for common microbiological techniques, and UNIT 10B.1 for those specific to M. marinum).

4. Prepare anesthetic bath of 0.02% tricaine in fish water (~100 ml) in a 250-ml beaker, and a recovery bath of fish water (containing no tricaine) in a spare tank.

5. Put fish to be injected, five at a time, into the anesthetic bath. When the fish roll onto their dorsal sides, immediately spoon from the beaker into a petri dish and orient ventral side inward, gently holding fish in place (Fig. 10B.2.3).

   IMPORTANT NOTE: *Fish should roll within ~1 min. If fish remain in the anesthetic bath too long, they will not recover. It is recommended that the fish not remain in anesthetic any longer than necessary.*

6. Insert needle above the pelvic fin and left of the midline, holding the needle at a 45° angle (see Fig. 10B.2.3). Insert needle just enough to penetrate peritoneal cavity and inject 50 µl bacterial suspension.

   A good guideline is to insert the needle until the bevel tip is completely inside the fish.

7. When all five fish are injected, transfer to recovery bath and begin another group until fifteen fish have been injected. Wait to see that all fish have recovered and then transfer back into holding tank.

   Working rapidly helps to reduce immediate mortalities; however, some loss should be expected. An inexperienced researcher may lose a significant number of fish due to injury, anesthesia overdose, or keeping fish out of the water for too long. With experience, mortality may be kept fairly low (<5% loss). A novice may want to anesthetize only one or two fish at a time to start.

   It is not necessary to sterilize the injection site; in addition to the potentially toxic effects of antiseptic, keeping fish out of water for a prolonged period of time increases the chance of immediate mortality.

8. Check infected fish twice daily and record mortalities for determination of a survival curve.

   Alternatively, fish may be removed and killed according to a desired time course for bacterial enumeration (see Basic Protocol 3) and/or histology (see Basic Protocol 4), depending on the goals of the experiment and the scientific question(s) being addressed.

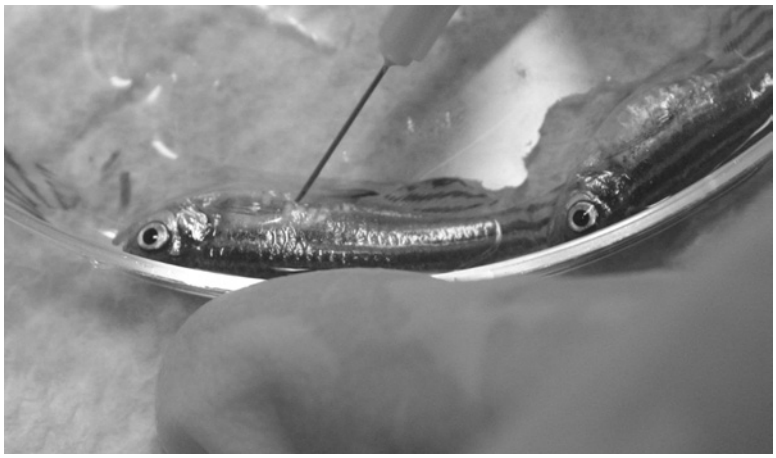

Figure 10B.2.3 Intraperitoneal injection of adult zebrafish. For color version of this figure, see *http://www.currentprotocols.com*

Enumeration of *M. marinum* from Adult Zebrafish

To enumerate the number of mycobacteria per animal, fish are homogenized in their entirety (intestines included). Therefore, additional steps must be taken to isolate the mycobacteria and prevent outgrowth of intestinal flora. It is also possible to dissect out individual organs aseptically, however, due to their small size, the authors prefer to simply enumerate cfu from the entire animal. Bathing the fish in kanamycin prior to euthanizing, and use of 7H10 agar plates supplemented with antibiotics for plating homogenates, prevents much, but not all, of the outgrowth by the intestinal flora. Thus far, this determination has only been accomplished using strains of *M. marinum* specifically resistant to kanamycin (in the form of a kanamycin-resistance cassette introduced on a plasmid, or integrated into the chromosome). However, it may be possible to use alternative antibiotics, to which *M. marinum* is naturally resistant (Aubry et al., 2000), to minimize contamination with normal flora. Note that *M. marinum* colonies take longer to grow on the antimicrobial supplemented medium described here than on 7H10 agar plates without such additions. Growth of wild-type bacteria (marked with a kanamycin-resistance cassette) should begin to appear after 10 to 14 days. Some mutant strains of *M. marinum* are compromised for growth on the supplemented agar (Cosma et al., 2006); therefore, it is important to confirm in advance whether the strains being used will grow under these conditions. Refer to Support Protocol 1 and to *UNIT 10B.1* for more information about growth of *M. marinum* in culture.

Materials

Infected fish (Basic Protocol 2)
PBS (*APPENDIX 2A*), sterile
Fish water (see recipe) supplemented with 1.5 mg/ml kanamycin sulfate
Fish water (see recipe) supplemented with 1.5 mg/ml kanamycin sulfate and 0.05% (w/v) tricaine
70% ethanol
7H10 agar plates (see recipe), 10 mg/liter amphotericin B, 25 mg/liter polymyxin B sulfate, 20 mg/liter trimethoprim, and 50 mg/liter carbenicillin disodium salt (see recipe for supplements to 7H10 agar plates)
17 × 100–mm sterile polystyrene tubes
Petri dish
Omni homogenizer (model TH-115; Omni International) with sterile Omni tips (disposable generator probes)
1-ml serological pipet with filter plug
Sterile glass test tubes
33°C incubator

1. For each fish, pipet 1.5 ml PBS into a sterile 17 × 100–mm polystyrene tube and weigh each tube to an accuracy of 0.1 g.
2. Bathe all fish to be processed in fish water supplemented with 1.5 mg/ml kanamycin for 1 hr.
3. To euthanize fish, transfer them to fish water containing 1.5 mg/ml kanamycin and 0.05% tricaine for 10 to 15 min to ensure death.

   Do not leave dead fish floating in tricaine solution longer than is necessary.

4. Transfer fish to sterile petri dish and spray with 70% ethanol to disinfect. Allow ethanol to air dry then transfer fish individually to the polystyrene tubes (one fish per tube).

5. Weigh each tube again to determine the weight of each fish.

   At this point, all fish should be in tubes containing PBS.

6. Homogenize 5 min using an Omni homogenizer at full speed.

7. Adjust volume to 2.5 ml by adding sterile PBS.

8. Prepare 1:10 serial dilutions of the homogenate in glass culture tubes containing PBS.

   Since the homogenate will be somewhat clumpy, the first two serial dilutions should be performed by transferring 1 ml homogenate to 9 ml PBS using a 1-ml serological pipet with filter plugs. Subsequent dilutions may be performed as desired with a pipettor. The number of cfu per ml homogenate depends greatly on the strain used, the dose, and the duration of the infection. The authors have observed counts ranging from <2500 up to 10^7 cfu/ml (Swain et al., 2006).

9. Plate 100 µl of the desired dilutions onto antimicrobial-supplemented 7H10 agar plates in triplicate.

   Due to the presence of contaminating intestinal flora, it is desirable to plate in triplicate to ensure obtaining plates with little enough contamination to provide meaningful data.

10. Incubate at 33°C until colonies can be counted.

    As some plates will likely be contaminated, it is advisable to check the plates regularly, and remove those with heavy contamination so as to avoid spread to other plates. Some molds or other bacterial contaminants may affect the growth of M. marinum, so considerable discretion should be exercised in determining whether the counts from any given plate may be reported with any confidence.

BASIC PROTOCOL 4

Histology of Adult Zebrafish Infected with *M. marinum*

If histological examination of infected fish is desired, parallel animals may be infected as described in Basic Protocol 2 and euthanized as described in Basic Protocol 3, step 3. Fixed fish may be sent to a commercial histology laboratory for embedding in paraffin, sectioning, and histochemical staining. Typical stains used are hematoxylin and eosin (H&E) to examine tissue and granuloma structure, or stains for acid-fast organisms (i.e., mycobacteria) such as Ziehl-Neelsen to visualize *M. marinum*. When requesting acid-fast stained slides, be sure that the provider includes a known positive control so that the quality of staining can be assessed.

Materials

Infected fish (Basic Protocol 2)
Dietrich's fixative (see recipe)
70% ethanol
Embedding cassettes (e.g., Tru-Flow, Fisher Scientific)

1. Fix fish whole by submerging in Dietrich's fixative for a minimum of 3 days.

   Before fixing fish, they must either be dead of natural causes, or must first be euthanized as described in Basic Protocol 3, step 3, or by another humane method consistent with local institutional or governmental guidelines.

2. Transfer fish to embedding cassette in 70% ethanol for storage and process for histology by standard techniques.

   Always use pencil to label the cassettes. Ink will be erased by the chemicals used for processing.

ANALYSIS OF TISSUES INFECTED WITH *M. MARINUM* BY FLUORESCENCE MICROSCOPY

The use of fluorescent bacteria allows for efficient visualization in infected tissues. Fluorescence makes possible the use of multiple, differentially marked strains. Also, promoter fusions to fluorescent proteins can be used as reporters of bacterial gene expression in vivo. Bacteria expressing GFPmut3 (Cormack et al., 1996), dsRed (Clontech), and ECFP (Clontech) can be easily visualized in tissues using appropriate filter sets on a standard fluorescence microscope (see references for spectral properties of the fluorescent proteins). Frog liver granulomas can be visualized as dense foci of DAPI-stained nuclei, which may contain bacteria. Frogs produce a dark pigment, which is taken up by macrophages, and at times may obscure some bacteria. Adult zebrafish granulomas often show a nuclear-free area of central necrosis (caseum), surrounded by a dense area of flattened, spindle-shaped nuclei and an outer epithelioid layer (Gauthier et al., 2003; Cosma et al., 2004).

Similar procedures are described for analysis of frogs and adult zebrafish.

Materials

Infected frog tissues (see Basic Protocol 1, step 12), or infected whole fish (see Basic Protocol 2)
4% (w/v) paraformaldehyde in 1× PBS (see *APPENDIX 2A* for PBS)
5%, 15%, and 30% (w/v) sucrose in 1× PBS (see *APPENDIX 2A* for PBS)
OCT compound (Tissue-Tek)
Antifade reagent with DAPI (e.g., Slow-Fade, Molecular Probes; or similar antiquenching reagent)
Nail polish
17 × 60–mm (8-ml) glass vials with screw caps (VWR)
Razor blade
Aluminum foil boats for freezing of tissue sections (Figure 10B.2.4)
Microtome/cryostat
Fluorescence microscope

Figure 10B.2.4 Aluminum foil boat used for freezing tissue samples.

Dissect tissue

1a. *For frogs:* While dissecting the animal (see Basic Protocol 1), remove a piece of liver ~5-mm × 5-mm × 5-mm and place it in a 17 × 60–mm glass vial.

This tissue should be removed first, taking care not to mangle it or in any other way compromise its integrity.

1b. *For fish:* Cut whole zebrafish transversally into two or three sections using a sharp razor blade.

Before processing fish, they must either be dead of natural causes, or must first be euthanized as described in Basic Protocol 3, step 3, or by another humane method consistent with local institutional or governmental guidelines.

Fix and rehydrate tissue

The main purpose of steps 2 to 5 is to sterilize the tissues so that samples may safely be cut and analyzed without fear of contaminating the microtome or other shared equipment with viable *M. marinum*.

2. Add 4% paraformaldehyde to the glass vial, enough to fully submerge the tissue sample (~2 ml). Leave for 2 hr at room temperature to sterilize the sample.

This step is performed to kill mycobacteria, allowing the user to perform subsequent steps under nonbiosafety conditions.

3. Remove paraformaldehyde and add 5% sucrose solution, enough to fully submerge the tissue, which may float. Incubate at room temperature until tissue sinks, or for a minimum of 30 min.

4. Repeat once more with 15% sucrose, and again with 30% sucrose.

It may be convenient to leave tissue in 30% sucrose overnight at 4°C.

Steps 3 and 4 are performed to rehydrate the tissue after fixation.

Embed tissue in OCT

5. Remove tissue from sucrose. Blot off as much liquid as possible and trim further if desired.

Excess sucrose will interfere with the bonding between the tissue and the OCT.

6. Prepare an aluminum foil boat (double layer) with a 1-cm deep × 1-cm wide well and fill with OCT compound.

The glass vials make useful molds for making foil boats (Fig. 10B.2.4).

7. Place the tissue in the well, submerging it in OCT, with the surface to be cut facing the bottom.

For fish, this should be the cut surface so that when sections are cut, whole body transverse sections are obtained.

8. Place boat in a dry ice/ethanol bath to freeze tissue. Carefully remove the boat and wipe off excess ethanol. Store tissue block at −80°C until use.

Ethanol interferes with the freezing of OCT, so it is important to avoid getting ethanol on the tissue block or in the OCT.

Perform cryosectioning

9. Prepare frozen sections using a microtome/cryostat using standard techniques, or according to the manufacturer's instructions.

Alternative tissue preparation and cutting protocols may be found in Watkins (1989).

It is ideal to use 5- to 10-μm sections for examination of fluorescent bacteria in granulomas.

Mount and examine tissue

10. Place the sections on the slides and allow them to dry 1 hr.

11. Add one to three small drops of antifade reagent containing 1.5 μg/ml DAPI. Cover with a glass coverslip, blotting excess reagent from the edges.

    DAPI is a nuclear stain that has been used to help identify granulomas as described in the introduction to Basic Protocol 5. If the use of an alternative nuclear stain is desired, Antifade reagent may be purchased without DAPI, and the alternative dye added.

12. Seal with nail polish, and examine using a fluorescence microscope (see Commentary).

    Typically 400× total magnification allows for careful examination of bacteria in the tissue. Store slides indefinitely at −20°C or colder.

PREPARATION OF *M. MARINUM* STOCKS FOR INFECTION OF ADULT ANIMALS

SUPPORT PROTOCOL 1

This support protocol describes the preparation of bacterial stocks to be used for infection of adult frogs and zebrafish. Due to the clumping nature of this organism, cfu do not necessarily represent the number of organisms present unless steps are taken to isolate single-cell suspensions. The procedures described here do not utilize single-cell suspensions, yet are fairly reproducible if cultures of similar density are prepared as described each time. Several methods exist to isolate single-cell suspensions of *M. marinum*, including passage of cultures through a 5-μm filter, sonication, or passing cultures through a syringe fitted with a needle to break up clumps. However, the impact of these procedures has not been evaluated in the adult animal models of infection described here. It is possible that planktonic bacteria isolated by filtration may possess different infectious properties than bacteria growing in clumps (akin to biofilms). Similarly, injection of excessive amounts of cell debris generated by sonication or passage through a syringe may impact infection in ways that have not been evaluated to date. See *UNIT 10B.1* for further information on the culture and characteristics of *M. marinum* grown in vitro.

Materials

7H9 liquid culture medium (see recipe)
M. marinum (ATCC # BAA-535 or other strain of interest), frozen stock (*UNIT 10B.1*)
Antibiotics as required
LB agar plate
25-cm^2 tissue culture flasks
33°C incubator

1. Add 5 ml fresh 7H9 liquid culture medium supplemented with OADC, Tween 80, and antibiotics (if required) to a 25-cm^2 tissue culture flask, and inoculate with a small scraping of *M. marinum* frozen stock or a bacterial colony. Incubate flask at 33°C in a horizontal position until it reaches an OD_{600} between 0.3 and 0.6.

2. After inoculating cultures, transfer a few microliters of each culture, as well as uninoculated medium onto an LB agar plate. Incubate this plate alongside the cultures and check for the presence of any contaminating growth.

   Some M. marinum may grow on the LB plate, however, if growth of any other bacteria or molds is observed, this is an indication that the culture and/or medium are contaminated. Do not use cultures that are contaminated.

   There is no need to spread the inoculum.

3. Dilute culture 1:100 in 10 ml of fresh medium and incubate as in step 1.

   If multiple strains are being used, it may be desirable to start several cultures at different dilutions so as to ensure that cultures of similar density are obtained for each strain.

4. Vortex culture, then maintain static in a vertical position on the benchtop for 10 to 15 min to allow the largest clumps to settle. Pull suspension of dispersed cells from the top and place in a separate sterile tube. Measure OD_{600}.

   While this step removes the largest of clumps, most bacteria will still be found growing in aggregates.

5. Normalize cultures to comparable densities by addition of fresh, sterile medium.

   Cultures of OD_{600} 0.3 to 0.6 generally contain 10^6 to 10^7 cfu/ml. Cultures may be diluted in fresh, sterile medium as necessary to obtain a desired dose.

BASIC PROTOCOL 6

INFECTION OF ZEBRAFISH EMBRYOS WITH *M. MARINUM* BY MICROINJECTION

This protocol describes how to infect 1- to 5-day-old embryos using a microinjector. Injection needles are pulled, loaded, and prepared. Embryos are anesthetized with tricaine, transferred to a dissecting microscope, and injected at the desired site. Methods for estimating the number of bacteria injected are described in Support Protocol 3. The care and use of zebrafish embryos in the laboratory is well established, and the reader is directed to *The Zebrafish Book* (Westerfield, 2000), in print or online at *http://www.zfin.org*, for basic techniques and recipes not specific to these protocols. See Figure 10B.2.5 for relevant points of zebrafish embryo anatomy. While the embryos themselves are not sterile, sterile zebrafish embryo medium is used as an additional precautionary measure to minimize risk of non-experimentally induced infections and mortality. Furthermore, 1-phenyl-2-thiourea (PTU), which is used to suppress melanocyte development, may be omitted depending on the purpose of the experiment (see Critical Parameters). If bacteria are to be visualized they should express a fluorescent marker.

Materials

M. marinum (ATCC # BAA-535 or other strain of interest, usually marked with a fluorescent marker)

1.5× EMPT (see recipe)

Dechorionated zebrafish embryos at 30 hr post-fertilization or later (see Support Protocol 4 for dechorionation details)

Sterile zebrafish embryo medium (see recipe)

Micropipettor with microloader tips for loading needles (Eppendorf)

Injection needles: needles made from aluminosilicate capillaries (SM100F-10, Harvard Apparatus) pulled on a Sutter P-2000 micropipet puller, with the following settings: Heat = 350, FIL = 4, VEL = 50, DEL = 225, PUL = 150

Microscope with Microinjector (e.g., Eppendorf 5246) and Micromanipulator (e.g., Narishige GJ-1)

Deep-well microscope slides (VWR)

Extra fine jeweler's forceps (Miltex no. 17-305 or Dumont no. 5)

Slide warmer, optional (e.g., Barstead no.26005 or 26020)

Microprobe (2 to 3 cm of 32-G platinum wire mounted on the end of a Pasteur pipet)

Multi-well culture plates, or small petri dishes

Large-bore glass pipets (Chase Scientific Glass, Inc. #63AS3WT, distributed by Fisher #XC-9993639, note that these are not typical Pasteur pipets)

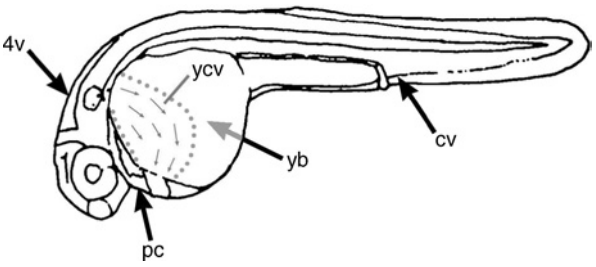

Figure 10B.2.5 Zebrafish embryo anatomy: The most-used injection site for infection has been the caudal vein (cv). Other useful sites have been the fourth ventricle of the brain (4v), the pericardium (pc) and the yolk ball (yb). The yolk circulation valley (ycv) is an excellent place to view macrophages both infected and uninfected during the early hours of blood circulation (Herbomel et al., 1999). Adapted from Westerfield (2000).

NOTE: Standard borosilicate capillaries may also be used, but the authors find clumping of mycobacteria to be less problematic when using aluminosilicate capillaries. If only borosilicate is available, note that the settings on a Sutter P-2000 micropipet puller are different for producing the same shape needle. Use: Heat=300, FIL=4, VEL=40, DEL=150, PUL=125.

Prepare needle for injection

1. Prepare bacteria for injection as described in Support Protocol 2.
2. Using a micropipettor with a microloader tip, draw up ~5 µl bacterial suspension and fill the injection needle to within 1 cm of its rear end.

   Take care not to leave air bubbles in the middle of the needle.
3. Mount the injection needle into the microinjector/micromanipulator and position it at the microscope where injection will take place (Fig. 10B.2.6).
4. Place the deep-well slide in the microscope base, filled with 1.5× EMPT.
5. Allow the needle to make an ~30° angle with the horizontal plane, and use the micromanipulator to move it almost in contact with the bottom of the well of the slide.

   Take care not to break the needle tip by lowering too far.
6. Using jeweler's forceps, break off the very tip of the needle while applying injection pressure to the needle (Fig. 10B.2.7A).

   Break off enough to allow free but not excessive flow. It is better to break off too little than too much.
7. Adjust the constant pressure on the needle to produce a faint, continuous stream out of the needle.

   Constant pressure at 5 to 20 hPa is usual.

   Because of the tendency for mycobacteria to clump and clog the needle, it is necessary to break off a bit more of the needle than is typically done when injecting other liquids. If excessive clogging is encountered, resyringe the culture (see Support Protocol 2), and load a new needle.

Prepare embryos for injection

Use the slide warmer on the benchtop to keep animals and solutions at 28.5°C.

8. Transfer a small number (2 to 10, depending on operator speed) of dechorionated embryos to 1.5× EMPT and wait at least 2 min for full anesthesia.

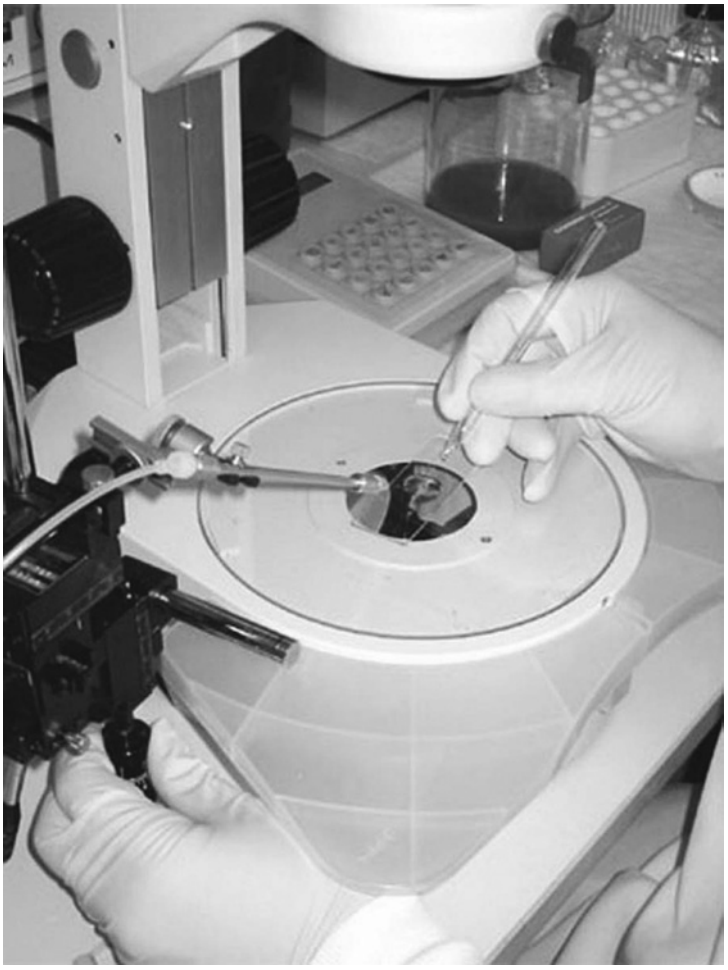

Figure 10B.2.6 Standard injection setup. Right-handed operators prefer to use the micromanipulator on the left, since more delicate work must be done with the microprobe.

9. Transfer one embryo to the microscope slide and, using the microprobe, position it gently so that the needle points to the intended injection target.

   For caudal vein injections, try lowering the needle onto the flank near the urogenital opening until it begins to press down on the embryo. With the embryo thus partially immobilized, tap the dorsal edge of the tail opposite the injection site to impale the embryo. See Figure 10B.2.7B and C.

Infect embryos with bacterial suspension

10. Apply injection pressure to drive the bacterial suspension into the fish.

    Adjust injection pressure and time according to the experiment. Begin with injection pressures of 120 to 140 hPa for 1-day-old embryos, and up to 170 hPa or more for older embryos. Typical injection times range from 0.3 to 0.5 sec. Some microinjectors can be set to inject for a set amount of time, allowing more consistent dosages.

    Use the accumulation of phenol red in the fish for a visual estimate of the amount of suspension injected (Fig. 10B.2.7C).

    For caudal vein injections, three useful signs of injection volumes are 'vein only' (Fig. 10B.2.7), 'blush' (a slight red color in the yolk circulation valley), and 'full red' (obvious red dye in the circulation valley). These, in combination with the concentration of the injection mix, can help to control the injection dose. Once appropriate settings have been determined, use them consistently for all embryos and when plating the injection dose (see Support Protocol 3).

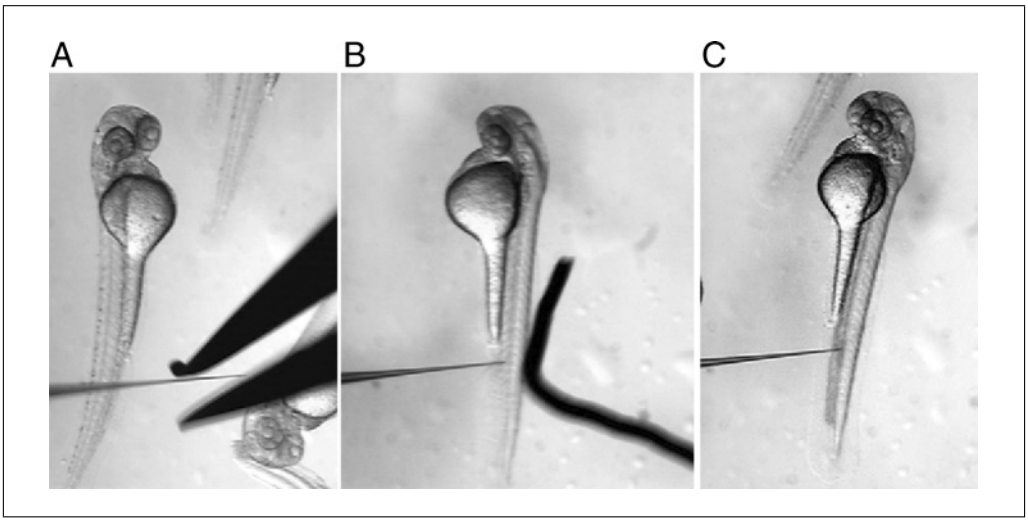

Figure 10B.2.7 Injection procedures. (**A**) Opening the end of the needle with jeweler's forceps (use a different set than those used for dechorionation). The higher the concentration of bacteria in the needle, the greater the length of needle that must be broken off to prevent clogging. (**B**) Caudal vein injection—inserting the needle. The platinum wire probe provides the force necessary to penetrate the periderm. Most operators find that moving the needle up and down, and setting its initial position, are the only times the micromanipulator is moved. (**C**) Successful caudal vein injection—phenol red dye is visible in the caudal vein, flowing toward the heart. With practice, a skilled operator can perform ~100 to 150 such injections per hour. For color version of this figure, see *http://www.currentprotocols.com*

11. Transfer the injected embryo to an appropriate container or multi-well plate containing sterile zebrafish embryo medium using a large-bore pipet.

    For holding embryos after injection, wells of 6-well tissue culture plates, or single 60 × 15–mm petri dishes, hold 10 to 25 embryos each. Long-term survival of embryos is enhanced by lower densities (10 per well/dish).

    To avoid contaminating embryos with a different bacterial strain, use a separate large-bore pipet for each infection group, and also separate pipets for injected and uninjected embryos.

12. At a time point 1 to 2 hr after injection, place the embryos in fresh sterile zebrafish embryo medium.

ENUMERATION OF *M. MARINUM* FROM INFECTED EMBRYOS

BASIC PROTOCOL 7

This protocol is designed to determine total *Mycobacterium* cfu per infected embryo. This procedure has been used for embryos up to 6 days post-fertilization. As described in Basic Protocol 3, this determination has only been done for kanamycin-resistant strains of *M. marinum*.

Materials

Sterile zebrafish embryo medium (see recipe) supplemented with 20 μg/ml kanamycin
Infected embryos (see Basic Protocol 6)
Fish water (see recipe) supplemented with 200 μg/ml tricaine
Trypsin-EDTA (Invitrogen)
PBS (*APPENDIX 2A*)
1% (v/v) Triton X-100
7H10 agar plates supplemented with OADC (see recipe) and antimicrobial agents (see recipe for supplements to 7H10 agar; see Basic Protocol 2)
30° and 33°C incubators
Water bath ultrasonicator (Bransonic, model no. 1510R-NT)

1. Pipet 500 µl sterile zebrafish embryo medium supplemented with 20 µg/ml kanamycin into 1.7-ml microcentrifuge tubes. Prepare one tube for each embryo to be lysed.
2. Transfer one infected embryo into each tube. Allow embryos to soak for 1 hr at room temperature.

   As for lysis of adult fish, this step greatly reduces contamination from natural flora.
3. Remove most of the zebrafish embryo medium with a pipet tip, taking care that the embryo remains in the tube. Add 500 µl of 200 µg/ml tricaine, and place tubes for 1 hr on ice.

   The minimum time required to kill the embryo is 1 hr. Shorter incubations may result in the embryos reviving when brought back to room temperature.
4. Remove tricaine solution and add 150 µl of 1× trypsin-EDTA. Incubate samples 1 hr at 30°C.
5. Vortex tubes on maximum setting for 2 min and microcentrifuge briefly at maximum speed to ensure all the liquid is returned to the bottom of the tube.
6. Return tubes to 30°C. Vortex samples every hour until there are no visible signs of embryo debris (~4 to 5 hr total), ending with a brief microcentrifugation each time.
7. When embryo digestion is complete, add 30 µl PBS and 20 µl of 1% Triton X-100 to each tube and vortex 2 min.
8. Place samples in a water bath ultrasonicator for 10 min. Microcentrifuge samples to consolidate liquid.
9. Pipet entire volume of each tube onto 7H10 agar plates and spread evenly.
10. Incubate plates at 33°C until colonies can be counted.

    Expect anywhere from five to hundreds of colonies per plate, depending on factors discussed in Basic Protocol 6. If larger infection loads are anticipated, prepare serial dilutions of the embryo lysate.

SUPPORT PROTOCOL 2

PREPARATION OF *M. MARINUM* STOCKS FOR INFECTION OF LARVAE

This protocol describes the preparation of *M. marinum* suspension which is suitably concentrated and less noxious to the bloodstream of the zebrafish embryo. See introduction to Support Protocol 1 for additional discussion on the preparation of *M. marinum* for infection experiments.

Materials

M. marinum (ATCC #BAA-535 or other strain of interest) frozen stock (*UNIT 10B.1*)
Zebrafish embryo medium (see recipe), sterile, or PBS (*APPENDIX 2A*)
20% (w/v) phenol red dye stock solution in H_2O
1-ml tuberculin syringes with 27-G needles
Additional reagents and equipment for preparing *M. marimum* (see Support Protocol 1, steps 1 to 3) and loading the microinjector needle (see Basic Protocol 6, steps 1 and 2)

1. Prepare *M. marinum* as described in Support Protocol 1, steps 1 to 3. Grow to an OD_{600} between 0.3 and 0.9.
2. No more than 1 hr before injection, vortex the culture vigorously and pipet 200 µl into a sterile 1.7-ml microcentrifuge tube. Microcentrifuge 2 min at maximum speed.

3. Remove supernatant and resuspend the pellet in the appropriate volume of zebrafish embryo medium or PBS.

   IMPORTANT NOTE: *Bacterial viability appears to decrease with extended time in zebrafish embryo medium, therefore, prepare the suspensions as close to injection time as practical and leave in zebrafish embryo medium for no more than 1 hr.*

   Resuspending in the original volume (200 μl) produces a 1× solution, which will yield inoculates of roughly 100 to 300 bacteria per 'full red' injection. A 1:10 dilution of this resuspension produces a 0.1× bacterial stock. A 'full red' injection with a 0.1× stock yields roughly 20 to 100 bacteria per 'full red' injection. These numbers should only be taken as a recommended starting point. Each researcher develops a sense for how much injection time, and how thick a bacterial suspension will be required to achieve a desired dose. See Support Protocol 3 and Commentary for additional discussions of dosing.

4. Just before loading the microinjection needle, pass the bacterial suspension through a 27-G needle attached to a 1-ml syringe 5 to 20 times to reduce clumping.

   An appropriate balance must be struck between reducing clumps and lysing bacteria. Ideally, the suspension should be passed through the needle only enough to reduce clumping. The bacterial suspension will become clear with increased passages through the needle. See Support Protocol 3 for discussion on estimating doses.

5. Add 20 μl bacterial suspension to a fresh 1.7-ml microcentrifuge tube containing 1 μl of 20% phenol red stock solution. Mix well prior to loading the needle as directed in Basic Protocol 6, step 2.

PLATING INJECTION VOLUMES TO ESTIMATE *M. MARINUM* INOCULUM

SUPPORT PROTOCOL 3

A single needle will not be consistent in dosage for >10 to 15 injections (dosage drops considerably after this). For the best measure of dosage, prepare the needle as described in Basic Protocol 6, inject one fish and determine the injection time and pressure required. Plate the determined injection time (see below), then inject an additional five to six fish before plating again. Plate one additional plate after the last embryo with the same needle. Consistency between injections and platings is necessary for platings to reflect the inoculum given. Inocula of 200 to 250 cfu induce heavy infections, with granulomas forming as soon as 2 days post-injection. Inoculations with >300 cfu induce infections so severe that embryos may not be useful. If very low doses are needed (10 to 20 cfu), it may be useful to continue with a single needle for up to 50 fish to allow the dosage to decrease through a desired range. Using this method, it is necessary to plate intermittently, inject extra embryos, and screen through them for the desired level of infection (see Commentary).

Materials

PBS (*APPENDIX 2A*)
Middlebrook 7H10 agar plates (see recipe)
Injection needles loaded with bacterial suspension (see Basic Protocol 6 steps 1 to 10)
Micromanipulator (e.g., Narishige GJ-1) and microinjector (e.g., Eppendorf 5246)
33°C incubator

1. Apply a 20-μl drop of PBS to the middle of a Middlebrook 7H10 agar plate.
2. Elevate the injection needle containing the bacterial suspension with the micromanipulator, slide the 7H10 agar plate under it, and lower the needle near the top of the PBS droplet.

   Extreme caution must be taken so as not to break the needle tip during this manipulation.

3. Lower the needle into the PBS and *immediately* apply the standard amount of injection time, then *immediately* lift the needle out of the PBS.

4. Spread the PBS and bacteria evenly onto the plate and incubate for 5 to 7 days at 33°C, or until colonies can be counted.

If using fluorescent bacteria, an alternative method involves "injecting" the dosage amount onto a slide containing 20 μl distilled water. The droplet of liquid is allowed to dry and the number of fluorescent bacteria can be counted directly using a fluorescence microscope. Using a slide inscribed with a microscopic grid can be helpful. Hemacytometer slides work well. Of course, it is essential to back this method by plating for viable cfu, but the numbers have been found to correlate fairly well.

SUPPORT PROTOCOL 4

MANUAL DECHORIONATION OF ZEBRAFISH EMBRYOS

Growing zebrafish larvae will dechorionate themselves between 3 and 5 days post-fertilization. However, even if no procedures are planned until after 5 days post-fertilization, it is a good idea to remove the chorions and move the larvae into fresh medium on day 2 post-fertilization. Dechorionation on day 1 post-fertilization may cause embryos to stick to plastic without special treatment. The chorion is an excellent place for harmful microbes to grow during incubation. In developmental biology studies, dechorionation is often done enzymatically on the first day of life (Westerfield, 2000), but given the small number and more advanced age of the embryos used here, manual dechorionation with two pairs of extra-fine jeweler's forceps is preferable. This manipulation is best performed using a dissecting microscope at 20× total magnification.

Materials

Zebrafish embryos (see Strategic Planning)
Zebrafish embryo medium (see recipe), sterile
100 × 15–mm petri dishes (or similar size)
Two pair extra-fine jeweler's forceps (Miltex #17-305 or Dumont #5)
Dissecting microscope

NOTE: Keep forceps for dechorionation separate from those used for injections.

1. Place embryos in 100 × 15–mm (or similar size) petri dishes with just enough zebrafish embryo medium (with or without PTU, see Critical Parameters) to cover the bottom.

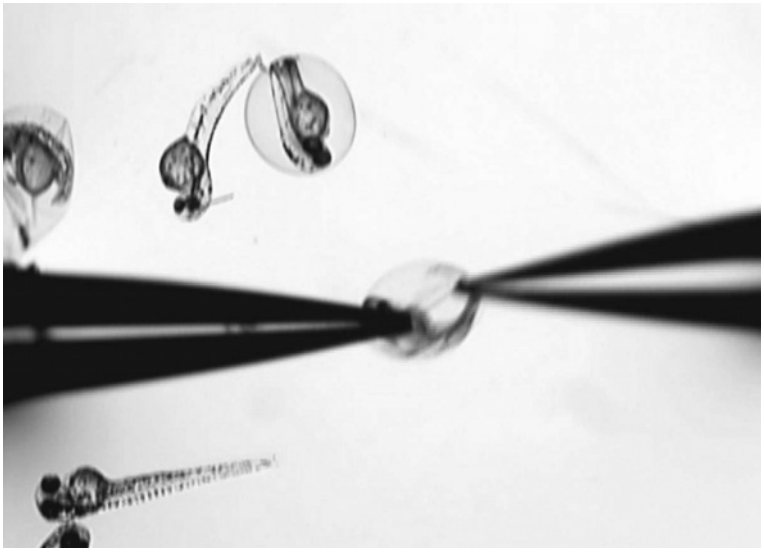

Figure 10B.2.8 Manual dechorionation of ~30 hr embryos. See Support Protocol 4 for details.

2. Gently swirl the medium inside the petri dish, allowing the embryos to collect in a cluster at the center.

3. Manually remove chorions one embryo at a time.

   Many techniques exist, but start by pinching a small portion of the chorion with one pair of extra-fine jeweler's forceps while steadying the embryo with the other pair of forceps. Next, pinch the chorion near the first pinch with the second pair of forceps, and pull apart (see Fig. 10B.2.8). It may help to pull the torn chorion up to the air/water interface, and gently tug the chorion upward, leaving the embryo in the water.

4. Separate embryos from chorions and transfer embryos to fresh medium.

   CAUTION: *Chorion fragments may harbor environmental microorganisms harmful to developing zebrafish. Hence, dechorionation and transfer to fresh water improves embryo survival.*

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

7H9 liquid culture medium

4.7 g Middlebrook 7H9 broth base (Difco)
4 ml 50% (v/v) glycerol

Dissolve base in water, add glycerol, bring to final volume of 900 ml, autoclave. Store up to 3 months at room temperature. Before use, add 100 ml OADC stock (see recipe) and 2.5 ml of 20% (v/v) Tween 80. Store up to 2 months at 4°C.

There is a great deal of variability in how 7H9 and 7H10 media are prepared in different laboratories. In fact, OADC does not mean the same thing in all laboratories. The authors have not compared M. marinum grown in subtly different media in side-by-side infection experiments and therefore cannot comment on the effect of using media other than those presented here.

7H10 agar plates

19 g Middlebrook 7H10 base (Difco)
10 ml 50% (v/v) glycerol
Dissolve base in water, add glycerol, and bring to final volume of 900 ml
Autoclave, allow to cool to ~42°C
Add 100 ml OADC supplement (see recipe)
Pour ~35, 100 × 15–mm plates (see *APPENDIX 4A*)
Store up to 2 months at 4°C

See comments regarding media variation above.

Dietrich's fixative

150 ml 100% ethanol
50 ml formalin
10 ml acetic acid
290 ml H_2O
Store indefinitely at room temperature

EMP

7.5 ml PTU stock solution (see recipe)
100 ml zebrafish embryo medium (see recipe)
Store up to 1 week at room temperature

EMPT, 1× (for visualization of embryos)

10 ml EMP (see recipe)
420 μl tricaine stock solution (see recipe)

Working concentrations last ~1 hr at room temperature.

EMPT, 1.5× (for injection of embryos)

10 ml EMP (see recipe)
630 μl tricaine stock solution (see recipe)

Working concentrations last ~1 hr at room temperature.

Fish water

Prepare water for fish by adding sea salt to a conductivity of 1500 μS. To a mesh bag, add 1 teaspoon (4.9 cm^3) crushed coral ($CaCl_2$) for every 10 gallons (38 liters) of water to be treated. Place in the spillway (plastic receptacle which receives all discharge water and channels it into the sump). Measure conductivity, pH, and temperature twice daily to be certain conditions are within range. Ideal operating parameters are: temperature = 27° to 28°C, pH = 7.4 ± 0.2, and conductivity = 1500 ± 100 μS). For tricaine solutions, tricaine is simply added to this solution in the desired amount.

If difficulties in anesthetizing occur, check the pH and correct to 7.2 as needed with sodium bicarbonate.

Frog water, 500×

250 g Instant Ocean (Aquarium Systems)
10 g KCl
15 g $CaCl_2$
5 liters ddH_2O
Store tightly sealed in a Nalgene carboy up to 6 months at room temperature
Dilute to 1× prior to use

Hanks' stock solutions

Stock #1:
8.0 g NaCl
0.4 g KCl
100 ml H_2O
Stock #2:
0.358 g Na_2HPO_4 anhydrous
0.60 g KH_2PO_4
100 ml H_2O
Stock #4:
0.72 g $CaCl_2$
50 ml H_2O
Stock #5:
1.23 g $MgSO_4 \cdot 7H_2O$
50 ml H_2O
Stock #6:
0.35 g $NaHCO_3$
10 ml H_2O

All of Hanks' stock solutions should be stored indefinitely at 4°C. Adapted from Westerfield (2000).

OADC supplement

Dissolve the following in ~700 ml dd H_2O:
50 g BSA, fraction V
50 ml 1% oleic acid (see recipe)
20 g dextrose
8.5 g NaCl
Bring to 1 liter final volume
Filter sterilize using a 0.22-μm filter
Store up to 6 months at 4°C

Oleic acid, 1%

Add 5 g oleic acid to 500 ml 0.2 N NaOH. Heat solution to 55°C to dissolve completely. Store at −20°C in 50-ml aliquots indefinitely. Thaw at 65°C prior to use with OADC.

PTU stock solution

0.06 g 1-phenyl-2-thiourea (PTU)
100 ml zebrafish embryo medium (see recipe)
Store up to 1 month at 4°C

PTU may take several hours to completely dissolve with constant stirring using a stir bar.

Supplements to 7H10 agar plates

40 ml 250 μg/ml amphotericin B stock
500 μl 50 mg/ml polymixin B sulfate
400 μl 50 mg/ml trimethoprim
1 ml 50 mg/ml carbenecillin, disodium salt
Store amphotericin B, polymixin B, trimethoprim and carbenecillin stocks up to 1 year at −20°C

Supplements are formulated per liter of 7H10 agar plates.

Tricaine stock solution

400 mg tricaine (Sigma)
97.9 ml H_2O
~2.1 ml 1 M Tris·Cl, pH 9 (*APPENDIX 2A*)
Adjust pH to ~7
Store up to 1 month at 4°C

Adapted from Westerfield (2000).

Zebrafish embryo medium

1.0 ml Hanks' stock solution #1 (see recipe)
0.1 ml Hanks' stock solution #2 (see recipe)
1.0 ml Hanks' stock solution #4 (see recipe)
95.9 ml H_2O
1.0 ml Hanks' stock solution #5 (see recipe)
1.0 ml Hanks' stock solution #6 (see recipe)
Use about ten drops 1 M NaOH to pH 7.2
Store indefinitely at 4°C

Adapted from Westerfield (2000).

COMMENTARY

Background Information

Mycobacteria are intracellular pathogens known for their ability to establish persistent infections. *Mycobacterium marinum*, a natural pathogen of poikilothermic species such as the frog and fish models described here, mediates infection in much the same way that species of the *Mycobacterium tuberculosis* complex cause infection in mammalian hosts (Cosma et al., 2003). *M. marinum* infection of ectotherms has gained popularity as a model for tuberculosis infections for a variety of reasons (Ramakrishnan, 2004). First, the lower growth temperature of this organism makes it less hazardous for human health (see Strategic Planning, Safety Considerations). Second, its faster generation time allows work to proceed more quickly than with *M. tuberculosis* complex organisms. For example, colonies appear on plates in 5 to 7 days as compared to 3 weeks for *M. tuberculosis*. Finally, *M. marinum* infection of zebrafish and frogs allows the study of two important aspects of mycobacterial infection that are not well replicated in the *M. tuberculosis*-mouse model. The first aspect is that infection of frogs leads to a chronic and asymptomatic infection for the lifetime of the animal, modeling the long latency seen in some humans infected with *M. tuberculosis* (Ramakrishnan et al., 1997). The second aspect is that the zebrafish model allows the study of caseating granulomas (Cosma et al., 2004), another important feature of human disease not replicated by the mouse model.

The zebrafish embryo model presents a unique opportunity to study early infection in unprecedented detail (Davis et al., 2002). Even before the onset of blood circulation, zebrafish embryos contain a population of embryonic macrophages derived from the 'rostral blood island' (Davidson and Zon, 2004; Fig. 10B.2.9) that participate in developmental processes such as the phagocytosis of apoptotic bodies. These embryonic macrophages are fully competent to participate in the control of infectious processes (Traver et al., 2003). In addition, there may be a second wave of myelopoiesis from near the caudal vein (or 'caudal blood island') beginning ~4 days post-fertilization (Herbomel et al., 1999), but it is uncertain the extent to which these macrophages participate in infection. Adult myelopoiesis is known to occur in the head kidney, although the precise timing of this process in development has not yet been ascertained. There is also a population of circulating neutrophil-like cells called heterophils, identified by myeloperoxidase expression as blood flow is initiated (reviewed in Davidson and Zon, 2003). Lymphocytes do not begin circulating until approximately day 9 post-fertilization (Danilova et al., 2004), allowing a window of opportunity in which to examine the contributions of innate immunity to mycobacterial infection in isolation. The second feature of the zebrafish embryo model is the fact that these embryos remain optically

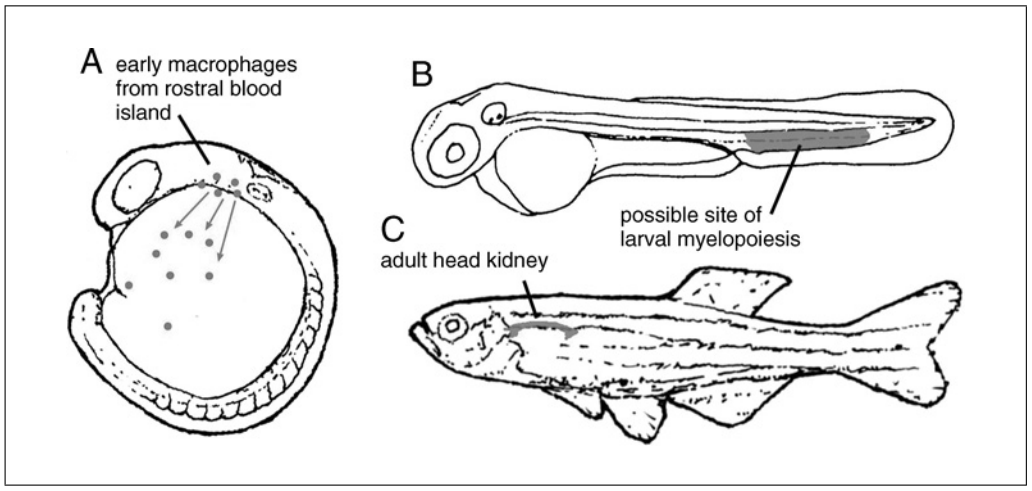

Figure 10B.2.9 Sources of macrophages in the developing zebrafish. (**A**) Early macrophages arise in the 'rostral blood island' around the 5-somite stage and begin to migrate into the future yolk circulation valley around the 14-somite stage. (**B**) There is a 'nest' of likely hematopoiesis near the caudal vein during early larval stages. It is suspected but not known if a distinct wave of macrophages is produced here. (**C**) The main site of myelopoiesis in the adult is the head kidney. Adapted from Westerfield (2000) and Davidson and Zon (2004).

transparent for the first week of life, allowing the visualization of an infectious process in vivo in real time. The embryos are best observed using a combination of differential interference contrast (DIC) and fluorescence microscopy when infected with fluorescent bacteria.

Critical Parameters

Adult frog infections

Rana pipiens used for experimental infection are wild-caught and outbred animals and, as such, exhibit variable responses to infection as measured by tissue cfu burden (Ramakrishnan et al., 1997). Therefore, a sufficient number of animals should be used to obtain statistically significant results. Immunosuppression using corticosteroids will result in fulminant infection and death of the animals.

Adult zebrafish infections

Mycobacteriosis in fish is a chronic, long-term infection relative to other fish diseases. It is characterized by histological observation of acid-fast bacilli and caseous granulomas occurring within the hematopoietic organs, namely the liver, kidney, thymus, spleen, and pancreas (Gauthier et al., 2003). Yet, *M. marinum* is clearly more pathogenic for zebrafish than for frogs, causing a slow but progressive illness that results in the death of the animal. The acute phase response to the formation of thousands of granulomas and internal organ remodeling causes several gross outcomes. Some fish may develop a large focalized lesion in the peritoneum, while others display a diffuse hemorrhagic condition that progresses into systemic ascites where the scales of the fish stand on end, like a "pine cone." This condition is typical in fish and results from a generalized bacteremia and kidney failure. Sick fish behave differently than healthy fish, sitting on the bottom of the tank or hiding in a corner to ward off attacks by healthier tankmates. Pecking at the weaker fish by healthier ones may operate as a mode of transmission among fish within a group. Mortalities are best collected immediately because healthier tankmates eat the gut of infected, fallen fish.

To minimize in-tank effects, each tank should be statistically evaluated in an independent manner and therefore, tank replication is essential. In-tank effects include group behavioral differences, sex ratio differences, and transmission variability.

Zebrafish embryos, general aspects of the infection

Microinjection can be used to infect embryos with doses ranging from <10 to >300 mycobacteria. Survival of embryos is dose dependent. For example, a small fraction of very lightly infected (10 to 20 cfu) embryos can survive 1 to 2 months (Volkman et al., 2004), although the vast majority will not survive more than 2 weeks (Davis et al., 2002). This does not appear to be due to the injection process itself, as mock-infected embryos, and those infected with attenuated strains of mycobacteria will survive to adulthood (Volkman et al., 2004). In contrast, extremely heavy doses (200 to 300 cfu) kill the embryos in a few days. The exact results are expected to vary from laboratory to laboratory and will depend upon the strains of zebrafish and mycobacteria used. The standard zebrafish strain used the authors' laboratory is the 'AB' strain, while the wild-type *M. marinum* strain used is strain M (ATCC #BAA-535).

It should be noted here that sustaining zebrafish larvae, infected or not, beyond 9 to 10 days post-fertilization is difficult. The methods necessary for rearing zebrafish are beyond the scope of this unit, but some general concepts should be considered. Larvae that are required for longer than 10 days should be housed at low concentrations (30 to 35 embryos per 100–mm petri dish) starting 24 to 48 hr post-fertilization, with daily water changes. On day 5 post-fertilization, feeding should commence with paramecia or other small-size food particles. Bear in mind that the larvae are visual feeders and therefore, require reasonably bright light to seek their food. For bench-top incubation of infected fish, use a low-wattage bulb in a desk lamp, or similar, and place the dishes on a white surface for maximum illumination. Water changes twice a day may be necessary once feeding begins to stem the growth of harmful paramecia such as *Coleps sp*. At 10 days post- fertilization, food should be shifted to larger fare such as *Artemia* (see Fish Care). The larvae must feed well from days 5 through 10 to develop jaws appropriate for eating larger food. Thus, the transition to eating *Artemia* is the point where many larvae die, and the point after which mortalities of uninfected fish begin to drop off.

Dosing for zebrafish embryo infections is as much an art as a science. New investigators should expect to observe considerable variability at the outset. Variation will be observed from culture to culture, and from injection to

injection, producing the need for operator consistency with injection time and periodic plating of the injection volume. The authors find that a given needle will give gradually decreasing doses, so for maximum reproducibility, it is best to plate the same needle before and after injecting the embryos. While consistent culture densities, resuspension volumes, and injection amounts ('vein only,' 'blush,' 'full red') can help control the applied dose, enough variety is still seen in doses to require constant assessment of the number of bacteria applied to each fish. Plating needles (on agar for cfu determination, and on microscope slides for visual inspection) and lysing fish to count bacteria soon after infection are the main ways to assess dosage (see Basic Protocol 4 and Support Protocols 2 and 3 for details). However, most researchers find that using fluorescent bacteria allows direct observation in each embryo of the relative level of infection within a few hours of injection. If a particular level of infection is desired, it is helpful to perform a range of injections and screen them afterwards for those individuals with the desired level of infection.

Visualizing the infection by microscopy

A typical light microscope fitted with DIC (differential interference contrast; see *UNIT 2A.1*) optics and capable of fluorescence microscopy is useful for many kinds of observations in zebrafish embryos and larvae. For screening purposes, individual embryos can be held in glass 10-well plates (Aquatic Eco-systems, Inc.) and viewed with a 10× or 20× objective on a compound microscope (note that a standard microscope eyepiece adds 10× more total magnification to the objective, yielding 100× or 200× total magnification, respectively. Herein, the authors will refer to the objective lens magnification only). A variable-magnification dissecting microscope equipped with epifluorescence is especially useful for screening embryos infected with fluorescent bacteria. For visualizations under 45 min, embryos should be anesthetized in 1× EMPT (less tricaine than for injections; see recipe in Reagents and Solutions) and placed under a 22 × 22–mm coverslip in a deep-well microscope slide (use deeper wells for injection and shallower wells for visualization; VWR). Details of this mounting technique are shown in Figure 10B.2.10. However, be aware that, although fine for fluorescence microscopy, the deep-well slide 're-lenses' focused light from the condenser, interfering with transmitted light microscopy such as DIC. Another mounting technique is described in Westerfield (2000). For longer viewing sessions, or for applications requiring absolute immobilization, embed the embryos in 1% low-melt agarose in zebrafish embryo medium. Small aliquots of this agarose in 1.7-ml microcentrifuge tubes will keep 3 to 4 days at ∼40°C. To mount an embryo, simply drop it into a tube of agarose and then pipet it back out and onto a warmed (40°C) slide. The warm slide allows time to position the embryo before it is immobilized. This agarose method can be used with deep-well slides or in the viewing chamber described in

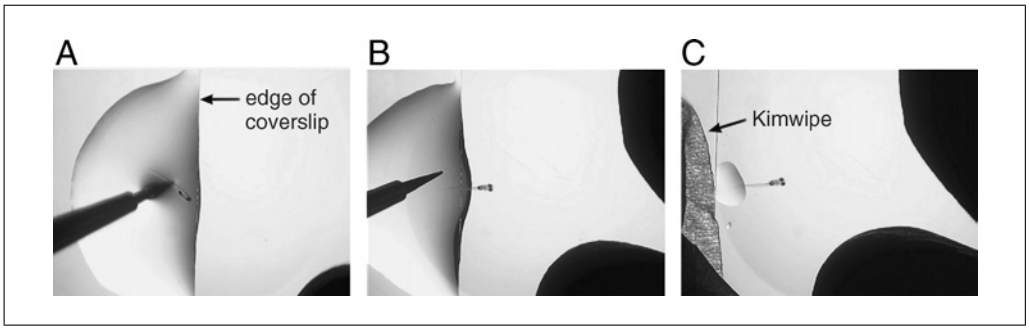

Figure 10B.2.10 Mounting an embryo on a deep-well slide, by a left-handed operator. (**A**) Using a fine paintbrush to manipulate the embryo, slide it under the edge of the coverslip, then gently slide the coverslip to cover the well. (**B**) Finish moving the embryo under the coverslip before it is forced too high up the side of the well. Embryos and larvae of various sizes can be held in place by adjusting how close to the edge of the well they are mounted. (**C**) Blotting away excess zebrafish embryo medium clamps the coverslip down securely onto the slide. Because of the shape of the embryo at its thickest point, a side view will either favor the dorsal or ventral view. The mount shown gives a better view of the ventral aspect of the left side, allowing a good view of the heart and pericardium, common locations for infected macrophages. This view also maintains the traditional pose—dorsal up, head to the left—when viewed under a compound microscope. Note that DIC microscopy is compromised when using deep-well slides—see text.

Westerfield (2000). Such visualizations are not fatal to the embryo if care is taken in removing them from the agarose; gently crushing the agarose around the embryo with a fine paintbrush, or prying the agarose apart with the tips of a fine pair of forceps both work well. More elaborate mounting methods for long-term viewing in agarose are described in Kamei et al. (2004) and Kamei and Weinstein (2005). For more information on general confocal microscopy, see *UNIT 2C.1*.

Because of its crystalline appearance, the zebrafish embryo can be easily viewed by DIC microscopy. The authors use 4× and 10× objectives for overall viewing of embryos, and 40× for quick, high-magnification detail views. Because of the short working distance, most conventional higher-magnification objectives do not allow for viewing more than portions of the head, tail, and yolk ball. However, long-working-distance water objectives are now available at 60× and 100×, allowing observation in exquisite detail as deep into the fish as the tissue allows. 'Dipping' lenses on confocal microscopes have similar advantages.

For fluorescence microscopy, 10× and 20× objectives are useful for overall viewing and imaging, while an objective of at least 40× is needed to detect single fluorescent bacteria in most cases. The authors also use the 60× (long-working-distance) water objective for fluorescence (such as, Nikon 93149 CFI W FLUOR 60× water immersion) although at these magnifications, either deconvolution or confocal microscopes become the obvious choice.

Confocal imaging of zebrafish embryos can also be performed and requires little additional comment, other than that the coverslip wells, available from Lab-Tek, with embryos mounted in agarose provide a fine mount for inverted microscopes. Also, for viewing with inverted microscopes, the embryo can be immobilized in agarose (as noted above) in a petri dish with a coverslip bottom, such as Wilco Wells #GW-3522. An embryo or larva immobilized in this way and covered with 1× EMPT may be visualized for hours (similar to the results of Kamei et al., 2004). Newer confocal microscopes capable of 'spectral unmixing,' which narrows the range of emitted light detected, can reduce problems associated with the green autofluorescence seen in zebrafish tissue. Long-working-distance 'dipping lenses' are also available for confocal microscopes (see above). Such lenses can be used on inverted microscopes by the careful application of a latex cuff (often a segment from a condom) to the end of the lens (Cody and Williams, 1997). For more details concerning confocal imaging of the developing zebrafish embryo, see Kamei et al. (2004) and Kamei and Weinstein (2005).

It should be noted that keeping the embryos in a solution of PTU (1-phenyl-2-thiourea) starting ~12 hr post-fertilization is essential to delay the development of melanocytes for maximum optical clarity (Westerfield, 2000). Putting them in PTU later than 24 to 28 hr post-fertilization is less effective. The effects of PTU on long-term development are unknown. Some researchers choose to keep all embryos in PTU if they are to be visualized by microscopy. Those being reared to adulthood, or being followed for other purposes may be better off without PTU. The albino *(alb*^{b4}) zebrafish (Streisinger et al., 1986) develop without melanophores, and although mycobacterial infection has not been studied in this line, it is an alternative to the use of PTU (Lawson and Weinstein, 2002).

Anticipated Results

Adult frog infections

Experimental inoculation of adult *Rana pipiens* with *M. marinum* results in a life-long, but asymptomatic infection (Ramakrishnan et al., 1997). Inoculation with as few as 10 to 100 bacteria will result in a chronic infection; however, bacteria may not be detectable in all animals until 4 to 6 weeks post-infection. Granulomas are reliably observed at doses of $>10^4$ cfu. Fairly low levels of infection (10^2 cfu/g tissue) can be detected by cfu determination, although 10^5 to 10^6 cfu/g tissue are needed for consistent visualization in sections (Cosma et al., 2004).

Adult zebrafish infections

Injecting bacteria is the most effective route of experimental inoculation and is most reliable for replicating consistent infections. There appears to be a range of survival responses depending on the initial dose administered. When a low number of bacteria is delivered (e.g., ~5 cfu), the acute phase of pathogenesis may take 6 to 8 weeks to develop. When >200 cfu are delivered, the acute phase results in ~1 week. An intermediate inoculum strikes an optimal pathogenesis for experimental observation. At moderate to high doses, hallmark clinical signs of disease manifest within 3 weeks, and culminate in 100% mortality of the tank population in ~3 months time. At low doses, some fish may

clear the infection while others go on to develop acute disease. For further details, consult Swaim et al. (2006).

Zebrafish embryo

In general, the infection proceeds as follows (Davis et al., 2002; Volkman et al., 2004): free bacteria can be seen in the blood for as long as 3 hr post-injection, but the majority are taken into macrophages quickly, a process that can be viewed in the yolk circulation valley. After 3 to 4 hr, infected macrophages are seen in various tissues. Over the next 2 to 5 days (depending on infection dose) the bacteria multiply within the macrophages, and aggregates of infected cells will form. Likely sites for aggregation are ventral to the caudal vein (a site of early hematopoiesis), in muscle near the injection site, in the pericardium, and in the brain. Bacterial dose contributes to the rate of aggregate formation. The authors have produced aggregation in as few as 2 to 3 days with heavy doses, while lightly infected embryos may not form aggregates for 5 to 7 days. In general, fish that form aggregates early in larval development die of the infection more quickly, but the vast majority will die within 2 weeks regardless of dose.

Time Considerations

Frog infections

Acclimatization requires 2 to 4 weeks and culture preparation will take 1 week. Injection time for 60 frogs is 2 hr. The duration of infection depends on the experimental goals. The authors have observed mycobacteria in the liver as early as 3 days following infection, and have isolated mycobacteria from livers of frogs infected for as long as 17 months. Up to 60 frogs can be killed, tissues harvested, and homogenates plated by a team of three researchers in a (grueling) 12-hr day. Allow 7 to 14 days for complete colony counts, depending on the vigor of the bacterial strains being used. To freeze tissues, allow 4 to 24 hr for fixation and rehydration, and 10 to 15 min to freeze each tissue.

Infection of zebrafish embryos

Start cultures from frozen stocks 5 to 7 days before the planned injection (if diluting from an existing stock, try a 1:100 dilution 3 days before, or a 1:10 dilution 2 days before) to obtain the appropriate growth density. Either process requires 15 to 20 min. Set up adult fish to spawn 1 day before evening injections, or 2 days before morning injections. This requires 15 to 30 min depending on the fish facility. Dechorionate embryos on day 2 post-fertilization, this will take 1 to 2 hr per 300 embryos, depending on the speed of the researcher. Measuring bacterial density, diluting, and preparing bacteria will require ~1 hr. Preparing EMPT, embryos, and injection needle will take ~0.5 hr. Depending on the injector, 50 to 150 embryos should be injected per hour. With two people, one to inject and one to load the needles and prepare the plates, it is possible to perform ≥300 injections per hour. Plating bacterial inoculum from a microinjection needle requires 1 to 2 min per plate. Allow 7 to 10 days for colonies to grow. Counting colonies requires 1 to 10 min per plate, depending on the number of colonies. The time to aggregate is 2 to 7 days formation depending on inoculum.

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Cultivation and Laboratory Maintenance of *Chlamydia trachomatis*

Chlamydia species are obligate intracellular bacteria that require growth inside a mammalian host cell for propagation and survival. As a result, *Chlamydia* cannot be grown on conventional bacteriological medium. This property makes *Chlamydia* difficult organisms to grow and maintain in the laboratory. Up until 1965, passage in the yolk sack of the embryonated hen egg was the only way to isolate and propagate the organism. Since then, a tissue culture system has been available that allows easier laboratory culture and maintenance of the *Chlamydia* species. The advent of tissue culture growth systems have allowed researchers to isolate, culture, and purify large quantities of chlamydial organisms from both clinical specimens and laboratory stock cultures, and has made possible the tremendous advancements in chlamydial research that have occurred during the last 30 years.

With the exception of lymphogranuloma venereum (LGV) serovars, most *Chlamydia trachomatis* strains do not readily infect tissue culture cells. Infectivity can be enhanced by either chemical or mechanical assistance. Basic Protocol 2 describes the procedure used for infecting monolayer tissue culture cells with *C. trachomatis* by pretreating tissue culture cells with diethylaminoethyl (DEAE)-dextran, whereas the Alternate Protocol describes the procedure for infecting monolayer tissue culture cells by centrifugation of the bacterial inoculum onto the host cell monolayer. Although *Chlamydia* can be easily passaged in tissue culture without direct purification, most researchers purify the bacteria away from host cell components for almost all research applications. Basic Protocol 3 describes a large-scale density gradient purification of chlamydial elementary bodies (EBs) that have been propagated in monolayer tissue culture cells. By altering the time that *Chlamydia* are harvested and the homogenization method performed, the replicative form of *Chlamydia* (reticulate bodies or RBs) can also be purified away from host cell components. Basic Protocol 4 describes the procedure for density gradient purification of RBs. *Chlamydia* can be obtained from the American Type Tissue Culture (ATCC), individual chlamydial researchers, or directly from clinical specimens. The isolation of *C. trachomatis* from clinical specimens is described in Basic Protocol 1 and the procedure for calculating the number of infectious forming units of chlamydial stocks is described in Support Protocol 2. The procedure for determining the sterility of chlamydial stocks is described in Support Protocol 1. Finally, Support Protocol 3 describes the procedure for culture and passaging mammalian monolayer tissue culture cells that are needed for growth of *C. trachomatis*. Monolayer tissue culture cell lines can be continually passaged in culture and are the most common cell type used for propagating *C. trachomatis* in the laboratory. HeLa 229, McCoy, and L929 are the most common cell lines used because they are susceptible to all *C. trachomatis* strains. Other cell lines may be used, but the susceptibility to *C. trachomatis* infection and incubation times must be determined by each researcher.

CAUTION: When working with *C. trachomatis*, appropriate safeguards must be utilized in situations where aerosols can be generated (e.g., sonication and centrifugation steps), as these procedures may pose a greater risk for laboratory infections. During these procedures, wear protective clothing, including lab coat, gloves, safety glasses, and mask. All procedures should be performed in a BSL-2 biosafety cabinet unless otherwise indicated.

Contributed by Marci A. Scidmore

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CAUTION: Chlamydia trachomatis is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All solutions and equipment that come into contact with living cells must be sterile and aseptic technique must be used.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise indicated.

BASIC PROTOCOL 1

ISOLATION OF *C. TRACHOMATIS* FROM CLINICAL SPECIMENS

Although many laboratory adapted strains of *C. trachomatis* are readily available, *C. trachomatis* can also be isolated from a wide variety of clinical specimens, including urethral, urogenital, and rectal swabs, as well as from tissue samples. The following procedure is used to isolate *C. trachomatis* strains from clinical specimens by serially infecting McCoy monolayer cells and to prepare frozen stocks of amplified clinical isolates. McCoy cells are exclusively used for this procedure because they are permissive for all *C. trachomatis* strains. Since clinical specimens are generally less infective than laboratory adapted strains, centrifugation is always used to enhance infectivity when isolating clinical specimens. Once sufficient numbers of organisms are obtained, Support Protocol 2 can be used to calculate infectious forming units of the amplified stock. Density gradient–purified EBs can then be obtained by following Basic Protocol 2 and Basic Protocol 3. If using laboratory-adapted strains that are obtained from either the ATCC or a chlamydial researcher, Basic Protocol 1 can be omitted and the researcher can proceed directly to Basic Protocol 2 or the Alternate Protocol, as well as Basic Protocol 3, depending on the specific application or the quantity or purity of the organisms that is needed.

Materials

Freshly trypsinized McCoy cells (see Support Protocol 3)
Clinical specimens stored in chlamydial transport buffer (see recipe for chlamydial transport buffer), 4°C
Complete medium supplemented with 10% FBS (see Critical Parameters) supplemented with 1 μg/ml cycloheximide (see recipe), 37°C
Sucrose/phosphate/glutamate buffer (SPG; see recipe)
12-mm shell vials
48-well tissue culture plates
Sonicator
5-ml sterile tubes
Ten Broek tissue homogenizers (for tissue samples only)
Low-speed bench-top centrifuge with tissue plate adaptors
Additional reagents and equipment for fixing and staining cells (see Support Protocol 1)

1. Seed freshly trypsinized McCoy cells into 12-mm shell vials and 48-well tissue culture plates (1 vial and 1 well/sample). Add 1 ml of at least 1×10^5 cells/ml to each vial. Incubate cells 24 hr or until a monolayer is formed.

2a. *For samples containing heavy debris or from rectal swabs:* Sonicate specimens for 10 sec at a frequency that does not cause cavitation or bubble formation. Centrifuge 10 min at 175 × *g*, 4°C. Transfer supernatant to a 5-ml sterile tube and discard pellet, which contains cellular debris.

2b. *For tissue specimens:* Homogenize in a Ten Broeck tissue homogenizer and sonicate for 20 sec at a frequency that does not cause cavitation or bubble formation.

3. Vortex specimen tubes for 10 sec.

4. Inoculate McCoy cells with 300 µl of each sample (1 shell vial and 1 well/sample) and centrifuge 60 min at 900 × *g*, 37°C. Add 1.0 ml complete medium supplemented with 10% FBS and 1 µg/ml cycloheximide and incubate infected cultures for 48 hr.

   For tissue specimens, dilute sample 1:10 in 1× HBSS and inoculate McCoy cells with 300 µl of diluted sample.

   Incubation time varies among different C. trachomatis serovars and can range from 36 to 72 hr post infection. Infection times are only suggestions, and may need to be altered by individual researchers depending on the cell culture system used and the rates of infections that are achieved by each individual laboratory.

5. Fix and stain infected cell samples in 48-well tissue culture plates with *Chlamydia*-specific antisera by following Support Protocol 2, steps 7 to 13, except increase volumes of primary and secondary antibodies to 400 µl. If inclusions are present precede to step 6.

   This is done to check for inclusion formation to determine whether infectious Chlamydia were present in the specimen.

   If no inclusions are present, sonicate cells in shell vial for 20 sec, and repeat steps 3 to 5, except inoculate entire sample onto fresh McCoy monolayer seeded in 12-mm vials. This step is needed if original samples contain few organisms.

6. If specimen is positive, aspirate culture medium and add 1 ml SPG to each 12-mm vial. Sonicate for 20 sec.

   To maintain a stock of each clinical isolate, Chlamydia may be stored at −80°C at this point.

   At this point in the procedure, the sonicate, which contains a mixture of EBs and lysed cells, can be either stored at –80°C for several years (see Background Information) or used to reinfect fresh monolayers to propagate the organism. If large numbers of organisms are needed, continue passaging the infected tissue sonicate in tissue culture cells. Increase numbers of cells to be infected as needed.

   The number of passages needed to obtain high titers is dependent on numerous factors, including the chlamydial strain and the number of organisms in the original specimen.

CHLAMYDIAL INFECTION OF MONOLAYER TISSUE CULTURE CELLS PRETREATED WITH DEAE-DEXTRAN

BASIC PROTOCOL 2

C. trachomatis are human pathogens and in vivo epithelial cells are the primary target cells of *Chlamydia.* However, in vitro*, Chlamydia* can infect and replicate within both epithelial and fibroblast cell lines of both human and nonhuman origin. Although numerous cell lines are capable of supporting chlamydial growth and differentiation, those most commonly used for culture and maintenance of *C. trachomatis* are McCoy, L929, and HeLa 229 cell lines. Other cell lines may be used, but should be tested for their susceptibility to *C. trachomatis* infection prior to any large-scale experiment. This protocol can be used for tissue culture cells grown in a wide variety of plastic tissue culture vessels, including all sizes of tissue culture plates and flasks. Refer to Table 11A.1.1 for guidelines to help determine appropriate cell concentrations, and inoculation and culture volumes for each of the commonly used tissue culture plates and flasks. In this protocol, infectivity of the non-LGV serovars is enhanced by pretreatment with DEAE-dextran. For large-scale preparations of purified organisms, carry out this protocol prior to proceeding with Basic Protocol 3 or Basic Protocol 4. For small-scale experiments in which tissue culture cells are seeded onto tissue culture plates (with or without coverslips), carry out this protocol or the Alternate Protocol.

Table 11A.1.1 Culture Conditions for Commonly Used Tissue Culture Plates and Flasks

Size of plate or flask	Cell concentration (cells/ml)	DEAE-dextran (ml)	Inoculum volume (ml)	Final culture volume (ml)
6-well plate	4.0×10^5	2	0.5	3
12-well plate	2.0×10^5	1	0.35	2
24-well plate	1.0×10^5	0.5	0.2	1
48-well plate	4.0×10^4	0.35	0.15	0.8–1.0
150-cm^2 flask	1.0×10^7	10	2.0	40–50

Materials

Freshly trypsinized monolayer tissue culture cells (see Support Protocol 3)
Complete tissue culture medium (see Critical Parameters) supplemented with 10% FBS and 10 μg/ml gentamycin, 37°C
10× DEAE-dextran (see recipe)
HBSS (*APPENDIX 2A*)
Stock of titered *C. trachomatis* EBs, −80°C (see Support Protocol 2)
SPG (see recipe)
Complete medium (see Critical Parameters) supplemented with 10% FBS, 10 μg/ml gentamycin, and 1 μg/ml cycloheximide
Platform rocker at 37°C
Inverted phase-contrast microscope

NOTE: DEAE-dextran pretreatment of host cells (step 2) can be omitted with *C. trachomatis* LGV strains.

1. Dilute freshly trypsinized cells into complete medium supplemented with 10% FBS and 10 μg/ml gentamycin, and transfer into an appropriately sized plastic tissue culture vessel. Incubate cells 24 hr.

   Use culture medium that is recommended for each specific cell line.

   Seed cells in an appropriately sized tissue culture vessel for each experiment. See Table 11A.1.1 for the most common tissue culture vessels used and the cell concentration needed for each size.

2. Dilute 10× DEAE-dextran (450 μg/ml) 1:10 to a final concentration of 45 μg/ml with HBSS. Remove culture medium from each tissue culture plate or flask. Add an appropriate amount of diluted DEAE-dextran to each plate or flask. Rock back and forth several times to cover the entire monolayer of cells. Incubate 15 min at room temperature.

   Tissue culture cells are not easily infected with the ocular and genital serovars of C. trachomatis. Therefore, to improve infection of tissue culture cells with these serovars, tissue culture cells are either pretreated with DEAE-dextran, which is thought to neutralize surface charge allowing for more efficient attachment, or the inoculum is centrifuged onto the monolayer (see Alternate Protocol). For large-scale preparations in which the cells are grown in 150-cm^2 tissue culture dishes, centrifugation is not an option, therefore, pretreatment with DEAE-dextran is utilized.

   The lymphogranuloma venereum (LGV) serovars do not require pretreatment with DEAE-dextran nor centrifugation to infect tissue culture cells efficiently. Therefore, step 2 can be eliminated when using LGV serovars.

3. During the DEAE-dextran treatment of the tissue culture cells, remove the frozen stock of *Chlamydia* EBs from the −80°C freezer and transfer immediately to a 37°C water bath. Gently agitate the tube continuously until EBs are thawed. To maintain sterility, wipe the tube with 70% ethanol and store on ice until ready to use.

   EBs should be thawed in <60 sec.

   EBs rapidly lose viability, therefore, thaw stock immediately before use and store on ice. In addition, do not thaw and reuse chlamydial stocks. Discard unused Chlamydia according to appropriate biosafety protocols dictated by each institution.

4. Dilute chlamydial EBs to an appropriate concentration in sterile SPG.

   Monolayer tissue culture cells are commonly infected at a multiplicity of infection (MOI) of 1 to 2 EBs/host cell. The MOI can be altered depending on the specific experiment, however, some non-LGV C. trachomatis strains are cytotoxic to tissue culture cells when infected at an MOI >100. Cytotoxicity of chlamydial strains needs to be determined empirically by each researcher.

   HBSS may be used in place of SPG.

5. Wash cells with 1 to 5 ml HBSS. Remove HBSS by decanting or pipetting and add desired amount of diluted EBs to each flask. Rock the flask back and forth several times to ensure that the inoculum covers the entire monolayer. Incubate flasks on a large platform rocker 2 hr at 37°C.

   Tissue culture plates do not need to be rocked.

   To prevent contamination, ensure that the caps of the tissue culture flasks are tightly closed.

   If unable to rock cultures at 37°C, rock cultures 3 hr at room temperature. Inoculate LGV serovars for 60 min at room temperature or 30 min at 37°C.

   See Table 11A.1.1 for appropriate volumes of EBs used to inoculate each type of tissue culture vessel.

6. Add appropriate volume of complete medium supplemented with 10% FBS, 10 μg/ml gentamycin, and 1 μg/ml cycloheximide to each plate or flask and incubate for desired time (Table 11A.1.2).

   See Table 11A.1.1 for specific volumes of culture medium to be used for each type of tissue culture vessel.

   Incubation time varies among different C. trachomatis serovars and can range from 36 to 72 hr post infection. Infection times are only suggestions, and may need to be altered by individual researchers depending on the cell culture system used and the rates of infections that are achieved by each individual laboratory.

   Cycloheximide inhibits host protein synthesis and gives C. trachomatis a metabolic advantage, thus resulting in higher yields of infectious organisms. The genital and ocular serovars of C. trachomatis require cycloheximide in the culture medium, while the LGV serovars do not. Therefore, cycloheximide can be eliminated from the growth medium when using LGV serovars.

   Because Chlamydia are bacteria, antibiotics such as penicillin and streptomycin commonly used to inhibit bacterial contamination in tissue culture cells cannot be used since they will inhibit chlamydial development. However, Chlamydia replicate within a unique vacuole (the inclusion) that does not fuse with endocytic or lysosomal organelles. Therefore, although gentamycin is routinely used to inhibit extracellular bacterial growth, gentamycin-supplemented culture medium can be used when growing Chlamydia, because gentamycin cannot cross mammalian membranes and does not come into contact with internalized Chlamydia. Although gentamycin may be internalized into mammalian cells by pinocytosis, pinocytotic vesicles also do not fuse with chlamydial-containing vacuoles.

Table 11A.1.2 Culture Conditions for *Chlamydia trachomatis* Infections of Monolayer Tissue Culture Cells

Biovar	Centrifugation or DEAE-dextran	Total incubation time for RBs (hr)[a]	Total incubation time for EBs (hr)[a]
LGV	No	~18	~44
Trachoma	Yes	~24	~72

[a]Temperature is at 37°C.

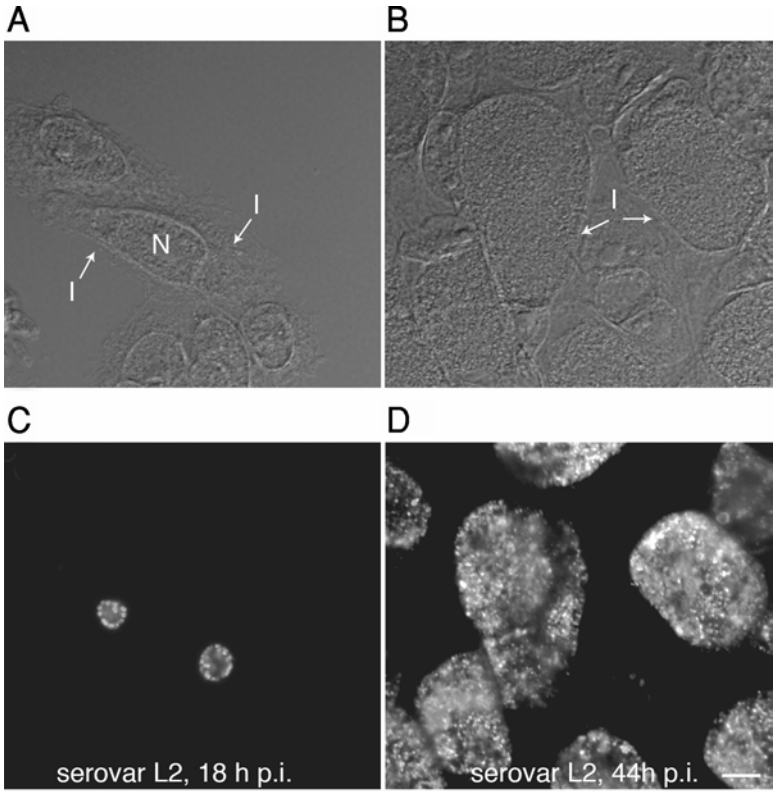

Figure 11A.1.1 Inclusion formation by *C. trachomatis* serovar L2. HeLa 229 monolayer cells were infected with *C. trachomatis*, serovar L2 at an MOI of ~1 and incubated at 37°C. At 18 and 44 hr post-infection, infected cells were fixed in methanol and stained sequentially with mouse anti-chlamydial LPS anti-sera and fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse antisera. Stained cells were viewed by Nomarski microscopy (**A** and **B**) and epi-fluorescence (**C** and **D**) to visualize inclusion formation. Inclusions (I) are indicated by arrows. Nucleus (N). Bar equals 10 μm.

7. Check for the infection efficiency by examining inclusion formation by phase-contrast microscopy.

   With experience, the percentage of infected cells can be determined by phase-contrast microscopy. By phase-contrast or Nomarski microscopy, inclusions will appear as large refractile vacuoles filled with numerous EBs, which sometimes can be seen moving caused by Brownian motion (Figure 11A.1.1). LGV serovars grow much faster than trachoma serovars. Therefore, depending on the serovar being used, inclusions will be visible ~18 to 24 hr post-infection.

   For large-scale preparations that will be used in Basic Protocol 3, >90% of cells should contain large inclusions.

INFECTION OF MONOLAYER TISSUE CULTURE CELLS BY CENTRIFUGATON

ALTERNATE PROTOCOL

A second method of enhancing infectivity of non-LGV *C. trachomatis* serovars is accomplished by centrifuging the bacterial inoculum onto the host cell monolayers. DEAE-dextran pretreatment of cells as in Basic Protocol 2 and centrifugation are equivalent methods for increasing the infectivity of non-LGV strains, and can be used interchangeably. This procedure can only be used for cells grown in shell vials or tissue culture plates because most centrifuges cannot accommodate tissue culture flasks. If using tissue culture flasks, carry out Basic Protocol 2.

Additional Materials *(also see Basic Protocol 2)*

Low speed bench top centrifuge with microplate adaptors

1. Dilute freshly trypsinized cells into complete medium supplemented with 10% FBS and 10 μg/ml gentamycin and transfer into an appropriately sized plastic tissue culture plate. Incubate cells 24 hr.

   Use culture medium that is recommended for the specific cell line being used.

   Seed cells in an appropriately sized tissue culture plate for each experiment. See Table 11A.1.1 for the most common tissue culture plates used and the cell concentrations needed for each type.

2. Remove the frozen stock of *Chlamydia* EBs from −80°C freezer and transfer immediately to a 37°C water bath. Gently agitate the tube continuously until EBs are thawed. To maintain sterility, wipe the tube with 70% ethanol and store on ice until ready to use.

   EBs thaw in <60 sec.

   EBs rapidly lose viability, therefore, thaw stock immediately before use and store on ice. In addition, do not thaw and reuse chlamydial stocks. Discard unused Chlamydia according to appropriate biosafety protocols.

3. Dilute chlamydial EBs to an appropriate concentration in sterile SPG.

   Monolayer tissue culture cells are commonly infected at a multiplicity of infection (MOI) of 1 to 2 EBs/host cells. The MOI can be altered depending on the specific experiment; however, some non-LGV C. trachomatis strains are cytotoxic to tissue culture cells when infected at an MOI >100. Cytotoxicity of chlamydial strains should be empirically determined by each researcher.

   HBSS may be used in place of SPG.

4. Remove culture medium from tissue culture cells and add desired amount of diluted EBs to each well. Centrifuge tissue culture plate 60 min at 900 × *g*, room temperature.

   See Table 11A.1.I for appropriate volumes of EBs used to inoculate each type of tissue culture plate.

   To prevent laboratory contamination, use a biohazard rotor when centrifuging C. trachomatis.

   Tissue culture cells are not easily infected with the ocular and genital serovars of C. trachomatis. To improve infection of tissue cultures with these serovars, tissue culture cells are either pretreated with DEAE-dextran (see Basic Protocol 2) or the inoculum is centrifuged onto the monolayer.

5. Add an appropriate volume of complete culture medium supplemented with 10% FBS, 10 μg/ml gentamycin, and 1 μg/ml cycloheximide, and incubate infected cells for desired time.

   See Basic Protocol 2, step 6, for a discussion of these parameters.

6. Check for the infection efficiency by examining inclusion formation by phase-contrast microscopy.

   With experience, the percentage of infected cells can be determined by phase-contrast microscopy. By phase-contrast or Nomarski microscopy, inclusions will appear as large refractile vacuoles filled with numerous EBs, which sometimes can be seen moving caused by Brownian motion (Figure 11A.1.1). LGV serovars grow much faster than trachoma serovars. Depending on the serovar being used, inclusions will be visible ~18 to 24 hr post-infection.

BASIC PROTOCOL 3

DENSITY GRADIENT PURIFICATION OF *C. TRACHOMATIS* ELEMENTARY BODIES

For most research applications, *C. trachomatis* EBs need to be free of host-cell contaminants. The most common method to purify EBs from infected tissue culture cells is by density gradient centrifugation, which is accomplished by first centrifuging infected tissue culture lysates through a 30% Renografin cushion, followed by centrifugation through a three-step Renografin density gradient. EBs obtained from the second density gradient are free of host-cell contaminants and can be stored for several years at −80°C without loss of viability. For large-scale preparations of density purified organisms, Basic Protocol 2 is performed prior to using this procedure to obtain sufficient quantities of *C. trachomatis*–infected monolayers. The Alternate Protocol cannot be used prior to this procedure, since most centrifuges do not accommodate tissue culture flasks. If purifying *C. trachomatis* RBs, follow Basic Protocol 4. After completing this procedure, Support Protocol 1 can be used to determine the asepsis of the purified organisms and Support Protocol 2 can be used to determine the infectious forming units of the density purified organisms.

Materials

C. trachomatis–infected monolayer tissue culture cells (20 infected 150-cm^2 flasks; see Basic Protocol 2)
HBSS (*APPENDIX 2A*)
70% and 95% (v/v) ethanol
95% (v/v) ethanol-soaked gauze strips
30%, 40%, 44%, and 54% (v/v) Renografin solutions (see recipe)
Disposable large cell scrapers, 3.0-cm blade width
10-ml disposable pipets, sterile
250- and 40-ml centrifuge tubes with sealing caps
Sonicator (e.g., Labsonic U Ultrasonic homogenizer with needle probe 40T, Ultrasonic Power)
Low-speed refrigerated centrifuge (Beckman JA-14 and JA-21 rotors or equivalent)
18-G metal cannulae
10-ml disposable syringes
25 × 89–mm ultraclear ultracentrifuge tubes (Beckman cat. no. 344058)
Ultracentrifuge with swinging bucket SW28 rotor
1.5-ml screw-cap microcentrifuge tubes

NOTE: For large-scale preparations of density gradient purified EBs, use approximately twenty 150-cm^2 flasks. Although volumes are given per 150-cm^2 flask, this procedure may be scaled up or down accordingly, depending on the numbers of infectious EBs desired.

NOTE: All solutions and centrifuge tubes should be pre-chilled on ice prior to use and kept on ice during use.

Prepare cells

1. Carefully decant culture medium from *C. trachomatis*–infected monolayer tissure culture cells and add 5 ml HBSS to each 150-cm^2 flask.

   CAUTION: *At this point in the procedure all culture medium, subsequent washes, and plasticware will be contaminated with infectious Chlamydia. All centrifuge tubes, pipets, and tissue culture plates will also be contaminated with infectious Chlamydia, and must be decontaminated or disposed of appropriately according to each institution's biohazard waste disposal procedures. Decontaminate nondisposable plasticware by soaking in a dilute bleach solution for 1 to 2 days. Wash and autoclave for re-use.*

   Large-scale purifications of chlamydial EBs are expensive given the large quantities of plasticware and FBS-supplemented media required. Rather than purifying Chlamydia from tissue culture cells grown in 150-cm^2 flasks, less expensive alternatives include propagating Chlamydia in tissue culture systems, such as roller bottles or suspension culture systems. If purifying EBs from infected cells grown in either of these two systems, begin this procedure with step 3.

   Decant infected culture medium into an appropriate waste container containing a dilute Roccal solution to kill any Chlamydia that may be present in the culture medium. Discard Roccal waste solution appropriately according to biohazard waste disposal protocols specific for each institution.

2. Dislodge infected cells from each flask with a large disposable sterile cell scraper. Pipet the cell suspension up and down several times with a sterile 10-ml pipet, flushing the bottom of flask to ensure that all cells have been displaced from flask. Combine and transfer cell suspension to a sterile 250-ml centrifuge tube that has been pre-chilled on ice.

   To prevent contamination of the centrifuge, use tubes with sealing caps.

   Depending on the size of the preparation, different-sized centrifuge tubes may be required.

3. Sonicate the cell suspension two times for 20 sec at 50 W each. Sterilize the probe by flaming and wiping with 95% ethanol before and after each sonication.

   CAUTION: *To prevent aerosol contamination that may occur during the sonication process, cover the opening of the centrifuge tube with three overlapping ethanol-soaked gauze strips.*

   Keep cell suspension on ice during sonication steps.

4. Centrifuge the sonicated cell suspension 15 min at 500 × *g* (e.g., 3000 rpm in a Beckman JA-14 rotor), 4°C. Decant the supernatant into a sterile 250-ml centrifuge tube and store on ice.

5. Resuspend the pellet in 5 ml HBSS/150-cm^2 flask. Repeat sonication and centrifugation as in steps 3 and 4. Pool the centrifuged supernatants and discard the pellet appropriately.

   The discarded pellet contains unbroken cells and nuclei.

6. Centrifuge the pooled supernatants 30 min at 30,000 × *g* (e.g., 14,000 rpm in Beckman JA-14 rotor), 4°C. Discard the supernatant and resuspend the pellet in 3 ml sterile SPG/150-cm^2 flask using an 18-G metal cannula attached to a 10-ml sterile syringe. Pipet up and down until the suspension is homogenous.

   The speed in which this centrifugation is carried out allows the EBs to pellet and become concentrated prior to ultracentrifugation in step 8.

7. Sonicate the suspension for 10 sec at 50 W. Keep cell suspension on ice.

   Sonication ensures that cells are completely resuspended.

Perform density gradient purification

8. Add 8 ml of 30% Renografin solution into each 25 × 89–mm ultraclear ultracentrifuge tube (one tube/ten 150-cm^2 flasks). Carefully layer 10 ml resuspended pellet over the 30% Renografin cushion in each centrifuge tube. Pour additional SPG containing resuspended pellet into centrifuge tube and swirl around several times. Carefully pipet SPG into ultracentrifuge tubes containing sample and 30% Renografin cushion until tubes are filled to 1/8 in. (3.175 mm) from the top.

   Weigh centrifuge tubes so that tubes will be appropriately balanced.

   Always fill ultracentrifuge tubes to 1/8 in. (3.175 mm) from the top before centrifuging.

9. Ultracentrifuge 30 min at 40,000 × *g* (18,000 rpm in Beckman SW-28 rotor with the brakes off), 4°C.

10. While the samples are in the ultracentrifuge, prepare discontinuous Renografin gradients containing 4 ml of 40% Renografin solution, 12 ml of 44% Renografin Solution, and 8 ml of 54% Renografin solution in clear ultracentrifuge tubes (one gradient/about five 150-cm^2 flasks).

    Make sure to carefully layer each Renografin solution over the previous layer such that each layer is not disturbed. Visualization of a well-defined step in the gradient at each interphase should be observed.

11. Carefully decant supernatant leaving the pellet undisturbed at the bottom of the ultracentrifuge tube.

    The pellet should be whitish in color. If it is yellowish in color and appears stringy, then it is contaminated with cellular material and DNA.

12. Resuspend each pellet in 1 ml SPG/150-cm^2 flask with an 18-G metal cannula attached to a sterile 10-ml disposable syringe. Make sure that the suspension is homogenous.

    The EB pellet can be difficult to resuspend; therefore, always use a cannula to resuspend EBs. Make sure the suspension is homogenous before proceeding.

    The pellet contains partially purified EBs and is termed 30% Renografin–purified EBs or seed stock. It can be used for passage of chlamydial stocks in culture and for isolation of additional density gradient–purified EBs. For seed stock storage, proceed to step 17.

    For most other applications, proceed with density gradient purification.

13. Carefully layer equal volumes of resuspended 30% Renografin–purified EBs over the preformed Renografin gradients in the ultracentrifuge tube from step 10. Add more SPG containing the 30% Renografin–purified EBs into the ultracentrifuge tube and continue to fill ultracentrifuge tubes until 1/8 in. (3.175 mm) from the top. Ultracentrifuge 60 min at 40,000 × *g* (18,000 rpm in Beckman SW-28 rotor with the brakes off), 4°C.

14. Carefully collect the white elementary body band at the 44%/54% Renografin interphase using an 18-G metal cannula attached to a sterile 10-ml syringe. Transfer collected EBs to a sterile 40-ml centrifuge tube with sealing cap and place on ice.

    Based upon the density of EBs (1.2 g/ml), EBs equilibrate at the 44%/54% interphase and appear as a cloudy white band.

    Collect EBs from the top of gradient by inserting the 18-G metal cannula attached to a sterile 10-ml syringe directly into the gradient and withdrawing the white EB band. It may be easier to remove and discard the top part of the gradient, and then insert a clean cannula to withdraw the EB band.

15. Dilute the density gradient–purified EBs with 10 vol sterile SPG and centrifuge 30 min at 30,000 × *g* (15,000 rpm in JA-20 rotor), 4°C, to remove the residual Renografin.

16. Carefully decant the supernatant and resuspend the pellet in 1 ml SPG/150-cm^2 flask. Keep on ice.

    The purified EBs can be directly passaged and used to infect fresh monolayer cultures or stored at −80°C for long-term storage.

***Store EB*s**

17. Dispense aliquots of density purified EBs into 1.5-ml screw-cap microcentrifuge tubes and store at −80°C.

    Frozen stocks cannot be refrozen and thawed without substantial loss in viability. Therefore, depending on the intended application, storage aliquot sizes may vary from 50 μl to 1 ml.

    Stocks kept at −80°C will retain titers for several years.

    EBs will become less infectious after storage. Therefore, to ensure an accurate titer, titer EBs after they have been stored at −80°C (see Support Protocol 2).

DETERMINING THE ASEPSIS OF ELEMENTARY BODY PREPARATIONS

SUPPORT PROTOCOL 1

Maintaining EB preparations aseptically is important, especially in cases where *Chlamydia* are isolated from clinical specimens obtained from the genital tract or rectum where large amounts of bacteria reside. Because *Chlamydia* are obligate intracellular bacteria and cannot be grown axenically, bacterial contamination of EBs can be examined by growth on blood agar plates. This procedure is used to test for bacterial contamination of density gradient–purified organisms obtained from Basic Protocol 3 or Basic Protocol 4 and of chlamydial stocks obtained from Basic Protocol 1.

Materials

BHI blood agar plates (see recipe), 37°C
Purified chlamydial organisms

1. Prepare BHI blood agar plates and pre-warm to 37°C.

   Blood agar plates permit the growth of most bacterial species.

2. Streak an aliquot of purified chlamydial organisms onto blood agar plates and incubate 24 hr at 37°C.

3. Check for bacterial growth on plates.

   Chlamydia will not grow on blood agar plates. Therefore, if the preparation is sterile, there will be no microbial growth on plates. If the preparation is contaminated, then bacterial growth, either individual colonies or swarming, will be present on plates. If contaminated, discard EBs.

TITERING INFECTIOUS FORMING UNITS BY INDIRECT IMMUNOFLUORESCENCE

SUPPORT PROTOCOL 2

For most applications, the precise number of infectious forming units (IFU) is required. The most common way to calculate IFU is by quantifying the number of inclusions formed from a given amount of starting material. This method is accurate if cells are infected at an MOI of ∼1, since a single infectious EB gives rise to a single inclusion. The easiest method to quantify inclusions is by indirect immunofluorescence using an antibody specific to *C. trachomatis.* This procedure is used to quantitate the number of IFUs present in density gradient–purified organisms (see Basic Protocol 3) or in amplified

stocks of clinical specimens (see Basic Protocol 1). It is also used before proceeding with Basic Protocol 2 or the Alternate Protocol in order to accurately infect cells with a desired MOI. If desired, this procedure, starting at step 7, can also be used to count inclusions formed upon completion of Basic Protocol 1, Basic Protocol 2, or the Alternate Protocol. Since RBs are noninfectious, purified RBs (see Basic Protocol 4) cannot be counted using this procedure.

Materials

Freshly trypsinized monolayer tissue culture cells (see Support Protocol 3)
Complete tissue culture medium (see Critical Parameters) supplemented with 10% FBS, 10 μg/ml gentamycin, and 1 μg/ml cycloheximide, 37°C
Frozen stocks of *C. trachomatis* EBs (see Basic Protocol 3) *or* infected cells (see Basic Protocol 2 or Alternate Protocol)
HBSS (*APPENDIX 2A*)
70% (v/v) ethanol
SPG (see recipe)
100% methanol
PBS (*APPENDIX 2A*)
PBS supplemented with 3% (w/v) BSA (see recipe)
Primary antibody specific to *C. trachomatis* (e.g., Abcam or Chemicon)
Fluorophore-conjugated secondary antibody
24-well tissue culture plates
Biohazard swinging-bucket rotor adapted for microtiter plates (e.g., Sorvall biohazard microtiter rotor, cat. no. 75006-444)
12-mm-diameter coverslips (no. 1 thickness), optional
37°C water bath
Inverted epi-fluorescence microscope with appropriate filter sets
Hand-held counter

Prepare cells

1. Seed monolayer tissue culture cells in 24-well tissue culture plates containing 1 ml complete tissue culture medium supplemented with 10% FBS, 10 μg/ml gentamycin, and 1 μg/ml cycloheximide, at a density of ~1–3 × 10^5 cells per well. Incubate 24 hr.

   To ensure accurate results, perform titer in triplicate. For each EB stock preparation to be titered, seed eighteen wells.

   HeLa 229 or L929 fibroblasts are commonly used to titer C. trachomatis EBs.

   If an inverted epi-fluorescence microscope equipped with long-working distance objectives is unavailable, culture cells on 12-mm diameter glass coverslips (no. 1 thickness) in 24-well tissue culture plates.

2a. *For isolated EBs:* Thaw an aliquot of EBs in a 37°C water bath. Agitate continuously until EBs are completely thawed. Keep EBs on ice.

   EBs should be completely thawed in <60 sec.

   To maintain sterility, wipe the storage tube with 70% ethanol before proceeding.

   EBs rapidly lose viability, therefore, thaw stock immediately before use and store on ice. In addition, do not thaw and reuse chlamydial stocks. Discard unused Chlamydia according to appropriate biosafety protocols dictated by each institution.

2b. *For infected cell monolayers:* Grow infected cells in 24-well plates. After 44-hr post-infection, wash cells one time in HBSS, and then lyse each well with 250 μl sterile water. Incubate cells 5 min at room temperature and pipet up and down several times to ensure complete lysis.

3. Make ten-fold serial dilutions of EBs (step 2a) or infected cell lysates (step 2b) in SPG or HBSS (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). Make at least 1 ml of each dilution. Keep on ice.

   SPG or HBSS may be used interchangeably in this step.

4. Remove culture medium from monolayers and add 200 µl of each dilution to three separate wells.

5. Centrifuge tissue culture plates 60 min at 900 × *g*, room temperature, in a biohazard swinging bucket rotor adapted for microtiter plates.

   A biohazard rotor (e.g., Sorvall) is used to prevent bacterial contamination of the centrifuge.

   If a centrifuge or rotor for microtiter plates is unavailable, cells can be pretreated with 45 µg/ml DEAE-dextran for 15 min prior to infection (see Basic Protocol 2).

   If titering LGV strains, remove culture medium and inoculate 200 µl of each dilution directly onto monolayers. Incubate cells for 30 to 60 min and proceed to step 6.

Infect cultures, wash and fix cells

6. Add 1 ml complete medium supplemented with 10% FBS, 10 µg/ml gentamycin, and 1 µg/ml cycloheximide, and incubate for 36 to 72 hr depending on the specific serovar being titered (Table 11A.1.1).

   For LGV serovars, cycloheximide can be omitted from the culture medium.

7. Remove infected culture medium and wash cells with HBSS two times.

   Dispose of infected culture medium appropriately.

8. Remove the wash solution and add 1 ml of 100% methanol to each well. Incubate 10 min at room temperature. Remove methanol and wash the cells three times in PBS. For each wash, add 1 ml PBS, incubate 5 min at room temperature, and then remove PBS. Add 1 ml PBS containing 3% BSA to fixed cells and incubate for 30 to 60 min to block nonspecific sites of antibody binding. Store up to several days at 4°C.

   Do not let cells dry out at any time during the procedure.

   Fixation conditions differ between different antibodies. If using a commercial antibody, follow manufacturer's recommendations for fixation conditions.

   Once infected cells are fixed, they are no longer considered infectious. Therefore, the remaining procedures can be performed on the bench top.

Add antibodies and view cells

9. Dilute primary antibody specific to *C. trachomatis* in PBS containing 3% BSA. Microcentrifuge antibody dilutions 5 min at maximum speed, room temperature, to pellet protein aggregates. Add 200 µl diluted primary antibody to each well. Incubate at least 60 min at room temperature with rocking or overnight at 4°C.

   Polyclonal antibodies that recognize chlamydial EBs or monoclonal antibodies that recognize components of EBs such as the major outer membrane protein (MOMP) are commercially available from several sources including Abcam and Chemicon. If using a commercial antibody, use the manufacturer's suggested dilutions. Typically, affinity-purified antibodies are diluted in the range of 1 to 10 µg/ml and rabbit polyclonal antibodies are diluted between 1:100 and 1:1000.

10. Dilute appropriate fluorophore-conjugated secondary antibody in PBS containing 3% BSA and microcentrifuge 5 min at maximum speed, room temperature, to pellet protein aggregates.

    Use manufacturer's recommended dilutions for conjugated secondary antibodies. Typically, conjugated secondary antibodies are diluted between 1:100 and 1:300.

11. Remove primary antibody from each sample and wash with PBS three times. For each wash, add 1 ml PBS, incubate 5 min, and remove PBS.

12. Add 200 µl of diluted secondary antibody to each well and incubate for at least 60 min at room temperature with rocking.

    Keep samples away from direct light to prevent fluorophore from bleaching.

13. Remove secondary antibody and wash with PBS three times. For each wash, add 1 ml PBS, incubate 5 min, and remove final PBS wash. Store fixed and stained cells in PBS at 4°C.

    Fixed and washed cells can be stored in PBS at 4°C for several days without loss of fluorescence.

    If cells were cultured on glass coverslips (see step 1), remove coverslips from tissue culture plate with forceps and 18-G needle, rinse by submerging in beaker of water, and dry coverslip by wicking with a Kimwipe. Place coverslip, cell-side down, on drop of antifading agent on a glass slide. Slides can be viewed with an inverted or upright epi-fluorescence microscope.

14. To calculate the number of infectious forming units (IFU), view fixed and stained cells with an inverted conventional epi-fluorescence microscope using appropriate fluorescence filter set for the specific fluorophore-conjugated secondary antibody that was used.

15. Using a hand-held counter, count all inclusions, which will appear as large fluorescent vacuoles, in each field (see Figure 11A.1.1). Count at least 15 fields per each dilution.

    Count appropriate dilution so that there are ~30 to 100 inclusions in each field.

16a. *To determine the infectious forming units:* Calculate the IFUs using the following equation:

$$\text{IFU/ml} = \frac{\text{average no. inclusions/field} \times \text{dilution factor} \times \text{no. fields/well}}{\text{volume inoculum}}$$

where

$$\text{fields/well} = \frac{\text{well surface area}}{\text{view area of field}}$$

and where

$$\text{area of field of view} = \pi r^2$$

and where

$$r = \frac{\text{field of view diameter (mm)}}{2} = \frac{\text{eye piece field no.}}{\text{total magnification}}$$

16b. *To determine the titer:* Calculate the titer using the following equation:

$$\text{total magnification} = \text{objective magnification} \times \text{optivar magnification} \times \text{eye piece magnification}$$

PURIFICATION OF *C. TRACHOMATIS* RETICULATE BODIES

BASIC PROTOCOL 4

Although reticulate bodies (RBs) are noninfectious, there are occasions where purified preparations of RBs may be required. Similar to EBs (see Basic Protocol 3), RBs are purified by Renografin density gradient centrifugation with the exception of two important changes. First, because of their unique developmental cycle, a pure population of RBs is only present for a short period early during the developmental cycle, therefore, RBs are harvested much earlier during the developmental cycle than are EBs. Second, RBs are much more fragile than EBs, therefore, RBs are released from the infected cells by lysis with a Dounce homogenizer as opposed to homogenization with a sonicator. This procedure is used following completion of Basic Protocol 2 and is similar to Basic Protocol 3 with the indicated modifications. Since RBs are noninfectious, Support Protocol 2, the Alternate Protocol, or Support Protocol 3 cannot be used following completion of this procedure.

Materials

Infected cells in 150-cm^2 flasks (see Basic Protocol 2)
10× K-36 buffer (see recipe)
Large cell scraper
250-ml centrifuge tube
40-ml Dounce homogenizer with a tight-fitting pestle
Glass microscope slides and coverslips
Phase-contrast microscope
Beckman JA-14 rotor or equivalent

Additional reagents and equipment for density gradient purification (see Basic Protocol 3)

1. Harvest infected cells in 150-cm flasks 18 to 24 hr post-infection. Dilute 10× K-36 buffer 1:10 in sterile water to make a final concentration of 1×. Add 5 ml of 1× K-36 buffer to each 150-cm^2 flask. Dislodge cells using a large cell scraper and transfer to a 250-ml centrifuge tube. Store on ice.

   Infected cells need to be harvested before infectious EBs are produced. Since the length of the developmental cycle varies between strains, harvest times should be determined empirically for each different chlamydial strain. Typically, LGV RBs are harvested at 18 hr post-infection while non-LGV RBs are harvested ∼24 hr post-infection.

2. On ice, transfer 30 to 40 ml of lysate into a 40-ml Dounce homogenizer and homogenize the lysate with 15 to 30 strokes using a tight-fitting pestle. Every five to ten strokes, transfer a small drop of lysate onto a glass slide and cover with a coverslip. View cellular lysate under a phase-contrast microscope to monitor cell lysis. Continue homogenizing until >90% of cells are lysed with the nuclei still intact. Transfer lysate to a 250-ml centrifuge tube. Repeat homogenization step with remaining lysate until it is homogenized.

   CAUTION: *Do not create any aerosols during homogenization procedure.*

3. Combine all homogenized lysate into a 250-ml centrifuge tube with sealing cap and centrifuge 15 min at 500 × *g* (3000 rpm in a Beckman JA-14 rotor), 4°C. Transfer supernatant into a clean 250-ml centrifuge and store on ice. Resuspend pellet in 30 ml K-36 buffer and transfer into Dounce homogenizer. Repeat homogenization with another 20 strokes. Transfer into a new 250-ml centrifuge tube and centrifuge 15 min at 500 × *g*, 4°C.

4. Pool the supernatants and centrifuge 30 min at 30,000 × *g* (14,000 rpm in Beckman JA-14 rotor), 4°C. Discard supernatant and resuspend pellets in 30 ml K-36 buffer.
5. Continue with Basic Protocol 3, step 8, and layer homogenate onto 30% Renografin cushions. Proceed with remainder of Basic Protocol 3 except substitute K-36 buffer for SPG.
6. Check purity of RB preparation by transmission electron microscopy (*UNIT 2B.1*).

Remember that RBs are not infectious and cannot be used to infect cells. If the preparation is a pure population of RBs with no EB contamination, no inclusions should be formed if the purified RBs were used to infect host cells.

RBs can be distinguished from EBs based upon the following parameters: (1) RBs are ~1 μm, while EBs are ~0.5 μm and (2) RBs have a decondensed nucleoid structure as opposed to EBs, which contain a highly condensed nucleoid structure that appears electron dense.

SUPPORT PROTOCOL 3

RECOVERY AND PASSAGE OF FROZEN MONOLAYER TISSUE CULTURE CELLS

Immortalized monolayer tissue culture cell lines can be continually passaged in culture and are the most common cell type used for propagating *C. trachomatis* in the laboratory. Hela 229, McCoy, and L929 are the most common cell lines used since they are susceptible to all *C. trachomatis* strains. Other cell lines may be used, but susceptibility to *C. trachomatis* infection and incubation times must be determined by each researcher. This protocol can be used to propagate frozen immortalized monolayer tissue culture cell lines to be used in Basic Protocol 1, Basic Protocol 2, Basic Protocol 3, Basic Protocol 4, Alternate Protocol, and Support Protocol 2.

Materials

Frozen immortalized tissue culture cell lines in liquid nitrogen: HeLa 229 (ATCC no. CCL-2.1), McCoy (ATCC no. CRL-1696), or L929 cell lines (ATCC no. CCL-1)
70% (v/v) ethanol
Complete medium (see Critical Parameters) supplemented with 10% FBS and 10 μg/ml gentamycin, 37°C
HBSS (*APPENDIX 2A*) without Ca^{2+} and Mg^{2+}
Trypsin/EDTA solution (see recipe), 37°C
37°C water bath
15-ml sterile centrifuge tubes
75-cm^2 tissue culture flasks
Inverted phase-contrast microscope
Hemacytometer slide with coverslip
Light microscope
Hand-held cell counter

Thaw and recover frozen monolayer tissue culture cell lines

1. Remove frozen tissue culture cells from liquid nitrogen freezer and immediately place vial into a 37°C water bath. Agitate continuously until vial is thawed.

Cells will usually thaw in <60 sec.

Thaw cells as quickly as possible to prevent lysis of cells that may occur due to ice crystal formation.

To maintain sterility of cells, avoid water droplets near the vial top.

2. To ensure sterility of cells, wipe top of the vial with 70% ethanol before opening. Transfer the thawed cell suspension into a sterile 15-ml centrifuge tube containing ~10 ml prewarmed complete medium containing 10% FBS supplemented with 10 μg/ml gentamycin. Centrifuge 5 min at 200 × *g*, room temperature. Carefully remove supernatant with pipet, resuspend cell pellet in 1 ml complete medium containing 10% FBS supplemented with 10 μg/ml gentamycin, and transfer to 75-cm^2 tissue culture flask containing 25 ml of complete medium containing 10% FBS supplemented with 10 μg/ml gentamycin.

   Cells must be pelleted and fresh medium must be added to remove DMSO, which is present in the freezing medium, before cells can be plated and cultured.

3. Check cultures after ~24 hr to ensure that cells have attached to plates.

4. Once monolayers have become confluent, detach cells from the tissue culture flask by trypsinization (step 5) and dilute into a clean tissue culture flask and fresh culture medium (see Table 11A.1.1).

   The time it takes a cell monolayer to become confluent depends on several factors, including the total number of cells thawed, the viability of cells, the doubling time, and the culture medium. Follow ATCC guidelines for each cell line and monitor monolayers by phase-contrast microscopy.

   In general, monolayer cultures are split every 3 to 4 days.

Trypsinize and subculture cells from a confluent monolayer

5. Decant culture medium from tissue culture flask containing confluent monolayer of cells and wash with 5 ml HBSS without Ca^{2+} and Mg^{2+} by swirling flask to remove any residual FBS. Remove HBSS by pipetting or decanting.

   Residual FBS will inhibit the action of trypsin.

6. Add appropriate volume of prewarmed trypsin/EDTA solution to cover the monolayer completely.

   If using a 75-cm^2 tissue culture flask, use 2.5 ml trypsin/EDTA solution.

   If using a 150-cm^2 tissue culture flask, use 5 ml trypsin/EDTA solution.

7. Incubate flask 5 to 10 min. Tap bottom of flask on countertop to detach cells. Check cultures with an inverted phase-contrast microscope to make sure that all cells are rounded up and detached from the surface. If not all of the cells are detached, continue incubating flask an additional 1 or 2 min.

8. Add 5 ml prewarmed complete medium containing 10% FBS and 10 μg/ml gentamycin to each flask containing trypsinized cells. Draw up cell suspension into a sterile pipet and rinse cell layer two to three times to dissociate any remaining adherent cells.

Determine cell number

9. Carefully clean the surface of a hemacytometer slide and coverslip with 70% ethanol.

   Use only a specially designed hemacytometer coverslip.

   A hemacytometer slide is needed to accurately count the number of cells. Each hemacytometer slide contains two separate counting chambers with etched 3 × 3–mm grids. Each grid is divided into nine 1-mm squares. The four corner and the central squares are used for counting.

10. Use a sterile pipet to transfer cell suspension (from step 8) to the edge of each hemacytometer counting chamber by holding the tip of the pipet under the coverslip and dispersing one drop of suspension. Allow cells to settle for several minutes before counting.

11. View hemacytometer slide under a light microscope. Use an appropriate objective lens such that the central area of the grid fills the entire microscope field.

12. Count the cells in all four corners and the central square with a hand-held cell counter.

    Count the cells that are touching the middle line of the triple line on the top and left of each square. Do not count the cells touching the middle line of the bottom or right side of each square.

13. To calculate the number of cells/ml of culture, use the following:

$$\text{Cells/ml} = \frac{\text{total no. cells counted}}{\text{no. squares counted}} \times \text{dilution factor} \times 10^4$$

    where 10^4 is the correction factor for the volume of cells counted.

    Each square is 1 × 1 mm, with a depth of 0.1 mm.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A*; for suppliers, see* **SUPPLIERS APPENDIX**.

Bacto heart infusion (BHI) blood agar plates

500 g beef heart infusion
10 g bacto-tryptone
5 g NaCl
15 g bacto-agar
dH_2O to 1 liter

Autoclave for 20 min and cool to 45° to 50°C. Add 5% (v/v) sterile, defribrinated, room temperature blood. Mix well. Pour into sterile petri plates and let dry at room temperature for 1 to 2 days. Store up to 30 days at 4°C.

Chlamydial transport buffer

Immediately prior to use, supplement 100 ml complete tissue culture medium with the following:
10 mg vancocin (100 μg/ml final concentration)
1 mg gentamycin (10 μg/ml final concentration)
1000 U mycostatin (10 U/ml final concentration)
180 mg glucose (3 μmol/ml final)
Adjust pH to 7.5 with HCl or NaOH

Cycloheximide, 1 mg/ml

Dissolve 10 mg cycloheximide into 10 ml 95% ethanol
Dispense into 0.5-ml aliquots and store up to 6 months at −20°C
Dilute 1:1000 into culture medium for growth of non-LGV *C. trachomatis* strains

DEAE-dextran, 10×

180 mg DEAE-dextran (450 μg/ml final concentration)
Adjust volume to 400 ml with H_2O
Filter sterilize through a 0.2-μm filter
Dilute 1:10 for pretreatment of tissue culture monolayers (45 μg/ml final concentration)
Store up to 1 year at room temperature

K-36 buffer, 10×

87.1 g K_2HPO_4
68.0 g KH_2PO_4
74.5 g KCl
8.7 g NaCl
H_2O to 1 liter
Adjust pH to 7.0 with 1 M KOH
Autoclave 20 min
Store up to 1 year at 4°C

PBS supplemented with 3% (w/v) BSA

Dissolve 3 g BSA into 100 ml PBS (*APPENDIX 2A*). Filter sterilize using a low-protein binding 0.22-μm filter. Store indefinitely at −20°C.

Renografin solutions, 30%, 40%, 44%, 54%

Prepare the following in a biological safety cabinet to maintain sterility:
30, 40, 44, or 54 ml Renocal-76 or Hypaque-76 (for 30%, 40%, 44%, or 54% solutions, respectively)
10 ml 10× K-36 buffer (see recipe)
Adjust volume to 100 ml with sterile H_2O
Store up to 6 months at 4°C

RenoCal-76 (E.R. Squibb and Sons) or Hypaque-76 (Nycomed) are the two most common sources of Renografin and they are supplied as sterile 76% solutions containing a 66% solution of diatrizoate meglumine and a 10% solution of diatrizoate sodium. Use directly from bottle when making dilutions.

Sucrose-phosphate-glutamate buffer

75 g sucrose
87 ml 0.2 M Na_2HPO_4 (dibasic sodium phosphate)
13 ml 0.2 M NaH_2PO_4 (monobasic sodium phosphate)
0.72 g L-glutamic acid
H_2O to 1 liter
Adjust pH to 7.4 with 2 N NaOH if necessary
Aliquot to 100-ml bottles and autoclave for 20 min
Store up to 1 year at 4°C

Trypsin/EDTA solution

0.25 g trypsin
0.2 g EDTA (disodium ethylenediamine tetraacetic acid)
H_2O to 100 ml
Filter sterilize
Store indefinitely at −20°C

Various concentrations of Trypsin/EDTA solutions are readily available from commercial manufacturers. The two most common sources are Invitrogen (GIBCO) and Mediatech.

COMMENTARY

Background Information

Maintenance of C. trachomatis

C. trachomatis are obligate intracellular bacteria that replicate within a nonacidified membrane-bound vacuole termed an inclusion (Moulder, 1991). Within the inclusion, all chlamydial species undergo a unique biphasic developmental cycle alternating between two morphologically and functionally distinct cell types (Moulder, 1991): the elementary body (EB) and the reticulate body (RB). The EB is infectious but metabolically inactive, while

the RB is noninfectious and metabolically active. Infection is initiated by the attachment and subsequent parasite-mediated endocytosis of the EB. During the first several hours post-infection, EBs begin to differentiate into RBs. Differentiation is characterized by the reduction of the disulfide cross-linked outer membrane, the decondensation of the bacterial nucleoid, and an increase in size of the organism. Depending on the specific strain or species, RBs begin to replicate somewhere between 10 and 18 hr post-infection. As some RBs continue to replicate, others will differentiate back into EBs resulting in a mixture of EBs and RBs within the inclusion. Eventually, the host cell will lyse, releasing the organisms and allowing the developmental cycle to continue upon infection of neighboring cells. The developmental cycle is conserved between all chlamydial species but differs in the time it takes to complete the cycle, which can range from 36 hr for *Chlamydia muridarum* to 96 hr for *Chlamydia pneumoniae* species. *C. trachomatis* developmental cycles range from 44 to 72 hr depending on the specific biovar or serovar. Completion of the developmental cycle is absolutely essential for the production of infectious EBs and the eukaryotic host cell is the only environment known to support chlamydial development and replication. Therefore, *Chlamydia* cannot be cultured on conventional bacteriological medium and must be maintained and propagated in tissue culture cell lines.

Although chlamydial infections are diseases of ancient times, laboratory culture of the organism was not achieved until 1957 when T'ang and co-workers successfully isolated the organism through inoculation of the yolk sack of an embryonated hen egg (T'ang et al., 1957). The ability to isolate and propagate the organism in the laboratory setting finally enabled researchers to begin to initiate studies outside the clinical setting, beginning with the development of potential trachoma vaccines. Passage of chlamydial organism eggs through yolk sacks is a very time-consuming and complex procedure that can be problematic due to bacterial and mycotic contamination. To overcome these difficulties, a tissue culture system was developed which allowed the isolation and growth of *Chlamydia* in monolayer tissue culture systems (Schachter and Wyrick, 1994). Although all chlamydial species are characterized by a common developmental cycle, they differ dramatically in their ability to infect and grow in tissue culture cells. Therefore, there is no single tissue culture system that will work for all chlamydial species and strains. *Chlamydia pneumoniae* strains are much more fastidious than *C. trachomatis.* Even between *C. trachomatis* strains there are huge differences: the trachoma biovars being much more fastidious than the LGV biovars. The non-LGV biovars do not readily infect tissue culture cells and usually require either mechanical assistance, such as centrifugation of the inoculum onto the monolayer, or chemical assistance, such as pretreatment of the monolayer with DEAE-dextran prior to chlamydial inoculation to enhance their infectivity. Although, the exact mechanism by which centrifugation enhances infectivity is not known, it is thought that it allows a more intimate interaction between the bacteria and the cell surface. DEAE-dextran pretreatment of cells is thought to enhance infectivity by neutralizing the surface charge of the host cell, thus allowing more efficient attachment of *Chlamydia*. In addition, after inoculation, the trachoma biovars require culture in medium supplemented with cycloheximide. Cycloheximide inhibits eukaryotic protein synthesis and is thought to allow *Chlamydia* to out compete the host for essential nutrients and biosynthetic precursors. Although the LGV biovars do not require either mechanical or chemical assistance in order to efficiently invade tissue culture cells or growth in medium supplemented with cycloheximide, these biovars may be grown under these conditions.

Infection of tissue culture cells can be used to isolate and propagate chlamydial organisms from clinical specimens including urethral, urogenital, and rectal swabs. *Chlamydia* survive storage for several years at −80°C, with minimal loss in viability, so permanent stocks of these organisms may be obtained by simply lysing infected cells, adding storage buffer, such as SPG, and storing the organisms at −80°C. For most research purposes, however, purified organisms are needed. Therefore, host contaminants need to be removed. This is achieved through two differential Renografin density gradient and cushion centrifugation steps (Caldwell et al., 1981). Renografin density centrifugation, as opposed to the more commonly used sucrose density centrifugation, is used for chlamydial purification for the following reasons. At the concentrations needed to achieve the appropriate densities, sucrose solutions are very viscous and exert extremely high osmotic affects. In contrast, Renografin is inert, nonionized, and less viscous. In addition, Renografin exerts very little osmotic effects and yields similar density ranges.

The most common way to purify EBs is to grow *Chlamydia* in monolayer tissue culture cells grown in 150-cm^2 tissue culture flasks; however, this can become very costly due to the large number of flasks and FBS-supplemented medium that are needed to obtain high-titered stocks. To reduce the cost, monolayer cultures grown in roller flasks can also be infected with *C. trachomatis*. In addition, suspension cultures of L929 or HeLa S3 cell lines can also be used to propagate some *C. trachomatis* strains such as the LGV serovars (Schachter and Wyrick, 1994). For the more fastidious *C. trachomatis* strains, a microcarrier bead culture technique has recently been developed to be used for non-LGV infection of McCoy cells (Schachter and Wyrick, 1994). Greater titers of infectious EBs can be obtained by this method.

Once purified, chlamydial EBs can be stored for several years at −80°C and can be used for a wide variety of research purposes, including molecular and cell biological studies, proteomic and genomic studies, in vivo animal studies, vaccine production, and DNA isolation and genomic sequencing projects.

Significance of C. trachomatis

Within the species *C. trachomatis*, there are two biovariants: the trachoma biovars and lymphogranuloma venereum (LGV) biovars. Although genetically similar, the trachoma and the LGV biovars can be differentiated by their clinical presentations and by their growth phenotypes in tissue culture cells and in animal model systems. The *Mouse Pneumonitis* agent, which was once classified as a *C. trachomatis* species, is now considered a separate species, *C. muridarum*.

Trachoma biovars. Within the trachoma biovars, there are at least twelve different serovars that can be serologically distinguished. The trachoma serovars are further divided into two groups based upon their anatomical distribution within the host.

Trachoma agents (Serovars A, Ba, Bb, and C). Serovars A, Ba, Bb, and C infect ocular mucosal surfaces, and are primarily associated with endemic blinding trachoma (Schachter, 1999). Although trachoma infections have been virtually eliminated in industrialized countries, the disease is still endemic in tropical and subtropical geographic areas, including North Africa, the Middle East, and Northern India. Close to 600 million people live in endemic areas and are at risk of developing trachoma (Thylefors et al., 1995). The disease is transmitted by human-to-human contact and can be potentiated by flies, which can transfer secretions from person to person. Infection begins as a conjunctivitis followed by follicle formation. Follicles may rupture, leading to scarring and distortion of the eyelids, which may cause an in-turning of the eyelid. As a result, the eyelashes scrape the cornea, ultimately resulting in blindness. Blindness does not result from an acute infection, but rather results from persistent and repeated infections. In the laboratory, a diagnosis of trachoma is made following the isolation of the organism from conjunctival scrapings or the presence of inclusions in epithelial cells obtained from conjunctival scrapings. Since trachoma is a disease of poverty and poor hygienic conditions, the current program in place to eradicate the disease is known as the SAFE strategy (surgery to repair damaged eyelids, antibiotic treatment, face washing to improve personal hygiene, environmental improvements to reduce the fly population). Tetracyclines such as doxycycline and azithromycin are currently being used to treat the disease.

Genital infections (Serovars D, E, F, G, H, I, J, and K). Genital serovars infect superficial columnar epithelial cells of the urogenital tract and the respiratory tracts of newborns (Schachter, 1999). Infection by these serovars is the leading cause of bacterially acquired sexually transmitted disease. Each year, close to 90 million new cases are reported, with ∼4 million of these cases occurring in the United States (WHO, 1997). *C. trachomatis* genital tract infections cause a wide variety of diseases in both men and women, including adult and infant inclusion conjunctivitis, cervicitis, salpingitis, female urethral syndrome, postpartum endometris, male urethritis, epididymitis, arthritis, and infant pneumonia (Schachter, 1999). Chlamydial infections elicit a strong pro-inflammatory response, which upon repeated or persistent infections, can lead to fibrosis and scarring. The physical consequences of scar formation, not a toxin produced by the bacteria, is the cause of the devastating sequelae that results from genital tract infections. In women, these can include pelvic inflammatory disease, ectopic pregnancy, and infertility. Unfortunately, the majority of cases, especially in woman, are asymptomatic and are therefore left untreated. Laboratory diagnosis is made by isolation of the organism from cervical or urethral swabs, or by direct fluorescent antibody tests, enzyme-linked immunoassays, or nucleic acid detection. As with ocular infections, genital tract infections are treated with tetracyclines.

Lymphogranuloma venereum (LGV, Serovars L1, L2, and L3). The LGV serovars also infect the mucosal surfaces of the genital tract, but in contrast to the trachoma biovars, which remain localized to mucosal surfaces, the LGV serovars are more invasive and infiltrate the submucosa resulting in lymphatic involvement (Schachter, 1999). Although infections by the LGV serovars have been virtually eliminated in the U.S., they are still prevalent in tropical areas of Africa, Central America, and Asia. The primary lesions develop on the penis, labia, vagina, and cervix, and may result in a mild proctitis. Left untreated, secondary lesions consisting of lymphoadenopathy may form. Within the nodes, abscesses form and may rupture resulting in chronic draining fistulas. At this stage, the abscesses may disappear or the disease may become systemic, resulting in fever, myalgia, and headache. In some cases, chronic inflammation and scarring may occur. Diagnosis is made following isolation of the organism from the primary lesion or from aspirated pus, or by serological assays such as the microimmunofluorescence tests.

Critical Parameters

Handling organisms

If beginning with clinical specimens, make sure that specimens are appropriately collected in chlamydial transport buffer and stored at 4°C. Chlamydia must be isolated from specimens within 24 hr post-collection. If beginning with frozen stocks of purified organisms, make sure to store organisms at −80°C immediately upon receipt and only thaw organisms immediately before use, otherwise, a loss in viability will result. *C. trachomatis* are considered BSL-2 organisms and must be handled according to appropriate federal and local biosafety standards (*UNIT 1A.1*). Wear appropriate protective clothing (gloves, laboratory coat, protective eye glasses) when handling the organism. In addition, wear a mask when sonicating infected cell lysates.

Cell type and culture medium selection

C. trachomatis is a human pathogen and in vivo they primarily infect epithelial cells. In addition, the LGV biovars but not the trachoma biovars will also infect macrophages. In vitro, *C. trachomatis* can infect epithelial as well as fibroblast cell lines of both human and nonhuman origin. The most common monolayer cell lines used today for isolation and propagation of *C. trachomatis* species are McCoy and L929 (mouse fibroblast), and HeLa 229 (human epithelial) cell lines. Monolayer cell cultures are the most common cell type used, however, L929 and HeLa S3 suspension tissue culture cells have been used successfully for at least some non-LGV *C. trachomatis* strains. For initial isolation of clinical specimens, McCoy cells are almost exclusively used. Use culture medium appropriate for each cell type. Common cell culture media include RPMI 1640 and minimal essential medium (MEM), both supplemented with 10% FBS. Media are most commonly purchased as either a liquid or a powder.

Culture vessel selection

Chlamydia can be cultured in cells grown in almost any type of plastic tissue culture vessel and the choice of vessel depends entirely on the application and the numbers of organisms needed. For initial isolation of clinical specimens, shell vials are used. No matter what type of vessel is used, the protocols are identical except for cell numbers and volumes. Refer to Table 11A.1.1 for appropriate cell numbers, inoculation, and culture volumes used for the different types of vessels.

Infection times

The infection or incubation period is not an absolute number and depends on numerous parameters, including the specific *C. trachomatis* serovar or strain being used, the MOI being used to infect the cells, the efficiency of infection, and the specific application. Approximate incubation times for large-scale purification of EBs and RBs are given in Table 11A.1.2. Note that these are just approximate times and the actual times may need to be experimentally determined by each researcher. To passage *Chlamydia*, they must have completed their developmental cycle so that sufficient numbers of infectious EBs will have been formed. Remember, RBs, which are formed during the middle of the developmental cycle, are not infectious and cannot be used to propagate the organism. When growing *Chlamydia* to be passaged into fresh monolayers or to be purified as stock EBs, carefully monitor the infected cells by phase-contrast microscopy. Collect the cells when the inclusions are large and are practically filling the entire cytoplasm of the host cell. At this stage in the developmental cycle, the inclusions will be easily visible by phase-contrast microscopy. Do not let the infection continue too long as this may result in premature host cell lysis.

Table 11A.1.3 Troubleshooting Guide for Laboratory Maintenance of *Chlamydia trachomatis*

Problem	Possible cause	Solution
No inclusion formation	No infectious EBs in starting sample	Blind passage sample two to three times before checking for inclusion development
	Improperly stored EBs	Store at −80°C and do not thaw until just before use
	Improperly stored clinical specimens	Store specimens in chlamydial transport buffer at 4°C
	Incorrect titer	Re-titer EBs or re-infect at higher concentration
Low inclusion formation	Improperly stored EBs	Store at −80°C and do not thaw until just before use
	Incorrect titer	Re-titer EBs or re-infect at higher concentration
	Cell type not permissible for strain being used	Try a permissive cell line
		Pretreat cells with DEAE-dextran or centrifuge inoculum onto monolayer
		Increase initial infection time
	Mycoplasma-contaminated tissue culture cell line	Check for mycoplasma contamination every couple of months
		Thaw and culture mycoplasma-free cell line
	Unhealthy or old tissue culture cells	Start new tissue culture passage
		Do not let tissue culture cells overgrow before splitting
		Check CO_2 levels in incubator
		Always infect cells 24 hr after seeding
Infected cells lysed	EBs toxic to cell	Decrease concentration of inoculum
	Harvested infected cells too late in developmental cycle	Decrease infection time
Low yields in purified EB preparations	Harvested infected cells too early in developmental cycle	Increase infection times
	Infected cells with too few EBs	Increase concentration of inoculum
		Scale up preparation and increase total number of infected cells
	Improperly prepared and stored reagents	Keep all solutions, lysates, and plasticware on ice during purification procedure
	Inefficient lysis of infected cells	Monitor cell lysis by phase-contrast microscopy and repeat homogenization steps
RBs contaminated with EBs	Harvested infected cells too late during the developmental cycle	Decrease infection times
Contamination	Mycoplasma-contaminated tissue culture cell line	Check for mycoplasma contamination every couple of months
		Thaw and culture mycoplasma-free cell line
	Improperly sterilized reagents	Prepare fresh reagents and properly sterilize by autoclaving or filtering

continued

Table 11A.1.3 Troubleshooting Guide for Laboratory Maintenance of *Chlamydia trachomatis*, *continued*

Problem	Possible cause	Solution
No inclusions detected by indirect immunofluorescence	Antibody does not react with chlamydial species being used	Use genus-specific antibody
	Incorrect dilution of primary antibody	Increase concentration of primary antibody
	Inappropriate fixation conditions for primary antibody	Follow suppliers recommended fixation conditions
		Try alternate fixation conditions
	Incorrect fluorophore-conjugated secondary antibody	Use secondary antibody specific for primary antibody used

Maintenance of viability and sterility of purified EBs

To maintain viability of EBs during purification procedures, make sure to keep all reagents, plasticware, and cultures on ice. EBs lose viability at room temperature and at 4°C, so try to work as quickly as possible during the purification procedure. Once the infected cells are lysed, the protocol must be completed in a timely manner. If the procedure is followed and no additional incubations on ice are added, EBs can and should be purified and frozen within 6 hr of lysing cells. To maintain sterility of purified EBs, sterilize all solutions and plasticware appropriately or use disposable plasticware when applicable. In addition, perform all work in a BSL-2 biological safety cabinet.

Troubleshooting

Table 11A.1.3 provides many of the common problems encountered with the methods detailed in this unit, as well as their possible causes and solutions.

Anticipated Results

From a large-scale density gradient preparation of *C. trachomatis* EBs, titers ranging from 1×10^8 to 1×10^{10} IFU/ml are usually obtained. Generally, higher titers of *C. trachomatis* LGV EBs are obtained compared to non-LGV EBs.

Time Considerations

The time it takes to complete a large-scale density gradient purification of *C. trachomatis* EBs depends on the specific chlamydial strain being purified. Starting with freshly trypsinized tissue culture cells, it will take 3 to 4 days before the infected cells can be harvested. Depending on the strain, infection of the tissue culture cells will take ~45 min to 3 hr. Finally, purification of the EBs by Renografin density gradient centrifugation will take ~5 to 6 hr.

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This is an excellent resource for understanding the basic techniques of mammalian cell tissue culture.

Schachter, J. and Wyrick, P.B. 1994. See above.

Chapter provides detailed protocols for culturing, and purifying C. trachomatis using several different tissue culture systems.

Richmond, J.Y. and McKinney, R.W. (eds.) 1999. Biosafety in Microbiological and Biomedical Laboratories, 4th ed. Government Printing Office, Washington, D.C.

Book is essential when working with infectious organisms such as Chlamydia trachomatis as it describes the principles of biosafety, the biosafety levels, primary containment, proper handling, and the transport and transfer of infectious agents.

Internet Resources

http://www.chlamydiae.com

A comprehensive site that discusses all aspects of Chlamydia biology including, cell biology, molecular biology, pathogenesis, taxonomy, immunology, laboratory diagnosis, treatment, and research methods.

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Analysis of Bacterial Membrane Proteins Produced During Mammalian Infection Using Hydrophobic Antigen Tissue Triton Extraction (HATTREX)

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ABSTRACT

Hydrophobic antigen tissue Triton extraction (HATTREX) provides a method to extract and identify hydrophobic bacterial membrane proteins from host-infected tissues. The non-ionic detergent Triton X-114 is used to solubilize host-infected tissues at 4°C. Subsequent phase partitioning of Triton X-114 extracted material at 37°C results in a "detergent poor" aqueous phase and a "detergent rich" detergent phase. While soluble proteins partition to the aqueous phase, hydrophobic proteins, such as proteins of the outer and inner membranes, can be found in the detergent phase. Characterization of the detergent phase sample provides insights into the proteome of bacterial membranes during infection. *Curr. Protoc. Microbiol.* 9:12.1.1-12.1.5. © 2008 by John Wiley & Sons, Inc.

Keywords: Triton X-114 • in vivo • hydrophobic • membrane • antigen

BASIC PROTOCOL

Although several pathogenic species of spirochetes can be cultured in vitro, the proteome of in vitro cultivated spirochetes often differs significantly from the proteome expressed during the course of an infection. This unit describes a methodology to provide samples enriched for the membrane proteomes of spirochetes during infection. Membrane proteins of the infecting spirochete can be preferentially extracted from infected mammalian tissues due to the hydrophobic nature of most outer membrane proteins and the phase partitioning properties of the non-ionic detergent Triton X-114 at 37°C. Infected host tissues are triturated into a suitable buffer containing Triton X-114 at 4°C. After extraction for a suitable length of time and removal of nonsoluble material, extracted samples are incubated at 37°C to induce phase partitioning and generate a "detergent rich" detergent phase and "detergent poor" aqueous phase. The more hydrophobic proteins of the bacterial membranes are recovered in the detergent phase, which can then be processed for further analyses, including separation by 1-D or 2-D SDS-PAGE and immunoblotting (Crother et al., 2003, 2004; Nally et al., 2007). The HATTREX procedure could potentially be adapted for analysis of other types of bacteria.

CAUTION: Follow all biosafety requirements relevant to the microorganism under investigation. Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

Materials

Tissue to be processed
Tissue suspension buffer (see recipe)
Triton X-114 (protein grade, Calbiochem)

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Trichloroacetic acid
Acetone
1-D SDS-PAGE solubilization buffer (see recipe)
2-D SDS-PAGE solubilization buffer (see recipe)

PowerGen 125 tissue grinder (Fisher Scientific) or similar device
Vortex
Shaking incubator
37°C water bath

Day 1

Tissue solubilization

1. Weigh tissue to be processed.
2. In an appropriately sized tube, add tissue suspension buffer to a concentration of 50 mg tissue per ml of buffer. Using a tissue grinder, triturate the tissue as rapidly as possible according to the manufacturer's instructions.

   If tissue type contains connective tissues, or skin, dicing into small pieces will aid in the grinding process. Ideally, fresh tissue should be used but similar results are generated using tissue which is snap frozen in a dry ice-ethanol bath at time of procurement and stored at −80°C until use.

3. Grind tissue until a homogeneous mixture is achieved, using caution not to cause foaming of the sample.

   CAUTION: *If working with BSL-2 or higher organisms, appropriate precautions should be taken. Always perform grinding in a Biosafety hood and wear protective outerwear, including gloves, labcoat, a mask, and safety glasses. For more information refer to* UNIT 1A.1.

   Do not overheat the sample. Incubate on ice between grindings.

   Different tissue types will vary in their ability to be triturated. Tissues such as skin can form large clumps during the grinding process. In addition to dicing the tissue into small pieces, increasing the volume of the tissue suspension buffer can aid in the grinding process.

Hydrophobic protein extraction

4. Add Triton X-114 to a final concentration of 1% to 3% to the sample (Cunningham et al., 1988; Radolf and Norgard, 1988; Brandt et al., 1990; Brusca and Radolf, 1994; Crother et al., 2003, 2004). Vortex the sample thoroughly and incubate overnight at 4°C while mixing gently.

Day 2

5. Centrifuge the sample for 30 min at 16,000 × *g*, 4°C. Transfer the supernatant to a fresh tube.

   This step will remove any insoluble material such as bone or connective tissues.

6. Incubate the sample in a 37°C water bath for 10 min to separate the detergent and aqueous phases.

   The sample should become cloudy and the two phases will become visible with time. If this is not the case, incubate the sample for another 10 min, as the larger the volume, the longer the incubation time.

7. Centrifuge the sample 15 min at 3400 × *g*, room temperature to separate the detergent and aqueous phases. Remove aqueous phase.

   The detergent phase will be the lower phase. If phases do not separate, try a longer incubation at 37°C as described above or add more ice-cold tissue suspension buffer with 1% to 3% Triton X-114. Vortex briefly and repeat from step 6.

8. Add ice-cold solubilization buffer (equal to the volume of aqueous phase removed) to the detergent phase and vortex until the solution is homogeneous. This will remove contaminating aqueous phase proteins. Proceed from step 6. Repeat this wash procedure twice.

Hydrophobic protein precipitation

9. After the final wash, add 3 vol of a 10% trichloroacetic acid solution in acetone and vortex the sample for 10 sec. Incubate 2 hr at −20°C.

   The acetone should be cooled to −20°C prior to use. Use acetone-resistant tubes.

10. Pellet the precipitate by centrifuging 30 min at 16,000 × *g*, 4°C. Carefully pour off the supernatant without disturbing the pellet and wash the precipitate with 3 vol of −20°C acetone.

11. Pellet the precipitate by centrifuging 30 min at 16,000 × *g*, 4°C. Carefully pour off the supernatant without disturbing the pellet and allow the protein precipitate to air dry ∼5 min.

    Overdrying results in a pellet which is more difficult to resuspend.

12. Resuspend the protein pellet in an appropriate amount of 1-D or 2-D SDS-PAGE solubilization buffer, depending on your needs.

    The protein pellet can be difficult to solubilize and may require overnight incubation.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Tissue suspension buffer

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM disodium EDTA
1 mM phenylmethylsulfonyl fluoride (PMSF)
Store up to 6 months at 4°C. PMSF should be added fresh.

CAUTION: *Phenylmethylsulfonyl fluoride is toxic.*

In place of EDTA and PMSF, a commercial protease inhibitor cocktail can be used.

1-D SDS-PAGE solubilization buffer

60 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
1% (w/v) SDS
Store up to 6 months at room temperature

2-D SDS-PAGE solubilization buffer

7 M urea
2 M thiourea
1% amidosulfobetaine-14 (Calbiochem, cat. no. 182750-5GM)
30 mM DTT
Store up to 6 months at −20°C

COMMENTARY

Background Information

Triton X-114 is a non-ionic detergent with a cloud point of 22°C. This allows for the enrichment of membrane proteins during the separation of the aqueous and detergent phases (Bordier, 1981). The field of spirochetology has made extensive use of this procedure to identify potential outer membrane proteins and vaccine candidates (Cunningham et al., 1988; Radolf et al., 1988; Radolf and Norgard,

1988; Brandt et al., 1990; Zuerner et al., 1991; Brusca and Radolf, 1994). More recently, the author's laboratory has used the detergent and aqueous phase partitioning ability of Triton X-114 to determine which hydrophobic antigens are present during infection of the Lyme disease spirochete, *Borrelia burgdorferi*, in rabbits and SCID mice (Crother et al., 2003, 2004). This process, termed hydrophobic antigen tissue Triton extraction (HATTREX), can be employed to determine the in vivo *hydrophobic* protein content of the antigenic proteins of the infecting spirochete. Due to the unique structure of the spirochete, HATTREX should be applicable to all spirochetal infections (Barbour and Hayes, 1986; Cox et al., 1992). The procedure may also be useful for analysis of other bacterial species during infection, although such possibilities have yet to be tested.

Critical Parameters and Troubleshooting

The success of the HATTREX process depends on several factors. HATTREX enriches for the antigenic hydrophobic proteins and allows these proteins to be analyzed by SDS-PAGE. However, in order to visualize these proteins, the bacterial presence in the tissue has to be of a sufficient quantity. The flip side of this parameter is the relative antigenicity of each protein. Finally, the quality of the antisera used to identify the individual antigens is extremely important due to the fact that the majority of proteins extracted by this process are of host origin.

Another important factor in the HATTREX procedure is the detergent to protein ratio. The amounts presented in this protocol are a general guideline due to the varying nature of different tissue types. Varying the solubilization volume or detergent concentration may be required to achieve a successful extraction and phase separation.

While this protocol uses TCA-acetone to precipitate the hydrophobic proteins, alternative precipitation procedures, such as chloroform-methanol precipitation, can be employed which can result in recovery of different proteins (Wessel and Flugge, 1984).

Identification of antigens in the sample can be achieved using several methods. Immunoblotting with monospecific sera can be used to look for the presence of a specific protein. Alternatively, more complex sera, such as serum from infected patients, can be used to identify a range of antigens. Two-dimensional SDS-PAGE of the detergent phase sample provides improved resolution of the antigen set. This proteome can then be compared to the proteome of in vitro cultivated spirochetes and antigen identifications made by aligning antigens with identical molecular mass and isoelectric points. An example of each of these identification procedures has been previously described (Crother et al., 2003, 2004; Nally et al., 2007).

Anticipated Results

Results will depend on several parameters including the numbers of spirochetes found in host tissue of interest, the relative antigenicity of individual proteins, and the quality of the antisera. Immunoblotting provides an additional level of sensitivity. In rabbits and SCID mice infected with the Lyme disease spirochete, *Borrelia burgdorferi,* antigens were detected in tissues which harbored as few as 10^4 to 10^5 *B. burgdorferi* per microgram of host DNA (Crother et al., 2003, 2004).

In addition, this protocol can be used to detect antigenic proteins in the aqueous phase of the sample preparation generated as described. However, these samples often contain large amounts of host proteins, making detection of spirochete proteins more difficult.

Time Considerations

This protocol involves an overnight incubation. Day 1 will involve minimal time of up to 30 min. Day 2 can vary but will ideally require ~4 to 5 hr. Precipitated protein pellets can be stored at −20°C indefinitely.

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Isolation and Laboratory Maintenance of *Treponema pallidum*

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ABSTRACT

The spirochetal bacteria that cause syphilis, yaws, and bejel cannot be cultivated in vitro. This unit describes methods for the isolation of subspecies of *Treponema pallidum* and other pathogenic treponemes from clinical specimens, the propagation of these isolates in rabbits, isolation of clonal populations of *T. pallidum*, and techniques for maintenance of frozen stocks of these treponemes. *Curr. Protoc. Microbiol.* 7:12A.1.1-12A.1.18. © 2007 by John Wiley & Sons, Inc.

Keywords: syphilis • treponeme • rabbit • chancre • cerebrospinal fluid

INTRODUCTION

Treponema pallidum is comprised of three subspecies: *T. pallidum* subsp. *pallidum*, which causes syphilis; *T. pallidum* subsp. *pertenue*, which causes yaws; and *T. pallidum* subsp. *endemicum*, which causes bejel or endemic syphilis. These organisms all cause human infection and cannot be propagated in vitro; experimental infection of rabbits is the preferred method for propagation of these subspecies. In addition to these treponemes, *T. paraluiscuniculi* causes a naturally occurring infection in rabbits, is not considered to be infectious for humans (Graves and Downes, 1981), and is closely related to the human treponemes described above. Another closely related treponeme, referred to as the Fribourg-Blanc (or simian) treponeme, was isolated from a baboon in Guinea and is propagated in rabbits (Fribourg-Blanc et al., 1963).

Because of the importance of syphilis to human health, considerable effort has been placed on the cultivation of *T. pallidum* subsp. *pallidum* in vitro. Although limited replication was accomplished in a tissue culture system 25 years ago (Fieldsteel et al., 1981; Norris, 1982), continuous culture has not been accomplished, and isolates must still be propagated in the rabbit. Routine propagation is accomplished by intratesticular passage, although rabbits can also be infected by intradermal, intravenous, and intracisternal routes. Similarly, isolation of new strains of pathogenic treponemes is accomplished by inoculation of clinical material (e.g., chancre exudates, cerebrospinal fluid, and blood) intratesticularly into rabbits (Turner et al., 1969). This unit describes methods for isolation, propagation, and maintenance (Basic Protocols 1, 2, 3, and 4) of these species of treponemes, hereafter referred to collectively as "noncultivable treponemes." A Support Protocol provides a dark-field microscopy method for quantifying the organisms. Additionally, this unit describes a method for the isolation of individual clones of treponemes in the rabbit (Basic Protocol 5).

CAUTION: Treponema pallidum and related treponemes are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. Review the Biosafety portion of Strategic Planning carefully prior to undertaking these procedures. Also see *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: These experiments require Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1*

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and other pertinent resources (*APPENDIX 1B*) for more information. See the Biosafety portion of Strategic Planning (below).

CAUTION: Personal protective equipment (gown, mask, eye protection, gloves) should be worn whenever there is risk of coming in contact with infected materials. See Biosafety (below) for more details.

IMPORTANT NOTE: T. pallidum does not survive for long periods outside of a host. Specimen preparations must be injected into the rabbit as rapidly as possible, at a maximum of 1 hour after collection. See Materials (below) for more details.

NOTE: Reagents and equipment should be ready in advance because rapid harvesting and re-inoculation of treponemes is essential for optimal survival and, thus, yield of organisms. See Materials (below) for more details.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: All solutions and reagents must be sterile.

STRATEGIC PLANNING

Biosafety

The noncultivable treponemes require BSL-2 laboratory and animal care facilities. All personnel handling *T. pallidum* or related organisms, including personnel caring for infected animals, should be carefully trained in such precautions. All personnel should wear appropriate personal protective equipment (PPE) including laboratory coat or gown, gloves, and eye splash protection. Masks are suggested for minimization of allergen exposure during handling of rabbits. Spontaneous aerosolization of *T. pallidum* from suspensions does not occur, although spraying and splashing of organisms from syringes is possible if a needle disengages from a syringe during injection. Disposable Luer-lok needles and syringes must be used for all injections, and recapping or removal of needles must be avoided.

T. pallidum infections of laboratory personnel can occur by accidental parenteral inoculation (needlestick) or by spillage or splashing of suspensions of treponemes (or infected rabbit or human blood and cerebrospinal fluid) on mucosal surfaces or open skin; these infections are not transmitted through intact skin. In case of suspected or known accidental exposure, laboratory personnel should be treated prophylactically with an antibiotic regimen known to be effective for incubating syphilis, and the individual should be monitored serologically (RPR or VDRL test; Larsen, 1999) at the time of treatment and every three months for one year. A prophylactic treatment protocol should be developed in advance of any such occurrence so that treatment is available immediately and at all hours. All persons handling infectious treponemes, even without known or suspected exposure, should be monitored serologically (by RPR or VDRL test) at 6 month intervals to identify inapparent infection. This testing should also be offered to animal care personnel. Whenever possible, all personnel who are isolating *T. pallidum* from human samples should be immunized for hepatitis.

Infected rabbits are not infectious during routine handling unless they have open skin lesions (resulting from intradermal or intravenous inoculation). Nonetheless, personnel handling all infected rabbits should wear the PPE as described above. Saliva, semen, urine, and feces from infected rabbits are not infectious. Therefore, bites or scratches should be washed and treated as they would for any injury caused by a rabbit, but specific treatment for syphilis is not warranted.

Tissues and carcasses, lesion exudates, blood, and cerebrospinal fluid from infected rabbits are potentially infectious and should be handled with care. All wastes should be disposed of in a well labeled biohazard container so that housekeeping or laboratory support personnel are not at risk of inadvertent exposure. Tissue wastes, including carcasses from infected rabbits, must be disposed of in a manner that ensures decontamination (e.g., autoclave, incineration). Suspensions of treponemes can be disinfected (using one of the agents listed below) or autoclaved prior to disposal in a sink drain. Reusable glass tubes containing blood or CSF from infected rabbits should be disinfected first before being subjected to normal glassware washing. *T. pallidum* is inactivated by 70% ethanol, Clidox (chlorine dioxide), phenol-based disinfectants (e.g., Vesphene), and 1% bleach (sodium hypochlorite). Spills or splashes should be contained immediately and the area disinfected with one of the above agents.

Materials

Propagation and maintenance of noncultivable treponemes requires several uncommon items and methods, including rabbits, a rabbit restraint board, and a specialized microscope for quantification of treponemes. In some instances, sedative agents or anesthetic agents are desirable or required (e.g., rabbits should be anesthetized before inoculation of HIV^+ fluids to decrease the likelihood of a needle stick injury to the technologist). Such materials are described below.

In addition, *T. pallidum* are extremely fragile and do not survive for long periods outside of a host. Thus, inoculation of clinical samples or strains should be performed within 1 hr of sample collection. This requires careful planning of all procedures and advance preparation of required reagents. In the case of propagation of treponemal strains, organisms must be harvested from tissues, quantified, and injected into rabbits within the specified time frame.

Rabbits

Noncultivable treponemes have been propagated in several rabbit strains, although most modern laboratories use adult male New Zealand White (NZW) rabbits. For optimal propagation of treponemes, rabbits should be healthy, adult (~3 months old, 2.5 to 3.0 kg for NZW), and have well developed testes. The rabbits should receive no antibiotics (either therapeutic or in their feed) before or after infection. Because of the possibility of inapparent infection of rabbits with *T. paraluiscuniculi*, all rabbits should be screened serologically for evidence of treponemal infection using both a nontreponemal (VDRL or RPR) test and a treponemal (e.g., FTA-ABS) test. The RPR and VDRL tests are available commercially and can be used directly with rabbit serum according to the manufacturer's directions. The FTA-ABS test must be adapted for use with rabbit serum by substitution of FITC-labeled anti-rabbit IgG for the FITC anti-human IgG in indirect immunofluorescence. Detailed instructions for these tests have been published (Larsen, 1999), including the adaptation of the FTA-ABS for rabbit serum (Lukehart, 1982). Although one could use a commercial hemagglutination or particle agglutination treponemal test for rabbit serum, these tests have not been used extensively for this purpose and their sensitivity for detecting *T. paraluiscuniculi* infection is unknown. Reactivity in either treponemal or nontreponemal test excludes a rabbit from use for treponemal infection or strain isolation.

Rabbits should be housed in an 18°C to 20°C temperature-controlled room to facilitate treponemal growth and lesion development. Despite careful temperature control, many laboratories experience less robust treponemal growth and lesion development during the summer months, perhaps due to hormonal changes in the rabbits. Note that all procedures involving rabbits must be approved in advance by the Institutional Animal Care and Use Committee (IACUC).

Rabbit Restraining Board

For intratesticular infection of rabbits, a restraining board is necessary (Fig. 12A.1). These boards are not commercially available, but can be manufactured by a machine shop from stainless steel (for ease of cleaning and disinfection). Ties can be made from soft leather and should be 28 in. in length.

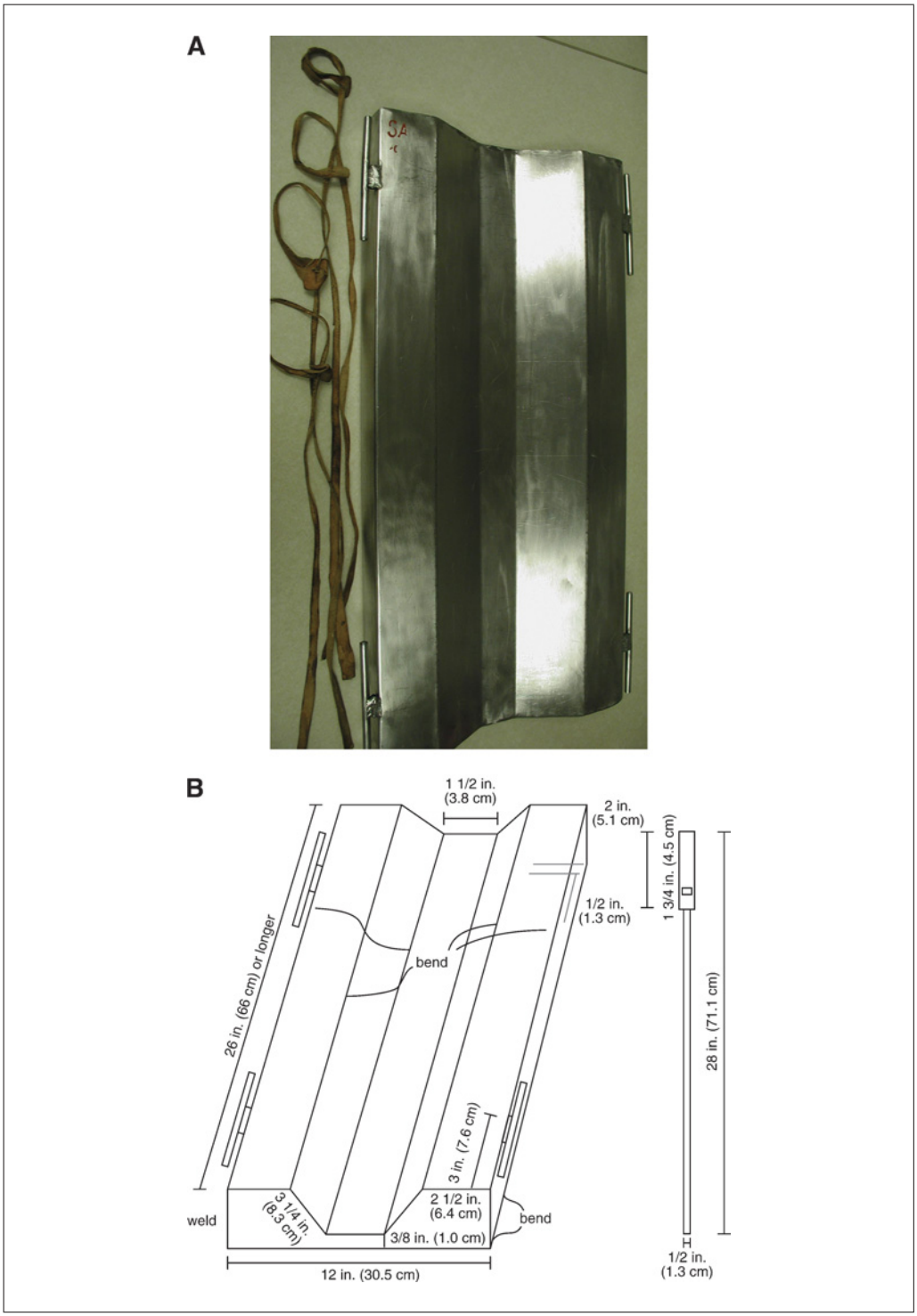

Figure 12A.1.1 Rabbit restraining board. (**A**) Photograph of a stainless steel board and leather ties. (**B**) Diagram with dimensions. Bend a $\frac{1}{2}$-in. lip around the bottom of the board (indicated by the pale gray lines and the word "bend"). Weld metal at corners to avoid sharp edges. The dimensions of the leather straps are shown at right.

ROUTINE PROPAGATION OF NONCULTIVABLE TREPONEMES IN RABBITS

The noncultivable treponemes are propagated most easily by serial passage in rabbit testes. The Nichols strain is the most widely used strain of *T. pallidum*. Since its isolation in 1912 (Nichols and Hough, 1913), this strain has been passaged in rabbit testes by injecting a suspension of treponemes into the body of the rabbit testis. The strain is now highly adapted for rapid growth and the resulting local cellular events have been well documented (Lukehart et al., 1980). The bacteria divide, increasing significantly in number in the interstitial spaces (between the seminiferous tubules) where they trigger a significant inflammatory response that is clinically evident as orchitis (firm, indurated testes). Histologically, this reaction is marked by evidence of edema and T lymphocyte infiltration. Within days of T cell infiltration, macrophages also infiltrate the interstitial spaces and the numbers of bacteria decline precipitously due to phagocytosis and destruction by macrophages. Resolution of the inflammatory response occurs and the testes clinically return to a normal appearance.

The goal in propagation is to harvest the organisms at peak bacterial numbers, before immune clearance occurs. This same series of events occurs following intratesticular infection with all of the noncultivable treponemes; however, the maximal bacterial numbers, time to development of orchitis, and severity of orchitis differ by strain and inoculum size. With the Nichols strain, routine propagation is accomplished by inoculation of 1–5 $\times$ 10^7 *T. pallidum* per testis, in a volume of 1 to 1.5 ml per testis. Using this inoculum size, peak bacterial numbers for the Nichols strain are obtained 9 to 11 days following infection. Clinically the rabbit's testes will be enlarged and firm at this time. The animal is euthanized, and the testes are aseptically removed. The testes are sliced and scored, placed into a saline/rabbit serum mixture, and agitated gently on a rotator. The numbers of organisms extracted into the saline can be monitored by dark-field microscopy (Support Protocol). When the desired treponemal concentration is reached, the suspension is inoculated intratesticularly into the naïve, seronegative recipient rabbit. The harvesting, extraction, and reinoculation of treponemes must be accomplished as rapidly as possible because these organisms do not survive well outside of an animal host. It is recommended that treponemes be reinoculated into the recipient rabbit within 1 hr of harvesting the donor testes. Strict aseptic techniques are required to prevent contamination of the treponemal inoculum with other bacteria.

Depending upon the frequency with which treponemes are needed for experiments, laboratories may develop different propagation schedules. For example, new rabbits may be infected on Monday and Friday of one week, then on Wednesday of the next week, then Monday and Friday of the following week, and so on. This is shown schematically in Figure 12A.1.2. The generation time for the Nichols strain in rabbit testes has been estimated to be 30 to 33 hr (Magnuson et al., 1948), and approximately 10^{10} treponemes can be harvested from the testes of a single rabbit. Other strains and subspecies may grow

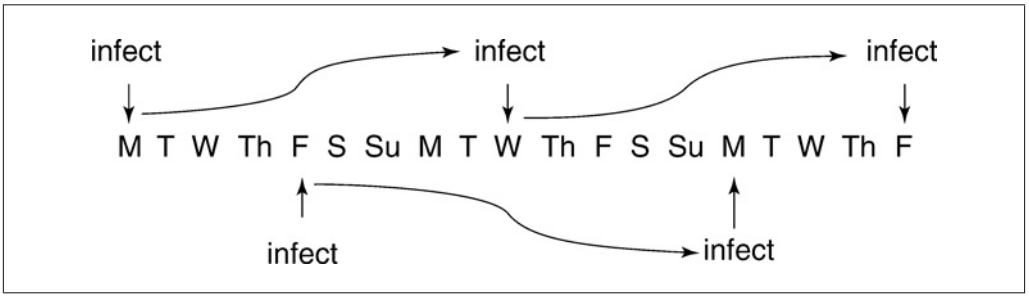

Figure 12A.1.2 Schematic showing a propagation schedule for the Nichols strain of *Treponema pallidum*, with passage to a new rabbit every 8 to 10 days.

at significantly slower rates, achieve substantially lower yields, and induce less dramatic orchitis (Turner and Hollander, 1957). When propagating strains other than the Nichols strain, the rabbit's testes should be palpated at least three times per week to identify the time of optimal orchitis. For propagation of unfamiliar strains, some investigators recommend infection of only one testis, so that the uninfected testis can serve as a control for assessing orchitis. The optimal passage time for individual treponemal strains ranges from 9 to 40 days. At the end of this protocol, the researcher will have isolated treponemes from rabbit testes, which are then immediately inoculated into another rabbit or frozen for storage (Basic Protocol 4).

Materials

T. pallidum sample, frozen (e.g., obtained from a colleague): up to 2 to 3 ml per injection
Rabbits: seronegative in VDRL/RPR or FTA-ABS tests, healthy, adult males with well developed testes and no history of antibiotic treatment: e.g., New Zealand White (NZW), ~3 months old (2.5 to 3.0 kg)
7.5% Betadine solution
70% (v/v) ethanol
Saline (0.14 M NaCl; see recipe)
Normal rabbit serum (NRS; see recipe)
Disinfectant (e.g., phenol based)

Rabbit restraining board (see Strategic Planning and Fig. 12 A.1.1)
3-cc sterile, disposable Luer-lok syringe
23-G × 1-in. sterile, disposable Luer-hub needle
Cotton balls or gauze
Sharps disposal unit
18°C to 20°C BSL-2 rabbit facility
5-in. surgical scissors and 6-in. Kelly hemostat forceps, sterile: packaged as a set prior to autoclaving; two sets required for each procedure
100 × 15–mm petri dishes, sterile
Closed transport container (e.g., plastic box with lid)
Scalpel, sterile
6- to 7-in. forceps, sterile
5- and 10-ml sterile disposable pipets and pipetting aid
Platform rotator with disposable adsorbent material on platform

Additional reagents and equipment for sedating and euthanizing rabbits (e.g., see *http://depts.washington.edu/auts/rabbit_handout_2007.pdf*) and quantifying treponemes by dark-field microscopy (Support Protocol)

Inoculate testes

1. Transport frozen sample to the vivarium on dry ice.
2. Sedate the rabbit (e.g., acepromazine by intramuscular or subcutaneous injection at 1 to 3 mg/kg body weight) according to an IACUC-approved protocol.
3. Place the rabbit securely on restraining board, using soft leather thongs to secure each leg.
4. Thaw the treponemal suspension gently by warming with the hands.
5. Draw the suspension immediately into 3-cc Luer-lok syringe with a 23-G × 1-in. needle.

   Generally, 1.0- to 1.5-ml volumes can be injected into each testis.

   If volume of suspension is small, it may be useful to draw the suspension into a syringe that already contains 1 ml of NRS.

If T. pallidum suspension has been frozen in a solution containing DMSO, it is essential to dilute the suspension immediately after thawing in an equal volume of NRS and to inject it into the rabbit as quickly as possible.

6. Press gently on the lower abdomen of the rabbit to cause the testes to descend; grasp above the body of one testis to prevent retraction.
7. Disinfect the scrotum area well with a cotton ball or gauze saturated with 7.5% Betadine solution.
8. Insert the needle near the distal end of the testis so that it reaches the middle of the testis and inject approximately one-quarter of the treponemal volume. Move the needle to another part of the testis and inject approximately one quarter of the volume. Remove the needle.

   Minor bleeding may occur at injection site. If so, squeeze rabbit's scrotum until bleeding stops.

9. Repeat steps 5 to 7 with the other testis. Discard the syringe in sharps discard container.
10. Place rabbit back in the cage and house in an 18°C to 20°C BSL-2 rabbit facility and observe three times per week for evidence of orchitis by palpating testes.

Dissect out testes

11. When orchitis is considered to be optimal (timing depends upon bacterial strain and inoculum dose), euthanize the rabbit (e.g., pentobarbital by intravenous or intraperitoneal injection at 90 mg/kg body weight) according to IACUC guidelines and secure on the rabbit restraining board.

    An anesthetic overdose is preferable to the use of a physical method.

    Phenobarbital and other euthanasia solutions do not have a detrimental effect on treponemes.

12. If necessary, press the lower abdomen of rabbit to cause testes to descend.
13. Thoroughly wet the scrotal area with 70% ethanol to control fur and to disinfect the area.
14. Grasp scrotum over one testis with Kelly forceps, and with sterile scissors cut longitudinally through scrotum along the length of the testis to expose the tunica albuginea. Repeat for the other testis.
15. Using a new set of instruments, grasp the tunica of one testis with the Kelly forceps and cut the tunica longitudinally with sterile scissors to expose the testis.
16. Grasp the testis with the Kelly forceps and lift the testis out of the tunica. Cut the testis free of the surrounding tissue by cutting the surrounding fat and spermatic vessels.

    Minimal fatty tissue should be left on the testis.

17. Place the testis in a sterile petri dish and replace lid. Repeat for the other testis. Maintain aseptic technique throughout this procedure
18. Clean all surfaces and the rabbit board with disinfectant.

Recover treponemes

19. Transport the petri dish containing testes to the laboratory using appropriate biosafety precautions (e.g., separate closed carrier).
20. If there is significant blood in the petri dish, move the testes to a clean dish before proceeding.

21. Grasp the testis with sterile forceps and slice with a scalpel longitudinally, but not completely through the testis (to form a butterfly shape).

22. Score the cut surfaces of the testis multiple times with the scalpel, then turn the testis cut side down in petri dish.

23. Repeat for the second the testis and place cut side down in the same petri dish.

24. Add 10 ml saline and 1 to 2 ml NRS to the petri dish. Squeeze the testes several times in the saline/NRS using forceps.

25. Place the covered petri dish on a platform rotator. Place a disposable adsorbent pad on the rotator (in case of spills) and rotate gently at room temperature.

    As an alternate approach, some investigators cut the testes into small pieces and place them into a 50-ml conical tube for extraction of treponemes.

Quantify treponemes

26. After rotating for 10 min, collect a sample (e.g., 10 µl) of the extraction and quantify by dark-field microscopy (see Support Protocol).

    If treponemes are present at 1–5 × 10^7/ml, the suspension is ready to be inoculated into a new recipient rabbit. If the concentration of treponemes is lower than 1 × 10^7/ml, the testes should be rescored and squeezed, then rotated for an additional 10 min.

    In some cases, particularly if using a strain other than T. pallidum Nichols, the number of organisms that can be extracted from the testes may be low. In such cases, it may be necessary to score the testes more vigorously, lengthen the extraction time (not more than 30 to 45 min), or decrease the extraction volume to maximize treponeme concentration.

Reinoculate treponemes

27. Draw 2 to 3 ml of suspension into a 3-cc Luer-lok syringe with 23-G × 1-in. needle.

    It may be necessary to move small pieces of tissue so that they do not clog the needle tip. Some investigators move the treponemal suspension to a tube using a disposable 10-ml pipet (remote pipetting) and allow the tissue to settle briefly to facilitate filling of syringe.

    In some instances with non-Nichols strains, where the testes must be vigorously manipulated to obtain organisms, the suspension is centrifuged 10 min at 250 × g and the supernatant used for subsequent inoculation.

28. Transport the syringe (or the treponemal suspension) to vivarium using appropriate biosafety precautions (e.g., separate closed container).

29. Infect both testes of a rabbit as described in steps 2 to 8.

30. Clean all surfaces, including the restraining board, with disinfectant.

    CAUTION: *All contaminated material must be decontaminated or disposed as biohazardous waste.*

SUPPORT PROTOCOL

DARK-FIELD MICROSCOPY FOR QUANTIFICATION OF *T. PALLIDUM* SUSPENSIONS

T. pallidum does not stain using typical bacteriological stains and is so thin that it is not visible by regular bright-field microscopy. The organisms may be readily visualized using a microscope fitted with a dark-field condenser (see *UNIT 2A.1*). Treponemal viability can be assessed by counting the proportion of treponemes that are actively motile. Generally, treponemes can be most accurately counted when there are 5 to 10 organisms per field. An alternative method for quantifying treponemes is through use of a Petroff-Hauser chamber and phase contrast microscopy. The authors use the darkfield-field quantitation method of described by Miller (1971).

NOTE: Refer to *UNIT 2A.1* for a general overview of dark-field microscopy.

Materials

Treponemes harvested from rabbit testes (Basic Protocol 1)
Dark-field microscope and condenser oil
Slide micrometer
Microscope slide and 22 × 22–mm coverslip

1. Measure the radius (r) of the 40× microscope field using a slide micrometer and calculate the area (πr^2).
2. Place a measured amount (e.g., 10 μl) of treponemal suspension on a microscope slide and cover with a 22 × 22–mm coverslip, being careful to avoid bubbles. Calculate the fluid depth by dividing the volume (ml or cm^3) by the area of the coverslip (cm^2):

   $0.010\ cm^3/4.84\ cm^2 = 0.0207$ mm deep

   Other suspension volumes and coverslips sizes may also be used.
3. Calculate the volume of fluid (ml) viewed in a microscope field by multiplying the field area (step 1) by the fluid depth (step 2).
4. Count the number of treponemes in 100 microscope fields, being certain to count organisms in all planes of focus in each field.
5. Calculate the number of treponemes per ml of suspension:

   treponemes/ml = (total treponemes counted in 100 fields)/(100 fields × field volume in ml)

ISOLATION OF *TREPONEMA PALLIDUM* FROM HUMAN SPECIMENS

BASIC PROTOCOL 2

New strains of *T. pallidum* may be isolated from blood, cerebrospinal fluid, chancre exudates, and biopsy tissue from patients with syphilis. Specimens are collected (with appropriate informed consent and approval by the relevant Human Subjects Review Committee) and injected intratesticularly into seronegative rabbits, as described in Basic Protocol 1.

CAUTION: Universal blood-borne pathogen precautions must be taken in the collection and handling of these human samples.

IMPORTANT NOTE: T. pallidum does not survive for long periods outside of a host. Specimen preparations must be injected into the rabbit as rapidly as possible, at a maximum of 1 hr after collection.

Materials

Saline/20% NRS: mix 10 ml saline (0.14 M NaCl; see recipe) and 1 to 2 ml normal rabbit serum (NRS; see recipe)

1-ml tuberculin syringe
100 × 15–mm petri dishes, sterile
Scalpel, sterile
Platform rotator
3-cc sterile, disposable Luer-lok syringe
23-G × 1-in. needle

Additional reagents and equipment for performing venipuncture, lumbar puncture, and punch biopsy (qualified personnel), anesthetizing rabbits (e.g., see *http://depts.washington.edu/auts/rabbit_handout_2007.pdf*), inoculating rabbits and harvesting treponemes (Basic Protocol 1), and performing VDRL/RPR and FTA-ABS screening (Lukehart, 1982; Larson, 1999)

1a. *To collect blood samples:* Collect aseptically by venipuncture into vacutainer tubes containing EDTA as an anticoagulant, per clinical protocol.

The blood of patients with early (primary or secondary) syphilis has the highest likelihood of successful T. pallidum isolation, although treponemes can be isolated from blood of persons with latent syphilis.

1b. *To collect cerebrospinal fluid (CSF):* Collect aseptically by lumbar puncture into a sterile tube, per clinical protocol.

The highest likelihood of isolation is from CSF of patients with primary or secondary syphilis, although treponemes can be isolated from CSF of persons with latent syphilis.

1c. *To collect chancre exudate:* Squeeze the chancre gently so that serous exudate accumulates in the ulcer; avoid blood contamination of the exudate. Collect the exudate using a tuberculin syringe (without needle). Place the tip of syringe into a tube containing 2 ml sterile saline/20% NRS and wash the exudate into the tube by drawing and ejecting 1 ml of saline/20% NRS into the tube several times.

1d. *To take a lesion biopsy:* Collect a 4-mm punch biopsy from a secondary lesion (rash or condylomata lata) or the leading edge of a primary chancre, per clinical protocol. Place the biopsy into a sterile petri dish containing 1 to 2 ml saline/20% NRS. Using a sterile scalpel, mince the tissue into very small pieces and squeeze the tissue into the liquid using forceps. Rotate ~10 min on a platform rotator at room temperature.

2. *Recommended:* Anesthetize the rabbit (serving as the recipient of human samples) and wear double gloves to minimize the risk of needlestick injury.

Anesthesia is recommended for short but potentially painful procedures.

For example, administer 3 to 5 mg/kg xylazine (0.45 to 0.75 ml of 20 mg/ml for 3-kg rabbit), wait 5 to 10 min, and then administer 35 to 50 mg/kg ketamine (1.0 to 1.5 ml of 100 mg/ml for a 3-kg rabbit), both given intramuscularly.

Alternatively, mix 10 ml of 100 mg/ml ketamine, 5 ml of 20 mg/ml xylazine, and 2 ml of 10 mg/ml acetylpromazine. Administer 0.5 ml/kg intramuscularly.

Additional useful information concerning rabbit use and handling may be obtained at http://depts.washington.edu/auts/rabbit_handout_2007.pdf.

3. Draw 2 to 3 ml of sample (blood, CSF, lesion exudate suspension, or biopsy suspension) into a 3-cc Luer-lok syringe with 23-G × 1-in. needle.

4. Place rabbit securely on restraining board, using soft leather thongs to secure each leg, and inject up to 1.5 ml of sample per testis, as described in the Basic Protocol 1, steps 6 to 9.

5. Observe and palpate testes three times per week to detect orchitis.

6a. *If orchitis develops:* Harvest treponemes from testes and transfer them to a new rabbit as described in Basic Protocol 1.

Orchitis is uncommon with the first passage of clinical samples. It is likely that there will be a low number of organisms in this first passage.

6b. *If no orchitis develops by 30 days:* Collect blood from the rabbit for VDRL/RPR and FTA-ABS screening and proceed to step 7.

7a. *If seronegative in both tests:* Continue to observe for orchitis and conduct monthly serological testing until 3 months post-inoculation.

If, after 3 months, the rabbit has not seroconverted in both tests, longer observation is unlikely to be fruitful.

7b. *If the rabbit becomes seropositive in both VDRL/RPR and FTA-ABS tests:* Harvest treponemes from testes (and ideally from popliteal lymph nodes; see Basic Protocol 3) and transfer them to a new rabbit as described in Basic Protocol 1.

It is possible that no treponemes will be visible in the suspension (called "blind passage"), but transfer of infection is likely to occur. Sometimes several blind or low-yield passages are required before the strain is growing well.

Orchitis does not always develop when the rabbits become seropositive (often the case in blind transfers).

8. Continue passage of treponemes as described in Basic Protocol 1.

RECOVERY OF *TREPONEMA PALLIDUM* FROM LYMPH NODES OF RABBITS

BASIC PROTOCOL 3

Sometimes infected rabbits will fail to develop orchitis, or one may wish to isolate treponemes from a rabbit with a latent infection. This is best accomplished by transfer of material obtained from the popliteal lymph nodes, because *T. pallidum* resides for years after infection in many tissues, including lymph nodes. This assay is sometimes used to assess the infection status of a rabbit (e.g., after infectious challenge of an immunized rabbit).

Materials

Infected rabbit (Basic Protocol 1)
70% (v/v) alcohol
Saline/20% NRS: mix 10 ml saline (0.14 M NaCl; see recipe) and 1 to 2 ml normal rabbit serum (NRS; see recipe)

Rabbit restraining board (see Strategic Planning and Fig. 12A.1.1)
4- to 5-in. curved scissors, sterile
100 × 15–mm petri dishes, sterile
Forceps, sterile
Stainless steel mesh screen (~2 × 2 in; e.g., from hardware store), sterile
3-cc sterile, disposable Luer-lok syringe
23-G × 1-in. needle

Additional reagents and equipment for euthanizing rabbits (e.g., see *http://depts.washington.edu/auts/rabbit_handout_2007.pdf*) and passaging treponemes in rabbits (Basic Protocol 1)

1. Euthanize the infected rabbit (e.g., pentobarbital by intravenous or intraperitoneal injection at 90 mg/kg body weight) and secure it on a restraining board at both forelegs and one hind leg. Secure the other hind leg to one of the brackets designed for the front legs so that the flexor side of the leg is exposed.
2. Saturate the fur on the back of the leg with 70% ethanol.
3. Using the sharp point of 4- to 5-in. sterile curved scissors, puncture the skin just behind the knee joint and extend the incision in both directions along the flexor side in each direction for approximately 2 to 3 in.
4. Grasp the popliteal node (see Miller, 1971), contained in a fatty mass just behind the knee joint, with forceps and cut it free. Place the node in a sterile petri dish.
5. Using sterile forceps and small curved scissors, trim the fat away from the node and remove node to a clean petri dish.
6. Repeat steps 2 to 5 for the other popliteal node, placing both nodes in the same petri dish.

7. Add 5 ml saline/20% NRS to the dish. Holding the sterile mesh screen securely with hemostat forceps and the nodes with regular forceps, scrape the nodes on the mesh so that the cells disperse into the saline/20% NRS.
8. Collect the fluid in a 3-cc Luer-lok syringe with a 23-G × 1-in. needle, and infect a seronegative recipient rabbit as per Basic Protocol 1, steps 1 to 10.
9. Observe the rabbit clinically and serologically as described in Basic Protocol 2, steps 5 to 7.
10. Passage organisms as described in Basic Protocol 1 or freeze cultures as in Basic Protocol 4.

BASIC PROTOCOL 4

LONG-TERM MAINTENANCE OF *T. PALLIDUM* STRAINS AS FROZEN STOCKS

It is often preferable to maintain *T. pallidum* strains as frozen stocks to avoid the need for continual passage in rabbits. Strains have been successfully recovered from stocks frozen at −70°C or in liquid nitrogen 20 years earlier. Frozen stocks can be successfully shipped with dry ice. Suspensions containing high concentrations of organisms (at least 10^7/ml) are preferable for freezing.

Materials

Saline/50% NRS: mix 10 ml saline (0.14 M NaCl; see recipe) and 10 ml normal rabbit serum (NRS; see recipe)
Sterile glycerol or DMSO
Dry ice
95% (v/v) ethanol

Sterile 2-ml cryovials, labeled with strain name, concentration, and date.
Liquid nitrogen freezer

Additional reagents and equipment for harvesting *T. pallidum* suspension (Basic Protocol 1)

1. Harvest a *T. pallidum* suspension as described in Basic Protocol 1, steps 11 to 26, except use saline/50% NRS for extraction.

   Ideally, the concentration of treponemes should be $>10^7$/ml.

2. Place 1 ml suspension in a cryovial. Add 1 ml sterile glycerol or 0.1 ml DMSO (10% final concentration). Dispense into aliquots in cryovials, close the cap, mix gently, and freeze immediately in a dry ice/ethanol slurry.

   CAUTION: *DMSO is hazardous. See the material safety data sheet (MSDS) for safety information.*

   Some investigators freeze T. pallidum suspensions with 10% DMSO (final concentration) instead of glycerol. Our laboratory prefers to use glycerol.

3. Allow suspensions to remain in the dry ice/ethanol slurry for 30 min, then move immediately to liquid nitrogen storage.

   T. pallidum can also be maintained frozen at −70°C for many years.

4. To propagate *T. pallidum* from frozen stock, follow Basic Protocol 1.

CLONING OF *T. PALLIDUM* IN THE RABBIT

Because *T. pallidum* cannot be grown in vitro, it is not possible to clone the organisms by selecting a single colony from an agar plate. One can, with considerable effort, successfully isolate clonal populations of *T. pallidum* in vivo using the rabbit model. The basic premise is that, following intravenous infection with *T. pallidum*, some organisms migrate to the skin and cause development of lesions. Each of the lesions is initially seeded by a single treponeme, thus a skin lesion is essentially the equivalent of a colony on an agar plate.

In *T. pallidum*, the *tprK* gene sequence is variable (even within a strain) and undergoes sequence variation, particularly under immune pressure in vivo (Centurion-Lara et al., 2000; LaFond et al., 2003; Centurion-Lara et al., 2004). This sequence can be used as a marker for clonality of *T. pallidum* populations and is used in this cloning protocol to assess the clonal characteristics of treponemal populations.

Materials

Rabbits: seronegative in VDRL/RPR or FTA-ABS tests, healthy, adult males with well developed testes and no history of antibiotic treatment: e.g., New Zealand White (NZW), ~3 months old, 2.5 to 3.0 kg
70% (v/v) ethanol *or* Betadine
Saline/20% NRS: mix 10 ml saline (0.14 M NaCl; see recipe) and 1 to 2 ml normal rabbit serum (NRS; see recipe)

15-ml sterile, conical, screw cap tubes
Low speed centrifuge with BSL-2 containment
Rabbit restraining box (restraining board can be used, but commercially available restraining box preferred)
Closed transport container (e.g., plastic box with lid)
3-cc sterile, disposable Luer-lok syringe
23-G × 1-in. needle
Animal clippers for keeping rabbit back free of fur (size 40 blade, optimal)
4-mm disposable biopsy punches (e.g., Miltex)
100 × 15–mm petri dishes, sterile
4- to 5-in. small curved scissors, sterile

Additional reagents and equipment for preparing *T. pallidum* suspensions (Basic Protocol 1), determining suspension concentration by dark-field microscopy (Support Protocol), sedating or anesthetizing and euthanizing rabbits (e.g., see *http://depts.washington.edu/auts/rabbit_handout_2007.pdf*), propagating organisms in rabbits (Basic Protocol 1), maintaining treponemes as frozen stocks (Basic Protocol 4), and sequencing DNA (e.g., see Centurion-Lara et al., 2000)

Prepare T. pallidum suspensions

1. Prepare a suspension of *T. pallidum* (as concentrated as possible but ideally at least 1×10^8/ml) in saline/20% NRS, as described in Basic Protocol 1, steps 11 to 26.
2. Place the suspension in sterile 15-ml conical, screw-cap tube. Centrifuge 10 min at 250 × *g*, room temperature to pellet gross cellular debris.
3. Pipet the supernatant into a sterile 15-ml conical, screw-cap tube.
4. Determine final concentration of *T. pallidum* suspension by dark-field microscopy (see Support Protocol).
5. Transport suspension to the vivarium using appropriate biosafety containment (closed container).

Inoculate rabbits

6. Withdraw sufficient volume of suspension into disposable Luer-lok syringe with 23-G × 1-in. needle to contain ~10^8 *T. pallidum*.

   If needed, as much as 10 ml of suspension can be injected, although smaller volumes are preferable.

7. Sedate (e.g., acepromazine by intramuscular or subcutaneous injection at 1 to 3 mg/kg body weight) or anesthetize (e.g., see Basic Protocol 2, step 2 annotation) a seronegative rabbit according to IACUC protocols (to minimize movement during injection) and place a in a restraining box.

8. Clean the lateral surface of the ear with 70% ethanol or Betadine.

9. Infect the rabbit by injecting the marginal ear vein with the suspension containing 10^8 *T. pallidum*. Gently squeeze the injection site with gloved fingers and gauze until bleeding stops.

10. Clip the rabbit's back free of fur. Clip daily or as needed to keep fur from growing back.

11. House the rabbit in a temperature-controlled room (18°C to 20°C) as described in Basic Protocol 1.

12. Observe rabbit daily for development of skin lesions (macules or papules) on the clipped back.

Collect organisms from lesions

13. When lesions appear, euthanize (e.g., pentobarbital by intravenous or intraperitoneal injection at 90 mg/kg body weight) or anesthetize (e.g., see Basic Protocol 2, step 2 annotation) the rabbit per IACUC protocol and clean a single lesion that is well separated from other lesions with 70% ethanol.

    It is important to collect biopsies of skin lesions soon after they appear; this minimizes the chance of diversification of organisms and loss of clonality.

    It is advisable to collect biopsies of several lesion and propagate treponemes from each one separately (steps 14 to 18). When tprK DNA sequences from a given biopsy demonstrate clonality (step 20), propagation of the clonal isolates can be continued.

14. Collect a 4-mm punch biopsy ideally encompassing the entire skin lesion.

15. Place the tissue in a sterile petri dish and trim off any subcutaneous fat.

16. Using sterile curved scissors, mince biopsy tissue into 2 to 4 ml saline/20% NRS.

17. Collect a 500-µl sample of suspension under clean PCR conditions for DNA sequencing (see step 20).

    The term "clean PCR conditions" means that the samples are prepared and amplified in a room in which there have never been PCR amplicons or even large amounts of target DNA. Extreme care must be taken to avoid contamination of the sample with extraneous DNA.

Propagate and freeze organisms

18. Inject remaining suspension intratesticularly into a seronegative recipient rabbit as in Basic Protocol 1 to propagate the clonal organisms.

19. Maintain aliquots of clonal treponemes as frozen stocks (Basic Protocol 4).

    When clonal treponemes are propagated from frozen stocks, however, they may undergo sequence diversification during testicular propagation and may need to be recloned by IV infection/biopsy again.

Assess clonality

20. While the organisms are being propagated in new rabbits (see Basic Protocol 1), assess the clonality of the inoculum derived from each biopsy by sequencing the *tprK* locus (e.g., see Centurion-Lara et al., 2000).

Occasionally, lesions contain multiple populations of treponemes, probably due to seeding of the skin by two treponemes in close proximity. These cases will be detected by tprK sequencing, and treponemes derived from them will not be clonal.

The tprK locus should be sequenced from treponemes at ***every*** *stage of the cloning process: inoculum, biopsy, and after propagation in testes. Because some strains have very high rates of sequence change, it may be necessary to repeat the intravenous infection/biopsy step to obtain a clonal population.*

The tprK gene is comprised of 7 regions of variable sequence (V regions) with intervening stretches of conserved sequence (Centurion-Lara et al., 2000). Although some V regions are more likely to vary in sequence, this is not absolute, and full ORF sequencing is necessary to assess clonality of a population. The gene is amplified by PCR, then the amplicons are cloned into E. coli for sequencing of individual E. coli clones. In our experience, direct sequencing of the amplicons fails to show the presence of sequence diversity unless the diverse subpopulations are quite prevalent. We routinely sequence ten clones from each amplification to assess clonality.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Normal rabbit serum (NRS)

Aseptically collect blood from the medial ear artery of seronegative (VDRL/RPR and FTA-ABS) rabbits. Allow blood to clot thoroughly, then centrifuge 10 min at 250 × *g*, 10°C to 23°C. Pipet separated serum into sterile tube. Pool serum from multiple animals at this stage. Heat 30 min at 56°C to inactivate complement. Divide into 1- to 2-ml aliquots and store up to 2 years at −20°C until used. Once thawed, do not refreeze.

In our experience, NRS that we prepare is better for supporting T. pallidum viability than commercially obtained rabbit serum.

Saline (0.14 M NaCl)

0.85 g NaCl
H_2O to 100 ml
Divide into 10-ml aliquots and autoclave
Store up to 6 months at room temperature

COMMENTARY

Background Information

Pathogenic treponemes

The pathogenic treponemes are among the most difficult microorganisms to study because of the inability to cultivate them in vitro and because of their fragility outside of an animal host. Yet, the diseases that they cause are among the most fascinating in terms of natural history and host-parasite interactions. The noncultivable members of the *Treponema* include three subspecies of *Treponema pallidum* (subsp. *pallidum*, *pertenue*, and *endemicum*) and *T. carateum*, which are natural human pathogens, as well as the rabbit pathogen *T. paraluiscuniculi*. These organisms are best described in a classic publication by Turner and Hollander (1957) and in a new book by Radolf and Lukehart (2006). In addition, there is a closely related treponeme that was isolated from a baboon in Guinea (Fribourg-Blanc et al., 1963).

T. pallidum subsp. *pallidum* causes the sexually transmitted infection syphilis. It is estimated that 12 million new cases of syphilis

occur globally each year (Gerbase et al., 1998), and the burden of disease is greatest in developing countries. In the United States and Western Europe, recent outbreaks of syphilis have been reported in large urban areas, particularly among homosexually active men. Syphilis is a chronic infection characterized by discrete clinical stages, including the localized chancre of the primary stage, the rash of secondary syphilis, the decades of asymptomatic latent infection, and the serious and destructive late or tertiary stages of disease. *T. pallidum* subsp. *pallidum* is highly invasive. It invades the central nervous system in a high proportion of patients, and it can cross the placenta in a pregnant woman to infect the fetus.

The remaining human pathogens, *T. pallidum* subsp. *pertenue*, *T. pallidum* subsp. *endemicum*, and *T. carateum*, cause the endemic treponematoses yaws, bejel, and pinta, respectively. Like syphilis, these are chronic infections that have distinct clinical and lengthy latent stages, including late destructive lesions. Unlike syphilis, however, these infections are transmitted by nonsexual, skin-to-skin or mucous membrane contact, often during childhood, and they do not to cross the blood-brain barrier or the placenta. *T. pallidum* subsp. *pertenue* and *T. pallidum* subsp. *endemicum* are likely very closely related genetically to *T. pallidum* subsp. *pallidum*, but careful comparative genomic studies have not been conducted. These two organisms can be propagated in rabbits, just as for *T. pallidum* subsp. *pallidum*. On the other hand, *T. carateum* has not been successfully grown in rabbits and, to the authors' knowledge, no isolates exist in laboratories. The prevalence of the endemic treponematoses is unknown, largely because of poor surveillance in the remote regions of developing countries where these infections exist. Fifty years ago, the prevalence was so high that the World Health Organization undertook a global eradication program in which over 460 million persons were examined, and more than 50 million were treated with penicillin, in 46 countries (Antal et al., 2002). This program significantly decreased the numbers of recognized cases but, despite reports of several outbreaks, surveillance and treatment of these diseases has not continued.

The Fribourg-Blanc simian isolate is predicted to be more closely related to *T. pallidum* subsp. *pertenue* than to *T. pallidum* subsp. *endemicum*, although extensive studies have not been conducted. *T. paraluiscuniculi* is a naturally occurring venereal pathogen of rabbits that causes crusting or ulcerative lesions most often on the genitals, lips, or nose. Like the other treponemal infections, untreated *T. paraluiscuniculi* infection is chronic. Significant gene sequence changes have been identified in *T. paraluiscuniculi*, compared to *T. pallidum* subsp. *pallidum* (Giacani et al., 2004).

Propagation of organisms

All of the pathogenic treponemes are extremely fragile outside of an animal host, often losing viability within hours. Under optimal culture conditions with eukaryotic cells, the Nichols strain of *T. pallidum* can undergo up to five or six rounds of cell division (Fieldsteel et al., 1981; Norris, 1982). Despite decades of effort, no improvement has been made on this number, and serial passage of *T. pallidum* in this tissue culture system has not been achieved. Even in vivo, the organism divides only every 30 to 33 hr (Magnuson et al., 1948). Analysis of the *T. pallidum* genome sequence reveals a striking lack of metabolic capacity including the absence of enzymes for the Krebs cycle, oxidative phosphorylation, and synthetic pathways for many amino acids, lipids, and nucleotides. To compensate, the organism has several transport systems that are thought to bring host-derived molecules into the bacterium.

In addition to serving as a "propagation vessel" for these organisms, the rabbit is the best animal model for syphilis. Like humans, rabbits develop clinical manifestations of primary and secondary syphilis (chancres and rash) and, without treatment, remain latently infected. Very old reports suggest that rabbits may also develop late lesions (e.g., gumma). Compared to the mouse, the use of the rabbit as an experimental model is very difficult; however, the mouse does not develop clinical syphilis after inoculation with *T. pallidum*. As of 2007, laboratory rabbits are not inbred, no "gene knockouts" are available, and immunological reagents are extremely limited. A low coverage genome sequence has recently become available for the rabbit (*http://www.ncbi.nlm.nih.gov/projects/genome/guide/rabbit*, and it is anticipated that this effort will stimulate the development of necessary tools and reagents.

Critical Parameters

Time

Minimizing the length of time that *T. pallidum* organisms are outside of an animal host

is critical to successful propagation. The addition of heat-inactivated normal rabbit serum significantly prolongs survival in vitro, and concentrations of 10% to 50% are efficacious. Most experienced laboratories harvest the treponemes and inoculate recipient rabbits within 1 hr. Several published studies outline conditions in which *T. pallidum* may remain viable in vitro for many hours, but prolonged incubations are not advisable for routine propagation.

Growth rate variation

The Nichols strain of *T. pallidum* subsp. *pallidum* has been maintained in rabbits since 1912, and it is highly adapted to growth in the rabbit testis. Other strains are less well adapted and are frequently difficult to grow, both in terms of bacterial numbers and rapidity of orchitis development. The time from inoculation to development of orchitis is dependent in part upon the inoculum dose, but it also varies by isolate. Investigators will need to regularly monitor rabbits infected with non-Nichols *T. pallidum* to learn the characteristics of each strain.

Rabbit quality

Rabbits used for propagating *T. pallidum* strains should be healthy adult males that have not received antibiotics therapeutically or in their food and water. The rabbit supplier should be chosen for having rabbits that are either specifically pathogen-free for *Pasteurella multocida* or with very low prevalence of symptomatic *Pasteurella* infection. Immature males are not as permissive to *T. pallidum* growth as fully mature rabbits.

Troubleshooting

Failure of infected rabbit to develop orchitis

Occasionally, infected rabbits will fail to develop an orchitis. This may be due to incorrect inoculation (e.g., inoculation into the scrotal sac rather than into the body of the testis). If replicate vials of the same strain are frozen, it is generally preferable to begin propagation again from a frozen stock. If no frozen stock is available, the strain may be recoverable by "blind transfer" of testes and popliteal lymph node extracts as described in Basic Protocols 1 and 3. Even after 1 to 2 years of infection, organisms can be recovered from testes and nodes. Successive rounds of rapid passage of treponemes in rabbits (short time between infection and transfer to the next rabbit) generally serves to select for more rapidly growing populations and higher apparent adaptation.

Unintentional bacterial contamination of the testis

If aseptic technique is not strictly followed during inoculation, the harvested testis may have visible whitish or purple areas at the site of needle insertion. These usually represent foci of secondary bacterial infection, and the testes should not be used for propagation of *T. pallidum*.

Anticipated Results

Well adapted strains of *T. pallidum* (e.g., Nichols strain) yield up to $\sim 10^{10}$ treponemes per rabbit (with lengthy extraction times), while several logs lower yield will be seen with some other strains. Generally, the non-pallidum subspecies and *T. paraluiscuniculi* will not grow to the same bacterial density as *T. pallidum* subsp. *pallidum*. Successful isolation of new *T. pallidum* strains from clinical specimens depends upon the nature of the specimen (i.e., blood, CSF, or lesion exudates), stage of infection in patient, specimen transport conditions, and speed with which the specimen is injected into the recipient rabbit. Generally, patients with primary and secondary syphilis are most likely to provide successful samples.

Time Considerations

The time required to harvest treponemes and re-inject them for routine propagation is 30 to 60 min. *T. pallidum* are extremely fragile and do not survive for long periods outside of a host. Thus inoculation of clinical samples or strains should be performed within 1 hr of sample collection. This requires careful planning of all procedures and advance preparation of required reagents.

Because it can take weeks to months for a new harvest of treponemes, investigators will have to plan very carefully for future needs. Rabbits may need to be ordered weeks to months in advance, time must be allowed for serological screening of the rabbits, and 2 to 6 weeks may elapse between infection and subsequent harvest.

Isolation of new strains will likely be more time consuming because one or even two blind passages are often necessary before the bacterial numbers reach concentrations that can be determined microscopically.

Derivation and maintenance of clonal *T. pallidum* populations is very time consuming. Many months of work may be required before it is possible to confirm that a population of organisms is clonal.

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Laboratory Maintenance of *Treponema denticola*

This unit describes methods, media, and equipment required for routine laboratory culture and handling of the oral anaerobic spirochete *Treponema denticola*. Topics discussed include nutrient requirements, recommended formulations for growth in liquid and solid media, and expected growth kinetics, as well as methods and equipment necessary to maintain anaerobic conditions. Protocols on isolation of *T. denticola* from clinical samples are included. With the notable exception of specific individual nutrient requirements and media formulations, these protocols are also appropriate for working with other species of cultivable commensal treponemes.

Basic protocols included in this chapter cover topics ranging from isolation of *T. denticola* from clinical samples (including isolation of pure cultures from single colonies) through culturing techniques applicable to biochemical analysis and genetic manipulation of *T. denticola*. The isolation protocols include a relatively standard method of dispersion and serial dilution on agar plates as well as a method using liquid medium in a 96-well plate format. Both of these methods employ rifampicin as a selective agent to enrich for treponemes in mixed clinical samples. Several commonly used liquid media and related solid media formulations are described, with comments on relative characteristics and performance of each. Methods, equipment, and conditions for inoculation, incubation, passage, and storage of cultures are discussed. Together, this information should provide the technical background required for routine growth and manipulation of *T. denticola* in a well-equipped microbiology laboratory.

CAUTION: *Treponema denticola* is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (see *APPENDIX 1B*) for more information.

NOTE: Most strains of oral spirochetes require strictly anaerobic conditions during transfer and growth of cultures. Unless otherwise specified, all incubations and, where feasible, manipulations described in this unit are to be performed under strictly anaerobic conditions (i.e., nonhumidified 85% N_2/10% H_2/5% CO_2 atmosphere) in an anaerobic glove box chamber at 37°C (see Strategic Planning).

STRATEGIC PLANNING

Media

General requirements

Several complex broth media formulations are in common use for growth of *T. denticola* strains (see Reagents and Solutions). These share common features including sources of peptides, amino acids, and trace nutrients (e.g., tryptone, brain heart infusion, yeast extract), as well as reducing agent(s) (L-cysteine and/or thioglycolate), volatile fatty acids, and heat-inactivated serum. All these media provide very low redox potential (Eh = −185 to −220), which is a critical factor in growth of *T. denticola* (Socransky et al., 1964). These media were originally devised for isolation of various species of oral spirochetes, and likely contain more nutrients than are required by *T. denticola*. For example, *T. denticola* does not require glucose for growth, which is required for growth of

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T. phagedenis (Smibert, 1973, 1976). Although serum stimulates growth of *T. denticola*, one study reports that it is not absolutely required for growth of some commonly used laboratory strains in complex medium (Cheng and Chan, 1983). *T. denticola* has been reported to grow reasonably well in serum-free broth media containing ceruloplasmin (Suzuki and Loesche, 1989) or α-globulin (Socransky and Hubersak, 1967). The media formulations listed here contain serum at 1% to 10% final volume: most laboratories use serum concentrations within this range. Although most original formulations called for rabbit serum, the serum source does not appear to be critical. Most, but not all, media formulations contain sodium bicarbonate; however, NOS medium without sodium bicarbonate does not support growth of several common laboratory strains of *T. denticola* (Cheng and Chan, 1983). As an alternative to the media described in Reagents and Solutions, good growth of *T. denticola* can be obtained using Spirolate Broth (BBL cat. no. 11636), supplemented with fatty acids, thiamine pyrophosphate, sodium bicarbonate, and serum, as described for NOS medium.

OMIZ-P4 medium

A chemically defined, serum-containing medium based on OMIZ-W1 (Wyss, 1992) has been used by several investigators. This medium, OMIZ-P4 (see Reagents and Solutions), supports the growth of all cultivable oral treponemes described to date. An earlier version of this medium has been particularly useful in isolating novel *Treponema* species from periodontal lesions (Wyss et al., 1999) and bovine digital dermatitis lesions (Schrank et al., 1999). OMIZ-P4 is listed by ATCC as Medium 2131 (*http://www.atcc.org*) and is available commercially by special order (Anaerobe Systems) at a price reflective of the complexity of its formulation. A chemically defined serum-free minimal medium for growth of *T. denticola* is under development and is likely to become available in the near future (C. Wyss, pers. comm.).

Growth of *T. denticola* in Solid Medium

Most investigators routinely cultivate *T. denticola* in broth medium; however, for many types of experiments, including growth studies and genetic transformations, growth of colonies in or on solid media is required. Difficulties encountered in growing *T. denticola* on solid media have been a continuing issue in the field, and have contributed to the rather slow progress of research compared with other organisms. To date, there have been no studies showing quantitative recovery of microscopically visible spirochetes as colony-forming units (CFUs) grown either in or on the surface of solid media, and studies of clinical samples suggest that the recovery rate on any solid medium is quite low. Several factors contribute to the difficulty of growing *T. denticola* on solid media. The extremely low oxygen tolerance of *T. denticola* appears to be a primary obstacle to growth on the surface of plate media, with most strains intolerant of oxygen concentrations >0.1% (Loesche, 1969). Colonies that do grow from surface inocula tend to grow down into the agar, except under extreme (95% H_2/5% CO_2) anaerobic conditions (Socransky et al., 1959). Growth on agar media is inhibited by the toxicity of standard Bacto agar to *T. denticola*, presumably due to trace impurities in this relatively crude product (Chan et al., 1993b). Traditionally, cultivation of oral spirochetes on solid media has been done by suspending cells in a molten Noble agar medium, so that subsurface colonies are formed; however, viability of *T. denticola* is greatly reduced by heating to temperatures (45° to 50°C) required to maintain Noble or Bacto agar in a molten state (Chan et al., 1993b). These factors have led to the use of alternate solidifying reagents, including low-melting-point agarose and mixtures of Noble agar and gelatin, certain formulations of which remain molten at or near 37°C (see Reagents and Solutions). Although there is one report suggesting that *T. denticola* can be grown under aerobic conditions in broth cultures (Syed et al., 1993), this has not been the experience of most laboratories.

Several complex media formulations have been used for oral spirochete cultivation as either surface or subsurface colonies. Generally, media formulations and considerations are the same as for broth media: provision of required nutrients, near-neutral pH, and low Eh. Earlier investigators often used various formulations of blood agar. More recently, blood in the medium has been replaced by serum, usually from rabbit. The recipe given in this unit for MTYGVS plate medium (see Reagents and Solutions) describes a solid medium for plating spirochetes (or clinical samples) in an anaerobe chamber. For use in isolation of spirochetes from clinical samples, this medium is supplemented with rifampicin (Leschine and Canale-Parola, 1980; Salvador et al., 1987). The other two solid media, NOS semisolid medium with low-melting-point (LMP) agarose and NOS-GN (see Reagents and Solutions), were developed for growth of subsurface colonies without overheating the spirochetes. These last two media, which utilize a pour-plate method, are routinely used for selecting *T. denticola* genetic transformants (*UNIT 12B.2*).

Microscopy

Contamination of *T. denticola* cultures with other more rapidly growing organisms is always a concern, especially when working with clinical isolates. Visualization of cultures by either dark-field or phase-contrast microscopy (see *UNIT 2A.1*) is a rapid and reliable method for routine validation of culture purity. The unique morphology and active motility of *T. denticola* enables the investigator to easily detect the presence of nonspirochete contaminants. If species specificity is required, this can be determined by PCR amplification and DNA sequencing of appropriate 16S rRNA sequences. Sequences of genes encoding 16S rRNA are available in Genbank. Universal, spirochete-specific and *Treponema*-specific oligonucleotide primer sets and appropriate PCR conditions have been reported by several authors (Paster et al., 1991; Choi et al., 1994; Paster et al., 2001).

Equipment Required for Maintenance of Anaerobic Conditions

Two anaerobic systems are in common usage: the anaerobic glove box (Rosebury and Reynolds, 1964; Aranki et al., 1969), and the GasPak and related products, which consist of a container for a few plates or tubes, and a disposable CO_2 and hydrogen generator (Brewer and Allgeier, 1966). The choice of which anaerobic system to use depends on the technical capability of the laboratory staff, space available, scale of the project, and budgetary concerns. Glove box systems are major laboratory equipment, whereas GasPak systems are small, portable, and much less expensive. With either system, providing an active catalyst to remove residual oxygen is particularly important (Dowell, 1972).

Anaerobic glove box chambers

Anaerobic glove box chambers maintain a 0- to 5-ppm oxygen atmosphere by means of hydrogen gas reacting with a palladium catalyst to remove oxygen. The typical gas mix is 85% N_2/10% H_2/5%CO_2, though some investigators use an 80:10:10 mixture. Samples are introduced to the chamber through an airlock port that is evacuated by vacuum and flushed with nitrogen gas several times and then filled with the chamber gas mix.

Vinyl glove box chambers

Flexible, vinyl anaerobic chambers and glove boxes, such as those supplied by Coy Laboratory Products, provide operational advantages over rigid glove box designs when used for anaerobic microbiology. This is due to the flexibility of the chamber organization and the relatively large space available. This allows for cultures on plates or in various tubes or flasks as required. In addition, this design allows for installation of several small instruments in the chamber, such as a microcentrifuge, vortexer, and pipettors.

This particular system was developed specifically for isolation and maintenance of strict anaerobes (Aranki et al., 1969; Rosebury and Reynolds, 1964).

Acrylic glove box chambers

This system, composed of a rigid acrylic glove box with transfer compartment, maintains controlled temperature and atmosphere, has integral lights, and measures ~104 (L) × 71 (W) × 66 (H) cm. The smaller size and rigid construction of this type of unit makes it somewhat less convenient for multiple users or large-scale experiments. These boxes can be obtained from suppliers such as Plas Labs.

Portable anaerobe jars or pouches with gas-generating envelopes

Oxoid AnaeroGen and BBL GasPak systems

The two basic components of these envelopes are a carbon dioxide generator envelope (with or without a hydrogen generator), a room temperature palladium catalyst, and a sealed container. Agar plates or broth tubes are placed in a specifically designed container along with a gas generator/catalyst envelope. The generator is activated and the container is sealed to generate the anaerobic environment by consuming oxygen, either by generating CO_2 or H_2O. Variations on this system are available from several suppliers. The Oxoid AnaeroGen system was compared with the BBL GasPak (Becton Dickinson) for the production of an anaerobic atmosphere and was evaluated for its ability to support the growth of 135 clinically significant anaerobic bacteria. Both of these systems performed essentially equally well compared with an anaerobe chamber for supporting the growth of anaerobes, as long as the gas generating system was used correctly (Miller et al., 1995).

AnaeroPack

The AnaeroPack system (Mitsubishi Gas Chemical America, distributed by REMEL) requires no catalyst or water, produces no hydrogen, and is oxygen absorbing and carbon dioxide generating (20% CO_2/0.1% O_2 within 1 hr, according to the manufacturer's information). The system consists of a sachet packet that is opened just before sealing the bacterial culture in the included container (sealable polystyrene box or disposable bag). Containers are available in several sizes to accommodate 2 to 36 standard agar plates. The disposable bag format easily handles several 15-ml culture tubes. Depending on container volume, one to three sachets are recommended. Performance of this system is similar to the GasPak system (Delaney and Onderdonk, 1997).

Common sense in the anaerobic glove box chamber

The anaerobic glove box chamber is a key piece of equipment in many laboratories that routinely cultivate anaerobic spirochetes, and is especially useful for growth of larger-volume cultures. In many laboratories, the chamber is used by several investigators; in fact, two people can work at the larger models simultaneously. As with any "common" or shared equipment, it is necessary to train all potential users in safe and efficient operation. The following list of "common sense" rules is designed as an example of a supplement to standard lab training that might be posted adjacent to the chamber.

1. Never open the outer entry lock door before checking to see whether the inner door is securely closed.
2. Conversely, never open the inner door before checking to see whether the outer door is securely closed. If the entry lock has an "anaerobic/aerobic" warning light, always check that the "anaerobic" light is on before opening the inner door.

3. Remove rings and watches before working in the chamber to avoid puncturing gloves and chamber sleeves.
4. Avoid using sharp items in the chamber except as necessary.
5. Use absorbent bench paper in the chamber to contain spills and debris. Change regularly.
6. Minimize the number of chamber entries by careful scheduling and planning of in-chamber procedures, including having the necessary supplies and equipment in the chamber as needed.
7. Maintain a regular schedule for changing desiccant and catalyst as appropriate to the level of use of the anaerobic chamber.
8. Use white cotton gloves (available from photographic suppliers) to absorb perspiration while working in the chamber. This is for your comfort as well as for the comfort of subsequent users.
9. Keep a roll of vinyl tape in the chamber, both for emergency repairs and for periodic routine glove replacement.
10. Use standard laboratory safety practices for changing and handling gas tanks, with special attention to ensuring that tanks are secured at all times.

ROUTINE GROWTH OF *T. DENTICOLA* IN LIQUID MEDIUM

BASIC PROTOCOL 1

Many, if not most, common laboratory strains of *T. denticola* have been in long-term or continuous culture for extended periods of time. While it is likely that multiple passaging of *T. denticola* strains results in phenotypic changes compared with isogenic low-passage isolates, there have been no studies demonstrating this. This is in part due to the fact that systems for modeling the contribution of *T. denticola* to periodontal disease pathogenesis are extremely limited. The author has not observed major differences between common laboratory strains of *T. denticola* obtained from ATCC and recent *T. denticola* clinical isolates. In any case, good microbiological practice suggests that passaging be limited, especially if one is studying characteristics or behavior believed to be related to pathogenicity. In the author's laboratory, strains recovered from frozen stocks are passaged fewer than ten times.

T. denticola can be revived from stored stock cultures by growth in one of the broth media listed below. All of these media formulations provide nutrients required for growth of *T. denticola*. Of the three, OMIZ-P4 is the closest to a "defined" medium. Even so, OMIZ-P4 contains neopeptone and serum. OMIZ-P4 will support growth of most cultivable oral spirochetes. Although this medium is extremely useful for metabolic and biochemical studies, it is very expensive for routine growth of *T. denticola* laboratory strains. NOS and TYGVS are complex media with significant amounts of brain heart infusion and yeast extract. TYGVS supplies higher concentrations of several nutrients, including nutrients (such as gelatin) that are absent in NOS. Though NOS is practical for routine growth of laboratory strains, growth is marginally faster and final densities typically higher in TYGVS. Some recent clinical isolates will grow in TYGVS but will not grow in NOS.

Materials

NOS, TYGVS, or OMIZ-P4 broth medium (see recipes)
T. denticola stock culture (e.g., ATCC #35405)
100% glycerol, sterile, at 37°C, prereduced
Dry ice/ethanol bath or liquid nitrogen
Preservation medium (see recipe)

15-ml glass or plastic (polystyrene or polypropylene) anaerobic culture tubes
Anaerobic chamber (see Strategic Planning)

2-ml cryovials (Corning or equivalent)

Additional reagents and equipment for lyophilizing *T. denticola* (Cheng and Chan, 1983; optional) or storing in liquid nitrogen (Syed et al., 1993; optional)

1. Freshly prepare supplemented medium and transfer into an anaerobic chamber. Equilibrate overnight with the cap loosened.

   Ideally, freshly autoclaved medium is introduced into the anaerobic chamber and supplements are added inside the chamber. In practice, this is rather cumbersome and does not allow for preparation of media stocks. This step provides a practical alternative.

2. Thaw or rehydrate a vial of stored stock culture under anaerobic conditions.

   The optimal concentration of the stock culture will depend upon whether any selective pressure is being applied. Serial dilutions to obtain individual colonies may be required.

3. Add contents of the vial to not more than 20 vol prereduced broth medium.

   For typical strain growth, 10-ml cultures are grown in 15-ml glass or plastic (polystyrene or polypropylene) tubes with caps loosened. Depending on the volume required, culture volumes may be stepped up to 1 liter or more in large flasks or bottles.

   The high level of inoculum helps to ensure recovery of the culture. Maintenance of excellent aseptic technique is crucial at this step (and in step 6 below). T. denticola grows more slowly than most environmental contaminants in rich medium.

4. Incubate at 37°C until active growth becomes apparent (2 to 5 days).

   Depending on the strain, growth medium, and serum concentration, the generation time of T. denticola is ~12 hr, yielding ~1 × 10^9 cells per ml after 4 days growth (late log phase) in NOS medium.

5. If determining growth kinetics and using a low-viscosity broth medium, briefly mix or vortex the culture gently to suspend the bacteria, then measure OD_{600}.

   T. denticola cells accumulate at the bottom of the tube by late log phase even though they retain motility as visualized by microscopy.

6. If desired, maintain cultures by weekly passage in fresh, prereduced medium at dilutions of between 1:20 and 1:100.

   Unlike other oral anaerobes such as Porphyromonas gingivalis, viability of T. denticola does not decrease rapidly after logarithmic growth in liquid media.

7a. *For long-term storage by freezing:* Add prereduced, prewarmed glycerol to actively growing cultures to a final glycerol concentration of 15% (v/v). Aliquot to 2-ml cryovials. If working in an anaerobic chamber, cap the vials at this point, remove from chamber, and freeze quickly in a dry ice/ethanol bath or by immersion in liquid nitrogen. Store vials at −70°C.

   As with all procedures with T. denticola, viability is optimal when manipulations are conducted under strict anaerobic conditions as much as is practicable, regardless of the storage method used.

7b. *For long-term storage by lyophilization:* Lyophilize by the method of Cheng and Chan, (1983).

7c. *For long-term storage in liquid nitrogen:* Store in liquid nitrogen in preservation medium (Syed et al., 1993).

PLATING *T. DENTICOLA* ON SEMISOLID MEDIA FOR VIABILITY COUNTS

Either 25-cm^2 tissue culture flasks (Corning) or deep petri dishes may be used for plating *T. denticola*.

Materials

NOS-GN 1% agar semisolid medium (see recipe) or NOS 1.5% LMP agarose semi-solid medium (see recipe)
T. denticola cell suspension (see Basic Protocol 1)

37° to 39°C water bath
25-cm^2 tissue culture flasks (Corning or equivalent)
6-well tissue culture plates (Corning or equivalent)
100 × 25–mm extra-deep petri dishes (Nalgene Nunc or equivalent)
Ice packs
Anaerobic chamber (see Strategic Planning)

For tissue culture flasks

1a. Prepare semisolid medium, aliquot to tissue culture flask(s), and cool to 37° to 39°C in a water bath. Add *T. denticola* cell suspension (up to 3 ml) to 25 ml medium in a 25-cm^2 tissue culture flask.

2a. Mix by gentle inversion and incubate at room temperature until the medium solidifies.

The medium must be brought to 25° to 30°C to solidify.

3a. When the medium has solidified, overlay with a few milliliters of noninoculated semisolid medium if desired.

Chan et al. (1993b) suggest this overlay as a means of sealing the medium from contact with atmospheric gases. Refer to Chan et al., 1993b, for more information concerning this technique.

For petri dishes or tissue culture plates

1b. Prepare semisolid medium in an appropriate-size bottle or flask and cool to 37° to 39°C in a water bath.

2b. Add *T. denticola* suspension (up to 5 ml per 50 ml medium), mix gently, and pour into one or more 100 × 25–mm extra-deep petri dishes (~50 ml per dish) or 6-well tissue culture plates.

3b. Precool the anaerobic glove box chamber entry port with two ice packs for a few minutes.

4b. Transfer the inoculated petri dishes or tissue culture plates to the cooled anaerobic chamber entry port to solidify the medium.

Once in the chamber port, the medium should solidify within an hour or less.

5b. Return inoculated petri dishes or plates to the chamber for incubation until growth is observed.

T. denticola growth is typically visible as diffuse subsurface colonies after one week, though genetic transformants sometimes take two weeks or longer to form colonies (also see UNIT 12B.2). Colonies will continue to grow larger as the organisms move through the medium. The diffuse "fuzzy" appearance of T. denticola colonies in agar media is due to spirochete motility through the semisolid medium. Subsurface colonies will be smaller and less diffuse in media with higher concentrations of solidifying agent.

ISOLATION OF *T. DENTICOLA* FROM CLINICAL SAMPLES

Although spirochetes are typically the predominant microscopically visible organisms in subgingival plaque samples, they are rarely isolated using standard methods of sample dispersal and culturing on agar media, even under strictly anaerobic conditions. This section provides two variations on a method for gentle dispersion of subgingival dental plaque samples and selective cultivation of oral spirochetes including *T. denticola*.

Two methods have been reported that work well for selective isolation of oral spirochetes: serial dilution and plating in an anaerobic glove box chamber (Salvador et al., 1987), and serial dilution in 96-well microtiter plates followed by incubation in a GasPak anaerobic jar (Wyss et al., 1996). Both of these techniques rely on the fact that oral spirochetes are naturally resistant to rifampicin (Leschine and Canale-Parola, 1980) and phosphomycin. Data on comparative efficiency of these methods have not been reported.

Subgingival plaque samples should be obtained by a qualified clinician from patients with active periodontal lesions. Typically, following removal of a supragingival plaque, a subgingival plaque from a periodontal pocket is sampled with a curette or paper point and immediately transferred to a vial containing 0.5 ml reduced transfer fluid (RTF). Plaque samples should be processed in the laboratory as soon as practicable, at least within a few hours.

BASIC PROTOCOL 3

Plating *T. denticola* in an Anaerobic Chamber

This method is essentially a standard inoculation of an agar plate under anaerobic conditions. This plating technique relies on (1) the distinct colonial morphology and (2) constitutive resistance to rifampicin and/or phosphomycin of oral spirochetes. As noted below in step 2, although there have been no studies directly comparing plating efficiency of various techniques described in this unit, the fact that *T. denticola* colonies tend to grow into or under the agar surface suggests that the in-agar or plate-dilution methods may be more efficient.

Materials

Subgingival plaque sample
Reduced transfer fluid (RTF; see recipe)
MTYGVS plates containing 5 μg/ml rifampicin (see recipe)
TYGVS medium (see recipe)
Anaerobic chamber (see Strategic Planning) containing 5 μg/ml rifampicin
Vortex mixer
Disposable cell spreaders (Fisher or equivalent)
Inoculating turntable
Semiautomatic spiral plater (Spiral Biotech Spiral Plater or equivalent; optional)
Pasteur pipets
15-ml glass or plastic (polystyrene or polypropylene) anaerobic culture tubes

1. Disperse subgingival plaque sample in RTF by vortexing 10 sec. Dilute serially in RTF and plate on MTYGVS agar plates containing 5 μg/ml rifampicin using a turntable and sterile cell spreader to spread the inoculum evenly over the agar surface. This entire process can be done in an anaerobic chamber.

   Rifampicin inhibits growth of the vast majority of nonspirochete members of the oral flora. Rifampicin resistance in most bacteria is the result of a single point mutation in the rpoB gene (Carter et al., 1994). Thus, simple selection with rifampicin can result in isolation of resistant organisms.

For inoculating large numbers of plates, it is convenient to use a semiautomatic plating device. While this is an expensive piece of equipment, it is very useful for rapid, standardized inoculation in high-throughput research laboratories and in clinical laboratories. Most smaller research laboratories will find a standard inoculating turntable will give adequate results if proper care is taken in standardizing inocula and spreading techniques.

2. Incubate anaerobically at 37°C until the diffuse subsurface colonies become visible (7 to 14 days).

3. Push the tip of a sterile glass Pasteur pipet down through the colony so that agar including a portion of the colony enters the end of the pipet.

   This is known as punch collection.

4. Expel the agar from the pipet into a culture tube containing 2 to 5 ml liquid TYGVS medium containing 5 μg/ml rifampicin.

   It is important that each colony selected be grown in broth and monitored by dark-field microscopy (UNIT 2A.1) for culture purity and typical spirochete morphology (motile helical organisms).

Limiting-Dilution Isolation in 96-Well Microtiter Plates Incubated in an Anaerobic Jar

BASIC PROTOCOL 4

This procedure has been modified from that of Wyss et al. (1996).

Materials

Subgingival plaque sample
Reduced transfer fluid (RTF; see recipe)
OMIZ-P4 medium with 5 μg/ml rifampicin and 100 μg/ml phosphomycin (see recipe; also see Strategic Planning)
OMIZ-P4 1.5% LMP agarose semisolid medium: prepare as described for NOS 1.5% LMP agarose semisolid medium (see recipe), substituting OMIZ-P4 basal medium (see recipe) for NOS basal medium (see recipe)

96-well microtiter plates
Anaerobic jar (Becton Dickinson GasPak or equivalent; see Strategic Planning)
37°C incubator
Disposable cell spreaders, sterile (Fisher or equivalent)
Inoculating turntable

1. Disperse subgingival plaque sample in 0.5 ml RTF by vortexing 10 sec. Dilute serially in 96-well microtiter plate with 0.2 ml per well OMIZ-P4 medium containing rifampicin and phosphomycin.

   Typically, dilutions of 1×10^{-5} or 1×10^{-6} result in growth in 50% of wells (Wyss et al., 1996).

   Conduct these manipulations on the benchtop with deliberate speed.

2. Incubate microtiter plates in an anaerobic jar 10 days at 37°C.

3. Determine cell morphology in wells containing growth by phase-contrast or dark-field microscopy (*UNIT 2A.1*). Select wells containing cells with typical spirochete morphology (motile helical organisms) for further analysis. If mixed cultures are observed, subject these to further limiting dilution or spirochete colony isolation as described in step 4 below, if desired.

   With access to an inverted microscope having dark-field or phase-contrast optics, this can be done without removing any of the culture from the plate.

4. Streak samples from wells containing spirochetes on plates containing OMIZ-P4 1.5% LMP agarose semisolid medium. Spread the inoculum evenly over the surface using a sterile cell spreader. Alternatively, suspend samples in prewarmed semisolid medium and inoculate as in Basic Protocol 2.
5. Incubate until diffuse colonies are visible within the agarose (5 to 10 days).
6. If further characterization is desired, pick colonies by punch collection using sterile glass Pasteur pipets, streak again to ensure purity, and transfer to liquid medium.

BASIC PROTOCOL 5

LABELING OF *T. DENTICOLA* WITH RADIOISOTOPES

Whole cells or specific cell components of *T. denticola* have been radioactively labeled for studies of adherence behavior (Sela et al., 1999), nutrient uptake (Blakemore and Canale-Parola, 1976), and phospholipid metabolism (Kent et al., 2004). For most experiments, it is preferable to grow broth cultures of *T. denticola* in an anaerobic glove box chamber (see Strategic Planning); however, handling, monitoring, and cleanup of radioisotopes is relatively cumbersome in the chamber. For this reason, it is much easier to conduct labeling experiments using an anaerobic jar system such as a GasPak or similar product. This is especially true when using radioisotopes such as ^{32}P that require shielding.

CAUTION: Radioactive materials require special handling. See *APPENDIX 1C* and the institutional Radiation Safety Office for guidelines concerning proper handling and disposal.

Materials

T. denticola broth culture (see Basic Protocol 1)
^{3}H-, ^{14}C-, or ^{32}P-labeled substrate
Phosphate-buffered saline (PBS; see *APPENDIX 2A*)

15-ml polypropylene centrifuge tubes with caps (Corning or equivalent)
Anaerobic jar (Becton Dickinson GasPak or equivalent; see Strategic Planning)

1. Grow *T. denticola* broth cultures to early logarithmic phase in 15-ml polypropylene tubes in an anaerobic chamber.

   This type of tube is used for growth of cultures in these experiments in order to minimize transfer and handling of radioactive materials.

2. Remove the cultures from the chamber, uncap the tubes, and add the radioactive label using conditions appropriate to the particular radioisotope.

   The choice of radiolabeled substrate will depend on the cell component being studied.

3. Recap the tubes, place them in an anaerobic jar or pouch system and activate the gas/catalyst system.
4. Incubate at 37°C in a standard incubator for 12 to 24 hr as required for efficient labeling.

   Time courses for more complex experiments such as pulse-chase studies must be determined empirically.

5. Harvest *T. denticola* cells by centrifuging 15 min at 4000 × *g*, 4°C.
6. Remove medium and wash the pellet by addition of 1 ml PBS followed by gentle resuspension. Repeat step 5.
7. Assay radioactivity in the pellet or cell fraction of interest as appropriate

   This protocol has been used for labeling various cell components with ^{3}H, ^{14}C, and ^{32}P (Kent et al., 2004).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

MTYGVS plate medium

To TYGVS basal medium (see recipe), add 10 g Noble agar (Difco) per liter prior to adjusting pH and autoclaving. Add supplements aseptically as for TYGVS broth medium after autoclaving and cooling to 50°C. For isolation of spirochetes from clinical plaque samples, add rifampicin to 5 μg/ml before pouring plates, in order to suppress the growth of most other oral organisms without affecting spirochete viability (Leschine and Canale-Parola, 1980; Salvador et al., 1987). Pour plates as for standard agar medium. Prereduce plates in the anaerobic chamber before use. Use plates within 2 weeks.

This medium was originally described by Salvador et al. (1987).

NOS basal medium

To ~950 ml distilled water add:
12.5 g brain heart infusion (Difco)
10 g trypticase peptone (BBL)
2.5 g yeast extract (Difco)
0.5 g sodium thioglycolate
1.0 g L-cysteine hydrochloride
0.25 g L-asparagine
2.0 g glucose
Adjust pH of basal medium to 7.4 with NaOH and sterilize by autoclaving
Store basal medium up to 6 months at 4°C
Prior to use, add the following NOS supplements (sterilize supplement stocks by passing each through a 0.2-μm filter) aseptically to medium cooled to 50°C:

3 ml 0.2% (w/v) thiamine pyrophosphate (TPP; store at 4°C)
2 ml fatty acids mix (0.5 ml each of isobutyric, isovaleric, valeric, and D,1-2-methylbutyric acid in 100 ml 0.1N KOH; store at 4°C)
20 ml 10% (w/v) sodium bicarbonate (store at room temperature)
20 ml heat-inactivated rabbit serum (see recipe)
Adjust volume to 1 liter with distilled water
Store complete NOS medium up to 1 month at 4°C

The original formulation for NOS (Leschine and Canale-Parola, 1980) calls for 2% rabbit serum. Other investigators have substituted horse or bovine serum with little difference in growth of laboratory strains.

Originally described by Leschine and Canale-Parola (1980), modified by Chan et al. (1993b).

NOS-GN 1% agar semisolid medium

To NOS basal medium (see recipe), add 5 g Noble agar (Difco) and 5 g gelatin (Bio-Rad) per liter final volume prior to adjusting pH and autoclaving. After autoclaving mixture and cooling to 50°C in a water bath, add NOS supplements aseptically. Cool to 37° to 39°C in a water bath before adding *T. denticola* cell suspension.

The gelling temperature of NOS-GN is somewhat higher than that of NOS-LMP agarose, and it will remain molten at 37° to 39°C. When rewarmed to 37°C, it is less solid than NOS-LMP agarose.

The Noble agar-gelatin agents may be used similarly in TYGVS or OMZ-P4 to make solid versions of these media.

NOS semisolid medium with Noble agar-gelatin (NOS-GN) was originally described by Chan et al. (1997).

NOS 1.5% LMP agarose semisolid medium

To NOS basal medium (see recipe) add 7 g low-melting-point (LMP) agarose (SeaPlaque, Cambrex Bio Science) per liter prior to adjusting pH and autoclaving. After autoclaving mixture and cooling to 50°C in a water bath, add NOS supplements aseptically, and dispense medium into 25-cm^2 tissue culture flasks (Corning). Cool to 37° to 39°C in a water bath before adding *T. denticola* cell suspension. Alternatively, pour into 100 × 25–mm extra-deep petri dishes (Nalgene Nunc or equivalent) as for standard agarose medium.

This medium, originally described by Chan et al. (1993b), is molten at 37°C, will solidify at 26° to 30°C, and will remain solid when rewarmed to 37°C. LMP agarose may be used similarly in TYGVS or OMZ-P4 to make solid versions of these media.

OMIZ-P4 basal medium

OMIZ-P4 basal medium is formulated as a 2× concentrate, which is supplemented and brought to a final concentration of 1× prior to use. OMIZ-P4 medium supports growth of most cultivable oral *Treponema* species, including *T. denticola*, *T. pectinovorum*, *T. vincentii*, *T. maltophilum*, *T. socranskii*, *T. parvum*, *T. putidum*, *T. lecithinolyticum*, and *T. amylovurum*.

Complete formulation for OMIZ-P4 basal medium and supplement stock solutions (see recipe) has been provided here courtesy of C. Wyss, Department of Oral Microbiology and General Immunology, Dental Institute, University of Zurich, Switzerland (pers. comm.). This recipe has been modified from OMIZ-W1 described by Wyss (1992). See Strategic Planning for more information.

OMIZ-P4 basal medium, 2×

Add the following ingredients to 500 ml H_2O in a 1.0-liter volumetric flask:
45.0 mg L-alanine
174.0 mg L-arginine
150.0 mg L-asparagine
133.0 mg L-aspartic acid
352.0 mg L-cysteine HCl
294.0 mg L-glutamic acid
680.0 mg L-glutamine
75.0 mg glycine
620.0 mg L-histidine
131.0 mg L-isoleucine
131.0 mg L-leucine
182.0 mg L-lysine·HCl
149.0 mg L-methionine
168.0 mg L-ornithine·HCl
200.0 mg D,L-carnitine
10.0 mg phenol red
10.0 µl 2-methylbutyric acid
1.0 ml $CaCl_2$ solution (see recipe for OMIZ-P4 supplement stock solutions)
1.0 ml Ni-Sn-V-Mo solution (see recipe for OMIZ-P4 supplement stock solutions)
165.0 mg L-phenylalanine
115.0 mg L-proline
525.0 mg L-serine
119.0 mg L-threonine
102.0 mg L-tryptophan
90.0 mg L-tyrosine
117.0 mg L-valine
968.0 mg KCl

continued

203.3 mg $MgCl_2 \cdot 6H_2O$
140.0 mg $NaH_2PO_4 \cdot H_2O$
1.6 g NH_4Cl
2.0 g D-galacturonic acid
1.4 mg hypoxanthine
2.4 mg thymidine
5.0 mg putrescine·2HCl
1.4 ml 30% (w/v) lactic acid
10.0 µl valeric acid
1.0 ml Cu-Mn-Zn solution (see recipe for OMIZ-P4 supplement stock solutions)
1.0 ml vitamin solution (see recipe for OMIZ-P4 supplement stock solutions)
2.0 g D-maltose
2.0 g D-mannose
2.0 g D-ribose
2.0 g D-xylose
2.0 g L-fucose
200.0 mg sodium citrate
500.0 mg *N*-acetylglucosamine
550.0 mg sodium pyruvate
500.0 mg sodium fumarate
300.0 mg sodium formate
1.4 mg adenine
2.0 g D-arabinose
2.0 g D -glucose
1.1 mg uracil
1822.0 mg ACES (*N*-(2-acetamido)-2-aminoethanesulfonic acid; Sigma)
5.0 mg spermidine
10.0 µl isobutyric acid
10.0 µl isovaleric acid
1.0 ml selenium solution (see recipe for OMIZ-P4 supplement stock solutions)
1.0 ml hemin solution (see recipe for OMIZ-P4 supplement stock solutions)
1.0 ml lipoic acid solution (see recipe for OMIZ-P4 supplement stock solutions)
Adjust pH to 6.0 with NaOH solution
Pass through a 0.22-µm pore-size filter to sterilize
Store 2× OMIZ-P4 basal medium in closed bottles up to 6 months at 4°C

Filter sterilization using nitrogen gas pressure rather than vacuum will minimize dissolved oxygen content of the medium and may improve performance.

OMIZ-P4 basal medium, 1×

Prior to use, add the following solutions, then bring the volume to 1 liter:
10 ml sodium bicarbonate solution (see recipe for OMIZ-P4 supplement stock solutions)
1 ml DHNA solution (see recipe for OMIZ-P4 supplement stock solutions)
10 ml heat-inactivated human serum (Sigma H-1388)
10.0 ml vitamin C solution (see recipe for OMIZ-P4 supplement stock solutions)
1.0 ml cholesterol solution (see recipe for OMIZ-P4 supplement stock solutions)
10.0 ml glutathione solution (see recipe for OMIZ-P4 supplement stock solutions)
20.0 ml yeast extract solution (150 g/liter; Invitrogen 18180-059)
1.0 ml ferrous sulfate solution (see recipe for OMIZ-P4 supplement stock solutions)

continued

10.0 ml neopeptone solution (see recipe for OMIZ-P4 supplement stock solutions)
Adjust pH to 6.9 with NaOH
Filter sterilize using a 0.22-μm pore-size filter
Complete OMIZ-P4 medium can be stored for at least 4 weeks at 4°C before use

Human serum is more physiologically relevant for human-associated organisms. Other media recipes use rabbit serum for convenience and economy with, in most reports, little apparent difference in T. denticola growth behavior.

Equivalent amounts of yeast extract powder may be substituted.

OMIZ-P4 stock solutions

Unless otherwise noted, stock solutions are to be filter sterilized using a 0.22-μm pore-size filter. Solutions are stable indefinitely when stored as indicated.

Complete formulation for OMIZ-P4 basal medium (see recipe) and supplement stock solutions has been provided here courtesy of C. Wyss, Department of Oral Microbiology and General Immunology, Dental Institute, University of Zurich, Switzerland (pers. comm.).

$CaCl_2$ solution
14.7 g $CaCl_2 \cdot H_2O$
100.0 ml 10 mM HCl
Store at −20°C

Cholesterol solution
20.0 mg cholesterol
20.0 ml ethanol
Store at −20°C

Cu-Mn-Zn solution
0.798 mg $CuSO_4$
0.169 mg $MnSO_4 \cdot H_2O$
0.287 g $ZnSO_4 \cdot 7H_2O$
1.0 liter 10 mM HCl
Store at −20°C

DHNA solution
40.8 mg 1,4-dihydroxy-2-naphthoic acid
100 ml ethanol
Store at −20°C

Ferrous sulfate solution
278.0 mg $FeSO_4 \cdot 7\ H_2O$
100 ml 10 mM HCl
Store at 4°C

Glutathione solution
30.73 g glutathione, reduced (Sigma)
150.0 ml distilled water
Adjust pH to 7.0 with NaOH
Adjust volume to 200.0 ml with distilled water
Store at −20°C

Hemin solution
12.5 mg hemin
100.0 ml 10 mM NaOH
Store at −20°C

continued

Lipoic acid solution
10.0 mg D,L-α-lipoic acid
1.0 ml 2-mercaptoethanol
9.0 ml ethanol
Store at −20°C

Neopeptone solution
10.0 g neopeptone (Difco, BD 211681)
100.0 ml distilled water
Store at −20°C

Ni-Sn-V-Mo solution
0.131 mg $NiSO_4 \cdot 6H_2O$
0.118 mg $SnCl_2 \cdot 2H_2O$
0.61 mg $NaVO_3$
12.4 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
1.0 liter 10 mM HCl
Store at −20°C

Selenium solution
17.3 mg $NaSeO_3$
1.0 liter 10 mM HCl
Store at −20°C

Sodium bicarbonate solution
10 g $NaHCO_3$
100 ml distilled water
Store at room temperature

Vitamin solution
500.0 mg calcium D-(+)-pantothenate
500.0 mg thiamine·2HCl
500.0 mg pyridoxal phosphate
100.0 mg folinic acid (calcium salt)
1.0 mg riboflavin
100.0 mg coenzyme A sodium
5.0 g choline chloride
2.5 g thiamine pyrophosphate
5.0 mg D(+)-biotin
50.0 mg nicotinamide
5.0 mg vitamin B12
100.0 mg FAD
5.0 g myo-inositol
500.0 mg pyridoxal·HCl
5.0 mg folic acid
100.0 mg nicotinic acid
100.0 mg β-NAD
1.0 g 2-mercaptoethanesulfonic acid
100.0 ml distilled water
Store at −20°C

Vitamin C solution
10.0 g ascorbic acid
70.0 ml distilled water
Adjust pH to 7.0 with NaOH
Store at −20°C

Rabbit serum, heat-inactivated

Inactivate serum by heating at 56°C for 1 hr. Clarify by centrifuging 15 min at 5000 × *g*, 4°C in a conical tube. Convenient tubes include 15- or 50-ml polypropylene tubes (Corning or equivalent). Pass the supernatant through a 0.2-μm filter to sterilize. Store in 10-ml aliquots up to 1 year at −20°C.

Reduced transport fluid (RTF)

Per 500 ml final volume, combine:
2.5 ml 8% (w/v) Na_2CO_3 in water
5.0 ml 0.1 M EDTA
37.5 ml RTF mineral stock solution (see recipe)
Autoclave and cool to 4°C
Add 10.0 ml of 1% (w/v) DTT (0.2-μm filter-sterilized solution in distilled water)
Store at 4°C up to 6 months before use

This reagent is used for collection and transport of clinical samples. Because samples collected under clinical or field conditions are often held for several hours before introduction to an anaerobe chamber, take precautions to minimize oxygen exposure during transit. In the case of subgingival plaque samples, place collected plaque in vials of RTF (typically 0.5 ml) with minimal headspace. Some investigators transport these samples in a GasPak anaerobic jar (see Strategic Planning). Once in the laboratory, introduce the vials into the anaerobic chamber where all subsequent manipulations will be performed.

For more information on use of RTF, refer to Loesche et al. (1972).

RTF mineral stock solution

Add to 500 ml distilled water:
3 g KH_2PO_4
6 g NaCl
6 g $(NH_4)_2SO_4$
1.25 g $MgSO_4 \cdot 7H_2O$
Sterilize by filtration using a 0.2-μm filter
Store at 4°C

T. denticola preservation medium

Combine three parts heat-inactivated rabbit serum (see recipe), one part broth medium in which culture was grown, and 7.5% (w/v) glucose (final). Store up to 1 year at −20°C.

TYGVS basal medium

Add to approximately 900 ml distilled water:
10.0 g tryptone (Difco)
5.0 g brain heart infusion (Difco)
10.0 g yeast extract
10.0 g gelatin
0.5 g $(NH_4)_2SO_4$
0.1 g $MgSO_4 \cdot 7H_2O$
1.13 g K_2HPO_4
0.9 g KH_2PO_4
1.0 g sodium chloride
Adjust pH of basal medium to 7.2 with 4 N KOH and sterilize by autoclaving
Store basal medium up to 6 months at 4°C
Prior to use, aseptically add TYGVS supplements (see recipe)

When using freshly prepared medium, cool below 50°C before adding supplements.

TYGVS supplements

To 1 liter TYGVS basal medium add:
0.0125 g thiamine pyrophosphate (TPP)
1.0 g glucose
1.0 g L-cysteine·HCl
0.25 g sodium pyruvate
0.27 ml acetic acid (glacial)
0.1 ml propionic acid
0.064 ml *n*-butyric acid
0.016 ml *n*-valeric acid
0.016 ml isobutryic acid
0.016 ml isovaleric acid
0.016 ml D,L-methylbutyric acid
100 ml heat-inactivated rabbit serum (see recipe)
Mix well and adjust pH to 7.0 with 1 N KOH
Filter sterilize through a 0.2-μm filter

The original reference (Ohta et al., 1986) calls for 10% rabbit serum. Horse or bovine serum can also be used with little difference in growth.

COMMENTARY

Background Information

Oral spirochetes, most notably *Treponema denticola*, are associated with aggressive forms of periodontal diseases (Loesche et al., 1982; Loesche et al., 1985; Socransky and Haffajee, 1991). Periodontal lesions contain a highly complex microflora in which proteolytic Gram-negative anaerobes predominate. Spirochetes alone often comprise up to 50% of total bacteria in periodontal lesions, particularly in subgingival plaques directly adjacent to gingival tissue (Listgarten, 1976). Inflammatory responses to this microbial challenge, modulated by several bacterial factors, leads to tissue destruction, loss of attachment at the junctional epithelium between the gingiva and the tooth, alveolar bone resorption, and tooth loss. The formation of a deep periodontal pocket between the tooth and the gingiva, coupled with the inflammatory response, further enhances the environment for growth of proteolytic Gram-negative anaerobes, and the process continues. The overall picture of periodontal disease is complex, involving numerous bacterial factors and host inflammatory responses, but it is an eventual result of bacterial challenge (Socransky and Haffajee, 1992).

A consortium of three proteolytic Gram-negative organisms, *Porphyromonas gingivalis (see UNIT 13C.1)*, *Tannerella forsythia (formerly Bacteroides forsythus)*, and *Treponema denticola*, has the highest association with periodontal disease severity (Socransky et al., 1998), especially in refractory cases. While characterization of oral spirochetes as specific periodontal pathogens has been problematic due to the inability to grow all but a very few species in vitro, their numerical prevalence, high association level, and spatial location within diseased sites suggest that they play an important role in disease progression (Listgarten and Levin, 1981; Loesche et al., 1985). The complexity of the oral spirochete population is illustrated by a recent study of spirochetal 16S rRNA amplified from a single subgingival plaque sample from a patient with severe periodontitis (Choi et al., 1994). Twenty-three distinct *Treponema* species were detected, which fell into eight major groups. Of these eight genetic groups, only two represented named species (*T. denticola* and *T. vincentii*). Clinical, biochemical, and molecular characterization studies have focused on *T. denticola*, in part because this species can be most readily isolated from clinical samples and grown in culture (Chan et al., 1993a). While spirochetes other than *T. denticola* may eventually be identified as key organisms in periodontal diseases, the demonstrated and potential virulence factors of this organism appear to be representative of the genus; therefore, the study of the virulence factors of *T. denticola* is important for understanding this chronic inflammatory disease.

Several recent reports have implicated spirochetes related to human oral treponemes in the etiology of bovine digital dermatitis, a major cause of lameness in dairy cattle. In addition to clinical symptoms including

painful ulcerations of infected sites, the disease results in considerable economic loss due to decreases in body weight and milk production (Blowey and Sharp, 1988). Spirochetes of the genus *Treponema* are among the most predominant organisms isolated from these lesions (Walker et al., 1995). Of five distinct *Treponema* phylotypes in digital dermatitis lesions identified by 16S rRNA analysis, *T. denticola*-like spirochetes predominated (Choi et al., 1997). The strong association of these organisms with chronic ulcerative diseases of both humans and cattle is reflected in increased interest in characterization of their involvement in disease progression.

Critical Parameters and Troubleshooting

Although spirochetes are typically the predominant microscopically visible organisms in subgingival plaque samples, they are rarely isolated using standard methods of sample dispersal and culturing on agar media, even under strictly anaerobic conditions. Of the greater than fifty species identified by genetic methods, only about ten have been grown in culture (Paster et al., 2001). The choice of which particular *T. denticola* strain or strains to work with is dependent on the experimental goals. *T. denticola* ATCC #35405 is the most widely studied strain, and its genome has been completed and annotated (Seshadri et al., 2004); however, experiments requiring the introduction of a replicating plasmid have been successful only in *T. denticola* ATCC #33520 (*UNIT 12B.2*). Several other strains are also in use, either because of particular traits of interest or for purely historical reasons.

Nutrient requirements of *T. denticola* are rather complex, and have not yet been clearly defined. Presumably, analysis of the genome sequence will provide some guidance in this area to complement the ongoing efforts that have gone into characterizing the nutrient requirements of this and other treponemes. Most media formulations in current use are derived from complex media formulations containing animal organs and serum. Key factors, in addition to a source of peptides and free amino acids, are short- and long-chain fatty acids, near neutral pH, and very low redox potential (Eh). Variations in media formulations are primarily due to subtractive removal or substitution of certain constituents by different investigators working with different strains or species of treponemes. For example, TYGVS medium contains a higher serum concentration and a greater variety of volatile fatty acids than are found in NOS medium (see Reagents and Solutions). Not surprisingly, *T. denticola* growth is somewhat more rapid in TYGVS. Some strains (especially recent clinical isolates) that do not grow well in NOS medium grow very well in TYGVS or OMIZ-P4. On the other hand, some laboratories have been able to maintain *T. denticola* strains with fewer volatile fatty acids than in the standard NOS recipe, or with serum replaced by alternate carriers of long-chain fatty acids (Socransky and Hubersak, 1967; Suzuki and Loesche, 1989); however, omitting or reducing concentrations of these or other components (such as serum) can be expected to result in slower growth rates and lower overall cell yields.

As noted above (see Strategic Planning), standard Bacto agar is inhibitory to growth of *T. denticola*, and probably to that of other oral treponemes. The higher cost and inconvenience of working with the alternative Noble agar, gelatin, or agarose formulations described here is well rewarded by increased viability of *T. denticola* plated on or in these solid media.

Dispersion by sonication, though the most effective method in terms of total numbers of bacteria recovered from highly aggregated plaque samples, results in extremely low recovery of oral spirochetes from clinical samples. Transport of freshly collected plaque samples in RTF and gentle dispersion by vortexing can result in greatly increased recovery of cultivable spirochetes (Salvador et al., 1987) when combined with selection with rifampicin (or rifampicin plus phosphomycin) and plating on appropriate rich media.

Unless otherwise specified, all incubations and (where feasible) all manipulations described here are performed under strictly anaerobic conditions in an anaerobic glove box chamber (37°C, in a nonhumidified, 85% N_2/10% H_2/5% CO_2 atmosphere; see Strategic Planning). While *T. denticola* can survive some exposure to oxygen, and media formulations described here provide considerable protection from oxidative processes, prudence suggests minimizing this exposure. The alternative of using anaerobic jars is certainly feasible and is preferable for experiments involving radioactive labeling (see Basic Protocol 5). This is due to the relative difficulty of handling and monitoring radioactivity in an anaerobic glove box chamber versus an anaerobic

jar system. Other than this special case, however, the advantages of the glove box system in terms of atmospheric control and experimental scale are considerable.

Anticipated Results

Isolation of *T. denticola* and other oral treponemes from clinical samples is typically very low-yield. In one well designed study in which spirochetes were ~30% of microscopic counts in subgingival dental plaque samples, spirochetes (primarily *T. denticola*) comprised <0.05% of total cultivable organisms (Salvador et al., 1987). Even with rifampicin selection against the hundreds of other species present in plaque samples, "contaminants" are very common, and new spirochete isolates must be cloned and characterized extensively for culture purity.

T. denticola cultures may be maintained by weekly passage in fresh, prereduced broth medium at dilutions of between 1:20 and 1:100. Although there have been no studies documenting changes in *T. denticola* physiology or behavior attributable to long-term passaging, prudent microbiological practice would encourage periodic replacement of stock cultures. This is likely to be more relevant when working with recent clinical isolates as opposed to common laboratory strains.

Growth rates vary between strains and in different media. Of the two most commonly used lab strains, ATCC #35405 tends to grow at a slightly more rapid rate than does ATCC #33520. This difference is more notable when the strains are grown in NOS medium, and less apparent in the more nutrient-rich TYGVS medium. Unlike other oral anaerobes such as *Porphyromonas gingivalis*, viability of *T. denticola* does not decrease rapidly after logarithmic growth in liquid media, and it is possible (though not recommended) to recover strains that have been incubated in broth for over a week. Growth of *T. denticola* on solid media is comparably slow, with subsurface colonies appearing in ~1 week, depending on the strain and the medium. Colonies are seldom observed growing on top of the medium. Plates inoculated by spreading on solid agar medium tend to show spirochete colonies growing down into the medium. The diffuse appearance of spirochete colonies is due to motility through the agar. As might be expected, colonies are more diffuse and spreading in lower-concentration agar formulations.

In addition, it is important to note that environmental conditions are rather more difficult to control for *T. denticola* than for organisms such as *E. coli*. *T. denticola* responds to "minor" variations in atmospheric conditions. These (especially gas composition) can be difficult to control, either in an anaerobic chamber or in GasPak-type systems. Similarly, temperature is often more difficult to control in an anaerobic chamber than in a standard incubator. Inability to replicate experiments can often be attributed to uncontrolled variations in environmental conditions.

Time Considerations

As noted in Basic Protocol 1, the generation time of *T. denticola* is ~12 hr, yielding $\sim 1 \times 10^9$ cells/ml after 4 days growth (late log phase) in NOS medium. Media formulation and serum concentration influence growth rate, and there is considerable strain-dependent variability in growth rates. If possible, pilot experiments should be conducted to validate growth conditions and kinetics for individual strains and individual laboratories.

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Key References

Chan et al., 1993b. See above.

Chan et al., 1997. See above.

These two articles describe efficient and repeatable methods of growing T. denticola as subsurface colonies.

Cheng and Chan, 1983. See above.

Loesche, 1969. See above.

This article documents oxygen sensitivity and other growth requirements of T. denticola.

Salvador et al., 1987. See above.

These two articles describe detailed methods for isolating Treponema species from clinical samples.

Smibert, 1973. See above.

Smibert, 1976. See above.

These two classic reviews by an early investigator of anaerobic bacterial metabolism provide an excellent introduction into microbial physiology and the biochemistry of spirochetes. It is important to note that nomenclature of oral spirochetes was rather fluid before the advent of ribotyping. Most strains reported as T. microdentium, T. dentium, or T. oralis are currently classified as T. denticola.

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This article describes in great detail a defined medium capable of supporting growth T. denticola.

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Genetic Manipulation of *Treponema denticola*

Treponema denticola is a gram-negative anaerobic spirochete associated with one of the most common infectious diseases that afflict mankind, periodontitis (Socransky et al., 1998). In addition, this organism, as well as other microorganisms involved in the polymicrobial disease periodontitis, may also induce systemic complications such as atherosclerosis (Okuda et al., 2004). Nevertheless, until very recently little information has been available regarding the molecular basis for the pathogenicity of the oral spirochetes. One limitation in defining the virulence of oral spirochetes has been the difficulty in cultivating many of them. An essential component of virulence analysis of cultivable treponemes such as *T. denticola* has been the development of gene-transfer techniques in these organisms (Li et al., 1996; Chi et al., 2002). To investigate the possibility of genetic manipulation of *T. denticola*, two strains, ATCC #35405 and ATCC #33520, were initially selected for investigation. Both strains were isolated from human plaque samples and represent two different serotypes of these organisms (Cheng et al., 1985). Strain 33520, but not 35405, was demonstrated to harbor an endogenous plasmid, pTD1 (Ivic et al., 1991). The construction of monospecific mutants has led to the identification of potential virulence factors in these organisms (Ishihara et al., 1998; Fenno et al., 1998; Ikegami et al., 2004). In addition, the development of shuttle plasmids for expression of homologous or heterologous genes in *T. denticola* has further helped define the pathogenic properties of treponemes. These developments, together with the recent sequencing of the genome of *T. denticola* ATCC #35405 (Scshadri et al., 2004), should greatly accelerate understanding of the role of treponemes in pathogenicity.

This unit presents protocols for the use of electroporation in transformation of *T. denticola* with plasmid DNA (see Basic Protocol 1) and gene silencing (see Basic Protocol 2), as well as protocols for the preparation of competent cells for each of these procedures (see Support Protocols 1 and 2, respectively).

CAUTION: Treponema denticola is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

TRANSFORMATION OF *T. DENTICOLA* WITH PLASMID DNA

BASIC PROTOCOL 1

The initial demonstration of the genetic manipulation of an oral treponeme was accomplished following electroporation of the broad-host-range shuttle plasmid pKT210 into *T. denticola* ATCC #33520 (Li and Kuramitsu, 1996). Subsequently, since plasmid pKT210 was shown to be unstable in strain 33520, a more stable shuttle plasmid, pKMR4PE, based upon the naturally occurring treponeme plasmid pTS1, was constructed (Chi et al., 1999). In the authors' laboratory, this plasmid could be introduced into strain 33520 following electroporation but not into strain 35405. However, the reason for this apparent strain difference has not yet been elucidated. In addition, this shuttle plasmid has been modified to harbor a coumermycin-resistance gene (*Cou*), pKMCou, for complementation of Erm-resistant mutants of strain 33520 (Chi et al., 2002). Very recently, a similar shuttle plasmid, pBFC, with a chloramphenicol-resistance gene (*Chl*) in place of *Erm* or *Cou* has been constructed (Sliviensky-Gebhardt et al., 2004). This latter plasmid has the advantage that there are markedly fewer background Chl^r colonies following transformation than Cou^r spontaneous mutants.

Contributed by Howard K. Kuramitsu, Bo Chi, and Akihiko Ikegami

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The development of shuttle plasmids for use in *T. denticola* now allows for the potential expression of heterologous spirochete genes in the oral spirochete. This is now possible even for organisms that cannot be cultivated in the laboratory, e.g., *T. pallidum* (Chi et al., 1999; Sliviensky-Gebhardt et al., 2004). Even though such genes can be expressed in *E. coli*, their expression in treponemes should be advantageous in terms of functional analyses.

Materials

Electrocompetent *T. denticola*, ATCC #35405 or ATCC #33520 (see Support Protocol 1)

Appropriate shuttle plasmid: pKMR4PE (Chi et al., 1999), pKMCou (Chi et al., 2002), or pBFC (Sliviesnki-Gebhardt et al., 2004)

TYGVS medium (*UNIT 12B.1*), with and without appropriate antibiotic (Sigma) for growth: erythromycin at 40 μg/ml, chloramphenicol at 10 μg/ml, or coumermycin A1 at 10 μg/ml

TYGVS medium (*UNIT 12B.1*) containing 0.8% (w/v) SeaPlaque agarose (VWR) and appropriate antibiotic (Sigma): erythromycin at 40 μg/ml (for pKMR4PE), chloramphenicol at 10 μg/ml (for pBFC), or coumermycin A1 at 10 μg/ml (for pKMCou)

Gene Pulser (Bio-Rad)

0.1-cm-gap electroporation cuvettes (Bio-Rad)

15-ml plastic screw cap test tubes (VWR)

Anaerobic chamber (*UNIT 12B.1*)

100 × 15–mm petri dishes

Sterile inoculation loops or sterile Pasteur pipets

Additional reagents and equipment for cultivation of *T. denticola* (*UNIT 12B.1*)

1. Chill 80 μl competent *T. denticola* on ice. Add 1 to 10 μg of the appropriate shuttle plasmid (pKMR4PE, pKMCou, or pBFC). Mix the suspension well by gentle pipetting and incubate 30 min on ice.

2. Transfer the mixture to an ice-cold electroporation cuvette, with a 0.1-cm gap, placed in a Gene Pulser. With the pulse controller set at 1.8 kV, capacitance at 25 μF, and resistance at 200 Ω, administer a single pulse to the cells with a time constant of ∼4.1 msec.

3. Immediately add 1.0 ml TYGVS medium without antibiotics. Mix gently by pipetting up and down a few times, then transfer to a 15-ml plastic screw-cap test tube. Add an additional 1.0-ml aliquot of medium. Incubate 18 hr at 37°C in an anaerobic chamber with the cap only loosely applied to allow for gas exchange (also see *UNIT 12B.1*).

4. Place 1.0 ml of the suspension from step 3 into a 100 × 15–mm petri dish, then add 35 ml precooled (37°C) 0.8% SeaPlaque agarose in TYGVS medium containing the appropriate antibiotic (40 μg/ml erythromycin for pKMR4PE, 10 μg/ml coumermycin A1 for pKMCou, or 10 μg/ml chloramphenicol for pBFC). Mix well by gentle rotation of the dish and allow to solidify 1 hr at room temperature.

5. Incubate the plates at 37°C face-up for 18 hr in an anaerobic chamber, then turn face-down and incubate an additional 5 to 7 days.

6. Remove individual colonies along with plug of agarose using a sterile loop or sterile Pasteur pipet. Transfer to 10 ml TYGVS medium containing the appropriate antibiotic. Incubate at 37°C to expand, then characterize for subsequent experiments.

   For example, examine under phase contrast or dark-field microscopy to check for purity, or extract DNA for agarose gel analysis to confirm plasmid transfer.

PREPARATION OF ELECTROCOMPETENT *T. DENTICOLA* FOR PLASMID TRANSFORMATION

SUPPORT PROTOCOL 1

The electroporation protocol for *T. denticola* is based upon general procedures developed previously for other bacteria such as *E. coli*, with some modifications. In addition, a heat-treatment step was introduced in order to minimize the effects of host restriction-modification systems on heterologous transforming plasmids.

Materials

T. denticola, ATCC #35405 or ATCC #33520
TYGVS medium (*UNIT 12B.1*)
10% (v/v) glycerol, ice-cold

Anaerobic chamber
50°C water bath
Sorvall RC5 or equivalent refrigerated centrifuge

Additional reagents and equipment for cultivation of *T. denticola* (*UNIT 12B.1*)

1. Grow *T. denticola* (also see *UNIT 12B.1*) in an anaerobic chamber at 37°C to mid- to late-log phase (48 to 72 hr).
2. Take a 100-ml aliquot of the cell suspension. Heat to 50°C for 30 min, then chill on ice for 30 min.

   Heating increases the rate of plasmid transformation, probably by inactivating host restriction-modification enzymes.

3. Wash cells three times, each time by centrifuging 10 min at 4000 × *g*, 4°C, resuspending cells in 10 ml of 10% ice-cold glycerol, centrifuging again as before, then removing the supernant. Resuspend pellet in 700 µl 10% glycerol.
4. If desired, divide into 80-µl aliquots and store indefinitely at −70°C.

ELECTROTRANSFORMATION OF *T. DENTICOLA* FOR ALLELEIC-EXCHANGE MUTAGENESIS

BASIC PROTOCOL 2

Following the initial demonstration of the transfer of a broad-host-range shuttle plasmid, pKT210, into several different strains of *T. denticola* (Li and Kuramitsu, 1996) by electroporation, it was possible to develop a system for gene inactivation in these organisms utilizing an erythromycin-resistance (*Erm*) cassette derived from plasmid pVA2198 as a selectable marker (Li et al., 1996). This allows for isolation of monospecific mutants that can then be used in either in vitro or in vivo assays to define the virulence factors of these organisms. Such mutants are crucial in confirming the role of a gene product in the pathogenicity of the organisms. As detailed below, this procedure requires care in selecting the proper strategy for maximizing target gene mutagenesis.

Materials

Electrocompetent *T. denticola*, ATCC #35405 or ATCC #33520 (see Support Protocol 2)
Plasmid pVA2198 (Fletcher et al., 1995; contact H. Fletcher, Loma Linda University, Loma Linda, Calif.)
T. denticola (ATCC #35405 or ATCC #33520)
TYGVS medium (*UNIT 12B.1*) with and without 40 µg/ml erythromycin (Sigma)
TYGVS medium (*UNIT 12B.1*) containing 0.8% (w/v) SeaPlaque agarose (VWR) and 40 µg/ml erythromycin (Sigma)

Gene Pulser (Bio-Rad)
0.1-cm-gap electroporation cuvettes (Bio-Rad)
15-ml plastic screw cap test tubes (VWR)
Anaerobic chamber (*UNIT 12B.1*)
100 × 15–mm petri dishes
Sterile inoculation loops or sterile Pasteur pipets

Additional reagents and equipment for cultivation of *T. denticola* (*UNIT 12B.1*)

1. Mix 80 μl of competent *T. denticola* (~4.7×10^{10} cells/ml, final concentration) with 5 μl of a 2-μg/ml suspension of linearized plasmid pVA2198. Incubate 30 min on ice.
2. Transfer the mixture to an ice-cold electroporation cuvette, with 0.1-cm gap, placed in a Gene Pulser. With the pulse controller set at 1.8 kV, capacitance at 25 μF, and resistance at 200 Ω, administer a single pulse to the cells with a time constant of ~4.1 msec.
3. Immediately add 2.0 ml TYGVS medium without antibiotics. Mix gently by pipetting up and down a few times, then transfer to a 15-ml plastic screw-cap test tube. Incubate 18 hr at 37°C in an anaerobic chamber with the cap only loosely applied to allow for gas exchange (also see *UNIT 12B.1*).
4. Place 1.0 ml of the suspension from step 3 into a 100 × 15–mm petri dish, then add 35 ml precooled (37°C) 0.8% SeaPlaque agarose in TYGVS medium supplemented with 40 μg/ml erythromycin. Mix well by gentle rotation of the dish and allow to solidify 1 hr at room temperature.
5. Incubate plates at 37°C face-up for 18 hr in an anaerobic chamber then turn face-down and incubate an additional 8 to 10 days.
6. Remove individual colonies along with plug of agarose using a sterile loop or sterile Pasteur pipet. Transfer to 10 ml TYGVS medium containing 40 μg/ml erythromycin. Incubate at 37°C to expand, then characterize for subsequent experiments.

   For example, examine under phase contrast or dark-field microscopy to check for purity, or perform molecular genetic analysis by PCR or Southern blots to confirm target gene inactivation.

SUPPORT PROTOCOL 2

PRODUCTION OF ELECTROCOMPETENT *T. DENTICOLA* FOR USE IN ALLELEIC EXCHANGE MUTAGENESIS

The procedure for the development of competent *T. denticola* cells for gene inactivation is basically identical to that for plasmid transformation. However, for the former procedures it is not necessary to introduce a heat-treatment step to ensure the isolation of targeted mutants.

Materials

T. denticola, ATCC #35405 or ATCC #33520
TYGVS medium (*UNIT 12B.1*)
10% (v/v) glycerol, ice-cold

Anaerobic chamber
50°C water bath
Sorvall RC5 or equivalent refrigerated centrifuge

Additional reagents and equipment for cultivation of *T. denticola* (*UNIT 12B.1*)

1. Grow *T. denticola* 35405 in an anaerobic chamber (also see *UNIT 12B.1*) at 37°C in 500 ml of TYGVS medium (using a 10% inoculum) for 48 to 72 hr.

   Strain 33520 has also been successfully used for gene inactivation.

2. Place the cells on ice for 15 min. Centrifuge 10 min at 4000 × *g*, 4°C. Wash by resuspending cells in 500 ml ice-cold distilled water, centrifuging again as before, and removing the supernatant.
3. Resuspend cells in 250 ml ice-cold distilled water. Centrifuge as in step 2, then resuspend cells in 10 ml ice-cold 10% glycerol.
4. Centrifuge cells and wash twice more, each time with 10 ml of 10% glycerol using the technique described in step 2, to ensure complete removal of the medium salts. After the last centrifugation, resuspend the cells in 1.0 ml of 10% glycerol.
5. Divide into 80-µl aliquots and store indefinitely at −70°C.

GENERATION OF A TARGETED GENE-DELETION CONSTRUCT

SUPPORT PROTOCOL 3

A DNA fragment corresponding to the target gene should be initially cloned into an appropriate *E. coli* plasmid (Li et al., 1996). This is conveniently accomplished following PCR amplification using two primers, which would result in an amplicon containing a unique restriction site for subsequent introduction of the *Erm* cassette.

The *Erm* cassette from plasmid pVA2198 (Fletcher et al., 1995), containing the *ermF* and *ermAM* genes, is isolated from the plasmid following *Pvu*II digestion and inserted into the target gene fragment following cleavage (and blunt-ending with Klenow DNA polymerase if necessary). The resulting plasmid is then linearized with a restriction enzyme that cleaves a unique restriction site outside of the mutagenized target gene for transformation.

It should also be possible to generate monospecific mutants using either the chloramphenicol or coumermycin resistance cassettes (Chi et al., 2002; Slivienski-Gebhart et al., 2004) via the same approach as that utilized for the generation of erythromycin-resistant mutants. In addition, these cassettes will allow for the isolation of mutants defective in multiple genes of *T. denticola*.

COMMENTARY

Background Information

T. denticola strains are among the most commonly occurring oral bacteria associated with adult periodontitis (Socransky et al., 1998). More recently, these organisms have also been identified in cardiac tissue, and it has been suggested that they are associated with atherosclerosis (Okuda et al., 2004). Therefore, the identification of potential virulence factors in these organisms is of interest. The development of techniques to construct monospecific mutants of these organisms is crucial for identifying some of these factors. In addition, the ability to express genes from shuttle plasmids in these organisms would allow for complementation of these mutants for further verification of their respective roles in pathogenicity. Moreover, these shuttle plasmids are also useful in expressing genes from heterologous treponemes, including those that cannot be readily cultivated in the laboratory (Chi et al., 1999; Slivienski-Gebhardt et al., 2004). Finally, these techniques might also be applicable to other cultivable treponemes, as has been recently shown in the authors' laboratory for *T. phagedenis* (Yamada et al., unpublished results).

At present, there are no treatment strategies for periodontitis that specifically target oral spirochetes. Instead, general approaches to eliminate subgingival plaque bacteria by physical removal (scaling and root planing), and/or by the use of antibiotics such as tetracycline, are commonly employed. Therefore, since a large portion of the bacteria associated with periodontitis are spirochetes, the development of strategies directed against these organisms could have a major impact on the progression of these polymicrobial diseases.

The current availability of at least two readily selectable antibiotic resistance markers (*Erm* and *Chl*) makes it convenient to construct mutants and complement them with the wild-type genes in *T. denticola* 33520. Coumermycin could also be used, but the high background associated with this

antibiotic makes the other two antibiotics more suitable. The inability to transform strain 35405 with the shuttle plasmids somewhat compromises this approach, since this strain appears to be much more virulent than 33520 in regard to some potential pathogenic properties (Ikegami et al., 2004). Nevertheless, the development of additional markers with low backgrounds following transformation would further enhance genetic approaches in these organisms. The expression of heterologous treponeme genes in *T. denticola* may also be somewhat limited by low-level expression rates (Chi et al., 1999) and by the overall low transformation rates of strain 33520; however, sufficient expression for complementation of a strain 33520 knockout mutant has been accomplished using a *T. pallidum* gene (Slivienski-Gebhardt et al., 2004). The utilization of heat-treated competent cells increases the plasmid transformation rates (Chi et al., 2002), but the expression problem is still a significant hurdle that needs to be overcome.

Critical Parameters and Troubleshooting

The relative fragility of treponemes such as *T. denticola* requires that care be taken in handling these organisms during manipulation of the cultures. For example, relatively gentle washing procedures should be utilized (e.g., no vigorous vortexing during resuspension). In addition, the procedures should be carried out rapidly, without excessive delay in between steps. In this regard, it is important to add the TYGVS medium to the electroporated cells immediately after removal from the Gene Pulser. Moreover, the cultures should be placed into the anaerobic chamber immediately after treatment.

For gene inactivation, it is important to have sufficiently long stretches of *T. denticola* DNA (>400 bp) flanking the erythromycin-resistance cassette inserted into the target gene. This will help ensure double cross-over recombination, required for gene inactivation. In addition, it is crucial that the *ermF* gene present in the Erm cassette be properly oriented such that its direction of transciption is identical with the targeted gene. This is necessary because it is likely that most of the transcription of the *ermF* gene is initiated from the promoter of the targeted gene. Finally, competent cells used for transformation should be harvested from mid- to late-log phase cultures (<72 hr incubation in TYGVS medium) of *T. denticola* for optimal transformation rates.

Because of the relatively low frequency of transformation of *T. denticola* (Li et al., 1996; Chi et al., 1999), it is not unusual for investigators using these procedures for the first time to experience difficulty in isolating transformants. As mentioned above, it is important that the electroporation steps be carried out quickly. For shuttle plasmid transformation of *T. denticola*, the frequency of transformation can be increased ~100× by using plasmids isolated from strain 33520 relative to the same shuttle plasmid isolated from *E. coli* (Chi et al., 1999). This likely reflects the presence of restriction-modification systems in *T. denticola*, as suggested by an examination of the genome sequence of strain 35405 (Scshadri et al., 2004).

Anticipated Results

The frequency of transformation of *T. denticola* 33520 or 35405 was found to be approximately one colony per microgram of transforming DNA for gene inactivation (Li and Kuramitsu, 1996; Chi et al., 1999), but almost one-hundred fold higher following plasmid transformation (Chi et al., 2002). Depending upon the antibiotic marker selected, some spontaneous mutant colonies might also be detected. This is especially a problem using plasmid pKMCou and Cou as a selective marker (Chi et al., 2002; Slivienski-Gebhardt et al., 2004). The utilization of Erm- or Chl-resistance-expressing plasmids should minimize the isolation of spontaneous antibiotic resistant colonies. However, if another readily detectable phenotypic property can be detected for the transformants on the agarose plates (i.e., swarming), it is still possible to utilize plasmid pKMCou and the coumermycin selection strategy (Chi et al., 2002).

Time Considerations

Because of the relatively slow growth rate of *T. denticola*, ~2 weeks are necessary from initiation of the growth of the cells until transformants can be detected following electroporation. In addition, because of the relatively low frequency of transformation of *T. denticola*, it is recommended that multiple electroporation experiments be carried out over the span of several days prior to examining the results of the initial transformation.

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Laboratory Maintenance of *Borrelia burgdorferi*

This unit describes the methods for growth and storage of *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis. It provides the recipe of the standard culture medium, Barbour-Stoenner-Kelly-II (BSK-II) and details its use in broth and solid culture. Methods for short and long-term storage of *Borrelia* cells are also provided.

CAUTION: B. burgdorferi is considered a Biosafety Level 2 (BSL-2) category organism. Work should be performed in a BSL-2 biosafety hood. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. The use of a biosafety hood also further protects cultures from potential contaminants, which will readily outgrow the spirochetes in the rich culture medium (see Commentary).

STRATEGIC PLANNING

Borrelia burgdorferi characteristics

Borrelia burgdorferi are slender, highly motile spirochetes 0.2- to 0.5-μm in diameter and up to 30 μm in length. They can be visualized by phase-contrast or dark-field microscopy (*UNIT 2A.1*). *B. burgdorferi* stain weakly Gram negative; other stains such as Giemsa or Wright are more appropriate. *B. burgdorferi* are motile in both low- and high-viscosity media due to periplasmic bundles of seven to eleven flagella attached at each end of the cell cylinder. Intriguingly, these flagella also determine the spirochete's flat-wave cell shape (Motaleb et al., 2000). Opaque white colonies form within or on the surface of solid medium (see Basic Protocol 3). In general, colonies are regularly shaped, up to 3 mm in diameter, with a dense center and a diffuse border. However, colony size and morphology can vary from strain to strain or even within clones of the same isolate (Elias et al., 2002).

Borrelia sensitivity to detergents

The use of disposable labware is recommended since *Borrelia* cells are sensitive to detergents, residues of which might be present on reusable plastic and glass labware. All reusable labware, such as beakers for medium preparation, should be rinsed with double-distilled water before use.

Stock solutions

The formulation for the standard *Borrelia* growth medium BSK-II, provided in Reagents and Solutions, follows the recipe described by Barbour in 1984. The medium is made in two steps: an incomplete or basal medium, which can be stored indefinitely at 4°C or lower, and a complete medium, which is stored up to 2 to 3 weeks at 4°C. In addition to the basal BSK-II medium, gelatin or agarose stock solutions for liquid or solid BSK-II, respectively, are prepared beforehand and stored indefinitely at room temperature. Rabbit serum is aliquoted in ready-to-use volumes and stored indefinitely at −20°C or lower.

CMRL-1066

CMRL-1066 without L-glutamine is available as liquid or powder from a limited number of companies, e.g., Invitrogen (1× solution), or U.S. Biological (powder). The 10× solutions previously sold by different suppliers (e.g., Invitrogen and Sigma) have been discontinued. In powder form, CMRL-1066 should be stored protected from light and humidity (e.g., in a dessicator) at room temperature. Solutions should be stored protected from light at 4°C.

Contributed by Wolfram R. Zückert
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Batch effect on components of BSK-II

It is suggested that batches of complex components of BSK-II, such as CMRL-1066, albumin and rabbit serum be tested individually for supporting the growth of *Borrelia*. This is particularly important when switching to a new supplier, as differences in growth medium composition, including different batches of commercially available BSK-H (Sigma), can significantly affect downstream experiments (see Background Information).

BASIC PROTOCOL 1

CULTIVATION AND STORAGE OF *BORRELIA BURGDORFERI* IN BSK-II

B. burgdorferi are best cultured in sterile, disposable plastic tubes (see Strategic Planning). The tube volumes depend on the ultimate purpose of the culture—e.g., growth inhibition assays (Sadziene et al., 1993b) can be carried out in 200 µl volumes in 96-well microtiter plates, and cells grown for generating electrocompetent cells (Samuels, 1995) can be directly inoculated in the 500-ml filter flasks used for medium preparation. Since Borreliae are microaerophilic, tubes should be filled to 70% to 90% of capacity for optimal growth. While BSK-II supports the outgrowth of a single spirochete, *Borrelia* cultures are routinely passed at dilutions of ~1% to 10% (v/v) to reduce the potential for selection of a subpopulation of cells.

Materials

Complete BSK-II medium (see recipe)
Late-exponential-phase *B. burgdorferi* culture or frozen stock (see Basic Protocol 2)

8-ml polystyrene round-bottom screw cap tubes (e.g., Falcon 352027)
34°C incubator
Petroff-Hausser counting chamber (optional; *APPENDIX 4A*)

1. Place 6 ml fresh complete BSK-II medium into a sterile 8-ml screw cap tube.
2. Inoculate with 50 to 500 µl of a late-exponential-phase *B. burgdorferi* culture (about 5×10^7 cells/ml) or with entire 1 ml of stock (Basic Protocol 2) immediately after thawing it at room temperature or in 34°C incubator.

   Microscopic assessment of thawed stock culture viability (i.e., motility) is suggested prior to inoculation. Thawed stocks should not be refrozen to preserve optimal cell viability. Cultures can also be inoculated with scrapings from a frozen stock surface using a sterile toothpick or platinum loop. Yet, inoculation with the entire stock culture is suggested to prevent selection of a subpopulation of cells (see Commentary).

3. Incubate the tightly capped tube at 34°C without agitation.

   The use of an anaerobic chamber and tubes permitting gas exchange is not necessary. Nonmotile B. burgdorferi mutants grow better with agitation (Motaleb et al., 2000).

   A gradual color change of the medium from red to orange to yellow (due to the phenol red pH indicator in CMRL-1066) indicates bacterial growth. The presence of Borreliae can be confirmed by microscopy. One spirochete per 40× field of view equals about 2×10^5 cells/ml. A Petroff-Hausser counting chamber (APPENDIX 4A) can be used for more precise culture density determinations.

   Cultures should reach their previous cell density within 3 to 7 days, depending on the dilution of the inoculum. Temperatures of 30° to 37°C support adequate growth. Generation times of strains range from 5 to 18 hr. Growth at ambient temperature (23° to 24°C) is very slow, with generation times of ~24 to 48 hr (Stevenson et al., 1995). Growth and recovery of viable spirochetes is not supported by temperatures of 40°C and above (Barbour, 1984). Growth at temperatures <23°C has not been assessed.

PREPARATION OF FROZEN *BORRELIA* STOCKS

BASIC PROTOCOL 2

Borrelia cultures can be stored short to intermediate term without cryoprotectant at 4°C, although recovery of viable cells is reduced beyond 2 months. For indefinite storage, cultures are best frozen at −80°C in 10% dimethyl sulfoxide (DMSO) or 20% glycerol. Either cryoprotectant yields equivalent results. The preparation of multiple vials per strain is suggested (see Basic Protocol 2). For consistency of multiple identical stocks, cell culture and cryoprotectant are best mixed in bulk and then aliquoted in sterile cryogenic vials. The following protocol is for the preparation of 1 ml frozen aliquot stock, but can be scaled, e.g., to store 200-µl aliquot stocks in 96-well microtiter plates.

Materials

Late-exponential-phase *B. burgdorferi* BSK-II culture (Basic Protocol 1)
Sterile 100% DMSO or 80% (v/v) glycerol solution
2-ml polypropylene cryogenic vial (Corning cat no. 430659), sterile

1a. *To cryopreserve with DMSO:* Add 900 µl of a late-exponential-phase *B. burgdorferi* BSK-II culture to 100 µl of sterile DMSO into a sterile 2-ml polypropylene cryogenic vial.

1b. *To cryopreserve with glycerol:* Add 750 µl of a late-exponential-phase *B. burgdorferi* BSK-II culture to 250 µl of a sterile (i.e., autoclaved) solution of 80% (v/v) glycerol in distilled water into a sterile 2-ml polypropylene cryogenic vial.

2. Mix thoroughly by pipetting or vortexing.

3. Transfer tubes to a −80°C freezer for indefinite storage.

Standard protocols for freezing bacterial cultures on dry ice or in liquid nitrogen prior to storage can be followed, but no empirical benefit for cell survival has been observed.

PLATING OF *B. BURGDORFERI*

BASIC PROTOCOL 3

The following protocol details the modification of BSK-II for subsurface plating of *Borrelia burgdorferi* isolates as described by Hinnebusch and Barbour (1992). This technique, in addition to limiting dilutions in liquid broth, allows for the cloning of *B. burgdorferi* isolates and represents an essential step in molecular genetics experiments. The plates consist of a bottom and top agar containing agarose and BSK-II medium, with the bacteria embedded in the top agar. In contrast to the air-tight culture tubes, the air-permeable plates need to be incubated in a CO_2 atmosphere.

Three stock solutions need to be made beforehand: a 2× BSK-II solution, and two separate agarose solutions. In comparison to the liquid BSK-II medium, the 2× solution lacks gelatin. All stock solutions need to be prewarmed prior to use and can be kept at their appropriate temperatures for several hours.

Plates are poured in two steps: First, the bottom agar is poured and allowed to solidify. Next, *B. burgdorferi* cells are added to the top agar and plated on top of the bottom agar. Solidified culture plates are then incubated in a CO_2 atmosphere.

Materials

2% (top) and 3% (bottom) agarose stock (see recipe)
2× BSK-II (see recipe), 37° and 45°C
B. burgdorferi cell suspension (in BSK-II or PBS; Basic Protocol 1)

45° and 65°C water baths
50-ml disposable polypropylene tube
10-cm diameter polystyrene petri dishes
34°C, 5% CO_2 incubator *or* anaerobic jar with hydrogen- and CO_2-generating packs (e.g., BD GasPacks Plus)
15-ml disposable polypropylene tubes

Prepare bottom agar

1. Melt 3% agarose in a microwave oven and equilibrate to 65°C.

   Do not allow the viscous solution to boil out of the vessel. Complete melting of the 3% agarose might require 2 to 3 heat-and-mix cycles.

2. Mix an equal volume of 2× BSK-II (kept at 37°C) and melted 3% bottom agarose (kept at 65°C) in a 50-ml disposable polypropylene tube by inverting four to five times. Pour quickly into 10-cm diameter polystyrene petri dishes.

   The volume per a standard 10-cm diameter polystyrene petri dish is 10 ml. The final 1.5% agarose solution solidifies within 30 sec or less. Pouring four plates at a time from a 40 ml solution mixed in a 50-ml disposable polypropylene tube is suggested.

   Air bubbles can be avoided by gently mixing and careful pouring of the agar. The standard remedy for plate air bubbles, i.e., a Bunsen burner flame, is not adequate in this case since the bottom agar will solidify too quickly.

3. Allow bottom agar to solidify at room temperature.

   Solidified bottom agar plates can be stored overnight at 34°C in a 5% CO_2 atmosphere incubator or anaerobic jar, or be used directly for top agar plating.

Prepare top agar

4. If necessary, melt 2% agarose in a microwave oven and equilibrate to 45°C.

5. Add a maximum volume of 1 ml *B. burgdorferi* cell suspension (in BSK-II or PBS) to 5 ml 2× BSK-II (kept at 45°C) in a 15-ml disposable polypropylene tube.

   The cell suspension volume used for plating should yield about 100 cfu and is dependent on the plating efficiency of a strain. B. burgdorferi plating efficiencies usually range from 50% to 100%, but can be several logs lower for mutants or highly passaged strains (Sadziene et al., 1995). If the plating efficiency is unknown, cells should be plated at several concentrations, e.g., 10^2 to 10^5 cfu/ml.

6. Add 5 ml melted 2% top agarose and mix by inverting. Pour immediately on top of bottom agar and allow to solidify.

   The final 1% low melt top agar solidifies much slower than the bottom agar, i.e., within about 2 min. This allows for a less frantic pace of plate pouring.

7. After ~10 min, incubate solidified plates lid-up at 34°C in a 5% CO_2 atmosphere, or anaerobic jar with a BD GasPack Plus.

   Depending on the strain background, opaque colonies should be visible within the top agar in 5 to 14 days.

8. Pick agarose plugs containing the bacterial colonies using sterile 1000-µl pipet tips or Pasteur pipets.

9. Eject plugs into medium to inoculate liquid cultures or squash under coverslips on glass slides for microscopic observation (*UNIT 2A.1*).

   Alternatively, sterile toothpicks can be used for inoculation of liquid medium.

REAGENTS AND SOLUTIONS

Use double-deionized, e.g., Milli-Q water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose stock, 2% (top) or 3% (bottom)

Add 2 g (2%) low-melt (e.g., SeaPlaque) or 3 g (3%) standard agarose (e.g., SeaKem) to 100 ml double-deionized water. Autoclave 20 min at 121°C on the liquid cycle. After autoclaving, make sure that the agarose is in solution by swirling. For immediate use, equilibrate to 45°C (for 2% agarose) or 65°C (for 3% agarose) in a water bath. Otherwise, store indefinitely at room temperature and melt by microwaving before equilibrating.

The use of ultrapure-grade agarose equivalent to SeaKem and SeaPlaque (Cambrex) is suggested. The use of microbiology-grade agar (a mixture of agaropectin and agarose) instead of purified agarose has not been evaluated.

BSK-II medium, complete

Thaw a 30-ml aliquot of trace-hemolyzed rabbit serum (e.g., Pel-Freez Biologicals; stored indefinitely at −20°C in 30-ml aliquots) at 37°C. Warm both incomplete BSK-II (see recipe) and 7% gelatin solution (see recipe) to 37°C. Add 100 ml of 7% (w/v) gelatin and 30 ml rabbit serum to 500 ml incomplete BSK-II. Mix and pass through a 0.22-μm filter unit. Store medium in the dark at 4°C, up to 2 weeks. Warm to 34° to 37°C before use. Before first use, incubate a small sample of each new batch of medium at 34°C to exclude contamination (see Troubleshooting).

Final volume is 630 ml, which should be accommodated by filtration-bottle units. Warming the gelatin and rabbit serum together with the use of polyethersulfone 0.22-μm filter-bottle systems (e.g., Millipore Stericup-GP PES) allows for fast filtration and further lowers the potential for clogging. No prefilters are necessary.

The recipe above is for 1× medium used for liquid culture (Basic Protocol 1). For 2× medium used for solid medium (Basic Protocol 3), add 13.6 ml rabbit serum to 100 ml 2× incomplete BSK-II (see recipe). Addition of gelatin, which increases the viscosity of liquid medium, is not necessary for solid medium. The smaller batches of complete 2× BSK-II are appropriate, since it is likely to be used less frequently. Store at 4°C and use within 2 weeks for optimal growth. Before first use, incubate a small sample of each new batch of medium at 34°C to exclude contamination. Warm to 37°C in a water bath or incubator before use.

Also see Strategic Planning.

BSK-II medium, incomplete (basal), 1× and 2×

Prepare 1× incomplete medium by mixing the following in a 2-liter beaker previously rinsed with Milli-Q water. For 2× medium, use the same recipe but half the amount of added water (i.e., 500 ml).

1000 ml H_2O
9.7 g CMRL-1066 (see Strategic Planning)
50.0 g BSA fraction V
5.0 g neopeptone
6.6 g HEPES, sodium salt
0.7 g sodium citrate
5.0 g glucose
2.0 g yeastolate
2.2 g sodium bicarbonate
0.8 g pyruvic acid
0.4 g *N*-acetyl-glucosamine

Adjust pH to 7.6 with 1 M NaOH

continued

Filter solution through a 0.45-μm filter unit. Place a glass-fiber prefilter on top of the 0.45-μm cellulose acetate filter unit to reduce clogging. Apply vacuum and wet the glass-fiber prefilter with ~10 ml medium before pouring the remaining volume into the filter unit. Change filter unit if it becomes clogged and flow rate declines significantly. Store indefinitely in 500-ml filter-bottle units protected from light at 4°C or lower.

The use of low-protein-binding cellulose acetate 0.45-μm filter-bottle systems in combination with glass fiber prefilters (e.g., Corning no. 430770 with no. 431353) is suggested to prevent clogging during medium filtration. Prewetting attaches the prefilter to the filter and prevents it from floating when the rest of the medium is poured into the filter unit.

The use of HEPES sodium salt instead of HEPES free acid leads to a final pH of the medium that is close to the desired 7.6. Thus, adjusting the pH with 1 M NaOH is usually not necessary when HEPES salt is used.

Although this recipe uses CMRL-1066 in powder form, the water and CMRL-1066 powder can be replaced with 1× CMRL-1066 solution, available commercially. The addition of solid medium components, mainly BSA, will increase the volume of prepared "incomplete" BSK-II to about 1200 ml.

Also see Strategic Planning.

Gelatin solution, 7%

Dissolve 7 g gelatin in 100 ml double-deionized water in a 250-ml glass bottle previously rinsed with distilled water (to remove potential detergent residues; see Strategic Planning). Autoclave 20 min at 121°C on liquid cycle. For immediate use, cool to 37°C in a water bath. Alternatively, 100-ml aliquots can be stored indefinitely at room temperature. Warm to 37°C before use.

Heating in a microwave or on a heating plate can be used to dissolve the gelatin, but autoclaving is the most effective way to get it fully into solution.

COMMENTARY

Background Information

Epidemiology

Borrelia spirochetes are the causative agents of vector-borne Lyme borreliosis and relapsing fever (Barbour, 2003). Tick-borne Lyme borreliosis, caused by *B. burgdorferi* sensu lato (Burgdorfer et al., 1982; Steere et al., 1983), is the most common vector-borne disease in the United States and is endemic in moderate climates of the Northern hemisphere, including Europe and Asia. Tick-borne relapsing fever, caused by a variety of *Borrelia* species, is a relatively rare infectious syndrome in the Western United States from Texas to Washington State, but more common in sub-Saharan Africa, the Middle East, and Central Asia. A louse-borne form of relapsing fever has surfaced in areas affected by war, poverty, and overcrowding (Barbour, 2003).

Genome and genetics

B. burgdorferi type strain B31 was the first spirochetal genome to be sequenced (Fraser et al., 1997; Casjens et al., 2000). With 1.6 megabases, its genome is relatively small and the most fragmented of any prokaryote analyzed to date. A 1-megabase linear chromosome is accompanied by multiple linear and circular plasmids ranging in size from about 5 to 50 kb. The recent genome analysis of other *Borrelia*, including another *B. burgdorferi* sensu lato strain (*B. garinii* Pbi; Glöckner et al., 2004), as well as two relapsing fever *B. hermsii* and *B. turicatae* strains (Guyard et al., 2005), confirmed earlier studies (Casjens et al., 1995; Palmer et al., 2000; Stevenson et al., 2000) that the overall genome structure of *Borrelia* spirochetes is conserved.

Most of the *B. burgdorferi* plasmids provide genes essential for survival in the infectious tick-mammal cycle and are readily lost upon in vitro propagation (Schwan et al., 1988; Sadziene et al., 1993a; see Critical Parameters and Troubleshooting, *Borrelia* Stocks: Plasmid Instability). Yet, at least one of these extrachromosomal elements, a 26-kb circular plasmid, carries genes essential for in vitro growth (Byram et al., 2004), such as a telomere resolvase essential for the replication of the bacterium's linear replicons (Kobryn

and Chaconas, 2002). This extrachromosomal presence of essential genes has led to the proposed term "minichromosome" (Barbour, 1993) for some of these plasmids.

Although the genetic tools to manipulate *B. burgdorferi* are not as numerous and refined as for other pathogens, they are sufficiently advanced to allow for detailed studies of bacterial physiology and virulence (comprehensively reviewed in Rosa et al., 2005). *B. burgdorferi* can therefore serve as a model organism for this bacterial phylum and as a "transgenic" expression host to study other spirochetal virulence factors (Schwan et al., 2003; Zückert et al., 2004).

Cultivation of Borrelia and other spirochetes

The cultivation of spirochetes has been a significant challenge for researchers. As a particularly stark example, the syphilis spirochete *Treponema pallidum* can only be recovered in significant numbers from the testicles of experimentally infected rabbits (Stamm et al., 1987; also see *UNIT 12A.1*). *Borrelia lonestari*, the suspected causative agent of Southern Tick-associated rash illness (STARI; Armstrong et al., 1996; Barbour et al., 1996), long considered "uncultivable" due to lack of growth in BSK-II and other media, was recently recovered from infected ticks in a coculture with an embryonic tick cell line (Varela et al., 2004), an approach that was previously used to study *B. burgdorferi*-tick cell interactions (Kurtti et al., 1988, 1993). In vitro cultivation methods are available for many other spirochetes (see other units in this chapter).

The current BSK-II medium is based on a formulation by Kelly, which supported the continual growth of *B. hermsii* (Kelly, 1971). The recipe was subsequently enriched by Stoenner to enable the outgrowth of a single *B. hermsii* cell (Stoenner et al., 1982). Ultimately, this "fortified" Kelly medium was modified by Barbour (Barbour, 1984) to reliably achieve high spirochete densities (1 to 4 $\times$ 10^8/ml), generation times of 11 to 12 hr and outgrowth of low inocula, i.e., one to two infectious organisms of both Lyme disease and relapsing fever spirochetes. Modifications of the BSK-II recipe also showed that relapsing fever borreliae are more fastidious than *B. burgdorferi* (Barbour, 1984).

Variations of the above described *Borrelia* culture medium, e.g., modified Kelly medium and BSK-H, have been used in different laboratories. Modified Kelly (MK) medium is detailed by Preac-Mursic et al. (1986) and is identical to the modified Kelly-Pettenkofer Medium (MKP) or modified Kelly Medium (MKM) mentioned in subsequent publications. MK differs slightly from BSK-II in its glucose, sodium bicarbonate, rabbit serum, and BSA concentrations. In BSK-based solid media, CMRL-1066 can be at least partially replaced by other tissue culture media components (Frank et al., 2003).

BSK-H, a commercially available version of *Borrelia* culture medium (Sigma Aldrich B-3528, B-8291), was originally developed to avoid the potential variations in undefined BSK-II components such as bovine serum albumin, neopeptone, and yeast extract (Pollack et al., 1993). BSK-H lacks gelatin and further differs from BSK-II in the proportions of other components. Like BSK-II, it supports the growth of both Lyme disease and relapsing fever spirochetes. Yet, several studies have shown that compositional variations in different batches of BSK-H, like the differences between BSK-II and BSK-H, can affect growth characteristics and gene regulation (Babb et al., 2001; Yang et al., 2001; Wang et al., 2004). It is therefore suggested that the same precautions are taken for individual BSK-H lots as for the individual components of BSK-II.

Critical Parameters and Troubleshooting

BSK-II components

As already stated in Strategic Planning, the quality control of individual components of BSK-II is crucial before large-scale use. This is particularly important for undefined and potentially variable medium components, such as BSA and rabbit serum. As an instructive example for this, the relapsing fever spirochete *Borrelia duttoni* grew only in BSK-II medium containing a subset of albumin batches from the same supplier (Cutler et al., 1999).

Contamination

Good sterile technique is paramount, since bacterial contaminants will readily outgrow *Borrelia* in the rich culture medium environment. Contaminated cultures will likely turn yellow and turbid within 24 hr of inoculation. In this unfortunate case, the source of contamination should be determined and fresh cultures should be started from uncontaminated sources. If this is not an option, an attempt can be made to eliminate the contaminants. *Borrelia* are intrinsically resistant to the antibiotics phosphomycin and rifampicin, and

cultures can be passaged through BSK-II containing these two antibiotics. Final concentrations are 50 μg/ml rifampicin (from a stock of 50 mg/ml in DMSO) and 100 μg/ml phosphomycin (from a stock of 100 mg/ml in distilled water). Other compounds that might help in removing contaminants are nalidixic acid (100 μg/ml) and 5-fluorouracil (200 μg/ml; Barbour, 1984). If needed, 100 μg/ml of the fungicide amphotericin B (e.g., Fungizone) can be used to supress fungal growth. Although these compounds should be superfluous during standard laboratory maintenance of *B. burgdorferi*, they can also be added to BSK-II to prevent contamination.

***Borrelia* stocks: plasmid instability**

Researchers should be keenly aware of the well-documented phenomenon of plasmid instability: the majority of *Borrelia burgdorferi* plasmids (see Commentary above) are dispensable for in vitro growth and readily lost upon extended cultivation. Therefore, passages should be kept to a minimum and cell stocks should be generated as early as possible.

Anticipated Results

Properly prepared BSK-II should support the outgrowth of one to two *Borrelia* cells to an approximate density of 10^8 cells. Assuming exponential growth conditions and cell divisions every 5 to 18 hr, this would require <30 generations, i.e., 6 days to 3 weeks. Microscopic observation of a wet mount under phase-contrast or dark-field microscopy (*UNIT 2A.1*) should show motile spirochetes. Large aggregates of bacterial cells (clumps) have been observed, especially in early passages of original isolates (Barbour, 1984). These aggregates can be temporarily dissolved by vortexing or pipetting and have no significant impact on further cultivation.

Borrelia grow in solid BSK-II in white, opaque colonies. Depending on the strain, they are small (<1 mm) and pointed, to larger (2 to 4 mm) and diffuse. Colonies should appear within 1 to 4 weeks of plating.

Time Considerations

The time-limiting step in the preparation of BSK-II is the solubilization of bovine serum albumin for the preparation of incomplete BSK-II, which usually takes 1 to 2 hr. This time can be used to prepare other stock solutions such as gelatin or agarose. Preparation of complete BSK-II only involves the warming and filtration of stock solutions and should be accomplished in <30 min.

The time-limiting step in the plating of *Borrelia* on solid BSK-II is the preparation and equilibration of the BSK-II and agarose stock solutions at their appropriate temperatures. Plate pouring should take <1 hr for ten plates.

Cultivation times depend on the inoculum size, strains, and cultivation conditions (see Anticipated Results).

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Key References

Barbour, 1984. See above

Describes the cultivation of Borrelia in BSK-II medium, including its original recipe.

Hinnebusch & Barbour, 1992. See above

Describes the cloning of B. burgdorferi by subsurface plating in BSK-II agar.

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Cultivation of *Borrelia burgdorferi* in Dialysis Membrane Chambers in Rat Peritonea

The dialysis membrane chamber (DMC) model is an animal model for studying changes in bacterial gene expression in response to growth within the mammalian host environment, a process referred to as "host adaptation." Briefly, diluted bacterial cultures are placed within chambers constructed from sterile dialysis membrane tubing (DMCs) and implanted into the peritoneal cavities of rats. Following a 9- to 12-day incubation, DMCs are recovered by explantation and the resulting host-adapted bacteria are harvested for subsequent analyses. Bacteria cultivated in this manner are suitable for use with a wide range of molecular techniques including, but not limited to, quantitative PCR/RT-PCR (*UNIT 1D.3*), microarray analyses, immunoblotting, and proteomics. This unit outlines a method for preparing bacteria-filled dialysis membrane chambers, surgically implanting them into rat peritoneal cavities, and then isolating the resulting host-adapted bacteria (see Basic Protocol). A method for preparing sterile dialysis tubing is also described (see Support Protocol).

PREPARATION OF HOST-ADAPTED *B. BURGDORFERI* USING DIALYSIS MEMBRANE CHAMBER PERITONEAL IMPLANTS

BASIC PROTOCOL

In this technique, a small length of sterile dialysis membrane tubing, filled with a diluted culture aliquot of *Borrelia burgdorferi*, is tied off at both ends to form a chamber that is then surgically implanted into the peritoneal cavity of a small mammal, typically a rat. The spirochetes inside the DMC are then cultivated within the mammalian host for a period of 9 to 12 days, following which the DMC is explanted and the dialysate containing the host-adapted spirochetes is harvested. Although spirochetes have been shown to survive for as long as 6 months post-implantation, the overall viable spirochetal density does not appear to increase above that observed at the 9- to 12-day incubation period. The spirochete material resulting from normal culture turnover will be retained within DMCs and may reduce the quality of the explanted bacterial cultures obtained. This protocol describes preparation of bacteria-filled DMCs and the subsequent surgical implantation procedure. In addition to being used in numerous studies to examine various aspects of Lyme disease pathogenesis, this procedure has been used to cultivate the pathogenic spirochete *Leptospira interrogans*, as well as *Pseudomonas aeruginosa* (Mashburn et al., 2004). It may also be modified for use with other bacterial pathogens.

CAUTION: Borrelia burgdorferi is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: This experiment requires Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

IMPORTANT NOTE: The success of this protocol is dependant on the proper use of aseptic techniques during all stages of both dialysis membrane chamber preparation and animal surgery.

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Materials

Female Sprague-Dawley rats weighing ~150 to 174 g upon arrival
70% ethanol in spray bottle
BSK-H (or BSK-II) medium (Sigma), prewarmed to room temperature
Borrelia burgdorferi growing in culture (*UNIT 12C.1*)
Ketamine/xylazine anesthetic cocktail (see recipe)
Ophthalmic ointment, sterile (e.g., Puralube from Pharmaderm Animal Health)
0.3 mg/ml buprenorphine·HCl (e.g., Buprenex Injectable)
Betadine surgical scrub solution

10-ml individually wrapped, sterile disposable pipets
Surgical drape, cut into ~18-in. (~46-cm) squares, sterile
Battery-operated pipet filler (e.g., Pipet-Aid, Drummond Scientific)
Petroff-Hausser counting chamber (Hausser Scientific)
50-ml conical polypropylene centrifuge tubes
Surgical gloves, sterile, individually wrapped
Sterile blunt-end forceps
Sterile dialysis tubing (see Support Protocol)
Sterile petri dishes
0.5-ml syringes with 28-G, 1/2-in. (~1.3-cm) needles
Sterile 4 × 4–in. (~10.2 × 10.2–cm) gauze
Surgical instruments, sterilized and kept within sterile packaging inside surgical field:
  Scalpel blade and holder, no. 10
  4-in. (~10.2-cm) iris scissors (optional)
  Tissue forceps (1 × 2–tooth dissecting or Adson-Brown, available from Henry Schein Medical)
  5 1/2-in. (~14-cm) needle holder with built-in scissors (e.g., Olsen Hegar, available from Henry Schein Medical)
  Ethicon 4-0, SH-1, 27-in. (~68.6-cm) coated Vicryl, violet-braided suture (available from Henry Schein Medical)
  Auto-clip 9-mm stainless steel wound closure clips and applicator (Fisher Scientific)
10-ml syringe with 18-G, 1-in. (~2.54-cm) needle, sterile

Additional reagents and equipment for injection (Donovan and Brown, 1995a) and euthanasia (Donovan and Brown, 1995b) of rodents

1. Acclimate female Sprague-Dawley rats, weighing 150 to 174 g upon arrival, to the animal facility environment for no less than 1 week prior to surgical procedure.

   Animals should be acclimated under standard lighting and temperature conditions with food and water ad libitum.

Prepare sterile surgical field

2. Place several disposable, individually wrapped pipets within the surgical field. Open but do not remove the sterile wrapping of each pipet.
3. Lay down a sterile surgical drape as a workspace within the surgical field.
4. Clean the outer surface of a battery-operated pipet filler with 70% ethanol and place on the sterile drape.

Prepare diluted bacterial culture

5. Count bacteria using a Petroff-Hausser counting chamber. Dilute *B. burgdorferi* culture in room temperature BSK-H medium to a final density of 3000 spirochetes/ml in a 50-ml conical centrifuge tube.

   BSK-II medium may also be used.

Each dialysis membrane chamber will require 8 ml medium containing spirochetes, and each bacterial isolate should be implanted in a minimum of two rats per experiment.

6. Place tubes containing the diluted culture(s) within the surgical drape area (see step 2). Spray the outside of each tube with 70% ethanol and wipe with a Kimwipe. Gently loosen the lid of each tube to allow for easy removal during subsequent steps

7. Place one of the disposable 10-ml pipets that were previously placed in the surgical field (step 1) into the nosepiece of the cleaned pipet filler (step 3), but do not remove from the sterile wrapping until just prior to use.

Fill dialysis membrane chambers with diluted bacterial culture

8. Once all required materials have been placed within close reach, put on a fresh pair of sterile surgical gloves.

   The open (inside) surface of the glove packaging material can be used to provide an additional sterile work area if needed.

9. Using sterile blunt-end forceps, remove a tubing strip from its storage container and place between the index finger and thumb of the free hand. With the other hand, remove the cap from the tube containing the diluted culture and transfer up to 8 ml to the interior of the tubing.

   Dialysis membrane tubing must be prepared as described in the Support Protocol. One end of the tubing will already have been tied off at this point (Support Protocol, step 2).

10. While continuing to hold the open end, flatten the unfilled part of the tubing to eliminate any air bubbles that may have been introduced during filling. Gently twist the filled tubing until essentially all remaining void volume has been eliminated and the tubing becomes taut.

    Try to make the dialysis membrane chamber as taut as possible. This will help to maneuver the chamber into the rat peritoneal cavity during surgery.

11. Begin tying off the top of the tubing strip by holding the twisted tubing tightly between thumb and index finger, and wrapping the loose tubing end one time around the index finger.

12. Bring the open tubing end through the loop that was made around the index finger so as to create a knot, moving the index finger just enough to allow the open tubing end to pass through the loop. Hold the twisted tubing closed by switching the grasp to thumb and middle finger if necessary.

13. Once the open tubing end has been pulled through the twisted tubing loop, pull down on the knot while holding the open end to tighten.

    It may be easier to use blunt-end forceps to manipulate the open tubing end as it is passed through the loop.

    The resulting filled, tied dialysis membrane chamber should be no more than ~1.5 in. (~3.8 cm). It is important that the maximum culture volume (i.e., 8 ml) not be exceeded when filling the dialysis tubing. Larger DMCs may result in animal discomfort due to interference with normal intestinal or bladder functions.

14. Once a dialysis membrane chamber has been prepared, place it into a sterile storage container (e.g., petri dish) containing enough BSK-H (or BSK-II) medium to partially cover each chamber.

    It is not recommended that filled dialysis membrane chambers be stored in this manner for extended periods of time, as this may increase the risk of contamination. Once filled, proceed directly to preoperative preparation of animals.

    Perform preoperative preparation of animals.

15. Anesthetize rat by intramuscular or intraperitoneal injection (Donovan and Brown, 1995a) with an anesthetic cocktail of ketamine/xylazine, administered using a 0.5-ml syringe with a 28-G, 1/2-in. (~1.3-cm) needle, to achieve a dose of 80 to 100 mg ketamine and 10 mg xylazine per kg body weight at time of injection.

    This dosage should provide effective anesthesia for approximately 30 to 40 min. The dosages represent a recommended guideline and may need to be adjusted to ensure that each animal receives an effective level of anesthesia. See Donovan and Brown (1995a), and Fox et al. (2002) for more information.

16. Peform a toe pinch on the animal to ensure anesthesia. If the animal flinches or otherwise responds to the pinch, wait 5 min before checking again. If the animal continues to be responsive, administer a second dose of anesthetic cocktail equivalent to half of the original dose, wait 10 min, then retest by toe pinch.

    If difficulties are experienced even after a second dosage, contact institutional veterinary staff for technical assistance.

    Once animal becomes unresponsive to toe pinch, it is safe to begin.

17. Transfer the anaesthetized animal to the surgical field, placing it supine on a fresh sterile drape. Apply a small amount of ophthalmic ointment to each eye. Check that the rat's tongue has not fallen back into the throat, potentially obstructing its airway.

18. Administer preoperative analgesic medication (i.e., buprenorphine·HCl, Buprenex) by subcutaneous injection at 0.05 to 0.1 mg/kg body weight using a 0.5-ml syringe with a 28-G, 1/2-in. (~1.3 cm) needle.

19. Test the level of anesthesia by performing a second toe pinch and begin preparing the implant site by cleaning with 4 × 4 sterile gauze soaked in Betadine surgical scrub solution. Once finished cleaning the implant site, put on a fresh pair of surgical gloves. From this point on, avoid contact with any nonsterile areas.

20. Open a fresh sterile surgical instrument pack within the surgical field, leaving the instruments in their sterile packaging until needed.

Place DMC within peritoneal cavity

21. Using a scalpel blade, cut a 2- to 3-in. (~5.1- to 7.6-cm) incision beginning ~1 in. (~2.54 cm) below the xiphoid process (Figure 12C.3.1) through the skin to expose the abdominal wall. Using tissue forceps, gently pull up each side of the incision and use the scalpel to dissect the tissue connecting the skin to the abdominal wall.

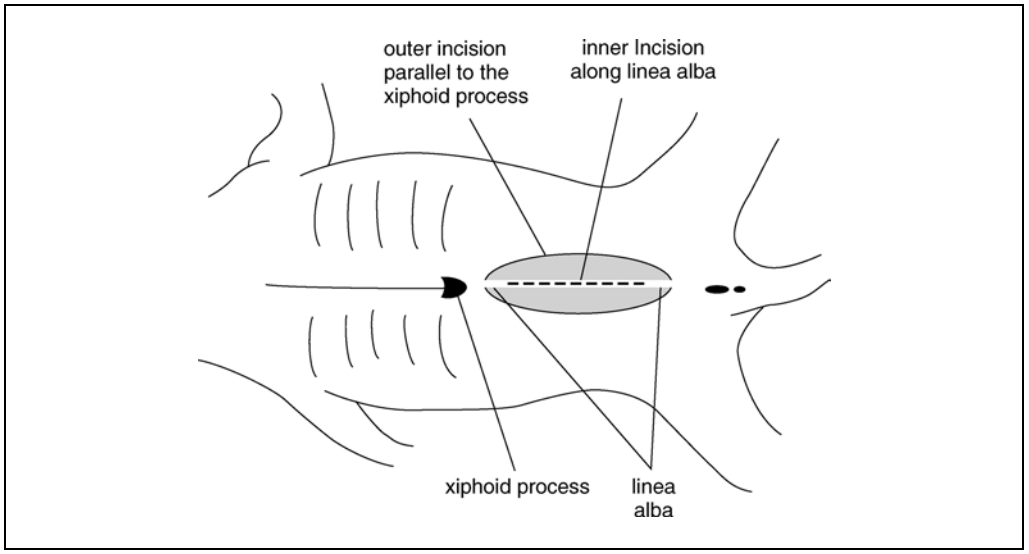

Figure 12C.3.1 Diagram of rat abdomen indicating surgical site.

22. Using tissue forceps, hold up a section of abdominal muscle to avoid injuring any internal organs, then make a small vertical midline incision using either the scalpel blade tip or, alternatively, 4-in. (~10.2-cm) iris scissors.

23. Open the abdominal wall to expose the peritoneal cavity by extending the length of the incision to ~2 in. (~5.1 cm), or slightly smaller than the outer (first) incision (Figure 12C.3.1).

    Use the white line along the abdomen (linea alba) as a guide to keep the incision straight and clean.

24. Using tissue forceps, hold up one side of the abdominal incision and, with the other hand, use blunt-end forceps to remove one dialysis membrane chamber from the medium in which it was placed at step 14. Carefully situate the DMC within the rat peritoneal cavity by pushing it to either side of the peritoneal cavity.

Close peritoneal implant incision and allow animal to recover

25. Using sterile suture with attached needle (e.g., Ethicon 4-0, SH-1, 27-in. (~68.6 cm) coated Vicryl, violet-braided suture), close the peritoneal cavity incision using square knots, closing both ends of the incision first to help keep the suturing line straight and even. Place suture closures ~3 to 4 mm apart along the length of the incision. Be sure to lift the edge of the incision with each suture to avoid puncturing the DMC.

    Although not required, this step is made easier by using Olsen-Hegar needle holder forceps with built-in scissors.

26. Use sterile 9-mm wound closure clips to close the outer incision.

    It is helpful to grasp both sides of the skin together with tissue forceps held in the right hand while applying the wound closures with the left, moving down the incision in one contiguous line until the incision is completely closed.

27. Return the rat to a cage containing fresh bedding, overlaid with a sterile surgical drape. To help maintain body temperature, apply gentle heating to the cage bottom using a circulating water pad until animals are alert and responsive.

    If a circulating water pad is not available, one may also use an incandescent lamp (50-to 75-W) placed 12 to 14 in. (~30.5 to 35.6 cm) away from the animal to provide supplemental heat.

    Be sure to closely monitor the heat being applied to ensure that the animal does not become overheated.

Apply post-operative treatment of animals

28. Once animals have recovered (i.e., alert and responsive), place two animals per cage and return to animal facility housing.

29. Administer analgesic medication (i.e., Buprenex) by subcutaneous injection at a dosage of 0.01 to 0.05 mg/kg body weight, twice daily for 2 days post-operatively.

30. Monitor animals twice daily for signs of distress, stitch abscesses, peritoneal infection, or other complications for the duration of the experimental protocol.

    If animals show any signs of distress or discomfort, consult institutional veterinary staff immediately.

Explant implanted chambers

31. At 9 to 12 days following implantation, euthanize animal by CO_2 asphyxiation (Donovan and Brown, 1995b).

32. With the animal placed supine, use sterile surgical scissors to cut just under and along the line of wound closures to expose the abdominal wall.

33. Use tissue forceps to grasp the sutured inner incision and scissors to cut an opening larger than the original implant opening, to give a better view of the peritoneal cavity.

34. Locate and remove the dialysis membrane chamber using blunt-ended forceps. Transfer to a sterile, empty petri dish.

35. To ensure death, bisect the thoracic cavity using surgical scissors following DMC explantation.

    CAUTION: *Dispose of the animal as infectious material.*

36. Remove the chamber dialysate using an 18-G, 1-in. (~2.54-cm) needle attached to a sterile 10-ml syringe by holding one end of the explanted dialysis membrane chamber with blunt-ended forceps. Gently transfer the fluid from the syringe to a centrifuge tube.

    CAUTION: *Be careful to avoid needle stick with infectious material.*

37. Analyze DMC-cultivated spirochetes using methodologies similar to those for in vitro–cultivated spirochetes (e.g., one- and two-dimensional SDS-PAGE, immunoblot, immunofluorescence, RT-PCR, microarrays).

    Since spirochetes will revert to an in vitro expression profile once placed back into artificial medium, host-adapted spirochetes should be processed immediately following explantation without further manipulation or cultivation ex vivo.

SUPPORT PROTOCOL

PREPARATION OF STERILE DIALYSIS MEMBRANE

One of the most critical aspects of the DMC procedure for cultivating *B. burgdorferi* is the preparation of suitable dialysis membrane tubing. Although a 6000 to 8000 MWCO membrane is recommended, smaller (3500-MWCO) pore size has also been successfully used It is strongly recommended that one start with sterile regenerated dialysis membrane tubing (i.e, Spectra/Por 6 regenerated cellulose) that has been stored in the presence of 0.1% sodium azide as a preservative. Once opened, remaining dialysis membrane should be stored in the cold in its original packaging, including preservative. One important aspect of the washing steps described below is the removal of residual sodium azide preservative to ensure that the bacterial culture used for implantation is not affected. It is critical, therefore, that the volumes of the washing solutions are not reduced. The second important aspect of this protocol is to ensure that the dialysis membraning tubing is effectively sterilized. The use of aseptic technique at all stages of this procedure is absolutely essential for success. Every effort should be taken to minimize contact of the tubing with nonsterile instruments during the cutting and transfer of tubing. All beakers should remain (loosely) covered with sterile aluminum foil prior to and following transfer of strips.

Materials

1 mM EDTA, pH 8.0
Three 2-liter sterile Pyrex beakers
Regenerated dialysis membrane tubing (Spectra/Por 6, 6000 to 8000 MWCO, 32-mm width; Spectrum Labs)
Surgical gloves, sterile, individually wrapped
5 1/2 in. (~14 cm) straight surgical scissors, sterile
11 13/16 in. (~30 cm) extra-long blunt-end forceps, sterile
250-ml sterile Pyrex beakers or 150-mm sterile petri dishes with lids

Cut dialysis membrane strips

1. Fill three 2-liter Pyrex beakers with the following solutions:

   Beaker 1: 1.5 liters Milli-Q water
   Beaker 2: 1.5 liters 1 mM EDTA
   Beaker 3: 1.5 liters Milli-Q water.

   Add a stir bar to each beaker, cover with heavy-duty aluminum foil, and autoclave. Allow to cool briefly.

   Beakers should be prepared the same day as the implantation procedure to ensure sterility.

2. Wearing sterile surgical gloves, cut a strip of dialysis tubing 18- to 20-cm (6- to 7-in.) in length using sterile surgical scissors. Using fingers, tie off one end of the tubing. Remove any excess tubing at the tied end using sterile scissors.

   When first learning to handle the tubing, it may be helpful to cut longer strips. Any excess tubing will be removed from the open end during subsequent steps.

3. Transfer the tied strip of dialysis membrane tubing to one of the beakers containing sterile Milli-Q water (Beaker 1). Replace the aluminum foil cover to maintain sterility.

4. Cut and tie off additional tubing strips as required, preparing one strip of tubing for each rat to be implanted. Transfer to Beaker 1, replacing the aluminum foil lid after each addition.

   It is recommended that at least one extra bag be added in case a bag becomes contaminated during subsequent steps.

Sterilize dialysis membrane strips

5. Once the required number of tubing strips has been transferred to Beaker 1, place that beaker on a magnetic stirrer and rinse the strips by gently stirring at room temperature for 20 min.

6. While tubing strips are rinsing, place the beaker containing EDTA (Beaker 2) on a hot plate/magnetic stirrer and bring to a rolling boil with constant stirring.

7. Gently transfer rinsed tubing from Beaker 1 to Beaker 2 using sterile extra-long blunt-end forceps. Replace aluminum foil cover and continue boiling for 20 min with constant stirring.

   It is important to closely monitor this step to ensure that each dialysis membrane tubing strip remains submerged within the boiling solution at all times. It may be necessary to vent the beaker by briefly lifting one corner of the aluminum foil lid, to release built-up steam.

8. While strips are boiling, place Beaker 3 (containing water) on a hot plate/magnetic stirrer and bring to a rolling boil with constant stirring.

9. Transfer tubing strips from Beaker 2 to Beaker 3 using sterile extra-long blunt-end forceps. Replace sterile aluminum foil cover and continue to boil tubing strips for 20 min with constant stirring.

10. Turn off hot plate/magnetic stirrer and allow tubing to cool until the outside surface of Beaker 3 is warm to the touch.

    The tubing is now ready to use. Although it is preferable to use freshly prepared dialysis membrane tubing, strips may also be prepared several days in advance and stored at 4°C submerged in sterile Milli-Q water. If prepared in advance, however, dialysis membrane tubing should be stored in small quantities of tubing per container (i.e., 5 to 9 strips per container), then opened only once using strict aseptic technique to avoid contamination.

11. If desired for convenience, transfer tubing to a smaller container (e.g., 250-ml sterile Pyrex beaker or 150-mm sterile Petri dish with lid) containing freshly autoclaved Milli-Q water.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ketamine/xylazine anesthetic cocktail

Stock solutions (purchase, e.g., from Fort Dodge Animal Health):
100 mg/ml ketamine hydrochloride
20 mg/ml xylazine hydrochloride
At time of injection, combine 3.75 ml of 100 mg/ml ketamine·HCl with 1.25 ml of 20 mg/ml xylazine·HCl for an anaesthetic cocktail of 75 mg/ml ketamine and 5 mg/ml xylazine.

Xylazine is also available from Vedco, Inc. (http://www.vedco.com).

COMMENTARY

Background and Information

There is now substantial evidence that *Borrelia burgdorferi*, the Lyme disease spirochete, undergoes extensive changes in its transcriptome and proteome as it cycles between its arthropod and mammalian hosts. Most notably, *B. burgdorferi* exhibit numerous changes in gene expression and antigenic composition following the initiation of the tick bloodmeal that continue throughout the infectious process. For recent reviews, see Anguita et al. (2003) and Pal and Fikrig (2003). The ability to host-adapt prior to transmission from tick vector to mammalian host, therefore, is presumed to be central to the *B. burgdorferi*'s pathogenic programs, and likely involves the differential expression of genes encoding factors that prepare the bacterium for both the physiological (i.e., environmental and nutritional) and immunological stresses imposed by growth within the mammalian host milieu. Analysis of differential gene expression in *B. burgdorferi* in the context of the mammalian host, therefore, has been the focus of numerous studies examining the underlying mechanisms contributing to Lyme disease pathogenesis.

One of the best studied examples of differential gene expression by *B. burgdorferi* is the reciprocal expression of outer surface protein A (OspA) and C (OspC) during tick feeding (Schwan and Piesman, 2000). In recent years, a number of other borrelial proteins—e.g., DbpA, Mlp, and OspE/OspF/Elp (Erp), as well as lipoproteins and oligopeptide permease proteins (Opps)—have similarly been shown to be differentially expressed in the context of the arthropod vector and/or mammalian host (Akins et al., 1998; Hefty et al., 2001; Wang et al., 2002; Miller et al., 2003; Yang et al., 2003). Although in vitro studies have demonstrated that temperature and pH are key environmental signals influencing borrelial differential gene expression (Stevenson et al., 1995; Yang et al., 2000), other, as yet unidentified in vivo signals appear to be required for some aspects of mammalian host adaptation (Hefty et al., 2001; Brooks et al., 2003). Investigators have thus far been unable to reproduce the environmental conditions required to induce many of these changes by manipulating in vitro growth conditions. The identification and characterization of borrelial genes preferentially expressed within the mammalian host has been stymied, in large part, by the paucity of spirochetes found in infected host tissues. As a consequence, borrelial gene-expression studies have traditionally relied on more conventional molecular biology techniques (i.e., northern blotting and RT-PCR) and immunological methodologies (immunoblotting) to examine differential expression patterns related to Lyme disease pathogenesis. The majority of these techniques, however, allow for the simultaneous analysis of only a limited number of genes within a given sample, or require the use of previously identified antigens as targets. Although recent advances in molecular biology, such as quantitative real-time RT-PCR (*UNIT 1D.2*), have been successfully applied to the study of *B. burgdorferi* isolated from infected ticks and tissues (Hodzic et al., 2003), the small numbers of organisms that can be obtained from infected ticks and tissues still remains a barrier to

the use of more extensive genome-wide molecular approaches, such as microarrays and proteomics.

In order to circumvent the technical limitations imposed by the paucity of spirochetes within infected tissues, a number of alternate animal models have been developed to cultivate and, more importantly, recover *B. burgdorferi* in a host-adapted state. Several of these models have explored the use of various growth chamber implantation techniques, including the use of steel net (Jonsson et al., 1995) and Teflon chambers (Hurtenbach et al., 1995) containing *B. burgdorferi*, implanted subcutaneously into mice. Each of these strategies, however, is hampered by the relatively small number of spirochetes that can be analyzed. Thus, their use has been primarily restricted to immune protection assays, antibody profiling studies, or both. In order to isolate the quantities of "host-adapted" spirochetes required for global expression profiling, Akins et al. (1998) developed a model for cultivation of *B. burgdorferi* within dialysis membrane tubing implanted into the peritoneal cavities of rats. This method was based, in part, on a modification of an earlier murine model using implanted plastic ring chambers (de Silva et al., 1998). This new animal model, described within this unit, has since been used successfully to examine the expression profiles and functions of a number of borrelial loci, including *ospA*, *ospC*, *dbpBA*, *vlsE*, *oppA*, and *pncA* (Akins et al., 1998; Elias et al., 2002; Parveen et al., 2003; Purser et al., 2003). The cellular material generated using this methodology has proved to be sufficient for both microarray analyses (Revel et al., 2002; Brooks et al., 2003) and proteomics (Akins et al., 1998).

One significant additional benefit of this model lies in its ability to help distinguish between the physiological and nonphysiological demands placed upon spirochetes during growth within the mammalian host (Elias et al., 2002; Purser et al., 2003; Caimano et al., 2004). The pore size of the dialysis tubing used to make the DMC chambers allows for the free diffusion of small molecules and nutrients, but does not permit the entry of immune effector cells. The use of DMCs, therefore, allows for an analysis of growth in vivo, independently of the ability to disseminate or evade host immune defenses. For example, isolates containing mutations within loci whose gene products are involved in generalized metabolism (e.g., Opps and PncA) exhibit partial or complete growth defects within DMCs (Elias et al., 2002; Purser et al., 2003), while isolates containing mutations within loci involved in more traditional virulence factors, e.g., antigenic variations such as VlsE (Elias et al., 2002; Purser et al., 2003; Caimano et al., 2004) grow in a manner that is indistinguishable from that of their wild-type parent. Use of the DMC model, or any animal model for that matter, to define a phenotype associated with a genetic mutation requires a note of caution. Given that the ability to survive within DMCs, and subsequently host-adapt, is almost certainly a complex process involving multiple loci, it is critical that any mutant (or complemented mutant) isolate be examined in the context of its wild-type isogenic parent to ensure that any phenotypes observed are due to the mutation of interest and not a spontaneous secondary mutation.

Critical Parameters

Pilot experiments

The DMC model should be suitable for use with any low-passage, virulent, *B. burgdorferi* isolate. Prior to beginning use of this technique, however, a number of variables should be considered.

Although little strain-to-strain variation has been observed when the DMC procedure has been employed to examine virulent low-passage isolates of strains 297, B31, and N40, these isolates represent *B. burgdorferi* whose in vitro growth characteristics, antigenic composition, and virulence properties have all been well established in numerous published studies. When using a strain other than these, a small pilot experiment should be considered. This is particularly important when contemplating the use of uncharacterized isolates, such as those obtained from clinical specimans. To facilitate the design of such a pilot experiment, it is highly advisable that one first determine the growth kinetics of each isolate in BSK medium following temperature shift from 23°C to 37°C (Fig. 12C.3.2). This preliminary in vitro study will help take into account any significant variations in growth kinetics between isolates when estimating the implantation time course. Growth kinetics within DMCs will likely be similar to that of cultures grown in vitro at 37°C, although the DMC-cultivated cultures do not appear to be able to attain the same maximal density as their in vitro–grown counterparts (Fig. 12C.3.2).

A second variable to consider is that at least one borrelial locus, *pncA* (*bbe22*), encoding a nicotinamidase, has been shown to be required

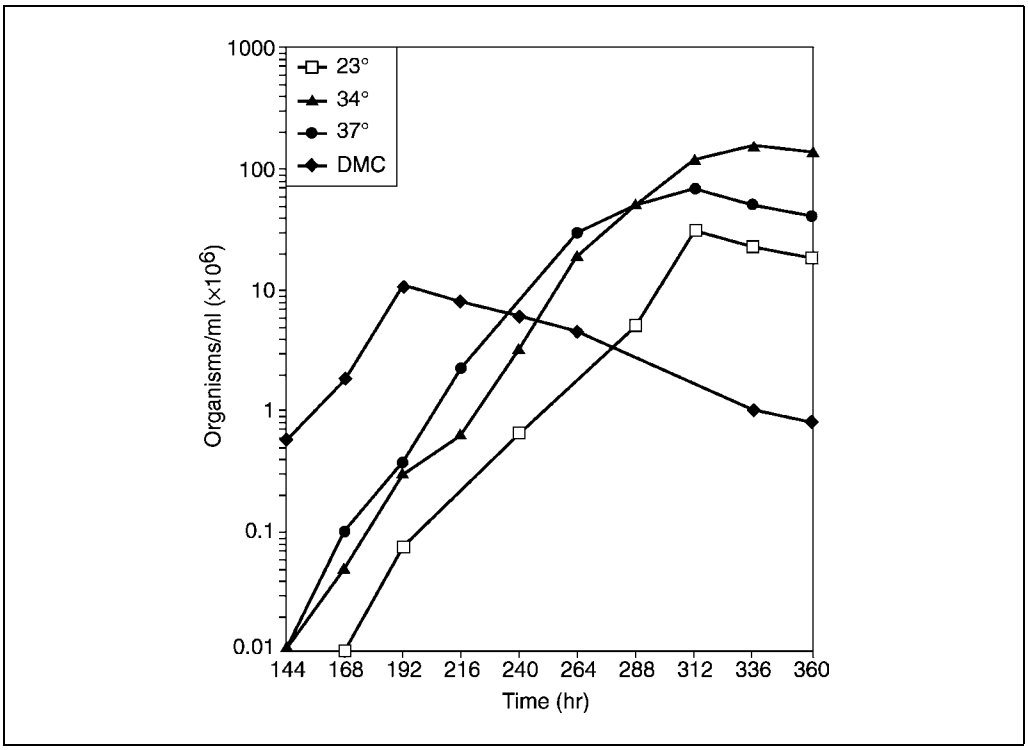

Figure 12C.3.2 Growth kinetics of *B. burgdorferi* strain 297 cultivated in vitro or within DMCs. Spirochetes cultivated in BSK-II medium at 23°C (open squares), 34°C (triangles), or 37°C after temperature-shift (circles), or within DMCs (diamonds), were enumerated by dark-field microscopy over a 15-day period after inoculation. In all four cultures, 5-ml volumes were inoculated with 103 mid-logarithmic-phase organisms. Data points represent the means of duplicate samples. Adapted with permission of the publisher from Akins et al. (1998).

for DMC cultivation (Purser et al., 2003). Since the plasmid encoding this locus, lp25, is easily lost during in vitro cultivation, it is critical that the borrelial isolate of interest be tested for the presence of *pncA* prior to use in DMCs.

Lastly, growth of *B. burgdorferi* in vivo almost certainly involves as yet unidentified borrelial loci in addition to *pncA*; it is therefore recommended that, once tested for the ability to host-adapt within DMCs, the isolates be subjected to minimal passaging in vitro to reduce the loss of plasmids (in addition to lp25) or the acquisition of spontaneous mutations that may affect host adaptation.

Verification of host adaptation

In general, mammalian-host adaptation by *B. burgdorferi* is characterized by the differential expression of a number of key antigens, most notably the up-regulation of OspC, DbpA, and DbpB, and the down-regulation of OspA and Lp6.6 (Fig. 12C.3.3). To ensure that an isolate of interest has actually undergone host adaptation, however, it is important to compare the polypeptide profile of DMC-cultivated spirochetes to that of the same isolate cultivated in vitro at 37°C following temperature shift. Lysates prepared from bacteria cultivated in vitro and within DMCs may be compared visually for well expressed proteins such as OspA and OspC using SDS-PAGE gel electrophoresis. Alternatively, immunoblotting may be used to examine borrelial antigens whose expression is not readily apparent by gel electrophoresis (e.g., DbpA, Lp6.6). When testing lysates by immunoblot, however, it is important to keep in mind that strain-to-strain sequence variability may result in limited to no antigenic cross-reactivity. Thus, one should be sure to use antisera that are known to react against the corresponding antigens within the isolate of interest.

Troubleshooting

The most common problem associated with the procedure is contamination of the DMC during the implant surgery. It is critical that aseptic technique be maintained at all times during both the preparation of the dialysis membrane tubing strips and the implant surgery. To reduce contamination, be sure that the surgical area is thoroughly cleaned with a surface sterilant immediately prior to the procedure. Ideally, instruments and drapes should

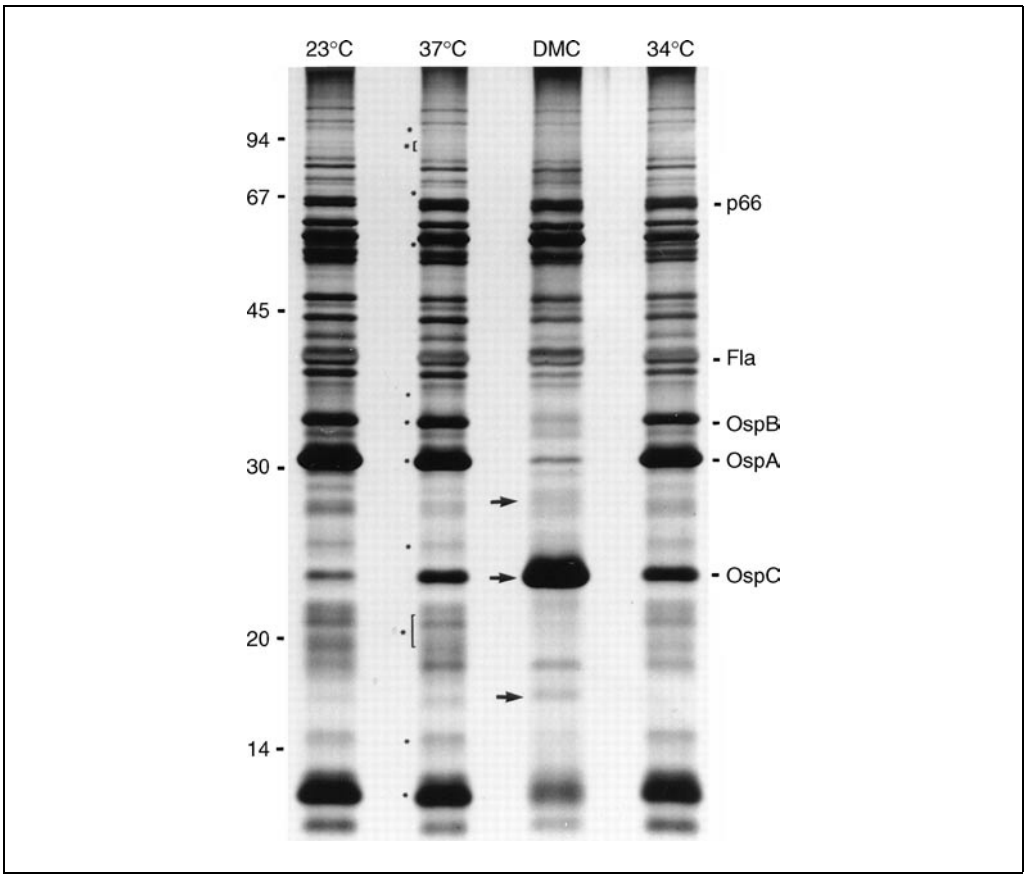

Figure 12C.3.3 Wild-type *B. burgdorferi* strain 297 ($\sim 10^7$ per lane) was cultivated in vitro in BSK-H medium at 23°C, at 37°C following temperature shift, at 34°C, and within dialysis membrane chambers (DMC). The whole-cell lysates were separated by SDS-PAGE, then stained with silver. Molecular mass markers (kDa) are indicated. Asterisks indicate polypeptides that were absent or dramatically down-regulated in chamber-grown spirochetes. Arrows designate proteins either dramatically up-regulated or observed only in chamber-cultivated organisms. Well characterized borrelial proteins are indicated on the right and molecular mass markers in kilodaltons are shown on the left. Adapted with permission of the publisher from Akins et al. (1998).

be autoclaved on the day of the procedure or on the day before, but they may be stored in a closed container or cabinet for $\leq$2 weeks prior to use. Also, for best results, the sterile dialysis membrane tubing strips should be prepared within 24 hr of the implant procedure. If the bags become contaminated following implantation, the animals may exhibit symptoms associated with peritonitis, most notably pronounced abdominal swelling. If such symptoms occur, notify institutional veterinary staff immediately.

When first beginning to use the DMC protocol, it is recommended that one test the isolate of interest in parallel with a virulent low-passage isolate whose ability to host-adapt has been previously tested (i.e., 297, B31-Medimmune, or N40), to rule out technical issues. If the isolate of interest fails to multiply within DMCs despite multiple trials, but does not appear to be lacking lp25 (see above), it is recommended that the isolate be tested for survival alone by implanting spirochetes at higher density (1×10^7 spirochetes per ml) for a short incubation period ($\sim$5 days), and then evaluating for spirochete viability, following explantation, by either dark-field microscopy to assess motility or by limiting-dilution plating.

Anticipated Results

The average recovery volume per DMC chamber following explantation will vary between 6.5 to 8 ml dialysate-containing spirochetes. The theoretical yield of spirochetes contained within the DMCs should be between 2×10^6 and 2×10^7 spirochetes per ml, or an average 700- to 3000-fold increase in bacterial number (Fig. 12C.3.2). Following explantation, spirochetes should be examined using dark-field microscopy to assess spirochetal viability. Spirochete density should be calculated

by enumeration using a Petroff-Hauser counting chamber.

While elevated growth temperature has been shown to result in increased expression of a number of borrelial lipoproteins, including OspC, DbpA, and DbpB, expression of each of these, as well as other borrelial proteins (e.g., Mlps), has been shown to be further enhanced during DMC cultivation (Akins et al., 1998; Hefty et al., 2001; Parveen, et al., 2003; Yang et al., 2003). Of equal significance is the decreased expression of numerous borrelial genes during cultivation within DMCs, compared to their in vitro–cultivated counterparts (Revel et al., 2002; Brooks et al., 2003). Collectively, these studies suggest that mammalian host signals, in addition to temperature, contribute to differential gene expression in vitro. The DMC model, therefore, provides a facile methodology for studying changes in gene expression and antigenic composition associated with growth within the mammalian host that could not otherwise be reproduced by manipulation of in vitro growth conditions.

Time Considerations

Animals should be received on site at least 1 week prior to surgery to allow for acclimatization. The preparation of sterile dialysis membrane tubing strips should take ~1.5 to 2 hr. The total time needed for surgery is ~20 to 40 min per animal, depending on the investigator's level of experience. Full recovery of animals from anesthesia following surgery takes ~4 to 6 hr; animals should not be returned to housing until bright, alert, and responsive. The incubation time of DMCs can vary and should be tested empirically with any previously uncharacterized isolates. Generally, 9 to 12 days have been found to be sufficient for most isolates tested. A pilot experiment examining bacterial density daily over a 6-day period, beginning with day 8 and explanting 1 to 2 rats per day, may help establish the optimal explant time course for the isolate of interest. Once maximal density has been reached, longer incubation times do not generally result in higher spirochete densities. On the contrary, the overall spirochete density may remain the same (or decline) and the proportion of nonviable organisms in the recovered supernatant following explantation may increase. Viable spirochetes, however, have been recovered from DMCs explanted as long as 6 months after implantation.

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Key References

Akins et al., 1998. See above.

Original report describing use of dialysis membrane chamber technique with B. burgdorferi.

Fox et al., 2002. See above.

Contains practical advice administering anesthetics and analgesics in common laboratory animal species.

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Laboratory Maintenance of Pathogenic *Leptospira*

Leptospira are long, thin, helically shaped bacteria. Pathogenic *Leptospira* utilize fatty acids and fatty alcohols as primary carbon and energy sources (Baseman and Cox, 1972). These bacteria consume, preferentially, saturated long-chain fatty acids by β-oxidation. This requirement for fatty acids as a critical component of *Leptospira* media poses difficulties for reliable cultivation of cells in vitro, because, at elevated levels, these compounds are toxic to bacterial growth. This toxicity is often circumvented by the addition of albumin. Both defined and complex media have been formulated for *Leptospira* growth. The purpose of this unit is to describe the preparation of various *Leptospira* growth media and methods for bacterial propagation (see Growth of Pathogenic *Leptospira*; Basic Protocols 1 through 3). Methods for the long-term storage (see Storage and Recovery of Pathogenic *Leptospira*; Basic Protocols 4 and 5), isolation (see Isolation of Pathogenic *Leptospira*; Basic Protocols 6 and 7), and detection of pathogenic *Leptospira* by immunofluorescence (see Basic Protocol 8) are also described.

CAUTION: *Leptospira interrogans sensu lato* is a Biosafety Level 2 (BSL-2) pathogen that can cause acute, fatal infections in humans. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

IMPORTANT NOTE: Water for all media solutions must be sterilized by autoclaving prior to use to remove saprophytic *Leptospira* that can pass through microbiological filters. Saprophytic *Leptospira* are ubiquitous in water supplies and are a common contaminant of improperly prepared media. When preparing the media supplements, it is important to follow the order of constituent additions.

STRATEGIC PLANNING

Recipes for Media

Recipes are provided for standard media commonly used to propagate pathogenic *Leptospira* (see Reagents and Solutions). The order in which media components are added is critical for successful preparation.

Additional media recipes are described elsewhere (Faine, 1994). Commercially prepared media are available and are often suitable for routine maintenance of some strains, but some strains either do not grow well in commercial media or have altered growth characteristics.

Stock Solutions

Several stock solutions must be prepared before assembling media components. All solutions are made with glass-distilled water that must be sterilized by autoclaving 20 min at 121°C and allowed to cool before used. The complete medium is prepared by mixing an albumin supplement with a phosphate-buffered basal salt solution, which is filter-sterilized before use.

EMJH Medium

Reagents and Solutions describes the preparation of the common, defined base media used for isolation and growth of *Leptospira*. Many laboratories utilize the Johnson and Harris (1967) modification of Ellinghausen McCullough (1965) medium (EMJH) to cultivate *Leptospira*. The EMJH base medium can be used with or without serum for the

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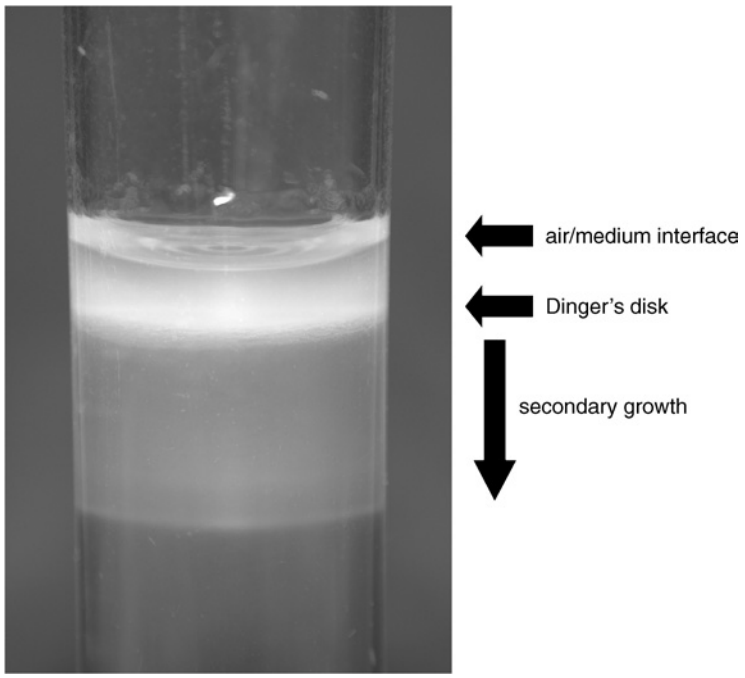

Figure 12E.1.1 Growth of *Leptospira* in a semisolid agar medium. The formation of a Dinger's disk is typical of *Leptospira* grown in semisolid media. After prolonged incubation, growth extends deeper into the tube and may form several disks farther from the air/medium interface. Photograph courtesy of Richard Hornsby.

propagation of leptospires, and has been subjected to minor modifications to support the isolation of various strains from clinical samples. Notes for common alterations of the base media are included. Modified Stuart medium (see recipe in Reagents and Solutions) is rich in rabbit serum and is a convenient alternative to EMJH for routine propagation of laboratory-adapted strains of *Leptospira*.

GROWTH OF PATHOGENIC *LEPTOSPIRA*

Optimal growth of *Leptospira* occurs at 29° to 30°C. Although several strains of pathogenic *Leptospira* grow at 37°C, cultures grown at the elevated temperature shift quickly from logarithmic growth to stationary phase, and enter into death phase rapidly with the sudden onset of autolysis. Cultures will grow slowly at room temperature, and this is useful for maintenance and storage of cultures. Saprophytes grow at low temperatures (e.g., 13°C), which is helpful in differentiating them from pathogenic *Leptospira*, which do not grow at low temperature (Johnson and Harris, 1967). In addition, saprophytic *Leptospira* grow in the presence of 225 μg/ml of 8-azaguanine, while pathogenic strains do not (Johnson and Rogers, 1964).

These bacteria have a slender morphology that eludes detection in wet mounts under light microscopy. Therefore, observation of live cultures in suspension requires a dark-field microscope.

BASIC PROTOCOL 1

Growth in Semisolid Media

Semisolid media tend to support stable growth of pathogenic *Leptospira* better than liquid cultures. Semisolid media (see recipe) have the consistency of a slurry and can be easily dispensed using a pipet. In semisolid media, bacteria form a dense zone of growth, commonly referred to as a Dinger's disk (Lawrence, 1951; Fig. 12E.1.1). During prolonged growth in semisolid media, the growth zone extends towards the bottom of the

tube. With some cultures, little growth is seen near the medium/air interface, and limited growth is seen near the bottom of the tube.

For growth in a semisolid medium, transfer a small amount of culture, ~100 to 250 µl, using a pipettor fitted with a sterile filtered tip or a 1-ml sterile pipet to a fresh tube containing 6 to 8 ml of semisolid medium. To keep cells active, transfer most strains to fresh medium weekly. Some strains grow very slowly, requiring transfers less frequently, occasionally as infrequently as once every 6 months. Typically, transfers are made when a Dinger's disk forms with visible density. The time between transfers is determined empirically because of differences in a variety of factors, including strain differences and type of media being employed to cultivate these bacteria. The critical factor in determining when to transfer cultures is density. Some strains achieve sufficient density within 1 week, while comparable culture density with other strains may take months. Continuous in vitro passage of pathogenic *Leptospira* often results in reduced virulence. However, carefully controlled studies have not been performed that demonstrate how many passages lead to a reduction of virulence, and the mechanisms causing these phenotypic changes are uncharacterized.

Growth on Solid Media

BASIC PROTOCOL 2

Historically, *Leptospira* strains were rarely purified by plating onto solid media. The growth of isolated colonies of pathogenic *Leptospira* is not always possible, and when possible requires a great deal of patience. To isolate single colonies of saprophytic and fast-growing pathogenic *Leptospira*, streak cultures or spread limiting dilutions on solid EMJH medium (see recipe for solid *Leptospira* medium in Reagents and Solutions). Many strains will form isolated colonies in 10 to 21 days. Fastidious strains of *Leptospira* may take 4 to 6 weeks before isolated colonies appear, or may fail to grow on solid medium. To minimize exposure to contaminants, reduce desiccation, and to prevent inadvertent exposure to pathogenic *Leptospira*, inoculate media in a biological safety cabinet, seal plates with Parafilm, and place in a tightly sealed plastic bag. Incubate plates inverted and observe at approximately weekly intervals. Colonies typically are embedded just below the surface of the agar, appear white in color, and have a diameter of ~1 to 2 mm. Extended growth may result in diffusion of the bacteria into the agar. In contrast, saprophytic *Leptospira* form slightly larger colonies (~2 to 3 mm in diameter) and show substantial diffusion radiating outward (1 to 2 cm) from the central colony upon extended incubation.

To isolate individual colonies, remove the colony using either a sterile Pasteur pipet or a micropipet (e.g., Rainin Pipetman 200) fixed with a sterile, filtered tip and gently aspirate the colony into the tip. Place the colony in a semisolid medium and incubate until a Dinger's disk is obtained (7 to 10 days). Direct passage of pathogenic *Leptospira* on a solid medium often fails and recovery of isolated colonies in a semisolid medium is recommended.

Growth in Liquid Media

BASIC PROTOCOL 3

Adaptation of pathogenic *Leptospira* to a liquid medium from a semisolid medium requires a transition period whereby a portion of the semisolid medium containing the bacteria is suspended in a small amount of liquid medium. After the initial suspension of the semisolid culture in liquid, the culture (containing a mixture of liquid and semisolid media) is passed to a tube containing liquid medium, and then the bacteria may be passed directly as liquid cultures. Pathogenic *Leptospira* inoculated directly from a semisolid medium to liquid medium often fails to replicate to sufficient densities for continued propagation; therefore, this adaptation process is essential for most strains.

Materials

Starter cultures of *Leptospira*
Semisolid *Leptospira* medium (see recipe)
Liquid medium (see recipes for EMJH medium, modified Stuart medium, or T80/40/LH medium)

29° to 30°C incubator
50-ml culture tubes with loosely fitting caps

1. Grow starter cultures of *Leptospira* in semisolid medium until a Dinger's disk forms.
2. Gently suspend the Dinger's disk in ~2 to 3 ml of fresh liquid medium, and incubate for several days at 29° to 30°C.

   The liquid portion of the culture should appear slightly turbid.
3. Transfer liquid culture and some semisolid medium (~3 ml) to a 50-ml culture tube containing 8 ml fresh liquid medium.

   Culture tubes should have either Morton closures or loosely fitting caps.

   At this point, the culture may be gently shaken during growth (at ~50 rpm).
4. Follow the growth of the culture until it becomes turbid.

   Typically, this will take from 7 to 14 days.
5. To propagate liquid cultures, transfer 0.5 to 1 ml to 8 ml fresh liquid medium.

   Because the cultures are less stable in liquid, they should be observed more often than semisolid cultures.

   Growth can be measured in liquid using standard laboratory spectrophotometers (A_{400}) or by nephelometry.

STORAGE AND RECOVERY OF PATHOGENIC *LEPTOSPIRA*

Long-term storage of pathogenic *Leptospira* can be achieved in semisolid media at room temperature. However, strains stored under these conditions often have reduced viability and may lose virulence. Most *Leptospira* strains are best stored frozen in liquid nitrogen. Viability of each strain through the freezing protocol should be tested before discarding strains stored at room temperature; some strains may not tolerate the freezing process well.

BASIC PROTOCOL 4

Storage of Pathogenic *Leptospira*

Materials

Fresh culture
Leptospira storage medium (see recipe)
Liquid nitrogen

2-ml cryogenic vials, sterile
Styrofoam rack

Freeze cultures

1. Mix equal volumes of fresh culture with *Leptospira* storage medium.
2. Dispense in 2-ml sterile cryogenic vials.
3. Place vials in a styrofoam rack for ~4 hr at −70°C.
4. Store vials in liquid nitrogen.
5. Test for viability after ~1 month.

If the culture material to be frozen is grown in a semisolid medium (preferred), use a freshly grown cultured Dinger's disk. If the culture material is prepared from liquid culture, use at least 1 × 10^8 cells/ml in the late logarithmic growth phase.

Recovery of Frozen Cultures

BASIC PROTOCOL 5

Materials

Frozen cultures in cryogenic vials
Semisolid *Leptospira* medium (see recipe) in 16 × 25–mm tubes
Capped glass specimen jar

1. Remove a cryogenic vial containing the frozen culture from liquid nitrogen storage directly into a capped glass specimen jar containing sterile distilled water.
2. Allow the culture to thaw in a biological safety cabinet and then remove cryovial from specimen jar.
3. Immediately transfer 100 to 300 µl culture contents into two or three tubes containing 6 ml semisolid medium, and incubate at 30°C.

ISOLATION OF PATHOGENIC *LEPTOSPIRA*

Isolation of Pathogenic *Leptospira* from Urine

BASIC PROTOCOL 6

Infection with pathogenic *Leptospira* usually results in a systemic infection and localization of the bacteria in kidneys. The bacteria are often shed by urine into the surrounding environment, thereby contributing to the spread of this disease to naïve hosts. Urine is typically collected by clean catch into a sterile vessel. When collecting from animals with suspected cases of leptospirosis, wear appropriate personal protective gear to avoid infection. Injection of the diuretic furosemide intravenously can facilitate urine collection. These samples may be used directly for isolation of viable bacteria using the following protocol.

Materials

Urine sample
Leptospira storage medium (see recipe)
Semisolid *Leptospira* culture medium (see recipe)
16 × 125–mm capped tubes
29°C incubator
Dark-field microscope

1. Place 1 ml urine in 9 ml *Leptospira* storage medium to make a 10^{-1} dilution of the sample.
2. Cap tube and vortex for 10 sec.
3. Transfer 1 ml of the diluted sample to a second tube containing 9 ml of storage medium to make a 10^{-2} dilution.
4. Cap tube and vortex for 10 sec.
5. Transfer 0.3 ml of each dilution to a tube containing 6 ml semisolid *Leptospira* culture medium. Incubate at 29°C.
6. Check for growth after 7, 14, 21, and 28 days of incubation and thereafter monthly for 6 months. Examine for growth using dark-field microscopy (*UNIT 2A.1*).

BASIC PROTOCOL 7

Isolation of Pathogenic *Leptospira* from Tissues

Tissue samples collected post-mortem from necropsy can also be used to isolate pathogenic *Leptospira*. The most common sources of tissue used for primary isolation are kidney and liver, although other samples including blood can provide viable bacteria for culture. If samples are collected in the field, it is important to spray the surface of the animal and of the organ from which the tissue will be collected with 70% alcohol to minimize growth of contaminants, prior to collecting tissue samples.

Materials

Tissue sample
Leptospira storage medium (see recipe)
Culture medium

Whirlpak bag
Tissue homogenizer (e.g., Stomacher 400, Seward Medical)
29°C incubator
Dark-field microscope

1. Place ~1 g tissue sample in 9 ml *Leptospira* storage medium in a Whirlpak bag and homogenize for 5 min with a Stomacher tissue homogenizer.
2. Transfer 1 ml tissue homogenate to 9 ml storage medium to make a 10^{-2} dilution.
3. Cap tube and vortex for 10 sec.
4. Transfer 1 ml tissue homogenate (from step 3) to 9 ml storage medium to make a 10^{-3} dilution.
5. Transfer 0.1 ml of each dilution to 6 ml culture medium. Incubate at 29°C.
6. Check for growth after 7, 14, 21, and 28 days of incubation and thereafter monthly for 6 months. Examine for growth using dark-field microscopy (*UNIT 2A.1*).

BASIC PROTOCOL 8

FLUORESCENT ANTIBODY STAINING OF *LEPTOSPIRA*

One commonly used method for the detection of *Leptospira* in clinical or experimentally generated samples is by immunofluorescence microscopy (Fig. 12E.1.2). For this assay, a sample thought to contain pathogenic *Leptospira* is treated with a broadly cross-reacting antiserum that is conjugated with a fluorescent tag, often fluorescein isothiocyanate (FITC), which has an excitation wavelength of 488 nm and peak emission at 520 nm. After binding the antibody to samples fixed to a microscope slide, excess antibody is removed by washing and the sample examined using a standard fluorescent microscope having a transmission filter in the 515 to 550 nm wavelength range. FITC-conjugated anti-*Leptospira* antisera prepared in rabbits can be obtained from Mr. Mark Wilson, NVSL, Ames, IA 50010, (515) 663-7595. Check with Mr. Wilson for current pricing. Orders may be sent by FAX to NVSL at (515) 663-7402 (reference item LEP-FAC).

Materials

Urine sample (see Basic Protocol 6) or tissue homogenate (see Basic Protocol 7)
PBS (*APPENDIX 2A*)
Acetone
Rabbit anti-*Leptospira* antisera conjugated with fluorescent tag (available from Mark Wilson, NVSL, Ames, IA 50010, 515-663-7595)
Flazo Orange counter stain (diluted 1:10 to 1:20 with PBS)
Buffered glycerol

2-ml microcentrifuge tubes
Microscope slides

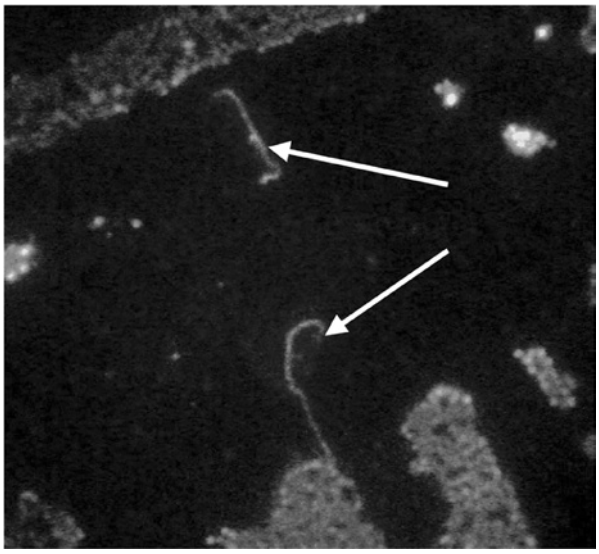

Figure 12E.1.2 Fluorescent antibody detection of *L. interrogans* serovar Pomona. Bacteria obtained directly from urine of an infected steer were fixed and stained with anti-*Leptospira* antisera conjugated with a fluorescent dye. The resulting micrograph shows characteristic green fluorescent staining of the *Leptospira* (arrows) with an orange and black background. Photograph courtesy of Richard Hornsby. For the color version of this figure go to *http://www.currentprotocols.com.*

Moist chamber: petri dish with lid, containing a circle of filter paper moistened with water
37°C incubator
Coverslips
Fluorescent microscope

To prepare urine samples

1a. Concentrate 1.5 ml urine sample in a 2-ml microcentrifuge tube by microcentrifuging 10 min at 13,000 × *g*, room temperature. Remove all but the final 100 µl of supernatant and discard.

2a. Add 500 µl distilled water and resuspend the pellet. Centrifuge 10 min at 13,000 × *g*, room temperature, and remove all but the final 25 to 50 µl of supernatant.

3a. Resuspend pellet by adding 150 µl distilled water. Spot 10 to 15 µl of sample on a clean glass slide. Proceed to step 4.

To pepare tissue samples

1b. Prepare tissue homogenate as described in Basic Protocol 7, diluting tissue at a 1:10 ratio with PBS.

2b. Dilute tissue homogenate 1:10 in PBS.

3b. Spot 10 to 15 µl of the 1:100 dilution of tissue homogenate on a clean glass microscope slide. Proceed to step 4.

Process slides

4. Air dry samples on microscope slides at room temperature, typically overnight.

5. Place slides in acetone for 10 min to fix. Air dry samples.

6. Place each slide into a moist chamber (supported by two parallel toothpicks laid flat upon the moist filter paper) and add 15 µl of rabbit anti-*Leptospira* antisera conjugated with fluorescent tag to each sample. Incubate for 1 to 1.5 hr at 37°C.

7. Rinse slides briefly with PBS, then wash well with PBS for 10 min with stirring using a magnetic stir bar and plate at room temperature.
8. Blot away excess moisture, then spot 15 μl of Flazo Orange counter stain (diluted 1:10 to 1:20 with PBS) for ~20 sec.
9. Rinse slide briefly with PBS, and blot away excess moisture.
10. Apply a small amount of buffered glycerol as a mounting medium and attach coverslip.
11. Observe samples using a fluorescent microscope (Figure 12E.1.2).

REAGENTS AND SOLUTIONS

Use glass-distilled water for the preparation of all solutions. Sterilize the water by autoclaving for 20 min at 121°C and allow time for cooling before preparing any filtered media to eliminate contaminating saprophytic Leptospira. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Basal salt solution

1 g Na_2HPO_4
0.3 g KH_2PO_4
1 g NaCl
1 ml ammonium chloride stock solution (25 g NH_4Cl/100 ml)
1 ml thiamine stock solution (0.5 g thiamine/100 ml)
1 ml glycerol stock solution (10 g glycerol/100 ml)
Add components to ~800 ml glass-distilled water with stirring
Adjust pH to 7.4 with dilute NaOH or diluted HCl
Bring volume to 1 liter with glass-distilled water
Sterilize by autoclaving
Store up to 1 month at 4°C

Volumes given are for completing a 1 liter solution.

BSA stock solution

Weigh out 10 g bovine serum albumin, fraction V, and add to 50 ml sterile, distilled water with constant stirring. Stir slowly to prevent foaming. If necessary, gently heat (<50°C). Once the BSA is dissolved, use immediately to prepare the complete BSA supplement (see recipe).

It is often convenient to start dissolving the BSA in the afternoon, then set overnight at 4°C.

The quality of BSA for use in media is important and must be determined empirically (see Critical Parameters and Troubleshooting).

Several vendors supply satisfactory BSA, fraction V, for use in media preparation. Before purchasing a large amount of BSA from an individual vendor, it is critical to test it by preparing sample albumin supplements and used in test media.

Complete BSA supplement

To 50 ml BSA stock solution (see recipe), add the following stock solutions with constant stirring in the following order:

1 ml calcium chloride (1 g $CaCl_2 \cdot 2H_2O$/100 ml)
1 ml magnesium chloride (1 g $MgCl_2 \cdot 2H_2O$/100 ml)
1 ml zinc sulfate (0.4 g $ZnSO_4 \cdot 7H_2O$/100 ml)
0.1 ml copper sulfate (0.3 g $CuSO_4 \cdot 5H_2O$/100 ml)
10 ml ferrous sulfate (0.5 g $FeSO_4 \cdot 7H_2O$/100 ml)
1 ml vitamin B_{12} (0.02 g/100 ml)

continued

12.5 ml Tween 80 (10 g/100 ml)
Adjust pH to 7.4 with the addition of 2 N NaOH (~0.4 ml)
Bring the final volume to 100 ml with H_2O
Sterilize by filtration through a 0.22-μm filter
Store indefinitely at −20°C

Although the final solution is filter-sterilized, it is critically important that water for all stock solutions be sterilized by autoclaving prior to their preparation to eliminate potential contamination by saprophytic Leptospira.

Leptospira require long-chain fatty acids or alcohols for growth, which can be toxic for growth in prepared media. Addition of albumin or animal serum can bind excess fatty acids, to enable growth of these bacteria in vitro.

EMJH medium (liquid medium)

Aseptically add 100 ml completed BSA supplement (see recipe) and 10 ml 5-FU stock (1 g/100 ml) to 890 ml basal salt solution (see recipe). If rabbit serum is added, reduce the volume of basal salt solution accordingly to maintain a final volume of 1 liter. Aliquot as needed for experiments. Store up to 1 month at room temperature.

This medium is quite stable for routine propagation of cultures. However, for isolation from clinical samples, fresh medium should be prepared monthly.

Leptospira storage medium

Dissolve 10 g BSA slowly (overnight at 4°C) in 90 ml phosphate buffer (see recipe). Adjust pH to 7.4 using 10% NaOH and then bring volume to 100 ml with phosphate buffer (see recipe). Filter-sterilize medium through a 0.22-μm filter. Store in 10-ml aliquots up to 1 month at 4°C.

Modified Stuart medium

1.93 g NaCl
0.34 g NH_4Cl
0.19 g $MgCl_2 \cdot 6H_20$
0.13 g L-asparagine
0.67 g $NaHPO_4$
0.087 g KH_2PO_4
Dissolve components in 1 liter glass-distilled water
Adjust pH to 7.5 with dilute NaOH or dilute HCl
Sterilize by autoclaving for 20 min at 121°C, cool
Add inactivated, sterile rabbit serum, aseptically to 10% final concentration
Store up to 1 year at room temperature

This recipe was modified by Faine in 1984 from the original formulation by Stuart (1946). The recipe given here is from Faine (1994).

Phosphate buffer

0.087 g KH_2PO_4
0.664 g Na_2HPO_4
Bring volume to 1 liter with glass-distilled, sterile water
Mix by stirring
Prepare fresh

Historically, a combination of sodium and potassium salts has been used to prepare this buffer, presumably to limit exposure of the bacteria to higher concentrations of either cation.

Semisolid Leptospira medium

Primary isolation, propagation, and freezing of *Leptospira* cultures are often done in semisolid media. Modify a liquid medium (e.g., EMJH or T80/40/LH medium, see recipes) by adding 1.5 g purified agar per liter of basal salt solution (see recipe)

continued

before sterilization. Cool basal salt solution to ~50°C before adding 100 ml complete BSA supplement (see recipe) per liter. The semisolid medium has properties similar to a suspended slurry, and can be stored in 1-liter bottles and dispensed as needed. Store up to 1 year at room temperature.

Solid Leptospira medium

Prepare solid agar plate medium by adding 8 g agar per liter EMJH or T80/40/LH medium (see recipes), before autoclaving the basal salt solution. Cool the autoclaved solution to ~50°C before adding 100 ml complete BSA supplement per liter, and then pour individual petri dishes with ~40 ml of medium. If possible, pour plates in a biological safety cabinet to minimize contamination. The plates are incubated for a long time, so potential sources of contamination must be kept to a minimum. Once plates have solidified, place them in the original petri dish bags and store, inverted, at 4°C until needed.

T80/40/LH medium (liquid medium)

To support the growth of more fastidious strains of *Leptospira*, Ellis and Thiermann (1986) modified the EMJH medium, resulting in the T80/40/LH medium. The essential changes to the EMJH medium are as follows.

Add the following to 100 ml complete BSA supplement (see recipe):

1 g lactalbumin hydrolysate
0.1 g superoxide dismutase
0.04 g sodium pyruvate

During preparation of the complete BSA supplement (see recipe), add:

0.1 ml manganese sulfate stock solution (0.3 g/100 ml) after the addition of zinc sulfate
9 ml Tween 80 per 100 ml of supplement
3.5 ml Tween 40 per 100 ml of supplement
Upon final assembly of the medium, add 4 to 10 ml fresh rabbit serum (heat inactivated 30 min at 56°C) per liter of complete BSA supplement.

For primary isolation of pathogenic Leptospira, suppression of contaminants is aided by the addition of 10 ml of 5-FU stock solution (5-FU; 1 g/100 ml) (optional) per liter.

COMMENTARY

Background Information

Pathogenic *Leptospira* are the causative agents of leptospirosis, one of the most common zoonotic diseases in the world. Over 100,000 leptospirosis cases occur annually worldwide. The mortality rate in humans infected with *Leptospira* is ~5% (WHO, 1999). In addition to the importance to human health, leptospirosis causes problems in the livestock industry, resulting in significant economic loss and creating a health hazard to animal care workers. The difficulty in identifying cases of leptospirosis has often resulted in an under estimation of the frequency of its occurrence. The symptoms of leptospirosis are diffuse. Often, patients have flu-like symptoms with variable severity. Leptospirosis can also manifest as a sudden onset of fever, headache, and muscle aches (Levett, 2001). In the more serious cases, death occurs from cardiac, renal, or hepatic failure (Faine et al., 1999). Antibiotic treatment can be successful, and in potential laboratory exposures, prophylactic antibiotic treatment is usually recommended.

Relatively few laboratories have successfully isolated *Leptospira* from clinical samples, yet successful culture of isolates is critical to determining the prevalence of different antigenic types (serovars) of pathogenic *Leptospira*. There are >200 recognized serovars of pathogenic *Leptospira* (Brenner et al., 1999), and each appears to form a close association with one mammalian host species (Faine et al., 1999). While a variety of techniques are available to detect leptospiral DNA or anti-leptospiral antibodies, these

methods lack sufficient specificity to accurately determine serovar prevalence in a given geographical location. Barriers to successful cultivation of these bacteria from clinical specimens include the requirement of specialized media for successful isolation and propagation, long incubation times, and the potential biohazard.

The growth characteristics of *Leptospira* are similar to other bacteria, i.e., inoculation of few cells in culture typically results in a pronounced lag phase, followed by logarithmic growth, reaching a plateau in the stationary phase. During logarithmic growth, the generation time for pathogenic *Leptospira* varies greatly depending on the growth medium and the strain used, but many strains double in number every 14 to 18 hr. The lag phase during primary isolation can be quite long, and in practice, propagation of active cultures in the laboratory typically uses larger inocula compared to many other bacterial species (Faine, 1994). Upon entry into the stationary phase, the cells can undergo considerable changes resulting in rapid degradation of genomic DNA and autolysis. Thus, it is critical that mid- to late-log phase cells be used for routine preparation of cellular material for analysis.

Three different methods can be used for measuring *Leptospira* growth in liquid cultures: (1) direct counting using a Petroff-Hauser chamber and a dark-field microscope; (2) determining the percent transmittance at 400 nm in a spectrophotometer; or (3) use of a nephelometer to measure changes in turbidity. Because pathogenic *Leptospira* are slender cells that do not often grow to high cell densities, growth measurement using a spectrophotometer is the least accurate of these methods.

Leptospira are typically observed using dark-field microscopy. These bacteria are too slender for resolution by light microscopy using wet mounts. In essence, the condenser of a dark-field microscope redirects the path of light so that it hits the bacteria from an oblique angle, thereby illuminating the cell. Thus, the bacteria appear light against a dark background. For additional information, a detailed description of the principles of dark-field microscopy can be found in Faine (1994; also see *UNIT 2A.1*).

Critical Parameters and Troubleshooting

Use of glass-distilled water that has been sterilized by autoclaving is essential for preparation of all media components that will be filter-sterilized during processing. This point cannot be emphasized enough because contamination by saprophytic *Leptospira* is a chronic problem when this step is ignored, and has led to mistaken diagnoses.

The primary source of difficulty in propagating pathogenic *Leptospira* is the medium, and typically, the source and quality of the BSA in the complete BSA supplement. Trial analysis of different lots of BSA is essential if isolation of bacteria from clinical material is attempted in the EMJH-base media. Contact the manufacturer and arrange to obtain samples from several different lots of BSA. Ensure that the manufacturer has sufficient material from each lot until the testing is completed. Test the strains that are most difficult to grow in the collection in media made with albumin supplements made from different lots or manufacturers. Successful and healthy growth of bacteria after several passages is seen as an indication that the BSA is suitable for use. Once the suitable lot is identified, acquire enough BSA to last so that it is not necessary to have to repeat this process.

Another potential problem in media preparation is the presence of inhibitory substances or antibodies in rabbit sera. This is a common problem when sera from only a few rabbits are used for growth media. Commercial availability of pooled rabbit sera is a suitable alternative to preparing in-laboratory sera for media preparation.

Anticipated Results

Pathogenic *Leptospira* do not grow to the same cell densities as many other bacteria. Under optimal conditions using laboratory adapted cultures, cell densities of $\sim 1 \times 10^9$/ml may be obtained. Clinical isolates typically reach saturation approximately one to two logs lower cell densities. Therefore, it is often necessary to use considerably more volume of *Leptospira* culture to obtain equivalent quantities of cells as compared to other bacteria.

Routine passage of *Leptospira* cultures in vitro in semisolid media (see Basic Protocol 1) will result in reliable growth rates and zones of growth (Dinger's disks) on average in 7 to 14 days with most strains.

Cultures of pathogenic *Leptospira* grown on solid media (see Basic Protocol 2) should result in the formation of small white colonies that appear partially embedded in the agar.

Growth of pathogenic *Leptospira* in liquid media (see Basic Protocol 3) should result in

cultures with cell densities of ~1–5 × 10^8 cells/ml at saturation. Extended incubation of liquid culture may result in lysed cells unless continuous passage is maintained. Transfer of a small volume of bacteria into a much larger volume, e.g., 10 ml into a 500-ml culture, may result in an extensive lag phase, or growth in the larger volume may not succeed.

Culture samples should be easily contained frozen in suitable cryovials for storage (see Basic Protocol 4), and cultures should retain viability for several decades. Pathogenic *Leptospira* cultures should recover from the frozen state and form Dinger's disks in a semisolid medium in 1 to 2 weeks (see Basic Protocol 5).

Primary isolation of pathogenic *Leptospira* from urine (see Basic Protocol 6) may take up to 6 months of incubation before growth is observed. Many clinical samples yield growth within 2 to 4 weeks, but some of the more fastidious strains take considerably longer to isolate. Although vaccination may not prevent infection of an animal, successful isolation of viable bacteria from vaccinated animals is more difficult than from unvaccinated animals.

Primary isolation of pathogenic *Leptospira* from tissues (see Basic Protocol 7) may take up to 6 months of incubation before growth is observed. Many clinical samples may yield growth within 2 to 4 weeks.

Well prepared samples will result in green fluorescent staining of *Leptospira* with an orange background (see Basic Protocol 8), as shown in Fig. 12E.1.2. Fragments of bacteria can cause difficulty in the interpretation of the sample.

Time Considerations

For the preparation of media, it is easiest to autoclave the glass-distilled water to be used for making filter-sterilized components the day before to allow it to cool. Likewise, suspension of BSA can be initiated the day prior to preparing media.

Culturing *Leptospira* requires a great deal of patience and several months may pass before clinical isolates grow.

Routine passage of pathogenic *Leptospira* in semisolid media (see Basic Protocol 1) takes ~20 to 30 min depending on the number of cultures. Culture transfers should be done in a biological safety cabinet, and thus some time should be allotted for preparing the work area (wiping down surfaces with 70% ethanol and UV irradiation of the cabinet) prior to and after culture transfer. The time between culture transfers varies greatly between different strains and can range from 7 days to ~6 months. Often, primary isolates grow slower than strains that have been maintained in vitro.

The preparation time to prepare and streak cultures of pathogenic *Leptospira* on solid media (see Basic Protocol 2) typically takes ~20 to 30 min. Sufficient time is needed to prepare the biological safety cabinet for transfers, and for cleaning up the work area. The length of time required for different strains to form colonies varies from ~3 to 6 weeks.

Routine culture of pathogenic *Leptospira* in liquid media (see Basic Protocol 3) requires ~20 to 30 min, depending on the number of strains to be inoculated. As noted above, sufficient time is needed to prepare the work area before transfers, and to decontaminate the area after finishing the culture work. The time between subcultures varies but most cultures can be transferred weekly. Expansion of cultures from small (~10 ml) volumes to preparative volumes (e.g., 500 ml) should be done gradually over a 1- to 2-week period.

Preparation and freezing of pathogenic *Leptospira* (see Basic Protocol 4) requires a time commitment of ~5 hr. Dispensing cultures into cryogenic tubes should take ~10 to 20 min but the cultures should be moved from −70°C to liquid nitrogen after ~4 hr to maintain viability. Recovery of frozen cultures of pathogenic *Leptospira* (see Basic Protocol 5) requires ~30 min to prepare samples and initiate growth.

Isolating pathogenic *Leptospira* from urine (see Basic Protocol 6) should take ~45 min. Routine examination of cultures should require ~10 to 20 min. Additional time will be required if collecting clinical samples prior to culturing.

Isolating pathogenic *Leptospira* from tissues (see Basic Protocol 7) should take ~45 min. Routine examination of cultures should require ~10 to 20 min. Additional time will be required if collecting clinical samples prior to culturing.

Fluorescent antibody staining of *Leptospira* requires ~3 to 4 hr. A 1- to 1.5-hr incubation period is included in this protocol, followed by ~20 min of processing. Careful examination of slides will take varying amounts of time depending on experience of the investigator and clarity of the samples.

Acknowledgement

This unit was prepared with the assistance of Richard Hornsby (National Animal Disease Center, U.S. Department of Agriculture, Ames, IA).

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Key References

Faine, S. 1994. See above.

Faine et al. 1999. See above.

These two books provide comprehensive coverage of the organism, metabolism, and the disease.

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Hamster Model of Leptospirosis

Animal inoculation remains essential for many aspects of leptospirosis research. Although pathogenic leptospiral strains may be able to infect a wide variety of animals, the Golden Syrian hamster is the preferred model because its susceptibility to infection and the reproducibility of the results. Common applications include determination of strain infectivity, restoring virulence to culture-attenuated strains, assessing usefulness of potential vaccines or diagnostic antigens, and examining pathology of mammalian infection. In many respects, acute leptospirosis in the hamster reproduces the severe form of human leptospirosis (see Commentary).

This unit provides a detailed description of the procedures involved in the hamster model of leptospirosis, including housing and handling of hamsters and intraperitoneal challenge (Basic Protocol 1), and monitoring response to challenge (Basic Protocols 2 and 3, and Alternate Protocol). Methods are provided for demonstrating infection, including culture isolation of leptospires from blood and tissues (Basic Protocol 2) and serologic studies and histopathology (Basic Protocol 3). Quantitative PCR is provided as an alternative detection method (Alternate Protocol). Although leptospires disseminate to many organs, the tissues of the liver and kidney are the primary targets of infection. The liver is heavily infected during the initial stage of hematogenous dissemination. Persistent infection occurs primarily in the kidneys of animals that survive acute disease. Infection of the renal tubules leads to shedding of infectious organisms in the urine, which is the primary mode of transmission in nature. A safety policy memorandum is provided (see Strategic Planning) that should be read and signed by all staff.

In the Commentary section, a detailed rationale is presented for use of hamsters as an animal model of leptospirosis. The effects of challenge dose on the hamster model, LD_{50}, in vitro passage of leptospiral strains, and immunological maturity are discussed. Alternative animal models are evaluated with reference to the concept of accidental versus reservoir hosts. The advantages and disadvantages of the hamster model are considered, with reference to use of alternative host animals to address specific research or vaccine-development issues.

CAUTION: Pathogenic *Leptospira* species are Biosafety Level 2 (BSL-2) pathogens. *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. borgpetersenii*, *L. santarosai*, and *L. weilii* are known to be pathogenic for humans. Certain strains of *L. inadai*, *L. meyeri*, *L. fainei*, and *L. alexanderi* may also be pathogenic. *L. biflexa*, *L. wolbachii*, and *L. parva* are thought to be nonpathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals. This experiment requires Animal Biosafety Level 2 (ABSL-2) conditions. Follow all appropriate guidelines for the use and handling of infected animals. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Fluids and tissues of infected animals are highly infectious. Numerous laboratory-associated infections, including deaths, have been reported (Miller et al., 1987; Pike, 1976). A safety policy memorandum is provided that should be signed by all staff (see Strategic Planning).

Contributed by David A. Haake
Current Protocols in Microbiology (2006) 12E.2.1-12E.2.13

STRATEGIC PLANNING

Successful challenge studies require the simultaneous availability of Golden Syrian hamsters and virulent leptospiral cultures. Hamsters typically require a 1-week quarantine after shipment and should be no more than 4 weeks old on the day of challenge. Virulent leptospires typically require at least 1 week of growth after inoculation of culture medium from frozen stocks. However, leptospiral growth varies depending on the strain and the quality of the medium (*UNIT 12E.1*).

Prior to initiating animal challenge studies, the principal investigator should ask all personnel to read and sign a safety policy memorandum stating that they understand and agree to the following:

a. Leptospirosis is a well-documented laboratory hazard. Virulent *Leptospira* may be present in urine, blood, and tissues of infected animals and humans. Ingestion, accidental parenteral inoculation, and direct and indirect contact of skin or mucous membranes with cultures or infected tissues or body fluids—especially urine—are the primary laboratory hazards.
b. All personnel must submit a baseline serum sample to employee health to test for leptospiral antibodies.
c. All personnel must wear a gown, eyeware, gloves, mask, hat, and shoe coverings while in any room containing infected animals.
d. In addition to standard microbiological safety practices, cultures and infected materials will be handled in a laminar flow biological safety cabinet that has been designated for use exclusively by the principal investigator and personnel in his or her laboratory. Infected materials will be disinfected with bleach or autoclaved before disposal.
e. In the event of an accidental percutaneous or mucous membrane exposure to an infected animal or its tissues, the principal investigator and the employee health department should be contacted immediately for consideration of antibiotic prophylaxis with amoxicillin (500 mg, orally, three times daily) or doxycycline (100 mg, orally, twice daily) for 7 days.

BASIC PROTOCOL 1

INTRAPERITONEAL CHALLENGE OF HAMSTERS WITH *LEPTOSPIRA*

Intraperitoneal inoculation is the standard route for challenging animals with pathogenic leptospires. The primary advantage of intraperitoneal inoculation is the ease of delivering a reproducible volume of inoculum. A number of other, more biologically relevant inoculation routes have been reported, including subcutaneous (Truccolo et al., 2002) and conjunctival (Bolin and Alt, 2001) challenge models. These alternative routes more accurately reproduce aspects of natural transmission, which may be important in vaccine studies, but are less well studied than the intraperitoneal challenge route and may be more difficult to reproduce.

CAUTION: Wear appropriate personal protective equipment (see Strategic Planning) during handling of animals. Personnel should be trained in proper handling of hamsters. Golden Syrian hamsters are generally docile but may scratch or bite if startled. To avoid injury, some researchers prefer to handle hamsters with tongs and/or chain mail gloves (optional). These safety precautions are usually not necessary if researchers reach into the cage slowly and exercise patience. See Donovan and Brown (2006a) for additional instruction in handling and restraining hamsters.

Materials

3- to 4-week-old Golden Syrian hamsters (Harlan Bioproducts for Science)

Log-phase *Leptospira* culture for challenge (*UNIT 12E.1*): low-passage, virulent strain (e.g., *L. interrogans* serovar Copenhageni strain L1-130 or *L. kirschneri* serovar Grippotyphosa strain RM52)

EMJH liquid medium (see recipe)

Hamster cages, solid-bottom, with filter top to prevent escape of contaminated bedding (e.g., Ancare; *http://www.ancare.com*)

Hamster bedding (Sani-Chips; P.J. Murphy Forest Products, *http://www.pjmurphy.net/*)

Dark-field microscope (*UNIT 2A.1*)

Petroff-Hausser counting chamber or equivalent (also see *APPENDIX 4A*)

1-ml syringes

22-G needles

Additional reagents and equipment for intraperitoneal injection (Donovan and Brown, 2006b)

1. House hamsters in a room separated from the corridor by an anteroom for donning and discarding of personal protective equipment (see Strategic Planning).

   CAUTION: *Because infected animals shed virulent leptospires in their urine, the bedding and entire contents of the cage should be considered contaminated after challenge. Cages should be fitted with a filter top or other protective covering to prevent bedding from escaping as animals move about the cage.*

   To prevent cross-contamination of cages during bedding changes, animals should be moved directly to new cages; a transfer cage should not be used. Double gloves should be worn during handling of infected animals to allow replacement of the outer glove between cages. If forceps are used to transfer animals, the instrument must be disinfected between cages. The author disinfects by dipping the forceps in Clidox-S (Indulab, http://www.indulab.ch), prepared by combining 1 part activator with 18 parts water and 1 part Base).

   Consider ordering litters of 2- to 3-week-old weanling hamsters to allow time for a ≥1-week quarantine before use, so that animals are less than 4 weeks of age on the day of challenge.

2. Determine the density of the leptospiral challenge strain by dark-field microscopy using a Petroff-Hausser or similar counting chamber (also see *APPENDIX 4A*).

   The leptospiral challenge strain should be in the log phase of growth (density >10^8 cells/ml) and should have been passaged less than ten times in vitro after isolation from a mammalian host. See UNIT 12E.1 for Leptospira storage and maintenance.

3. Prepare serial 10-fold dilutions in liquid EMJH medium to yield concentrations ranging from 10^9 to 10^0 cells/ml.

4. In a biological safety cabinet, inject each designated hamster intraperitoneally (Donovan and Brown, 2006b) with 1.0 ml of the appropriate dilution using a 1-ml syringe and 22-G needle. Use three hamsters per dilution.

   CAUTION: After inoculation, syringes should be discarded in a sharps disposal container without recapping the needle, since most needle-puncture injuries occur during recapping.

   Intraperitoneal injection of hamsters with liquid EMJH containing BSA or rabbit serum does not appear to interfere with these experiments.

5. Examine animals at least twice per day after challenge.

   There may be a relatively short interval (several hours) between onset of symptoms and death. Typically, animals demonstrate decreased activity, assume a hunched posture, and have ruffled fur. Appropriate endpoint criteria include loss of interest in food or water and weight loss of >10% of expected weight. Animals that survive acute infection should be euthanized 21 to 28 days after challenge to collect tissues (Basic Protocol 2) to determine whether sublethal infection is present.

HARVESTING BLOOD AND TISSUES FOR ASSESSMENT OF *LEPTOSPIRA* INFECTION

At the desired time following challenge, the hamsters are anesthetized with isoflurane and blood and tissues are harvested; this is a terminal surgical procedure. Inhaled anesthesia is required to ensure that animals are unconscious during the surgical procedure.

CAUTION: Use of volatile anesthetics, such as isoflurane, requires a well ventilated area and/or an appropriate scavenging system.

Materials

Hamsters infected with *Leptospira* (see Basic Protocol 1)
Isoflurane
Small, sealable container (e.g., Tupperware) to accommodate hamster for anesthesia
Gauze or cotton sponges
50-ml conical polypropylene centrifuge tubes
Dissecting equipment: two sets of dissecting scissors, tweezers, and scalpel
Additional reagents and equipment for blood collection by cardiac puncture (Donovan and Brown, 2006c)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

1. Place animal in a small container (Tupperware works well) containing gauze or cotton sponges soaked with isoflurane to induce anesthesia.
2. When the animal's breathing rate has slowed to less than one breath per 3 sec, remove the animal from the container and place the open end of a 50-ml centrifuge tube containing gauze or cotton sponges soaked with isoflurane over the nose of the animal to maintain anesthesia throughout the procedure. Ensure a snug fit by wrapping gauze around the animal's nose.

   CAUTION: Surgery on infected animals should be performed inside a biological safety cabinet to prevent exposure to infectious material.

   The anesthesia should be deep enough that the animal does not respond to toe pinch but not so deep that the heart stops. Optimal blood collection is performed when the heart is still beating.

3. With animal placed on its back, open the skin via a vertical midline incision with one set of dissection scissors and tweezers.
4. To minimize risk of contaminating specimens with the animal's skin flora, retract the skin and use a second set of dissection scissors, tweezers, and scalpel to open the peritoneal and chest cavities.

   Opening of the chest cavity exposes the heart and lungs, preventing the animal from breathing and regaining consciousness.

5. Obtain blood for culture and serology by cardiac puncture (Donovan and Brown, 2006c).
6. Obtain liver and kidney tissue for culture and histopathology.

   These tissues contain the highest densities of organisms.

   The liver is the dark red multilobed organ in the right upper quadrant of the peritoneal cavity. Note any areas of hemorrhage. Using scissors or scalpels, obtain 1-cm × 1-cm segments of liver for culture and histopathology. To avoid crush artifacts, grasp only the margin of the organ segment with tweezers rather than the body of the organ.

Hamster kidneys are normally the size, color, and shape of kidney beans. The kidneys are located on the left and right posterior walls of the peritoneal cavity and are visualized by moving the loops of intestine to one side or the other. Note any areas of hemorrhage or heterogeneity. Cut the vascular structures with scissors. To avoid crush artifacts, remove the organ by grasping only the hilum with tweezers, rather than the organ itself. One kidney should be cultured and the other submitted for histopathology.

7. Consider collecting other tissues (lung or spleen) depending on the goals of the study.

   The lungs are found in the chest cavities and appear as collapsed white structures after the chest cavity has been opened. Note any areas of hemorrhage. Remove the lungs by cutting the hilum with scissors. Examination of the lung is of interest because pulmonary hemorrhage is an important cause of morbidity and mortality in human disease.

   The spleen is a dark, red, ribbon-like structure in the left, upper peritoneal cavity and is visualized by moving the loops of intestine to the hamster's right.

DETECTION OF LEPTOSPIRAL INFECTION

BASIC PROTOCOL 3

Three following three methods should be carried out in parallel to assess leptospiral infection.

Materials

Blood and organs from *Leptospira*-infected hamster (and blood from uninfected hamster as control in serological procedure)
Semisolid *Leptospira* medium with 5-fluorouracil (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Culture of late logarithmic growth–phase *Leptospira* (*UNIT 12E.1*)
Neutral buffered formalin (e.g., Fisher)
70% (v/v) ethanol
Steiner-Steiner silver staining kit (optional; e.g., Sigma)
Stomacher bags (e.g., Thomas Scientific)
Microscope with dark-field optics (*UNIT 2A.1*)
Dedicated histopathology facility (optional)
Additional reagents and equipment for paraffin-embedding and sectioning of tissues (Zeller, 1989), hematoxylin/eosin staining (Zeller and Rogers, 1993), and Steiner-Steiner silver staining (Steiner and Steiner, 1944; reagents available as kit from Sigma and other histology suppliers)

NOTE: All reagents and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

To assess infection by standard bacteriological methods

1a. Inoculate blood into semisolid *Leptospira* medium with 5-fluorouracil at dilutions of 1/100 (by adding 0.1 ml blood to 10 ml semisolid medium) and 1/1000 (by adding 1.0 ml of the 1/100 dilution to 10 ml semisolid medium).

   5-fluorouracil is added to this medium to prevent overgrowth of contaminants (Faine et al., 1999).

2a. Inoculate tissue sample as follows. Place a 1 × 1–cm segment of liver tissue or one kidney in 1 ml EMJH liquid medium in a sterile Stomacher bag. Homogenize, then inoculate into semisolid medium with s-fluorouracil at dilutions of 1/100 (by adding 0.1 ml homogenized tissue to 10 ml semisolid medium) and 1/1000 (by adding 1.0 ml of the 1/100 dilution to 10 ml semisolid medium).

A simple way to homogenize tissue is to place the tissue and medium in a Stomacher bag, fold over the open end of the bag, and role a 100-ml cylindrical flask over the bag, forcing the contents between the flask and the hard surface of the counter top inside the biological safety cabinet.

3a. Examine a sample from each culture biweekly by dark-field microscopy for growth of leptospires.

To assess infection by serological examination

The microscopic agglutination test (MAT) described in steps 1b to 4b is a sensitive serological method of detecting exposure to Leptospira species (Faine et al., 1999).

1b. Prepare serum from the blood of the infected hamster by placing the blood in a sterile microcentrifuge tube, incubating 1 hr at 37°C, microcentrifuging 5 min at maximum speed, and transferring the medium to a separate tube. Similarly prepare serum from the blood of an uninfected hamster as control. Prepare serial two-fold dilutions of the serum in PBS.

2b. Mix each dilution with an equal volume of late log-phase *Leptospira* culture, then incubate 2 to 4 hr at room temperature.

3b. Examine each culture by dark-field microscopy for agglutination.

4b. Determine the titer endpoint, which is defined as the highest dilution resulting in 50% agglutination, measured by comparison with the density of leptospires incubated with negative control sera from uninfected hamsters (no agglutination).

Formation of agglutinating antibody indicates infection with leptospires. 50% agglutination is determined by comparing the density of organisms in the control sample (serum from uninfected hamster) versus the density of the organism in the test sample. Density of the leptospires is determined as described in UNIT 12E.1.

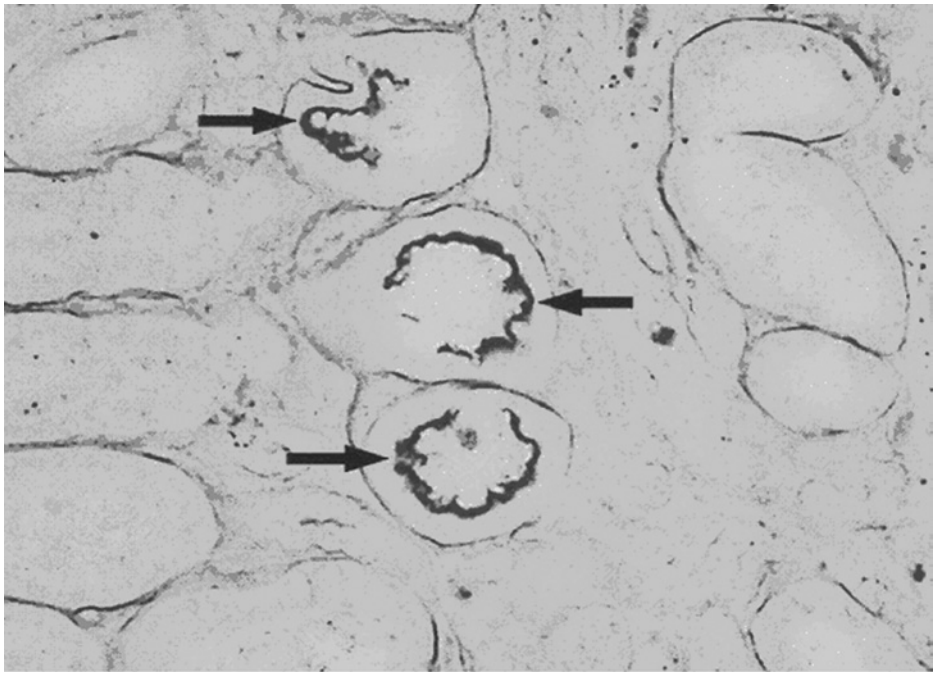

Figure 12E.2.1 Silver stain of hamster kidney tissue obtained 28 days after challenge. Arrows show positively-staining aggregates of leptospires lining the epithelial surface of several adjacent renal tubules. For the color version of this figure go to *http://www.currentprotocols.com.*

To assess infection by histopathology

1c. Fix a 1 × 1–cm square segment of liver tissue or one kidney in neutral buffered formalin for 8 hr, then transfer to 70% ethanol.

2c. Embed in paraffin and cut 4-μm sections according to standard procedures (e.g., Zeller, 1989).

The authors submit fixed tissues to a commercial pathology laboratory for embedding, sectioning, and staining.

3c. Stain sections with hematoxylin and eosin (Zeller and Rogers, 1993) to characterize the histopathology of the organs. Perform silver staining using the technique of Steiner and Steiner (1944) to detect organisms.

Steiner-Steiner silver staining kits are available from Sigma and other suppliers.

4c. Have the sections analyzed by a person trained in histopathology to identify the organisms and characterize the extent and severity of lesions, including assessment and grading of the location, number, and types of inflammatory cells present.

Figure 12E.2.1 shows a silver stain of hamster kidney tissue obtained 28 days after challenge showing leptospires (arrows) lining the epithelial surface of several adjacent renal tubules.

QUANTIFICATION OF INFECTION LEVELS USING REAL-TIME PCR

ALTERNATE PROTOCOL

Culture, serology, and histopathology are sensitive methods of detecting sublethal leptospiral infection and are appropriate for most applications, including vaccine protection studies. However, these methods are not quantitative. For some types of research, such as antibiotic prophylaxis and treatment studies, a quantitative measure of leptospiral tissue burden is desirable. Amplification of a 87-bp region of the leptospiral 16S gene by quantitative (TaqMan) PCR has been shown to have a mean detection limit of two cells (Smythe et al., 2002).

Amplification and fluorescence detection is performed in an ABI Prism 7700 sequence detector. Use of other equipment may require use of different amplification conditions which should be determined empirically.

Additional Materials *(also see Basic Protocols 1 and 2)*

QIAamp Blood Kit (Qiagen)
DNeasy Tissue Kit (Qiagen)
TaqMan Universal PCR Master Mix (Applied Biosystems)
PCR primers:
  Lepto F (5′-CCCGCGTCCGATTAG-3′)
  Lepto R (5′-TCCATTGTGGCCGRACAC-3′)
TaqMan probe (Applied Biosystems):
  [5′ (FAM)-CTCACCAAGGCGACGATCGGTAGC-(TAMRA) 3′]
Negative control: genomic DNA (Wilson, 1997) extracted from the nonpathogen *L. biflexa* (ATCC # 23582)
Positive control dilution series: genomic DNA (Wilson, 1997) extracted from serial dilutions containing 10^8 to 10^0 cells/ml of the *Leptospira* challenge strain
TE buffer
96-well PCR plate
ABI Prism 7700 Sequence Detection System (Applied Biosystems) with dedicated real-time PCR software

1. Challenge mice with *Leptospira* (Basic Protocol 1) and harvest blood and tissues (see Basic Protocol 2).

2. Extract total DNA from the blood and tissues of challenged hamsters using the QIAamp Blood Kit (Qiagen) and the DNeasy Tissue Kit (Qiagen), respectively.

3. For each sample DNA, negative control DNA, and positive control DNA dilution, prepare a PCR reaction mix (total volume, 50 µl; prepare all samples and controls in duplicate) in a PCR plate well as follows:

   a. First prepare 45 µl TaqMan Universal PCR Master Mix containing:
      3 pmol/µl Lepto F PCR primer
      3 pmol/µl Lepto R PCR primer
      2 pmol/µl TaqMan probe
   b. Add 5 µl template DNA—i.e., sample DNA, negative control (*L. biflexa*) DNA, or positive control DNA dilution—to the corresponding wells.
   c. Add 5 µl TE buffer to the designated wells in place of the DNA for a "no-template" negative control.

4. Conduct amplification and fluorescence detection in the ABI Prism 7700 dedicated thermal cycler using the following program, collecting the data throughout:

40 cycles:	15 sec	95°C
	1 min	60°C.

5. Using the dedicated real-time PCR software supplied with the ABI Prism 7700 Sequence Detection System, quantify the leptospiral DNA in the samples using the positive control dilution series of DNA from the *Leptospira* challenge strain as a standard curve.

UNIT 1D.3 provides more detail on the theory and practice of real-time PCR.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BSA stock solution

Weigh out 10 g bovine serum albumin, fraction V, and add to 50 ml sterile, distilled water with constant stirring. Stir slowly to prevent foaming. If necessary, gently heat (<50°C). Once the BSA is dissolved, use immediately to prepare the complete BSA supplement (see recipe).

It is often convenient to start dissolving the BSA in the afternoon, then allow the solution to set overnight at 4°C.

The quality of BSA for use in media is important and must be determined empirically (see UNIT 12E.1).

Several vendors supply satisfactory BSA, fraction V, for use in media preparation. Before purchasing a large amount of BSA from an individual vendor, it is critical to test it by preparing the complete BSA supplement and using it to prepare the medium for a pilot experiment.

Complete BSA supplement

To 50 ml BSA stock solution (see recipe), add the following stock solutions with constant stirring in the following order:

1 ml calcium chloride stock solution (1 g $CaCl_2 \cdot 2H_2O$/100 ml)
1 ml magnesium chloride stock solution (1 g $MgCl_2 \cdot 2H_2O$/100 ml)
1 ml zinc sulfate stock solution (0.4 g $ZnSO_4 \cdot 7H_2O_7$/100 ml)
0.1 ml copper sulfate stock solution (0.3 g $CuSO_4 \cdot 5H_2O$/100 ml)
10 ml ferrous sulfate stock solution (0.5 g $FeSO_4 \cdot 7H_2O$/100 ml)

1 ml vitamin B_{12} stock solution (0.02 g/100 ml)
12.5 ml Tween 80 stock solution (10 g/100 ml)
Adjust pH to 7.4 with 2 N NaOH (~0.4 ml)
Bring the final volume to 100 ml with H_2O
Sterilize by filtration through a 0.22-μm filter
Store indefinitely at –20°C

Although the final solution is filter sterilized, it is critically important that water for all stock solutions be sterilized by autoclaving prior to their preparation to eliminate potential contamination by saprophytic Leptospira.

Leptospira require long-chain fatty acids or alcohols (provided by the Tween 20 in the above mixture) for growth, which can be toxic for growth in prepared media. Addition of albumin or animal serum can bind excess fatty acids, to enable growth of these bacteria in vitro.

EMJH basal salt solution

1 g Na_2HPO_4
0.3 g KH_2PO_4
1 g NaCl
1 ml ammonium chloride stock solution (25 g NH_4Cl/100 ml)
1 ml thiamine stock solution (0.5 g thiamine/100 ml)
1 ml glycerol stock solution (10 g glycerol/100 ml)
Add components to ~800 ml glass-distilled water with stirring
Adjust pH to 7.4 with dilute NaOH or diluted HCl
Bring volume to 1 liter with glass-distilled water
Sterilize by autoclaving
Store up to 1 month at 4°C

Volumes given are for completing a 1 liter solution.

EMJH liquid medium

Aseptically add 100 ml complete BSA supplement (see recipe) and 10 ml of 0.01 g/ml 5- fluorouracil (5-FU) to 890 ml EMJH basal salt solution (see recipe). Divide into aliquots as needed for experiments. Store up to 1 month at room temperature.

This medium is quite stable for routine propagation of cultures. However, for isolation from clinical samples, fresh medium should be prepared monthly.

Semisolid Leptospira medium with 5-fluorouracil

Add 1.5 g agar to 890 ml basal salt solution (see recipe) and autoclave. Cool to ~50°C, then add 100 ml sterile complete BSA supplement (see recipe) and 10 ml of 0.01 g/ml 5-fluorouracil (5-FU), both sterilized by filtration through a 0.22-μm filter. Store in 1-liter bottles up to 1 year at room temperature.

Primary isolation, propagation, and freezing of Leptospira cultures are often done in semisolid medium. The semisolid medium has properties similar to a suspended slurry, and can be stored in 1-liter bottles and dispensed as needed. The semisolid medium prepared in this recipe is a modified EMJH medium; by addition of the appropriate supplements, a modified, semisolid T80/40/LH medium (UNIT 12E.1) may also be prepared.

COMMENTARY

Background Information

The Golden Syrian hamster model of leptospirosis is well characterized and has a number of important advantages, including excellent reproducibility and susceptibility to a broad range of pathogenic strains (Stavitsky, 1945; Ferguson and Hamdy, 1957; Miller and Wilson, 1966; van den Ingh and Hartman, 1986). The susceptibility of hamsters to leptospiral infection may be related to the fact that these animals evolved in a dry desert habitat where they would rarely have been exposed

to the moist climatic conditions conducive to transmission of leptospirosis. The fulminant, disseminated infection seen in hamsters is typical of the accidental host pattern of disease. As such, the hamster model is an appropriate model of the severe form of human leptospirosis.

Guinea pigs (Stavitsky, 1945; Faine, 1957a,b; Nally et al., 2004) and gerbils (Lewis and Gray, 1961) have also been found to be suitable animal models of fulminant-type accidental infection. The guidelines presented here are generally applicable to these alternative models. By contrast, rats and mice are reservoir or maintenance hosts in nature. Once rats and mice become immunologically mature, they are typically resistant to experimental leptospiral challenge, but may develop a sublethal infection of the renal tubules (Faine, 1962). Very young, C3H/HeJ mice can develop disseminated infection (Pereira et al., 1998). However, mice have an extremely short window of susceptibility, which can be a technical or logistical hurdle to overcome. Development of a mouse-lethal leptospiral strain by repeatedly challenging and reisolating the organism from the blood stream of mice has been reported (Koizumi and Watanabe, 2004). Leptospiral vaccine studies have been described using a mouse-adapted challenge strain obtained after more than ten passages in C3H/HeJ mice (Koizumi and Watanabe, 2004). Leptospiral vaccine studies have been described using a mouse-adapted challenge strain obtained after more than ten passages in C3H/HeJ mice (Koizumi and Watanabe, 2004).

The hamster model is not without some disadvantages. Hamsters are not allowed in certain parts of the world, such as Australia. Consequently, much of the early work by Solly Faine and colleagues was done in guinea pigs (Faine, 1957a,b) and mice (Faine, 1962). Larger animals such as dogs may be more appropriate for studies examining renal physiology of interstitial nephritis due to leptospirosis. Vaccines developed in hamsters must be validated in the target animal. Relatively few hamster-specific reagents are commercially available, making it difficult to examine the immunologic and cytokine response to infection. Likewise, hamster microarrays are not available to examine the transcriptional response of host tissues to leptospiral infection. Ultimately, gene knockouts that render mice predictably susceptible to leptospiral infection or, better yet, mice with critical genes replaced by human counterparts may become better models of human infection.

Critical Parameters

Based on animal husbandry issues and the reproducibility of the model, hamsters are the animal model of choice for leptospiral vaccine

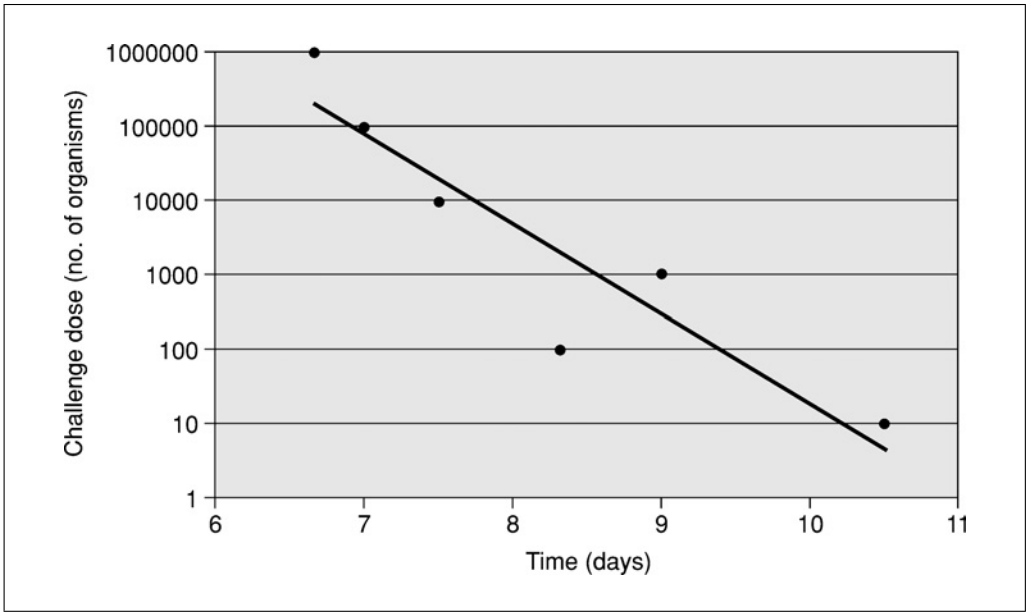

Figure 12E.2.2 Time from challenge to 50% mortality. 3-week-old Golden Syrian hamsters in groups of four were challenged by intraperitoneal inoculation with serial 10-fold dilutions of *Leptospira kirschneri* serovar Grippotyphosa, strain RM52. Each dose was administered to hamsters in groups of four. Each point represents the time to death of the second of four hamsters. The LD_{50} of this strain is <10 organisms.

and antibiotic treatment studies. As shown in Figure 12E.2.2, the correlation between the time from challenge to 50% survival and the challenge dose is remarkably linear. When establishing the hamster model using a new challenge strain, it is essential to determine its LD_{50} by serial dilution. In organisms that have been passaged in vitro less than ten times after isolation from an animal host, the LD_{50} for intraperitoneal inoculation of 3- to 4-week-old hamsters may approximate one organism.

Troubleshooting

Leptospires rapidly lose virulence during in vitro cultivation. Loss of the ability to produce lethal infection in hamsters can occur in as few as ten passages in culture medium. Stocks of primary cultures isolated from animals should be stored in liquid nitrogen, as described in *UNIT 12E.1*, and thawed out when needed for animal-challenge studies. Culture attenuation can also be minimized by passaging cultures frequently enough to avoid onset of stationary phase. More highly passaged strains and strains of unknown passage number may be reisolated from hamsters to obtain a challenge strain with improved virulence.

Anticipated Results

Lethal infection may occur in <100% of hamsters that are older than 4 weeks (immunologically more mature) or that have been immunized as part of a vaccine trial. Paradoxically, increased survival can be observed when older hamsters (>4 weeks) are challenged with higher doses of certain strains (Barnett et al., 1999). Animals that survive challenge may have sublethal infection, particularly involving the kidneys (Barnett et al., 1999; Haake et al., 1999). When carried out correctly, there is generally an excellent correlation between culture isolation, serology, and histopathology for detection of sublethal infection.

Another benefit of hamsters is that an alternative, and possibly more biologically relevant, subcutaneous challenge route has been demonstrated (Trucollo et al., 2002).

Time Considerations

Successful challenge studies require coordinating the simultaneous availability of Golden Syrian hamsters and virulent leptospiral cultures. If hamsters are being shipped from another institution, contact the provider weeks to months in advance to ensure that sufficient numbers of animals of the proper age and sex will be available for shipping on the desired date. After arrival, hamsters typically require a 1-week quarantine period for acclimatization and screening for infection. Virulent leptospires typically require at least 1 week of growth after inoculation of culture medium from frozen stocks. However, leptospiral growth rates vary depending on the strain and the quality of the medium (see *UNIT 12E.1* for details).

In experienced hands, intraperitoneal inoculation of hamsters (see Basic Protocol 1) takes 5 min per animal. Putting on personal protective equipment (gown, gloves, eyeware, mask, cap, and shoe covering) and preparing the work area requires 20 to 30 min. Another 20 to 30 min are required after the inoculations are completed to decontaminate the work area, discard syringes, and remove personal protective equipment. Animals should be checked 1 hr after intraperitoneal inoculation to ensure that no complications have ensued. Checking the health of animals on the day of inoculation and on subsequent days typically requires less than 1 min per cage.

Harvesting blood and tissues from hamsters (see Basic Protocol 2) takes 15 min per animal. Depending on strain virulence and challenge dose, hamsters may reach endpoint criteria as early as 3 days post challenge or as late as 21 days post challenge. Blood and tissue are immediately inoculated into semisolid culture medium (see Basic Protocol 3), which takes 5 min per specimen. Blood not used for culture takes 1 hr to clot, after which 5 min are required for centrifugation, separation of serum from cells, and storage. Cultures of primary isolates should be examined weekly and may take weeks to months to become positive. Dark-field microscopy of cultures takes 5 min per sample. The microscopic agglutination test of serum from hamsters requires 20 to 30 min to set up, followed by 2 to 4 hr of incubation time and 20 to 30 min to read the results by dark-field microscopy, depending on the number of samples. Examination of the histopathology slides takes 30 to 45 min, depending on the number of slides and the experience of the observer.

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Printed version of important safety document, also available online (see Internet Resources).

Internet Resources

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Web site for the guide to Biosafety in Microbiological and Biomedical Laboratories, 4th Edition (Richmond and McKinney, 1999; see Key References).

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Genetic Manipulation of *Leptospira biflexa*

The genus *Leptospira* belongs to the order *Spirochaetales* and is composed of both saprophytic and pathogenic members, such as *Leptospira biflexa* and *L. interrogans*, respectively. Both saprophytic and pathogenic *Leptospira* have been classified into serovars (more than 220 serovars are recognized as pathogens; Levett, 2001) which are defined by agglutination cross-absorption, and more recently have been classified into 17 species based on DNA-DNA hybridization studies. Knowledge of the genetics of *Leptospira* remains at a very early stage in comparison to that of other bacterial species. The lack of genetic tools in pathogenic *Leptospira* does not allow for the full-characterization of genes of interest. Only recently has the first evidence of gene transfer been demonstrated in the pathogen *L. interrogans* by random transposition of *Himar1*, a transposon of eukaryotic origin (Chiang and Rubin, 2002). In contrast, numerous tools for genetic manipulation of saprophytic *Leptospira* have been developed in the authors' laboratory, as will be described in this unit. These studies enable the use of *L. biflexa* as a model bacterium. Among spirochetes, *L. biflexa* is a relatively fast-growing, aerobic bacterium: 1 week is necessary to obtain colonies on solid medium. It could therefore allow the functional analysis of genes from slow- or noncultivable spirochete members.

In this section, several approaches are described that are useful for genetic manipulation of the saprophyte *L. biflexa*. Basic Protocol 1 describes the preparation of electrocompetent cells of *L. biflexa* and the procedure for transforming them with plasmid DNA. *L. biflexa* can be transformed at a high rate using a replicative plasmid containing a kanamycin- or spectinomycin-resistance cassette as a selectable marker (see Basic Protocol 2). Replicative plasmids are suitable for heterologous gene expression and complementation studies. Targeted mutagenesis can be achieved in *L. biflexa* by using a suicide plasmid delivering the inactivated allele in the targeted chromosomal gene (see Basic Protocol 3). Use of the counter-selectable marker *rpsL* can improve the recovery of allelic exchange (see Basic Protocol 4). Finally, the authors recently developed an in vivo transposon mutagenesis system in *L. biflexa* that could allow large scale mutagenesis studies (see Basic Protocol 5).

CAUTION: Leptospira biflexa is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

IMPORTANT NOTE: Since penicillin is the recommended treatment against leptospirosis, antibiotic resistance cassettes such as ampicillin should not be used.

PREPARATION AND TRANSFORMATION OF ELECTROCOMPETENT *LEPTOSPIRA BIFLEXA*

BASIC PROTOCOL 1

This protocol describes the preparation of electrocompetent cells of *L. biflexa* and how to transform them with plasmid DNA. Replicative plasmids are suitable for heterologous gene expression and complementation (see Basic Protocol 2), while suicide plasmids, i.e., plasmids that are not replicative in *L. biflexa*, are currently used to perform allelic exchange (see Basic Protocol 3) and random transposon mutagenesis (see Basic Protocol 5). Briefly, *L. biflexa* is grown to exponential phase, and the cells are then washed

Contributed by Hélène Louvel and Mathieu Picardeau
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and concentrated in water. A mixture of cells and DNA are electroporated, allowing the DNA to enter into the cells. Finally, transformants are grown and then plated onto antibiotic-containing (selective) medium.

Materials

L. biflexa serovar Patoc strain Patoc1 (National Reference Center for Leptospira, Institut Pasteur, Paris, France)
EMJH liquid medium (see recipe)
Sterile water (B. Braun)
Plasmid DNA (see Basic Protocols 2, 3, 4, and 5; Fig. 12E.4.1), salt-free
EMJH agar plates supplemented with the appropriate antibiotic (see recipe)

30°C shaker and incubator
50-ml polypropylene centrifuge tubes (Corning)
0.2-cm electroporation cuvettes (Bio-Rad), chilled
Electroporator (Bio-Rad, Gene Pulser)
15-ml polypropylene tubes (Falcon), sterile

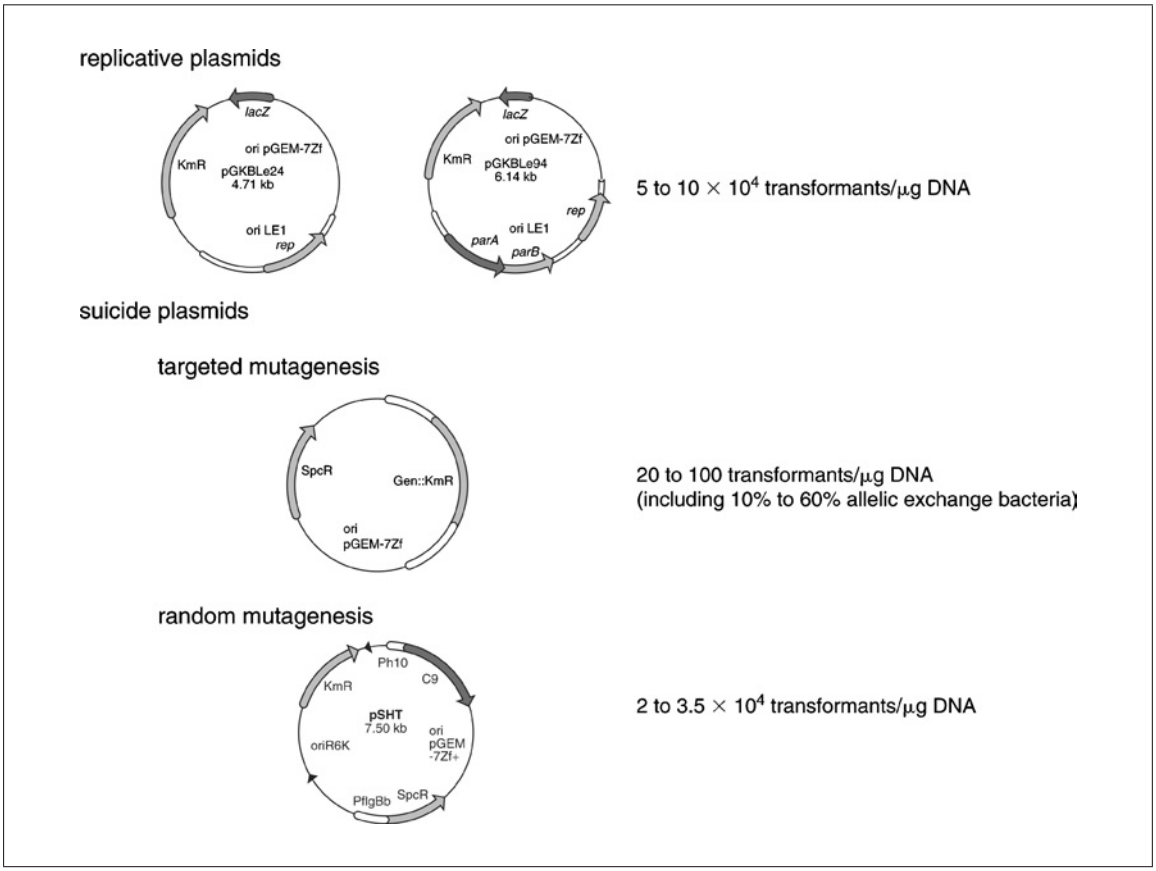

Figure 12E.4.1 Schematic representation of some plasmid vectors used for the transformation of *L. biflexa*. C9, hyperactive transposase; Ph10, *L. interrogans hsp10* promoter; PflgBb, *B. burgdorferi flgB* promoter; KmR, kanamycin-resistance cassette; SpcR, spectinomycin-resistance cassette. Small arrowheads on pSHT represent the borders of the transposon. A suicide vector used to inactivate a *L. biflexa* gene is represented (white bars flanking KmR indicate the sequences of the targeted gene used for homologous recombination). Expected transformation efficiencies are indicated. Plasmids can be requested from the authors (mpicard@pasteur.fr).

Prepare electrocompetent cells

1. Inoculate 1 ml exponential-phase culture of *L. biflexa* into 100 ml EMJH medium. Incubate at 30°C until the culture reaches an OD_{420} of 0.3 to 0.4 (6 to 9 × 10^8 bacteria/ml).

   At 30°C with shaking (80 rpm), the cells in the 100 ml EMJH medium may reach the exponential phase (OD_{420} of 0.1 to 0.4) in 3 to 4 days. Harvesting cells at other stages (early exponential phase or stationary phase) results in reduced transformation efficiency. A 100-ml culture should be enough for ~10 electrotransformations.

2. Transfer the culture to 50-ml centrifuge tubes. Centrifuge 20 min at 4000 × *g*, room temperature. Aspirate and pour off the supernatant.

   Because Leptospira are sensitive to cold temperatures (i.e., <~10°C), cells should be kept at room temperature for all subsequent steps.

3. Add an identical volume of sterile water to the cell pellet and gently resuspend cells (do not vortex). Centrifuge tubes again as in step 2.

   Vortexing may damage the leptospiral cells.

4. Resuspend cells in 1 ml sterile water (~10^{11} bacteria/ml)

   Competent cells cannot be stored in glycerol at −80°C; therefore, electrotransformation must be undertaken with freshly prepared competent cells.

Introduce plasmid DNA by electroporation

5. Chill the desired number of 0.2-cm electroporation cuvettes. Turn on the electroporator and set to 1.8 kV, 25 μF, and 200 Ω.

   Electroporation cuvettes should be stored at −20°C.

6. In 15-ml polypropylene microcentrifuge tubes, mix 10 to 100 ng salt-free plasmid DNA with 100 μl competent cells (step 4). Transfer into the prechilled electroporation cuvettes.

   Include controls such as competent cells without plasmid DNA. Test different DNA concentrations to evaluate the best transformation efficiency. The volume of DNA added to the cells should be kept as small as possible.

7. Introduce the DNA into the cells by electroporation.

   See Basic Protocols 2, 3, 4, and 5 for the different plasmids that can be used for electroporation (Fig. 12E.4.1). Electroporation may result in a time constant of ~5 msec.

8. Immediately after electroporation, add 1 ml EMJH medium to each cuvette using a Pasteur pipet. Transfer the electroporation mix to a 15-ml sterile tube and incubate with shaking at 30°C overnight to allow expression of the antibiotic resistance gene.

Analyze transformants

9. Following overnight growth, spread 10 to 250 μl of the cells on EMJH plates supplemented with the appropriate selective marker. Incubate the plates at 30°C for one week.

   The spectinomycin- and kanamycin-resistance cassettes are the only two selective markers currently known to be functional in Leptospira species. Depending of the selective marker carried by the plasmid vector (Fig. 12E.4.1), EMJH plates should be supplemented with 40 μg/ml kanamycin or 40 μg/ml spectinomycin. At 30°C, colonies may take 5 to 10 days to come up on EMJH medium. Plates should be sealed with Parafilm to avoid desiccation. Not all colonies appear simultaneously, suggesting that several maturation stages may exist (Fig. 12E.4.2A).

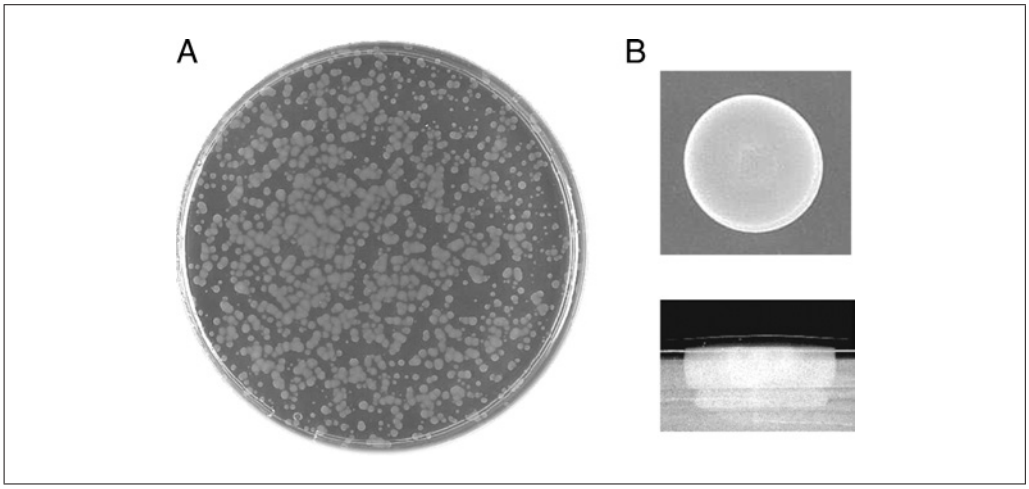

Figure 12E.4.2 Colony morphology of *L. biflexa*. (**A**) Colonies on EMJH plates after 1 week of incubation at 30°C. (**B**) Top (upper panel) and side (lower panel) views of an *L. biflexa* colony. Note that *Leptospira* cells grow into the solid medium, forming subsurface colonies.

10. Pick transformants using a micropipettor and subculture in 5 ml EMJH liquid medium containing appropriate selection antibiotic. Maintain antibiotic selection during subsequent growth and manipulation of strains.

    Leptospira species do not grow on the surface of solid EMJH medium, but in subsurface colonies (Fig. 12E.4.2B).

BASIC PROTOCOL 2

INTRODUCTION OF *E. COLI-L. BIFLEXA* SHUTTLE VECTOR INTO *L. BIFLEXA* FOR COMPLEMENTATION OF MUTANTS OR HETEROLOGOUS GENE EXPRESSION

This protocol allows the introduction of an *E. coli-L. biflexa* shuttle vector (Saint Girons et al., 2000) in *L. biflexa* to perform complementation of mutants or heterologous gene expression. This plasmid vector contains the replication origin of the leptophage LE1 (Saint Girons et al., 2000) and it is the only plasmid vector found to be replicative in *Leptospira* species so far.

Begin by performing Basic Protocol 1: introduce a plasmid containing the LE1 replication origin (Saint Girons et al., 2000; Bourhy et al. 2005a; Fig. 12E.4.1) by electroporation, then select for drug resistance (i.e., kanamycin or spectinomycin depending on the resistance cassette).

Note that the replicative vector may contain partition genes, i.e., the *parAB* operon of the replication origin of LE1 (Saint Girons et al., 2000). In this case, recombinant clones harboring the *parAB*-containing plasmid derivatives can be subsequently maintained without antibiotic (see Commentary section). A replicative vector containing a counterselectable marker can also be used in a streptomycin-resistant strain of *L. biflexa* (see Basic Protocol 4). Since the *L. biflexa* replicative plasmid contains the pGEM7Z-f+ ori (Fig. 12E.4.1), direct transfer of vector DNA from *L. biflexa* into *E. coli* is possible after genomic DNA extraction of *L. biflexa* transformants and subsequent transformation of *E. coli*.

BASIC PROTOCOL 3

TARGETED MUTAGENESIS BY HOMOLOGOUS RECOMBINATION

Insertion of a DNA fragment into a specific gene on the chromosome, thereby rendering it inactive, has been widely used for targeted mutagenesis in both eukaryotes and prokaryotes. In this protocol, transformation is used to introduce an inactivated allele, which replaces the wild-type copy by homologous recombination (Fig. 12E.4.3). However, the frequency of double cross-over events may be low in comparison to single cross-over

events, which usually result in a wild-type phenotype. In *L. biflexa*, UV treatment of the transforming DNA prior to electroporation is critical for obtaining a high number of recombinants, including bacteria with allelic exchange (Picardeau et al., 2001). To mediate homologous recombination, nucleotide sequences of the targeted gene flanking the antibiotic cassette are usually 0.5 to 1 kb in length.

Materials

Suicide plasmid (for example, a pGEM7Z-f+ Promega derivative plasmid that replicates in *E. coli* but not in *Leptospira* species) containing the gene of interest interrupted by a kanamycin or spectinomycin resistance cassette (Fig. 12E.4.1)

Tris·Cl, pH 8.0 (*APPENDIX 2A*)

PCR primers flanking the insertion site of the resistance cassette in the targeted gene (Fig. 12E.4.3)

UV chamber (GS Gene linker, Bio-Rad)

100°C water bath

Additional reagents and equipment for preparation and transformation of electrocompetent *Leptospira biflexa* (Basic Protocol 1) and PCR (Kramer and Coen, 2001)

Grow the strain to be mutagenized and prepare electrocompetent cells

1. Perform Basic Protocol 1 steps 1 to 4.

Induce homologous recombination

2. Subject 100 ng suicide plasmid containing the inactivated allele to 5 to 15 sec UV treatment (254 nm, 400 μW/cm^2) using a UV chamber.

   Alternatively, to induce homologous recombination, plasmid DNA can also be denatured by alkali treatment. To do this, denature the DNA in a 50-μl solution containing 0.2 mM EDTA/0.2 M NaOH for 15 to 30 min at 37°C, and then purify using Qiaquick minicolumns (Qiaquick PCR purification kit, Qiagen).

3. Introduce plasmid DNA by electroporation (see Basic Protocol 1, steps 5 to 7)

   Since a low number of recombinants is expected, >200 μl of the electroporation mix can be spread onto agar medium.

Screen for double cross-over mutants

4. Pick resistant colonies using a micropipettor and subculture in 5 ml EMJH liquid medium.

   Since double homologous recombination are rare events, subculture at least 20 colonies.

5. To prepare thermolysates, resuspend cell pellets in 100 μl of 10 mM Tris·Cl, pH 8.0, and heat 10 min at 100°C, followed by cooling on ice for at least 10 min. Microcentrifuge 10 min at maximum speed, room temperature. Retain the supernatants which will be used as crude DNA extracts to detect double cross-over events in the targeted gene by PCR.

   Alternatively, extract DNA by the phenol/chloroform method.

6. Perform PCR (Kramer and Coen, 2001) to amplify the insertion of the resistance cassette in the target gene.

   Use standard PCR conditions for the gene to be amplified. In transformants, the increase in size of the amplified product in comparison to the wild-type strain is due to the insertion of the resistance cassette (double cross-over event) into the target locus. Transformants resulting in single cross-over events (plasmid insertion into the target locus) exhibit one amplified product identical to the one obtained from the wild-type strain and, when PCR conditions are optimal for the amplification of large DNA fragments, a larger one corresponding to the allele interrupted by the resistance cassette (Fig. 12E.4.3).

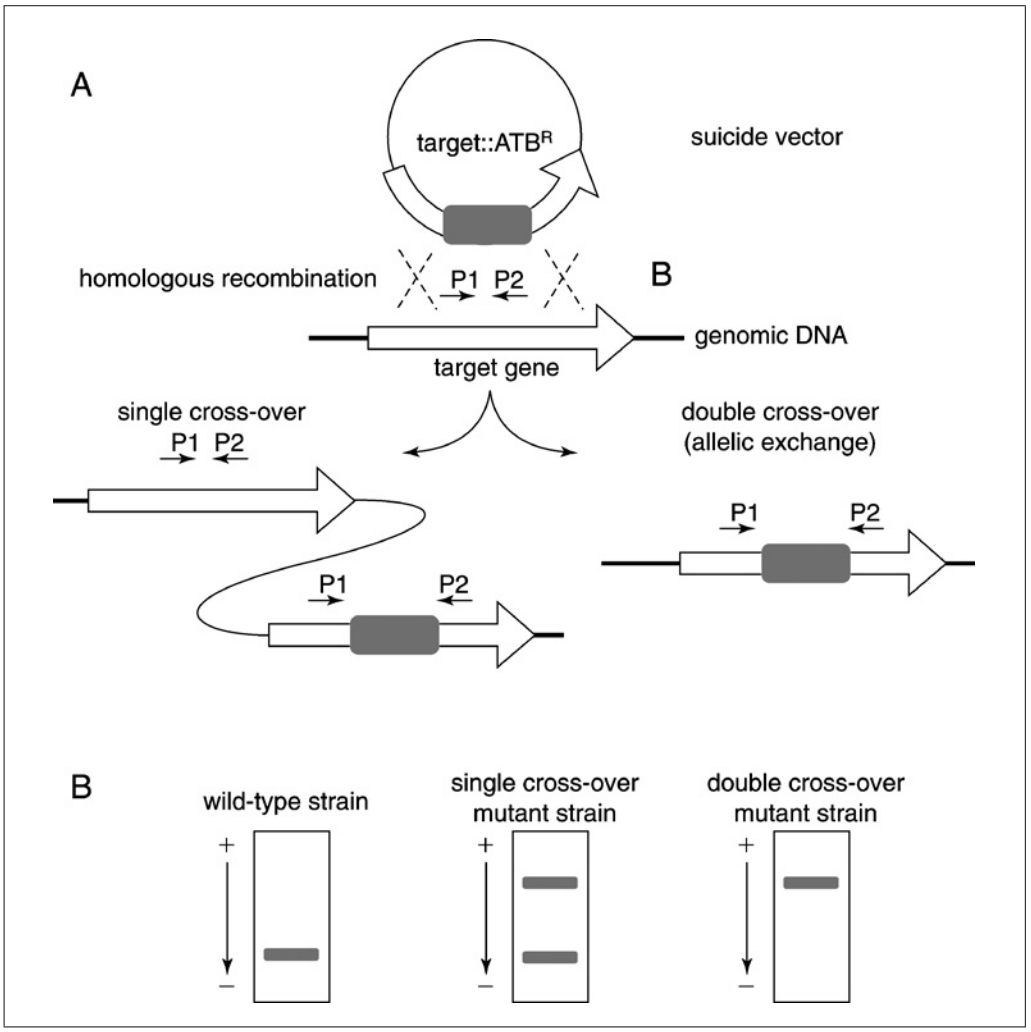

Figure 12E.4.3 Schematic diagram of the procedure for targeted mutagenesis by homologous recombination. (**A**) The target gene in a suicide plasmid (unable to replicate in *Leptospira* species) is interrupted by a kanamycin- or spectinomycin-resistance cassette, shown in grey. The suicide plasmid is used to transform *L. biflexa* by electroporation. The transformants resulting from homologous recombination are then selected on EMJH supplemented with the appropriate antibiotic. The genotypes of the transformants are characterized by PCR with primers P1 and P2 flanking the insertion site of the resistance cassette. (**B**) Gel electrophoresis of amplified products with primers P1 and P2. An increase in size with respect to the wild-type strain is observed for the transformants resulting from the integration of the resistance cassette by double cross-over recombination in the target gene. The single cross-over recombinants, resulting from the integration of the suicide plasmid, were characterized by a wild-type amplified product and a larger product.

BASIC PROTOCOL 4

COUNTERSELECTION OF *LEPTOSPIRA BIFLEXA* USING *rpsL*$^+$

In this protocol, the *L. biflexa rpsL* wild-type allele is cloned into a suicide or replicative plasmid DNA. The gene *rpsL* encodes the S12 ribosomal protein, which is the target of streptomycin, but bacteria become resistant to this antibiotic if *rpsL* is mutated. The resistant phenotype is recessive in a merodiploid, i.e., a streptomycin-resistant strain (due to a mutation in *rpsL*) and becomes sensitive to streptomycin when the *rpsL* wild-type allele is present in the cell. Using a streptomycin-resistant strain of *L. biflexa*, it is therefore possible to select mutants that have lost the plasmid DNA containing the *rpsL* wild-type allele by plating the transformants on streptomycin (Picardeau et al., 2001). The use of counterselection can, for example, enhance the recovery of bacteria with allelic exchange events.

Materials

L. biflexa serovar Patoc strain Patoc1 resistant to streptomycin (National Reference Center for Leptospira, Institut Pasteur, Paris, France)
Primer LpR1 (5′- TACAAAATGTCGCTATCTGG-3′)
Primer LpR2 (5′-TGTATTTAGGATCGCCTTCG-3′)
EMJH agar plates supplemented with 40 μg/ml streptomycin and another appropriate antibiotic (see recipe)
Sterile toothpicks

Additional reagents and equipment for preparing thermolysates (Basic Protocol 3), PCR (Kramer and Coen, 2001), cloning (Ausubel et al., 2007, Chapter 3), and transformation of *Leptospira* (Basic Protocol 1)

Construct plasmid DNA for transformation

1. Prepare thermolysates of the *L. biflexa* wild-type strain as described in Basic Protocol 3, step 5.
2. Perform PCR (Kramer and Coen, 2001) to amplify the *rpsL* wild-type allele using primers LpR1-LpR2 and crude DNA extract of *L. biflexa* wild-type strain as a template.
3. Clone the PCR product into the plasmid vector (Ausubel et al., 2007, Chapter 3) used to deliver the inactivated allele.

   After a vector containing a streptomycin-resistant mutant rpsL gene has been produced, one may chose to use that vector as the basis for all subsequent allelic exchange mutagenesis experiments.

Grow the streptomycin-resistant strain of L. biflexa and transform with plasmid DNA

4. Perform Basic Protocol 1 with the streptomycin-resistant strain of *L. biflexa* and transform competent cells with *rpsL*$^+$-containing plasmid derivatives.

Counterselect transformants

5. When colonies appear on kanamycin or spectinomycin plates (to 10 days), replica plate using sterile toothpicks onto EMJH plates supplemented with streptomycin.
6. Restreak colonies growing on plates supplemented with streptomycin on plates supplemented with streptomycin and kanamycin or spectinomycin to confirm the streptomycin resistance.

   Double cross-over mutants should be resistant to streptomycin.

   Transformants can be directly spread on plates supplemented with streptomycin and kanamycin or spectinomycin. However, the two-step procedure generally enhances the recovery of double cross-over events. Perform PCR with primers flanking the insertion site of the resistance cassette to identify double cross-over mutants (Fig. 12E.4.3).

GENOME-WIDE TRANSPOSON MUTAGENESIS

BASIC PROTOCOL 5

This protocol allows the generation of large numbers of *L. biflexa* mutants by random transposon mutagenesis using a *mariner* transposon, *Himar1*. This mutagenesis approach yields a randomly distributed set of insertion mutations throughout the genome, which can be screened for specific phenotypes. The screening of mutants on medium with and without hemin is described here, but this approach can be adapted for use with other screens.

Materials

pSC189 derivative plasmids carrying both the hyperactive transposase C9 and the *Himar1* transposon containing a kanamycin resistance cassette (Fig. 12E.4.1, plasmid vectors are available upon request from authors: mpicard@pasteur.fr)

EMJH agar plates supplemented with 40 μg/ml kanamycin and 50 μM hemin (see recipe)

Additional reagents and equipment for introducing the plasmid by electroporation (Basic Protocol 1), replica plating, extraction of genomic DNA (Wilson, 1997), and ligation-mediated PCR (Prod'hom et al., 1998)

Grow the strain to be mutagenized and prepare electrocompetent cells

1. Perform Basic Protocol 1 to introduce the *Himar1*-delivering plasmid by electroporation.

   It has to be noted that due to the presence of a hyperactive transposase, pSC189 derivative plasmids may not be stable in E. coli. Each plasmid preparation should therefore be done with freshly transformed E. coli cells. In contrast, since the plasmid vectors are not replicative in Leptospira species, after transposition of Himar1 into the L. biflexa genome, the transposase gene which is adjacent to Himar1 but not included into Himar1 is lost together with the plasmid. This avoids subsequent transposase-mediated events.

Screen for mutants, example of screening for hemin-requiring mutants

2. After electroporation with plasmid carrying *Himar1*, spread transformants on plates containing kanamycin and 50 μM hemin (see Basic Protocol 1, step 9).

   L. biflexa and other Leptospira species use hemin as an iron as well as a heme source. Addition of hemin ensures the isolation of mutants with auxotrophic defects. Other screening tests such as amino acid auxotrophy can be used.

3. Screen for auxotrophs by replica plating onto hemin-EMJH and EMJH plates.

   Mutants should be purified by streaking for single colonies and retested for their phenotypes.

4. Extract genomic DNA of *L. biflexa* mutant strains by phenol/chloroform (Wilson, 1997), amplify the *Himar1* insertion site by using ligation-mediated PCR (Prod'hom et al., 1998), and sequence the amplified products.

   Identification of the Himar1 insertion site in the chromosome may facilitate subsequent identification and analysis of mutated genes. Alternatively, the presence of the oriR6K in Himar1 may be used to recover sequences flanking the transposon insertion site (Chiang and Rubin, 2002).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Albumin supplement

Dissolve 10 g BSA into 50 ml distilled water with stirring (~30 min), being careful to avoid foaming. Add the following:

1 ml1.5% (w/v) $MgCl_2 \cdot 6H_2O$
1 ml 0.4% (w/v) $ZnSO_4 \cdot 7H_2O$
1 ml 1.5% (w/v) $CaCl_2 \cdot 2H_2O$
1 ml 0.02% (w/v) vitamin B12
1 ml 10% (w/v) pyruvic acid (prepared from sodium salt)

continued

0.4 g glycerol (~0.4 ml)
1.25 g Tween 80 (~1.25 ml)
Adjust final volume to 100 ml with H_2O
Store indefinitely at −20°C

All glassware must be washed with distilled water before use (it is recommended to make the mixture in sterile plastic containers). It is also critically important that water be sterilized by autoclaving prior to all preparations to eliminate potential contamination by saprophytic Leptospira (see UNIT 12E.1).

Stock solutions should be autoclaved and stored up to 6 months at 4°C.

EMJH, base

1 g Na_2HPO_4
0.3 g KH_2PO_4
1 g NaCl
0.25 g NH_4Cl
0.005 g thiamine
Add H_2O to 1 liter

Base EMJH medium is commercially available in powder form (Leptospira Medium Base EMJH; BD Biosciences).

EMJH medium, liquid

For 1 liter of liquid EMJH medium:
Dissolve 2.3 g base EMJH (see recipe) into 800 ml distilled H_2O
Add 100 ml albumin supplement (see recipe)
Add 100 ml 0.5% (w/v) $FeSO_4 \cdot 7H_2O$ to final concentration of 0.05% (w/v)
Adjust pH to 7.4 with NaOH or HCl as necessary
Bring the final volume to 1 liter with H_2O
Sterilize by filtration through a 0.22-μm filter
Divide into aliquots as needed for experiments
Store up to 2 months at 4°C

EMJH agar plates

Dissolve 2.3g base EMJH (see recipe) into 500 ml distilled H_2O
Add 100 ml albumin supplement (see recipe)
Add 0.05% $FeSO_4$ (prepared from a 0.5% $FeSO_4 \cdot 7H_2O$ solution)
Adjust pH to 7.4 with NaOH or HCl as necessary
Bring the final volume to 660 ml with H_2O
Pass through a 0.22-μm filter to sterilize
Warm the solution to ~50°C (~30 min)
Add 340 ml 12% Noble Agar solution (see recipe), at ~50°C
Add antibiotics as specified in protocols

Pour ~30 ml medium into individual petri dishes under a hood to minimize contamination

Once plates have solidified, place them in the original petri dish bags and store, inverted, up to 1 month at 4°C until needed.

Noble Agar solution, 12%

Prepare a 12% Noble Agar solution by dissolving 12 g Noble Agar (BD Biosciences) in 340 ml distilled water. Sterilize by autoclaving. Cool the autoclaved solution to ~50°C and pour plates as instructed in recipe for EMJH agar plates.

COMMENTARY

Background Information

E. coli-L. biflexa shuttle vector

A major breakthrough in *Leptospira* genetics was the first report of genetic transformation by electroporation of an *E.coli-L. biflexa* shuttle vector. It contains the replication origin of the circular replicon of the *L. biflexa* LE1 prophage, a kanamycin-resistance cassette from the Gram-positive bacterium *Enterococcus faecalis*, and an *E. coli* plasmid origin (Saint Girons et al., 2000). Replicative vectors containing a gene encoding resistance to spectinomycin can also be used (Bauby et al., 2003; Bourhy et al., 2005a). Two types of replicative plasmids are available, plasmids with the minimal replication origin of LE1 (~1-kb in length) and plasmids containing both the minimal replication origin of LE1 and the *parAB* partition operon, therefore conferring higher stability in absence of selective pressure (Bauby et al., 2003; Bourhy et al., 2005a).

Targeted mutagenesis

In *L. biflexa*, homologous recombination between the inactivated allele and the wild-type allele is greatly enhanced after UV irradiation of the delivery vector (Picardeau et al., 2001). This method allowed the inactivation of several genes such as *flaB*, *recA*, *metX*, *trpE*, and *hemH* in the saprophytes *L. biflexa* and *L. meyeri* (Picardeau et al., 2001, 2003; Tchamedeu Kameni et al., 2002; Bauby et al., 2003; Guégan et al., 2003). Single cross-overs (integrated plasmids) can be easily differentiated from double cross-overs (allelic exchange) by PCR analysis of colonies. Since the proportion of allelic exchange mutants is low, and single recombination is the general rule, the use of a plasmid vector containing a counter-selectable marker adjacent to the inactivated gene should eliminate clones harboring the plasmid vector and/or clones that have integrated the vector by a single cross-over event. The presence of the *rpsL* wild-type gene in streptomycin-resistant transformants promotes bacterial death in the presence of streptomycin and facilitates the recovery of double cross-over mutants. Spontaneous streptomycin resistant colonies appear at a frequency of 10^{-5}, which is low enough to permit the use of *rpsL* as a counter-selectable marker (Picardeau et al., 2001).

Random transposon mutagenesis

Transposons of the *mariner* family have been used successfully for random mutagenesis of a diverse range of organisms, including eukaryotes, archaea, and both Gram-positive and -negative bacteria. In order to generate a library of mutants in *L. biflexa*, a random mutagenesis system based on the *mariner* transposon *Himar1* was developed. The transposition frequency was increased when the expression of the hyperactive transposase is controlled by spirochetal promoters (Bourhy et al., 2005b). The transposon randomly inserts into the chromosome and has allowed the identification of amino acid auxotrophs and hemin-requiring mutants (Louvel et al., 2005).

Critical Parameters and Troubleshooting

Electrotransformation of L. biflexa

Electroporation is routinely used to transfer DNA into eukaryotic and prokaryotic cells. Basic Protocol 1 is relatively uncomplicated to perform and does not require special equipment.

The preparation of competent cells with high transformation efficiency depends on (1) harvesting bacterial cultures in exponential phase of growth, (2) keeping cells at room temperature throughout the procedure, and (3) the quality of the EMJH medium used for experiments. Each batch of solid and liquid EMJH media must be checked for wild-type growth of *L. biflexa*. Reduced transformation efficiency and/or delays in the appearance of colonies on plates are often due to low-quality EMJH medium. It has to be noted that, for the authors long-term storage of competent cells dramatically reduces the transformation efficiencies. The transformation frequency is linear to the amount of DNA added, up to 100 ng: higher plasmid concentration do not result in better transformation efficiency. Although this method was developed relatively recently, no spontaneous resistance of *L. biflexa* under kanamycin selection has been identified so far. Since penicillin is the recommended treatment against leptospirosis, antibiotic resistance cassettes such as ampicillin should not be used.

Targeted mutagenesis

UV treatment is the more successful and technically easiest method, generating larger numbers of transformants than alkali treatment. However, different conditions of UV treatment should be tested to optimize transformation efficiency. Optimal conditions may depend on the equipment used for UV treatment and the intensity of the UV lamp. Mutating essential genes should only lead to

the detection of single cross-over events (Fig. 12E.4.3). When no transformants are obtained, higher DNA concentration and/or longer UV treatment should be tested. The *rpsL* system can permit the positive selection of double recombinants in a streptomycin-resistant strain. Although double recombinant mutants can be directly selected on kanamycin plus streptomycin plates after electroporation, a two-step procedure can be applied in cases where the double recombination frequency is too low. In the first step, recombinants are selected according to the antibiotic gene marker used for gene disruption. These recombinants are grown to stationary phase, thus allowing secondary recombination events to accumulate, then plated on media containing streptomycin. UV treatment of the delivery vector prior to electroporation may lead to the appearance of spontaneous streptomycin-resistant colonies because of mutation within *rpsL*.

Random transposon mutagenesis

Due to the presence of the hyperactive transposase in pSC189 derivative plasmids, care should be taken when preparing plasmid DNA in *E. coli*. The use of modified plasmids carrying the hyperactive transposase under the control of spirochetal promoters increase *L. biflexa* transformants yield 10-fold, in comparison to pSC189 carrying the hyperactive transposase with its native promoter (Chiang and Rubin, 2002). Transforming the cells with <100 ng of plasmid DNA usually results in better transformation efficiency. Further analysis of mutants should confirm that mutant phenotypes are due to a single transposition event.

Anticipated Results

Using Basic Protocol 1, transformation efficiencies can reach 10^5 bacteria/μg plasmid DNA with pGKBLe24 derivatives. In targeted mutagenesis, the number of transformants should range from 20 to 100 transformants/μg DNA, including 10% to 60% allelic exchange bacteria. However, these results greatly vary from gene to gene. Using *Himar1*, transformation efficiency can reach 10^4 transformants/μg DNA (Fig. 12E.4.1).

Time Considerations

L. biflexa is a slow-growing organism with a generation time of ~4 hr. Typically, strains should be grown 3 to 4 days in EMJH liquid medium to reach the exponential phase (see *UNIT 12E.1*). Once the culture of the bacterial cells is ready to be harvested, the cells are washed and concentrated in 1 hr, then transformed with DNA. Plating transformed cells occurs the day after. At least 1 week is necessary to obtain colonies on solid medium. If required, an additional 2 days will be needed to isolate genomic DNA for PCR, Southern blot analysis, and/or DNA sequencing reactions.

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Laboratory Maintenance of Fusobacteria

INTRODUCTION

Fusobacterium are Gram-negative, anaerobic, nonspore-forming bacilli. While some type strains are fusiform (spindle-shaped), others are not. The bacteria form either speckled, bread-crumb-like colonies, or smooth colonies. This unit presents the basic techniques for growing pure cultures of fusobacteria on blood agar plates or in broth (Basic Protocol 1), enriching fusobacteria from a mixed population such as dental plaque (Basic Protocol 2), and storing fusobacteria on a short- or long-term basis (Basic Protocol 3).

CAUTION: *Fusobacterium* are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All incubations are performed anaerobically at 37°C in 90% N_2, 5% CO_2, and 5% H_2. Manipulations outside the anaerobic chamber should be limited to <30 min whenever possible (see Strategic Planning and Critical Parameters).

STRATEGIC PLANNING

Blood agar plates are best prepared 1 to 2 days before use. After the agar solidifies, the plates can be left closed on the laboratory bench overnight or with lids off in a laminar flow hood for 10 to 20 min to dry off excessive moisture. The unused plates can then be stored, well wrapped, at 4°C and used within 1 to 2 weeks. Agar and broth media should be placed inside the anaerobic chamber one day before use to reduce the oxygen content. The plates should be unwrapped and the test tube caps loosened to allow for anaerobic equilibration.

GROWTH OF PURE FUSOBACTERIAL CULTURES

BASIC PROTOCOL 1

This protocol describes media preparation and growth of pure fusobacterial cultures on blood agar plates and in broth.

Materials

Columbia blood agar plates (see recipe)
Columbia broth (see recipe)
Fusobacterium cultures (clinical isolates or ATCC strains)
Anaerobic incubator: 37°C anaerobic chamber, desiccant, palladium catalyst pellets, nitrogen gas tank, gas mix (90% N_2, 5% CO_2, and 5% H_2) tank—e.g., Coy or Forma *or*
Anaerobic jar system (e.g., 2.5-liter Pack-Rectangular Jar, AnaeroPack System; Mitsubishi Gas Chemical Co.; *http://www.mgc-a.com/Pages/anaeropac.html*)
Sachets for anaerobic jar system (Mitsubishi Gas Chemical Co.)
Anaerobic indicator strips (Becton Dickinson)

1. Place Columbia blood agar plates and broth inside the 37°C anaerobic chamber or anaerobic jar one day before use to reduce the oxygen contained in the medium.
2. Using a 3-mm inoculating loop, inoculate fusobacteria by streaking onto the blood agar plates or by mixing into the broth.

   It is important when preparing broth cultures that four to five colonies instead of only one are inoculated into the broth to ensure sufficient inoculum.

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3a. *If using an anaerobic chamber*: Transfer the plates and tubes inside the chamber; place plates upside down and loosen the caps of the tubes.

To ensure anaerobicity, the desiccant and catalysts in the anaerobic chamber need to be recycled frequently. The anaerobicity should be checked periodically with anaerobic indicator strips.

3b. *If using anaerobic jar system*: Place plates and tubes in the jar along with a newly opened sachet; place plates upside down and loosen the caps of the tubes. Close the lid and place the anaerobic jar in a regular 37°C incubator.

The sachet must be replaced each time the jar is opened.

4. Incubate 2 to 7 days or until the desired growth is obtained.

BASIC PROTOCOL 2

ENRICHMENT OF FUSOBACTERIA FROM A MIXED POPULATION

The following protocol describes enrichment of fusobacteria from mixed cultures such as dental plaques (Loesche et al., 1972; Walker et al., 1979; Morgenstein et al., 1981).

Materials

Reduced transport fluid (RTF; see recipe)
FEA or CVE plates (see recipes)
Dental plaque (provided by a dental care professional)
Anaerobic incubator: 37°C anaerobic chamber, desiccant, palladium catalyst pellets, nitrogen gas tank, gas mix (90% N_2, 5% CO_2, and 5% H_2) tank—e.g., Coy or Forma *or*
Anaerobic jar system (e.g., 2.5-liter Pack-Rectangular Jar, AnaeroPack System; Mitsubishi Gas Chemical Co.; *http://www.mgc-a.com/Pages/anaeropac.html*)
Sachets for anaerobic jar system (Mitsubishi Gas Chemical Co.)
Anaerobic indicator strips (Becton Dickinson)

1. Place FEA or CVE plates inside the 37°C anaerobic chamber or jar one day before use to reduce the oxygen contained in the medium.
2. Suspend dental plaque (from either individual sites or pooled from different sites in the same patient) into 1 ml RTF. Vortex 60 sec.
3. Plate 10- or 100-fold serial dilutions of the RTF suspension onto prereduced FEA or CVE plates from step 1.
4. Incubate anaerobically at 37°C for 5 to 7 days.

CVE permits almost total recovery of pure cultures of fusobacteria when compared to nonselective medium and suppresses pure cultures of non-fusobacteria by six orders of magnitude. Crystal violet inhibits Gram-positive organisms. Fusobacteria are relatively resistant to erythromycin, which can inhibit a number of other oral bacteria at low concentrations.

FEA yields no suppression of F. nucleatum relative to nonselective medium and may allow growth of fewer contaminating organisms than CVE because the combination of vancomycin, neomycin, and josamycin inhibits an even broader spectrum of oral bacteria. The differential susceptibility of Fusobacterium and Bacteroides to josamycin is greater than erythromycin. In addition, the egg yolk in FEA allows for differentiation of F. necropherum from other fusobacteria due to its lipase activity.

For more information see Walker et al. (1979) and Morgenstein et al. (1981).

STORAGE OF FUSOBACTERIA

Fusobacteria grown on plates or in broth should be stored anaerobically, either in an anaerobic chamber or an anaerobic jar at room temperature. The cultures on plates should be passaged every 2 weeks. The broth cultures can be passaged three to four times. For long-term storage, glycerol stocks should be made and maintained at −80°C, as described below.

Materials

Fusobacterium cultures
Columbia broth (see recipe)
Glycerol (autoclaved)

Anaerobic chamber or anaerobic jar (see Basic Protocol 1)
1.5-ml microcentrifuge tubes, sterile

1. Grow fusobacteria in Columbia broth as in Basic Protocol 1 to early stationary phase (OD_{600} ~1).

   At a doubling time of 5 to 6 hr during growth, it may take 24 to 72 hours to reach this density, depending on the inoculum.

2. Place Columbia broth and glycerol inside the 37°C anaerobic chamber or anaerobic jar one day before use to reduce the oxygen contained in the medium.

3. Mix 500 µl fusobacterial culture with 500 µl prereduced 50% glycerol in Columbia broth in a sterile 1.5-ml microcentrifuge tube. Invert the tube several times to mix well and store immediately at −80°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Columbia blood agar plates

Suspend 44 g Difco Columbia blood agar base in 1 liter of water and autoclave 15 min at 121°C. Cool to 50°C and add 50 ml defibrinated whole sheep blood (Cleveland Scientific), prewarmed to room temperature. Pour into sterile 100 × 15–mm Petri plates (~30 ml per plate). After the agar solidifies, leave the plates on the laboratory bench overnight or in a laminar flow hood with lids off for 10 to 20 min to dry excess moisture. Store, well wrapped, up to 2 weeks at 4°C.

Other agars, e.g., brain heart infusion agar (Becton Dickinson) and trypticase soy agar (Becton Dickinson; also see UNIT 4B.1 for recipe for tryptic soy agar), can also be used to grow fusobacteria. The agars should all be supplemented with ~5% defibrinated sheep blood (no supplement needed for the broth).

Columbia broth

Suspend 35 g Difco Columbia broth base (see recipe) in 1 liter of water, autoclave in 100-ml aliquots 15 min at 121°C. As needed, dispense 5 ml into sterile 15-ml polypropylene screw-cap tubes. Store up to 6 months at room temperature.

Columbia broth base

28.57% (w/w) pancreatic digest of Casein (10 g)
14.29% (w/w) yeast extract (5 g)
14.29% (w/w) proteose peptone #3 (5 g)
8.57% (w/w) tryptic digest of beef heart (3 g)
0.29% (w/w) L-cysteine HCl (0.1 g)
7.14% (w/w) glucose (2.5 g)
14.29% NaCl (w/w) (5 g)

0.29% (w/w) anhydrous magnesium sulfate (0.1 g)
0.06% (w/w) ferrous sulfate (0.02 g)
1.71% (w/w) sodium carbonate (0.6 g)
2.37% (w/w) Tris base (0.83 g)
8.17% (w/w) Tris·Cl (2.86 g)

The final pH of this medium when dissolved in water is 7.5 ± 0.2.

Numbers in parenthesis represent the mass needed to prepare 1 liter of Columbia broth (see recipe).

Columbia broth powder is commercially available from Difco.

CVE plates

Dissolve the following in 1 liter H_2O with mixing:
10 g trypticase base (e.g., Becton Dickinson)
5 g yeast extract (e.g., Fisher)
5 g NaCl
2 g glucose
0.2 g tryptophan
5 mg crystal violet

Autoclave 15 min at 121°C. Cool to 50°C. Add 4 mg erythromycin and 50 ml defibrinated whole sheep blood, prewarmed to room temperature. Pour into sterile 100 × 15–mm Petri plates (~30 ml per plate). After the agar solidifies, leave the plates on the laboratory bench overnight or in a laminar flow hood with lids off for 10 to 20 min to dry excess moisture. Store, well wrapped, up to 2 weeks at 4°C.

Refer to Walker et al. (1979) for more information.

FEA plates

Dissolve the following into 1 liter H_2O with mixing:
41 g Brucella agar base (BBL)
5 g Na_2HPO_4
1 g K_2HPO_4
0.1g $MgSO_4$
5 mg hemin (Sigma)
1 ml polysorbate 80 (Sigma)

Adjust to pH 7.6 with NaOH, autoclave at 121°C for 15 min, and cool to 50°C. Add 5 mg vancomycin, 100 mg neomycin, 3 mg josamycin (Sigma), and 25 ml sterile egg yolk suspension (Fisher). Pour into sterile 100 × 15–mm Petri plates (~30 ml per plate). After the agar solidifies, leave the plates on the laboratory bench overnight or in a laminar flow hood with lids off for 10 to 20 min to dry excess moisture. Store, well wrapped, up to 2 weeks at 4°C.

Refer to Morgenstein et al. (1981) for more information.

Reduced transport fluid (RTF)

Combine the following:
0.5 ml of 8% w/v Na_2CO_3 stock solution (sterilize by passing through a 0.22-μm filter)
1.0 ml of 0.1 M EDTA, pH 8.0, stock solution (sterilize by autoclaving)
2.0 ml of 1% w/v DTT stock solution (sterilize by passing through a 0.22-μm filter)
7.5 ml of 0.6% w/v K_2HPO_4 stock solution (sterilize by autoclaving)
7.5 ml RTF stock mineral solution (see recipe)
Adjust volume to 100 ml with sterile H_2O
Store in 2-ml aliquots up to 6 months at −20°C

Final concentrations are 0.04% Na_2CO_3, 0.001 M EDTA, 0.02% dithiothreitol, 0.045% K_2HPO_4, 0.045% KH_2PO_4, 0.09% NaCl, 0.09% $(NH_4)_2SO_4$, and 0.018% $MgSO_4$

Refer to Loesche et al. (1972) for more information.

RTF mineral stock solution

0.6% w/v KH_2PO_4
1.2% w/v NaCl
1.2% w/v $(NH_4)_2SO_4$
0.25% w/v $MgSO_4$
Autoclave and store up to 6 months at room temperature

COMMENTARY

Background Information

Fusobacteria are a group of Gram-negative obligate anaerobic bacilli belonging to the phylum Fusobacteria, class Fusobacteria, order Fusobacteriales, family Fusobacteriaceae, and genus *Fusobacterium* (Garrity, 2001). The type strain of the genus is *Fusobacterium nucleatum*, which includes three subspecies, i.e., subsp. *nucleatum*, subsp. *polymorphum*, and subsp. *vincentii* (Dzink et al., 1990). The type strains of each subspecies are *F. nucleatum* subsp. *nucleatum* ATCC25586, *F. nucleatum* subsp. *polymorphum* ATCC10953, and *F. nucleatum* subsp. *vincentii* ATCC49256, respectively. Based on 16S RNA sequences, *F. nucleatum* is most closely related to two other oral species, *F. periodonticum* and *F. simiae*, forming a distinct cluster within the genus (Lawson et al., 1991). The fusoabcteria genome is highly AT rich, with the GC content ranging from 26% to 34% (Bennett and Eley, 1993). The genome sequences of several *F. nucleatum* strains have been published and consist of approximately two million base pairs encoding ~2000 open reading frames (Kapatral et al., 2002, 2003).

The primary colonization site for *F. nucleatum* is in the oral cavity. It is frequently isolated from subgingival plaque associated with both healthy and diseased periodontal sites (Moore and Moore, 1994). During periodontal infection, its quantity can increase more than 10,000 fold (Moore and Moore, 1994). In addition, although *F. nucleatum* is normally found only in the oral cavity, it is one of the most common oral species implicated in systemic infections, having been isolated from infections and abscesses in many other parts of the body (Moore and Moore, 1994). *F. nucleatum* is prevalent in preterm birth and has been isolated from the amniotic fluid and placenta (normally sterile environments) of women in preterm labor (Hill, 1993, 1998). When intravenously injected into pregnant mice to mimic transient bacteremia during periodontal infection, the organism colonized and proliferated specifically in the placenta and induced preterm and term stillbirths (Han et al., 2004).

F. nucleatum is known as an "adhesive" organism. It adheres to a variety of host mammalian cells, including epithelial and endothelial cells, polymorphonuclear leukocytes (PMNs), monocytes, erythrocytes, fibroblasts, and HeLa cells, as well as salivary macromolecules, extracellular matrix proteins, and human IgG (Winkler et al., 1987; Ozaki et al., 1990; Tuttle and Mangan, 1990; Xie et al., 1991; Babu et al., 1995; Han et al., 2000, 2004;). It also coaggregates with all oral bacterial species tested so far and, thus, is a key player in dental plaque formation (Kolenbrander and Andersen, 1989; Kolenbrander et al., 1989; George and Falkler, 1992; Kolenbrander et al., 1995; Grimaudo and Nesbitt, 1997; Andersen et al., 1998; Bradshaw et al., 1998; Jabra-Rizk et al., 1999; Metzger et al., 2001; Rosen et al., 2003). It has been suggested that *F. nucleatum* possesses multiple adhesins including both lectin-like and nonlectin-like types (Murray et al., 1988; Mangan et al., 1989; Tuttle et al., 1992; Takemoto et al., 1995; Shaniztki et al., 1998; Weiss et al., 2000). Investigation of the adherence properties of *F. nucleatum* is a major focus of fusobacterial research. Three components, a 40- to 42-kDa major outer membrane porin protein, FomA, a 39.5-kDa polypeptide, and a 30-kDa polypeptide, have been suggested as possible adhesins from *F. nucleatum* involved in inter-bacterial coaggregation (Kaufman and DiRienzo, 1989; Kinder and Holt, 1993; Shaniztki et al., 1997). FomA was also found to bind to the human IgG Fc fragment (Guo et al., 2000). A high

molecular-weight component, ranging from 300 to 330 kDa, has also been suggested as a galactose-binding agglutinin (Murray et al., 1988). Recently, a novel 13.6-kDa adhesin peptide from *F. nucleatum* was found to be involved in attachment to mammalian cells (Y. W. Han, unpub. observ.).

F. nucleatum also invades endothelial and epithelial cells. It is a strong stimulator of the production of IL-8 from epithelial cells, indicating its ability to induce inflammation (Darveau et al., 1998; Han et al., 2000). *F. nucleatum* causes apoptosis of human peripheral white blood cells and is able to suppress T-cell responses (Shenker and Datar, 1995; Jewett et al., 2000). This organism also induces production of innate natural antibiotics, human β-defensins (hBD), in gingival epithelial cells and has been shown to be resistant to hBD-1 and hBD-2 (Krisanaprakornkit et al., 2000). This is presumably a mechanism for inhibiting the growth of competitive species and to interfere with the host innate and adaptive immune responses.

Critical Parameters

The most critical parameter in laboratory maintenance of fusobacteria is to minimize oxygen exposure. All media and solutions should be prereduced in the anaerobic chamber. The cultures should not remain outside the anaerobic chamber for more than $^1/_2$ to 1 hr at any given time, and they should be returned to an anaerobic environment immediately following necessary manipulation at the bench. To maintain anaerobicity in the chamber, the desiccant and palladium catalyst pellets need to be recycled frequently, i.e., from once every 2 weeks to twice a week, depending on the load in the chamber.

Troubleshooting

If the bacterial culture does not grow as expected, the temperature and the anaerobicity in the chamber should be examined. The anaerobicity can be tested using a BBL disposable anaerobic indicator strip (Becton Dickinson). Alternatively, the oxygen and hydrogen levels in the chamber can be monitored by an oxygen and hydrogen analyzer (Coy Laboratory Products). If oxygen is detected, the air in the chamber needs to be recycled. The presence of oxygen may indicate a leak in the chamber, which can be detected using a leakage detector (Coy Laboratory Products). The rubber gloves are more prone to leakage than other parts of the chamber.

If the bacteria grow on the blood agar plates but not in the broth, it may be that the inoculums in the broth are too small. It is important to inoculate several colonies, instead of just one, into the broth.

Anticipated Results

Most *F. nucleatum* cultures can reach an OD_{600} of >2 when grown in broth. When using selective media such as CVE or FEA, contaminating species will also grow, although most oral species will be inhibited 10^6- to 10^8-fold. Frozen stocks of fusobacteria can usually last >10 years if stored properly at −80°C, although loss of viability may occasionally occur with some clinical isolates.

Time Considerations

Depending on the strain, it usually takes 2 to 7 days for fusobacteria to grow up on blood agar plates or in broth. When subculturing from broth to broth, however, the bacteria may reach the stationary phase within less than 24 hr if the dilution factor is low enough (e.g., 1:5 or 1:10).

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Laboratory Maintenance of *Flavobacterium psychrophilum* and *Flavobacterium columnare*

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ABSTRACT

Bacteria in the genus *Flavobacterium* are highly heterogeneous and are comprised of pathogenic and non-pathogenic species. They are found in a wide variety of environments that include fresh and marine water, soil, and ocean sediments. This unit focuses on laboratory maintenance of two important fish pathogens within this genus. *Flavobacterium psychrophilum* and *F. columnare* are fastidious, slow-growing organisms that require specialized medium for optimal growth in the laboratory. Methods are described for the preparation of media, broth and agar culture, cryopreservation, and enumeration of viable cells for these bacteria. In addition, the diseases caused by these pathogens are described along with methods of diagnosis and treatments. This unit serves as a general guide for the cultivation of *F. psychrophilum* and *F. columnare* and discusses some of the difficulties that have been encountered for each. *Curr. Protoc. Microbiol.* 6:13B.1.1-13B.1.12.

Keywords: *Flavobacterium* • bacterial culture • pathogen • soil bacteria • aquatic bacteria

INTRODUCTION

The genus *Flavobacterium* is a highly heterogeneous and diverse group of Gram-negative bacteria found everywhere from a variety of soil and water environments to plants, food stuffs, and even distilled water lines. This unit presents general protocols for the laboratory maintenance of *Flavobacterium* species, with an emphasis on the fish pathogens *F. psychrophilum* and *F. columnare*, which greatly impact fisheries and aquaculture worldwide. *Flavobacterium psychrophilum* is the etiological agent of bacterial cold-water disease (CWD) and rainbow trout fry syndrome (RTFS), while *F. columnare* is the etiological agent of columnaris disease.

Flavobacterium psychrophilum and *F. columnare* achieve optimal growth in vitro on specialized selective low-nutrient medium. Tryptone yeast extract salts (TYES) is one medium that promotes good growth for both species (see Basic Protocol 1) and is routinely used in many laboratories. Several other media formulations have been shown to support good growth for these species (see Reagents and Solutions). The process of culturing these bacteria is described from frozen and lyophilized stocks as well as routine maintenance of cultures in broth or agar medium (see Basic Protocol 1). The procedure is identical for *F. psychrophilum* and *F. columnare* with the exception of temperature. *Flavobacterium psychrophilum* grows best at 15°C, while the optimum temperature for *F. columnare* is 25°C. Cryopreservation of *Flavobacterium* species can be achieved by preparing glycerol stocks and storing at −80°C (see Basic Protocol 2); using this method, bacteria can remain viable for over 30 years. Enumeration of viable cells for *Flavobacterium* can be achieved using a spread plate method (see Basic Protocol 3). However, characteristics can differ between isolates and some may clump or auto-agglutinate during growth, making this method of enumeration difficult. In such

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cases, direct counting of cells by microscopy, which is briefly described in the Critical Parameters and Troubleshooting section, can be used to estimate the number of cells present but will not provide viable cell counts.

CAUTION: Flavobacterium psychrophilum and *Flavobacterium columnare* are Biosafety Level 1 (BSL-1) organisms. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

TYES Medium

Flavobacterium psychrophilum and *F. columnare* do not grow effectively on standard bacteriological medium such as tryptic soy agar (TSA; *APPENDIX 2C*); therefore, several specialized low-nutrient media have been developed. One of the most common is TYES (Holt et al., 1993), which has proven to be effective for the cultivation of numerous isolates in the authors' laboratory. The procedure for the preparation and sterilization of 1 liter of TYES broth or agar is described in Reagents and Solutions. Other media commonly used for the cultivation of these species is discussed below, with formulations also provided in Reagents and Solutions.

Alternative Media: Anacker and Ordal, Hsu-Shotts, Shieh, and MAT

There have been numerous media and enhancements proposed for the growth of *F. psychrophilum* and *F. columnare*. Three alternative media that are commonly used include Anacker and Ordal (Anacker and Ordal, 1955), Hsu-Shotts (Bullock et al., 1986), and Shieh (Shieh, 1980). It may be possible to obtain better growth of *F. psychrophilum* and *F. columnare* using these media. Additionally, the use of MAT medium (Crump et al., 2001) has been useful for reducing agglutination during broth culture (see Critical Parameters and Troubleshooting). It is recommended that the growth of the isolate of interest be analyzed in each of these and TYES to determine which medium is optimal for the isolate being used. See Reagents and Solutions for formulations of media.

Avoiding contamination

The most common type of contamination during the culture of *Flavobacterium* species is contamination from environmental bacteria present in the laboratory. The best way to avoid contamination is prevention. When preparing culture media and other solutions for use with bacteria, it is essential that they be sterilized prior to use. The use of sterilization monitors during autoclaving is a good practice to ensure sterility of the solutions (see Critical Parameters and Troubleshooting).

When preparing liquid media, it is best to sterilize the liquid medium in the culture flasks, tubes, or other containers in which the bacteria will be grown. This prevents the possibility of contaminating the medium while transferring it from a medium bottle into a sterile culture flask or tube. When pouring petri plates for solid medium, it is best to pour the plates in a biological safety cabinet or near a Bunsen burner. The use of a Bunsen burner creates a draft of air that rises, which will reduce the possibility of bacterial contamination.

The use of aseptic technique is critical when working with bacteria (see Basic Protocols 1, 2, and 3). All materials that will be used need to be sterile and it is good practice to keep the area in which the work will be performed as sterile as possible by cleaning the surface with 10% bleach or 70% ethanol. Passage of bacteria can be conducted near a Bunsen burner as described above or in a biological safety cabinet. The utilization of

sterile medium and solutions, aseptic technique, and being cautious at all times when working with bacteria will greatly reduce the potential for contamination.

BROTH CULTURE OF *FLAVOBACTERIUM PSYCHROPHILUM* AND *FLAVOBACTERIUM COLUMNARE*

BASIC PROTOCOL 1

Flavobacterium psychrophilum and *F. columnare* growth occurs readily in TYES broth and the alternative media described in Strategic Planning. Stock cultures are aseptically plated onto TYES agar (or alternative media) and incubated 4 to 7 days. Isolated colonies are then picked, inoculated into TYES broth (or alternative media), and cultured for 3 days. Following confirmation of purity, this culture can be used for further passage in appropriate medium or for subsequent analyses of the bacteria. Bacteria obtained may be used for preparing frozen stocks, isolate characterization, electrophoresis, etc.

Materials

Lyophilized or frozen stock of *F. psychrophilum* or *F. columnare* (see Basic Protocol 2)
Sterile KPBS (*APPENDIX 2A*)
TYES agar plates and broth (or alternative medium broth and plates; see recipe for TYES agar and broth)

Sterile inoculation loops
Sterile 1-ml syringes and needles
Parafilm
Low-temperature incubator (15° to 25°C)
15-ml tubes

Additional reagents and equipment for Gram staining (*APPENDIX 3C*) and light microscopy (*UNIT 2A.1*)

1a. *For frozen stocks:* Remove frozen stock of *Flavobacterium* isolate, aseptically remove cap, and gently scrape the frozen stock with a sterilized inoculation loop. Aseptically streak for isolation on a petri plate containing TYES agar (or alternative medium), use duplicate plates per isolate.

Keep the frozen stock cold by placing in an ice bucket when not in use.

1b. *For lyophilized stocks:* If the stock of *Flavobacterium* is lyophilized in a glass vial, aseptically reconstitute the bacteria by injecting 1 ml sterile KPBS into the vial using a sterile 1-ml syringe and needle. Remove the reconstituted bacteria and aseptically pipet ~30 µl onto a petri plate containing TYES agar (or alternative medium) and streak to isolate colonies, use duplicate plates per isolate.

2. Seal the agar plates with Parafilm, and incubate at 15°C for *F. psychrophilum* or 25°C for *F. columnare*.

The growth of F. psychrophilum and F. columnare isolates vary; however, growing for 4 to 7 days should be sufficient to obtain growth for most isolates.

3. Following incubation, observe the plates for isolated colonies typical of *F. psychrophilum* or *F. columnare*.

Colonies of F. psychrophilum are generally characterized as bright yellow, shiny, non-adhering, raised convex colonies with a thin spreading irregular margin, sometimes referred to as a "fried-egg" appearance. However, some strains of the bacterium may produce convex yellow colonies with smooth margins or a mixture of the two types of colonies (Fig. 13B.1.1). Flavobacterium columnare colonies are characterized as yellowish, strongly adhering, flat rhizoid colonies with an irregular margin, however, some isolates may exhibit a smooth margin (Fig. 13B.1.1).

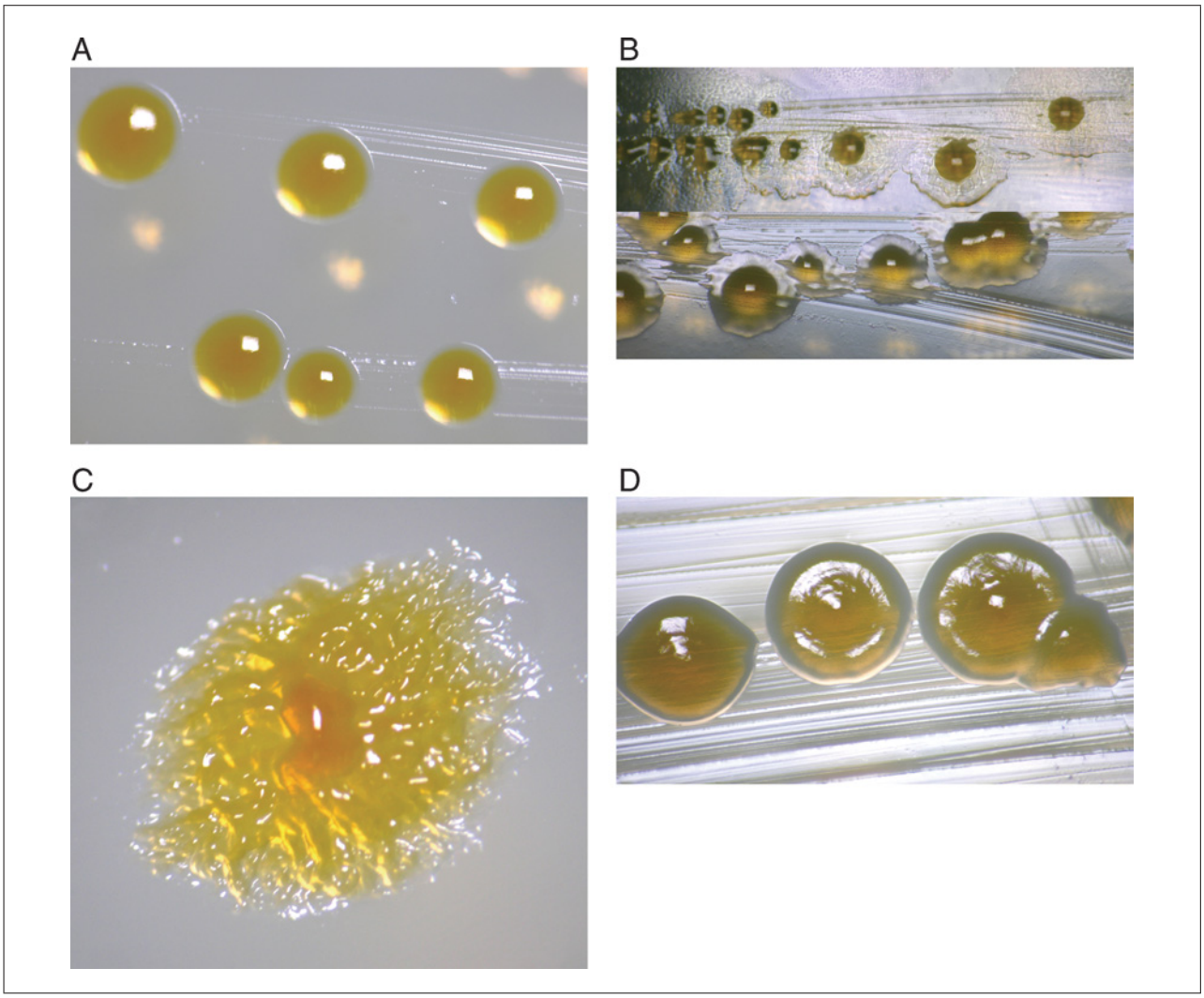

Figure 13B.1.1 Morphology of *F. psychrophilum* colonies exhibiting a smooth margin (**A**), *F. psychrophilum* colonies exhibiting thin spreading margins ("fried-egg") (**B**), *F. columnare* colony exhibiting a characteristic flat rhizoid colony with an irregular margin (**C**), and *F. columnare* colonies exhibiting smooth margins (**D**). For a color version of this figure, see *http://www.currentprotocols.com*

4. Aseptically pick an isolated colony using a sterilized inoculation loop and inoculate a sterile 15-ml culture tube containing 15 ml TYES broth (or alternative medium). Lightly cap the culture tube and gently vortex.

   Due to the adherent nature of F. columnare colonies, it may be necessary to excise or scrape colonies from agar using the sterile inoculation loop.

5. Incubate the inoculated broth statically or on an orbital shaker for 3 days at 15°C for *F. psychrophilum* or 25°C for *F. columnare*.

6. Confirm the purity of the culture using Gram staining (*APPENDIX 3C*) and light microscopy (*UNIT 2A.1*) and/or aseptically plating a loop of culture for isolation onto TYES agar (or alternative medium; *APPENDIX 4A*) and incubate for 4 to 7 days at 15°C for *F. psychrophilum* or 25°C for *F. columnare*.

   Flavobacterium psychrophilum cells are characterized as Gram-negative, long slender rods with rounded edges. Cells from young cultures generally range from 2 to 7 μm and 0.5 to 1.1 μm in length and width, respectively (Fig. 13B.1.2). Filamentous forms are also common with lengths ranging from 10 to 70 μm. Flavobacterium columnare cells are characterized as long slender Gram-negative rods. Cells from young cultures generally range from 0.3- to 0.7-μm wide and 3- to 10-μm long (Fig. 13B.1.2).

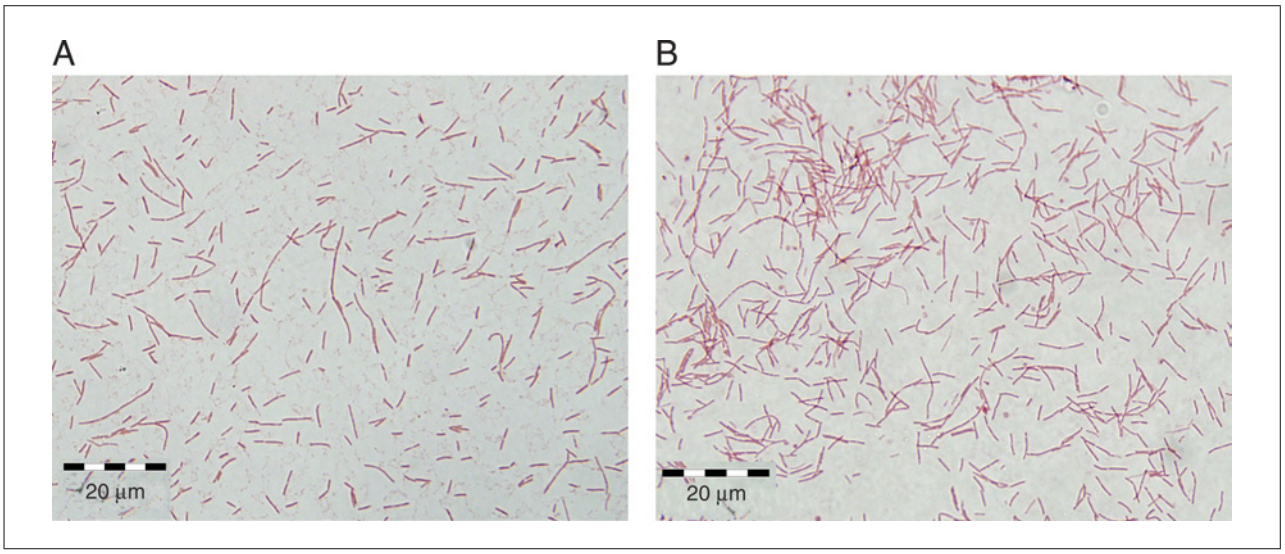

Figure 13B.1.2 Cell morphology of *F. psychrophilum* (**A**) and *F. columnare* (**B**) following Gram staining and light microscopy. For a color version of this figure, see *http://www.currentprotocols.com*

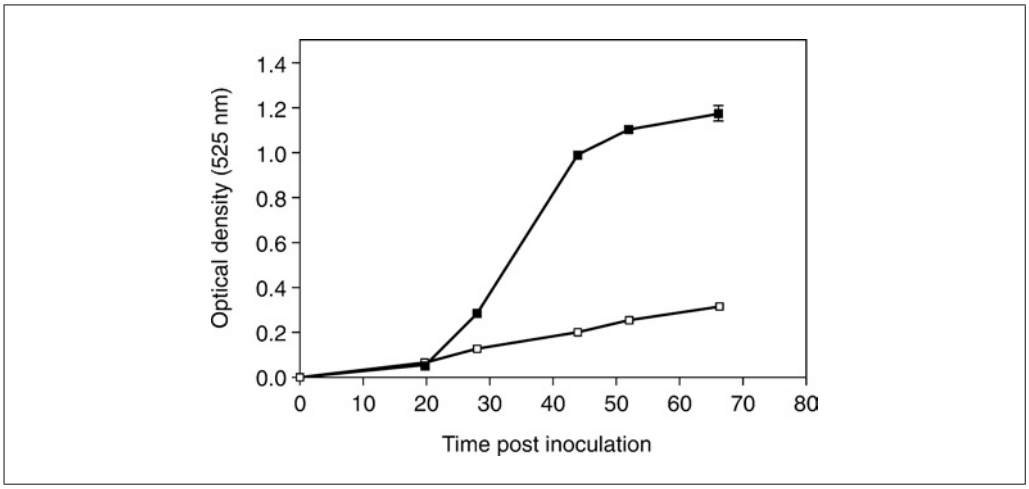

Figure 13B.1.3 Growth of *F. psychrophilum* strain ATCC #49418 in TYES broth for ~72 hr at 15°C. Duplicate 500-ml flasks containing 200 ml TYES were inoculated with 0.5 ml of a 72-hr culture (1.25×10^7 cfu ml^{-1}) of *F. psychrophilum* and cultured either statically (open squares) or with the use of an orbital shaker at ~100 rotations min^{-1} (closed squares).

7. Aseptically obtain a loop of 3-day culture using a sterilized inoculation loop and inoculate a sterilized culture tube or flask containing the desired volume of TYES broth (or alternative medium).

   The volume of broth required will vary depending on the desired use of the cultured bacteria. For routine maintenance of the bacteria, it is recommended to use 15 ml broth. If larger quantities of bacteria are needed, it is recommended to use 200 to 250 ml broth in a 500-ml culture flask and inoculated with a larger volume of culture.

8. Incubate the inoculated broth for 2 to 7 days at 15°C (*F. psychrophilum*) or 25°C (*F. columnare*) statically or on an orbital shaker.

   Growth of Flavobacterium isolates varies, therefore, it is recommended that a growth curve be developed for each isolate to approximate when log-phase growth occurs. Growth is also enhanced using a shaker platform (Fig. 13B.1.3).

9. Once log-phase growth is achieved, confirm the purity of the culture (see step 6). If the culture is confirmed to be pure, then use for subsequent analyses.

The optical density of the Flavobacterium cultures can be determined using a spectrophotometer at a wavelength of 525 nm. Sterile TYES or an alternative medium broth should be used as a blank. It should be noted that for some strains of F. columnare, OD_{525} readings may be inaccurate if significant auto-agglutination is observed.

BASIC PROTOCOL 2

CRYOPRESERVATION OF *FLAVOBACTERIUM*

Flavobacterium species can be cryopreserved in glycerol with good viability. Log-phase *Flavobacterium* culture is mixed with sterile glycerol stock and 1-ml aliquots are frozen at −80°C in cryovials.

Materials

Sterile glycerol stock (see recipe)
Log-phase growth of *Flavobacterium* (see Basic Protocol 1)

Sterile 15-ml polypropylene tubes
1.0-ml cryovials
Vortex
Cryovial storage box

1. Label a 15-ml polypropylene tube with the isolate number and ten 1.0-ml cryovials with the isolate number, date, and any other information deemed necessary.
2. Aseptically add 4 ml sterile glycerol stock to the tube.
3. Aseptically add 6 ml log-phase growth of *Flavobacterium* to the 15-ml polypropylene tube.

   This procedure will give a final concentration of 20% glycerol.

4. Mix by gently vortexing 10 to 15 sec using a setting of 3 or 4 until a homogenous solution is obtained.
5. Loosen lids of cryovials and aseptically add a 1.0-ml volume of the bacterial stock into each tube.
6. Tighten lids, place in cryovial storage box, and freeze up to 30 years at −80°C (see Background Information).

BASIC PROTOCOL 3

ENUMERATION OF VIABLE *FLAVOBACTERIUM* CELLS

The spread-plate method is a technique for determining the number of viable cells in a bacterial culture, i.e., cfu ml^{-1}. This method involves plating aliquots of cell dilutions, culturing bacteria for 7 days, and counting the number of colonies on each plate. From these data, the number of viable cells in the original culture can be estimated. It should be noted that clumping and auto-agglutination of some isolates (especially for *F. columnare*) may prevent accurate estimation of viable cells. Therefore, direct enumeration of cells may be necessary (see Critical Parameters and Troubleshooting).

Materials

Petri plates with TYES or alternative medium agar (see recipe)
Sterile KPBS (*APPENDIX 2A*)
Flavobacterium culture (see Basic Protocol 1)
95% ethanol

Sterile culture tubes (13 × 100–mm)
Cell spreader
Parafilm

1. Label nine sterile 13 × 100–mm culture tubes from 10^{-1} to 10^{-9}. Also label petri dishes containing TYES agar (or alternative medium) in an identical manner and include isolate number, date, and any other information deemed necessary.

   It is recommended that duplicate or triplicate agar plates be used per dilution to accurately determine the viable cell count.

2. Add 900 µl sterile KPBS into each tube.

3. Serially dilute the bacterial culture from 10^{-1} to 10^{-9} by aseptically removing 100 µl from the *Flavobacterium* culture and add to the tube labeled 10^{-1}. Mix well by gentle vortexing.

4. Remove 100 µl of the 10^{-1} dilution and add to the tube labeled 10^{-2}. Mix well by gentle vortexing. Continue this procedure until the final 10^{-9} dilution has been prepared.

   The number of dilutions required will depend on the concentration of bacteria in the culture.

5. Gently vortex the 10^{-1} dilution, aseptically remove 50 µl, and dispense the liquid onto the TYES agar (or alternative medium) plate labeled 10^{-1}.

6. Sterilize a cell spreader by immersing in 95% ethanol and flaming. Allow to cool, and then spread the liquid over the entire surface of the plate. Repeat with the duplicate (or triplicate) plates and remaining *Flavobacterium* dilutions.

7. Seal the agar plates with Parafilm, invert, and incubate 3 to 7 days at the optimum temperature for the *Flavobacterium* species being used (see Basic Protocol 1).

8. Once observable colonies have grown, count the number of colonies on each plate and average the duplicates (or triplicates) for each dilution.

   The use of a dissecting microscope or colony counter will allow for accurate colony counts. It is also suggested that only plates containing between 30 and 300 colonies be used for enumeration to obtain statistically relevant numbers.

9. Estimate the number of viable cells in the original bacterial culture by using the following equation for the dilution(s) that resulted in the growth of between 30 and 300 colonies:

$$\text{cfu ml}^{-1} = \frac{(\text{no. of colonies})(1/\text{dilution})}{(\text{plated volume in ml})}$$

For example, if 50 µl of a dilution of 10^{-5} yields an average of 35 colonies per plate:

$$\text{cfu ml}^{-1} = \frac{(35)(1/0.00001)}{(0.05)}$$

$$\text{cfu ml}^{-1} = 7 \times 10^{7}$$

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anacker and Ordal liquid or solid medium

Dissolve the following into ~900 ml deionized water:
0.5 g tryptone
0.5 g yeast extract

continued

0.2 g sodium acetate
0.2 g beef extract
11.0 g agar (for solid medium only)
Prepare and store as described for TYES medium (see below)

Also see Strategic Planning.

Glycerol stock, 50% (v/v), sterile

Combine equal volumes of KPBS (*APPENDIX 2A*) and glycerol into a medium bottle and mix until a homogenous solution is obtained. Sterilize by autoclaving for 15 min at 121°C. Store up to 1 year at room temperature.

Hsu-Shotts liquid or solid medium

Dissolve the following into ~900 ml deionized water:
2.0 g tryptone
0.5 g yeast extract
3.0 g gelatin
15.0 g agar (for solid medium only)
Prepare and store as described for TYES medium (see below)

Also see Strategic Planning.

MAT liquid or solid medium

Dissolve the following into ~900 ml deionized water:
0.5 g $MgSO_4$
0.5 g $CaCl_2$
10.0 g maltose
0.2 g sodium acetate
0.4 g yeast extract
4.0 g tryptone
15.0 g agar (for solid medium only)
Prepare and store as described for TYES medium (see below)

Also see Strategic Planning.

Shieh liquid or solid medium

Dissolve the following into ~900 ml deionized water:
5.0 g peptone
1.0 g glucose
0.01 g sodium acetate
0.1 g sodium pyruvate
0.01 g citric acid
0.5 g yeast extract
0.01 g $BaCl_2 \cdot H_2O$
0.1 g K_2HPO_4
0.05 g KH_2PO_4
0.3 g $MgSO_4 \cdot 7H_2O$
0.0067 g $CaCl_2 \cdot 2H_2O$
0.001 g $FeSO_4 \cdot 7H_2O$
0.05 g $NaHCO_3$
15.0 g agar (for solid medium only)
Prepare and store as described for TYES medium (see below)

Also see Strategic Planning.

TYES solid or liquid medium

Dissolve the following into ~900 ml deionized water:
0.5 g $MgSO_4$
0.5 g $CaCl_2$
0.4 g yeast extract
4.0 g tryptone
15.0 g agar (for solid medium only)

To prepare liquid medium, adjust the pH to 7.2 with 1 M NaOH or 1 M HCl, bring the volume up to 1 liter with deionized water using a graduated cylinder, divide the medium equally into 2 medium bottles (or dispense into culture flasks and/or tubes), and then sterilize by autoclaving for 15 min at 121°C. Store liquid medium up to 1 month at room temperature.

To prepare solid medium, follow the protocol for liquid medium, add half the amount of agar to each medium bottle, and then autoclave for 15 min at 121°C. Following sterilization, allow the medium to cool to ~60°C and then aseptically pour ~15 ml into petri plates and allow the agar to solidify. Following solidification, invert the petri plates and store up to 2 weeks at 4°C.

Also see Strategic Planning.

COMMENTARY

Background Information

Protocols vary greatly for the laboratory maintenance of *Flavobacterium* species due to the heterogeneous nature of this genus. For example, the American Type Culture Collection (ATCC) suggests the use of nutrient broth for *F. pectinovorum* and lactose medium for *F. hibernum*. Jooste and Hugo (1999) reviewed cultivation methods for the genera of the family *Flavobacteriaceae*. The culture of the fish pathogens, *F. psychrophilum* and *F. columnare*, requires a specialized medium such as TYES (see Strategic Planning) due to their fastidious nature. Other medium formulations are available (see Strategic Planning) and numerous improvements to these standard medium have been recommended (Song et al., 1988; Lorenzen, 1993; Daskalov et al., 1999; Michel et al., 1999; Cepeda et al., 2004). A growth analysis should be performed utilizing the medium described in this unit to obtain optimal growth of the species and isolate of interest.

The preparation of glycerol stocks is a standard method for the cryopreservation of bacteria. Bacteria preserved in this manner remain viable for over 30 years. Another method of cryopreservation commonly used for *Flavobacterium* species is lyophilization. This also results in a high level of viability following preservation. Several methods of lyophilization and suspending medium have been tested for *F. psychrophilum* and *F. columnare* and it was concluded that the use of 67% Difco Bacto Brucella broth supplemented with 33% horse or fetal calf serum was optimal for these species (Desolme and Bernardet, 1996). When working with pathogenic bacteria such as *F. psychrophilum* and *F. columnare*, it is important that the method of cryopreservation does not reduce the virulence of the bacterium. Michel and Garcia (2003) demonstrated that long-term storage of *F. psychrophilum* in frozen glycerol stocks and by lyophilization did not result in a loss of pathogenicity 2 years post-preservation. The same likely applies to *F. columnare*.

The enumeration of viable cell counts for *F. psychrophilum* and *F. columnare* can result in large variations in cfu ml^{-1}. Michel et al. (1999) hypothesized that the variation for *F. psychrophilum* could be due to lack of effective medium, culture conditions, or susceptibility of the bacteria to stress. To reduce this variation, they recommended using isotonic medium for suspension of the bacteria and avoidance of strong mixing and centrifugation (Michel et al., 1999).

Flavobacterium

The genus *Flavobacterium* is a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group and encompasses a diverse group of over 300 species of bacteria that are frequent inhabitants of soil and ocean sediment,

fresh and marine water, and foods and food processing plants. The description of this genus has been revised multiple times and has resulted in a complex history. Taxonomically, much of the confusion has been resolved and *Flavobacterium* represent Gram-negative pigmented bacteria primarily possessing gliding motility, a DNA G + C content of 32 to 37 mol %, and menaquinone-6 being the primary respiratory quinone (Bernardet et al., 1996). *Flavobacterium psychrophilum* and *F. columnare* are the focus of this unit and represent significant pathogens of both coldwater and warm-water fish species.

Flavobacterium psychrophilum

Flavobacterium psychrophilum is the etiological agent of CWD and RTFS, and causes significant economic losses to trout and salmon aquaculture worldwide. *Flavobacterium psychrophilum* causes an acute septicemic infection and the bacterium can usually be isolated from all internal organs of heavily infected fish (Wood and Yasutake, 1956). Clinical signs of disease in fry and fingerlings can include yellow-pigmented lesions (Holt et al., 1993), frayed and eroded fins (Schmidtke and Carson, 1995), and dark coloration (Evenson and Lorenzen, 1996). Presumptive diagnosis is based on clinical signs and isolation of the disease agent exhibiting the cellular and colony characteristics described in Basic Protocol 1. Definitive diagnosis is based on serological techniques such as fluorescent antibody test or agglutination (AFS-FHS, 2006). Polymerase chain reaction (PCR) protocols are also available for confirmation (Taylor and Winton, 2002). Control methods for CWD and RTFS include external treatment with microbicides and antibiotics. Florfenicol (AQUAFLOR; Schering-Plough Animal Health Corp.) is approved by the FDA in the United States for controlling mortality in freshwater-reared salmonids due to *F. psychrophilum*. Treatments may be ineffective and the development of resistant strains is a concern; therefore, research efforts are increasing in the area of vaccine development.

Flavobacterium columnare

Flavobacterium columnare is the etiological agent of columnaris disease. Most species of freshwater fish are susceptible to *F. columnare*, and susceptibility is greatly increased if fish have been exposed to predisposing conditions such as stress. Columnaris disease is the second leading cause of mortality in the channel catfish (*Ictalurus punctatus*) aquaculture industry in the southern United States (Thune, 1993). *Flavobacterium columnare* can cause both acute and chronic infections of the skin, gills, and fins. Acute infections involving highly virulent strains may cause no external signs; however, clinical signs of chronic infections include lesions of the skin (particularly around the dorsal fin), oral cavity, and gills (Plumb, 1999). A presumptive diagnosis is based on clinical signs and isolation of the disease agent exhibiting the cellular and colony characteristics described in Basic Protocol 1. Definitive diagnosis is based on serological techniques, biochemical testing, or molecular methods (AFS-FHS, 2006). Control methods for columnaris disease include external treatment with microbicides and antibiotics. Hydrogen peroxide (35% PEROX-AID, Eka Chemicals) is approved by the FDA in the United States for controlling mortality due to external columnaris disease in freshwater-reared coolwater finfish and channel catfish. Additionally, an avirulent live vaccine is approved by the USDA-APHIS for use in channel catfish (AQUAVAC-COL, Intervet).

Critical Parameters and Troubleshooting

The use of aseptic technique and sterile medium is critical for culture and maintenance of any bacteria. When autoclaving freshly prepared medium, it may be advisable to include a monitor to ensure proper sterilization. One example may consist of a chemical pellet in a glass tube that will only melt at a specific temperature (121°C). Placement of a sterilization monitor into the medium during autoclaving will ensure adequate heat penetration and proper sterilization.

It is essential that frozen glycerol stocks of *Flavobacterium* be maintained frozen when using these to resuscitate an isolate from cryopreservation. Maintaining the stock on ice during use will greatly reduce the possibility of freeze/thaw cycles, which will decrease the viability of the cells.

Some isolates of *F. psychrophilum* and *F. columnare* tend to agglutinate during growth, hampering effective cultivation. In the authors' laboratory, it has been found that the use of MAT medium (see recipe) may help to alleviate problems associated with agglutination. Problems have also been encountered when determining viable cell counts with some isolates of *F. columnare* due to the growth of this bacterium in clumps and autoagglutination. Therefore, direct enumeration

of cells may be the best way to quantify certain isolates. The method consists of staining suspensions of bacteria with crystal violet and counting cells in a Petroff-Hauser bacterial counting chamber. It is important to realize that this method provides only total numbers of cells and not viable cell counts. However, a possibility is to couple direct counting with the use of bacterial viability kits to estimate the number of viable cells by direct enumeration. Such methods have been used with *F. psychrophilum* (Michel et al., 1999; Vastos et al., 2003).

Anticipated Results

The growth of different isolates of *F. psychrophilum* and *F. columnare* can vary greatly and growth curves should be generated for the isolate being used. A typical growth curve for *F. psychrophilum* grown statically or on an orbital shaker is illustrated in Figure 13B.1.3.

The methods of cryopreservation described in this unit should yield viable stocks for greater than 30 years. Enumeration of cells via the spread plate method will allow for quantification of viable cells from most cultures of *Flavobacterium*.

Time Considerations

Preparing medium for *Flavobacterium* should take ∼10 min for solid and liquid media. Sterilization of the medium by autoclaving should take ∼30 min, depending on the type of autoclave being used. Cooling medium to a useable temperature for broth (room temperature) and agar medium (∼60°C for pouring) will vary depending on the ambient temperature of the laboratory.

The entire process of obtaining a culture of *Flavobacterium* from a frozen or lyophilized stock will take ∼12 to 18 days. Preparing streaks of bacteria from frozen or lyophilized stocks will take ∼15 min, followed by 4 to 7 days of incubation to allow for isolated colonies to grow. Inoculating the obtained isolated colonies to broth medium will take <15 min, followed by 3 days of incubation. Confirmation of the purity of the culture will take ∼20 min for Gram staining (*APPENDIX 3C*) and microscopic evaluation, or 4 to 7 days for plating bacteria and examining for colonies with the phenotype of the species being cultivated. Inoculating the desired volume of medium with the 3-day culture will take <15 min, followed by 2 to 7 days of incubation to obtain log-phase cultures that can be used for subsequent analyses. At this point the cultures should be examined for purity. The time required for this would be the same as previously described.

Cryopreserving an isolate of *Flavobacterium* as glycerol stocks will take ∼15 min. Labeling tubes and cryovials will take 5 min, mixing culture and glycerol stock will take ∼5 min, and dispensing aliquot stocks and freezing will take <5 min.

Enumerating viable cells by the spread plate method will take ∼8 days. Set-up, tube and plate labeling, and serial dilutions will take ∼30 min. Aseptically plating bacterial dilutions will take ∼30 min. Inoculated plates need to be incubated for up to 7 days or until sufficient growth is achieved to visualize colonies. Following incubation, counting colonies and calculating cfu ml^{-1} will require ∼30 min depending on the number of plates requiring counts.

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Internet Resources

http://aquanic.org/publicat/usda_rac/efs/srac/479bfs.pdf

Website link to an extension bulletin of the Southern Regional Aquaculture Center (SRAC) on columnaris disease.

http://www.fish.washington.edu/wrac/Coldwater_bacterial_disease.pdf

Website link to an extension bulletin of the Western Regional Aquaculture Center (WRAC) on coldwater disease.

Laboratory Maintenance and Cultivation of *Bacteroides* Species

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ABSTRACT

Bacteroides species are Gram-negative, obligate anaerobic bacteria. They are the predominant indigenous bacterial species in the human intestinal tract, where they play an important role in the normal physiology of the host, but they can also be significant opportunistic pathogens. The fact that these are obligate anaerobes is the prevailing feature that affects the methodology used for their cultivation. Several techniques are described for anaerobic culturing; the anaerobic glove box and anaerobic jars are the two techniques most adaptable to a range of research needs. Straightforward methods are presented for propagation on solid media and in broth cultures, and for the long-term storage and maintenance of stock cultures. The *Bacteroides* species are saccharolytic, which is the second feature of their physiology that impacts cultivation methodology. Several flexible media formulations, including a defined minimal media, are provided that allow the researcher to choose the carbon source best suited for his or her work. *Curr. Protoc. Microbiol.* 9:13C.1.1-13C.1.21. © 2008 by John Wiley & Sons, Inc.

Keywords: anaerobic culturing • *Bacteroides* • anaerobic jars • anaerobic chambers

INTRODUCTION

This unit describes methods for the culturing, storage, and maintenance of the Gram-negative, anaerobic *Bacteroides* species. Members of the genus *Bacteroides* play important roles in human and animal health (Smith et al., 2006). They are the predominant members of the indigenous intestinal flora in humans where they contribute to normal intestinal development, physiology, and function. Largely due to their proximity to the human host, several *Bacteroides* species can be opportunistic pathogens and they are frequently isolated from a range of anaerobic infections including intraabdominal and pelvic abscesses, soft tissue, female genital tract, and bacteremia. Their ability to successfully colonize mammals is due in part to their extended aerotolerance, simple nutritional requirements, and ability to utilize diverse carbohydrate substrates for carbon and energy. All of these factors are important considerations when culturing these organisms. Due to a combination of innate and acquired mechanisms the *Bacteroides* species are resistant to a wide array of commonly used antibiotics which can complicate antimicrobial therapy of anaerobic infections. Although there has been considerable interest in the mechanisms responsible for the transmissible, acquired antibiotic resistance, it is their innate resistance to aminoglycosides and vancomycin that has proven to be important for development of selective media for their isolation (see Smith et al., 2006).

The single most important consideration when culturing the *Bacteroides* is that they are obligate anaerobes and they will not divide in the presence of oxygen. That said, they are one of the most aerotolerant anaerobic species known and a recent report (Baughn and Malamy, 2004) has suggested that they may actually slowly divide in the presence of nanomolar concentrations of oxygen. Thus, while it is appropriate that this unit include a discussion of anaerobic culturing techniques, the methods discussed here may not be suitable for many other anaerobic bacteria since they are not as rigorous in their

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requirement for exclusion of oxygen at all stages of media preparation and inoculation. In this regard, it is important to note that anaerobic culturing requires some specialized equipment. It is our intention to describe sufficient equipment options so that those new to the field will be able to easily initiate *Bacteroides* culturing without a large initial investment in expensive equipment. However, this unit is not intended to be a comprehensive treatise on anaerobic culturing methods and the reader will be directed to several excellent reviews and manuals for detailed descriptions of anaerobic techniques.

CAUTION: Bacteroides are considered Biosafety Level 1 (BSL-1) organisms in the USA although in some European countries they are BSL-2. These organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. However, given that some procedures described in this unit require the use of syringes for the transfer of cells, it is recommended that the standards for BSL-2 be followed. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

Culturing *Bacteroides* in Liquid Media

Bacteroides cultures must be incubated anaerobically to allow growth, but since they are aerotolerant many manipulations can be done conveniently on the benchtop. The choice of whether to work on the benchtop or in an anaerobic chamber/glove box depends on the needs of the experiment. Culturing performed entirely in the glove box will result in more consistent growth patterns with shorter lag periods, which are necessary for some physiological studies but may be of no consequence when simply inoculating a series of overnight cultures for DNA isolation.

Another important consideration when growing cultures in broth is the type of tube or growth vessel to be used. For experiments in which the culture will remain in the anaerobic chamber or jar, any type of closure that maintains sterility will suffice. However, it often is desirable to grow cultures outside of the chamber due to limited space or the need to frequently monitor growth. In these situations tubes or flasks which can be tightly sealed with a butyl rubber stopper are used. Thus, for long term incubation of culture tubes outside of the glove box the culture tubes must be inoculated in the chamber and then sealed with a sterile rubber stopper (#00 for 13 × 100–mm tubes and #1 for 16 × 150–mm tubes). Screw-cap tubes with a rubber cap liner are useful culture tubes and will maintain an anaerobic atmosphere for a short time when removed from the anaerobic chamber. Another type of tube suitable for anaerobic culturing outside of the glove box is the Hungate tube (Bellco Biotechnology; see Internet Resources). These are standard screw-cap tubes that have a specialized cap (with a 9-mm opening) that is used to hold a flanged, septum-type butyl rubber stopper in place in the top of the tube. When using Hungate tubes inoculation and sampling are done directly through the stopper with a syringe or the cap is taken off and the rubber stopper removed with a hemostat. Another advantage of Hungate tubes is that they can be autoclaved with the caps securely fastened (see Support Protocol 5).

Culturing *Bacteroides* on Solid Media

Solid media are always prepared outside of the anaerobic chamber for the convenience of pouring agar plates on the benchtop and to avoid introducing high levels of moisture into the chamber. The plates are poured in a traditional manner (*APPENDIX 4A*) and then allowed to dry prior to use or are transferred into the anaerobic chamber for storage. Generally resazurin, a redox indicator, is used in the media and it will turn from pink to the reduced colorless form ~2 hr after being put into the chamber. Plates should not be stored outside of the chamber for more than several hours.

In general, spreading or streaking of agar plates is accomplished as described in *APPENDIX 4A* with the following modifications. The inoculation of agar plates either by spread plating or streaking usually is performed outside of the anaerobic chamber and plates are transferred into the chamber for incubation. In this regard the procedures for using anaerobic jars are no different than those used with an anaerobic glove box.

Some genetic or physiological studies require that the organisms not be exposed to oxygen at any time due to their extreme oxygen sensitivity or reduced viability. In these cases it is necessary to perform all plating in the chamber. For spread plating experiments, we have made a large number of bent glass rods which are sterilized in beakers covered with aluminum foil and stored in the chamber until needed. Commercially available sterile plastic spreaders also can be used. Streak plates are done with disposable, plastic inoculating loops. Several packs of sterile loops are brought into the chamber and used as needed.

When storing or incubating plates in an anaerobic glove box it is important to protect them from the drying atmosphere created by the silica gel desiccant. Plates will dry out in several days but this can be avoided by placing cultures in plastic bags for both storage and during incubation.

Bacteroides are robust organisms and grow rapidly in complex media. In most applications we expect to see colonies forming on plates within 24 hr and they will be large enough to count in 36 to 48 hr. *Bacteroides* colonies are distinctive with a smooth rounded morphology, frosty surface, and dull white to pale beige color.

Antibiotics Used in the Culturing of *Bacteroides*

Antibiotics not only play an important role in the management of infectious diseases but they also are critical tools used during the course of genetic and physiological studies of microbes. For example, antibiotic selection of plasmid cloning vectors or for gene disruption techniques are essential applications of antibiotics in modern microbiology but care must be taken in choosing suitable drugs that will work with the *Bacteroides*. These anaerobes are inherently resistant to a wide range of antibiotics typically used in genetic engineering. Most anaerobes, including the *Bacteroides,* are naturally resistant to high levels of aminoglycoside antibiotics and to common β-lactam antibiotics such as ampicillin. Thus some of these drugs, such as gentamicin and kanamycin, would not work well as genetic tools but they are typically used to select for *Bacteroides* from mixed populations that contain facultative anaerobes. Other drugs such as rifampicin, fusidic acid, and nalidixic acid are effective against *Bacteroides* but resistant mutants are available for use in different experimental genetic protocols.

The *Bacteroides* also have acquired antimicrobial resistance in the form of antibiotic resistance genes associated with plasmids and transposons (Smith et al., 1998; Hecht, 2006). These acquired resistance genes have been identified in clinical isolates and they are commonly employed in most of our genetic tools. The most frequently used drugs are tetracycline (*tetQ*) and erythromycin (*ermF*), one or the other of which are found on most of the cloning/shuttle vectors. These drug resistance markers also have been used successfully in transposon mutagenesis strategies and for targeted gene inactivation. The drug cefoxitin (third-generation cephalosporin) also has found some use in genetic engineering applications since the discovery of a gene, *cfxA*, encoding a β-lactamase which degrades it. The carbapenem drugs are one of the "drugs of choice" in treating *Bacteroides* infections and resistance is rare (Hecht, 2006). Although several carbapenem resistance genes encoding metalo-β-lactamases have been identified, they have not found widespread use as genetic tools.

Long-Term Storage of *Bacteroides*

Two approaches are used to store *Bacteroides* strains; they are either flash frozen and stored at −70°C or grown in chopped meat medium and stored in the dark at room temperature. Chopped meat cultures remain viable for more than 10 years but there is some variation in survival depending on the phenotype of the strains. Due to the good longevity and rapid recovery of cells from chopped meat cultures they also are used as "working stocks" for short- or mid-term storage. These cultures will last for months depending on the amount of material taken for each subculturing.

The method of choice for long-term storage is to maintain frozen cultures at −70°C. These frozen stocks are prepared using either glycerol or skim milk as the cryoprotectant and they remain viable for at least 20 years without significant loss in viability. The glycerol stocks are easy to prepare and the medium has a longer shelf life compared to the skim milk medium, but the skim milk medium is a superior cryoprotectant for *Bacteroides*.

BASIC PROTOCOL 1

PREPARING LIQUID CULTURES OF *BACTEROIDES* IN AN ANAEROBIC GLOVE BOX

The following is a typical protocol for starting cultures from a working stock culture in an anaerobic glove box.

Materials

Supplemented brain heart infusion medium (BHIS; see recipe) *or* Tryptone yeast extract glucose medium (TYG; see recipe), 5 ml in 13 × 100–mm Hungate screw-cap tubes

Chopped meat stock culture of *Bacteroides* strain in chopped meat medium (see Basic Protocol 3)

Glove box (Support Protocol 3)
Neoprene gloves
Disposable antiseptic wipes (Allegiance Antiseptic Towelettes)
6″ hemostat
1-ml disposable sterile pipet

1. Prepare a clean work space in the glove box and gather the materials together.

   It is good practice to bring all glassware, plasticware, pipets, and other materials into the glove box at least 4 hr (preferably overnight) prior to use. This is especially important for plasticware which tends to bind oxygen and releases it slowly, which can lead to oxidation of the media.

2. Arrange two tubes of BHIS medium in a rack and fully loosen the caps. Make sure the tubes are well separated in the rack so that there is room to maneuver with the neoprene gloves.

   TYG medium is a versatile, complex medium that can be substituted for BHIS in situations where glucose is not desirable (also see Critical Parameters and Troubleshooting).

3. Gently mix the contents of the chopped meat culture in the Hungate tube, then swab the top of the tube with an antiseptic wipe. Take off the screw cap and carefully remove the stopper with the hemostat. Place the stopper upside down in a clean location.

4. Using a 1-ml pipet remove 0.6 ml from the culture, then lift the cap to one BHIS tube, dispense 0.4 ml of the culture, and then repeat with the second BHIS tube adding 0.2 ml to it.

   Growth of cultures from chopped meat stocks can vary depending on the age of the culture, types of antibiotics present in the media (Table 13C.1.1), or other factors. Thus, usually two amounts of inoculum are used to optimize the results. If equal growth is observed in both tubes, then the one that received the lower inoculum volume is used for subsequent operations.

Table 13C.1.1 Commonly Used Antibiotics in Genetic Studies

Antibiotic	Resistance gene	Stock solution (mg/ml)	Working concentration (μg/ml)
Antibiotics used in plasmid and transposon vectors			
Cefoxitin	*cfxA*	20 in dH_2O, filter sterilize	20-25
Chloramphenicol	*cat*	25 in 95% ethanol	25
Clindamycin	*ermF*	10 in dH_2O, filter sterilize	5
Erythromycin	*ermF*	50 in 95% ethanol	10-20
Tetracycline[a]	*tetQ*	10 in 70% ethanol	4-10
Other useful antibiotics			
Gentamicin		50 in dH_2O, filter sterilize	50-200
Nalidixic acid[b]		20 in 0.1 M NaOH	40-50
Rifampicin[a]		20 in 100% methanol	20
Fusidic acid		25 in 95% ethanol	50
Trimethoprim		Add powder to cooled media	100

[a]Light sensitive and unstable.

[b]In the sodium salt form, make stock in dH_20 and filter sterilize.

An alternate method to prepare cultures from chopped meat stocks in Hungate tubes is to simply wipe the top of the tube with an antiseptic wipe and then insert a 1-ml syringe (20-G needle) into the tube. Remove the indicated amount of culture to transfer to the new tubes.

One also can inoculate broth cultures from plates. The only difference is that disposable inoculating loops are used to pick colonies and are then swirled in the tubes to dislodge the cells. These tubes can be vortexed to further break up the cell clumps and ensure even growth in the tube.

5. Screw the caps into place and then replace the stopper on the chopped meat culture making sure that it is well seated in the tube.

6. Place the inoculated tubes at 37°C in the glove box incubator.

Dense growth (A_{550} > 0.8) of the cultures should be observed following overnight incubation.

Growth will be slower if antibiotics (Table 13C.1.1) are added to the medium and the generation time varies greatly with different media.

PREPARING LIQUID CULTURES OF *BACTEROIDES* IN AN ANAEROBIC JAR

BASIC PROTOCOL 2

The following is a typical protocol for starting cultures from a petri plate followed by incubation in an anaerobic jar.

Materials

BHIS medium (see recipe) or TYG medium (see recipe), 5 ml in 13 × 100–mm screw cap tubes in rack
Bacteroides strain streaked on BHIS or TYG plate (see Strategic Planning)

Sterile culture tubes
Inoculating loop
Bunsen burner
Anaerobic jar (Support Protocols 1 and 2)
37°C incubator

1. Dispense freshly prepared BHIS medium into sterile culture tubes. Gather other materials and cultures.
2. Sterilize loop, allow to cool, and pick up cells from a large colony.
3. Remove cap from culture tube, flame lip of tube, and swirl loop in medium.
4. Replace cap on tube, mix well, and place in round test tube rack.

   Ensure that the caps on the culture tubes are sufficiently loose to allow exchange of gasses.

5. Place rack in jar and then process jar as described below using GasPak gas-generating envelopes or as described for the vented jars.
6. Place jar upright in a 37°C incubator.

ESTABLISHING AND MAINTAINING AN ANAEROBIC ENVIRONMENT

There are two fundamental conditions that must be met for reproducible culturing of *Bacteroides*. That is, one must establish a relatively low redox potential in the media and oxygen must be removed from the atmosphere. The redox potential is generally controlled by using freshly prepared media to which a reducing agent, usually cysteine, has been added. Due to the limited capacity of reducing agents, media should be used on the day of preparation unless it can be stored under anaerobic conditions. Immediately upon inoculation of either solid or liquid media, the cultures must be placed under appropriate anaerobic conditions with oxygen concentrations <0.5%. The atmosphere most commonly used for culturing *Bacteroides* contains 5% or 10% CO_2 with the balance made up of N_2; however, depending on the system used to generate anaerobic conditions, the atmosphere may also contain 5% or 10% H_2 which is utilized by catalyst systems in the removal of oxygen. The hydrogen reacts with oxygen in the presence of a palladium catalyst to form water effectively removing the oxygen from the atmosphere. Investigators should not use H_2 concentrations >10% since these are explosive and must be carefully monitored.

The four most common systems used to establish the anaerobic conditions needed for *Bacteroides* culturing are sealed Brewer's jars, vented anaerobe jars, anaerobic chambers, and the gassing cannula. These methods apply similar principles of oxygen removal but differ in the implementation of those principles. Each method will be discussed briefly below but the reader is encouraged to review the references provided and the Internet Resources for a more detailed account of the techniques and their theoretical basis.

SUPPORT PROTOCOL 1

Sealed Brewer's Jars

The routine use of sealed anaerobic jars for cultivation of anaerobes was made possible by the development of a self-contained, disposable H_2/CO_2 generation system and it still remains one of the most cost-effective methods in anaerobic microbiology (Brewer and Allgeier, 1966). In these commercially available systems hydrogen is produced when water activates a sodium borohydride tablet; the hydrogen then reacts with oxygen and the palladium catalyst present in the lid of the jar. Carbon dioxide (4% to 10%) is generated from a sodium bicarbonate/citric acid tablet and an anaerobic atmosphere suitable for growth of *Bacteroides* species is produced in ~90 min.

Materials

Palladium catalyst (Coy Laboratories; in wire mesh basket attached to jar lid)
Cultures (rack of culture tubes or streaked petri plates)
10 ml tap or dH_2O

Polycarbonate GasPak jar or equivalent with lid
GasPak anaerobic system envelope

10-ml pipet or syringe
GasPak anaerobic indicator
37°C incubator

NOTE: GasPak is a trademark for the Becton, Dickinson and Company (BD). The Oxoid Company also supplies Brewer-type jars and anaerobic gas-generating envelope systems that work equally well for the growth of *Bacteroides* species and the Mitsubishi Gas Chemical-America Company supplies gas-generating envelopes.

1. Fill the wire mesh basket with 2 to 3 g of palladium catalyst pellets and attach it to the jar lid.

   The palladium catalyst must be rejuvenated after each use by heating at 160° to 170°C for at least 2 hr. After rejuvenation the pellets should be stored desiccated or in a dry environment. Catalyst pellets can become contaminated by hydrogen sulfide over time and must be replaced. Replacement is indicated when the anaerobic indicator fails to turn colorless within 6 hr.

   GasPak envelopes are now available that have a disposable palladium catalyst sachet attached to the envelope. This avoids the need to monitor the palladium catalyst but greater expenses will be incurred.

2. Cut off the corner of a GasPak envelope and place it upright in the jar together with the rack of petri plates or tubes containing the cultures. Add 10 ml of water to the gas generation packet with a pipet or syringe.

   When placing any liquid cultures in the jar, the caps of the culture tubes must be loose to allow adequate circulation of the atmosphere. Also, avoid stacking petri plates directly under the catalyst basket since extreme heat is generated during the reaction.

   Two jar sizes are available. The smaller 2.5 liter jar can accommodate one rack of 12 standard petri plates and requires one GasPak envelope. The 9.5 liter jar uses three envelopes and can accommodate 36 standard petri plates.

3. Open an anaerobic indicator strip and place it in the jar. The strip is saturated with methylene blue and turns from blue to colorless in the absence of oxygen.

   The strip should begin to turn colorless within 60 min of closing the jar and it should be completely colorless within a few hours.

4. Quickly place the lid on top of the jar and secure with the lid clamp. Place the jar in a 37°C incubator for 24 to 48 hr. The jar must remain upright at all times in order to avoid spillage of the water.

   Within an hour of incubation the user can check progress of the anaerobic system by examination of the indicator strip visible through the clear polycarbonate jar and by the appearance of condensation on the inside of the jar indicating the formation of water vapor as the free oxygen is removed. The use of the clear polycarbonate jars also is advantageous since cultures can be inspected periodically without having to open the jar.

Vented Anaerobe Jars

SUPPORT PROTOCOL 2

The use of vented anaerobe jars is essentially the same in principle as the sealed Brewer's jars except that instead of using a gas generating system the atmosphere of the jar is evacuated and replaced with an anaerobic gas mixture, usually consisting of 85% N_2, 10% CO_2, and 5% H_2. Any oxygen remaining in the atmosphere is removed by reaction with hydrogen in the presence of a palladium catalyst. The advantage to this method is that an anaerobic atmosphere is generated much more quickly due to the considerably lower concentrations of oxygen remaining after replacement of the jar atmosphere with the gas mixture. The use of vented jars is somewhat more cumbersome and requires a slightly greater initial investment than the sealed Brewer's jars, but the

supply costs remain relatively low since it does not require purchase of the gas-generating envelopes.

Materials

Palladium catalyst (in wire mesh basket attached to jar lid)
Cultures (rack of culture tubes or petri plates)

Polycarbonate GasPak jar or equivalent with lid
GasPak anaerobic indicator
Standard vacuum pump with gauge, tubing, and three-way stopcock (Fisher Scientific)
Gas Mixture cylinder (85% N_2, 10% CO_2, and 5% H_2) and regulator
37°C incubator

NOTE: An automatic evacuation/replacement system, Anoxomat (see Internet Resources), that precisely evacuates and fills jars by a microprocessor controlled pumping unit is now available. The standard Anoxomat configuration has one gas connection and one jar connection but options are available for connecting up to 5 jars at one time.

1. Fill the wire mesh basket with 2 to 3 g of palladium catalyst pellets and attach it to the jar lid.

   The same considerations for care and maintenance of the palladium catalyst exist as stated in Support Protocol 1.

2. Place all cultures in the jar, open an anaerobic indicator strip, place it in the jar, close the lid and secure with the lid clamp.

   The jars used in this method are essentially the same as those described in Support Protocol 1 except that they are vented with a hose nipple to which is attached a short length of rubber tubing and a screw clamp to control air flow. Commercially available jars all have a vent option.

3. Attach the jar to a vacuum pump via the vent nipple and ensure the screw clamp is fully open. Evacuate the jar to ~25″ Hg. Using the stopcock direct the air flow away from the vacuum pump to the gas cylinder and fill the jar slowly with gas being careful not to overfill the jar. Repeat the evacuation and fill again.

   The anaerobe jars are designed to hold a vacuum but cannot hold positive pressure. Thus, there is little danger of overfilling a jar to the point where it will burst; however, close attention must be paid to the gas replacement step so that the jar lid does not become damaged.

   It is generally convenient to construct a small gassing manifold which has the stopcock valve and tubing fixed to a wooden platform together with a vacuum gauge to monitor pressure in the jar (Fig. 13C.1.1). The idea is to design the manifold so that the user can control direction of the air flow at the proper time between the vacuum pump and the gas cylinder. The vacuum gauge should be connected to the manifold by a T connector and should be located between the jar and the first stopcock valve. The manifold greatly simplifies the evacuation and filling process and ensures that the jars will not be overfilled with gas.

4. Finally, evacuate one more time and then use the stopcock to direct airflow to the gas mix cylinder and proceed to fill the jar with the anaerobic gas mixture. Close the screw clamp and disconnect the jar from the vacuum pump assembly.

5. Incubate the jar 24 to 28 hr at 37°C. Inspect the anaerobic indicator after several hours to ensure proper functioning of the catalyst system.

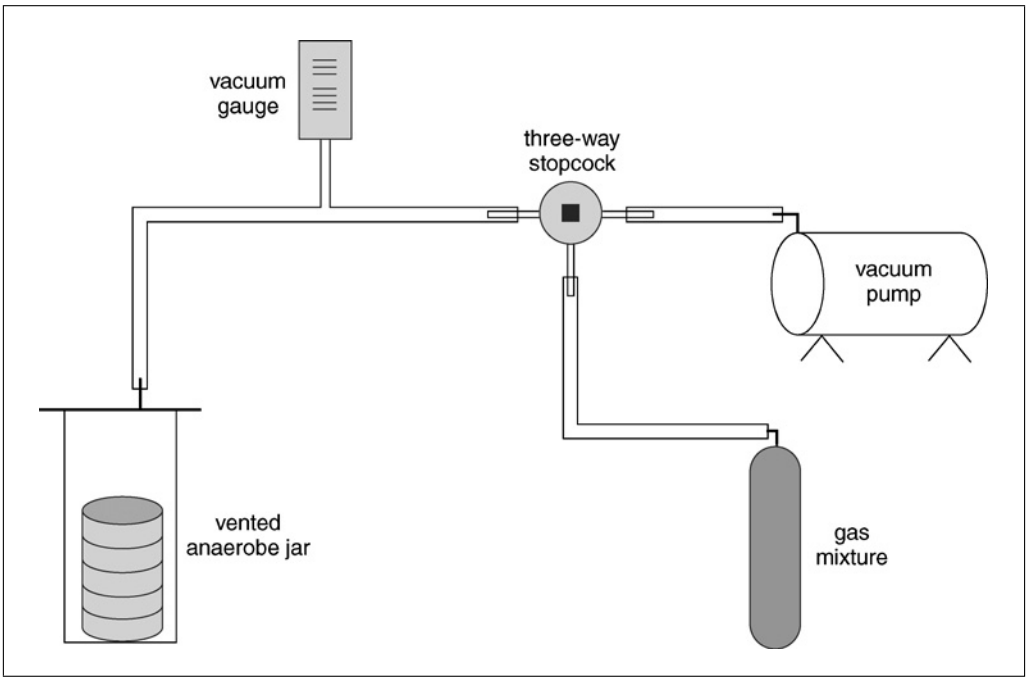

Figure 13C.1.1 Typical gassing manifold for the filling and evacuation of vented anaerobic jars using a standard vacuum pump and gas mixture.

Anaerobe Chamber

SUPPORT PROTOCOL 3

The anaerobic chamber or anaerobic glove box is a self-contained, closed system providing the investigator with an anaerobic laboratory bench where nearly any experiment can be performed under anaerobic conditions. The only disadvantage of the anaerobic chamber is in the initial cost of the investment but the experimental benefits far outweigh the cost for a laboratory routinely engaged in anaerobic microbiology. Anaerobic chambers accommodate an incubator, allowing the culture of plates, flasks, and tubes without the need for jars. There also is shelving space to allow the storage of media and other materials, thus eliminating the need to freshly prepare media before use. Cultures also can be stored in the anaerobic chamber on plates but care must be taken to ensure that they do not dry out.

As with the vented anaerobic jars, the glove box atmosphere contains 5% to 10% CO_2, 5% to 10% H_2, and the balance will be nitrogen or argon. An anaerobic atmosphere with <5 ppm of O_2 is maintained in the glove box by circulation through wire screen filters containing palladium catalyst. All materials (e.g., cultures, media, and tubes) enter the chamber through a double door airlock which is purged with anaerobic gas and thus functions to limit the amount of oxygen that enters the glove box. Most modern anaerobic chambers possess an automated airlock that simplifies their use and makes if difficult to accidentally introduce large amounts of air into the chamber by inappropriate opening of the airlock doors. Once one becomes at ease with the inconvenience of working with the neoprene gloves they will find the anaerobic chamber to be a convenient, efficient way to culture the *Bacteroides*. There are several types of chambers available, from rigid to flexible, and gloved to gloveless (see Internet Resources). By far the most adaptable and cost effective are flexible vinyl glove boxes manufactured by Coy Laboratory Products and based on the original design by Aranki and Freter (Aranki et al., 1969). Only use of the flexible glove box will be considered in this unit.

Materials

Palladium catalyst, 4 packs (wire screen enclosure containing ~165 g catalyst)
Silica gel desiccant type IV, 2 trays each containing ~ 2.5 kg desiccant (Sigma Chemical)
Bacterial cultures

Coy flexible glove box (2 person) with 37°C forced air incubator (Coy Laboratories)
Oxygen/hydrogen meter (Coy Laboratories)
Nitrogen gas cylinder and regulator (Coy Laboratories)
Gas mixture cylinder (80% N_2, 10% CO_2, and 10% H_2) and regulator (Coy Laboratories)

1. Gather materials together and open the outer airlock door. Place the catalyst, trays of desiccant, and other materials inside the airlock. Close the outer airlock door and initiate the purging cycle to lower the oxygen level in the entry lock.

   CAUTION: *Hot liquids should not be brought through the airlock since they will boil over during the vacuum cycle. Allow them to cool to at least 60°C.*

2. The purging cycle will evacuate and replace the airlock atmosphere with N_2 twice and then following a final evacuation the air lock is filled with the anaerobic gas mixture. When the cycle is complete, check the front panel of the airlock to determine that the anaerobic indicator light is on and then open the inner airlock door. Remove all items from the airlock and then place in any outgoing materials before closing the inner door.

   The anaerobic indicator light is used to signal the status of the airlock and is activated following the purging cycle and remains lit until the outer airlock door is opened. The indicator light should always be checked prior to opening the inner airlock door in order to prevent accidental contamination of the chamber atmosphere with oxygen.

3. Place the catalyst packs into the two catalyst fan boxes and set the trays of desiccant on the shelving unit. Check the oxygen/hydrogen meter to ensure safe, efficient operation of the glove box.

   Four catalyst packs (two in each fan box) are needed in very active chambers and they should be changed about twice a week. Humidity can get high in active chambers due to oxygen removal so the silica gel desiccant should be changed twice a week or more according to the level of humidity as shown by the indicator in the desiccant.

   To rejuvenate the catalyst, heat in an oven at 160°C for 2 hr, then store with a desiccant until needed. The life expectancy of the catalyst pellets is ~1 year but can vary depending of the level of usage and exposure to hydrogen sulfide which poisons the catalyst. Refer to Critical Parameters and Troubleshooting for instructions on how to determine if the catalyst is still active. Assessing changes in the color indicator present in the media is not a useful indicator of the catalyst state as there are many possible causes to such a change. Desiccant is dried before use in a 110°C oven and stored in an airtight container.

   A chamber using a 10% hydrogen gas mixture should maintain internal levels of hydrogen between 4% to 6%. Higher hydrogen levels suggest a decrease in catalyst efficiency and lower hydrogen levels indicate a recent exposure to oxygen or infrequent use of the chamber. The hydrogen in the chamber atmosphere is only replaced during entry through the airlock and the levels can become low over time in an inactive chamber. If hydrogen levels fall < 1% to 2% additional gas mixture should be added through the airlock.

4. Place bacterial cultures in the incubator and other materials on the shelving unit.

   When first bringing cultures into the glove box loosen the caps to allow circulation of the anaerobic atmosphere in the tubes or flasks. Likewise, fresh media should be stored overnight with the caps loosened to ensure that all oxygen diffuses out of the media and that there is full exchange of the head space of the media vessels with the anaerobic atmosphere.

The redox indicator resazurin (final concentration 0.0001%) is routinely incorporated into many of our culture media. This allows easy monitoring of the media to ensure that it is fully reduced prior to inoculation and it provides a backup check on the atmosphere in the glove box. The indicator is colorless when fully reduced at −110 mV and red when oxygen is present and the redox potential goes above −50 mV.

5. Open the outer door and remove items from air lock. Recycle the airlock leaving it in the anaerobic state if not planning additional use of the glove box.

Anaerobic Gassing Cannula

SUPPORT PROTOCOL 4

Not every anaerobic culturing situation that arises can be handled in a glove box or with anaerobic jars. There will be times that air must be excluded from a culture vessel on the benchtop during the course of specialized media preparation, inoculation, or sampling. These circumstances require the use of a gassing cannula attached to a source of oxygen-free gas. Oxygen is excluded by directing a stream of anaerobic gas into the culture vessel thus maintaining anaerobiosis.

The basic use of a gassing cannula is described here in order to support other protocols described in this unit. The reader is directed to Holdeman et al. (1977), Bryant (1972), and Breznak and Costilow (1994) for more detailed descriptions of using gassing cannulas and the preparation of prereduced media.

Materials

Tube of chopped meat medium (Support Protocol 5)
Bacteroides strain streaked on BHIS or TYG plate (see Strategic Planning)

Gas mixture cylinder (90% N_2, 10% CO_2, 10% H_2) and regulator (Coy Laboratories)
Gassing cannula with 6″, 16-G blunted needle bent at 90° (Popper & Sons)
Bunsen burner
Test tube rack
Inoculating loop
6″ hemostat
37°C incubator

1. Open the regulator for the gas mixture and adjust the gas flow so that a gentle stream of gas flows from the end of the cannula with a force great enough to put a small dent into the Bunsen burner flame.

   The gassing cannula is a 2-ml Luer-Lok glass syringe. The syringe barrel is filled with cotton and then sterilized. The syringe is then attached to the gas source via 1/4″ rubber tubing.

   It is not desirable to have such a strong flow of gas from the cannula that the culture media will splatter or bubble up from the vessel.

2. Flame the top of the chopped meat medium tube and quickly remove the rubber stopper with the hemostat, being careful to not contaminate the rubber stopper. Place the tube in the rack.

3. Quickly sterilize the cannula by heating the bent needle in the Bunsen burner until it is red, then place it in the tube of medium so that it rests on the lip of the tube.

4. Sterilize the inoculating loop and then pick a colony from the plate. Transfer the colony to the tube of medium by gently stirring the loop in the medium.

   Antibiotics (Table 13C.1.1) and other additions to the medium can be made while the tubes are under the flow of gas.

5. Retrieve the rubber stopper, briefly flame it, and reseal the tubes quickly by pulling up on the cannula as the stopper is being pushed down in place. Once the tube is closed twist the stopper to ensure a good seal and the turn off the gas.

   Care must be taken when closing tubes and other culture vessels with the rubber stoppers. Do not use too much force or push down excessively since the tubes will break and injury can result. Use a twisting motion to ensure a good seal with minimal force.

6. Incubate the tube for 18 to 24 hr at 37°C.

BASIC PROTOCOL 3

LONG-TERM STORAGE OF *BACTEROIDES* AT ROOM TEMPERATURE

The long-term storage of stock cultures at room temperature provides a useful backup for frozen stock cultures. These stock cultures do not require any special storage conditions (no refrigeration is necessary) and they remain viable for >10 years.

Materials

Chopped meat medium stock cultures (Support Protocol 5)
Bacteroides strain streaked on BHIS or TYG plate (see Strategic Planning)
37°C incubator

1. Inoculate chopped meat medium stock cultures with a single-well isolated colony from an agar plate.
2. Incubate the culture 36 to 48 hr at 37°C. Store the cultures in the dark at room temperature.

   Due to the inherent turbidity of the chopped meat medium it is not always easy to determine if adequate growth has occurred; thus, cultures are usually incubated for the prescribed length of time to ensure adequate growth. Cultures grown in this way will remain viable for ~10 years.

   The inoculation of chopped meat cultures can be done as described above in the gassing cannula protocol or they can be inoculated from plates in the anaerobic chamber as described under the inoculation of liquid cultures protocol.

   The growth in chopped meat cultures is not as vigorous as in some other media due to the low levels of carbohydrate present in the medium, but it is this very fact that makes chopped meat a good storage medium. The low carbohydrates result in low acid production which consequently does not stress the cells.

SUPPORT PROTOCOL 5

CHOPPED MEAT MEDIUM

This medium is used for long-term and short-term storage of *Bacteroides* strains; thus, it needs to be well reduced and free of any oxidized components. To ensure the most consistent results this medium is prepared under more stringent anaerobic conditions during all steps.

Materials

Extra lean ground beef
1 M NaOH
Tryptone
Yeast extract
KH_2PO_4
K_2HPO_4
Hemin solution (see recipe)
Resazurin solution (see recipe)
Cysteine (free base, Sigma)
10% $NaHCO_3$ (see recipe)

2-liter beaker
Stir bar
Bunsen burner
Aluminum foil
Cheese cloth
Graduated cylinder
2-liter flask
Gassing cannula (Support Protocol 4)
Gas cylinder (either a gas mix or 100% N_2) and regulator (Coy Laboratories)
Laboratory tape
Anaerobe chamber
Hungate tubes (Bellco Biotechnology), 16 × 125–mm with septum stoppers and screw caps

1. In a 2-liter beaker put one liter dH_2O, a stir bar, 500 g extra lean ground beef, and 25 ml of 1 M NaOH. Bring to a boil with slow stirring and let boil for ~5 min.
2. Cover the beaker with aluminum foil and place at 4°C overnight or until fat solidifies. Remove ALL of the fat and discard it.

   The fat comes to the surface and is easily removed.
3. Strain the meat broth through several layers of cheese cloth, retain both the broth and the meat particles. Spoon off any remaining fat floating on the surface of the strained broth.
4. Pour the broth into a graduated cylinder and bring up volume to one liter with dH_2O.
5. Pour the broth into a 2-liter flask and add 30 g tryptone, 5 g yeast extract, 2.5 g KH_2PO_4, 2.5 K_2HPO_4, 10 ml hemin solution, and 1 ml resazurin solution.
6. Using the gassing cannula, bring the broth to a boil under a stream of N_2 or N_2 + CO_2 gas and boil for ~2 min. Cool the broth on ice while still gassing. When cooled stopper flask tightly, secure the stopper with laboratory tape, and then put the flask into the anaerobe chamber.

   Boiling the medium under a stream of oxygen-free gas will drive off all oxygen from the medium and as it cools under the stream of anaerobic gas there will be little or no oxygen contamination. This mixture is high in organic material and can rapidly boil over if care is not taken to adjust the flame as the boil begins.
7. While the broth is cooling prepare about 100 Hungate culture tubes in racks by adding ground beef meat particles so that they are evenly distributed among the tubes. Place chopped meat tubes, rubber stoppers, and screw caps into the anaerobe chamber and allow them to equilibrate for a short time.

   Placing the meat into the tubes can be facilitated by pressing the tubes into the meat and then using a 1-ml disposable pipet or glass rod to push the meat to the bottom of the tube.
8. Add 1.0 g of cysteine and 20 ml of $NaHCO_3$ (10% solution) to the meat broth and mix by swirling.
9. When cysteine is dissolved dispense 10 ml of broth into each tube, then secure a stopper into place, and screw on the cap. Ensure that the cap is tightened to prevent leakage of the anaerobic atmosphere during autoclaving.
10. Remove tubes from chamber and autoclave for 30 min.

    The sealed Hungate tubes with the tightened cap are designed to be autoclaved directly in the test tube racks. These tubes will easily withstand the internal pressure resulting from the autoclave temperatures and they will maintain their anaerobic atmosphere.

BASIC PROTOCOL 4

FROZEN GLYCEROL STOCK CULTURES OF *BACTEROIDES*

The preservation of *Bacteroides* by deep freezing is the preferred choice for preparing long-term stock cultures. These organisms retain high viability, providing that the cultures are flash frozen, as described below. In addition, it is advisable to minimize the number of times a culture is thawed and refrozen, since this also can reduce viability of the cultures. This protocol utilizes glycerol as the cryoprotectant.

Materials

BHIS medium and plate (see recipe)
Glycerol
Overnight culture (Basic Protocol 1)

125-ml bottle
Glass rod or rubber spatula
2-ml Cryogenic vials (Nalgene)
Dry ice/ethanol bath

1. Prepare 70 ml of BHIS medium without cysteine, resazurin, or $NaHCO_3$. Add 30 ml of glycerol, filter sterilize, and store in a 125-ml bottle.
2. Inoculate a BHIS plate containing the appropriate antibiotics (Table 13C.1.1) with 0.1 ml of an overnight culture. Incubate overnight or up to 48 hr at 37°C.
3. Label cryogenic vials and then cover label with clear tape.
4. Pipet 2 ml of BHIS/glycerol onto the surface of the plate, harvest cells from plate using a glass rod or rubber spatula and place in cryogenic vial.
5. Flash freeze in a dry ice/ethanol bath and store at −70°C.

BASIC PROTOCOL 5

FROZEN SKIM MILK STOCK CULTURES OF *BACTEROIDES*

This is an alternative method for cryopreservation of *Bacteroides*. Here the glycerol is replaced with skim milk as the cryoprotectant.

Materials

Bacto skim milk (Difco)
BHIS plate containing the appropriate antibiotics (see recipe)
Overnight culture (Basic Protocol 1)

Glass rod or rubber spatula
2 ml Cryogenic vial (Nalgene)
Dry ice/ethanol bath

1. Add 150 g Bacto skim milk to 1 liter dH_2O. Stir ~30 min or until powder is dissolved. Autoclave for 10 min (longer sterilization times will result in curdled milk).
2. Store medium at 4°C until needed.
3. Inoculate a BHIS plate containing the appropriate antibiotics (Table 13C.1.1) with 0.1 ml of an overnight culture. Incubate overnight and up to 48 hr at 37°C.
4. Label cryogenic vial and then cover label with clear tape.
5. Pipet 2 ml of skim milk medium on to the lawn of cells and harvest with a glass rod or rubber spatula.
6. Place cell mixture into the cryogenic tube.
7. Flash freeze in a dry ice/ethanol bath and store at −70°C.

DETERMINATION OF THE GROWTH CURVE

The growth curve is an essential protocol used to compare basic physiological properties of different *Bacteroides* species or mutant strains. These basic features are often best determined under defined conditions; thus a minimal, defined medium is best suited for these studies. The *Bacteroides* have simple nutritional needs and the defined medium of Varel and Bryant (1974) has been used for several decades with only slight modification. In this protocol the basic defined medium with glucose as the carbohydrate source and ammonia as nitrogen source will be described but this medium can be easily modified to meet any nutritional requirements that an experiment demands.

It is possible to perform growth rate measurements with a variety of culture tubes using this protocol. The only stipulation is that if screw-cap tubes are to be used they must be returned to the incubator inside of the anaerobic glove box between OD readings. This is necessary since screw-cap tubes will not maintain an anaerobic atmosphere for extended periods.

Materials

100 ml bottle of defined minimal medium (see recipe)
Chopped meat stock culture of *Bacteroides* in chopped meat medium (see Basic Protocol 3)

Anaerobic glove box
13 × 100–mm sterile screw cap tubes in rack
Antiseptic wipes
Hemostat
37°C incubator
125-ml side arm flask (sterile, aluminum foil cap)
1- and 10-ml pipets, disposable
Sterile #3 rubber stopper (in covered beaker)
Spectrophotometer

1. Gather materials together in the anaerobic glove box and prepare work space by placing all items within easy reach.
2. Arrange four tubes in the rack, completely loosen caps, and dispense 5 ml of defined minimal medium into the tubes immediately replacing the caps.
3. Gently mix the chopped meat culture, clean the top with an antiseptic wipe, unscrew cap, and remove the stopper with a hemostat.
4. Remove 1 ml of broth from the chopped meat culture and add 0.5 ml, 0.3 ml, and 0.2 ml respectively to the tubes containing medium. Tighten the caps and mix the tube. Place in the 37°C incubator overnight.

   The fourth tube of medium will be used to zero the spectrophotometer during the growth curve determination.

   Initial growth of the inoculum in defined medium can be variable when coming from a stock culture thus it is necessary to use several inoculum amounts to ensure adequate growth.

5. Carefully remove the foil from the top of the 125-ml flask and place upside down in a clean location. Add 50 ml of medium using a 10-ml pipet, replace the foil cap and place in the incubator.
6. The following day remove flask and tubes from incubator and inspect tubes for growth.

7. Choose the tube with the most growth (most turbid). If all tubes have equal growth then pick the tube with the lowest initial inoculum size and place it in a rack. Loosen the cap, remove 1 ml and add to the flask.
8. Using a hemostat, carefully grasp a sterile #3 stopper from the beaker and seal the flask. Twist the stopper to ensure a tight seal and then remove from the anaerobic chamber together with the tube of sterile medium.
9. Set the spectrophotometer to A_{550} and zero with the tube of sterile medium. Record the initial OD from the side arm and then place the flask in a 37°C water bath.
10. Continue to record OD readings as needed until growth has completed ~20 to 30 hr.

REAGENTS AND SOLUTIONS

Defined minimal medium (Varel and Bryant, 1974)

Ingredients per liter:	
Mineral 3B solution (see recipe)	50 ml
Glucose (20% in H_2O, filter sterilized)	25 ml (0.5%, 28 mM)
L-cysteine (free base)	1 g
Hemin solution (see recipe)	10 ml
L-methionine (0.2%, in H_2O, filter sterilized)	10 ml
$FeSO_4$ solution (see recipe)	1.5 ml
$NaHCO_3$ (10% sterile solution; see recipe)	20 ml
H_2O	885 ml
Agar (when required)	15 g

Mix together components except for $NaHCO_3$ and adjust the pH to 7.1 using 1 N NaOH. Autoclave for 20 min. Cool in water bath at 50° to 55°C before adding 20 ml of sterile 10% $NaHCO_3$.

Other carbohydrates can be substituted for glucose and 1 ml of 0.01% vitamin B_{12} solution can be substituted for methionine.

$FeSO_4$ solution

For 100 ml:

Dissolve 0.278 g of $FeSO_4 \cdot 7H_2O$ in 100 ml distilled water. Add two drops of concentrated HCl.

Hemin solution

For 200 ml:

Dissolve 100 mg of hemin in 2 ml of 1 M NaOH and then bring up volume to 200 ml with dH_2O. Store in an amber bottle up to 1 year at 4°C.

Mineral 3B solution

Ingredients per liter:	
KH_2PO_4	18 g
NaCl	18 g
$MgCl_2 \cdot 6H_2O$	0.4 g
$CaCl_2 \cdot 2H_2O$	0.52 g
$CoCl_2 \cdot 6H_2O$	0.02 g
$MnCl_2 \cdot 4H_2O$	0.2 g
NH_4Cl	10.0 g
Na_2SO_4	5.0 g

Dissolve salts in 800 ml dH_2O, then bring volume up to 1 liter. Autoclave for 20 min.

$NaHCO_3$, 10%

Dissolve 50 g into 500 ml H_2O, filter sterilize.

Resazurin, 0.1%

Dissolve 0.1 g of resazurin (Sigma Chemical) in 100 ml distilled water.

Salts solution "A"

Ingredients per liter:	
$CaCl_2 \cdot 2H_2O$	0.26 g
$MgSO_4 \cdot 7H_2O$	0.48 g
KH_2PO_4	1 g
K_2HPO_4	1 g
NaCl	2 g

Mix $CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$ in 300 ml H_2O until dissolved. Then add 500 ml H_2O and add remaining salts. When dissolved add 200 ml H_2O and store indefinitely at 4°C.

Supplemented brain heart infusion medium (BHIS)

This medium is used for routine culturing of *Bacteroides* and allows for rapid reproducible growth.

Ingredients per liter:	
Brain Heart Infusion Broth powder (Difco)	37 g
Cysteine (free base, Sigma)	1.0 g
Hemin solution (see recipe)	10 ml
Resazurin solution (0.1%, optional; see recipe)	1 ml
$NaHCO_3$ solution (sterile 10%; see recipe)	20 ml
H_2O	970 ml
Agar (if required)	15 g

Add all dry components to a flask containing the water, swirl until dissolved and then add the hemin and resazurin solutions. Aliquot the medium to 125-ml bottles (or any appropriate size bottle) and autoclave immediately for 20 min. When medium has cooled to ~60°C place in the anaerobic chamber for storage.

Add 2 ml of $NaHCO_3$ solution to each 100 ml of medium prior to use and then aliquot medium into sterile screw-cap tubes as needed. Do not add $NaHCO_3$ prior to autoclaving as it will be converted to CO_2 and lost from the medium. Antibiotics (Table 13C.1.1) that are required must be added just prior to inoculation of tubes.

For solid medium prepare as above, autoclave, and then place in a 55°C water bath. When the medium is cool, add the sterile $NaHCO_3$ and antibiotics if needed. Pour plates on the benchtop or in a Biosafety hood. Allow the plates to dry for several hours and then use immediately or place in the anaerobic chamber for storage.

Tryptone yeast extract glucose (TYG) medium

Ingredients per liter:	
Tryptone	20 g
Yeast Extract	10 g
Glucose	5 g
Cysteine (free base)	1 g
Salts solution "A" (see recipe)	40 ml

continued

Hemin solution (see recipe)	10 ml
Resazurin solution (0.1%; see recipe)	1 ml
Agar (if required)	15 g
NaHCO3 (10% solution; see recipe)	20 ml

All of the components except $NaHCO_3$ are added to 930 ml of H_2O. Then adjust pH to 7.0 and autoclave for 20 min. When medium has cooled to 55°C add sodium bicarbonate solution.

COMMENTARY

Background Information

In 1989 the genus *Bacteroides* was restricted and included just species associated with the human intestinal tract forming a phylogenetically and physiologically uniform group of Gram-negative, nonsporeforming, nonmotile, anaerobic rods. The current *Bacteroides* species are *B. fragilis, B. thetaiotaomicron, B. ovatus, B. uniformis, B. vulgatus, B. distasonis, B. eggerthii, B. caccae, B. merdae,* and *B. stercoris.* The *Bacteroides* are the most abundant of the indigenous intestinal flora making up to 30% of the total cultivable microbes in the gut. Although these organisms are capable of limited survival outside the mammalian host, they are best considered as obligate symbionts where they contribute to the normal intestinal development and physiology. During the past few years, interest in the *Bacteroides* has been rekindled by new discoveries of their ability to directly communicate with the host and influence development of the gut (Hooper et al., 2001). Other studies have shown that they also may have a fundamental role in the normal development of the immune system where it is thought that they modulate CD4 T cell expansion in the spleen (Mazmanian et al., 2005). These host-associated organisms also are potential opportunistic pathogens when they are translocated to normally sterile sites such as the peritoneal cavity and *B. fragilis* is the most commonly isolated anaerobic pathogen in such situations. The *Bacteroides* do not possess a wide array of virulence factors and it is becoming more clear that the same properties that allow them to evade the immune system and cause infection also permit them to successfully colonize the gut as normal flora.

The intestinal tract is a unique environment which has shaped the growth and culturing characteristics of *Bacteroides*. This is an anaerobic environment with carbon dioxide levels up to 10% and oxygen averaging $<1\%$. Other gases such as hydrogen and methane also are present, indicating that the gut atmosphere is the product of a complex anaerobic metabolism. This is a highly reducing environment and the redox potential usually runs in the range of -150 to -350 mV. The pH varies along the length of the tract from slightly acidic along the ascending colon to neutral or mildly alkaline conditions at the distal colon. The temperature is constant at $\sim$37°C and there is a constant influx of nutrients. It is not surprising that this is a highly competitive environment and home to some 500 species of bacteria. The *Bacteroides* have proven to be the most successful microbes in the human gut and the renewed interest in their interactions with the host promises to reveal new insight into their roles in human health. For example, a colonic microflora with increased numbers of *Firmicutes* and a smaller population of *Bacteroides* has been associated with obesity in animal models (Turnbaugh et al., 2006).

Critical Parameters and Troubleshooting

Anaerobic conditions

Most problems encountered with culturing the *Bacteroides* can be attributed to a lack of adequate anaerobic technique. Anaerobic culturing is as much a philosophy as it is a technique and the researcher needs to scrutinize all aspects of an experiment to ensure that reasonable consideration has been given to maintaining anaerobiosis at all stages of growth. Even low levels of oxygen contamination can inhibit growth of these organisms and influence experimental outcome. Thus, it is important to use anaerobic indicators whenever possible as these will allow the investigator to eliminate aerobiosis as a source of error when troubleshooting an experiment. The three indicators described in the unit, methylene blue strips (BD), resazurin, and the oxygen/hydrogen meter, will cover all routine culturing needs.

The anaerobic techniques described in this unit require an active palladium catalyst for the removal of oxygen from the atmosphere and it

is this catalyst that is the source of most problems. The catalyst must be heated regularly as described in the protocols above in order to drive off the moisture on the catalyst surface. It also is important to replace the catalyst on a regular schedule (every 9 months to a year) since it eventually becomes permanently poisoned from volatile sulfides produced by bacterial metabolism. Although the *Bacteroides* do not produce large amounts of H_2S it is still useful to have in the glove box a solution of 90 mM lead acetate or trays of activated charcoal to adsorb the sulfides.

A simple test will determine if the catalyst is still active. Rejuvenate the catalyst by heating at 160°C for 2 hr and let cool. Then using the gassing cannula direct a stream of an anaerobic gas mix containing H_2 over the surface of the catalyst. The catalyst pellets should immediately become hot and if the stream of gas is maintained the pellets will begin to glow red from the heat. If the pellets do not heat up then the catalyst must be replaced.

A second common source of oxygen contamination results from air leaks in the culturing equipment. The rubber gaskets that seal anaerobic jar lids to the jar must be inspected frequently to make sure they are free of defects or debris. Likewise the gasket around the airlock entry of an anaerobic chamber must be intact to maintain anaerobiosis.

Anaerobic glove box

The anaerobic glove box has become the method of choice for cultivation of anaerobes in most research laboratories so it may be beneficial to review some of the important features and maintenance requirements. The clear, flexible, vinyl glove box is the most practical for the average laboratory and unless space is of paramount importance always choose a 2-person (4 glove) chamber for the extra storage space and convenience of having an extra pair of hands for some procedures. The flexible style is preferred for a number of reasons but the fact that these chambers are always under a slight positive pressure is a great advantage for detecting leaks and maintaining anaerobiosis when there is a leak. Leaks are immediately detected whenever the chamber deflates more quickly than usual.

Two required accessories for the glove box are a 37°C incubator and an oxygen/hydrogen gas meter. The incubator will tend to limit the number of entries and exits from the chamber since cultures can be readily monitored. However, if a smaller glove box is purchased and one does not wish to give up the "bench space" for an incubator, it is possible to use anaerobic jars in conjunction with the glove box. Simply bring the jars with a fully charged catalyst into the chamber, add the cultures, secure the lid, and remove for incubation. Only the smaller size jars will fit into the entry lock. The oxygen/hydrogen meter is useful to keep watch on oxygen levels but more importantly it readily allows the investigator to monitor hydrogen. Changes is hydrogen levels can be indicative of a degenerating catalyst or a slow leak in the glove box. In our experience we have found that redox indicators in the media are more sensitive to changes in oxygen in the chamber than the meter but the meter is important to monitor aerobiosis following accidental contamination with oxygen.

All anaerobic chambers will eventually leak and the frequency of problems is directly related to the rate of use. The majority of problems occur with the neoprene gloves and the seams where the arms are joined to the body of the chamber. The gloves endure the most abuse and eventually wear out or get punctured. Likewise, the arm holes endure a significant amount of stress and the seams can sometimes split. Repairs on the vinyl chambers are relatively simple and anaerobic conditions can be maintained in the chamber for all repairs.

Media

Media formulation is straightforward for the *Bacteroides* and a range of media are used for their cultivation. One versatile medium not employed in any of our protocols but added to the Reagents and Solutions section is Tryptone yeast extract glucose medium, which is used by many laboratories for their routine culturing needs. This is a complex, rich medium to which any carbohydrate of choice can be added and it was initially designed for analysis of sugar utilization in anaerobes. Thus, it can readily replace BHIS in most situations.

Although the *Bacteroides* have relatively simple growth requirements, several parameters must be met to permit abundant growth. First, these are saccharolytic organisms and require a source of fermentable carbohydrate. Simple sugars are readily assimilated but these organisms also have sophisticated enzyme systems for the utilization of complex polysaccharides such as starch, pectin, and hemicellulose. It is this capacity to adapt to a wide range of carbohydrates that is thought to be a key to their success in the gut.

Another growth requirement is for a source of hemin. Although it is possible to cultivate these organisms for an extended period

without hemin, the typical generation time is only ~1/10 of normal growth. Thus, *Bacteroides* are essentially heme auxotrophs and must be provided with a source of hemin, protoporphyrin IX, or blood in the media. Among other things hemin is required for synthesis of the b-type cytochrome, important for succinate production and anaerobic electron transport.

The *Bacteroides* also require a source of vitamin B_{12}. Cobalamin is an important cofactor for many enzymes but the ability of L-methionine to be substituted for B_{12} suggests that a key enzyme in methionine synthesis is the most critical defect in the absence of B_{12}. The addition of $NaHCO_3$ has two functions; it provides a ready source of CO_2, which is a growth requirement for these organisms, and it is good buffer against a CO_2 atmosphere.

The preferred nitrogen source is ammonia and the *Bacteroides* have very little capacity to gain nitrogen for growth from single amino acids. They can however readily utilize peptides for growth if no ammonia is present but the growth yield is decreased. These nitrogen requirements are well suited for the gut environment where there are little or no free amino acids but ammonia is plentiful. Finally, *Bacteroides* require a reduced form of sulfur. That is they cannot utilize sulfate as the sole source of sulfur. In laboratory media the source of sulfur generally comes from cysteine, which is added to the media as a reducing agent, but they also have the ability to utilize H_2S and to a lesser extent they can use thioglycolate.

Time Considerations

The *Bacteroides* are robust organisms and can double at a relatively high rate under optimal conditions. Generation times for *B. fragilis* and *B. thetaiotaomicron* in BHIS media is ~40 to 60 min but the presence of antibiotics can have a significant affect even in resistant organisms. In defined medium growth rate will increase from 1.75 to 2 hr when glucose is the carbon/energy source. When inoculating a BHIS plate with a frozen or chopped meat stock of *Bacteroides*, one should expect to see colonies within 36 hr.

Acknowledgement

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Varel, V.H. and Bryant, M.P. 1974. Nutritional features of *Bacteroides fragilis* sp. *fragilis. Appl. Microbiol.* 28:251-257.

Internet Resources

http://www.anoxomat.com/english/standardpage.php[?ArtikelID=46

http://www.anaerobesystems.com

http://www.bellcoglass.com/product_detail.php?product_id=276

The above three Web sites contain information about anaerobic systems.

http://www.bd.com/ds/productCenter/BblGaspakProductsAndAccessories.asp

http://www.mgc-a.com/Pages/anaeropac.html

http://www.oxoid.com/us/index.asp?mpage=ipsearch&c=US

The above three Web sites provide information on commercially available gas packs.

http://www.coylab.com/anaerobic_chamber.html

http://www.shellab.com/bactron.html

http://www.biotrace.com/content.php?hID=2&nhID=75

http://www.800ezmicro.com/productImages.asp?mb=01&ez=9

The above four Web sites provide information on anaerobic chambers.

Genetic Manipulation of *Porphyromonas gingivalis*

This unit describes current molecular genetic approaches using the suicide vector pPR-UF1 (see Basic Protocol 2) and the transposon Tn*4351* (see Basic Protocol 6 and Alternate Protocol 2) to perform mutant construction in *Porphyromonas gingivalis*. Plasmid pPR-UF1 is a modified form of plasmid pVA3000, a suicide vector previously used for construction of single cross-over mutations. Plasmid pPR-UF1 contains two multiple cloning restriction sites into which inserts from the target gene can be cloned. Techniques using $MgCl_2$ to prepare *P. gingivalis* competent cells for electroporation purposes (see Basic Protocol 1) and conjugation protocols (see Basic Protocol 3 and Alternate Protocol 1) are described in this unit. While a mutation using a suicide vector is specific to a gene of interest, transposon mutagenesis is more or less random. Plasmid R751::Tn*4351*Ω4 is used to introduce transposon Tn*4351* into *P. gingivalis*. This plasmid carries two copies of Tn*4351* and can be transferred from *E. coli* to *P. gingivalis* via conjugation. Since R751::Tn*4351*Ω4 is unable to replicate in *P. gingivalis* cells, any erythromycin-resistant (Em^r) *P. gingivalis* transconjugants should be generated by transposition of Tn*4351* from R751::Tn*4351*Ω4 to the *P. gingivalis* chromosome, as described in Basic Protocol 1. In order to rule out polar mutation effects during phenotype studies of a particular gene, complementation should be performed; a technique to restore the wild-type phenotype of the mutant by complementation in *trans* using shuttle vector pT-COW is provided (see Basic Protocol 4). Finally, a description of a noninvasive reporter system using the xylosidase/arabinosidase enzyme to study gene expression and regulation in *P. gingivalis* (see Basic Protocol 5) completes this unit on genetic manipulations of *P. gingivalis*.

CAUTION: Porphyromonas gingivalis and *Escherichia coli* are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Potentially hazardous chemicals will be used throughout the experiments described in this unit. Readers should refer to *UNIT 1A.3* for proper handling and disposal of chemical hazards.

NOTE: Because *P. gingivalis* has special nutrient requirements, hemin and vitamin K_1 need to be added to TSB. Therefore, throughout this unit, supplemented TSB as described in this recipe is used for *P. gingivalis* cultivation. Supplemented TSB can be prereduced and prewarmed by incubating a bottle of fresh medium in the anaerobic chamber overnight before using it (also see Reagents and Solutions).

NOTE: Refer to *UNIT 12B.1* for information about equipment needed to maintain anaerobic conditions.

GENETIC TRANSFORMATION OF *PORPHYROMONAS GINGIVALIS*

BASIC PROTOCOL 1

The protocol is an adaptation of the methods described by Smith (1995) and by Fletcher et al. (1995). Modifications were done according to Dr. Gena Tribble (pers. comm.) of the University of Florida.

Materials

Supplemented tryptic soy broth (TSB), prereduced and prewarmed (see recipe)
3- to 4-day blood agar plate culture of *P. gingivalis* W83 (ATCC #BAA-308)
Electroporation (EP) buffer (see recipe), cold

Contributed by Myriam Bélanger, Paulo Rodrigues, and Ann Progulske-Fox
Current Protocols in Microbiology (2007) 13C.2.1-13C.2.24

DNA for transforming *P. gingivalis* (see Basic Protocol 2)
Blood agar plates (see recipe) containing appropriate antibiotics (e.g., 50 μg/ml gentamicin plus 5.0 μg/ml erythromycin; see Basic Protocol 2)

Refrigerated centrifuge and sterile 50-ml centrifuge tubes
0.5-ml microcentrifuge tubes
0.2-cm-gap electroporation cuvettes, sterile and prechilled
Electroporation apparatus: gene pulser and pulse controller (e.g., Bio-Rad)

Additional reagents and equipment for Southern blotting (*UNIT 14B.1*; also see Brown, 1993, 1999)

Prepare electrocompetent P. gingivalis

1. Inoculate 10 ml supplemented TSB with *P. gingivalis* from a 3- to 4-day-old blood agar plate. Incubate at 37°C anaerobically overnight without agitation.

   Broth cultures inoculated with P. gingivalis do not require agitation for growth.

2. Inoculate 100 ml supplemented TSB with sufficient overnight culture to obtain an initial OD_{550} of 0.1. Incubate at 37°C anaerobically until culture reaches a final absorbance of 0.55 to 0.65 at OD_{550} (~5 to 6 hr).

3. Centrifuge cells 10 min at 3000 × *g*, 4°C, in sterile centrifugation tubes. Remove supernatant.

4. Gently resuspend cells in 100 ml cold electroporation (EP) buffer; then centrifuge again as in step 3 and remove the supernatant.

5. Gently resuspend cells in 50 ml cold EP buffer, then centrifuge again as in step 3 and remove the supernatant.

   Step 5 can be omitted and the pellet directly resuspended in 1 ml of EP buffer (step 6); however, the additional wash performed in step 5 will help remove salts that may interfere with the electroporation process, and may improve transformation efficiency.

6. Gently resuspend the final pellet (now containing electrocompetent *P. gingivalis* cells) in 1.0 ml EP buffer.

7. Divide electrocompetent *P. gingivalis* cells in 100-μl aliquots into 0.5-ml microcentrifuge tubes and store at –80°C up to 1 year. Proceed to step 9 or freeze cells for convenience (step 7; thawing instructions in step 8).

   Electrocompetent cells should be used when freshly prepared. Alternatively, they can be frozen for convenience up to 1 year, as described in step 7.

Transform P. gingivalis with DNA of interest by electroporation

8. Thaw a vial containing 100 μl electrocompetent *P. gingivalis* on ice.

9. Pipet transforming DNA (see Basic Protocol 2) into a prechilled, sterile gene pulse cuvette (0.2-cm gap). Pipet 100 μl electrocompetent *P. gingivalis* cells into the same cuvette. Mix gently by tapping the cuvette.

10. Place the cuvette into the electroporation chamber and pulse with controller set to 2.5 kV, 5.0 msec, 400 Ω, and 25 μF.

11. Immediately after the electroporation, add 1.0 ml supplemented, prereduced, prewarmed TSB. Transfer the cell suspension to a microcentrifuge tube.

12. Incubate the cell suspension anaerobically at 37°C overnight.

13. Spread the entire 1.0 ml from the transformation onto ten blood agar plates (100 μl per plate) containing appropriate antibiotics (see Basic Protocol 2).

14. Incubate anaerobically until *P. gingivalis* transconjugant colonies are visible (up to 10 days).

15. Confirm the putative transformant by Southern blot (*UNIT 14B.1*; also see Brown, 1993, 1999) or colony PCR analysis.

MUTANT CONSTRUCTION IN *PORPHYROMONAS GINGIVALIS* USING SUICIDE VECTOR pPR-UF1

BASIC PROTOCOL 2

Plasmid pVA3000 has been previously used as a suicide vector for mutant construction (Wu et al., 2002). Plasmid pVA3000 (Lee et al., 1996) was created by a modification in the *Bacteroides-E. coli* shuttle vector pVA2198 (Fletcher et al.,1995). The origin of replication for *Bacteroides* in pVA2198 was removed by *Kpn*I digestion and the remaining 5.3-kb fragment was self-ligated, giving rise to pVA3000. This plasmid carries two erythromycin markers (*ErmAM* for *E. coli* and *ErmF* for *P. gingivalis*) and a small multiple cloning site (only five restriction enzyme sites). Recently, the authors have made improvements to plasmid pVA3000. New restriction enzyme sites were designed and cloned into the *Sal*I/*Sph*I double-digestion site in the previously existing multiple cloning site. Also, a new multiple cloning site was designed and inserted into the *Pvu*I/*Kpn*I double-digestion site upstream of the *ErmAM-F* markers, giving rise to plasmid pPR-UF1 (Fig. 13C.2.1). Using plasmid pPR-UF1, it is possible to construct double cross-over mutations and, therefore, achieve a stable disruption of the target gene. In addition, to select for double cross-over mutants, it is important to linearize the constructed plasmid by restriction enzyme digestion before introducing it into the recipient microorganism. In this case, the linearized plasmid is introduced by electroporation. This step is necessary to avoid selection of single cross-over mutants. Examples of restriction enzymes that can be used to linearize recombinant pPR-UF1 are shown in Figure 13C.2.1.

To perform a double cross-over mutation, a fragment located at the 5′ end and another fragment located at the 3′ end (see Fig. 13C.2.2) of the target gene need to be amplified by PCR and then cloned into multiple cloning sites (MCS) 1 and 2 of plasmid pPR-UF1 (see Figs. 13C.2.1 and 13C.2.2). Each of the two resulting amplified fragments will be cut by restriction enzymes that are unique to the targeted gene and that are also present in the specified MCS of the plasmid. Therefore, each fragment must include two restriction enzyme sites to enable it to be inserted correctly into the plasmid. Furthermore, the amplified fragments should not contain sites for restriction enzymes that will be used to linearize the plasmid prior to electroporation. See Critical Parameters and Troubleshooting for more information.

Materials

Supplemented tryptic soy broth (TSB), prereduced and prewarmed (see recipe)
3- to 4-day blood agar plate culture of *P. gingivalis* W83 (ATCC #BAA-308) or other *P. gingivalis* strain of interest
Wizard Genomic DNA Purification Kit (Promega; use manufacturer's Isolation of Genomic DNA from Gram Positive and Gram Negative protocol)
Nuclease-free distilled deionized H_2O
PCR primers designed to amplify fragments A and B of the gene of interest
QIAquick PCR Purification Kit (Qiagen)
Restriction enzymes to cut fragments (inserts) A and B of the gene of interest (Fig. 13C.2.1)
LigaFast Rapid DNA Ligation System (Promega)
Competent *E. coli* cells (e.g., DH5α, Invitrogen)
Plasmid pPR-UF1 (can be obtained from the author's laboratory; *apfox@dental.ufl.edu*)

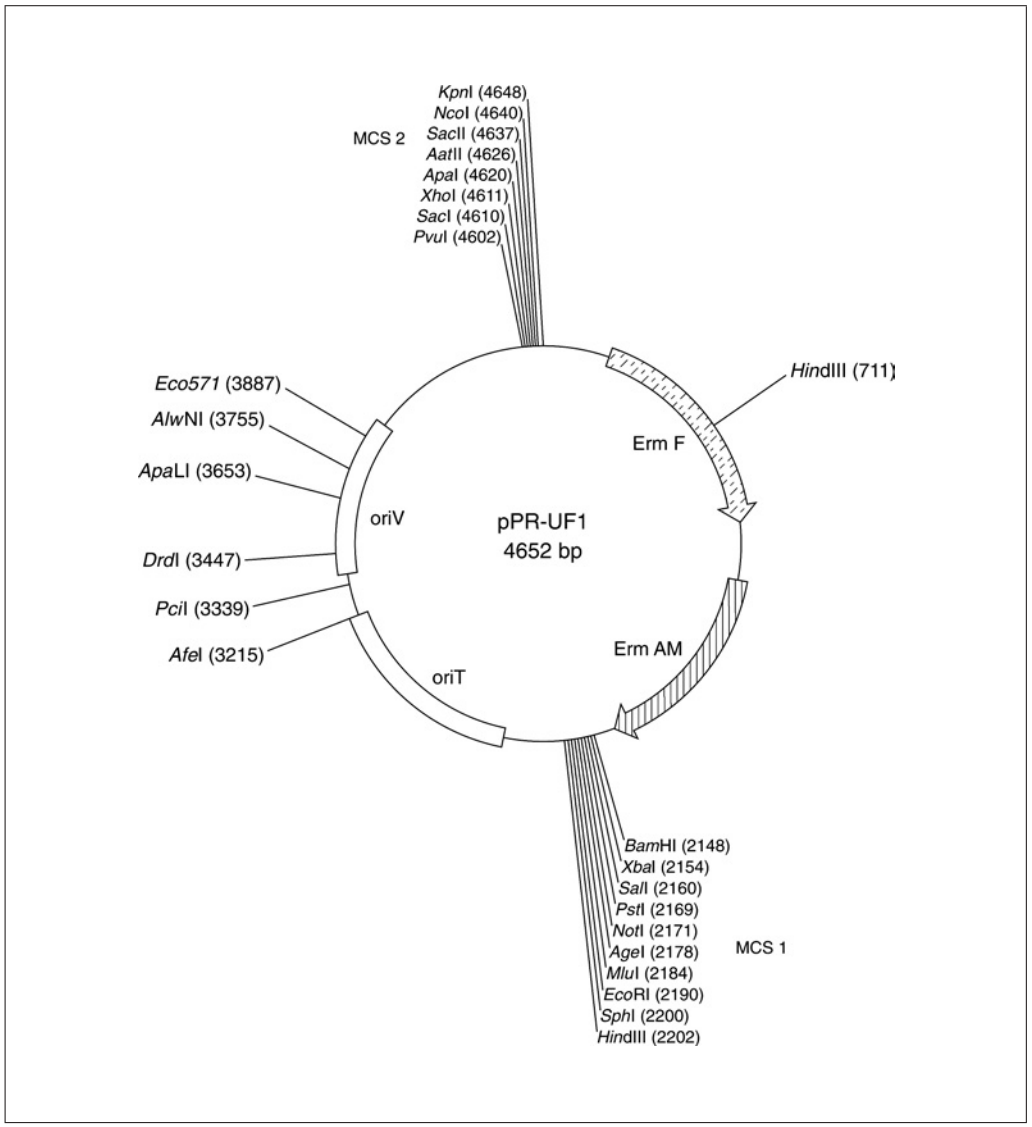

Figure 13C.2.1 Partial restriction map of plasmid pPR-UF1, the suicide vector used to construct mutants in *P. gingivalis*. Restriction enzymes *Afe*I, *Pci*I, *Drd*I, *Apa*LI, *Alw*NI, and *Eco*571 can be used to linearize the plasmid before transformation of *P. gingivalis* by electroporation. Restriction enzyme *Hin*dIII should not be used for cloning an insert. The ErmF cassette of pPR-UF1 also confers clindamycin resistance in *P. gingivalis*; therefore, 5.0 μg/ml clindamycin may be used instead of erythromycin to select *P. gingivalis* transconjugants.

LB plates (*APPENDIX 4A*) containing 300 μg/ml erythromycin (add from 300 mg/ml erythromycin stock; see recipe)

QIAprep Spin Miniprep Kit (Qiagen)

1% agarose gel (Voytas, 2000)

Restriction enzyme *Hin*dIII for screening to confirm the presence of inserts in recombinant plasmids pPR-UF1A and pPR-UF1AB

Restriction enzyme *Afe*I, *Pci*L, *Drd*I, *ApaL*I, *AlwN*I, or *Eco*57I (Fig. 13C.2.1) for linearizing the recombinant plasmid, pPR-UF1AB

Electrocompetent *P. gingivalis* cells (see Basic Protocol 1, steps 1 to 7)

Blood agar plates (see recipe) containing 50 μg/ml gentamicin (add from 50 mg/ml stock; see recipe) and 5.0 μg/ml erythromycin (add from 5.0 mg/ml stock; see recipe)

Refrigerated centrifuge

DNase-free pipet tips and tubes

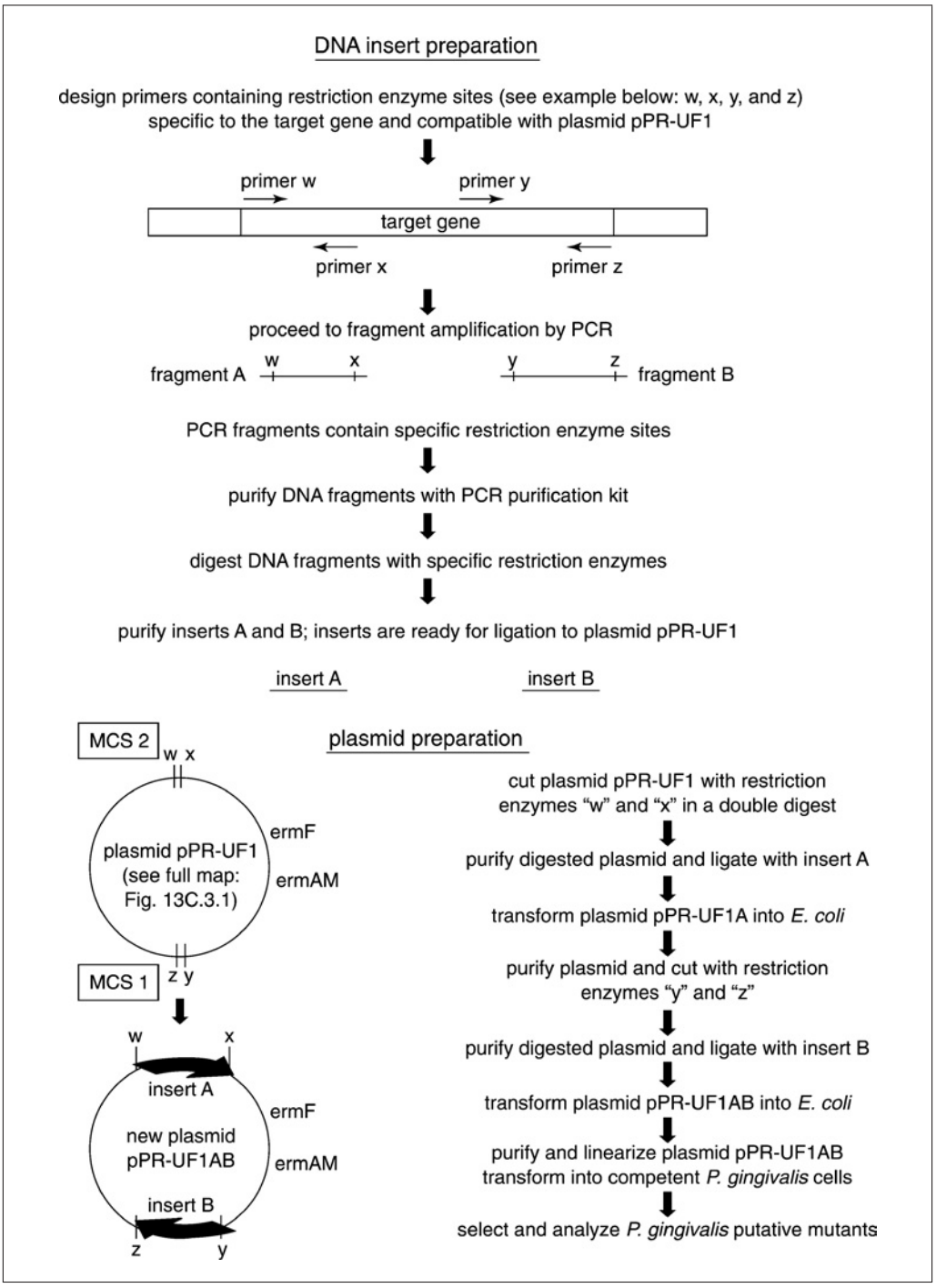

Figure 13C.2.2 Construction of *P. gingivalis* mutant by allelic or gene replacement (double cross-over mutant). Two fragments from the target gene are amplified by PCR, digested with restriction enzymes, and cloned into MCS 1 and MCS 2 of plasmid pPR-UF1.

Additional reagents and equipment for DNA purification (Moore and Dowhan, 2002), determining DNA concentration (Gallagher and Desjardins, 2006), the polymerase chain reaction (PCR; Kramer and Coen, 2001), restriction digestion (Bloch and Grossman, 1995), transformation of *E. coli* (Seidman et al., 1997), agarose gel electrophoresis (Voytas, 2000), transformation of *P. gingivalis* by electroporation (Basic Protocol 1), and Southern blotting (*UNIT 14B.1*; also see Brown, 1993, 1999)

Prepare P. gingivalis genomic DNA

1. Inoculate 5.0 ml supplemented TSB with *P. gingivalis* from a 3- to 4-day-old blood agar plate and incubate at 37°C anaerobically overnight without agitation.

   Broth cultures inoculated with P. gingivalis do not require agitation for growth.

2. Perform a 1/10 subculture in 30 ml fresh supplemented, prewarmed, prereduced TSB. Incubate at 37°C anaerobically overnight.

3. Centrifuge 25 ml of the culture 10 min at 3000 × *g*, 4°C. Purify *P. gingivalis* chromosomal DNA using a Promega Wizard Genomic DNA Purification Kit or other standard purification technique (e.g., Moore and Dowhan, 2002).

   In addition to the purification above, phenol extraction (e.g., Moore and Dowhan, 2002) or another procedure to further remove protein contaminants is desirable.

4. Resuspend DNA in 50 μl nuclease-free water and determine the DNA concentration (Gallagher and Desjardins, 2006).

Amplify fragment A of gene of interest by PCR

5. Perform PCR (Kramer and Coen, 2001) on *P. gingivalis* chromosomal DNA using primers designed to amplify the desired fragments A and B of the gene of interest.

   The conditions to be used in PCR (e.g., amount of starting DNA material, primer concentrations, thermal cycling program) will vary according to the DNA polymerase used. Consult the manufacturer's instructions.

6. Purify both PCR products (inserts A and B) from the PCR reaction using a QIAquick PCR purification kit or other standard purification technique (e.g., Moore and Dowhan, 2002). If using the QIAquick kit, elute DNA with 30 μl of the elution buffer (EB) provided with the kit.

7. Digest purified PCR fragment A using the two restriction enzymes chosen to cut this fragment (Bloch and Grossman, 1995).

   If the chosen enzymes function under the same buffer conditions, a double digest can be performed as described above. Otherwise, two separate enzymatic digests will needed.

8. Purify the digested fragment from the enzymatic reaction (using the QIAquick PCR Purification Kit; also see Moore and Dowhan, 2002) and determine the DNA concentration (Gallagher and Desjardins, 2006).

Clone fragment A of gene of interest into plasmid pPR-UF1 to prepare plasmid pPR-UF1A

9. Digest plasmid pPR-UF1 using the same two restriction enzymes used to cut fragment A. Purify the digested fragment from the enzymatic reaction (using the QIAquick PCR Purification Kit; also see Moore and Dowhan, 2002) and determine the DNA concentration (Gallagher and Desjardins, 2006).

10. Ligate insert A into plasmid pPR-UF1 using the Promega LigaFast Rapid DNA Ligation System.

    The LigaFast rapid DNA ligation system is based on T4 DNA ligase (also see Tabor, 1987).

    Various molar ratios of vector:insert can be used to enhance the efficiency of the ligation. A 1:3 vector to insert molar ratio and a starting quantity of 0.12 μg of plasmid pPR-UF1 is typically used in ligation reactions in the authors' laboratory. To determine the amount of insert necessary to add to a ligation reaction with plasmid pPR-UF1, see Critical Parameters and Troubleshooting.

11. Transform the ligation reaction into chemically or electrocompetent *E. coli* cells such as DH5α (Seidman et al., 1997).

12. Select on LB agar plates containing 300 μg/ml erythromycin. Incubate at 37°C overnight.

13. Screen resulting colonies for correct ligation of insert A and pPR-UF1 by either colony PCR (using the same primers with which insert A was produced) or purification of plasmid DNA (using the QIAprep Spin Miniprep Kit; also see Moore and Dowhan, 2002) followed by digestion with *Hin*dIII (Bloch and Grossman, 1995) and agarose gel electrophoresis on a 1% agarose gel (Voytas, 2000).

    HindIII digestion of pPR-UF1 will yield two bands on a 1% agarose gel: 1491 and 3161 bp. However, with pPR-UF1A, one of the bands will be of higher molecular weight since insert A is cloned into the plasmid. The HindIII site of pPR-UF1 should not have been used for cloning (see Critical Parameters and Troubleshooting). Furthermore, the presence of HindIII sites within insert A or B will change the band pattern expected during gel analysis.

Clone fragment B of gene of interest into plasmid pPR-UF1A to prepare plasmid pPR-UF1A

14. Prepare a miniprep of the plasmid DNA (using the QIAprep Spin Miniprep Kit; also see Wilson, 1997) from the transformed cells to obtain the plasmid containing insert A (pPR-UF1A). Repeat steps 7 to 13 with insert B, using the appropriate restriction enzymes for that fragment at step 7, and ligating insert B into the second multiple cloning site of pPR-UF1A at step 10. After transformation into competent *E. coli* (step 11), selection incubation on LB agar plates containing 300 μg/ml of erythromycin (step 12), and screening of colonies by digestion with *Hin*dIII, prepare a miniprep of the resulting plasmid using the QIAprep Spin Miniprep Kit.

    The resulting plasmid, pPR-UF1AB, can now be used to create a mutation in P. gingivalis.

15. Before proceeding to the electroporation of *P. gingivalis*, linearize ~1 μg recombinant plasmid per transformation using *Afe*I, *Pci*L, *Drd*I, *ApaL*I, *AlwN*I, or *Eco*57I (Fig. 13C.2.1) to avoid generation of single cross-over mutants. Use ~1 μg of linearized DNA per transformation.

    The authors' experience is that the use of the enzymes (described above) to linearize the recombinant plasmid will increase the number of putative mutants recovered.

Transform P. gingivalis with plasmid pPRUF1AB to construct mutant

16. Purify the digested fragment from the enzymatic reaction to remove any salts and other undesired components (using the QIAquick PCR Purification Kit; also see Moore and Dowhan, 2002).

17. On ice, thaw a vial containing 100 μl electrocompetent *P. gingivalis*.

18. Pipet the entire volume of the digested plasmid pPR-UF1AB (from step 15) into a prechilled sterile gene pulse cuvette (0.2-cm gap). Pipet the electrocompetent *P. gingivalis* cells into the same cuvette. Mix gently by tapping the cuvette.

19. Perform electroporation to transform the bacteria (Basic Protocol 1, steps 8 to 12).

20. Spread the entire 1.0 ml transformation onto ten blood agar plates containing 50 μg/ml of gentamicin plus 5.0 μg/ml of erythromycin, using 100 μl per plate.

    Gentamicin is solely used to prevent contamination from growing on blood agar plates. Thus, it is not necessary for the selection of mutants and could be omitted from the medium.

21. Incubate anaerobically up to 10 days until *P. gingivalis* colonies are visible. Confirm mutants by Southern blot (*UNIT 14B.1*; also see Brown, 1993, 1999) or colony PCR analysis.

INTRODUCING DNA INTO *PORPHYROMONAS GINGIVALIS* BY CONJUGATION (AGAR PLATE METHOD)

Conjugation is the transfer of DNA from one bacterial cell to another by direct cell-to-cell contact. It was first described by Lederberg and Tatum in 1946 and it usually involves conjugative plasmids. These plasmids encode genes required for their transfer, including those for the conjugative pili necessary for establishing cell-to-cell contact. It is likely that many genetically distinct conjugation systems exist, since plasmids from one incompatibility group or family do not complement transfer-deficient mutants of another. Conjugation can be used with Gram-negative or Gram-positive bacteria, and is commonly done between different bacterial species. The following procedure, or that described in Alternate Protocol 1, can be used to introduce DNA into *P. gingivalis*. Basic Protocols 4 and 5 utilize either of these conjugation protocols. Note that Basic Protocol 6 and Alternate Protocol 2 (Mutant Construction Using Transposon Tn*4351*) utilize somewhat different methods of conjugation. The following protocol is an adaptation of the methods described by Dyer et al. (1992) and Lee et al. (1996).

Materials

Recipient *P. gingivalis* strain W83 (ATCC #BAA-308) or other *P. gingivalis* strain of interest
Blood agar plates (see recipe) without antibiotics
Donor *E. coli* strain (S17-1; not available commercially; can be obtained from authors' laboratory, *apfox@dental.ufl.edu*)
LB plates (*APPENDIX 4A*) containing appropriate antibiotics (see Critical Parameters and Troubleshooting)
Supplemented tryptic soy broth (TSB), prereduced and prewarmed (see recipe)
Blood agar plates (see recipe) containing 150 μg/ml gentamicin and appropriate antibiotic to select for transconjugants (see Critical Parameters and Troubleshooting)
Blood agar plates (see recipe) containing 50 μg/ml gentamicin and appropriate antibiotic to ensure antibiotic resistance and purity of transconjugants (see Critical Parameters and Troubleshooting)

1. Grow the recipient *P. gingivalis* strain on a blood agar plate without antibiotics (or containing 50 μg/ml gentamicin) at 37°C anaerobically for 2 to 3 days.
2. Grow the donor *E. coli* containing the recombinant plasmid aerobically on a LB agar plate with the appropriate antibiotic (see Critical Parameters and Troubleshooting) at 37°C overnight.

   In conjugation experiments, the authors prefer to use the mobilizing E. coli strain S17-1 as a donor strain for P. gingivalis.

   Steps 3 to 5 of this protocol can be modified as described by Maley et al. (1992) and by Park and McBride (1993). See Alternate Protocol 1.

3. Using a sterile loop, scrape *E. coli* and *P. gingivalis* from plates obtained in steps 1 and 2, and mix on a 3- to 4-cm^2 area of a blood agar plate containing no antibiotics.
4. Incubate at 37°C anaerobically overnight.
5. Collect the mixed cells and resuspend in 1.0 ml supplemented TSB.
6. Spread 0.1 ml cell suspension onto blood agar plates containing 150 μg/ml gentamicin and the appropriate antibiotic to select for transconjugants (see Critical Parameters and Troubleshooting).
7. Incubate at 37°C anaerobically up to 10 days until *P. gingivalis* transconjugant colonies are visible.

8. Streak several colonies onto fresh blood agar plates containing 50 μg/ml of gentamicin and the appropriate antibiotic to ensure antibiotic resistance and purity (see Critical Parameters and Troubleshooting).

INTRODUCING DNA INTO *PORPHYROMONAS GINGIVALIS* BY CONJUGATION (BROTH CULTURE METHOD)

ALTERNATE PROTOCOL 1

This protocol is an adaptation of the methods described by Maley et al. (1992) and by Park and McBride (1993).

***Additional Materials** (also see Basic Protocol 3)*

LB liquid medium (*APPENDIX 4A*) without antibiotics
Sterile filter membrane (cellulose esters membrane; Millipore)
Anaerobic chamber (*UNIT 12B.1*)

1. Perform steps 1 and 2 of Basic Protocol 3.
2. Inoculate 3.0 ml supplemented TSB with recipient *P. gingivalis* and incubate at 37°C anaerobically until the culture reaches a final absorbance of 0.2 at OD_{550} (~60 min).
3. Inoculate 1.0 ml LB medium without antibiotic with the donor *E. coli* and incubate at 37°C aerobically until the culture reaches a final absorbance of 0.2 at OD_{550} (~10 min).
4. Mix 0.2 ml *E. coli* culture with 1.0 ml of the *P. gingivalis* culture. Centrifuge 2 min at 9600 × *g*, room temperature.
5. Resuspend the resulting cell pellet in 0.1 ml LB medium and spot onto a sterile filter membrane placed on blood agar plate without antibiotics.
6. Incubate aerobically 2 hr at 37°C.
7. Transfer to an anaerobic chamber and incubate 72 hr at 37°C.
8. Remove the membrane from the blood agar plate. Using a sterile inoculating loop, scrape the cells off the membrane and resuspend in 3.0 ml of supplemented TSB.
9. Perform steps 6 to 8 of Basic Protocol 3.

COMPLEMENTATION OF *PORPHYROMONAS GINGIVALIS* MUTANTS

BASIC PROTOCOL 4

To design complementation experiments, it is necessary to clone the entire gene of interest as well as any transcriptional regulatory elements (TRE) upstream of the target gene. A problem that may arise in complementation experiments is that the transcriptional regulatory elements may be significantly upstream of the target gene.

An undesirable feature of pT-COW is that there are no multiple cloning sites and few restriction enzyme sites available (Fig. 13C.2.3). It is important to choose restriction sites that are not located within critical DNA regions of the plasmid, e.g., the replication and mobilization (rep/mob) regions. However, restriction enzyme sites that exist in one of the antibiotic resistance markers for *E. coli* can be used. Later, when selecting *E. coli* cells carrying the recombinant plasmid (pT-COW plus gene), use the selective antibiotic with a complete copy of its gene. In Figure 13C.2.3, all known unique restriction enzyme sites are shown.

Refer to Basic Protocol 2 and its Critical Parameters and Troubleshooting section for information on designing primers that introduce restriction sites upon amplification.

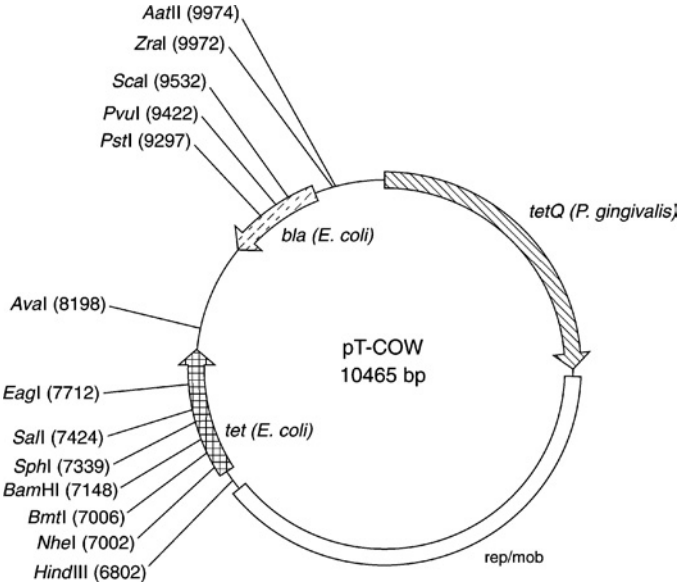

Figure 13C.2.3 Partial restriction map of plasmid pT-COW, shuttle vector used for complementation of *P. gingivalis* mutants and reporter gene analysis. When using pT-COW, grow *E. coli* in media containing 50 μg/ml carbenicillin or 5.0 μg/ml tetracycline, and grow *P. gingivalis* in media containing 1.0 μg/ml of tetracycline.

Materials

PCR primers designed to amplify the gene of interest

P. gingivalis chromosomal DNA, purified (see Basic Protocol 2)

Restriction enzymes appropriate for cutting the insert and for cutting the plasmid pT-COW (see Bloch and Grossman, 1995; also see Fig. 13C.2.3)

QIAquick PCR Purification Kit (Qiagen)

Plasmid pT-COW (Shoemaker et al., 1986; not available commercially; contact author at *apfox@dental.ufl.edu* for more information)

LigaFast Rapid DNA Ligation System (Promega)

Electrocompetent *E. coli* strain S17-1 cells (can be obtained from the author's laboratory; *apfox@dental.ufl.edu*)

LB agar plates (*APPENDIX 4A*) containing 50 μg/ml carbenicillin (add from 50 mg/ml stock; see recipe) *or* 5.0 μg/ml tetracycline (add from 10 mg/ml stock; see recipe)

QIAprep Spin Miniprep Kit (Qiagen)

1% agarose gel (Voytas, 2000)

Additional reagents and equipment for the polymerase chain reaction (PCR; Kramer and Coen, 2001), DNA purification (Moore and Dowhan, 2002), determining DNA concentration (Gallagher and Desjardins, 2006), the polymerase chain reaction (PCR; Kramer and Coen, 2001), restriction digestion (Bloch and Grossman, 1995), transformation of *E. coli* (Seidman et al., 1997), agarose gel electrophoresis (Voytas, 2000), transformation of *P. gingivalis* by conjugation (Basic Protocol 3 or Alternate Protocol 1), large-scale preparation of plasmid DNA (Heilig et al., 1998), and Southern blotting (*UNIT 14B.1*; also see Brown, 1993, 1999)

Prepare insert DNA by PCR

1. Perform PCR (Kramer and Coen, 2001) using primers specific for the desired locus with purified *P. gingivalis* chromosomal DNA as the template, under the appropriate

conditions. Purify as described in Basic Protocol 2, or by using another purification technique (e.g., Moore and Dowhan, 2002).

It is important to emphasize the use of a high-fidelity DNA polymerase when amplifying the insert. The PCR experiment should be done according to the DNA polymerase manufacturer's instructions. Restriction enzyme sites may be incorporated into the primers used to amplify the TRE regions to ensure proper orientation upon ligation. PCR products may be purified using an appropriate technique. The authors routinely purify PCR amplification products using the QIAquick PCR Purification Kit (Qiagen).

2. Double digest the purified insert using the restriction enzyme(s) chosen for this insert (Bloch and Grossman, 1995). Purify the digested fragment from the enzymatic reaction using the QIAquick PCR Purification Kit (also see, e.g., Moore and Dowhan, 2002), and determine the DNA concentration (Gallagher and Desjardins, 2006).

Prepare recombinant plasmid

3. Digest plasmid pT-COW using the restriction enzyme(s) which will create compatible overhangs with the digested insert (see Fig. 13C.2.4). Purify the digested fragment from the enzymatic reaction using the QIAquick PCR Purification Kit (also see, e.g., Moore and Dowhan, 2002), and determine the DNA concentration (Gallagher and Desjardins, 2006).

4. Ligate the *P. gingivalis* locus into pT-COW using the Promega LigaFast Rapid DNA Ligation System.

The LigaFast rapid DNA ligation system is based on T4 DNA ligase (also see Tabor, 1987).

Various molar ratios of vector:insert can be used to enhance the efficiency of the ligation. A 1:3 vector- to-insert molar ratio and a starting quantity of 0.12 μg plasmid pT-COW is typically used in ligation reactions in the authors' laboratory. To determine the amount of insert necessary to add to a ligation reaction with plasmid pT-COW, see Critical Parameters and Troubleshooting.

5. Transform *E. coli* strain S17-1 with the ligation reaction (Seidman et al., 1997).

Other E. coli strains with greater transformation efficiency may be used to propagate the recombinant plasmid (e.g., DH5α). However, before proceeding to transformation of P. gingivalis, it is necessary to transform the E. coli strain S17-1 with the recombinant plasmid.

6. Spread 100 μl from the transformation onto an LB agar plate containing the appropriate antibiotic (50 μg/ml carbenicillin or 5.0 μg/ml tetracycline).

The antibiotic selection should take in consideration which antibiotic resistance gene for E. coli is still intact in the recombinant plasmid (see introduction Basic Protocol 5).

The remaining transformation mixture may be stored at 4°C and plated out the next day if necessary.

7. Incubate at 37°C overnight.

8. Pick several colonies and streak them onto LB agar plates containing the appropriate antibiotic (50 μg/ml of carbenicillin or 5.0 μg/ml of tetracycline). Incubate the plate at 37°C overnight and then keep at 4°C as the master plate.

9. Analyze colonies from the master plate for desired recombinant plasmid by either colony PCR or isolation of plasmid (using the QIAprep Spin Miniprep Kit; also see Moore and Dowhan, 2002) followed by digestion with an appropriate restriction endonuclease (Bloch and Grossman, 1995). Run digested product on 1% agarose gel (Voytas, 2000).

Transform P. gingivalis with recombinant plasmid by conjugation

10. Once it is determined that a colony of *E. coli* strain S17-1 colonies carries the desired recombinant plasmid, transform *P. gingivalis* with the recombinant plasmid via conjugation (Basic Protocol 3 or Alternate Protocol 1).

11. Confirm that the complemented mutant colonies are carrying the recombinant plasmid by isolating total DNA (Heilig et al., 1998) and performing a Southern blot (*UNIT 14B.1*; also see Brown, 1993, 1999) to verify the presence of the recombinant plasmid.

    P. gingivalis has no naturally occurring plasmids; thus, any plasmid isolated should be the plasmid used for complementation.

    Plasmid pT-COW is a low-copy-number plasmid in P. gingivalis. It has proven to be difficult to isolate it using common plasmid isolation protocols. Thus, the authors currently perform Southern blots to confirm the presence of the recombinant plasmid.

BASIC PROTOCOL 5

REPORTER GENE SYSTEM USING XYLOSIDASE/ARABINOSIDASE

The following protocol is an adaptation of methods described by Whitehead (1997) and Liu et al. (2000) using the bifunctional xylosidase/arabinosidase enzyme (XA) as the reporter gene. In this protocol, plasmid pXA1 (Whitehead, 1997) is used as a donor of the promoterless *xa* gene, and plasmid pT-COW is used as the carrier of the fused target gene/*xa* gene. The authors' approach, using pT-COW, a shuttle vector, is to avoid disruption of the target gene, since its phenotype can be important in the experimental procedures.

The following protocol will describe how to prepare a reporter gene system (Fig. 13C.2.4) for the target gene. It is critical to clone all transcriptional regulatory elements (TRE) upstream of the target gene. Restriction enzymes may be chosen based on buffer compatibility. It is important to check that the chosen enzymes do not cut within the TRE region of the gene of interest.

Materials

PCR primers designed to amplify the gene of interest
P. gingivalis chromosomal DNA, purified (see Basic Protocol 2)
High-fidelity *Taq* DNA polymerase
QIAquick PCR Purification Kit (Qiagen)
Restriction enzymes appropriate for cutting the insert and for cutting the plasmid pT-COW (see Bloch and Grossman, 1995; also see Fig. 13C.2.4)
QIAquick Gel Extraction Kit (Qiagen)
Plasmid pXA1 (Whitehead, 1997)
Restriction enzymes *Eco*RI and *Bam*HI for cutting plasmid pXA1 (also see Bloch and Grossman, 1995)
Plasmid pT-COW (Shoemaker et al., 1986; not available commercially; contact author at *apfox@dental.ufl.edu* for more information)
1% agarose gels (see Voytas, 2000)
LigaFastTM Rapid DNA Ligation System (Promega)Nuclease-free H_2O
Electrocompetent *E. coli* strain S17-1 cells (can be obtained from the author's laboratory; *apfox@dental.ufl.edu*)
QIAprep Miniprep Kit (Qiagen)
25°C water bath

Additional reagents and equipment for the polymerase chain reaction (PCR; Kramer and Coen, 2001), DNA purification (Moore and Dowhan, 2002), restriction digestion (Bloch and Grossman, 1995), agarose gel electrophoresis (Voytas, 2000), transformation of *E. coli* (Seidman et al., 1997), transformation of *P. gingivalis* by conjugation (Basic Protocol 3 or Alternate Protocol 1), and large-scale preparation of plasmid DNA (Heilig et al., 1998)

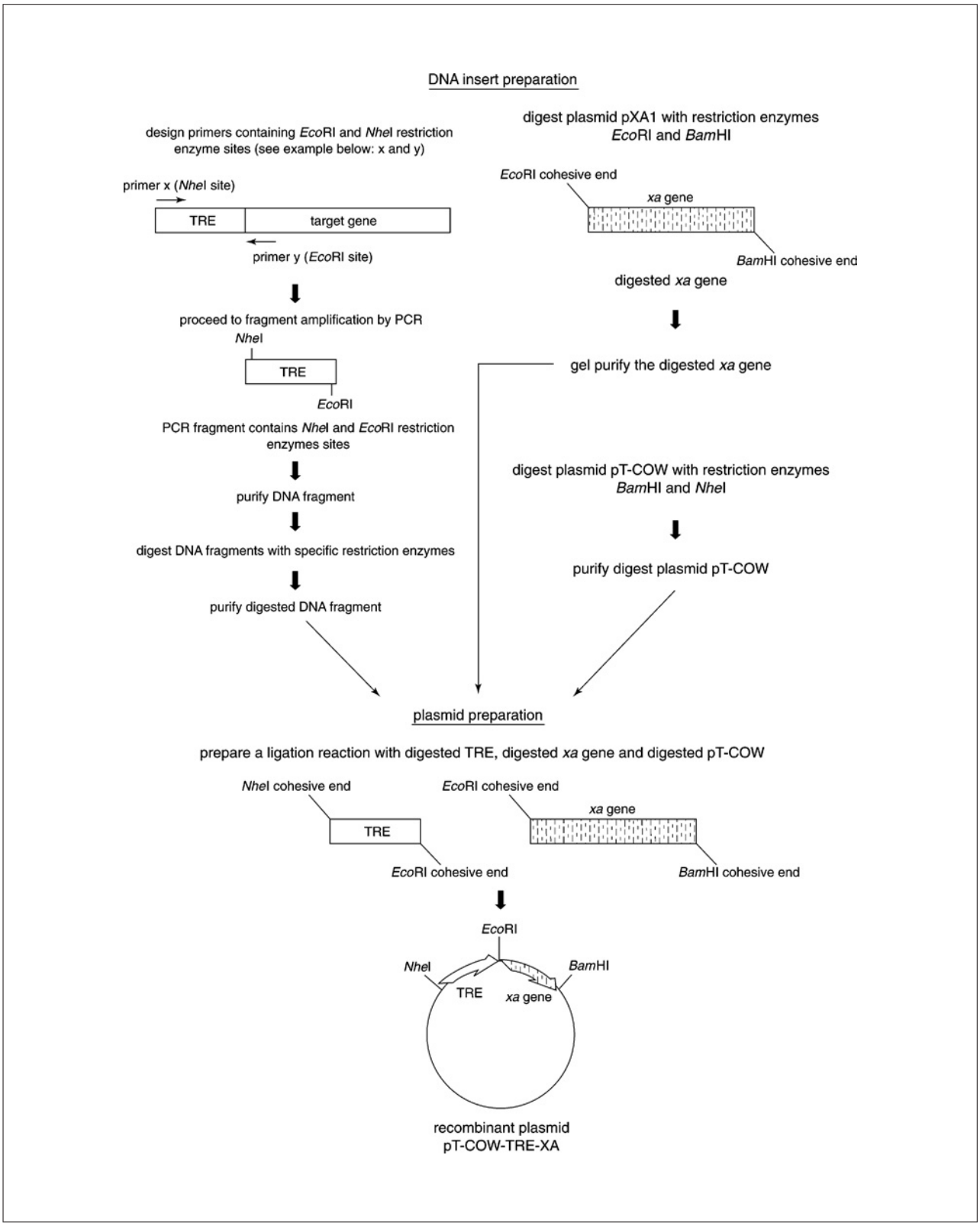

Figure 13C.2.4 Example of reporter gene system construction using the *xa* gene as the reporter gene and plasmid pT-COW as the shuttle vector.

1. Perform PCR (Kramer and Coen, 2001) using primers specific for the desired locus with purified *P. gingivalis* chromosomal DNA as the template, under the appropriate conditions. Purify as described in Basic Protocol 2 (using the QIAquick PCR Purification Kit) or using another appropriate purification technique (e.g., Moore and Dowhan, 2002).

   It is important to emphasize the use of a high-fidelity DNA polymerase when amplifying the insert. The PCR experiment should be done according to the DNA polymerase manufacturer's instructions. Restriction enzyme sites may be incorporated into the primers used to amplify the TRE regions to ensure proper orientation upon ligation. PCR products may be purified using an appropriate technique. The authors routinely purify PCR amplification products using the QIAquick PCR Purification Kit (Qiagen).

2. Digest the purified *P. gingivalis* PCR product using the restriction enzymes chosen for this insert (Bloch and Grossman, 1995). Purify the digested fragment from the enzymatic reaction using the QIAquick Gel Extraction Kit or other appropriate technique (e.g., Moore and Dowhan, 2002). If using the QIAquick kit, elute DNA with 30 µl of the elution buffer (EB) provided with the kit.

3. Remove the *xa* gene from plasmid pXA1 by restriction enzymatic digestion with restriction enzymes *Eco*RI and *Bam*HI. Run the entire reaction in a 1% agarose gel (Voytas, 2000). Purify the *xa* gene band (~1.4 kb) using the QIAquick Gel Extraction Kit and determine the DNA concentration (Gallagher and Desjardins, 2006).

4. Digest pT-COW using the same restriction enzymes chosen to cut the 5′ region of the TRE and to cut the 3′ region of the *xa* gene. Purify the digested plasmid from the enzymatic reaction using the QIAquick PCR Purification Kit and determine the DNA concentration (Gallagher and Desjardins, 2006).

   An undesirable feature of pT-COW is that there are no multiple cloning sites and only a few unique restriction enzyme sites are available (Fig. 13C.2.3). For cloning purposes, use restriction enzyme sites that are not located in critical DNA regions of the plasmid, e.g., replication and mobilization (rep/mob) regions. However, restriction enzyme sites that exist in one of the antibiotic resistance markers for E. coli can be chosen. Later, when selecting E. coli cells carrying the recombinant plasmid, use the selective antibiotic with a complete copy of its gene. In Fig. 13C.2.3, all known unique restriction enzyme sites are shown.

5. Prepare the following ligation reaction:

   15 µl of 2× buffer (from kit)
   3 U T4 DNA ligase (from kit)
   0.12 µg digested pT-COW
   x µg digested TRE insert
   y µg digested *xa* gene
   Add nuclease-free H_2O to a final volume of 30 µl.

   Ligate the TRE fragment and xa gene into pT-COW at 25°C for 20 min.

   Ligations of the digested inserts and the digested plasmid are routinely done in the authors' laboratory using LigaFastTM Rapid DNA Ligation System (Promega).Various molar ratios of vector:insert can be used to enhance the efficiency of the ligation. A 1:3 vector-to-insert molar ratio and a starting quantity of 0.12 µg of plasmid pT-COW is typically used in ligation reactions in the authors' laboratory. To determine the necessary amount of each insert to add to the ligation reaction with plasmid pT-COW, see Critical Parameters and Troubleshooting.

   The ligation of each fragment into the plasmid can be done separately. However, the ligation may be performed together if using directional cloning, as the digested TRE insert and digested xa gene will have different compatible cohesive ends.

6. Transform *E. coli* strain S17-1 with the recombinant plasmid (Seidman et al., 1997).

7. Confirm the presence of the inserts in the plasmid by colony PCR or by purification of plasmids using the QIAprep Spin Miniprep Kit (also see Moore and Dowhan, 2002) followed by cutting with an appropriate restriction enzyme (e.g., *Hin*dIII; also see Bloch and Grossman, 1995), and electrophoretic analysis on a 1% agarose gel (Voytas, 2000).

   Other E. coli strains with greater transformation efficiency may be used to propagate the recombinant plasmid (e.g., DH5α). However, before proceeding to the next step, it is necessary to transform the E. coli strain S17-1 with the recombinant plasmid.

8. Transform *P. gingivalis* by conjugation (Basic Protocol 3 or Alternate Protocol 1).

9. Confirm that the colonies obtained carry the reporter gene plasmid by performing a plasmid isolation procedure from those colonies (Heilig et al., 1998). After isolating the plasmids, run the samples on a 1% agarose gel (Voytas, 2000).

   P. gingivalis does not have any naturally occurring plasmids; thus any plasmid isolated should be the reporter plasmid.

MUTANT CONSTRUCTION USING TRANSPOSON Tn*4351* (BROTH CULTURE METHOD)

BASIC PROTOCOL 6

Transposon mutagenesis produces a transposon library containing thousands of mutants. The transposon insertions are relatively random; thus, it is usually necessary to screen hundreds of mutants to find the transconjugant carrying the phenotype of interest. Transposon Tn*4351* can be introduced into *P. gingivalis* using plasmid R751::Tn*4351*Ω4 (Shoemaker et al., 1986). This plasmid carries two copies of Tn*4351* and can be transferred from *E. coli* to *P. gingivalis* via conjugation. Tn*4351* contains two antibiotic resistance genes—one that confers resistance to erythromycin (Em^r) in *P. gingivalis* and another that confers resistance to tetracycline (Tc^r) in aerobically grown *E. coli*. Note that Em selection will work in *P. gingivalis* and Tc selection in *E. coli*; however, the reverse is not the case. Since R751::Tn*4351*Ω4 is unable to replicate in *P. gingivalis* cells, any Em^r *P. gingivalis* transconjugant should be generated by transposition of Tn*4351* from R751::Tn*4351*Ω4 to the *P. gingivalis* chromosome.

This protocol is an adaptation of the method described by Progulske-Fox et al. (1989).

Materials

Supplemented tryptic soy broth (TSB), prereduced and prewarmed (see recipe)
3- to 4-day blood agar plate culture of *P. gingivalis* W83 (ATCC #BAA-308)
E. coli strain HB101 containing plasmid R751::Tn*4351*Ω4 (not available commercially; see Shoemaker et al., 1986): culture overnight
LB liquid medium (*APPENDIX 4A*) containing 300 μg/ml erythromycin (add from 300 mg/ml stock; see recipe)
Blood agar plates (see recipe), antibiotic free
Blood agar plates (see recipe) containing 150 μg/ml gentamicin (add from 50 mg/ml stock; see recipe) and 5 μg/ml of erythromycin (add from 300 mg/ml stock; see recipe)
37°C shaking incubator

Additional reagents and equipment for Southern blotting (*UNIT 14B.1*; also see Brown, 1993, 1999)

1. Inoculate 3.0 ml supplemented TSB with *P. gingivalis* from a 3- to 4-day old blood agar plate and incubate at 37°C anaerobically overnight.

2. Inoculate 9.0 ml prereduced, prewarmed supplemented TSB with 1.0 ml of the overnight broth culture of *P. gingivalis* and incubate at 37°C anaerobically until the culture reaches an absorbance of 0.4 at OD_{550} (~2 hr).
3. Add 100 μl from an overnight culture of *E. coli* strain HB101 containing plasmid R751::Tn*4351*Ω4 to 2.0 ml LB liquid medium containing 300 μg/ml erythromycin. Place tube in a shaking incubator at 37°C under aerobic atmosphere until the culture reaches an absorbance of 0.2 at OD_{550} (~10 min).
4. Mix the entire volume of both cultures thoroughly and centrifuge the *P. gingivalis-E. coli* suspension 5 min at 5000 × *g*, room temperature. Resuspend the pellet in 1.0 ml prereduced, prewarmed supplemented TSB.

   The method of mixing is not important; however, it is important that the cultures be mixed thoroughly.
5. Spread 0.1 ml cell suspension onto antibiotic-free blood agar plates; incubate aerobically at 37°C for 1 hr, then under an anaerobic atmosphere at 37°C overnight.
6. Harvest all growing cells with an inoculation loop and resuspend in 1.0 ml prereduced, prewarmed, supplemented TSB.
7. Spread 0.1 ml cell suspension onto blood agar plates containing 150 μg/ml of gentamicin and 5 μg/ml of erythromycin. Incubate anaerobically until *P. gingivalis* transconjugant colonies are visible (up to 10 days).
8. Screen for the desired phenotype, e.g., via animal model or hemagglutination activity.
9. Confirm that the transposon-generated mutant has a single insertion by Southern blot analysis (*UNIT 14B.1*; also see Brown, 1993, 1999).

ALTERNATE PROTOCOL 2

MUTANT CONSTRUCTION USING TRANSPOSON Tn*4351* (AGAR PLATE METHOD)

This protocol is an adaptation of the method described by Dyer et al. (1992).

***Additional Materials** (also see Basic Protocol 6)*

E. coli strain HB101 containing plasmid R751::Tn*4351*Ω4 (not available commercially; see Shoemaker et al., 1986)
LB agar plates (*APPENDIX 4A*) containing 10 μg/ml tetracycline (add from 10 mg/ml tetracycline stock; see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Cellulose esters membrane (Millipore)

1. Grow *E. coli* strain HB101 containing plasmid R751::Tn*4351*Ω4 overnight on an LB agar plate containing 10 μg/ml tetracycline at 37°C, aerobically. Grow *P. gingivalis* on an antibiotic-free blood agar plate (or blood agar plate containing 50 μg/ml gentamicin) for 48 hr at 37°C anaerobically.
2. Harvest all growing cells from each plate and resuspend separately in 1.0 ml PBS.
3. Mix 0.1 ml *E. coli* suspension with 0.9 ml *P. gingivalis* suspension. Centrifuge 5 min at 5000 × *g*, room temperature, and remove the supernatant.
4. Place a cellulose esters membrane onto an antibiotic-free blood agar plate and spread the pellet onto the membrane. Incubate overnight at 37°C, aerobically.
5. Harvest all growing cells with an inoculation loop and suspend in 1.0 ml PBS.

6. Spread onto blood agar plates containing 150 μg/ml gentamicin and 5 μg/ml erythromycin.
7. Incubate anaerobically until *P. gingivalis* transconjugant colonies are visible (up to 10 days).
8. Screen for the desired phenotype, e.g., via animal model or hemagglutination activity.
9. Confirm that the transposon-generated mutant has a single insertion by Southern blot analysis (*UNIT 14B.1*; also see Brown, 1993, 1999).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blood agar plates

40 g tryptic soy agar (Difco)
5.0 g yeast extract
0.5 g L-cysteine hydrochloride
1.0 ml 5.0 mg/ml hemin (final concentration: 5.0 μg/ml)
0.2 ml 5.0 mg/ml vitamin K_1 stock (final concentration: 1.0 μg/ml)
H_2O to 1000 ml
Autoclave
Cool to ~55°C, then add 50 ml sheep blood (5% final concentration)
Add antibiotics if necessary
Store plates up to 1 month at 4°C

Menadione (vitamin K_3) can be used at a final concentration of 1.0 μg/ml instead of vitamin K_1.

Hemin and vitamin K are essential nutrients for P. gingivalis growth. L-cysteine is used to lower the oxygen tension in the medium.

See UNIT 8B.1 for additional, detailed discussion on the preparation and use of blood agar; also see Jousimies-Somer et al. (2002).

Carbenicillin stock solution, 50 mg/ml

Dissolve 0.5 g carbenicillin in 10 ml water (or in 50% ethanol/50% water). Filter sterilize through a 0.2-μm membrane. Divide into aliquots and store up to 1 year at –20°C.

Electroporation (EP) buffer

10% (v/v) glycerol
1 mM $MgCl_2$
Filter sterilize through 0.2-μm membrane
Prepare fresh
Cool to 4°C before use

Erythromycin stock solution, 300 mg/ml

Dissolve 3 g erythromycin in 70% ethanol (7.0 ml of 200 proof ethanol and 3.0 ml of sterile water). Make up volume to 10 ml final with 70% ethanol. Divide into aliquots and store up to 1 year at –20°C.

Gentamicin stock solution, 50 mg/ml

Dissolve 0.5 g gentamicin in 10 ml water. Filter sterilize through a 0.2-μm membrane. Divide into aliquots and store up to 1 year at –20°C.

Supplemented tryptic soy broth (TSB) to grow P. gingivalis

30 g tryptic soy broth (Difco)
5.0 g yeast extract
0.5 g L-cysteine hydrochloride
1.0 ml 5.0 mg/ml hemin (final concentration, 5.0 μg/ml)
0.2 ml 5.0 mg/ml vitamin K_1 stock (final concentration: 1.0 μg/ml)
H_2O to 1000 ml
Autoclave
Store up to 1 year at room temperature

Wrap each bottle in aluminum foil or keep bottle in the dark as hemin and vitamin K_1 are light sensitive and will be degraded.

Supplemented TSB can be prereduced and prewarmed by incubating a bottle of fresh medium in the anaerobic chamber overnight before using it.

Menadione (vitamin K_3) can be used at a final concentration of 1.0 μg/ml instead of vitamin K_1.

Tetracycline stock solution, 10 mg/ml

Dissolve 0.1 g tetracycline in 10 ml of 70% ethanol (7.0 ml of 200 proof ethanol and 3.0 ml of sterile water). Divide into aliquots and store up to 1 year at –20°C. (Tetracycline is light sensitive, therefore keep solution in the dark.)

COMMENTARY

Background Information

In order to determine and study the function of a particular gene, mutant construction is a critical step. Studies with mutant strains carrying the disrupted gene can elucidate important processes such as biochemical pathways, bacteria-bacteria interactions, and bacteria-host interactions.

Multiple techniques to construct mutants in various bacterial species are described in the literature. For studies with *P. gingivalis*, three methods to disrupt genes are routinely used: transposon mutagenesis, targeted insertional inactivation, and allelic replacement.

Transposon mutagenesis

In transposon mutagenesis, mutant construction can be achieved using the *Bacteroides fragilis* transposon Tn*4351* (Shoemaker et al., 1986). Usually, a clean knockout phenotype is obtained with transposons. Two desirable features of transposons are that they carry a selectable marker and that they can be used as a target for cloning the interrupted gene. Transposons are typically introduced into *P. gingivalis* by conjugation using a suicide plasmid.

Progulske-Fox et al. (1989) reported that plasmid pE5-2, a chimeric shuttle vector that replicates in *E. coli* and in some *Bacteroides* species (Shoemaker et al., 1985) could also be mobilized from *E. coli* to *P. gingivalis*. In addition, Progulske-Fox et al. (1989) showed that the transposon Tn*4351*, carried by plasmid pE5-2, seems to integrate into the *P. gingivalis* chromosome. Dyer et al. (1992) reported that another plasmid pVAL-1 carrying transposon Tn*4351* was successfully introduced into *P. gingivalis* by conjugation with *E. coli*. Subsequently, Hoover and Yoshimura (1994) reported the first mutation constructed using transposon mutagenesis. The authors described pigment-deficient mutants that also had altered trypsin-like protease and hemagglutination activity. Since then, several studies have been published describing the use of transposon Tn*4351* in mutant construction to analyze genes involved in pigmentation and adhesion of *P. gingivalis* (Genco et al., 1995; Onoe et al., 1995; Watanabe-Kato et al., 1998; Umemoto et al., 1999; Hayashi et al., 2000; Shoji et al., 2002). Recently, the use of another transposon in *P. gingivalis* was reported (Chen et al., 2000). The authors described a new transposon mutagenesis based on *Bacteroides* transposon Tn*4400*. The authors state that transposon Tn*4400* can generate a large number of mutants with single insertions and may be stable for many generations in the absence of antibiotics.

Targeted insertional inactivation and allelic replacement

The use of suicide vectors is also employed for targeted insertional inactivation, and many plasmids can be chosen to construct mutants. The suicide vector is usually transferred to the recipient bacterium by conjugation. Disruption of the gene is achieved by cloning an internal fragment of the target gene into the suicide vector. A suicide vector does not replicate in the recipient bacterium but integrates into the bacterial chromosome by homologous recombination between the portion of the gene cloned into the suicide vector and the target gene on the chromosome. In this case, the most common outcome is a single cross-over event into the target gene via the cloned internal fragment. Since the suicide vector integrates into the chromosome, the cloned internal fragment is duplicated. Thus, single cross-over insertions can be unstable. The disrupted gene may be recovered by homologous recombination between the duplicated fragments. It is therefore necessary to keep continuous antibiotic pressure on the mutant to avoid reversion.

Another approach to gene disruption that produces stable mutations is allelic or gene replacement. In this case, two noncontiguous internal fragments of the target gene are cloned into the plasmid, flanking a selectable marker. The construct is then introduced into the recipient microorganism. The result is usually a double cross-over event resulting in the replacement of the target gene in the chromosome with a disrupted form of the same gene.

In 1993, the first report describing a successful isogenic mutant construction in *P. gingivalis* was published (Park and McBride, 1993). Suicide vector pBY2-IN was used to disrupt the protease gene *tpr.* In 1994, several reports were published describing mutant constructions for other genes using suicide vectors (Joe et al., 1994; Hamada et al., 1994; Malek et al., 1994; Nakayama, 1994). Joe et al. (1994) constructed a glutamate dehydrogenase–deficient mutant using plasmid pJOE1, which is derived from plasmids pNJR5 and pBA3-3. Hamada et al. (1994) constructed a *fimA* deficient mutant using vector pGP704, which was previously described for mutant construction in *Vibrio cholerae* (Miller and Mekalanos, 1988). Malek et al. (1994) constructed a *fimA*-deficient mutant using plasmid pVAL-7. Nakayama (1994) constructed a new suicide plasmid (pKDCMZ) using plasmid pVAL-1 and plasmid pACYC184; the resulting mutant had a disruption in the gene encoding a superoxide dismutase (*sod*). In 2002, a new suicide vector was described (Wu et al., 2002). The authors reported the use of vector pVA3000 to create mutations by single cross-over insertion into the *P. gingivalis* chromosome. More recently, the authors of this unit have modified pVA3000 producing pPR-UF1, which is very amenable to the construction of mutations by allelic replacement.

Electroporation and conjugation

Electroporation and conjugation are two techniques used to transfer nucleic acids into eukaryotic and prokaryotic cells. In electroporation, a high-voltage electric field is applied briefly to cells, producing transient holes in the cell membrane through which external DNA enters. This method can be used with several bacterial species, including both Gram-negative and Gram-positive bacteria. In conjugation, a microorganism carrying the plasmid containing genetic information of interest (donor strain) transfers it into another microorganism (recipient strain) via a conjugation pilus. This method can also be used with Gram-negative and Gram-positive bacteria, and conjugation is commonly used between different bacterial species.

Complementation

Complementation studies are employed to restore the function of a disrupted gene created for mutant analysis. It is important to complement an isogenic mutant to rule out polar effects in the phenotype studies of that particular gene. Shuttle vectors are commonly utilized in complementation studies. Shuttle vectors are plasmids that replicate in both microorganisms used for cloning (usually *E. coli*) and the microorganism of interest (e.g., *P. gingivalis*).

For complementation studies in *P. gingivalis*, there are two shuttle vectors described. Park et al. (1997) used *Bacteroides* pNJR12 shuttle vector (Maley et al., 1992) to study the enzymatic activities of the *tpr* gene, a membrane-associated protease in *P. gingivalis*. O'Brien-Simpson et al. (2001) also used shuttle vector pNJR12 to study the role of RgpA, RgpB, and Kgp proteases in virulence of *P. gingivalis* in a murine lesion model. More recently, a second shuttle vector was reported for complementation studies in *P. gingivalis*. Park et al. (2005) used *Bacteroides* pT-COW shuttle vector to investigate the structure and binding activities of the short fimbriae of *P. gingivalis* to *Streptococcus gordonii*. Plasmid pT-COW was originally designed for use in *Prevotella ruminicola* (Gardner et al., 1996).

Reporter gene systems

Reporter gene systems are used to study genetic expression and regulation. DNA regions containing transcriptional regulatory elements (TRE) of the target gene are fused to a gene whose function can be readily assayed. Thus, this recombinant/fusion gene is transferred into bacteria where it can be monitored under experimental conditions.

In 1996, the first reporter gene system for *P. gingivalis* was described (Lee et al., 1996). The suicide vector pPGIVET contains a multiple cloning site upstream of two tandem reporter genes that confer tetracycline resistance and galactokinase activity. DNA regions of interest containing TRE of the target gene are cloned into the multiple cloning site; thus, the reporter genes are under control of cloned promoters. Using this approach, the authors demonstrated that *hagB* and *hagC* genes are actively regulated in vivo.

Whitehead (1997) proposed another reporter gene system. It was suggested that the use of a bifunctional xylosidase/arabinosidase enzyme (*xa*) as a reporter gene could be used for most *Bacteroides* species, *P. gingivalis* and *E. coli*. The activity of the *xa* gene can be easily assayed using ρ-nitrophenol derivatives as substrates in crude extracts or toluene-treated whole cells. Furthermore, the enzymatic activities can be detected on agar plates by fluorescence with methyllumbelliferyl derivatives. Liu et al. (2000) described the use of the *xa* gene as a reporter gene for *P. gingivalis*. In addition, the use of the *luxAB* gene as a new reporter gene was proposed. However, the activity of this gene was only detected in the presence of exogenously reduced flavin mononucleotide ($FMNH_2$; Liu et al., 2000).

Other reporter genes such as luciferase, green fluorescent protein (GFP), and β-galactosidase (*lacZ*) have been described. Unfortunately, luciferase and GFP reporter gene systems require oxygen for production of fluorescence, and therefore cannot be used for *P. gingivalis*, an anaerobic bacterium. However, the *lacZ* gene has been used as a reporter gene in *P. gingivalis* to study gene regulation of *fimA* (Xie et al., 1997), *rgpA* (Tokuda et al., 1998), and *tpr* (Lu and McBride, 1998), although β-galactosidase activity is better detected when the *lacZ* gene is cloned on a multiple-copy plasmid (Lu and McBride, 1998).

Critical Parameters and Troubleshooting

The purity of DNA is critical for the efficiency of restriction enzyme reactions as well as for ligation reactions. Please refer to *Current Protocols in Molecular Biology* for more details: Moore and Dowhan (2002) for DNA purification; Bloch and Grossman (1995) for restriction digestion; and Tabor (1987) for ligation. Useful information and tips to troubleshoot problems related to the purification kits used in this unit can be found in their manuals.

Handle all enzymes carefully according to the manufacturer's instructions. Enzymes should be kept on ice for a minimum amount of time and quickly returned to the freezer. Benchtop coolers should be used to hold the enzymes while they are in use. For more detailed information about restriction enzymes in general and other enzymes used for PCR reactions, see *Current Protocols in Molecular Biology*: Bloch and Grossman (1995) for restriction digestion and Kramer and Coen (2001) for PCR.

The following formula is used to determine the amount of insert to be added to a ligation reaction:

$$\frac{\text{ng of plasmid} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{\text{mol insert}}{\text{mol vector}} = \text{ng of insert}$$

Creating mutations in small genes (<1 kb) can be problematic. Therefore, the authors suggest using portions of the flanking genes to design the inserts A and B. Once a gene has been selected as a target for mutation, the complete sequence of the targeted gene and of the flanking genes (~500 bp from each flanking gene) should be analyzed to find the restriction enzyme sites that cut only a few times or that are unique to this region. Note that these restrictions sites should be present in MCS1 and MCS2 of pPR-UF1 (see Fig. 13C.2.1). To illustrate how to prepare the inserts, the upstream primer (w) should be designed within the 3′ region of the gene flanking the targeted gene (see Fig. 13C.2.2). A restriction site already present within the gene sequence and also present in pPR-UF1 should be found. The same applies for primer z, but it should be designed using the downstream gene sequence. Primers (x and y) located within the targeted gene need to have unique restriction sites as well. However, restriction sites can be added to the primer sequence and need not be present in the targeted gene. Note that a proof-reading polymerase should be used for the PCR to minimize the risk of generating point mutations in the genes flanking the targeted gene.

It is crucial to make a restriction map of the inserts A and B to determine restriction enzymes that can be used in the construction

of mutants using plasmid pPR-UF1. Inserts A and B should be inserted in the same direction in plasmid pPR-UF1. However, it does not matter if both the inserts are cloned in the sense or antisense orientations (see Fig. 13C.2.2). Restriction enzyme *Hin*dIII should not be used in the cloning processes, as there are two sites for this enzyme in pPR-UF1.

It is crucial to make a restriction map of one's insert to determine restriction enzymes that can be used in the complementation studies using plasmid pT-COW. Plasmid pT-COW has a low copy number in *E. coli* and *Bacteroides* species. In *P. gingivalis*, such features have not been described yet; however, the low concentration of tetracycline used in the blood agar plates may be an indication that pT-COW also has a low copy number in *P. gingivalis*. Such phenomena could also be due to a weak tetracycline gene promoter in *P. gingivalis*. Thus, it is important to use only 1 μg/ml tetracycline in blood agar plates when selecting *P. gingivalis* mutants carrying the recombinant plasmid.

When using transposon mutagenesis, it may be necessary to screen a large number of mutants before finding the desired phenotype. Furthermore, more than one transposon insertion can be present per mutant. When using transposon Tn*4351*, Chen et al. (2000) showed that only 20% of mutations were single-transposon insertions, and that there was also a high rate of cointegration of the delivery vector into the genome, which can complicate further analysis of the disrupted genes.

Always use fresh cultures when performing conjugation experiments as this increases the yield of transconjugant colonies. In addition, use freshly prepared media containing antibiotics to avoid loss of antibiotic activity. Loss of antibiotic activity becomes a problem, since it increases the number of putative mutants that must be screened.

The appropriate antibiotics used in the conjugation protocols are determined by the plasmid. If using plasmid pT-COW, the authors recommend 50 μg/ml carbenicillin or 5.0 μg/ml tetracycline for *E. coli*, and 1.0 μg/ml tetracycline for *P. gingivalis*.

The use of gentamicin in blood agar plates is to prevent the growth of possible contaminants and to restrict the growth of the donor *E. coli* strain in the conjugation protocols.

The antibiotic clindamycin may be used to select for transconjugants at the same concentration as that used for erythromycin in these protocols. Clindamycin and erythromycin have similar mechanism of action—both interfere with protein synthesis. In the authors' experience, erythromycin stock solutions tend to precipitate. Clindamycin stocks do not precipitate and they are more stable than erythromycin.

The product of the *bla* gene is a β-lactamase. Sufficient β-lactamase can be produced by a single transformed *E. coli* colony to hydrolyze the antibiotic in the surrounding medium and create a protected zone in which antibiotic-sensitive colonies can grow. Using carbenicillin instead of ampicillin can reduce this problem, because carbenicillin is more resistant than ampicillin to hydrolysis by β-lactamases.

Anticipated Results

The technique described for isolating *P. gingivalis* genomic DNA should result in good yield and high-quality DNA.

When starting with 3.0 ml culture, high isolation (5.0 to 9.0 μg) of plasmid pPR-UF1 should be obtained with the protocol detailed in this unit. In mutant construction using plasmid pPR-UF1, expect few transconjugant colonies. Transformation efficiency of electrocompetent *P. gingivalis* cells is not nearly as high as in *E. coli*, and the recombination rate in *P. gingivalis* is low.

On the contrary, expect thousands of mutants when using transposon Tn*4351*. However, screening to find the desired phenotype will be more tedious than if allelic replacement were used to generate the mutation.

The transformation efficiency of *P. gingivalis* has not yet been determined.

The yield of plasmid pT-COW is low (~2.0 μg from a starting culture of 10 ml in LB broth). Larger cultures of the *E. coli* strain carrying plasmid pT-COW are necessary to achieve higher concentrations of the plasmid. For this purpose, the authors suggest following the protocols detailed in Heilig et al. (1998).

Usually <50 *P. gingivalis* transconjugant colonies are obtained per conjugation procedure.

Time Considerations

Constructing a mutation by gene replacement is time consuming, and may take longer than a month to obtain and confirm. *P. gingivalis* usually grows overnight in TSB cultures; however, it takes 3 to 4 days to grow on blood agar plates. *E. coli* growth to isolate both plasmid pPR-UF1 and recombinant

plasmids require overnight cultures. PCR reactions are typically 3 to 5 hr long. It takes 7 to 10 days to visualize *P. gingivalis* transconjugants on blood agar plates after transformation by electroporation. Finally, Southern blot analysis may take 2 to 3 days to be completed.

Constructing a transposon library can also be time consuming. The growth of *P. gingivalis* transconjugant colonies may take 7 to 10 days. The screening of the putative mutants for the desired phenotype is variable. Southern blot analysis may take 2 to 3 days to complete. In addition, PCR reactions and DNA sequencing analysis are necessary to confirm disruption of the gene of interest.

It takes ~1 week to prepare electrocompetent *P. gingivalis* cells. It is necessary to cultivate *P. gingivalis* on a blood agar plate for 3 to 4 days before inoculation of TSB. An overnight growth period in TSB is necessary, followed by a full day for preparation of the electrocompetent cells.

The conjugation protocols may take longer than a week. Fresh cultures of *P. gingivalis* and *E. coli* are required. Overnight cultures for both bacterial species are required in Basic Protocol 3 and Alternate Protocol 1. Moreover, after the conjugation is performed, it might be necessary to wait up to 10 days until *P. gingivalis* transconjugants are visible on blood agar plates.

It should take ~1 month to obtain and to confirm a mutation. *P. gingivalis* usually grows overnight in TSB cultures; however, it takes 3 to 4 days to grow on blood agar plates. *E. coli* growth to isolate both plasmid pT-COW and recombinant plasmid require overnight cultures. PCR reactions typically require 3 to 5 hr. Finally, it takes up to 10 days to visualize *P. gingivalis* transconjugants on blood agar plates after transformation by conjugation.

Introduction and confirmation of a reporter gene in *P. gingivalis* cells should not take longer than 1 month.

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Kuramitsu, H. K. 2003. Molecular genetic analysis of the virulence of oral bacterial pathogens: An historical perspective. *Crit. Rev. Oral Biol. Med.* 14:331-344.

Describes genetic manipulations that can be performed with various bacteria, including P. gingivalis.

Lamont, R.J. and Jenkinson, H.F. 1998. Life below the gum line: Pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* 62:1244-1263.

Comprehensive review article describing the various virulence factors of P. gingivalis.

Nakayama, K. 2003. Molecular genetics of *Porphyromonas gingivalis*: Gingipains and other virulence factors. *Curr. Protein Pept. Sci.* 4:389-395.

Review describing the introduction of molecular genetics to analysis of pathogenesis of P. gingivalis. Special emphasis is placed on proteinases.

Internet Resources

http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpg

Access to whole genome sequence of Porphyromonas gingivalis strain W83 as well as genomic analysis tools.

http://www.tigr.org

The Institute for Genomic Research (TIGR) provides structural, functional and comparative analysis of genomes and gene products.

Contributed by Myriam Bélanger, Paulo Rodrigues, and Ann Progulske-Fox
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Cell Culture Assay for Transient Replication of Human and Animal Papillomaviruses

This unit describes an assay (see Basic Protocol) to assess the functionality of papillomavirus origins of replication during transient transfection of cultured cells. Papillomavirus genomes generally will not replicate autonomously in cultured cells because of poor endogenous expression of viral replication proteins (E1 and E2). Consequently, replication competence is typically evaluated after transient cotransfection of the cloned genome (or a cloned genome fragment) with expression vectors for the E1 and E2 proteins (Fig. 14B.1.1). This assay is widely used to map the genomic location and boundaries of the functional origin, to test replication activity of origin mutants, and to determine replicative capacity of E1 and/or E2 mutants.

CAUTION: Human papillomaviruses are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All reagents and buffers used should be of the highest available molecular-biology grade and free of nucleases. Disposable gloves should be worn at all times when handling samples and gels to avoid nuclease contamination from the skin.

ASSESSING THE FUNCTIONALITY OF PAPILLOMAVIRUS ORIGINS OF REPLICATION DURING TRANSIENT TRANSFECTION OF CULTURED CELLS

BASIC PROTOCOL

The plasmid vector used to clone the papillomavirus origin fragment should be devoid of eukaryotic *cis* regulatory sequences, such as transcriptional control elements or origins of replication that would function in mammalian cells, as either of these types of sequences could influence replication from the cloned papillomavirus origin. For studies in the author's laboratory, the pUC18 vector is used, though many similar vectors should function adequately in this assay. The choice of vector for expression of the papillomavirus E1 and E2 proteins is less constrained. Typically, all that is required for the expression vector is a strong, constitutive promoter that will function in the cell type being transfected. The pcDNA-series vectors (Invitrogen) have a cytomegalovirus (CMV) promoter that is widely active in many cell types, and is usually suitable for E1 and E2 expression. Note that the pcDNA vectors themselves will not replicate (except in COS cells which supply T antigen), though they will persist sufficiently during the time course of the assay to provide adequate E1 and E2 levels.

The cloned papillomavirus genomic or origin DNA must be prepared from *dam*$^+$ *E. coli* to allow methylation at adenine residues. Most commonly used strains of *E. coli* are *dam*$^+$, but the published genotypes should be consulted to ensure that a *dam* mutation is not present. The author's laboratory typically uses the TB1 strain which is available from New England Biolabs. Purified DNA is transfected into an appropriate cell line, and at 24-hr intervals after transfection, the low-molecular-weight episomal DNA is extracted (see Support Protocol 1), restriction digested with *Dpn*I, and analyzed by Southern blotting (see Support Protocol 2). *Dpn*I cleaves at the sequence 5′-GATC-3′ only when the A is methylated, so residual, unreplicated input DNA will be digested

Contributed by Van G. Wilson

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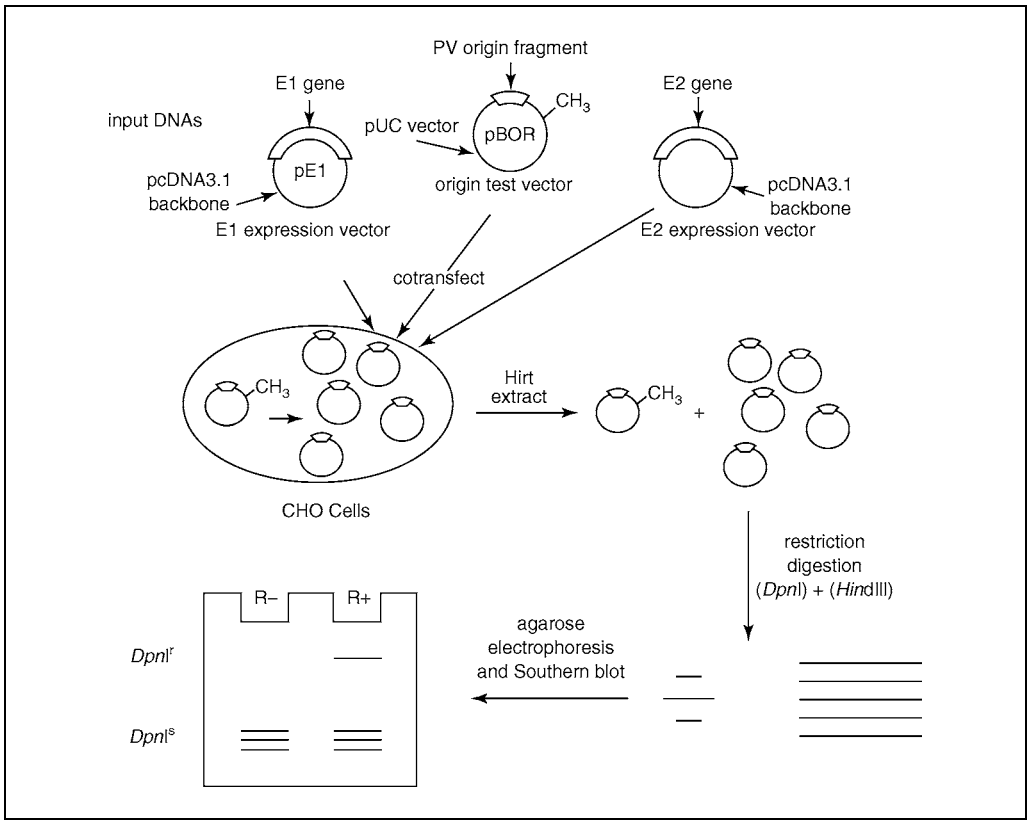

Figure 14B.1.1 Schematic overview of the transient replication assay. The input DNAs for the co-transfection consist of a papillomavirus origin-containing test vector (pBOR) and two expression vectors, pE1 and pE2, that provide the viral E1 and E2 proteins, respectively. All the input plasmid DNAs will normally be methylated at specific adenine residues due to the endogenous *dam* methylation system in *E. coli.* After cotransfection of the three plasmids into an appropriate cell line, the presence of a functional papillomavirus origin will allow E1- and E2-dependent replication of the test vector. During replication, the adenine methylation will be lost, as metazoan cells lack this activity. Subsequent Hirt extraction isolates the small nucleic acids, including the replicated and unreplicated plasmid DNAs. The input, methylated plasmid DNA is susceptible to *Dpn*I digestion and will be cleaved into small fragments. In contrast, replicated plasmid DNA will be unmethylated and, therefore, resistant to *Dpn*I cleavage. A simultaneous second digestion with a single-cut restriction enzyme (e.g. *Hin*dIII) facilitates subsequent quantitation by converting supercoiled and open circular forms of the plasmid into a single linear species. The replicated and unreplicated DNAs are separated by agarose gel electrophoresis and visualized by Southern blotting with a probe specific for the test vector. Details of this overall process are described in the text.

into small fragments. Since higher eukaryotic cells lack adenine methylation, papillomaviral DNA that replicates after transfection will produce progeny molecules that are unmethylated at adenine residues. This material will be resistant to *Dpn*I cleavage, resulting in a replication product the same size as the input DNA. The amount of replicated DNA can be quantitated and should increase over time. This assay can be used to confirm replication activity or to compare replication efficiency of mutant versus wild-type origins.

Materials

Chinese hamster ovary (CHO) cells (American Type Culture Collection)
Ham's F12 medium (e.g., Life Technologies) with and without 10% fetal bovine serum (FBS)
Trypsin, tissue culture grade

DNA stocks for transfection (store at −20°C), prepared in TE buffer, pH 7.0 (*APPENDIX 2A*):
500 ng/μl pE1 (E1 expression vector)
500 ng/μl pE2 (E2 expression vector)
100 ng/μl pBOR (origin positive vector)
100 ng/μl pUC (origin negative; parental vector for pBOR; New England Biolabs)
500 ng/μl pcDNA3.1 (parental vector for pE1 and pE2; Invitrogen)
Lipofectamine2000 (Invitrogen)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
70% (v/v) ethanol
TE buffer, pH 7.0 (*APPENDIX 2A*)
500 μg/ml RNase, DNase-free (Boehringer-Mannheim)
10× restriction enzyme buffer compatible with *Dpn*I and *Hin*dIII
≥20 U/μl *Dpn*I restriction endonuclease
≥20 U/μl *Hin*dIII restriction endonuclease
Molecular-biology-grade H_2O
10× agarose sample buffer (see recipe)
Molecular-biology-grade agarose
DNA quantitation standards (see recipe)
1% (w/v) ethidium bromide

60-mm and 100-mm tissue culture plates
68°C water bath or heating block
Ultraviolet lamp
Plastic sheet protector (i.e., folder for papers available from office supply store)

Additional reagents and equipment for counting cells using a hemacytometer (Strober, 1997), Hirt precipitation (see Support Protocol 1), agarose gel electrophoresis (Voytas, 1992), Southern blotting (see Support Protocol 2), and detection of radiolabeled DNA in blots by autoradiography or phosphor imaging (Voytas and Ning, 2002)

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Maintain cells at subclonfluency.

Prepare cells

1. Grow Chinese hamster ovary (CHO) cells in Ham's F12 medium supplemented with 10% FBS.

   The number of cells needed depends on the number of samples to be transfected. In a 75-cm^2 flask at 50% to 70% confluency, there are ~6 × 10^6 CHO cells; each transfected sample will require 1 × 10^6 cells. Other rodent or primate cell lines can also be used, as replication of papillomavirus origin-containing DNA is not species restricted, as long as the E1 and E2 proteins are expressed.

2. On the afternoon prior to transfection (day 1), harvest the subconfluent cells by trypsinization using tissue-culture-grade trypsin. Pool the harvested cells and quantitate with a hemacytometer (Strober, 1997).

3. Prepare new 60-mm tissue culture plates with 5 ml Ham's F12 medium/10% FBS per plate.

   There should be one plate for each sample to be transfected, though preparing a few extras is advisable to account for contamination or other loss.

   If antibiotics have routinely been included in the culture medium, they should be excluded from steps 3 to 11, as antibiotics will cause cell death during Lipofectamine2000 transfection.

4. Add 1 × 10^6 cells per plate, swirl gently to distribute evenly, and incubate overnight.

Table 14B.1.1 Assembly of DNAs for Transfection

Sample	pE1	pE2	pUC	pBOR	pcDNA3.1
1	2.0 μl (1000 ng)	2.5 μl (250 ng)	1.0 μl (100 ng)	—	—
2	2.0 μl (1000 ng)	2.5 μl (250 ng)	—	1.0 μl (100 ng)	—
3	—	2.5 μl (250 ng)	—	1.0 μl (100 ng)	2.0 μl (1000 ng)
4	2.0 μl (1000 ng)	—	—	1.0 μl (100 ng)	0.5 μl (250 ng)

Transfect cells

5. The next morning (day 2), check each plate for uniform cell distribution, cell health, and possible contamination.

   Confluency should be 50% to 80%.

6. Approximately 1 hr before transfection, aspirate the medium from each plate and replace with 3 ml fresh Ham's F12/10% FBS. Return plates to incubator until step 11.

7. Prepare the samples for transfection by mixing combinations of the appropriate DNA stock solutions in microcentrifuge tubes as indicated in Table 14B.1.1.

   Minimally, each reaction requires three DNAs: (1) the plasmid to be tested for replication activity (either a negative control plasmid or the cloned papillomavirus genomic or origin fragment); (2) an E1 expression vector; and (3) an E2 expression vector. While there is some degree of cross-species functionality of the origins and replication proteins, maximum activity is observed with a cognate origin/replication proteins mixture. The example provided is for the bovine papillomavirus (BPV)–cloned origin and E1+E2 proteins. The ratios of these three DNAs have been optimized for the BPV system, and ratios of plasmid DNAs for other papillomavirus origins with their cognate replication protein expression vectors should be empirically optimized by systematically varying the amounts of the three plasmids. The final quantity of total DNA per sample should be equal for all samples and should be between 2 to 3 μg.

   Samples 1, 3, and 4 in Table 14B.1.1 are negative controls with nonorigin DNA (pUC), no E1, and no E2, respectively. True replication should only occur with sample 2, which contains origin DNA (pBOR) and both the E1 and E2 replication factors. The pcDNA3.1 DNA, which is the parental vector for pE1 and pE2, is used to adjust each reaction to a constant total amount of DNA, since the DNA quantity affects the transfection efficiency.

8. Add 250 μl Ham's F12 medium without FBS to each tube of DNA and stir gently with the pipet tip.

9. Prepare sufficient master mix for all samples by combining 8 μl Lipofectamine2000 and 250 μl Ham's F12 without FBS for each sample. Stir thoroughly with a pipet tip and incubate 5 min at room temperature.

   For example, for ten samples, combine 80 μl Lipofectamine 2000 and 250 μl Ham's F12 without FBS. It is usually advisable to prepare enough volume for one extra sample, to account for pipetting losses.

10. Add 250 μl of the master mix to each DNA sample prepared in step 7. Stir gently and incubate 20 min at room temperature.

11. When incubation is complete, add all of each DNA sample to a separate plate from step 6. Swirl plates gently and return to incubator.

12. The next morning (day 3), prepare three 100-mm tissue culture plates for each original sample. Add 10 ml of Ham's F12 medium/10% FBS to each plate and place in an incubator for at least 1 hr before step 13.

13. At 24 hr post-transfection (see step 11), aspirate the medium from each transfected plate and harvest the cells by standard trypsinization. Divide the cells from each original plate equally into the three 100-mm plates prepared in step 12. Swirl gently to distribute the cells and return all the plates to the incubator.

Harvest transfected cells and extract DNA

At 48, 72, and 96 hr post-transfection (i.e., days 4, 5, and 6), harvest one of the plates for each sample and process as in steps 14 to 23.

14. Aspirate the medium from the plate, add 5 ml room temperature PBS, swirl gently, and then aspirate the PBS.

15. Add another 5 ml room temperature PBS, swirl, and aspirate.

16. Tilt plate and let drain 1 to 2 min to collect residual PBS, then aspirate the PBS.

17. Perform Hirt precipitation (see Support Protocol 1).

18. After the overnight incubation in the refrigerator (see step 14 of Support Protocol 1), microcentrifuge the tube 45 min at 12,000 × *g*, 4°C.

19. Carefully remove the supernatant with an automatic pipettor, being careful not to disturb the small pellet of nucleic acid. Discard the supernatants.

20. Add 200 µl of room temperature 70% ethanol and let stand at room temperature 1 to 2 min.

21. Microcentrifuge 45 min at 12,000 × *g*, 4°C, remove supernatant with an automatic pipettor, and discard. Drain the residual fluid from the tubes by inverting them for 10 min at room temperature. Aspirate any liquid that accumulates on the tube wall, being careful not to disturb the pellet. After aspiration, let tube stand upright another 5 min at room temperature to dry the pellet.

    It is critical that all liquid be removed at this step as residual alcohol will impair the solubility of the nucleic acid at the next step. In addition, even small volumes of remaining liquid can alter the sample volume significantly at step 22.

22. Redissolve each final pellet in 10 µl of TE buffer, pH 7.0, and pool the A and B tubes for each sample.

23. Add 1.6 µl of 500 µg/ml RNase and incubate 1 hr at 68°C.

    After the RNase digestion, the samples can be stored for several days at 4°C or indefinitely at −20°C. Typically, the samples from each time point (48, 72, and 96 hr) are stored until all of the time-point samples have been processed. Subsequent steps are performed in parallel on samples from each time point.

Perform restriction digestion

24. Transfer 5 µl of each sample from step 23 into a new microcentrifuge tube. Add 1.5 µl of an appropriate 10× restriction enzyme buffer, 10 U of *Dpn*I, 10 U of *Hin*dIII, and sufficient molecular-biology-grade water for a final volume of 15 µl.

    HindIII is used to linearize the test plasmids (pBOR or pUC) as it cuts each plasmid only once. Linearization facilitates quantification of replicated DNA as it converts all the supercoiled and open circular molecules to a single linear species. Any single-cut restriction enzyme would serve at this step, and the choice of enzyme should be based on the test plasmid being studied.

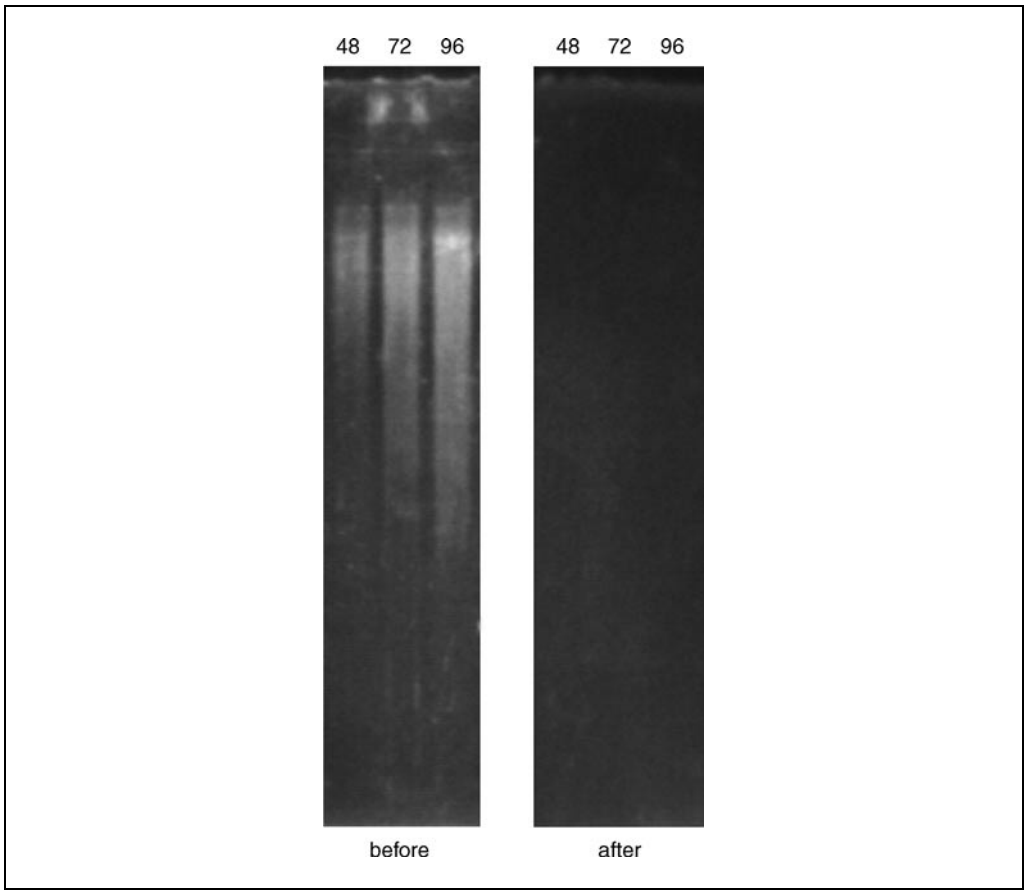

Figure 14B.1.2 Ethidium bromide–stained agarose gel before and after transfer of the DNA to the GeneScreen membrane. The gel was photographed under UV illumination. Numbers above the lanes indicate the hour post-transfection at which the DNA samples were harvested.

25. Incubate reactions at least 5 hr at 37°C, then add an additional 5 U *Dpn*I and continue incubation overnight.

    Extensive overdigestion with DpnI is critical, since any undigested input DNA would falsely contribute to the replicated DNA signal on the Southern blot. The control samples (1, 3, and 4; see step 7) should exhibit no signal at the position of linear, full-length plasmid.

26. At the end of the incubation (day 9), add 2 µl of 10× agarose sample buffer to each sample and store at 4°C until the agarose gel is run.

Perform agarose gel electrophoresis

27. Prepare an 0.8% agarose gel (see Voytas, 1992) containing 0.4 µg/ml of ethidium bromide (added from 1% w/v stock).

    The physical size of the gel should be tailored to the number of samples and the expected sizes of the replicated and unreplicated DNAs. Thinner gels facilitate DNA transfer to the membrane, but gel thickness must be sufficient for the wells to hold 15 µl of sample.

28. Load 15 µl of each sample (see step 26) on the gel. Also load 10 µl of each DNA quantitation standard.

29. Electrophorese at 20 mA (Voytas, 1992). Use the migration of the bromphenol blue in the sample buffer to monitor run time.

    Since it is necessary to retain the small DpnI digestion products on the gel, electrophoresis is typically stopped before bromophenol blue reaches the bottom of the gel. Bromophenol blue runs at ~400 to 500 bp in this gel.

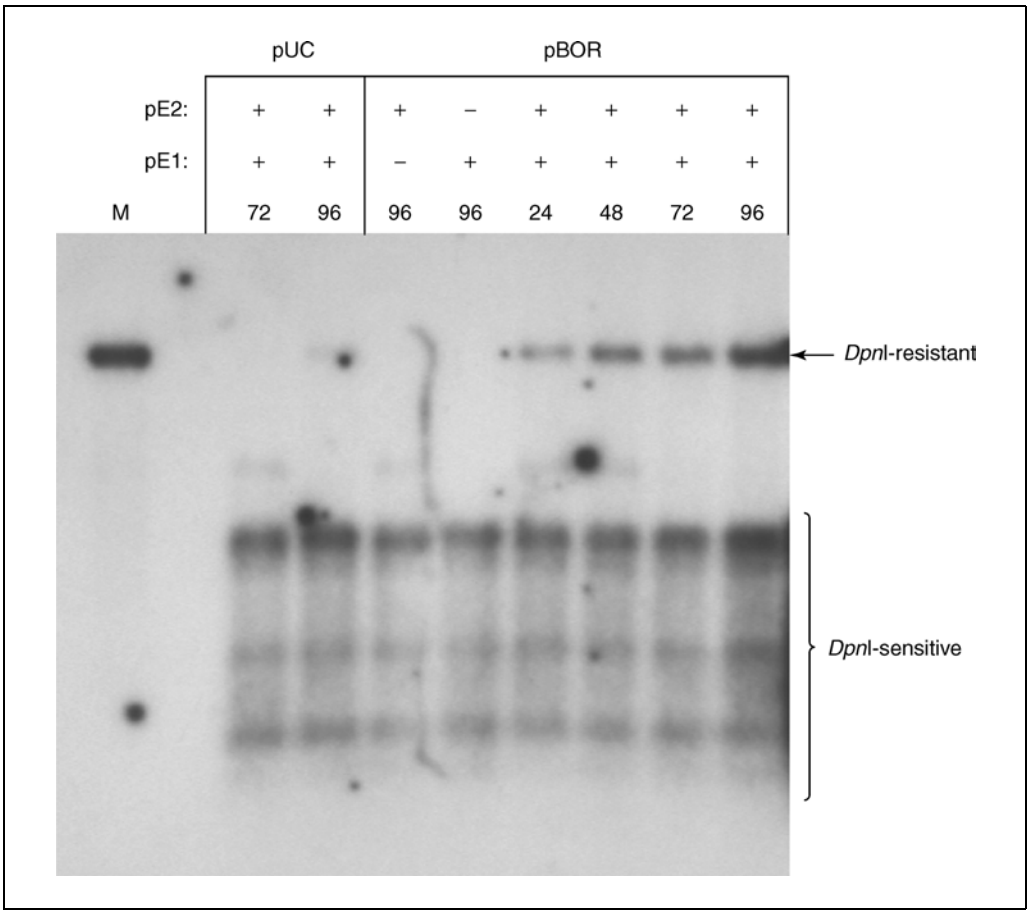

Figure 14B.1.3 Phosphordensitometer image of a typical replication experiment. Numbers above the lanes indicate the time (in hr) post-transfection at which each transfected cell culture was harvested. The presence (+) or absence (−) of the pE1 and pE2 DNAs in the original transfection mixture for each sample is as indicated. Samples also received either pUC (nonorigin DNA) or pBOR (bovine papillomavirus origin clone) as shown. Lane M is a marker containing 200 pg of linearized pBOR DNA. The first four transfection lanes are negative controls with the nonorigin DNA or the origin DNA lacking either E1 or E2. Note that there is a minor signal at the position of the marker DNA in the 96-hr pUC sample, indicating that the *Dpn*I digestion was slightly incomplete for this sample. The remaining transfection lanes show increasing replication of pBOR over time. The bracket indicates the digested input DNA (*Dpn*I-sensitive), and the arrow indicates the replicated pBOR DNA (*Dpn*I-resistant) that was linearized with *Hind*III.

30. Photograph the gel under ultraviolet illumination.

    There should be a visible smear of cellular DNA in each of the samples that increases in intensity from the 48- to 96-hr samples (Fig. 14B.1.2).

Perform Southern blotting and visualize by autoradiography

31. On day 10, transfer the DNA from the gel to an appropriate membrane and perform a Southern blot with a ^{32}P-labeled probe that will detect the origin plasmid (see Support Protocol 2).

32. Place the membrane inside a sheet protector and expose the blot to X-ray film (with an intensifier screen) or image using a phosphor imager (both techniques described in Voytas and Ning, 2002) to visualize the replicated and unreplicated DNAs (Fig. 14B.1.3).

The sheet protector contains the radioactivity so the membrane will not contaminate either the intensifier or phosphor imager screen. The protector also prevents the membrane from sticking to X-ray film and damaging the emulsion. Typically these blots require at least overnight exposure.

Samples 1, 3, and 4 (negative controls) should have only small fragments (DpnI digestion products), while sample 2 should have both small fragments (residual input DNA) and a band at the position of the linear pBOR DNA (replicated product).

SUPPORT PROTOCOL 1

HIRT PRECIPITATION

This is a standard protocol for extraction of low-molecular-weight nucleic acids, e.g., plasmids or small DNA virus genomes, from cultured mammalian cells. The procedure comprises four distinct operations: (1) cell lysis; (2) high-salt precipitation of cell debris and large, chromosomal DNA fragments; (3) removal of protein from the low-molecular-weight DNA fraction; and (4) concentration and recovery of the low-molecular-weight DNAs by alcohol precipitation. Direct lysis of the plated cells in an SDS-containing buffer inhibits cellular DNase activity and preserves DNA integrity while facilitating efficient recovery of all the cellular components. The subsequent addition of NaCl to the lysis buffer selectively precipitates the cellular debris and large chromosomal DNA fragments, while the smaller nucleic acids remain in solution. Contaminating cellular proteins remaining in the soluble fraction after the NaCl-precipitation step are removed by a combination of proteinase K digestion followed by phenol-chloroform extraction. Lastly, the plasmid DNA in the deproteinated soluble fraction is concentrated and recovered by alcohol precipitation. Note that this final fraction also includes other small nucleic acids such as RNAs and smaller chromosomal fragments that may be problematic for downstream applications other than Southern blotting.

Materials

Plates containing cells to be lysed (see Basic Protocol, step 16)
Hirt lysis buffer (see recipe)
5.0 M NaCl (sterilized by autoclaving)
20 mg/ml proteinase K stock in sterile H_2O (store up to 1 year at −20°C)
25:24:1 phenol/chloroform/isoamyl alcohol (Ambion)
Chloroform
TE buffer, pH 7.0 (*APPENDIX 2A*)
Isopropanol
50°C water bath or heating block

Lyse cells

1. Dispense 800 µl of Hirt lysis buffer into each plate and swirl gently for 2 min.

   The cell/buffer suspension should become viscous during this swirling process. It is important to ensure that the buffer covers the plate thoroughly, so that all cells are lysed and harvested.

2. Tilt the plates and incubate 10 min at room temperature to collect the lysed cell suspension.

3. Pour the suspension directly from each plate into a 1.5-ml microcentrifuge tube.

Precipitate cellular DNA

4. Add 200 µl of 5.0 M NaCl. Mix gently by inverting 5 to 10 times. Do the addition and mixing one sample at a time.

5. Place tubes on ice ≥1 hr.

   This incubation is usually extended overnight for convenience.

6. At the end of the incubation, centrifuge the tubes 30 min at 12,000 × *g*, 4°C. Transfer the supernatants to new tubes using a 1-ml automatic pipettor.

   After the centrifugation there should be a large, whitish, solid pellet. If the pellet is unstable or if there is unpelleted debris, then recentrifuge until the supernatant is free from debris. When removing the supernatant, it is better to leave a small amount of liquid behind than to transfer any of the debris material.

Remove cellular protein

7. Add 5 μl of the proteinase K stock and incubate for 5 hr at 50°C.
8. Add 500 μl of 25:24:1 phenol/chloroform/isoamyl alcohol, vortex for 10 sec, incubate 5 min at room temperature, then spin 1 to 2 min in a microcentrifuge.

   CAUTION: *Phenol and chloroform are hazardous and require use of protective gloves and eyewear. Steps 8 to 12 should be performed in a chemical hood to avoid inhalation of these reagents.*

9. Transfer the supernatants with an automatic pipettor to new microcentrifuge tubes and repeat step 8.
10. Transfer the supernatants to new microcentrifuge tubes and add 500 μl chloroform. Vortex for 10 sec and spin 1 to 2 min in a microcentrifuge.
11. Transfer the supernatants to new microcentrifuge tubes.
12. Carefully measure the volume of each supernatant with a an automatic pipettor, then adjust each sample to 1400 μl with TE buffer, pH 7.0.

Precipitate plasmid DNA

13. Split each sample into two 700-μl in two microcentrifuge tubes, A and B.
14. Add 42 μl of 5.0 M NaCl and 665 μl isopropanol to each tube. Vortex for a few seconds then incubate on ice in a refrigerator overnight.

    Samples are stable at this step and can be stored indefinitely at 4°C.

SOUTHERN BLOTTING

SUPPORT PROTOCOL 2

Southern blotting is commonly used to visualize and quantitate the plasmid DNAs that are extracted from the cells at intervals post-transfection. Hybridization with a probe specific for the target DNA allows selective detection of the target DNAs amidst an enormous background of residual cellular DNA fragments. There are numerous variations of the gel transfer procedure and the hybridization conditions for Southern blotting. The downward transfer protocol described here is simple, effective, and reliable, but any other standard transfer procedure would likely be suitable as well. Hybridization conditions may need to be optimized for the specific plasmid DNAs and probes employed.

Materials

Agarose gel containing separated proteins (see Basic Protocol, step 29)
0.25 M HCl
0.4 M NaOH
20× SSC (*APPENDIX 2A*)
0.4 mg/ml ethidium bromide
Probe DNA stock: prepare linearized pBOR as described for DNA quantitation standards (see recipe), but resuspend at 1 μg/ml in TE buffer, pH 7.0

Prime-A-Gene kit (Promega) containing:
dATP, dGTP, and dTTP stock solutions
5× buffer
Nuclease-free H_2O
Nuclease-free BSA stock solution
DNA polymerase I large (Klenow) fragment stock solution
10 mCi/ml [^{32}P]dCTP (3000 Ci/mmol)
Rapid-Hyb buffer (Amersham Biosciences)
0.1× SSC (*APPENDIX 2A*)/0.1% (w/v) SDS

Gel staining trays
GeneScreen Plus membrane (PerkinElmer Life Sciences)
Whatman 3MM filter paper
Platform rocker
Stratalinker UV cross-linker (Stratagene)
65° or 95°C water bath or heating block
Hybridization oven
Hybridization bottles
15-ml conical tubes

CAUTION: Radioactive materials require special handling. See *APPENDIX 1C*.

Perform DNA transfer

1. Trim away the top and sides of the gel to remove the wells and the unused lanes on either side of the samples; leave one blank lane on each side of the sample lanes. Measure the dimensions of the gel.

   Since the gel is fragile, it is left on the gel tray during steps 2 to 5 to facilitate handling. The gel will be slid off of the tray onto the membrane in step 8.

2. Submerge the gel in 0.25 M HCl and incubate 15 min.

   The HCl treatment nicks and denatures the DNA, which facilitates subsequent transfer to the membrane. The bromophenol blue band will generally turn yellow during this incubation, which is a good indication that the HCl has thoroughly penetrated the gel.

3. During the incubation in step 2, prepare the following:

   a. Cut a GeneScreen Plus membrane to the exact dimensions of the gel. Place a small mark on the upper right hand corner of the membrane with pencil to identify the orientation after transfer.

      Always handle the membranes with gloves, as skin oils will interfere with the binding capacity of the membrane.

   b. Cut six pieces of Whatman 3MM paper to the same dimensions as the membrane.

   c. Cut paper towels into pieces the same dimension as the membrane.

      A stack of these pieces that is at least 3- to 4-in. high will be needed.

   d. Cut two wicks from the Whatman 3MM paper of a width equal to the height of the trimmed gel (i.e. from the wells to the bottom of the gel) and ~2 feet in length (these can be shortened later as necessary).

4. After 15 min, remove the gel and gel tray from the HCl. Drain the excess HCl from the surface of the gel by tipping the gel tray on a paper towel. Hold the gel gently while tipping the tray to prevent it from sliding off the tray.

5. Submerge the gel in 0.4 M NaOH for 1 to 2 min to neutralize HCl, then remove the gel and let it stand while quickly assembling the following components.

   Do not allow the gel to dry. If necessary, return the gel to the NaOH solution to keep it moist.

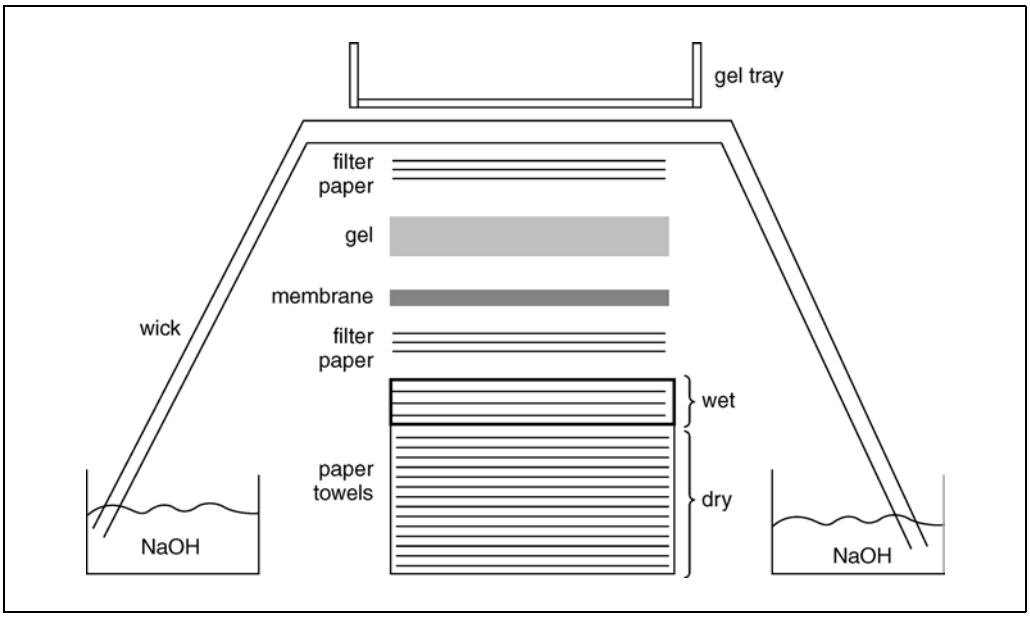

Figure 14B.1.4 Diagram of the organization of the gel transfer stacking setup. Details of the assembly and transfer are described in the text.

6. Immerse the GeneScreen membrane in 0.4 M NaOH until it hydrates.

   The membrane should wet uniformly within 1 min. Areas that remain unhydrated are defective and will not bind transferred DNA. If such areas are observed, prepare a new membrane.

7. Soak the 3MM pieces, the wicks, and an ~ 0.25-in. stack of the paper towels in 0.4 M NaOH until all are thoroughly wetted.

8. Assemble the stack as in Figure 14B.1.4 (transfer is performed at room temperature). Use a 5- or 10-ml plastic pipet to roll out air bubbles between each layer. The order of the stack from the bottom up is:

   Dry paper towels
   Wet paper towels
   3 sheets wet 3MM paper
   Membrane
   Gel
   3 sheets wet 3MM paper
   Wicks

   Each level must be carefully aligned with the one below. The intent is for the NaOH solution to flow up the wicks and straight down through the gel to diffuse the DNA out of the gel and onto the membrane. Poor alignment of the edges of each level may allow the NaOH to bypass a level and "short-circuit" the NaOH flow. Likewise, air bubbles caught between any of the pieces will prevent transfer through the region of the bubble and may result in blank spots on the membrane.

   DNA will begin to transfer from the gel to the membrane very quickly when gel and membrane are brought into contact. Moving the gel around on the membrane while trying to align the edges of these two components may result in a blurred image, as the pattern of the DNAs on the membrane will be shifted slightly during the alignment process. Consequently, great care should be taken to align the gel on the membrane accurately on the first attempt, so that it does not have to be moved and repeated.

Remember that the gel is fragile and is best transferred by sliding it off of the gel tray onto the membrane. Be sure to orient the gel so that the upper right-hand corner of the gel coincides with the pencil mark on the membrane.

Lastly, the setup is somewhat messy, so the authors typically assemble the stack on a glass plate or in a glass dish, though this is not strictly necessary.

9. Place ends of the wicks in trays of 0.4 M NaOH.
10. Set the gel tray on top of wick to weigh the wick down and ensure good contact.
11. Let the transfer proceed overnight.
12. Disassemble the stack and soak the membrane in 2× SSC for 10 to 15 min at room temperature with rocking.
13. Incubate the gel 5 min in 50 ml of 0.4 μg/ml ethidium bromide. Photograph the gel under UV illumination, then discard the gel and the ethidium bromide solution into an appropriate chemical waste receptacle.

    The gel will dehydrate and lose ethidium bromide during the transfer process, and will be quite thin. After restaining with ethidium bromide, there should be little or no DNA visible in the gel, indicating that the transfer was complete (see Fig. 14B.1.1).

14. Place the membrane on a piece of clean filter paper and let stand for several minutes until the excess liquid is absorbed.
15. Cross-link the DNA to the membrane by performing two cycles of UV irradiation with a Stratalinker set to full power.

    Alternatively, cross-linking can be achieved by baking the membrane for 1 hr at 80°C in a vacuum oven.

16. Let the membrane air dry at room temperature, then place between two sheets of fresh filter paper, wrap in aluminum foil, and store at 4°C until hybridization.

Radiolabel probe preparation

17. Thaw the probe DNA stock completely and transfer 25 μl to a new microcentrifuge tube.

    The probe DNA is usually the parental plasmid vector used for cloning the papillomavirus sequence.

18. Denature the probe DNA by heating 2 min at 95°C.
19. Quickly cool the DNA by transferring the microcentrifuge tube directly from the 95°C bath to an ice-water bath.
20. For ^{32}P-dCTP labeling, make a dNTP stock by mixing 1 μl each of the dATP, dGTP, and dTTP stock solutions from the Prime-A-Gene kit.

    For this radiolabeling procedure, the Promega Prime-A-Gene kit is used, though other products may give comparable results.

21. Remove the DNA from the ice-water bath and microcentrifuge briefly at maximum speed to collect the liquid at the bottom of the tube.
22. Add the following reagents to the tube:

    10 μl 5× buffer from Prime-A-Gene kit
    5 μl nuclease-free water from Prime-A-Gene kit
    2 μl dNTP stock from step 20
    2 μl BSA stock solution from Prime-A-Gene kit.

23. Add 5 μl of 10 mCi/ml [^{32}P]dCTP (3000 Ci/mmol).

    CAUTION: *Proper radiological safety procedures should be followed from this point on; see APPENDIX 1B.*

24. Add 1 μl of DNA polymerase I Klenow fragment stock from the Prime-A-Gene kit and stir gently to mix components.

25. Incubate 60 min at room temperature.

26. Denature for 2 min at 95°C.

27. Quickly cool by transferring to an ice-water bath. Leave in the bath until step 32.

    The probe can be used immediately for hybridization. If not used immediately, it should be stored at −20°C and always denatured/quick-cooled prior to use.

Hybridize blot to probe

28. Prewarm the hybridization oven to 65°C.

29. Add 15 ml Rapid-Hyb buffer to a hybridization bottle and place in oven for at least 30 min to prewarm the buffer.

30. Place the membrane from step 16 into the hybridization bottle.

31. Incubate the bottle for 60 min on the rotating wheel in the oven.

32. Withdraw 2 ml of buffer from the hybridization bottle, transfer into a 15-ml conical tube, and add 12.5 μl of the probe from step 27. Mix carefully by gently pipetting up and down.

33. Hold the hybridization bottle vertically and re-add the 2 ml that was withdrawn, now mixed with the probe, to the 13 ml of buffer in the bottom of tube. Do not touch the membrane with the pipet or cause spattering that will contact the membrane. Mix buffer in tube thoroughly by gentle stirring and return bottle to oven.

    It is important to prevent the probe from contacting the membrane until it has been diluted into the 15 ml of Rapid-Hyb buffer, to prevent intense blotches of nonspecific radioactivity on the membrane.

34. Rotate the bottle for 2.5 hr in the oven at 65°C.

35. Aspirate the buffer thoroughly into radioactive waste.

36. Add 100 ml of a room temperature solution of 2× SSC/0.1% SDS.

37. Rock the bottle for 20 min at room temperature and then aspirate the wash into radioactive waste.

38. Add 100 ml of a 65°C solution of 0.1× SSC/0.1% SDS.

39. Incubate 20 min on the rotating wheel in the hybridization oven at 65°C.

40. Aspirate the wash into radioactive waste and repeat steps 38 and 39.

41. Aspirate the final wash into radioactive waste.

42. Remove the membrane from bottle and lay on filter paper for a few seconds to blot off excess liquid.

43. Transfer the membrane to fresh filter paper and let air dry.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose sample buffer, 10×

50% (v/v) glycerol
0.5% (w/v) bromophenol blue
0.5% (w/v) xylene cyanol
Store up to 1 year at 4°C

DNA quantitation standards

pBOR standards: Linearize 1 μg of pBOR in a 25-μl reaction containing 2.5 μl of 10× NEBuffer 2 (New England Biolabs) and 5 U of *Hin*dIII. Incubate the reaction 1 hr at 37°C, then inactivate the *Hin*dIII by incubating 20 min at 65°C. Dilute the reaction to 1 ml with TE buffer, pH 7.0 (*APPENDIX 2A*; final pBOR stock will be at a concentration of 1000 pg/μl). Prepare standards by mixing 1, 2.5, 5, 10, and 20 μl of the digested pBOR stock each with 1 ml of TE buffer, pH 7.0, to give the following final concentrations: 1000, 2500, 5000, 10,000, and 20,000 pg/ml.

Hirt lysis buffer

10 mM Tris·Cl, pH 7.7 (*APPENDIX 2A*)
10 mM EDTA
Autoclave, then add 6 ml of 10% (w/v) SDS per 100 ml buffer
Store up to 1 year at room temperature.

COMMENTARY

Background Information

Papillomaviruses are the etiological agents of skin and mucosal warts in human and animals. While these viruses are quite specific for their natural host species and generally will not infect another, they nonetheless share very similar genome organizations and related proteins. Consequently, certain animal papillomaviruses, primarily the cottontail rabbit papillomavirus (CrPV) and the bovine papillomavirus (BPV), have been useful experimental model systems for molecular characterization of the viral life cycle and gene functions. Bovine papillomavirus type 1 was the first genome cloned and sequenced, and for many years was the prototype for papillomavirus studies. In subsequent years, numerous human papillomaviruses (HPVs) were isolated, and there are now over 100 subtypes of HPV that have been identified. While most HPV infections are inapparent or lead to benign lesions, several specific subtypes of HPV are associated with malignant disease such as cervical cancer.

Papillomaviruses cannot be propagated in standard cell cultures, as they will not produce virions under these conditions. Therefore, traditional virological assays for replication, such as plaque assays, cannot be performed. Consequently, assessment of viral genome replicative activity, mapping of functional origins of replication, and mutational analysis of requisite *cis* and *trans* factors required the development of an assay that measures viral DNA specifically and directly. The transient transfection approach described here was initially applied to bovine papillomavirus (BPV) in the mid 1980s by the Botchan laboratory (Lusky and Botchan, 1984, 1986), using *Dpn*I digestion of input DNA as developed by Peden et al. (1980). The assay was subsequently optimized and used to identify the authentic origin of replication for BPV (Ustav and Stenlund, 1991; Ustav et al., 1991). Shortly thereafter, it was demonstrated that a major restriction to human papillomavirus (HPV) DNA replication in cell culture is poor expression of the viral replication initiation proteins, E1 and E2, and that providing these two proteins from heterologous expression vectors allowed efficient replication of viral origin-containing DNAs (Chiang et al., 1992). These observations provided the conceptual framework whereby the transient assay can be applied to any

papillomavirus as a triple transfection system comprising an origin-containing plasmid, an E1-expressing vector, and an E2-expressing vector. This approach has been widely used to study critical *cis* and *trans* factors for both BPV and HPVs (Chiang et al., 1992; Lu et al., 1993; Gopalakrishnan and Khan, 1994; Sverdrup and Khan, 1994; Holt and Wilson, 1995; Piirsoo et al., 1996; McShan and Wilson, 1997; Lin et al., 2000; White et al., 2003). Typically the *Dpn*I-resistant, replicated DNA has been detected by Southern blotting using a probe specific for the target plasmid containing the papillomavirus origin. Alternatively, there have been recent studies that have utilized quantitative PCR to detect the target sequences after *Dpn*I digestion (Titolo et al., 1999; Taylor and Morgan, 2003; Wang et al., 2003), and these references should be consulted for this alternative approach.

Critical Parameters and Troubleshooting

Transfection

Efficient cotransfection of the three plasmids (origin, E1, and E2) is essential for adequate detection of the replication signal. The initial report applying this assay to BPV utilized electroporation for introduction of the DNAs (Ustav and Stenlund, 1991); however, the protocol reported here utilizes lipid-based transfection, which the authors of this unit find more consistent than electroporation. The choice of transfection protocol is likely dependent upon the cell type being used, and should be optimized via a convenient reporter system, such as a GFP (green fluorescent protein)–expressing construct. Any transfection protocol should be suitable as long as the DNAs are delivered effectively, i.e., at efficiencies of at least 30%.

E1 and E2 Expression

Failure to observe replication may be due to inadequate expression of the E1 protein, the E2 protein, or both. The expression vectors for these two viral proteins should have a suitable promoter for the cell type being used. Typically, a highly active constitutive promoter with broad host range, such as the cytomegalovirus immediate early (CMV IE) promoter, is adequate for expression of the viral proteins. In addition, optimum replication efficiency requires an appropriate ratio between the E1 and E2 expression vectors and the origin-containing vector. This ratio must be determined empirically by systematic variation of the quantities of the three DNAs used in the transfection. Utilization of a positive control set, such as the BPV system described here, will provide a reference sample for confirmation that replication can be detected.

Hirt extraction

Proper performance of the Hirt extraction procedure is critical for quantitatively equivalent recovery of plasmid DNAs from each sample being processed. Typically, samples are processed in sets of three to four plates for steps 1 to 3 of Support Protocol 1, to ensure complete lysis and to keep the timing consistent. For step 4, each individual sample should be mixed *immediately* after addition of the NaCl to prevent localized extremely high salt conditions at the top of the Hirt solution, as would result from adding the salt to all samples before beginning the mixing, which can lead to anomalous precipitation. In the subsequent phenol-chloroform extraction steps (steps 8 to 11) it is also important to remove the aqueous layers as thoroughly as possible to avoid sample-to-sample variation due to differences in recovery volume.

DpnI digestion

A major technical issue is failure to completely digest unreplicated input DNA with *Dpn*I, as incomplete digestion will lead to a false positive contribution to the replication signal. Consequently, it is critical that a negative control containing the E1 and E2 expression vectors and a non-origin plasmid be processed in parallel with the test sample(s) for every experiment—i.e. "historical controls" are not adequate. There should be no full-length plasmid band detected in the Southern blot for the negative control sample. If such a band is detected, the conditions for *Dpn*I digestion should be made more stringent by increasing the number of units of enzyme during each of the incubations.

Southern blot sensitivity

Hybridization and wash conditions may need to be optimized for the specific probe-target combination utilized. A series of dilutions of the linearized target plasmid DNA should be analyzed to determine the sensitivity limit; detection down to 50 to 100 pg of the target DNA is necessary to ensure that low levels of replicated DNA can be visualized. If this range of DNA cannot be detected using the conditions outlined in this protocol, consult Brown (1999) for details on Southern blotting.

Table 14B.1.2 Time Requirements for the Cell Culture Assay for Transient Replication of Papillomaviruses

Procedure	Time required	Protocol step
Growth of cells	7-14 days	Basic Protocol, step 1
Purification of plasmid DNAs	2 days	—[a]
Plating cells for transfection	2 hr	Basic Protocol, steps 2-4
Transfection	2 hr	Basic Protocol, steps 5-11
Splitting and reseeding transfected cells	1 hr	Basic Protocol, step 12-13
Incubation	2-4 days	Basic Protocol, step 13 (see note above step 14)
Harvesting DNA (Hirt extraction and alcohol precipitation)	2-3 days	Support Protocol 1, then Basic Protocol, steps 14-22
RNase digestion	1 hr	Basic Protocol, step 23
Restriction digestion	1 day	Basic Protocol, steps 24-26
Agarose gel electrophoresis	6 hr	Basic Protocol, steps 27-30
Preparation of agarose gel for blotting	1 hr	Basic Protocol, step 31
DNA transfer from gel to membrane	Overnight	Support Protocol 2, steps 1 to 16
Hybridization	6 hr	Support Protocol 2, steps 17 to 43
Exposure of membrane to film or phosphor imager	1-5 days	Basic Protocol, step 32

[a]Not described in this unit; see Ausubel et al. (2005) for plasmid purification techniques.

Anticipated Results

The protocol described here is useful for measuring replication activity of cloned papillomavirus genomes or origin-containing fragments.

Use of the Hirt extraction procedure enriches for low-molecular-weight DNA, i.e., the plasmid DNAs (either residual input or replicated molecules) and decreases the background in the Southern blot step by eliminating much of the cellular nucleic acid. There should be a small but visible pellet after the isopropanol precipitation step of the Hirt procedure. With the Southern blot procedure descibed here, detection down to 50 pg of DNA or less should be possible. Inclusion of a dilution series of known quantities of marker DNA can be used to assess the sensitivity of detection.

A replication-competent DNA should yield a *Dpn*I-resistant, full-size product that increases in quantity over time post-transfection (see Fig. 14B.1.2). Authentic replication should also be both E1- and E2-dependent. (Note that for some papillomaviruses the replication may not be absolutely dependent on E2, but E2 should always greatly enhance replicative activity.) Once authentic replication is established for the wild-type genome and cognate E1/E2 pair, this assay can be used to map the location and boundaries of the origin region, test the effect of origin mutants, and evaluate the functionality of E1 and/or E2 mutants.

Time Considerations

The assay described in this unit is a lengthy procedure that can take several weeks from start to finish, depending primarily on how long it takes to accumulate sufficient cells to plate for the transfections. For planning purposes, the durations of individual steps in the protocol are summarized in Table 14B.1.2. For all steps where optional overnight incubations are possible (see Basic Protocol for details), the times in the table include these prolonged incubation periods. Fortunately, there are several stopping points along the way where the samples can be stored indefinitely if necessary. Nonetheless, strategic planning is advisable prior to commencing the assay to ensure that mandatory steps occur at points where the experimenter has sufficient time and that needed reagents are available at the correct times. Blocking out a daily schedule for the entire protocol in advance of initiating the experiment will prevent unanticipated delays.

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Key References

Ustav and Stenlund, 1991. See above.

This is the first study to document that the viral E1 and E2 proteins were necessary and sufficient to support replication of a papillomavirus origin in a transient transfection assay.

Ustav et al., 1991. See above.

This study correctly identified and mapped the bovine papillomavirus origin of replication. This was the first authentic definition of a functional papillomavirus origin, and documented that a DpnI assay could be used to successfully detect transient replication of papillomavirus origin–containing DNA.

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Human Adenoviruses: Propagation, Purification, Quantification, and Storage

Human adenoviruses (Ads) have proven to be one of the most powerful tools for probing the molecular and cell biology of eukaryotic cells. Studies using Ad systems as models for virus replication and cell transformation have been pivotal to understanding transcription regulation and the mechanisms by which cells regulate their growth (Green, 1978; for reviews, see Phillipson, 1995 and Shenk, 2001). Thorough understanding of Ad early gene function has allowed the development of replication-deficient Ad vectors, which provide important new tools for probing gene function as well as for gene therapy and cancer treatment. One of the advantages for gene expression analysis and for gene therapy is that Ads and Ad vectors are able to efficiently infect most human cell types regardless of their cell cycle state.

Human adenoviruses, of which there are at least 49 distinct types, as well as numerous Ad expression vectors encoding cloned foreign genes, are easy to propagate, and can be purified in milligram quantities and to high titers, i.e., 10^{11} to 10^{12} infectious units/ml (Green and Pina, 1963; Pina and Green, 1965; Green et al., 1967a). It is important to prepare consistent-quality Ads and Ad vectors, and to accurately establish their titers in order to ensure meaningful and reproducible experimental results. The protocols that follow describe the propagation and purification of Ad and Ad vectors, as well as their quantification.

CAUTION: Human Ads are pathogens that can cause upper respiratory infection, gastroenteritis, and conjunctivitis, and therefore must be treated with respect and considered biohazard agents to some extent. Concentration and purification of Ads and Ad vectors should be conducted under at least BSL-2 conditions with the use of appropriate personal protective barriers, including gloves, lab coats, and eye protection. Manipulation of concentrated virus is best conducted in a Class II biosafety cabinet. Institutional Biosafety Committee review may be required at many institutions prior to work with Ads and Ad vectors. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Genetically modified human adenoviruses, especially variants expressing oncogenes, may under some circumstances be considered BSL-3 agents (refer to NIH Guidelines for working with recombinant DNA molecules and the local Institutional Biosafety Committee for further advice). Follow all Centers for Disease Control guidelines for the use and handling of pathogenic microorganisms (*http://www.cdc.gov/od/ohs/biosfty/bmbl/bmbl3toc.htm*). Experimental animals receiving Ads and Ad vectors should be handled and disposed of using recommended Animal Biosafety Levels (ABSL) for infectious agents according to vertebrate animal guidelines. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

Safety

Even in view of the abovementioned warnings, working with Ads is considered safe for nonimmunocompromised workers, especially in view of the extensive amount of research done worldwide with these viruses. What may be of greater concern is the use of Ad vectors. Here one should consider what effect the expression of the vector "package" will be in inadvertently infected cells of human workers. Another issue to consider in

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working with replication-deficient Ad vectors is that, in rare instances, it is possible for homologous recombination to occur between the E1A genomic region in 293 cells (discussed below) and the Ad vector DNA, causing the gene of interest to be carried in a replicative-competent Ad (Lochmuller et al., 1994). It is statistically possible for this event to occur during large-scale growth and multiple passages. Replication-competent Ad recombinants can be identified by plaque screening of the Ad vector preparation in a cell line such as A549 that is nonpermissive for replication-deficient Ad vectors (see Basic Protocol 2).

Propagation

There are 49 currently known human Ads (Ad types 1 to 49; see Commentary), all of which are available from the American Type Culture Collection (ATCC; *http://www.atcc.org/*). Protocols for the purification and storage of Ads, replication-competent Ad vectors, and replication-deficient Ad vectors are essentially identical. Protocols for the propagation of Ads and replication-deficient Ad vectors differ only in the cells lines that can be used for virus propagation. Ads and replication-competent Ad vectors can be infected into and grown in a wide variety of cells. Two cells lines that are commonly used for this purpose are the human cervical carcinoma cell line HeLa (ATCC #CCL-2) and the human lung carcinoma cell line A549 (ATCC #CCL-185). Replication-deficient Ad vectors, on the other hand, must be grown in human 293 cells (ATCC #CRL-1573) or 911 cells (E1-transformed human embryonic retinal cells; Fallaux et al., 1996).

The widely used 293 cell line was derived by transformation of primary human embryonic kidney cells with sheared human Ad type 5 DNA (Graham et al., 1977; Harrison et al., 1977; Louis et al., 1997). 293 cells express the gene products of the Ad5 early gene 1 (E1A and E1B).

One common characteristic among replication-deficient Ad vectors is the absence or inactivation of early genes E1A and E1B. Expression of these genes is necessary for activation of the other Ad genes and is essential for viral replication. E1A and E1B functions must therefore be provided by the 293 cell in order to propagate replication-deficient Ad vectors. The protocols in this unit, which refer generically to Ads, are meant to include both Ad viruses and replication-deficient Ad vectors.

Contamination

An important consideration while planning and conducting experiments and purification of Ads is their ability to easily and efficiently infect most cell types. Accidental infection of normal cell lines and cross-contamination of virus, virus mutants, and Ad vectors is a concern, and efforts must be made to prevent this from occurring. Common sense precautions should be observed at all times. Separate biosafety cabinets and incubators should be used for infected and noninfected cultures, if possible. Biosafety cabinets and work surfaces should be decontaminated with 70% ethanol after each use. Incubators should be disinfected as well. It is desirable to use plug-seal type tissue culture flasks when incubating Ad-infected cultures in a CO_2 incubator. The cap can be loosened when placed in the incubator and tightened after equilibration with the CO_2-enriched atmosphere. Whenever possible, only one strain of Ad or Ad vector should be prepared at any given time. Naturally, all tissue culture steps should be conducted using the best sterile techniques possible. This involves the use of disposable, sterile, plugged tissue culture pipets fitted with a filtered pipetting aid.

PROPAGATION, PURIFICATION, AND STORAGE OF Ads

Materials

One of the following cell lines growing in 75-cm^2 tissue culture flasks:
293A cells (Invitrogen #R705-07)
293 cells (ATCC #CRL-1573)
A549 cells (ATCC #CCL-185)
HeLa cells (ATCC #CCL-2)
Phosphate-buffered saline (PBS; see recipe), 4°C and room temperature
Trypsin-EDTA (Invitrogen cat. no. 25300-062)
Complete DMEM medium (*APPENDIX 4B*) containing 10% FBS(DMEM-10)
0.4% (w/v) trypan blue (Sigma)
Adenovirus (Ad) stock (ATCC; see Strategic Planning and Commentary)
10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*), 4°C
Glycerol (optional)
CsCl, optical grade (UltraPure, Invitrogen)
Tris-buffered saline (TBS; *APPENDIX 2A*) containing 30% glycerol (see recipe), cold
Inverted tissue culture microscope
150-cm^2 plug-seal-capped tissue culture flasks
15- and 50-ml conical polypropylene centrifuge tubes
Refrigerated low-speed centrifuge: e.g., Beckman J6-HC
Beckman Coulter Beckman Coulter Optima-L series ultracentrifuge with NV Ti 65 rotor (or equivalent ultracentrifuge and rotor)
Beckman Coulter OptiSeal ultracentrifuge tubes (Beckman Coulter cat. no. 362181)
Beckman Coulter OptiSeal tube rack assembly for NV Ti 65 rotor (Beckman Coulter cat. no 360538)
Beckman Coulter OptiSeal spacers (for NV Ti 65; Beckman Coulter cat. no. 362202)
Beckman Coulter OptiSeal vise assembly, wrench, and other necessary tools
18-G, 1-in. (~2.5-cm) needles
5-ml syringes
Slide-a-Lyzer dialysis cassettes, 3500 MWCO (Pierce)
2-ml sterile screw-cap tubes with gasket seals (Sarstedt)
Additional reagents and equipment for counting cells with a hemacytometer and determining cell viability (Strober, 1997a,b)

CAUTION: All procedures should be carried out on absorbable bench paper that can be autoclaved prior to disposal. All liquid wastes should be treated with bleach to a final concentration of 10%. Needles and syringes must be placed in an appropriate sharps container until autoclaved and disposed of. Plasticware should also be autoclaved prior to disposal.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

Prepare monolayer cultures for propagation of Ads

1. Wash a seed culture of cells growing in a 75-cm^2 tissue culture flask with 10 ml room temperature PBS, then add 4 ml trypsin-EDTA at room temperature. Observe cell layer using an inverted tissue culture microscope.

   HeLa and sometimes A549 cells are used to propagate Ads. 293 cells must be used to propagate replication-deficient Ad vectors (see Strategic Planning).

2. When cells become rounded (usually within 5 min), detach from flask surface using a mild tap of the open hand (be careful not to overtrypsinize 293 cells).
3. Add 16 ml complete DMEM-10 medium to the trypsinized cell suspension.
4. Remove a small aliquot of cell suspension with a 1-ml pipet and combine it with an equal volume of 0.4% trypan blue. Count the viable (nonstained) cells by loading ~10 μl on each side of a hemocytometer covered by hemocytometer coverslip and examining microscopically at 100× magnification (see Strober, 1997a,b).
5. Seed ~2×10^6 viable cells into a 150-cm^2 tissue culture flasks containing 30 ml DMEM-10. Incubate to 50% to 70% confluency.

   Moderate amounts of virus are readily produced in monolayer culture grown in 150-cm^2 tissue culture flasks. Five infected flasks will yield ~2 ml highly purified Ad with a titer of ~1 to 3 × 10^{11} pfu/ml.

Infect cell cultures

6. After 2 to 3 days, when the cells are 50% to 70% confluent, prepare a virus inoculum for each flask to be infected by adding 5×10^7 to 10^8 pfu (plaque-forming-units, see below) Ad stock culture to 10 ml complete DMEM-10.
7. Remove the medium from each flask and replace it with 10 ml virus inoculum.

   This should provide a final MOI of 10 to 20 pfu/cell.

8. Incubate the flasks at 37°C for 30 to 60 min to allow efficient adsorption of virus particles. Occasionally rock flasks to ensure even distribution of virus inoculum and to prevent drying of portions of the cell monolayer. After virus adsorption, add 20 ml DMEM-10 and continue incubation.

Harvest infected cell cultures

9. When most of the cells are detached or can be removed by a mild tap of the flask with the open palm, transfer cell suspension into a 50-ml conical centrifuge tube. Centrifuge 5 min at 200 × *g*, 4°C. Decant the supernatant and retain the cell pellet, which contains the vast majority of Ad.

   Virus-induced cytopathic effects (CPE) will be apparent under the microscope within 2 to 3 days. Cells will appear rounded and begin to detach from the plastic surface.

10. Resuspend the cell pellet in 10 ml cold PBS, centrifuge as in step 9, and discard the supernatant. If multiple flasks have been infected, pool the cell suspensions and centrifuge again as described in step 9. Store the cell pellet at −70°C until ready for CsCl purification of virus.

Prepare crude Ad stock

11. Thaw the Ad-infected cell pellet on ice for 10 min and resuspend in 12 ml cold 10 mM Tris·Cl, pH 8.0. Resuspend the pellet by pipetting up and down.
12. Freeze the cell suspension 30 min on dry ice, then thaw in a 37°C water bath with occasional shaking. Ensure that the temperature of the suspension does not rise above 4°C, i.e., remove the suspension from the water bath while some ice remains in the tube.
13. Repeat step 12 twice to promote complete cell lysis.
14. Transfer the cell lysate to a 15-ml high-speed centrifuge tube and clarify by centrifuging 10 min at 8000 × *g*, 4°C. Decant the viral supernatant into a 15-ml centrifuge tube. If crude virus stocks are to be stored for more than several months, add glycerol to a final concentration of 30% (v/v).

    The viral supernatant in the 15-ml centrifuge tube constitutes the "crude virus stock."

Purify Ads by CsCl equilibrium density gradient centrifugation

15. Measure the volume of the supernatant from step 14 and multiply it by 0.51 to yield the weight in grams of CsCl needed to provide a final density of 1.34 (the buoyant density of Ads; Green and Pina, 1963). Add the required amount of CsCl to the cell lysate at room temperature.

    Keep CsCl solution at room temperature to prevent precipitation.

    Ultracentrifugation of Ads in CsCl solution can be done in a variety of fixed-angle and swinging-bucket rotors (Green and Pina, 1963; Green and Wold, 1979). Time and speed will vary with each rotor. Check for the appropriate speed and centrifugation time at http://www.beckman.com/resourcecenter/labresources/centrifuges/rotorcalc.asp. Vertical or near-vertical rotors are the most efficient, in that the density gradient is rapidly formed in the width as opposed to the length of the centrifuge tube, thus permitting equilibrium and banding of the virus in a relatively short time. The steps that follows are for the Beckman Coulter near-vertical titanium NV Ti 65 rotor.

16. Place the needed number of NV Ti 65 OptiSeal tubes into the tube rack provided by the manufacturer (see Fig. 14C.1.1). Fill each OptiSeal tube to the neck (11.2 ml), ensuring that the stem is dry. If the sample does not fill the tube to the neck, add more CsCl solution of the same density (1.34 gm/cm^3 in 10 mM Tris·Cl, pH 8.0).

    CAUTION: *Tubes must not be underfilled; otherwise there is a danger of tube collapse during centrifugation.*

17. Place an OptiSeal plug into the neck of each tube (Fig. 14C.1.1A). Using the supplied plug-seating tool, seat the plugs into all of the tubes at the same time (Fig. 14C.1.1B).

    Use of the seating tool ensures that equal pressure is used on all plugs.

18. Wipe the outside of each tube with a Kimwipe and insert the tubes into the rotor.

    CAUTION: *The NV Ti 65 rotor contains eight positions for tubes. It is imperative that two, four, six, or eight tubes be used, to ensure dynamic balance for centrifugation.*

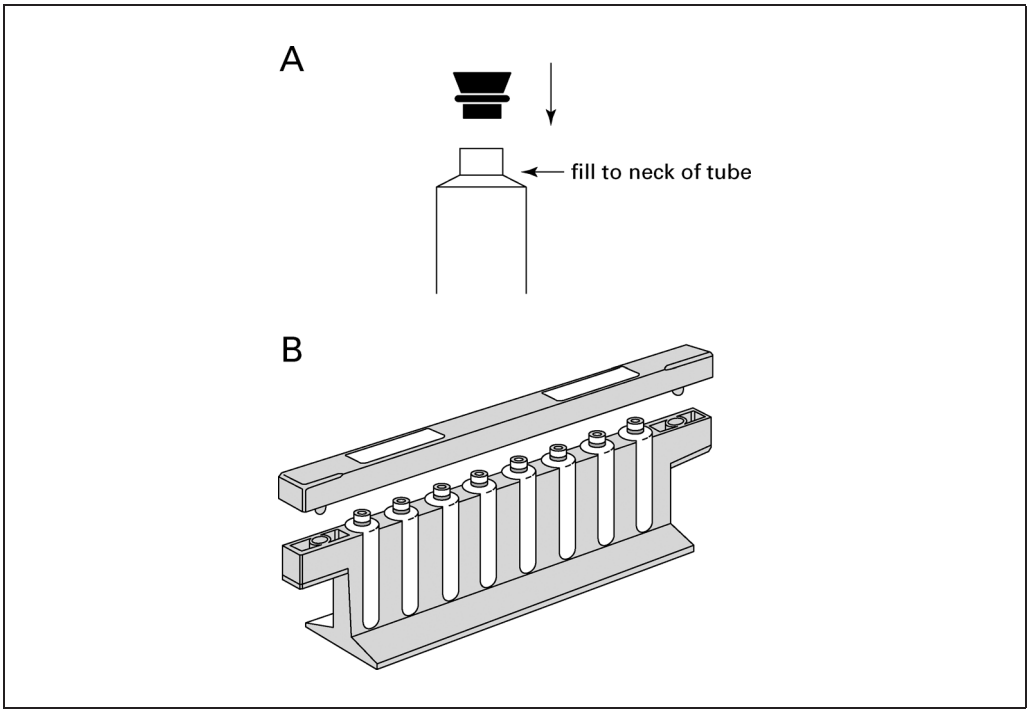

Figure 14C.1.1 (**A**) Schematic of an OptiSeal NV Ti 65 rotor tube for purification of Ads by CsCl density gradient equilibrium centrifugation. (**B**) Tube rack assembly used to seat plugs in the tubes.

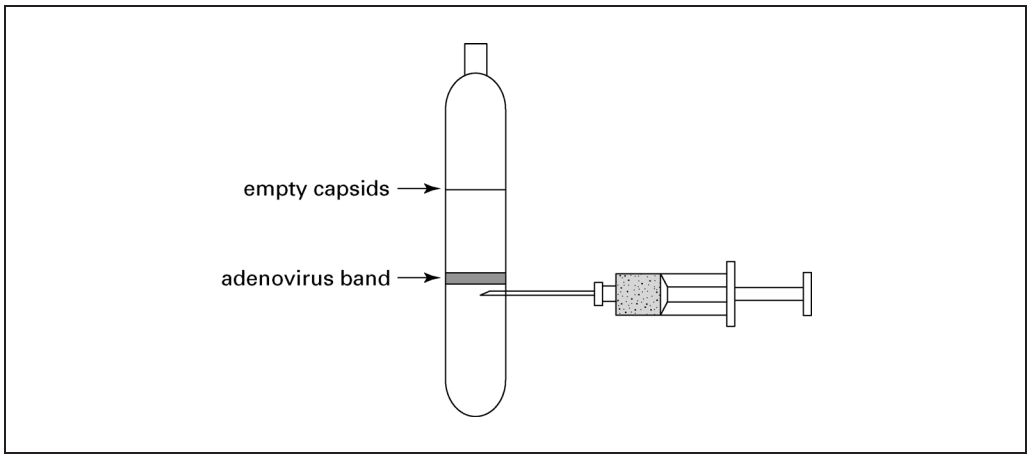

Figure 14C.1.2 Schematic of an OptiSeal tube after CsCl density gradient centrifugation of an Ad preparation. Indicated are the expected positions of the virus band and the position of a possible empty capsid band. Also shown is the optimal position for inserting the syringe needle prior to harvesting the virus band.

19. Insert an OptiSeal aluminum spacer on the top of each tube to provide support for the neck and shoulder of the tube during centrifugation. Insert an aluminum rotor plug (cap) into every one of the eight openings of the rotor, whether it contains a tube or not.

20. Use the manufacturer's supplied hex plug and torque wrench to tighten each cap to 120 in.-lb. while the rotor is held tightly by a rotor vise assembly (or by a strong colleague).

21. Centrifuge 5 hr (or overnight for convenience) at 321,000 × *g* (63,000 rpm in NV Ti 65 rotor), 22°C.

22. Terminate centrifuge run with minimal or no braking. Remove the rotor from the centrifuge. Using the supplied extraction tools, carefully remove the cap, spacer, and centrifuge tube from the rotor. Place the centrifuge tube in the tube rack and carefully remove the centrifuge tube plug.

    The opalescent virus band should be clearly visible near the center of the centrifuge tube (see Fig. 14C.1.2). Often there is a lighter band near the top of the tube that represents empty viral capsid.

Collect the purified Ad band

23. Insert an 18-G, 1-in needle attached to a 5-ml syringe ~5 mm below the position of the virus band, bevel up (Fig. 14C.1.2).

24. Once the needle has penetrated the tube wall, move the bevel upward so that it is positioned just below the band. Gently withdraw the virus into the syringe.

25. Remove the virus-filled syringe and needle from the centrifuge tube and inject the virus band (~2 ml) into a port of a 3-ml Slide-A-Lyzer dialysis cassette. Place the punctured centrifuge tube into a 50-ml tube for later disposal.

    Slide-A-Lyzer dialysis cassettes allow for convenient and rapid dialysis of macromolecules and viruses. They are supplied with foam floats that allow dialysis in a rapidly stirred beaker.

26. Dialyze the virus against 1 liter of cold TBS/30% glycerol for 3 hr in a 4°C cold room to remove CsCl.

27. Divide the dialyzed virus into ~300-μl aliquots in 2-ml gasket-containing, sterile screw cap tubes. Store up to 5 years at −70°C.

The stability of Ads varies according to group serotypes (Green et al., 1967a. In general, purified, concentrated virus preparations are stable for long periods of time (many years) when stored in TBS containing 30% glycerol at −35°C to −70°C (Green and Wold, 1979). Storage in aliquots helps preserve the stock titer from degradation by repeated freeze-thaw cycles. Valuable stocks should be titered periodically (every few years), passaged, and repurified if necessary.

PROPAGATION OF Ads IN SPINNER CULTURE

ALTERNATE PROTOCOL 1

Nonadherent cells grown in suspension are convenient for the preparation of large amounts of Ads and Ad vectors. Suspension cultures can be prepared by scraping cells from monolayer cultures into suspension with a rubber policeman, followed by growth in modified MEM medium containing no calcium ion (to prevent cell clumping) and a 10-fold increased level of phosphate buffer (increased buffering capacity). Commercially available spinner flasks that contain a suspended rotating magnetic bar for maintaining cells in suspension when used in conjunction with a magnetic stirring base are available from several sources. It is sometimes difficult and frustrating to adapt cells to spinner use. Further, commercial spinner flasks are prone to malfunction (often the day before a large infection is planned). Suspension cultures of both 293 and HeLa cells have been adapted to grow in nonadherent spinner culture using large Florence flasks and standard round-edged spinning bars resting at the bottom of the flasks (B. Stillman, pers. comm.). This, coupled with reliable low-heat-generating magnetic stirrer bases, has made the use of large-scale spinner cultures facile.

HeLa or 293 cells are grown in Joklik-modified MEM supplemented with 10% calf serum (instead of fetal calf serum) in volumes of 100 ml to 4 liters in Florence flasks that are loosely sealed with a rubber stopper wrapped with aluminum foil. The stopper and neck of the Florence flask are covered with a second layer of aluminum foil. Flasks are not filled beyond three-quarters of capacity, so that sufficient air exchange occurs at the surface of the medium. Flasks are incubated in a 37°C environmental room and stirred with a magnetic bar at a rate that prevents cells from settling but that does not result in cell death or frothing. Cultures adjusted to ~5×10^5 cells/ml will maintain pH values suitable for exponential growth in the absence of a CO_2-enriched atmosphere.

Additional Materials *(also see Basic Protocol 1)*

Complete spinner medium (see recipe)
250-ml centrifuge bottles
250-ml and 2-liter Florence flasks
Magnetic Stir 4 magnetic stirrer (Bellco Biotechnolog) and corresponding stir bars

NOTE: All spinner culture incubations should be performed in a 37°C environmental room unless otherwise specified.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

1. For infection of 1 liter suspension culture, centrifuge 3×10^8 exponentially growing HeLa or 293 cells (i.e., 600 ml cells at 500,000 cells/ml) 10 min at 200 × *g*, room temperature, in sterile 250-ml centrifuge bottles. Gently resuspend the cell pellet by pipetting up and down in 50 ml complete spinner medium.

2. Infect with 20 pfu per cell (6×10^9 total pfu) Ad stock. Transfer to a an aluminum-foil-wrapped 250-ml Florence flask, loosely seal with a foil-wrapped rubber stopper, then cover the stopper and neck of the flask with additional foil. Incubate with stirring on magnetic stirrer for 30 min at 37°C.

3. Transfer the infected cells to 950 ml prewarmed complete spinner medium in a 2-liter Florence flask. Loosely seal and wrap this flask in foil as described for the smaller flask and incubate at 37°C with stirring for 30 to 40 hr.

   When observed with a phase-contrast microscope, HeLa cells infected under these conditions are 2- to 3-fold larger and have prominent nuclei when compared to uninfected cells. This appearance is due to a lack of cell division and dramatic increases in protein, DNA, and RNA content (Green, 1962).

4. Transfer suspension to 250-ml centrifuge bottles and harvest infected cells by centrifuging 10 min at 200 × *g*, 4°C, and removing the supernatants. Wash each cell pellet by adding 15 ml cold PBS, centrifuging again as before, and removing the supernatant. Store infected cell pellet at −70°C.

   Over 90% of the virus remains cell-associated; Green, 1962.

5. Purify virus as described (see Basic Protocol 1).

ALTERNATE PROTOCOL 2

CONSTRUCTION AND PROPAGATION OF REPLICATION-DEFICIENT Ad VECTORS EXPRESSING A GENE OR SEQUENCE OF INTEREST

Replication-deficient Ad vectors are now used extensively and show great promise for multiple purposes, including fundamental studies on gene expression and medically significant applications such as vaccination, targeted cell killing for cancer therapy, and gene therapy. In the past, the most widely used strategy for Ad vector construction was homologous recombination in human 293 cells, which can complement Ad vectors defective in early gene 1 (which is required for replication of replication-deficient Ads). This approach has been limited by the low efficiency of recombination in 293 cells and the need for multiple rounds of plaque purification to isolate the final recombinant vector containing the inserted gene. In 1998, a simplified procedure for constructing Ad vectors (AdEasy system) was reported, which utilizes recombination in *E. coli* (He et al., 1998). This system has been successfully used and is now available commercially (Stratagene). Described below is a facile Ad vector construction system which is commercially available from Invitrogen (ViraPower Gateway Adenoviral Expression System). This simple two-step system uses lambda site-specific recombination in vitro. This system has several distinct advantages for a research laboratory, including short time requirements and the absence of restriction enzyme and DNA ligase reactions with their associated purification steps.

The ViraPower system involves the cloning of the gene or DNA sequence of interest into an "entry" vector (pENTR) followed by transfer from the entry clone to an Ad "destination" vector (pDEST). The first step is readily achieved by PCR cloning of the DNA insert into a topoisomerase-linked entry vector, ensuring highly efficient and rapid directional cloning into the vector cloning site, which lies between lambda *att*L recombination sites. The *att*L recombination sites allows for the subsequent facile transfer of the DNA insert into a variety of destination vectors that are designed to accept lambda recombination, including the ViraPower vector pAd/CMV/V5-DEST, utilized for constructing CMV promoter-containing Ad vectors. pAd/CMV/V5-DEST, like all replication-deficient Ad vectors, is deleted in E1A and E1B (see Strategic Planning). Additionally, this vector is deleted in early region 3 (E3). However, it contains elements and genes required for production and packaging of replication-deficient Ads.

Materials

DNA template of interest
Platinum High Fidelity *Taq* DNA polymerase (Invitrogen cat. no. 11304-011) or other good-quality proofreading *Taq* polymerase
pENTR/SD/D-TOPO cloning kit (Invitrogen cat. no. K2420-20) including:
  Salt solution
  Topoisomerase-tagged entry vector pENTR/SD/D-TOPO
  Competent *E. coli* TOP10 cells
  SOC medium (*APPENDIX 4A*)
LB agar plates (*APPENDIX 4A*) with 25 μg/ml kanamycin, 37°C
LB liquid medium (*APPENDIX 4A*) with 25 μg/ml kanamycin
QIAprep Spin Miniprep Kit (Qiagen) or equivalent
ViraPower pAd/CMV/V5-Dest System (Invitrogen cat. no. V493-20)
LR Clonase kit (Invitrogen) including:
  5× clonase reaction buffer
  Clonase enzyme
  Proteinase K solution
LB agar plates (*APPENDIX 4A*) with 100 μg/ml ampicillin
LB liquid medium (*APPENDIX 4A*) with 100 μg/ml ampicillin
Plasmid Maxi Kit (Qiagen)
TE buffer, pH 8.0 (*APPENDIX 2A*), sterile
*Pac*I restriction endonuclease and appropriate buffer (New England Biolabs)
293A cells (Invitrogen) growing in 75-cm^2 flask
Complete DMEM medium (*APPENDIX 4B*) containing 10% FBS (DMEM-10), with and without antibiotics
Dulbecco's Modified Eagle Medium (DMEM; Invitrogen)
OptiMem I medium (Invitrogen)
Lipofectamine 2000 transfection reagent (Invitrogen)
Trypsin-EDTA (Invitrogen cat. no. 25300-062)
42° water bath
12-ml snap-cap culture tubes, sterile
6-well tissue culture plates
75-cm^2 tissue culture flasks
Additional reagents and equipment for PCR (Kramer and Coen, 1997) and counting viable cells (Strober, 1997b)

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

Prepare a PCR product encoding the desired gene or DNA sequence of interest

1. Design and synthesize PCR primers appropriate for the template DNA insert.

   The forward primer must contain the sequence CACC at the 5′ end so that it can be directionally cloned using the topoisomerase-tagged entry vector. Additionally, it should contain an ATG in the context of a Kozak consensus sequence. If expression of the V5 tag in the intended Ad expression destination vector, pAdCMV/V5-DEST, is not desired, then a stop codon must be included in the reverse primer. If the V5 tag is desired, the absence of a stop codon will allow the expression of the protein containing the V5 peptide at its C terminus. In this case, the gene should be in frame with the V5 epitope tag after recombination (see detailed instructions for primer design provided with the ViraPower kit). The V5 Tag permits the sensitive detection of the protein expressed from the Ad vector in cells using commercially available antibody.

2. Prepare a 50-μl PCR reaction containing the DNA template of interest and the desired PCR primer set. Perform PCR (Kramer and Coen, 1997) using a good-quality proofreading *Taq* DNA polymerase (e.g., Platinum High Fidelity *Taq* polymerase) to ensure that the product is blunt-ended. Refer to the manufacturer's instructions for specific PCR conditions.

   Because the PCR primer set is dictated by the sequence of the DNA insert (usually a complete gene), it is not always possible to avoid the use of difficult, nonoptimal primer sets. It may be necessary in such cases to alter PCR conditions such as melting temperature or annealing time. It may also be effective to alter the PCR mixture by the addition of DMSO or betaine.

***Clone the PCR product into the entry vector* pENTR/SD/D-TOPO**

3. Using the pENTR/SD/D-TOPO cloning kit from Invitrogen, add 1 μl of the supplied salt solution to 4 μl of the unpurified PCR product. Initiate the reaction by addition of 1 μl of the topoisomerase-tagged entry vector, pENTR/SD/D-TOPO. Incubate at room temperature for 5 min.

   In this system, the PCR product is directionally cloned. The overhang in the pENTR/SD/D-TOPO entry vector invades the 5′ end of the PCR product and anneals to the CACC of the forward primer, thus stabilizing the PCR product at the cloning site. The topoisomerase inserts the PCR product into the vector at >90% efficiency.

Transform chemically competent E. coli cells with the* pENTR/SD/D-TOPO *recombinant

4. Incubate 2 μl of the reaction mixture from step 3 with 50 μl chemically competent *E. coli* TOP10 cells (provided with the pENTR/SD/D-TOPO kit) for 30 min on ice. Heat-shock the cells for 30 sec in a 42°C water bath without shaking, and immediately place on ice for 2 min.

5. Add 250 μl of SOC medium (also provided with the pENTR/SD/D-TOPO kit) and incubate at 37°C for 30 min with shaking. Plate 50 μl on prewarmed LB plates containing 25 μg/ml of kanamycin and incubate overnight at 37°C.

6. Pick four colonies and inoculate each into 5 ml of LB medium containing 25 μg/ml kanamycin in a sterile 12-ml snap cap tube and incubate at 37°C overnight with shaking.

7. Purify the plasmid DNA from 3 ml bacterial culture using QIAprep Spin Miniprep Kit. Sequence the purified plasmid DNA utilizing the universal M13 forward and reverse primer sequences present in the entry vector to ensure the correctness of the DNA insert.

   Any number of miniprep kits are available for purification of plasmid DNA. It is important that the plasmid DNA be of sufficient quality to support DNA sequencing protocols. Generally, all of the four clones that are picked will have the correct sequence.

Transfer the cloned sequence from the entry vector to pAd/CMV/V5 DEST using the LR clonase reaction

8. Mix 300 ng of a confirmed entry clone plasmid DNA from step 7 with 300 ng ViralPower pAd/CMV/V5 DEST and 4 μl of 5× clonase reaction buffer (from LR Clonase reaction kit) to give a final volume of 16 μl.

9. Add 4 μl of clonase enzyme (stored at −80°C and thawed on ice) and pipet gently up and down. Incubate at room temperature for 1 hr (or overnight for convenience). Add 2 μl of the proteinase K solution provided by the manufacturer and incubate 10 min at 37°C. Transform TOP10 (or other suitable *E. coli* cells) with 1 μl of the digest, as described in step 4, except use LB agar plates containing 100 μg/ml of ampicillin rather than kanamycin.

During this reaction, the clonase enzyme mixture catalyzes the 1:1 exchange of the DNA sequence between the two aatL sites of the entry vector (which include the cloned DNA insert) with the sequences located between the two aatR sites in the destination vector, which includes a suicide gene (ccdB) whose expression is toxic to most E. coli cells. The end result is that unexchanged destination vector will kill cells that it transforms. The only cells that will grow on an ampicillin-selection plate will be those that contain the destination vector which has exchanged its suicide gene for the cloned insert from the entry vector. This reaction is highly efficient. Because there is a direct exchange between the recombination sites of the entry vector and destination vector, theoretically there can be no alteration in the cloned insert. Therefore, one can proceed with only one or two clones from this point on. Note that clonase LR is guaranteed by the manufacturer for 6 months when stored at −80°C.

10. Inoculate two of the clones from the ampicillin plate into separate 100-ml quantities of LB liquid medium containing 100 μg/ml ampicillin and incubate overnight at 37°C with shaking.

11. Purify the Ad plasmid DNAs using a Plasmid Maxi Kit. Resuspend the purified Ad plasmid in sterile TE buffer, pH 8.0. Digest 5 μg of Ad plasmid DNA with 10 units of *Pac*I in the manufacturer's recommended buffer at 37°C for 1 hr.

    Digestion with PacI removes the Amp gene and the pUC ori from the pAdCMV/V5 DEST recombinant, thus exposing the Ad 5′ and 3′ inverted terminal repeats. Transfection of this linear plasmid into 293 cells will result in self annealing, which allows for Ad DNA replication and the formation of recombinant Ad vector virus particles containing the cloned DNA insert.

Establishment of Ad Vector Infection of 293 cells by DNA transfection

12. Trypsinize a 75-cm^2 flask of 293A cells and count viable cells (Strober, 1997b). Plate 5×10^5 cells in 2 ml complete DMEM-10 into each well of a 6-well plate. Incubate overnight at 37°C.

13. The next day, aspirate the medium and replace it with 1.5 ml fresh DMEM-10 medium lacking antibiotics.

14. For each clone to be transfected, prepare an Ad vector–Lipofectamine complex as follows.

    a. Dilute ~1 μg *Pac*I-digested pAdCMV vector containing the insert (step 11) into 250 μl of OptiMEM I medium in a sterile 1.5-ml microcentrifuge tube.
    b. Add 3 μl of Lipofectamine 2000 to 250 μl of Opti-MEM I medium in a separate microcentrifuge tube and incubate 5 min at room temperature.
    c. Add the diluted Lipofectamine 2000 to the corresponding tube containing the diluted DNA and incubate 20 min at room temperature.

15. After 20 min, add each mixture dropwise to a well of 293A cells (from step 13). Mix gently by rocking the plate back and forth and incubate at 37°C overnight.

16. At a time point 24 hr after transfection, aspirate the cell culture medium and feed each well with 2 ml complete DMEM-10.

17. At 48 hr after transfection, trypsinize the cells as in Basic Protocol 1, steps 1 and 2, except use 0.5 ml trypsin-EDTA per well. Transfer the contents of each well to a separate 75-cm^2 flask containing 15 ml of complete DMEM-10. Incubate at 37°C.

    At this point and at all subsequent steps, infectious replication-deficient Ad is present.

18. Replace the culture medium every 2 to 3 days until distinct areas of Ad-induced CPE (also see Basic Protocol 1 and Commentary) are visible. Allow infection to proceed until >50% of cells show CPE, typically 10 to 13 days post-transfection.

Prepare a crude viral lysate

19. Harvest the cells and medium by tapping or pipetting up and down. Freeze the suspension in dry ice for 15 min. Thaw in a 37°C water and repeat for a total of three freeze-thaw cycles.

20. Clarify the suspension by centrifugation for 10 min at 8000 × *g*. 4°C, and decant the supernatant into a fresh sterile centrifuge tube. Divide into 1-ml aliquots and freeze at −70°C.

    The infectivity of the clarified supernatant from this step is usually ∼10^7 pfu/ml.

Amplify and/or purify recombinant Ad vector

21. Amplify the virus by repeated rounds of infection of 293 cells growing in 75-cm^2 flasks using an MOI of 10 to 20 pfu/cell.

    This will provide crude virus preparations with titers of ∼10^8 to 10^9 pfu per ml. This is suitable for many experimental uses. At this stage, 150-cm^2 flasks can be infected as described in Basic Protocol 1, and high-titer purified recombinant Ad vector prepared by CsCl centrifugation.

ALTERNATE PROTOCOL 3

OTHER Ad PURIFICATION METHODS

Recently, kits have been developed for Ad purification that rely on ion-exchange chromatography, e.g., the AdenoX Virus Purification kit (BD Biosciences Clontech) and Vivapure adenoPACK purification system (Vivascience). These systems are easy to use but expensive. Laboratories that routinely prepare several different Ads or Ad vectors will probably find these systems cost-prohibitive. Use of large-capacity vertical rotors and CsCl centrifugation as described above (see Basic Protocol 1) is rapid and provides for high-capacity purification.

BASIC PROTOCOL 2

QUANTITATIVE PLAQUE TITRATION OF Ads ON 6-WELL TISSUE CULTURE PLATES

It is essential for meaningful studies that the amount of infecting virus in Ad stocks be accurately known. A plaque assay developed for the quantitation of the commonly used Ad type 2 (Rouse et al., 1963) has been shown to be effective for most Ad serotypes (Green et al., 1967a). A plaque is a cluster of infected cells produced by the infection of a single cell by one virus particle followed by secondary infection of contiguous cells. Antibody-based assays (see Alternate Protocol 4) rely on the production of viral proteins (eg., hexon) within cells, and may not adequately reflect infectious units. A disadvantage of the Ad plaque assay is that it requires from 7 to 11 days to reach a final titer. Described here is a facile plaque assay method using 293 cells, modified from a protocol described by Invitrogen. This protocol is very similar to the standard Ad plaque assays which have been used for many years (Green et al., 1967a). The use of a well suited subclone of 293 cells (293A) in this assay makes it ideal both for Ads and replication-deficient Ad vectors.

Materials

293A cells (Invitrogen) *or* 293 cells (ATCC #CRL-1573) growing in 75-cm^2 tissue culture flask
Complete DMEM medium containing 10% FBS (DMEM-10)
Adenovirus (Ad) stock (ATCC; see Strategic Planning and Commentary)
4% agarose stock (see recipe)
0.33% (w/v) Neutral Red stock solution, sterile (Sigma)
5 mg/ml 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT, also called thiazolyl blue; Sigma cat no. M-2128)

6-well tissue culture plates
65°C water bath
Light box

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

1. Trypsinize a 75-cm^2 flask of 293 cells and count viable cells (see Basic Protocol 1, steps 1 to 4). Plate 0.8 to 1 × 10^6 cells per well in a 6-well tissue culture plate. Feed cells in each well with 2 ml complete DMEM-10 and incubate overnight (should be 80% to 90% confluent the next day).

   One 6-well plate is sufficient for titration of each virus preparation. For a purified stock (generally titers of 10^{10} to 10^{11}), duplicate wells containing 10^{-7}, 10^{-8}, and 10^{-9} dilutions are adequate. For a crude virus preparation (generally a titer of ~10^8 pfu/ml), dilutions of 10^{-5}, 10^{-6}, and 10^{-7} are good starting points.

Infect 293 cells with appropriate dilutions of Ad stock

2. The next day, feed wells with 800 µl fresh complete DMEM-10. After 2 hr, prepare appropriate dilutions of Ad stock in sterile 1.5-ml microcentrifuge tubes, as described in Table 14C.1.1.

3. Add 200 µl of each dilution to duplicate wells of the 6-well plate. Gently rock the plate to disperse the inoculum. Incubate at 37° overnight.

4. The next day (1 day post infection), melt sterile 4% agarose stock (with cap loosened) in a microwave oven and cool in a 65°C water bath for 30 min. At the same time, warm complete DMEM-10 to 37°C.

5. Aspirate the medium from each well. For a 6-well plate, prepare an agarose overlay by adding 1.2 ml of the 4% agarose at 65°C (step 4) to 12 ml of 37°C complete medium. Pipet up and down twice to mix, then immediately add 2 ml of overlay to each well by carefully pipetting down the side of the well.

6. Allow the agarose to solidify on a level surface within the biosafety cabinet for 15 min, then transfer plates to an incubator and continue incubation.

   The agarose overlay provides nutrients for cells and physically limits intercellular spread of virus except to adjacent cells. To avoid premature solidification, bring the agarose and the medium to the biosafety cabinet in vessels containing water at the appropriate temperature.

7. At 4 to 5 days post-infection, add an additional 1 ml of agarose overlay.

Table 14C.1.1 Serial Dilution Scheme for Adenovirus Plaque Titration

Tube no.	Vol. virus	Vol. medium	Resulting dilution
1	10 µl virus stock	990 µl	10^{-2} dilution
2	10 µl from tube 1	990 µl	10^{-4} dilution
3	10 µl from tube 2	990 µl	10^{-6} dilution
4	100 µl from tube 3	900 µl	10^{-7} dilution
5	100 µl from tube 4	900 µl	10^{-8} dilution
6	100 µl from tube 5	900 µl	10^{-9} dilution

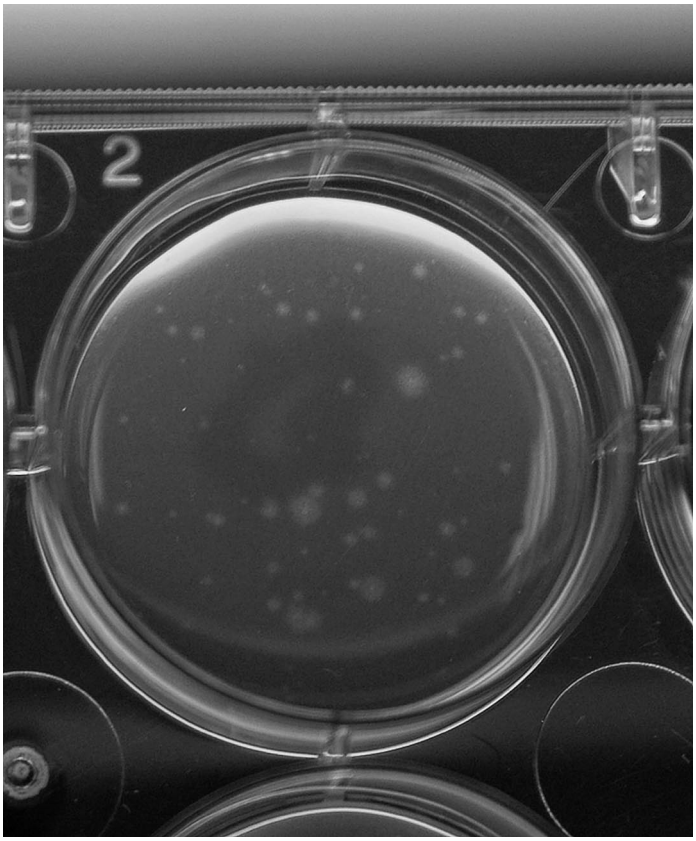

Figure 14C.1.3 Photograph of one well of a 6-well plaque titration plate showing plaques resulting from a plaque assay on 293A cells. This well contains 200 μl of a 10^{-9} dilution of a recombinant Ad 5 vector at 8 days post-infection.

Count Ad plaques

8. Observe wells at an oblique angle over a light box and count the number of plaques daily using a marking pen to dot each plaque on the bottom surface of the plate. Return plate to incubator.

   On 293 cells, plaques are visible to the naked eye starting at ~8 days post-infection (see Fig. 14C.1.3 for example).

9. When the plaque count does not increase significantly, make a final count on wells containing virus dilutions that yield at least 10 plaques (see steps 10a and 10b; count can be done without staining for 293 cells but is necessary for A549 cells).

   Plaque count will stabilize ~10 to 12 days post-infection depending upon the Ad serotype.

   Wells containing >100 plaques are often too difficult to count.

   The size and appearance of plaques vary with the Ad serotype (Green et al., 1967a).

10a. *For staining with Neutral Red:* Stain cells with Neutral Red (a traditional method) at 10 days post-infection as follows.

   With A549 cells, it is desirable to stain plates prior to the final plaque count.

   i. For each well, prepare an additional 2 ml agarose overlay as in step 5, except supplement with 180 μl of 0.33% Neutral Red stock solution per 12 ml medium/1.2 ml 4% agarose used.

ii. Count plaques the following day.

Neutral red will viably stain uninfected cells. Plaques of Ad infected cells are readily visualized as circular areas of non-stained cells (1 to 5 mm in diameter) surrounded by a background of stained cells. Plaques can be counted for several days after staining.

10b. *For staining with MTT:* Add 300 µl of 5 mg/ml of MTT solution to each well and incubate at 37°C for 1 hr.

In this case, plaques are visualized as clear areas on a dark background. Staining with MTT results in cell death and thus can only be used when the plaque assay is terminated.

11. Calculate the final titer by multiplying the plaques per well by the dilution factor and by five, since only 0.2 ml of the 1 ml dilution was used for the inoculum:

(no. plaques/well) × dilution factor × 5 = titer (pfu/ml).

For example, if a 10^{-9} dilution gave an average of 25 plaques, then the titer is $25 \times 10^9 \times 5 = 1.25 \times 10^{11}$ pfu/ml.

OTHER Ad TITRATION METHODS

ALTERNATE PROTOCOL 4

An alternate method for virus titration utilizes specific monoclonal antibody directed against the Ad hexon protein and an anti-mouse secondary antibody linked to horseradish peroxidase. A commercial version is available, i.e., Adeno-X Rapid Titer Kit (BD Biosciences Clontech). Briefly, 293 cells are plated in 12-well plates and immediately infected with Ad or Ad vector. Cells are stained for hexon 24 hr later using the supplied DAB reagent with the horseradish peroxidase, giving a dark brown color to hexon-positive cells. This kit works well and has the advantage of a rapid titration. A disadvantage is that it is expensive for labs that do multiple titrations. Furthermore, it is unclear whether this antibody can detect the hexon of all Ad serotypes.

REAGENTS AND SOLUTIONS

Use tissue-culture-grade water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose stock, 4%

4 g UltraPure agarose (Invitrogen cat. no. 15510-019)
100 ml tissue-culture grade H_2O
Autoclave 20 min on liquid cycle
Cool to 65°C prior to use
Store up to 2 months at 4°C

Complete spinner medium

Joklik's Modified Minimal Essential Medium (ICN Biomedicals; prepare per manufacturer's instructions; store up to 3 months at 4°C) containing:
10% calf serum (Invitrogen cat. no. 10371-029)
Cold sterilize under pressure through high-capacity tissue culture filter (Pall Corporation)
Store up to 3 months at 4°C or up to 1 year at −20°C

Use of fetal calf serum is unnecessary for this medium.

Phosphate buffered saline (PBS)

1.54 mM KH_2PO_4
155.17 mM NaCl
2.71 mM $Na_2HPO_4 \cdot 7H_20$
Filter sterilize
Store indefinitely at 4°C

TBS/30% glycerol

137 mM NaCl
20 mM Tris base
30% (v/v) glycerol
Adjust to pH 8.0 with HCl
Filter sterilize and store indefinitely at 4°C

COMMENTARY

Background Information

Human adenoviruses (Ads) have proven to be one of the most powerful tools available to molecular and cell biology. Studies using Ad systems as models for virus replication and cell transformation have been pivotal to understanding transcription regulation and the mechanisms by which cells regulate their growth (Green, 1978; for reviews, see Phillipson, 1995; Shenk, 2001). Early studies with Ads "provided the backbone which everyone could build in establishing the virus as the preferred tool to dissect gene expression in mammalian cells" (Phillipson, 1995). Furthermore, the thorough understanding of Ad early gene function has allowed the development of replication-deficient Ad vectors, which provide important new tools for probing gene function, as well as for gene therapy and cancer treatment.

Human adenoviruses are nonenveloped DNA viruses possessing a duplex, linear, noncircularly permuted DNA genome of 20–28 × 10^6 Da (Green et al., 1967b). The Ad virion is assembled from about thirteen viral polypeptides and the viral DNA genome into an icosahedral structure consisting of an external capsid and an inner core containing viral DNA and at least two basic proteins.

Human adenoviruses were first isolated in 1953 from tonsil and adenoid tissues (Rowe et al., 1953) as well as from patients with acute respiratory disease (Hilleman and Werner, 1954). Ad serotypes 1 to 31 were highly purified and classified into five groups (A to E) based on their DNA genome homologies in cross-hybridization studies (Green et al., 1979; 1980) and base composition (Pina and Green, 1965). At least 49 serotypes, classified into six groups, A through F (see Table 14C.1.2), are now recognized.

Adenoviruses replicate mostly in the upper respiratory tract or in the gut. Ad infections account for 3% to 5% of acute respiratory disease in the general population. Ad serotypes 1, 2, and 5 are the most common; however, epidemics of adenoviral infection have caused serious respiratory illness in military recruits, generally from Ad serotypes 4 and 7. In 1962, it was reported that crude preparations of some human Ads caused cancer when injected into newborn hamsters (Trentin et al., 1962). Subsequently, it was demonstrated that injection of microgram quantities of highly purified group A Ads (highly oncogenic Ads12, 18, and 31) rapidly induced cancers in weeks, group B Ads (weakly oncogenic Ads 3, 7, 11, 14, 16, and 21) induced cancer in months, and group C Ads (Ad 1, 2, 5, and 6) and Ad 4 were not tumorigenic in hamsters (Green, 1971). However, all Ads appear to possess oncogenic properties, since they can transform nonpermissive rodent cells in culture (see Table 14C.1.2).

Because of the oncogenic potential of Ads, concerns were raised that the military live Ad vaccine program could have a long-term negative impact. Extensive studies were carried out to determine whether human Ads had any role in human cancers. Using hybridization technology that could detect 1/10 of a copy of the transforming genes of groups A, B, C, and E, it was shown that no significant Ad transforming gene sequence was present in a wide variety of defined human tumor types (reviewed in Green et al., 1980).

The enteric, coated, live virus vaccine that was provided to new military recruits from ~1970 to 1995 was highly effective in preventing epidemics caused by Ad infection (Top et al., 1971; Ludwig et al., 1998). Detailed retrospective studies on military recruits who had received the vaccine showed that not only

Table 14C.1.2 Classification Scheme for Human Adenoviruses[a]

Subgroup	Serotypes	% DNA homology[b]	Percentage of G + C in DNA	Oncogenic potential	
				Tumorgenicity in animals	Transformation in cell culture
A	12, 18, 31	48-69/8-20	48-49	High	Yes
B	3, 7, 11, 14, 16, 21, 34, 35	89-94/9-20	50-52	Moderate	Yes
C	1, 2, 5, 6	99-100/10-16	57-59	Low or none	Yes
D	8, 9, 10, 13, 15, 19, 20, 22-30, 33, 36-39, 42-49	94-99/4-17	57-61	Low or none (mammary tumors)	Yes
E	4	4-23 with other types	57-59	Low or none	Yes
F	41	Unknown	Unknown	Unknown	Unknown

[a]Modified from Green et al. (1980) and Shenk (2001).
[b]Percentage within group/percentage with other types.

was the vaccine effective in preventing acute epidemics but there were no long-term negative effects of any sort that could be associated with the administration of the live Ad vaccines. The military vaccine program was terminated by the manufacturer in 1995 because of economic considerations. Unfortunately, this has resulted in recent serious Ad epidemics and even deaths among military recruits. Plans to renew the Ad vaccine program are currently in progress.

Troubleshooting

Tissue culture problems

Occasional bacterial, yeast, or mold contamination will occur from time to time in any cell culture laboratory. Strict adherence to sterile technique and the use of biosafety cabinets will minimize this problem. The inclusion of antibiotic/antimycotic agents in working cultures is recommended. It is often suggested that antibiotic/antimycotic agents be avoided in stock cultures to allow nascent contaminations to become apparent. Stock cultures of cell lines should be tested on a regular basis for contamination with mycoplasma.

The use of complete commercial liquid media whenever possible will minimize problems in cell culture that might occur due to a multitude of reasons, including, significantly, the quality of the water. Laboratories that prepare their own cell culture media should ensure that their cell culture water be pyrogen-free, with fewer than 10 parts per billion of total organic content.

Low yields of Ads

Low yields may be due to several causes. Various Ad serotypes, Ad mutants, and Ad vectors carrying different foreign genes may replicate at different rates and require longer periods for growth. Mycoplasma contamination will result in reduced virus yields (see *APPENDIX 3B*). It has been often found that late-passage cells (293 and A549) can become less efficient for virus replication, for unknown reasons. This problem is rectified by returning to an earlier passage of the cell line. Finally, the multiplicity of infection (MOI) is important. The use of over 100 to 200 pfu of Ad per cell can result in significant CPE with reduced virus replication.

Anticipated Results

Basic Protocol 1 and Alternate Protocols 1 and 2 should result in pure stable Ads and Ad vectors at concentrations, in general, of $\sim 10^{11}$ pfu/ml or more.

Time Considerations

Basic Protocol 1 and Alternate Protocol 1, from the time of harvesting Ad infected cells to the final aliquoting of purified virus, require only ~3 hr of labor, conveniently done over 2 days. This includes centrifugation time and dialysis. Accurate titrations using plaque assay methods (see Basic Protocol 2) require less than 2 hr of labor but take up to 12 days for the final plaque count. The construction and purification of replication-defective Ad vectors (see Alternate Protocol 2) expressing a

gene of interest, from the point of a DNA sequence–confirmed entry clone to a purified amplified recombinant Ad expression vector, requires relatively little labor but will take up to 15 to 23 days.

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Internet Resources

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

For CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition

http://www.atcc.org/

American Type Culture Collection.

http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html

For NIH Guidelines for Research Involving Recombinant DNA molecules

http://www.geocities.com/CapeCanaveral/Hangar/2541/

Joe Mymryk's home page; an excellent source of information concerning the Ad5 E1A oncogene, including a large database of Ad5 E1A mutants, E1A interactive proteins, and E1A resources

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Herpes Simplex Virus: Propagation, Quantification, and Storage

Human herpes simplex viruses (HSV) are large, double-stranded DNA viruses that are enveloped and contain structured capsids and a proteineous layer located between the envelope and the capsid referred to as the tegument (Roizman and Knipe, 2001). HSV-1 is a neurotropic herpesvirus that causes a variety of infections in humans. It remains latent in the neurons of its host for life and can be reactivated to cause lesions at or near the initial site of infection. Recurrent infections result from the lytic replication of the virus after reactivation from the latent state.

During productive lytic infection in cultured cells, glycoproteins in the viral envelope interact with receptors on the host cell, including heparan sulfate and the herpesvirus entry mediators. HSV-1 gene expression proceeds in a tightly regulated cascade, and changes in its levels during infection are usually the consequence of transcriptional regulation. The first viral genes expressed during infection, termed the immediate-early (IE) genes, are stimulated by incoming virion VP16 and are transcribed in the absence of de novo viral protein synthesis. The IE gene products 0, 4, 22, and 27 function cooperatively to regulate the expression of all classes of viral genes. The early genes are expressed next and encode proteins mainly involved in viral DNA synthesis. The last genes expressed are the late genes, and they mainly encode virion components such as VP16 and glycoproteins. The late genes are further divided into the leaky-late and true-late classes of genes. The true-late genes absolutely require viral DNA synthesis for their production. HSV-1 generally completes its replication cycle within 12 to 18 hr of infection.

This unit describes protocols required for the propagation and quantitation of HSV. These include splitting of cultures (see Support Protocol 1), freezing cells (see Support Protocol 2), and thawing cells (see Support Protocol 3), as well as preparing virus stocks (see Basic Protocol 1), determining virus titers (see Basic Protocol 2), and picking virus plaques (see Basic Protocol 3). Additionally, methods are detailed for preparing whole infected cell extracts (see Basic Protocol 4), detecting HSV proteins by separation on DATD-acrylamide gels (Basic Protocol 5) and subsequent immunoblotting (see Basic Protocol 6), and indirect immunofluorescence (see Basic Protocol 7).

These methods may be used for clinical isolates and laboratory strains of both HSV types 1 and 2. HSV-1 serves as the prototype of the *Herpesviridae*. There are three main laboratory strains of HSV-1 that are used throughout the world and are available from the ATCC. They are HSV-1(17syn+), HSV-1(F), and HSV-1(KOS). Note that HSV-1(17syn+) has been completely sequenced and is available in Genbank (Locus: HE1CG; Accession numbers: X141112, D00317, D00374, S40593).

CAUTION: Human herpes virus is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified, 37°C, 5% CO_2 incubator unless otherwise specified.

Contributed by John A. Blaho, Elise R. Morton, and Jamie C. Yedowitz
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BASIC PROTOCOL 1

PREPARING VIRUS STOCKS

Before any experiments that require virus can be done, the virus must be properly grown up into a stock, titered, and characterized. There are several ways to make a virus stock, as well as varying opinions on the best way to maintain the virus. Described below is a straightforward method for preparing a virus stock, which may be scaled up accordingly. The virus storage method used here allows for repeated freeze-thawings without significant reduction in virus titers. The virus concentration in a stock is expressed as plaque forming units (pfu) per milliliter. It should be recognized that unless infectious virions are purified by banding in a density gradient (Pomeranz and Blaho, 2000), all stocks will contain noninfectious particles along with the infectious virions.

Materials

Cell line of choice (e.g., Vero; see Table 14E.1.1) grown to confluence in 75-cm^2 tissue culture flasks (see Support Protocol 1 for culture techniques)
199V medium (see recipe)
HSV stock (ATCC), titered (see Basic Protocol 2)
DMEM/5% NBCS medium (see recipe)
Sterile milk (see recipe)
Freezer box, cardboard (optional)
15-ml conical tubes, sterile
Probe sonicator (e.g., Branson Sonifier) with microtip
Screw-capped cryovials
Additional reagents and equipment for titering virus (see Basic Protocol 2)

Infect cells with virus

1. Aspirate medium from a confluent 75-cm^2 flasks of the appropriate cell type.
2. Prepare 3 ml of 199V medium containing HSV at a multiplicity of infection (MOI) of 0.01 pfu/cell according to the following formula:

   $$0.01 \times (2 \times 10^7) \times (1/\text{titer in pfu/ml}) = \text{volume (ml) virus stock needed.}$$

   Here, 0.01 represents the desired MOI in plaque-forming units (pfu)/cell (see Basic Protocol 2) and 2×10^7 is the approximate number of Vero cells per confluent 75-cm^2 flask. For example, if the stock has a titer of 1×10^8 pfu/ml, then one must add 2 µl (0.002 ml) to infect one 75-cm^2 flask at an MOI of 0.01.

   199V medium contains less serum than cell maintenance medium and is used solely when inoculating cells with virus.
3. Add virus/medium inoculum to cells and incubate 2 hr at 37°C to allow cells to absorb virus.

Table 14E.1.1 Examples of Commonly Used HSV-Permissive Cell Lines

Cell line	ATCC	Growth conditions	Uses
Vero (African Green Monkey Kidney Cells)	#81-CCL	DMEM containing serum, passage every 3–4 days, 37°C, 5% CO_2	Growing virus stocks and titering Indirect immunofluorescence
HEp-2 (Human Epithelial Carcinoma Cells)	#23-CCL	DMEM containing serum, passaged every 3–4 days, 37°C, 5% CO_2	Preparation of infected cell extracts Indirect immunofluorescence

4. Aspirate 199V and add 3 ml fresh DMEM/5% NBCS medium to flask.
5. Incubate 2 to 3 days, until 100% of the cells display cytopathic effect (CPE).

   CPE caused by HSV on Vero cells is quite distinct and involves rounding up of the cells off the plate, along with numerous biochemical alterations to the cells themselves (Roizman and Knipe, 2001).

Harvest virus and store virus stock

6. When 100% CPE is observed, tighten the cap of the flask and place it at −80°C for at least 15 min.

   Use of a cardboard freezer box to hold the virus stock simplifies this procedure.

7. Carefully remove the flask from the freezer and carry it into a biosafety hood. Allow the flask to warm up slowly at room temperature in the hood. Loosen and then tighten cap to equilibrate the air pressure. Do not allow the flasks to warm up too quickly or to get knocked around. Be very careful not to break the flask.
8. As the flask warms, add 3 ml sterile milk to a fresh 15-ml sterile tube.

   The use of milk as a stabilizer is a more effective way of storing HSV, as compared to storage in medium alone. Powdered milk is cheaper than BSA, and it remains stable through the autoclaving procedure. Virus titers have been found to decrease dramatically when stock is stored in medium, while they are significantly retained with milk storage.

9. As the ice in the flask begins to thaw, shake the virus/cell suspension (often referred to as a "herpes-Slurpee") over the monolayer to resuspend all cellular material in the flask.
10. Remove the cell suspension from flask using a 5-ml pipet and add it to the 15-ml tube containing the sterile milk.
11. Gently resuspend cells in the milk. Maintain the virus on ice from this point on.
12. Sonicate three times, each time for 30 sec using a Branson Sonifier with microtip at an output setting of 4 (timer on "Hold"; % Duty Cycle on "Constant"; approximate Power Output of 15%), letting the cell suspension cool on ice for 1 min between sonications, to free virus particles from the cellular debris.
13. Split virus stock into smaller aliquots and transfer to sterile, screw-capped cryovials. Label stock with virus name, passage, and date, and store at −70°C or colder.

    Dividing the suspension into aliquots prevents excess freezing and thawing of the virus every time it is removed from the freezer. Label stock by virus name, passage, and date. It is imperative that virus be stored a temperatures of −70°C or colder. Storage at higher temperature rapidly leads to loss of infectivity.

DETERMINING VIRUS TITERS

BASIC PROTOCOL 2

Before viruses can be used in experiments, the titer of the virus stock must be determined. The protocol below utilizes the fact that HSV encodes a receptor that binds the Fc portion of human immunoglobulin molecules (Roizman and Knipe, 2001). Addition of pooled human immunoglobulin precludes the need to grow virus under solid supports, such as agar, and greatly facilitates rapid virus titering. This ensures that the observed plaques result strictly from cell-to-cell spreading and not secondary infection by extracellular virus particles.

Materials

Cell line of choice (e.g., Vero)
DMEM/5% FBS medium (see recipe)
199V medium (see recipe)

Virus stock (see Basic Protocol 1)
20 mg/ml pooled human immunoglobulin (Sigma) in distilled H_2O
Phosphate-buffered saline with potassium (KPBS; see recipe)
Methanol
KaryoMax Giemsa Stain stock solution (Invitrogen)

25-cm^2 tissue culture flasks
Plugged, sterile pipet tips
Fine-point marking pen
Inverted microscope

Additional reagents and equipment for splitting cells (see Support Protocol 1)

Prepare cells and virus dilutions

1. Split Vero cells (Support Protocol 1) into a sufficient number of 25-cm^2 tissue culture flasks for titration in duplicate (see step 3) using DMEM/5% FBS such that they will be ~90% confluent in 24 hr (e.g., split 1:5 for a concentration of $\sim 4 \times 10^6$ cells/25-cm^2 flask).

   The next day, use these cells for titering.

   The titration is performed in duplicate to ensure accuracy.

2. Add 1 ml 199V medium to each of a series of tubes. Using a sterile pipet, add 10 µl virus stock to the 1 ml of 199V in the first tube and vortex (this is now a 10^{-2} dilution). Transfer 10 µl from the 10^{-2} tube to the next tube containing 1 ml of 199V and vortex (this is now a 10^{-4} dilution). Transfer 100 µl from the 10^{-4} tube to the next tube and vortex to prepare a 10^{-5} dilution. Repeat until the 10^{-8} dilution has been reached. Discard 100 µl from last tube to keep the volumes constant.

   Make serial dilutions of virus at least 10^{-6}, 10^{-7}, and 10^{-8}.

   Make sure to use a clean, plugged pipet tip each time. This is crucial. Virus stocks are routinely of very high titer and repeated usage of a pipet tip for dilutions will affect the results.

3. Label each 25-cm^2 flask of confluent cells (from step 1) with the dilution of virus that it is to receive. Aspirate the medium. Add 1 ml of each diluted stock to the corresponding flask of cells.

4. To allow virus to absorb, incubate 2 hr with constant gentle rocking, or swirling every 30 min. During the incubation, combine 3.38 µl of 20 mg/ml pooled human immunoglobulin with 9 ml DMEM/5% NBCS medium to prepare a solution containing 7.5 µg/ml human immunoglobulin.

5. Aspirate the virus inoculum from each flask and add 3 ml of the DMEM/5% NBCS containing pooled human immunoglobulin prepared in step 4.

6. Incubate ~2 days, until plaques are visible.

Stain plaque dishes

The following steps need not be performed under sterile conditions.

7. Aspirate medium from dishes. Rinse each monolayer twice with KPBS.

8. Add 1 ml methanol to each dish and leave flat for 5 min at room temperature to fix cells.

9. Dilute the KaryoMax Giemsa Stain stock solution 1:10 with distilled water. Aspirate the methanol and add 2 ml of the diluted Giemsa stain to each flask. Incubate flat for 20 min at room temperature.

10. Discard the stain down a sink. Rinse cells gently with cold tap water until the liquid is clear. Dry inverted flask on a paper towel.

11. Count plaques (circle or mark each with a fine-point marking pen) viewing through an inverted light microscope at low (10×) magnification.

    While a phase-contrast microscope is not neccesary, it may help some investigators to more easily distinguish plaques.

    To determine titer of the stock, average the duplicate number of plaques counted for a given dilution. This is the number before the exponent. The dilution is the exponent. For example, if four plaques are counted on a 10^{-8} dish, the titer is 4×10^{8}. Since this is an infectious center assay, titers are expressed as pfu/ml.

PICKING VIRUS PLAQUES FOR PLAQUE PURIFICATION

BASIC PROTOCOL 3

To insure that the virus stock is not a mixture of different viruses, it is necessary to plaque-purify the isolate. Often, after a virus has been grown up, the stock needs to be tested for contamination. The protocol below provides a rapid method for screening several virus isolates by picking plaques. Once the plaques are picked, subsequent "plaque-pure" virus stocks are made. These should be used for characterizing the virus (see below).

Materials

Virus stock (see Basic Protocol 1)
DMEM/5% NBCS medium (see recipe)
199V medium (see recipe)
20 mg/ml pooled human immunoglobulin (Sigma) in distilled H_2O
Sterile milk (see recipe)
1% (w/v) agarose in KPBS (see recipe for KPBS; store at 4°C)
Phosphate-buffered saline containing potassium (KPBS; see recipe), sterile
25-cm^2 tissue culture flasks
Plugged, sterile pipet tips
Fine-point marking pen
15-ml snap-cap tubes
Bent Pasteur pipet (see recipe)
Hand-held battery-operated pipetting device (e.g., Pipet-Aid; Drummond Scientific)
Probe sonicator (e.g., Branson Sonifier) with microtip
Additional reagents and equipment for splitting cells (see Support Protocol 1)

Isolate plaques

1. Split Vero cells (Support Protocol 1) appropriately into 25-cm^2 tissue culture flasks such that they will be 90% confluent in 24 hr (see Basic Protocol 2, step 1).

2. To ensure that the plaques will be spread out in the dish, dilute the stock down to its estimated titer by making serial dilutions of virus stock in 199V medium in the same fashion as for virus titering (see Basic Protocol 2, step 2).

   Only ~20 to 50 plaques per dish are desirable.

3. Aspirate the medium from each flask of confluent cells prepared in step 1, replace with 1 ml diluted virus, and incubate 2 hr at 37°C to allow the virus to absorb, swirling every 30 min. During the incubation, combine 1.125 μl of 20 mg/ml pooled human immunoglobulin with 3 ml DMEM/5% NBCS medium to prepare a solution containing 7.5 μg/ml human immunoglobulin.

4. Aspirate medium from each flask and add 3 ml of the DMEM/5% NBCS containing pooled human immunoglobulin prepared in step 3. Incubate at 37°C until plaques are seen (usually within 2 days).

5. Choose and circle 5 to 10 plaques, preferably ones with space between them, using a fine-point marking pen.

   As noted below (see Commentary), HSV infection of monolayer cells yields distinctive phenotypes, including enlargement and rounding of cells. Bear in mind that certain mutant viruses will have plaque phenotypes that differ slightly from that of wild-type virus.

6. Label one 15-ml snap-cap tube per plaque to be picked, including plaque number, virus, date, and any other appropriate parameters. Add 2 ml 199V and 2 ml sterile milk to each tube.

7. Heat 1% agarose in KPBS in a microwave until agarose is completely dissolved. When the mixture is cool enough to touch (~45°C), aspirate the medium from each dish (step 4) and add 3 ml of the 1% agarose in KPBS.

8. After the agarose has hardened (~2 to 5 min), pick plaques using a bent Pasteur pipet in a hand-held battery-operated pipetting device (e.g., Pipet-Aid) by sucking up the agarose above the plaque and transferring it to the corresponding tube prepared in step 6, pipetting up and down to release the agar plug. Make sure that it enters the tube.

9. Freeze plaque stocks at −80°C for ≥15 min.

Prepare high-titer virus stocks

10. Thaw plaque stocks from step 9 on ice. Sonicate on ice three times, each time for 10 sec using a Branson Sonifier with microtip at an output setting of 4 (timer on "Hold"; % Duty Cycle on "Constant"; approximate Power Output of 15%), letting the virus suspension cool on ice for 10 sec in between sonications.

    Use equivalent settings if a different sonicator is used.

11. Use 0.5 ml sonicated plaque stock to infect a 90% confluent 25-cm^2 flask of Vero cells (prepared as in step 1). Incubate 2 hr at 37°C to allow the virus to absorb, swirling every 30 min.

12. Wash monolayer by first aspirating off the milk and then rinsing with 2 ml prewarmed sterile KPBS.

13. Aspirate KPBS and add 3 ml DMEM/5% NBCS medium. Incubate until nearly 100% CPE is observed.

    In order to obtain good yields, it is very important that the cells go as far along in the infection as possible. This can take up to 2 to 3 days.

14. When 100% CPE has been achieved, freeze flasks at −80°C for ≥15 min.

15. Thaw flasks in biological safety hood (work with no more than three to four flasks at a time). Swirl the virus/cell suspension (often referred to as "herpes-Slurpee") over the bottom of the flask to resuspend all cellular material.

16. When all of the ice is thawed, transfer the medium to a 15-ml tube containing 3 ml sterile milk. Mix by pipetting up and down a few times and place the tubes on ice. Label tubes with appropriate parameters.

17. Sonicate on ice three times, each time for 30 sec using the same instrument and settings as described in step 10, letting the virus suspension cool on ice for 30 sec in between sonications.

18. Store at −80°C or use ~0.5 ml directly for infections.

SPLITTING CELLS

Both Vero and HEp-2 cells are adherent cell lines that can be grown in almost any type of culture vessel. Covered dishes must always be kept in a CO_2 incubator. Capped (filterless) flasks (75-cm^2 or 25-cm^2) do not require such an incubator but require the injection of filtered CO_2 prior to tightening; these gassed flasks may be placed in any 37°C environment, including water baths.

Note that it is important to consider the relative confluency of the cell monolayer prior to splitting. Cells should be split once they reach between 95% to 100% confluency. The doubling times of some cell lines are significantly longer than other, standard lines. Therefore, such cells should be maintained at a higher cell density (e.g., never going below 60% confluency).

Materials

DMEM/5% FBS medium (see recipe)
Cells: Vero or HEp-2 (Table 14E.1.1)
Phosphate-buffered saline with potassium (KPBS; see recipe), sterile
70% ethanol
Trypsin-EDTA (Invitrogen; also see recipe)
Culture vessels: 75- or 25-cm^2 tissue culture flasks

Prepare flasks

1. Prewarm DMEM/5% FBS medium and sterile KPBS in a 37°C water bath for 10 to 15 min.

   In general, DMEM with fetal bovine serum (FBS) is used for maintain cells for passaging, whereas DMEM with newborn calf serum (NBCS) is used for cells to be infected with virus.

2. Before use, wipe down all bottles (medium, KPBS, and trypsin-EDTA), aspirator, pipetting devices, and biological safety hood with 70% ethanol.

3. Using sterile technique, transfer 10 ml DMEM/5% FBS medium into each new 75-cm^2 flasks into which the cells are to be split or transfer 3 ml of the medium into each new 25-cm^2 flask into which the cells will be split.

   If the cell line being passed contains a plasmid encoding a particular gene of interest, it will often also contain a gene encoding a protein for resistance to a particular drug or antibiotic. The presence of this drug ensures that only the cells containing the plasmid will divide. Thus, this antibiotic should be added to fresh culture medium prior to splitting cells.

Trypsinize cells

4. Remove the cells to be split from the incubator. Tilt flask so that medium pools in the corner of the flask away from the cell monolayer. Using a sterile Pasteur pipet, vacuum aspirate the medium at the corner such that the pipet tip does not come into contact with the cells.

5. Rinse cells with 5 ml sterile KPBS, being sure to wash entire surface in order to remove all serum-containing medium. Aspirate the KPBS with a sterile Pasteur pipet at the corner opposite the cells.

6. Repeat the wash described in steps 4 and 5.

7. Add 2 ml trypsin-EDTA and rock the flask back and forth, ensuring that all of the cells are coated. Remove the trypsin-EDTA quickly (within 30 sec) using a sterile Pasteur pipet.

8. Tighten the cap of the flask and incubate in 37°C incubator for 2 to 5 min.

   It is not necessary to place flasks in the incubator after applying trypsin, but doing so will speed up the process of detaching cells. Some cell lines are more sensitive to trypsin. Overtrypsinizing cells can lead to a loss of cell-surface factors from which the cells cannot recover. Some cells even require a rinsing step to remove any residual enzyme that is left behind. To do this, resuspend the detached cells in KPBS, centrifuge several minutes at 800 × g using a tabletop clinical centrifuge, and resuspend pellet in cell culture medium.

9. Tap flask to dislodge cells and check under microscope to make sure most cells are detached and floating.

10. Using sterile technique, add an appropriate quantity of prewarmed DMEM/5% FBS to the trypsinized flask, according to the desired split.

    For example, if splitting 1:4, add 4 ml to the cells at this step.

Split cultures

11. Using a 5-ml pipet, triturate the cells ~30 times by pipetting up and down, squirting the medium against the bottom of the flasks each time. Transfer the cells to the 75- or 25-cm^2 flasks according to the desired split.

    For the 1:4 example above, transfer 1 ml of the cell/medium mixture to the medium that was transferred to the labeled flask in step 3.

12. Put the flask on its side and rock it gently to fill the bottom surface of the flask with the cells.

13. Place the flask in the incubator and continue incubating. Loosen the cap of the flask if using a CO_2 incubator.

SUPPORT PROTOCOL 2

FREEZING CELLS

In order to maintain proper quality control, it is recommended that cells not be passaged more than 20 to 30 times. This ensures that they will always be at approximately the same passage during infection experiments, to help maintain reproducibility and consistency. It is therefore necessary to freeze the cell stocks in aliquots that can be rethawed as needed. As a general rule of thumb, it is suggested that each time a new stock is thawed, it be one of the older aliquots. Upon thawing, the cells should be expanded and then a portion frozen, before two to three passages, for future use.

Materials

Phosphate-buffered saline with potassium (KPBS; see recipe), sterile
DMEM/5% NBCS medium (see recipe)
Dimethylsulfoxide (DMSO), sterile
70% ethanol
Confluent flasks of Vero or HEp-2 cells (see Support Protocol 1)
Trypsin-EDTA (Invitrogen; also see recipe)
Liquid nitrogen

Tabletop clinical centrifuge
1.5-ml cryovials
Liquid nitrogen tank or freezer

Prepare CFM and rinse cells

1. Prewarm sterile KPBS in a 37°C water bath for 10 to 15 min.

2. Depending on the number of flasks that are to be frozen, combine DMEM/5% NBCS medium and DMSO as described in Table 14E.1.2 to prepare an appropriate amount of cell freezing medium (CFM). Place CFM on ice.

Table 14E.1.2 Recipes for CFM Based on Number of Flasks Used

Number of flasks	Amount of DMEM/5% NBCS (ml)	Amount of DMSO (μl)
2	2.7	300
3	3.6	400
4	4.5	500
5	5.4	600

3. Before use, wipe down all bottles (i.e., medium, KPBS, and trypsin-EDTA), aspirator, pipetting devices, and biosafety hood with 70% ethanol.
4. Remove the cells that are going to be frozen from the 37°C incubator. Using a sterile Pasteur pipet, aspirate the medium at the corner opposite the cell monolayer.
5. Rinse cell layer with 5 ml prewarmed sterile KPBS (step 1). Be sure to wash entire surface in order to remove all serum-containing medium.
6. Using a sterile pipet, aspirate the KPBS with a sterile Pasteur pipet at the corner opposite the cells
7. Repeat steps 5 and 6 for an additional wash.

Trypsinize cells

8. Add 2 ml trypsin-EDTA and rock the flask back and forth, ensuring that all of the cells are coated.
9. Remove the trypsin-EDTA quickly (within 30 sec) using a sterile Pasteur pipet.
10. Tighten the cap of the flask and incubate in 37°C incubator for 2 to 5 min.
11. Tap the sides of the flask to dislodge cells.
12. Resuspend cells in 2 ml DMEM/5% NBCS and transfer to a 5-ml tube. From this point forward, keep cells on ice.

Freeze cells

13. Centrifuge cells 3 to 4 min at 800 × *g*, 4°C, using a tabletop clinical centrifuge, and resuspend cell pellets in CFM at ∼1 × 10^7 cells/ml.
14. Distribute cells in CFM evenly between desired number of cryovials (∼750 μl/cryovial).
15. Transfer cells to a −80°C freezer as soon as possible and leave there overnight or up to 2 weeks to allow cells to equilibrate, then transfer to −150°C in a liquid nitrogen tank or liquid nitrogen freezer for long-term storage.

THAWING CELLS

SUPPORT PROTOCOL 3

Cells permissive for HSV are available from the ATCC and are provided as DMSO-containing frozen stocks which must be properly thawed to ensure recovery of viable cells. Choosing the type of cell in which to grow virus depends on the purpose of the infection. Certain cells are highly permissive for virus replication and are therefore used to prepare virus stocks. Others are more suited for the characterization of virus replication. Table 14E.1.1 presents some examples of commonly used cell lines.

Note that the conditions outlined below are suitable for Vero and HEp-2 cell lines, which are commonly used; however, media, culture vessel, optimal pH, and CO_2 concentrations may vary depending on the cell line being thawed.

Materials

DMEM/5% FBS medium with antibiotics (see recipe)
Cryovial containing frozen cells (see Support Protocol 2, or purchase from ATCC), maintained at −70°C or below
70% ethanol
75-cm^2 flask (or alternative culture vessel)
Inverted tissue culture microscope

1. Prewarm DMEM/5% FBS medium at 37°C for 15 to 20 min.
2. Prepare a 75-cm^2 flask containing 10 to 12 ml of the prewarmed culture medium adjusted to optimal pH with 5% CO_2/95% air.
3. Remove vial containing cells from the freezer and immerse in a 37°C water bath. Agitate so that cells thaw rapidly (within 1 min).
4. Wash outside of vial thoroughly with sufficient 70% ethanol to sterilize (~5 ml).
5. In a sterile hood, transfer thawed cells to the flask from step 2. Pipet up and down several times to ensure that all cells are distributed throughout the medium.
6. Place flask in 37°C incubator overnight.
7. On the following day, replace old medium (containing DMSO from the freezing medium) with fresh prewarmed DMEM/5% FBS medium.
8. Monitor cell growth using inverted microscope. Passage cells (see Support Protocol 1) when they reach confluency (cell foci are touching each other).

BASIC PROTOCOL 4

PREPARING WHOLE INFECTED CELL EXTRACTS FOR IMMUNOBLOTTING

One of the easiest and most reliable methods for documenting viral infection is to assay for immune reactivity against specific viral proteins. Numerous antibodies are now commercially available that recognize individual viral gene products. The protocol below, in conjunction with Basic Protocols 5 and 6, is a simple, straightforward method that can be used with almost any antibody raised against an HSV antigen.

Determining protein concentrations allows for equal loading of protein on denaturing gels (see Basic Protocol 5). Without equal loading, comparisons cannot be made on the basis of protein accumulations. This method uses a modified Bradford assay provided by Bio-Rad. It requires generating a standard curve using a known stock of bovine serum albumin (BSA). The slope of the graph of BSA concentration versus absorbance at 595 nm (OD_{595}) is the calibration factor. Measure protein concentration of each sample as described in step 17.

Materials

DMEM/5% FBS medium (see recipe)
199V medium (see recipe)
Phosphate-buffered saline with potassium (KPBS; see recipe), 4°C
Buffer A with protease inhibitors (see recipe), 4°C
Bio-Rad Protein Assay solution
Platform rocker
Cell scraper: preferably 12 in. (~30 cm) long
6-ml tubes

Tabletop clinical centrifuge
Probe sonicator (e.g., Branson Sonifier) with microtip
Spectrophotometer
Additional reagents and equipment for splitting cells (see Support Protocol 1), infecting cells (see Basic Protocol 1)

Infect cells

1. Split cells (see Support Protocol 1) into 25-cm^2 flasks using DMEM/5% FBS medium. Incubate cells overnight at 37°C/5% CO_2 to 95% to 100% confluency.
2. Aspirate medium. Infect confluent cells at an MOI of 5 to 10 in 1 ml prewarmed 199V medium (see Basic Protocol 1, step 2).

   Always keep the virus on ice and return the vials to −80°C as soon as the virus has been used. This will prevent a drop in virus titer upon refreezing.
3. Place flasks on a platform rocker and incubate 1 hr at 37°C to allow virus to absorb.
4. Aspirate virus and add 2 ml DMEM/5% NBCS to each flask. Incubate until it is time to harvest.

   HSV infections are usually completed between 18 to 24 hr post-infection (hpi). To perform a time course of infection, simply stop the infection at each hour post-infection (Pomeranz and Blaho, 1999).

Prepare whole infected cell extracts

5. To harvest, remove flasks from incubator and carefully begin scraping cells off the side of the flask using a cell scraper. Transfer cells and medium to a 6-ml tube on ice.
6. When all samples have been harvested into tubes, gently centrifuge for 5 min at 3500 rpm in a tabletop clinical centrifuge at 4°C.
7. Carefully aspirate supernatant and discard. Resuspend cells in 1 ml ice-cold KPBS and transfer samples to correspondingly labeled 1.5-ml microcentrifuge tubes.
8. When all samples have been transferred, microcentrifuge 10 to 15 sec at maximum speed, 4°C. Carefully aspirate supernatant and discard.
9. Gently resuspend cells in 300 μl of ice-cold Buffer A containing protease inhibitors.
10. Sonicate samples three times, each time for 10 sec using a Bronson Sonifier with microtip at an output setting of 2, letting the extract cool on ice for 10 sec between sonications.

    Always keep samples on ice while sonicating.

Determining infected cell protein concentrations

11. Perform a standard Bradford assay (also see *APPENDIX 3A*) using the following reaction:

    1590 μl H_2O
    400 μl Bio-Rad Protein Assay solution
    10 μl protein extract (substitute 10 μl Buffer A to prepare blank).

Measure absorbance in a spectrophotometer at 595 nm.

12. Proceed directly to gel electrophoresis (see Basic Protocol 5) followed by immunoblotting (see Basic Protocol 6) or store samples in aliquots up to 1 to 2 years at −80°C.

    The extracts should not be thawed and refrozen more than two times. Store in aliquots to ensure extract integrity.

BASIC PROTOCOL 5

PREPARING AND RUNNING DATD-ACRYLAMIDE GELS WITH HSV WHOLE INFECTED CELL EXTRACTS FOR IMMUNOBLOTTING

Denaturing gel electrophoresis (SDS-PAGE) is one of the most convenient assays for assessing HSV infection. It is preferable to use polyacrylamide gels that are cross-linked with *N*,*N*′-diallyltartardiamide (DATD). DATD has many advantages over bisacrylamide, as it allows fine resolution of post-translationally modified viral polypeptides, including glycoproteins (Brown and MacLean, 1998).

Materials

Mild laboratory detergent
70% ethanol
Petroleum jelly
1.4 μg/ml ammonium persulfate
Protein gel solutions A, B, and C (see recipe)
20% (w/v) SDS (*APPENDIX 2A*)
TEMED
30% acrylamide/bisacrylamide (see recipe)
1× protein running buffer (see recipe for 10×)
Whole infected cell extract (see Basic Protocol 4)
Buffer A (see recipe)
4× disruption buffer (see recipe)
Gel-forming apparatus:
  Glass plates for 20 × 20–cm gel
  2-mm spacers
  Combs
  Large binder clips
Syringes
Vertical electrophoresis apparatus, power supply, and cables
Bent needles for removing bubbles from electrophoresis chamber
Boiling water bath

Pour the gel

The gel must be made before the extract samples are prepared for loading.

1. Thoroughly wash glass gel plates with a mild detergent. Rinse, dry thoroughly, and wipe down with 70% ethanol.

2. Using a syringe, outline the larger glass plate with a thin line of petroleum jelly along both sides and the bottom.

   There are numerous gel systems available. This method guarantees gels that never leak.

3. Place spacers on top of the petroleum on the glass and adjust into place. Add a thin line of petroleum jelly on top of the spacers and carefully place the other glass on top. Using large binder clips, clip both sides and the bottom to prevent leaks.

4. Stand plates up vertically. Using the appropriate-sized comb, mark on the plate at ~1 cm from where the wells will end.

5. Prepare the separator gel in a 125-ml flask, according to the percentage of the gel desired, typically (for 20 × 20 gel with 2-mm spacers):

   4.8 ml H_2O
   15 ml 1.4 μg/ml ammonium persulfate
   7.5 ml protein gel solution A
   3.16 ml protein gel solution C

300 µl 20% (w/v) SDS
18 µl TEMED.

The percentage of the gel used should be determined by what protein the researcher is trying to identify. If the researcher is looking for a low-molecular-weight protein, a higher percentage gel should be used.

6. Upon mixing the above ingredients, immediately pour the gel, using a 10-ml pipet, up to the line marked with the comb (see step 4; approximately the bottom three-quarters of the gel).

   The dimensions of the separator gel will be needed when detecting HSV proteins by immunoblotting (see Basic Protocol 6).

7. Gently add a layer of water on top the separating gel using a Pasteur pipet and allow the separator gel to polymerize.

8. When polymerized, remove the top layer of water using a syringe. Tilt the glass plates until all of the water is removed.

9. Prepare the stacking gel (top 1/4 of the gel) in a 50-ml centrifuge tube, according to the percentage of the gel desired, typically (for 20 × 20 gel with 2-mm spacers):

   1.61 ml H_2O
   3 ml 1.4 µg/ml ammonium persulfate
   750 µl protein gel solution B
   638 µl 30% acrylamide/bisacrylamide
   2 µl TEMED.

10. Immediately pour the stacking gel using a 5-ml pipet. Put the comb into place and wait for the stacking gel to polymerize completely.

11. Remove the clips and bottom spacer. Use a Kimwipe remove any excess petroleum jelly.

    Failure to remove excess petroleum jelly may cause the gel not to run properly.

12. Stand the gel vertically and align so smaller plate and comb face inward. Clip plates onto a vertical electrophoresis apparatus.

13. Add 1× protein running buffer to the top and bottom chambers. Make sure that the top does not leak.

14. Remove the bubbles between the two plates in the bottom chamber with a curved (bent) needle on a syringe.

15. Mark the lanes on the glass plates.

16. Remove the comb and rinse the wells with buffer.

    Samples can now be loaded onto the gel.

Load samples on gel

17. Calculate the volumes of protein extract needed to ensure that there is 50 µg of extract protein loaded in each well. Add buffer A to the whole infected cell extract sample mixtures to make bring all samples to equal volumes.

18. Add 4× disruption buffer to each sample for a final concentration of 1×. Boil samples in a water bath for 5 min, then microcentrifuge 2 to 3 sec at maximum speed to pellet insoluble material.

19. Load samples onto the gel.

Perform electrophoresis

20. Run the gel at a constant current of 70 mA.

    Running a 2-mm 15% DATD-acrylamide gel at 70 mA typically takes 4 to 5 hr.

21. Perform immunoblot staining (see Basic Protocol 6).

BASIC PROTOCOL 6

DETECTION OF HSV PROTEINS BY IMMUNOBLOTTING

This method, performed on the gel that was run in Basic Protocol 5, utilizes an electrical transfer method in a tank apparatus, as this is the technique that yields the most efficient transfer of high-molecular-weight polypeptides. While other transfer methods are available, e.g., semi-dry and vacuum-based ones, these techniques suffer from low transfer efficienies. This method also utilizes an alkaline phosphatase-conjugated secondary antibody system, which is ideal for visualizing viral proteins. Chemiluminescence methods may also be used, but these are prone to high background levels due inherent cross-reactivites of the available anti-HSV primary antibodies.

Materials

Gel containing separated HSV proteins (see Basic Protocol 5)
Transfer buffer with and without SDS (see recipes)
0.1% Ponceau S (optional; see recipe)
Phosphate-buffered saline (KPBS; see recipe)
5% milk/KPBS (see recipe)
Tris-buffered saline (TBS; see recipe)
Primary antibodies: HSV-specific antibodies are available from the Rumbaugh-Goodwin Institute for Cancer Research (*http://www.rgicr.org/*)
Secondary antibody, alkaline phosphatase–conjugated (Sigma or Fisher); fluorescently tagged antibodies may be obtained from Molecular Probes
1% BSA/KPBS (see recipe)
Tris-buffered saline with Tween 20 (TBST; see recipe)
AP buffer (see recipe)
15 mg/ml nitroblue tetrazolium chloride (NBT) in 70% dimethylformamide (DMF)
30 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100% DMF
Whatman no. 1 filter paper
0.45-μm nitrocellulose transfer membrane
Plastic dish large enough to accommodate gel
Flat glass plate slightly larger than gel
Electroblotting apparatus: tank transfer system including transfer cassette and power supply (e.g., Bio-Rad)

Prepare the transfer stack

1. Measure the dimensions of the separator gel. Cut two pieces of Whatman no. 1 filter paper to a slightly larger size, i.e., allowing an extra centimeter on each side.

   Avoid wasting nitrocellulose and filter paper by being accurate in gel dimensions.

2. Cut a piece of 0.45-μm nitrocellulose transfer membrane according to the dimensions measured.

   Be careful in handling; nitrocellulose is delicate.

3. Add transfer buffer without SDS to a plastic dish and place a flat glass plate slightly larger than the cut filter paper in the buffer.

4. Write "top" on one piece of filter paper. Wet the nitrocellulose and filter paper in transfer buffer without SDS.

5. Place the bottom piece of filter paper on the glass plate, followed by the nitrocellulose. Center and smooth to make sure there are no air bubbles trapped under the paper.

6. At this point, turn off the power supply to the protein gel. Discard the protein running buffer in the top chamber. Unclip the gel and remove it from the chamber. Place the gel flat on a tabletop lined with paper towels. Slide out the side combs and gently separate the two plates using a metal spatula.

7. Using a razor blade, carefully remove the stacking gel, slide it onto a paper towel, and discard. Remove any gel below the blue buffer front line and slide it onto a paper towel to discard. Cut a corner of the gel for orientation.

8. Roll the gel off the glass plates using a razor blade, allowing it to fall onto the nitrocellulose. Gently move the gel into place (congruent with nitrocellulose membrane).

9. Put the second, "top" piece of filter paper onto the gel to form a "sandwich." Smooth out any air bubbles. Remove the sandwich from the buffer.

Perform the transfer

10. Place the sandwich into an appropriate transfer cassette.

    The top filter paper should be facing the black terminal (cathode) side of the cassette. Orientation of the gel is very important when transferring. If the "top" is not facing the black side and/or the cassette is placed in the transfer apparatus incorrectly, the gel will be transferred the wrong way and the proteins will be lost.

11. Assemble and close the transfer cassette. Place cold transfer buffer with SDS into the transfer apparatus rig. Insert the cassette into the rig nearest to the anode (red side), oriented so that the "top" side of the cassette faces the black pole of the apparatus (cathode).

12. Plug into power supply and run at 100 V in a cold room (4°C).

    It will take ~2 hr to completely transfer a 2-mm, 15% acrylamide gel.

13. Turn off the power. Take apart the cassette. Rinse cassette, sponges, and rig with tap water to remove salts before storing.

    Depending upon time available, the researcher may opt to air dry the nitrocellulose membrane or go directly to next step and block in 5% milk. The membrane may also be stained at this point using 0.1% Ponceau S (see recipe), which allows for visualization of almost all the proteins on the membrane. This gives the researcher the ability to see whether there are any air bubbles or if there was a problem with the transfer. This step is not necessary, but it is advised.

Probe for immune reactivity of viral proteins

14. If the blot was dried, rewet it completely in KPBS. Block the membrane by incubating in 5% milk at room temperature for 1 hr or overnight at 4°C.

    Blocking in milk reduces the background binding of antibodies. The blocked membrane may also be air dried and stored.

15. Rinse the blot six times, each time with 10 ml TBS.

16. If it is desirable to cut the blot into sections (e.g., to stain with more than one antibody), carefully cut the blot with a razor blade using the markers as a guide.

    Loading prestained molecular weight markers onto the gel is advised if one desires to cut the membrane.

17. Dilute primary antibody in 1% BSA/KPBS.

    It is important to know the required dilution for the primary antibody. A titration of antibody dilutions should be performed to determine the optimal dilution.

18. Add the appropriate primary antibody to each blot and incubate at 4°C.

    Incubation with primary antibodies against viral proteins should take only 2 hr and may be done at room temperature. However, detection of certain cellular proteins requires overnight incubation at 4°C.

19. Remove the primary antibody. Rinse the blot three times, each time with 10 ml TBST

    The antibody stock may be reused and should be stored at 4°C.

20. Dilute secondary antibody 1:1000 in 1% BSA/KPBS. Add the diluted secondary antibody to the blot and incubate for exactly 1 hr at room temperature.

    The secondary antibody must correlate with the primary. For example, if the primary antibody is from mouse, the researcher must make sure to use an anti-mouse secondary antibody. Antibodies should be diluted (e.g., 1:1000) in 1% BSA/KPBS. The authors generally suggest secondary antibodies coupled to alkaline phosphatase.

21. Discard secondary antibody and rinse three times, each time for 15 min with 10 ml TBST.

22. Rinse three times, each time for 5 min with 10 ml TBS.

23. Rinse once with 10 ml AP buffer for 1 to 2 min.

24. Prepare developer solution by diluting 15 mg/ml NBT and 30 mg/ml BCIP together 1:500 in AP buffer.

25. Add developer solution to the blot and wait for the bands to appear. Stop by rinsing with water before the blot becomes over-developed.

BASIC PROTOCOL 7

USING INDIRECT IMMUNOFLUORESCENCE TO LOCALIZE VIRAL PROTEINS WITHIN CELLS

Indirect immunofluorescence allows the researcher to visualize the localizations of various viral proteins within the infected cell. Refer to Pomeranz and Blaho (1999) for examples using specific antibodies to show that different viral proteins reside in different places in the infected cell. The following is a standard method for confirming productive viral infection.

Materials

Ethanol
Cells of choice, growing in tissue culture
DMEM/5% FBS medium (see recipe)
Virus stock (see Basic Protocol 1)
199V medium (see recipe)
DMEM/5% NBCS medium (see recipe)
Phosphate-buffered saline (KPBS; see recipe)
2.5% paraformaldehyde (see recipe)
Acetone, −20°C
10 μg/ml human immunoglobulin in 1% BSA/KPBS (see recipe for 1% BSA/KPBS)
Primary antibody
1% BSA/KPBS (see recipe)
Fluorescently conjugated secondary antibody
ProLong Antifade Kit (Molecular Probes)
Clear nail polish

25-mm^2 coverslips
6-well or 33-mm^2 tissue culture dishes
Dark plastic box
Glass microscope slides

Additional reagents and equipment for splitting cells (see Support Protocol 1)

Prepare cover slips with cells

1. Dip a coverslip in ethanol and flame to sterilize. Place carefully in dish or well.
2. Seed an appropriate number of the cells of choice (see Support Protocol 1 for splitting technique) in 2 ml DMEM/5% FBS onto the sterilized coverslips in the well/dish to achieve confluency on the next day. Incubate cells overnight.

Infect cells

3. *Optional:* Perform a "synchronized infection," by placing dishes on ice for 20 min prior to infection and maintaining them on ice during virus absorption to ensure that all cells are at the same point of the replication cycle throughout infection (Pomeranz and Blaho, 1999).

   This is especially useful when examining the kinetics of viral protein production.

4. Remove medium and infect cells by adding virus stock at an MOI of 15 in 199V medium. Absorb virus 1 hr at 37°C (or on ice for synchronized infection).

   Always keep the virus on ice and return the virus vials to −80°C as soon as virus has been used.

5. Aspirate virus and add 2 ml DMEM/5% NBCS to each well/dish. Incubate 1 to 2 hr.
6. Remove cells from the incubator and aspirate off the medium from the edge of the dish to ensure that the cells are not being removed from the coverslip.

   The remaining steps need not be performed under sterile conditions.

7. Rinse cells twice, each time with 2 ml KPBS at room temperature, aspirating each time.

Fix cells

8. Add 2 ml of 2.5% paraformaldehyde to each well and incubate at room temperature for 20 min.
9. Aspirate the paraformaldehyde and add 1 ml acetone, −20°C, to each well. Place at −20°C for 3 to 5 min, then aspirate and rinse cells twice as in step 7.

Perform staining

10. Add 2 ml/well of 10 μg/ml human immunoglobulin diluted in 1% BSA/KPBS and block by incubating 1 hr to overnight at 4°C.

    Treatment with this amount of human immunoglobulin was previously shown to be sufficient to neutralize Fc binding by the viral gE and gI proteins (Pomeranz and Blaho, 1999).

11. Dilute the primary antibody appropriately in 1% BSA/KPBS.

    This dilution varies depending on the antibody.

12. Remove dishes from the 4°C refrigerator and aspirate the blocking buffer. Rinse twice with 2 ml KPBS at room temperature.

13. Line the bottom of a dark box with Parafilm. Place 50 µl of primary antibody onto the Parafilm. Remove the coverslip containing the infected cells from the well/dish and very carefully place it cell-side-down on the antibody solution in the dark box. Incubate 1 hr at room temperature.

    Pay special attention to where the cells are located relative to the coverslip. It is extremely important to know the orientation of the coverslip in relation to being "cell-side-up" or "cell-side down."

14. Remove the coverslip from the box and place it cell-side up in a fresh well or dish. Rinse twice with KPBS at room temperature, leaving the second rinse in the well or dish.

15. Dilute the secondary antibody in 1% BSA/KPBS.

    This dilution varies depending on the antibody. For example, FITC-coupled antibodies usually use 1:300, while Texas Red–coupled antibodies use 1:150.

16. Remove the Parafilm lining from the dark box and replace with fresh Parafilm. Place 50 µl of the diluted, fluorescently-conjugated secondary antibody on the Parafilm.

17. Remove the coverslip from the well and very carefully place it cell side-down on the secondary antibody solution in the dark box. Incubate for exactly 45 min at room temperature, making sure that the dark box is closed.

18. Remove the coverslip from the box and place it in a fresh well/dish, cell-side-up. Rinse twice with 2 ml KPBS at room temperature, leaving the second rinse in the well or dish.

Mount coverslip on slide

19. While the secondary antibody is incubating, add 1 ml of Component B from the ProLong Antifade Kit to one of the brown vials from the kit that contain the antifade reagent.

    The mounting solution thus prepared can be stored up to 1 week at −20°C protected from light.

20. Drop ∼5 µl of the mounting solution onto a clean microscope slide. Remove the coverslip from the well or dish and very carefully place it cell-side-down on the mounting solution on the slide.

21. Seal the edges of the coverslip with clear nail polish and allow it to completely dry, keeping the slides in the dark box. Place slides at 4°C overnight.

    Slides will look the best when viewed the following day. Slides are typically viewed at 100× magnification with oil immersion using a fluorescence microscope.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acrylamide, 30%/bisacrylamide

Weigh out 145 g of acrylamide and 5 g bisacrylamide. Add 300 ml water and allow these reagents to go into solution with stirring. Adjust volume to 500 ml with water. Filter through a 0.45-µm membrane. Store up to 1 year at 4°C in a glass bottle wrapped with aluminum foil

CAUTION*: A dust mask should be worn when weighing out powdered acrylamide. Pre-dissolved liquid acrylamide may be substituted*

AP buffer

10 ml 5 M NaCl
50 ml 1 M Tris·Cl, pH 9.5 (*APPENDIX 2A*)
2.5 ml 1 M $MgCl_2$ (add 20.33 g of $MgCl_2$ to ~80 ml H_2O; stir and adjust volume to 100 ml with H_2O; store indefinitely at room temperature)
Adjust volume to 500 ml with H_2O
Store up to 1 month at room temperature

Bent Pasteur pipets (for sucking up plaques)

Apply heat near the tip of a Pasteur pipet over a low flame until it bends at a very short 45° angle.

It is important that the tip of the pipet not be allowed to close during the heating.

BSA, 1% in KPBS

Dissolve 1 g BSA in 80 ml KPBS (see recipe). Adjust volume to 100 ml with KPBS. Add 100 µl of 20% (w/v) sodium azide for a final concentration of 0.02%. Store up to 3 months at 4°C.

Buffer A

5 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
3 ml 5 M NaCl
1 ml 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)
4 ml 10% (v/v) Triton X-100 (Sigma)
H_2O to 100 ml
Store buffer with above ingredients up to 6 months at room temperature
Just prior to use, add the following protease inhibitors to an appropriate-sized aliquot of Buffer A (e.g., 1 ml):
0.1 mM phenylmethylsulfonylfluoride (PMSF; add from 0.1 M stock in methanol; *APPENDIX 2A* or Sigma)
0.01 mM L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK; add from 0.01 M stock in H_2O; Sigma)
0.01 mM L-1-chlor-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK; add from 0.01 M stock in ethanol; Sigma)

Disruption buffer, 4×

Combine the following in a 15-ml snap-cap tube:
4 ml 20% SDS (*APPENDIX 2A*)
2 ml of 1 M 2-mercaptoethanol
2 ml of 1 M Tris·Cl, pH 7.0 (*APPENDIX 2A*)
2 ml H_2O
~2 mg of bromophenol blue (Sigma)
Store in aliquots up to 3 months at −20°C

DMEM/5% FBS or DMEM/5% NBCS medium

Add 6.25 ml of 10,000 U penicillin/10,000 µg streptomycin (Pen/Strep), 25 ml fetal bovine serum (FBS) *or* newborn calf serum (NBCS), and 50 µl filter-sterilized 250 mg/ml Fungizone to 500 ml of Dulbecco's Modified Eagle's Medium (DMEM).

In general, FBS is used to maintain cells to be passsaged while NBCS is used for cells to be infected. While powdered DMEM may be used, premade liquid DMEM is preferred, since it precludes the need for a large-scale filter-sterilization apparatus.

Milk in KPBS, 5%

Dissolve 5 g nonfat dry milk in <100 ml KPBS (see recipe). Adjust volume to 100 ml with KPBS. Add 100 µl of 20% (w/v) sodium azide. Prepare fresh.

Paraformaldehyde, 2.5%

Combine 3 ml of 10% (w/v) paraformaldehyde, methanol-free (store in dark at room temperature) with 9 ml KPBS (see recipe). Prepare fresh.

Only make enough for the experiment to be performed.

Phosphate-buffered saline with potassium (KPBS)

0.14 M NaCl (add 4 g for 500 ml; add 16 g for 2 liters)
3 mM KCl (add 0.10 g for 500 ml; add 0.40 g for 2 liters)
10 mM Na_2HPO_4 (add 0.71 g for 500 ml; add 2.84 g for 2 liters)
1.5 mM KH_2PO_4 (add 0.10 g for 500 ml; add 0.40 g for 2 liters)
Store indefinitely at 4°C

Ponceau S, 0.1%

Add 495 ml water to a 500-ml glass bottle. Add 0.5 mg Ponceau S and 5 ml glacial acetic acid. Stir to mix. Store up to 1 month at room temperature

Protein gel solutions

Solution A (3 M Tris·Cl, pH 8.5):

Dissolve 66.3 g of Tris base and 130.8 g Tris hydrochloride in 300 ml water with mixing. Adjust pH to 8.5 using NaOH. Adjust volume to 500 ml.

Solution B (Tris/SDS):

Mix 96 ml 1 M Tris·Cl, pH 7.0 (*APPENDIX 2A*), and 4 ml 20% SDS (*APPENDIX 2A*). Store in a small glass bottle.

Solution C (DATD-Acrylamide):

Dissolve 140 g acrylamide and 3.68 g DATD (*N*,*N*′-diallytartardiamide) in 300 ml water by stirring. Adjust volume to 500 ml with water. Filter and store up to 6 months at 4°C in a bottle wrapped with aluminum foil.

CAUTION: *A dust mask should be worn when weighing out powdered acrylamide. Predissolved liquid acrylamide may be substituted. In this case, the DATD cross-linker must be added to the final solution.*

Protein running buffer, 10×

Dissolve 6 g Tris base, 28.8 g glycogen, and 2 g SDS in <2 liters water. When dissolved, adjust volume to 2 liters with water. Store up to 3 months at room temperature. Store up to 3 months at room temperature. Dilute to 1× for running gels

Sterile milk

Add 9 g nonfat dry milk to 100 ml water and transfer to a small glass bottle. Autoclave this solution three times for 20 min, removing it immediately each time and allowing it to cool so that the milk does not curdle. The final solution should have a light brown appearance similar to that of coffee with milk. Store up to 6 months at 4°C.

Tris-buffered saline (TBS)

10 ml 5 M NaCl
5 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Adjust volume to 500 ml with H_2O
Store indefinitely at room temperature

Tris-buffered saline with Tween (TBST)

10 ml 5 M NaCl
5 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
5 ml 10% (v/v) Tween 20
Adjust volume to 500 ml with H_2O
Store up to 1 year at room temperature

Transfer buffer with SDS

Add 4.61 g Tris hydrochloride, 5.55 g Tris base, and 43.28 g glycine to a beaker. Add 2 liters water and stir. Once solids are dissolved, add 600 ml methanol. Add 3.75 ml of 20% SDS (*APPENDIX 2A*). Using a graduated cylinder, adjust volume to 3 liters with water. Store up to 1 month at 4°C.

Transfer buffer without SDS

Dissolve 1.54 g Tris·Cl, 1.85 g Tris base, and 14.43 g glycine in 200 ml methanol. Adjust volume to 1 liter with water. Store up to 1 month at room temperature.

Trypsin-EDTA

Prepare a 0.5 g/ml trypsin stock solution by rehydrating 1 g lyophilized bovine trypsin in 20 ml sterile water. Divide into 1-ml aliquots and store up to 1 year at −20°C. To prepare the working solution, measure out 100 ml sterile Versene (see recipe), add 1 ml of the trypsin stock, and invert to mix. Sterilize by passing through a 0.22-μm filter. Store up to 3 months at 4°C

Note that trypsin-EDTA may also be purchased as a premade solution from Invitrogen.

199V medium

Combine the following in a sterile (autoclaved) 500-ml glass bottle:
430 ml sterile H_2O
50 ml 10× Hanks Balanced Salt Solution (HBSS; *APPENDIX 2A* or commercial supplier, e.g., Invitrogen)
6.25 ml 10,000 U penicillin/10,000 μg streptomycin (Pen/Strep; Invitrogen)
5 ml 67 mM L-glutamine (0.5 g L-glutamine, e.g., Invitrogen, in 50 ml sterile H_2O)
Filter sterilize using 0.22-μm filter
Add 9.35 ml 7.5% (w/v) $NaHCO_3$ (7.5 g $NaHCO_3$ in 80 ml sterile H_2O)
Stir and adjust volume to 100 ml
Filter sterilize in a biosafety hood using an 0.22-μm filter
If color of the solution is not cherry red (pH ∼7.0), add sterile 10 M NaOH dropwise to the bottle until the solution turns that color
Store up to 6 months at 4°C

Versene

Add 8.0 g NaCl, 0.2 g, KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 to 800 ml water. Stir until all chemicals are in solution. Add 0.2 g disodium EDTA and adjust the volume to 1 liter with water. Continue mixing until the EDTA is in solution. Autoclave, cool, and store indefinitely at 4°C.

COMMENTARY

Background Information

HSV-1 is the prototype of a family of highly cytolytic viruses, the *Herpesviridae*, whose lytic replication cycle ultimately leads to the destruction of infected cells. HSV-1 is a neurotropic herpesvirus that causes a variety of infections in humans ranging from benign lesions to fatal encephalitis. It remains latent in the neurons of its host for life and can be reactivated to cause lesions at or near the initial site of infection. Recurrent infections result from the lytic replication of the virus after reactivation from the latent state. The current licensed antiviral therapies recommended for treating

HSV-1 infections are nucleoside derivatives that target the viral DNA polymerase.

During productive HSV infection in cultured cells (Roizman and Knipe, 2001), glycoproteins in the viral envelope interact with receptors on the host cell including heparan sulfate and the herpesvirus entry mediators. HSV-1 gene expression proceeds in a tightly regulated cascade and changes in its levels during infection, usually as the consequence of transcriptional regulation. The first viral genes expressed during infection, termed the immediate-early (IE) genes, are stimulated by incoming virion transactivation protein and are transcribed in the absence of de novo viral protein synthesis. The IE gene products function cooperatively to regulate the expression of all classes of viral genes. The early (E) genes are expressed next and encode proteins mainly involved in viral DNA synthesis. The last genes expressed are the late (L) genes, which mainly encode virion components. The L genes are further divided into the leaky-late and true-late classes of genes. The true-late genes absolutely require viral DNA synthesis for their production. HSV-1 generally completes its replication cycle within 12 to 18 hr of infection.

The cytopathic effect (CPE) of wild-type HSV-1 infection is generally observed as the rounding up of cells. It occurs almost immediately upon infection and tends to become more severe as infection proceeds. Manifestations of wild-type HSV-1 infection include (i) the loss of matrix binding proteins on the cell surface, leading to detachment, (ii) modifications of membranes, (iii) cytoskeletal destabilizations, (iv) nucleolar alterations, and (v) chromatin margination/aggregation or damage, as well as (vi) a decrease in cellular macromolecular synthesis (Roizman and Knipe, 2001). These features will be readily detected during the course of the protocols outlined in this unit.

Critical Parameters and Troubleshooting

Sterility

Standard sterile technique for cell culture must be used when working with HSV. Microbial contamination of cells will cause significantly reduced virus yields and may result in contaminated virus stocks, which will then be passed along to the next set of cells to be used for infections. In the undesirable event that the virus stock becomes infected with bacteria, yeast, or mold, the following may be performed to cure the contamination. Using the spoiled virus sample, infect fresh cells to create a new stock (see Basic Protocol 1). Once the "herpes-Slurpee" (see Basic Protocol 1) has thawed, sonicate the sample at this point, pellet any insoluble material by low-speed centrifugation ($800 \times g$), remove the supernatant, and pass it through a 0.2-μm sterile filter prior to adding the milk stabilizer. Proceed with the protocols in the usual manner from this point on.

Storage temperature of virus

The single most frequent mistake made while working with HSV is to store virus stocks at the wrong temperature. Because the virus contains an envelope and tegument (the proteineous layer located between the envelope and the capsid), both of which are required to be intact in order to initiate a productive infection, the virus is extremely fragile. The virus should be stored at the coldest possible temperature and never above −70°C; storage of virus stocks at −20°C leads to a rapid loss of infectivity.

Quality of cell culture

The efficiency of HSV infection is directly related to the quality of the cells used. The cells recommended in this unit (see Support Protocol 3; also see Table 14E.1.1) are adherent cells. If the Vero and HEp-2 cells used for the infections have been left too long prior to infection (more than one day after reaching confluency), a decrease in productive virus replication will occur. It is important in the immunoblotting and indirect immunofluorescence studies (see Basic Protocols 6 and 7) that all cells in the monolayer be infected. When cells are overgrown, they tend to either grow on top of each other (e.g., HEp-2) or form tight, fibrous structures (e.g., Vero), and this hinders the ability of cells to become infected during the adsorption phase. Therefore, it is recommended that subconfluent cells be used for HSV infection.

Anticipated Results

Infection of Vero or HEp-2 cells with HSV will result in obvious CPE in less than a 24-hr period. When low-MOI infections are performed (see Basic Protocols 1 and 2), obvious, well isolated infectious centers (plaques) will be observed. When infections are performed at an MOI of 5 (see Basic Protocols 4 and 7), all cells in the monolayers will display CPE. Representative results from immunoblotting and indirect immunofluorescence analyses of HSV-infected cells may be found in Pomeranz and Blaho (1999, 2000).

Table 14E.1.3 Time Considerations for HSV Protocols

Protocol	Operation	Time required
Basic Protocol 1	Preparing virus stocks	3 days
Basic Protocol 2	Determining virus titers	5 days
Basic Protocol 3	Plaque purification	1 week
Support Protocol 1	Splitting cells	45 min
Support Protocol 2	Freezing cells	45 min
Support Protocol 3	Thawing cells	15 min
Basic Protocol 4	Preparing whole infected cell extract	3 days
Basic Protocol 5	Gel electrophoresis	2 hr
Basic Protocol 6	Immunoblotting	1 day
Basic Protocol 7	Indirect immunofluorescence to localize viral proteins	3 days

Time Considerations

One single-step replication cycle of HSV takes between 12 to 18 hr for completion. Therefore, all infections take a minimum of one day to perform. In situations where multiple rounds of replication are required (see Basic Protocols 4 and 7), it is necessary to leave the infections for multiple days. The immunoblotting and indirect immunofluorescence procedures (see Basic Protocols 6 and 7) are multifaceted methods containing several subprotocols. Accordingly, these techniques require more than 1 day for completion. Convenient stopping points are noted in each of these protocols. Time allotments for all protocols in this unit are summarized in Table 14E.1.3.

Literature Cited

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Pomeranz, L.E. and Blaho, J.A. 2000. Assembly of infectious Herpes simplex virus type 1 virions in the absence of full-length VP22. *J. Virol.* 74:10041-10054.

Roizman, B. and Knipe, D.M. 2001. Herpes simplex viruses and their replication. *In* Virology, 4th ed. (D.M. Knipe and P.M. Howley, eds.) pp. 2399-2459. Lippincott-Raven, Philadelphia.

Key References

Brown, S.M. and MacLean, A.R. 1998. Methods in Molecular Medicine, Vol. 10: Herpes Simplex Virus Protocols. Humana Press, Totowa, N.J.

This review volume provides additional technical information for the analysis of HSV in cell culture and animal systems

Internet Resources

http://www.stdgen.lanl.gov/stdgen

Web site maintained by the Los Alamos National Laboratory Bioscience Division that includes compilation and analysis of molecular sequence information pertaining to sexually transmitted bacteria and viruses. Dynamic graphics and extended analyses are available for all organisms. Annotation is accomplished by a combination of automation and hand review of each record.

http://darwin.bio.uci.edu/~faculty/wagner

Home page of Dr. Edward Wagner, Professor of Microbiology and Molecular Genetics at the University of California–Irvine. It provides an introduction to HSV. Here one can explore herpes virus research with Dr. Wagner on the following topics: herpes simplex virus (HSV); the HSV genome; HSV replication; use of DNA microarrays to analyze gene expression in HSV-infected cells; microarray analysis of cellular transcript abundance as a function of HSV infection; temporal patterns of HSV-2 transcripts; analysis of herpes simplex virus promoters; structural properties of herpes simplex virus promoters; and latent infections by HSV.

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Epstein-Barr Virus (EBV): Infection, Propagation, Quantitation, and Storage

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ABSTRACT

Epstein-Barr virus (EBV) was first reported as the etiological agent of Burkitt's lymphoma in 1964. Since then, EBV has also been associated with nasopharyngeal carcinoma, which is highly prevalent in Southeast Asia, as well as infectious mononucleosis, complications of AIDS, and transplant-related B cell lymphomas. This virus has further been linked with T cell lymphomas and Hodgkin's disease, establishing the concept of a wide spectrum of EBV-associated malignant disorders. So far, there are a number of EBV-infected cell lines established that can be induced for production of infectious viral progeny and that facilitate the study of the mechanism of EBV-related infection, transformation, and oncogenesis. This unit describes procedures for the preparation of EBV virion particles and in vitro infection of cells with EBV. In addition, procedures for quantitation and storage of the virus are provided. *Curr. Protoc. Microbiol.* 6:14E.2.1-14E.2.21. © 2007 by John Wiley & Sons, Inc.

Keywords: Epstein Barr virus • induction • infection • quantitation • B lymphocyte

INTRODUCTION

This unit focuses on techniques for infection, propagation, quantitation, and long-term storage of Epstein-Barr virus (EBV), as well as the immortalization of primary human B lymphocytes in vitro by EBV. A series of procedures are detailed that will be valuable to the novice as well as the more experienced virologist working with EBV. Numerous details that have been incorporated into the protocols over time, which are now part of the standard techniques used in working with EBV, are outlined. These techniques depend on the healthy growth of cell cultures and the length of time that individual cultures are kept growing or passaged for induction and harvesting of the virus. Most techniques are provided in great detail so that they can be easily established elsewhere; varying strategies are included, which can be of interest and can provide for some flexibility in the labs that will use these protocols.

CAUTION: Epstein-Barr Virus is a Biosafety Level 2 or 2+ (BSL-2) pathogen. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

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STRATEGIES FOR INDUCTION OF EBV REPLICATION

BASIC PROTOCOL 1

Induction of EBV Replication Using Ig Cross-Linking Agents

The use of cross-linking agents has been one of the long standing-strategies for inducing EBV from latency in B cells (Takada and Ono, 1989; Daibata et al., 1990; Miller et al., 1994; Longnecker and Miller, 1996). Treatment of latently infected B lymphocytes with anti-IgG antibodies will lead to cross-linking of the receptor and activation of the B cells, and thus induce production of viral progeny. Initial studies utilized concentrations that ranged from 50 to 100 μg IgG/ml medium. Cells are typically collected by centrifugation at slow speed, usually 800 × *g*, for 15 min at room temperature in a biohazard-containment centrifuge in a BSL-2 or BSL-3 tissue culture room (Fig. 14E.2.1A). All cells should be transferred and handled under BSL-2 conditions using sterile γ-irradiated conical polypropylene centrifuge tubes. 5×10^7 cells are collected in a 50-ml tube and the supernatant aspirated into a collection flask in a sterile environment. A class II–containment laminar flow hood, with a vacuum attachment and an in-line 0.2-μm filter between the vacuum system and the collecting trap, provides a suitable working environment. The collection flask should prefilled with bleach up to 20% of the volume of the flask before aspiration, so that the concentration of the bleach will be always >20% and the material

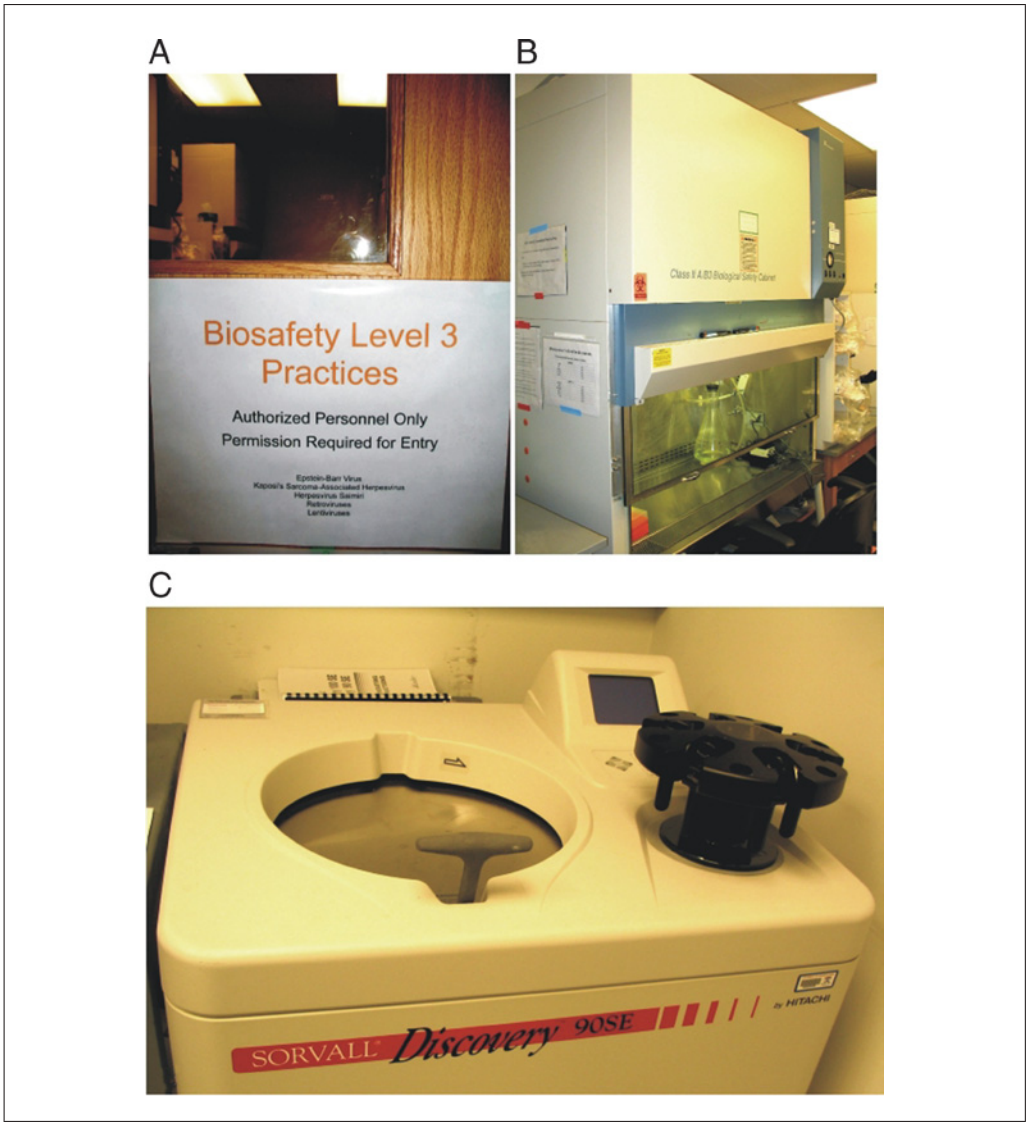

Figure 14E.2.1 (**A**) Entrance to a Biosafety Level 3 (BSL-3) laboratory. (**B**) Laminar flow hood with biosafety HEPA filter. (**C**) Ultracentrifuge with swinging-bucket rotor.

aspirated into the flask will be immediately lysed in hypochlorous acid (HClO). The setup is illustrated in Figure 14E.2.1B.

Cells collected for induction should be resuspended in ~10 ml complete medium and supplemented with the final concentration of antibody mentioned above. The resuspended cells should then be transferred to a culture flask and placed at 37°C in an incubator with 5% CO_2 for induction. The lytic reactivation of the virus can take as long as 4 to 5 days, but the induction of the lytic transactivator Zta can be seen within 24 to 48 hr post induction. Virus can then be collected by harvesting the supernatant and concentrated by high-speed centrifugation (Fig 14E.2.1C).

Materials

EBV-positive B cells (see Basic Protocol 7): Akata cells infected with GFP-tagged EBV are recommended (Kanda et al., 2004; to obtain cells, contact Dr. Kanda at *tkanda@igm.hokudai.ac.jp*)
Complete RPMI medium containing 10% FBS (see recipe)
Anti-IgG antibody
Centrifuge with swinging-bucket rotor

Additional reagents and equipment for harvesting EBV from cell supernatant (Basic Protocol 5)

1. Suspend logarithmically growing EBV-infected Akata cells to a final concentration of 5–10 × 10^6 cells/ml with fresh complete RPMI medium containing 10% FBS.
2. Add anti-IgG antibodies to a final concentration of 100 μg/ml.
3. After 48 hr culture at 37°C, harvest cells by centrifuging for 5 min at 800 × *g*, room temperature. Retain the supernatant, which includes the virus (also see Basic Protocol 5).

   The Akata cells in the pellet are activated and produce viruses. They can be used to check the expression of EBV early antigens by immunoblotting or immunofluorescence.

Induction of EBV Replication Using *BZLF1* from a Heterologous Promoter

BASIC PROTOCOL 2

The BZLF1 protein (also referred to as Zta), expressed from a heterologous promoter, is also an efficient strategy for induction of the virus from latency in vitro (Lear et al., 1992; Robertson and Kieff, 1995). The Zta protein, encoded by the *BZLF1* open reading frame (ORF), is the major intermediate early transactivator of lytic reactivation. The *BZLF1* ORF should be cloned downstream of a highly active promoter. The human cytomegalovirus intermediate early promoter (HCMV IE promoter), the promoter for the cellular elongation factor IFa (pBoss), and the Simian virus 40 (SV40) promoter are all relatively strong promoters and can produce high levels of transcripts (in this case that from *BZLF1*). These *BZLF1* expression constructs are available from a number of laboratories in the U.S.A. and Europe (Tomkinson et al., 1993a,b). Alternatively, for more efficient transduction of *BZLF1*, an adenoviral, retroviral, or lentiviral vector can be used. BSL-3 practices are required for use of high-titer viral expression vectors; however, it is also acceptable to use BSL-2+ technique if low-titer viral expression vectors are used.

EBV-positive cells can be induced for lytic reactivation by transfection with the *BZLF1* expression vector or transduction with a lentiviral vector encoding *BZLF1*. Briefly, cells are pelleted by centrifugation and washed once in phosphate-buffered saline or serum-free medium. The collected cells can then be transfected by electroporation or by using a chemical transfection agent (Tomkinson et al., 1993a,b; Robertson and Kieff, 1995). For human B lymphocytes, the electroporation strategy for transfection has been more successful. Typically, cells can be electroporated by resuspending in 400 μl of serum-free RPMI medium, transferred to a 0.4-cm gap electroporation cuvette, and electroporated

at 220 V and 975 µF. Here, a specific protocol performed in the authors' laboratory will be described; however the initial amount of cells infected with EBV can be varied depending on the amount of virus to be produced.

Materials

EBV-positive B cell line: B95.8 (available from ATCC) or LCL1 or LCL2 (generated in Dr. Erle Robertson's laboratory; *erle@mail.med.upenn.edu*)
Complete RPMI medium containing 10% FBS (see recipe) and serum-free RPMI 1640 medium (e.g., Invitrogen)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1µg/ml *BZLF1* expression vector in TE buffer, pH 8.0 (*APPENDIX 2A*) or distilled H_2O
Centrifuge with swinging-bucket rotor
0.4-cm-gap electroporation cuvette
Gene pulser II system (Bio-Rad)
Additional reagents and equipment for harvesting EBV from cell supernatant (Basic Protocol 5)

NOTE: B95.8, LCL1, and LCL2 are all EBV-positive B cells lines that can be used as a resources for production of the virus, with no significant difference between them.

1. Collect 1.0–1.5 × 10^7 cells in exponential growth phase by centrifuging 5 min at 216 × *g*, room temperature, in a swinging-bucket rotor.
2. Remove supernatant. Wash cells once by resuspending in PBS, then centrifuging 5 min at 216 × *g*, room temperature, and removing the supernatant.
3. Gently resuspend the cells in 400 µl serum-free RPMI 1640. Mix with 10 µg of a *BZLF1* expression construct.
4. Transfer the cells to a 0.4-cm gap electroporation cuvette.
5. Electroporate the cells at 220 V and 975 µF using a Bio-Rad gene pulser (Fig. 14E.2.2).

Figure 14E.2.2 Bio-Rad Gene Pulser II System.

6. Transfer the cells into 10 ml complete RPMI medium containing 10% FBS. Incubate the cells at 37°C in a 5% CO_2 incubator for 3 to 5 days, allowing the production of viral progeny.
7. Harvest cells by centrifuging for 5 min at 800 × *g*, room temperature. Retain the supernatant, which contains the virus. Also see Basic Protocol 5.

Induction of EBV Replication Using Phorbol Ester (12-*O*-Tetradecanoyl-Phorbol-13-Acetate; TPA)

BASIC PROTOCOL 3

TPA is a well-known activator of protein kinase C (PKC), which has been shown to induce the reactivation of herpesviruses such as EBV and KSHV in certain latently infected cells (Lazdins et al., 1987; Davies et al., 1991; Gradoville et al., 2002). In the case of EBV, TPA activates viral lytic replication by inducing the expression of the major viral intermediate-early gene *BZLF1*.

Materials

EBV-positive B cell line: B95.8 (available from ATCC) or LCL1 or LCL2 (generated in Dr. Erle Robertson's laboratory; *erle@mail.med.upenn.edu*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Complete RPMI medium containing 10% FBS (see recipe)
2000× (40 μg/μg/ml) 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma), sterilized by filtration through 0.22-μm membrane
Centrifuge with swinging-bucket rotor

Additional reagents and equipment for harvesting EBV from cell supernatant (Basic Protocol 5)

NOTE: B95.8, LCL1, and LCL2 are all EBV-positive B cells lines that can be used as a resources for production of the virus, with no significant difference between them.

1. Harvest 1.0 × 10^6 EBV-positive cells by centrifuging 5 min at 216 × *g*, room temperature, in a swinging-bucket rotor.
2. Remove supernatant. Wash cells once by resuspending in PBS, then centrifuging 5 min at 216 × *g*, room temperature, and removing the supernatant.
3. Resuspend the cells in 10 ml fresh complete RPMI medium containing 10% FBS. Add 2000× (40 μg/ml) TPA to a final concentration of 1× (20 ng/ml).
4. Incubate the cells at 37°C in a 5% CO_2 incubator for 3 to 5 days, allowing the production of viral progeny.
5. Harvest cells by centrifuging for 5 min at 800 × g, room temperature. Retain the supernatant, which contains the virus. Also see Basic Protocol 5.

Induction of EBV Replication Using a Combination of TPA and Sodium Butyrate

BASIC PROTOCOL 4

TPA does not always efficiently reactivate viral production in certain EBV-infected cells. In these cases, TPA can be combined with sodium butyrate (SB) to improve the efficiency of viral production. SB is a potent inhibitor of histone deacetylases which silence gene expression (Kruh, 1982). A number of studies have shown that SB can trigger the lytic replication of a number of herpesviruses, including EBV (Cen et al., 1993).

Materials

EBV-positive B cell line: B95.8 (available from ATCC) or LCL1 or LCL2 (generated in Dr. Erle Robertson's laboratory; *erle@mail.med.upenn.edu*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Complete RPMI 1640 medium containing 10% FBS (see recipe)
2000× (40 μg/μg/ml) 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma), sterilized by filtration through 0.22-μm membrane
3 M sodium butyrate (SB), sterilized by filtration through 0.22-μm membrane
Centrifuge with swinging-bucket rotor

Additional reagents and equipment for harvesting EBV from cell supernatant (Basic Protocol 5)

NOTE: B95.8, LCL1, and LCL2 are all EBV-positive B cells lines that can be used as a resources for production of the virus, with no significant difference between them.

1. Harvest 1.0 to 1.5 × 10^7 EBV-positive cells by centrifuging 5 min at 216 × *g*, room temperature, in a swinging-bucket rotor.
2. Remove supernatant. Wash cells once by resuspending in PBS, then centrifuging 5 min at 216 × *g*, room temperature, and removing the supernatant.
3. Resuspend the cells in 10 ml fresh complete RPMI medium containing 10% FBS. Add 2000× (40 μg/ml) TPA to a final concentration of 20 ng/ml and add 3 M SB to a final concentration of 3 mM.
4. Incubate the cells at 37°C in a 5% CO_2 incubator for 3 to 5 days, allowing the production of viral progeny.
5. Harvest cells by centrifuging for 5 min at 800 × *g*, room temperature. Retain the supernatant, which contains the virus. Also see Basic Protocol 5.

ISOLATING EBV

BASIC PROTOCOL 5

Harvesting of EBV from Cell Supernatant

The induction of EBV by the methods described in Basic Protocols 1 to 4 leads to lytic replication to produce viral progeny. Eventually, the cells harboring EBV are lysed and the infectious virions are released into the culture supernatant. A portion of the virion pool, however, may also be trapped within cells that are not lysed, or may be associated with cellular organelles. Usually, three cycles of freeze-thaw lead to further release of virions from intact cells and organelles, and thus increase viral production (Lee et al., 2001; Lan et al., 2005).

Materials

Cells induced for EBV replication (Basic Protocols 1 to 4)
Dry ice
Isopropanol

Centrifuge with swinging-bucket rotor
15-ml conical centrifuge tubes
Container suitable for resisting extremely cold temperatures
0.45-μm syringe filter

1. Centrifuge induced cells 12 min at 300 × *g*, room temperature.
2. Transfer most of the supernatant into a fresh 15-ml tube.

   For instance, transfer 9 ml if the cell culture is 10 ml total.

3. Resuspend the cells in the remaining supernatant and perform three cycles of freeze-thaw in the following manner.

   a. Add an appropriate amount of dry ice to a container capable of resisting extremely cold temperature changes and mix with isopropanol at a 1:1 ratio.

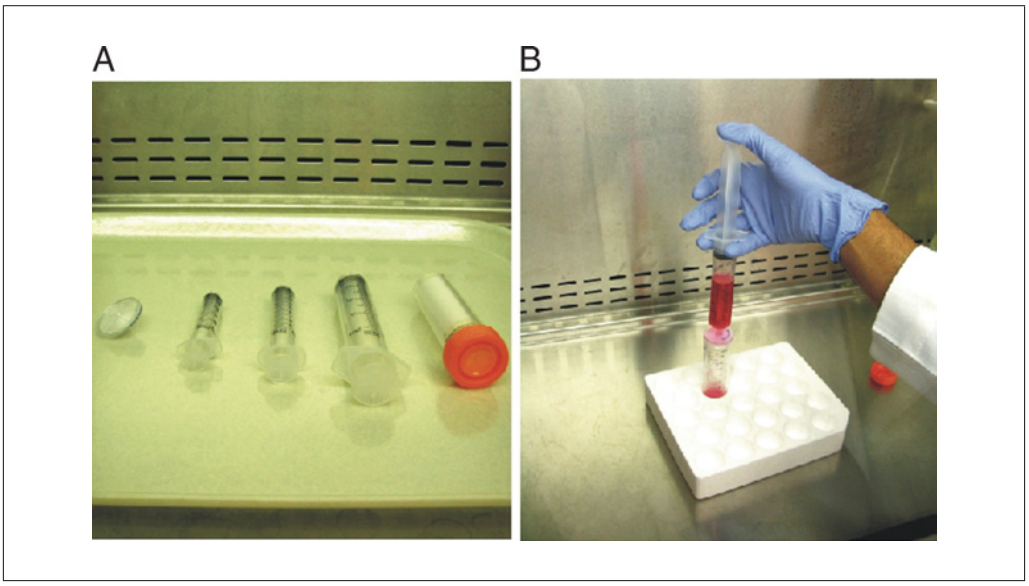

Figure 14E.2.3 (**A**) Components used in this unit, including 0.45-μm filter, different size syringes, and collecting tube for filtering virus. (**B**) Filtering virus in a laminar-flow hood.

b. Incubate the tube containing cells in the dry ice/isopropanol mixture for 5 min.
c. Immediately transfer the tube to a 37°C water bath and incubate for ~5 min or until it is thawed.
d. Repeat the freeze-thaw cycle described in substeps "b" and "c," above, three times.
e. Remove debris by centrifuging 10 min at 1000 × *g*, room temperature. Recover supernatant.

4. Combine the supernatant with the previous 9 ml (see step 2).
5. Filter the supernatant using a 0.45-μm syringe filter (Fig. 14E.2.3).

Concentration of EBV by Ultracentrifugation

BASIC PROTOCOL 6

Once the supernatant containing EBV infectious virions is obtained, it can be directly used to infect cells. If the chemical inducers interfere with the experimental system, the viral particles can be pelleted by ultracentrifugation to remove the inducing agents in the supernatant. In addition, if the titer of virus in supernatant is low, the virus can also be concentrated by ultracentrifugation.

Materials

70% (v/v) ethanol
EBV-containing supernatant (Basic Protocol 5)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)

10-ml ultracentrifuge tube
UV light source for sterilization
Ultracentrifuge with swinging-bucket rotor (Fig. 14E.2.1C)
Refrigerated centrifuge with fixed-angle rotor accommodating 1.7-ml microcentrifuge tubes

1. Sterilize a 10-ml ultracentrifuge tube by overnight exposure to UV light.
2. Carefully wash out the ultracentrifuge rotor with 70% ethanol.
3. Transfer the supernatant into a 10-ml ultracentrifuge tube.

4. Ultracentrifuge 2 hr at 75,000 × *g*, 4°C, in a swinging-bucket rotor.
5. Aspirate supernatant carefully (be careful not to aspirate the virus pellet).
6. Resuspend pellet using 1 ml PBS, and transfer to a 1.7-ml microcentrifuge tube.
7. Centrifuge 20 min at 25,000 × *g*, 4°C.
8. Resuspend viral particles in 1/100 of the original volume of PBS.

INFECTING CELLS WITH EBV

BASIC PROTOCOL 7

Infection of Human Cells with EBV

Once EBV is made as per the protocols above, it can be used to infect human cells in order to address a range of experimental questions. EBV has tropism for human B cells, and a number of other cell types such as epithelial cells, with varying degrees of efficiency. Here, the de novo infection method of EBV for suspension cells as well as adherent cell lines is introduced; a method is also provided to infect primary B lymphocytes in order to create a lymphoblastoid cell line (LCL). These cell lines can be used for exploring the mechanism of EBV latent infection, and can also be used as a reservoir for producing infectious virions.

Materials

Human B cell lines (suspension), human epithelial cell lines (adherent), or primary human B cells (*APPENDIX 4C*)
Complete RPMI medium containing 10% FBS (see recipe)
Supernatant containing EBV infectious virions (Basic Protocol 5 or 6)
1× trypsin/EDTA solution (e.g., Invitrogen)
Complete DMEM medium containing 10% FBS (see recipe)

15-ml conical polypropylene centrifuge tubes
Centrifuge with swinging-bucket rotor
75-cm^2 tissue culture flasks or 100-mm tissue culture plates
96-well tissue culture plate (optional; for producing lymphoblastoid cell line)

NOTE: Medium containing 10% FBS is not always necessary; lower serum concentrations have also been shown to work well for a number of cell lines.

For EBV infection of suspension cell lines

1a. In a 15-ml centrifuge tube, harvest 1.0 × 10^7 cells in exponential growth phase by centrifuging 5 min at 300 × *g*, room temperature, and removing the supernatant.

Exponential-phase cells are most permissive for infection.

2a. Wash the cells once by adding 10 ml PBS, centrifuging as in step 1, and removing the supernatant.

3a. Resuspend pellet in 5 ml complete RPMI containing 10% FBS.

4a. Mix supernatant containing EBV virions with cells.

Although there is currently no method for titering EBV, based on the authors' experience, a quantity of virions collected from 1 × 10^7 induced EBV-infected cells should be used to infect every 1 × 10^7 cells.

5a. Incubate at 37°C for 3 or 4 hr.

6a. Centrifuge 5 min at 300 × *g*, room temperature, in a swinging-bucket rotor.

7a. Discard the supernatant, resuspend the cells at 15 ml with complete RPMI containing 10% FBS, and incubate the infected cells in a 75-cm^2 tissue culture flask or 100-mm plate.

For EBV infection of adherent cell lines

1b. Wash one 60% to 80% confluent flask of cells once with PBS.

2b. Harvest 1.0×10^7 cells by trypsinization. Transfer to a 15-ml centrifuge tube.

3b. Centrifuge cells 5 min at 300 × *g*, room temperature. Wash once by adding 10 ml PBS, centrifuging again as before, and removing the supernatant.

4b. Resuspend the cells in 5 ml complete DMEM containing 10% FBS.

5b. Mix supernatant containing EBV virions with cells.

Although there is currently no method for titering EBV, based on the authors' experience, a quantity of virions collected from 1 × 10^7 induced EBV-infected cells should be used to infect every 1 × 10^7 cells.

6b. Incubate at 37°C for 3 or 4 hr.

7b. Centrifuge 5 min at 300 × *g*, room temperature, in a swinging-bucket rotor.

8b. Discard the supernatant, resuspend the cells at a desired volume with complete DMEM containing 10% FBS, and incubate the infected cells into a 75-cm^2 tissue culture flask or 100-mm plate.

For EBV infection of primary B cells

1c. In a 15-ml centrifuge tube, harvest 1.0×10^7 primary B cells by centrifuging 5 min at 300 × *g*, room temperature, and removing the supernatant.

2c. Resuspend the cells in 5 ml complete RPMI containing 10% FBS.

3c. Mix supernatant containing EBV virions with cells.

Although there is currently no method for titering EBV, based on the authors' experience, a quantity of virions collected from 1 × 10^7 induced EBV-infected cells should be used to infect every 1 × 10^7 cells.

4c. Incubate at 37°C for 3 or 4 hr.

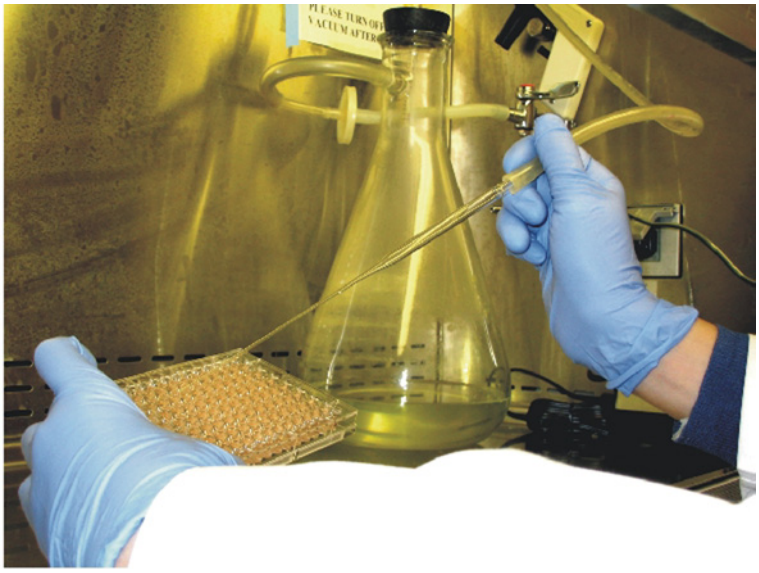

Figure 14E.2.4 Changing the medium in a 96-well plate by aspirating with vacuum filtration system.

5c. Centrifuge 5 min at 300 × *g*, room temperature, in a swinging-bucket rotor.

6c. Discard the supernatant, resuspend the cells at a desired volume with complete RPMI containing 10% FBS, and put the cells into flask or plate.

For producing lymphoblastoid cell line, the following steps (7c, 8c, and 9c) will need to be performed.

7c. Plate the cells into a 96-well plate at 100 μl for each well

The desired cell number is ~20,000 per well.

8c. Incubate the cells at 37°C with 5% CO_2 for 1 week, then add another 100 μl fresh medium to each well (for a total volume of 200 μl).

9c. Every third day, aspirate out about half the medium from each well and replace with fresh medium (Fig. 14E.2.4). Repeat this process for about 4 to 8 weeks until lymphoblastoid cells are clearly visible by microscopy (see Fig. 14E.2.7; cells typically look like a clump).

BASIC PROTOCOL 8

Microcell-Mediated EBV Genome Transfer to Mouse Cells

Most immunosuppression-associated malignant lymphomas are driven by oncogenic viruses, e.g., EBV or Kaposi's sarcoma-associated herpesvirus (KSHV). The underlying mechanisms driving these associated viral lymphomas, however, are yet to be fully elucidated. An experimental model system of viral infection would be extremely helpful in this regard for both in vitro and in vivo studies. EBV infects cells through two independent mechanisms. In one case, the viral glycoproteins gp350/220 interact with their corresponding receptor, CD21 (human complement receptor type 2; CR2; Fingeroth et al., 1984), and in the other, viral glycoproteins gp42/gH/gL interact with MHC class II (Haddad and Hutt-Fletcher, 1989; Molesworth et al., 2000). CR2 is the receptor for the C3d component of the complement factor which complexes with CD19, CD81, and Leu-13 (Levy et al., 1998). CR2 is predominantly expressed on mature human B cells, but is also found on some human T cells, follicular dendritic cells, and certain epithelial cells (Fingeroth et al., 1988; Fischer et al., 1991). Human cells that lack hCR2 are refractory to EBV infection (Carel et al., 1989; Li et al., 1992). Mouse cells express a CR2 receptor that does not bind EBV, and are therefore not susceptible to EBV infection (Fingeroth et al., 1989). Mouse lymphoid cells transfected with the *hCR2* gene acquire the capacity to bind EBV (Carel et al., 1989; Cantaloube et al., 1990). These cells can then be infected by the virus, but the stability of infection and the replication potential of the virus in these cells remain to be investigated.

Figure 14E.2.5 outlines the overall procedure described in detail below.

Materials

EBV-infected (Basic Protocol 7) and EBV-negative human lymphoid cell lines
Serum-free RPMI 1640 medium (e.g., Invitrogen)
EBV-hyg (hygromycin resistance gene–tagged EBV) *or* EBV-neo (neomycin resistance gene–tagged EBV)
Lipofectamine 2000 transfection reagent (Invitrogen)
Fetal bovine serum (FBS; *APPENDIX 2A*), heat inactivated
EBV-A9 selection medium A (see recipe)
Colcemid (demecolcine; e.g., Invitrogen or Sigma)
Percoll microcell gradient mixture (see recipe)
Serum-free DMEM medium (e.g., Invitrogen) containing 100 μg/ml phytohemagglutinin (PHA)

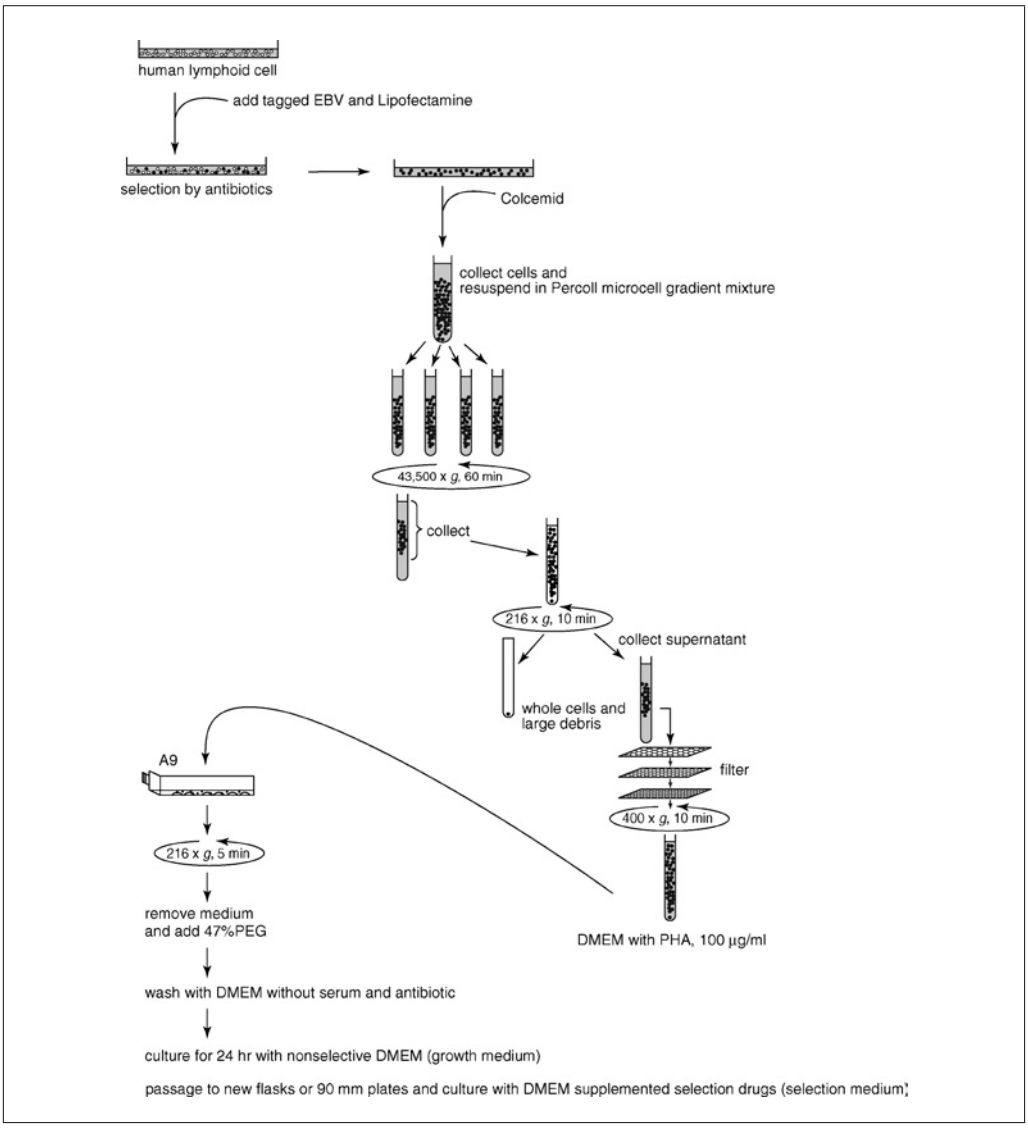

Figure 14E.2.5 Schematic diagram showing steps of EBV genome transfer to mouse cells.

A9 cells (mouse B lymphocyte cell line used here as recipient cells; ATCC #CRL-1811) grown as monolayer in 100-mm culture dish
47% (w/v) PEG (mol. wt. 8000)
Serum-free DMEM medium (e.g., Invitrogen)
Growth medium for A9 cells: complete DMEM medium containing 10% FBS (see recipe)
1× trypsin/EDTA solution (e.g., Invitrogen)
EBV-A9 selection medium B (see recipe)

6-well tissue culture plates
15- and 50-ml centrifuge tubes
Centrifuge and plate carrier
8-, 5-, and 3-µm pore-size Nucleopore polycarbonate filters (Whatman)
25-cm^2 tissue culture flasks
100-mm tissue culture plates

Prepare EBV-infected human lymphoid cells

1. Wash exponentially growing EBV-infected and EBV-negative (control) human lymphoid cells once with serum-free RPMI 1640 medium. Dilute to 2.5 × 10^5 cells/ml in the same medium.

The EBV-negative cells cannot make microcells, which are associated with the presence of the virus. The difference between the EBV-positive cells and EBV-negative controls will become apparent at step 13.

Some cells, e.g., fibroblasts, are resistant to micronuclei induction by Colcemid treatment (see below). In such a case, a different cell line should be used (Kelleher et al., 1998; Kugoh et al., 1999).

2. Add 5×10^5 cells in 2 ml medium to each well of a 6-well plate.
3. In a separate microcentrifuge tube corresponding to each well of cells prepared, dilute 1 μg EBV-hyg or EBV-neo DNA in 100 μl serum-free RPMI 1640 medium, and mix by gentle inversion. Incubate 5 min at room temperature.

   These can be created by the individual investigator or requested from the original creator (some papers using these tagged EBV are Fujiwara and Ono, 1995; Borza and Hutt-Fletcher, 1998; Delecluse et al., 1998; Kelleher et al., 1998; Kiss et al., 2003).

4. In a separate microcentrifuge tube corresponding to each well of cells prepared, dilute 1 μl Lipofectamine 2000 in 100 μl serum-free RPMI 1640 medium and incubate 5 min at room temperature.
5. After 5 min incubation, combine each DNA solution prepared in step 3 with the corresponding Lipofectamine solution prepared in step 4. Mix gently and incubate for a further 20 min at room temperature.
6. After the 20-min incubation, add each 200 μl of mixture to the center of the corresponding cell-containing well, and swirl plate to mix.
7. Incubate the plate at 37°C for 4 to 6 hr, and then add heat-inactivated fetal bovine serum (FBS) to each well for a final concentration of 10%.
8. The next day, passage the cells into fresh EBV-A9 selection medium A in a 6-well plate.

   The selection medium includes specific selection drugs that will depend on the drug-resistance marker (see Reagents and Solutions).

9. Grow to a total of 2×10^8 EBV-infected lymphoid cells (EBV-hyg or EBV-neo). Maintain cells between 3×10^5 and 8×10^5 cells/ml in EBV-A9 selection medium.

Induce formation of micronuclei and prepare microcell suspension

10. Add 0.1 mg/ml (final concentration) Colcemid to the EBV-infected lymphoid cell cultures and incubate 2 hr.

    Colcemid induces formation of micronuclei.

11. Collect cells and resuspend in 60 ml Percoll microcell gradient mixture.
12. Divide into four 15-ml centrifuge tubes and centrifuge 60 min at 43,500 × *g*, 33°C.
13. Collect the top 12 ml from each tube into two 50-ml tubes and vortex to mix.
14. Centrifuge 10 min at 216 × *g*, room temperature.
15. Collect the supernatant and sequentially filter through 8-, 5-, and 3-μm Whatman Nucleopore polycarbonate filters.

    Syringe filtration is the easiest way to accomplish this; gravity and vacuum filtration are alternatives.

16. Centrifuge 10 min at 400 × *g*, room temperature, to pellet the purified microcells. Resuspend pellet in 5 ml of serum-free DMEM medium containing 100 μg/ml phytohemagglutinin (PHA).

Incubate microcells with recipient cells

17. Add the microcell suspension from step 17 to a monolayer of recipient cells (A9 cells) in a 25-cm^2 culture flask at 37°C and incubate 15 min.

18. Centrifuge the dish 5 min at 216 × *g*, room temperature. Remove the supernatant. Add 2 ml of 47% PEG, then incubate 1 min. Wash the monolayer once with serum-free DMEM medium.

19. Add 4 ml complete DMEM medium containing 10% FBS (nonselective growth medium for A9 cells). Incubate the cells 24 hr, then trypsinize and split into three 100-mm plates containing 10 ml EBV-A9 selection medium B each.

QUANTITATION OF EBV

Quantitation of EBV Virions by Quantitative PCR Using DNA of Known Copy Number as Standard

BASIC PROTOCOL 9

Epstein-Barr virus (EBV) virions can be quantified by determining the amount of DNA encapsulated within the viral particles. Quantitation of virions based on the DNA content is more accurate, compared to the conventional plaque assay; also, plaque assay has not proven to be successful in quantitation of EBV virions. In order to isolate viral DNA, virions are purified by ultracentrifugation as described previously. Since the centrifugation may also pellet proteins as well as other DNAs that may interfere with the exact quantitation of the virus particles, these purified virions are subjected to further purification by density-gradient centrifugation. Density-gradient centrifugation is done either on a linear sucrose or Nycodenz step gradient. Both gradients are equally good for the purification of virions.

Materials

Concentrated EBV virion suspension (Basic Protocol 6)
30% and 60% (w/v) sucrose solutions
20% and 35% (w/v) Nycodenz (e.g., Sigma)
0.5× and 1× phosphate-buffered saline (PBS; see *APPENDIX 2A* for 1× concentration)
Lysis buffer (see recipe)
12:12:1 (v/v/v) phenol:chloroform:isoamyl alcohol
3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)
100% ethanol, ice-cold
Primers for quantitative PCR (Table 14E.2.1)
DNA of known copy number as standard for quantitative PCR (Bookout et al., 2006)

15-ml ultracentrifuge tubes
Gradient maker
Ultracentrifuge
18.5-G needles and syringes
MWCO 10,000 dialysis tubing, and dialysis clips
60°C water bath

Additional reagents and equipment for quantitative PCR (Bookout et al., 2006)

Purify virions

To purify concentrated virions on a linear sucrose gradient.

1a. Prepare a 10-ml linear 30% to 60% sucrose gradient in a 15-ml ultracentrifuge tube using a gradient maker. Add 0.5 ml concentrated virion suspension to the top of the gradient.

2a. Centrifuge the tubes 2 hr at 30,000 × *g*, 4°C, which separates the virions from cell debris. Collect the band containing virions by puncturing the tube with 18.5-G needle. Discard the remaining gradient solution as biohazard waste.

The band is generally located at the middle of the gradient and is clearly visible, resembling a white ring.

3a. Transfer the contents of the band into dialysis tubing and dialyze 3 to 6 hr against 4 liters of 0.5× PBS to remove sucrose. Pellet the purified virions by centrifugation for 1 hr at 40,000 × *g*, 4°C.

To purify concentrated virions on a Nycodenz step gradient

1b. Prepare a 10-ml Nycodenz step gradient by layering 5 ml 20% Nycodenz over 5 ml 35% Nycodenz in a 15-ml ultracentrifuge tube. Add 0.5 ml concentrated virion suspension to the top of the gradient and centrifuge 2 hr at 30,000 × *g*, 4°C.

Titer is not very important as this is a concentration step. Optimally, 10^7 to 10^8 particles (virions produced from 1–2 × 10^7 EBV-positive cells) can be used.

2b. Collect the band containing virions by puncturing the tube with 18.5-G needle. Discard the remaining gradient solution as biohazard waste.

The band is generally located at the middle of the gradient and is clearly visible, resembling a white ring.

3b. Transfer the contents of the band into dialysis tubing and dialyze 3 to 6 hr against 4 liters of 0.5× PBS to remove the Nycodenz. Centrifuge 1 hr at 40,000 × *g*, 4°C, to pellet the virion particles. Discard supernatant as biohazard waste.

Isolate genomic DNA from virion particles

4. Resuspend purified virions in 0.4 ml lysis buffer. Incubate at 60°C for 2 hr.

5. Extract virion DNA by treating the lysates with 0.4 ml of 12:12:1 phenol:chloroform:isoamyl alcohol, then centrifuging 10 min at 15,000 × *g* and transferring the aqueous layer to a fresh tube.

6. Precipitate DNA by adding 3 M sodium acetate to a final concentration of 0.3 M and adding 2.5 vol of ice-cold 100% ethanol. Incubate at −80°C for 15 min or at –20°C for 1 hr.

Quantitate viral DNA

7. Quantitate viral DNA yield by quantitative PCR amplification (Bookout et al., 2006) of a specific region of the genome (see Table 14E.2.1 for appropriate primers) followed by comparison with the Ct values form DNA of known size and amount (see steps 8 to 10) using the following equation:

Number of copies = [amount of DNA (in g)/molecular weight of the DNA (in Da)] × [Avogadro's number (6.023×10^{23})].

Table 14E.2.1 Primers Used For the Quantitation of EBV

Regions	Sense (5′-3′)	Antisense (5′-3′)
Bam C	GCAGGGCTCGCAAAGTATAG	TGCGGAAGTGACACCAAATA
Bam E	TACTGCCACCAGTACCACAACA	GGCCGACATTCTCCAAGATAA
EBNA2	CTCTGCCACCTGCAACACTA	ATTTGGGGTGCTTTGATGAG
LMP2	TGCAATTTGCCTAACATGGA	TGGACATGAAGAGCACGAAG
Bam W	CCAGACAGCAGCCAATTGTC	GGTAGAAGACCCCCTCTTAC

Prepare standard curve

8. Amplify a series of 10-fold dilutions of the known DNA using real-time quantitative PCR (Bookout et al., 2006).
9. Calculate the number of copies at every dilution of known DNA and plot against Ct values (Bookout et al., 2006).
10. Calculate the copies of the viral genome using the Ct values of virion DNA (Bookout et al., 2006).

Since each viral particle is expected to have a single copy of its genome, the number of genome copies would represent the number of viral particles.

Quantitation of EBV Virions by Quantitative PCR via Comparison to Cell Line with Known Viral Copies

ALTERNATE PROTOCOL

Alternatively, comparison of the Ct values (see Basic Protocol 9 and Bookout et al., 2006) of viral DNA and a cell line carrying known copy number of the EBV genome may also be used for the quantitation of virion. For example, Namalwa cells (request from Dr. Erle S. Robertson; *erle@mail.med.upenn.edu*) carry two copies of EBV genome (Speck et al., 2003), and thus can be used as a standard for quantitation of EBV virions. The Ct value from episomal DNA of known number of Namalwa cells, multiplied by 2, will represent the total EBV copies. Calculation based on the comparison of Ct values of virion DNA with the Ct value of the control Namalwa EBV will give an approximate number of virion particles (Fig. 14E.2.6).

LONG-TERM STORAGE OF EBV

EBV-infected cells and supernatant containing virus particles are stored frozen for long term at temperatures lower than –140°C, which is achieved either by the use of ultracold freezers or liquid nitrogen.

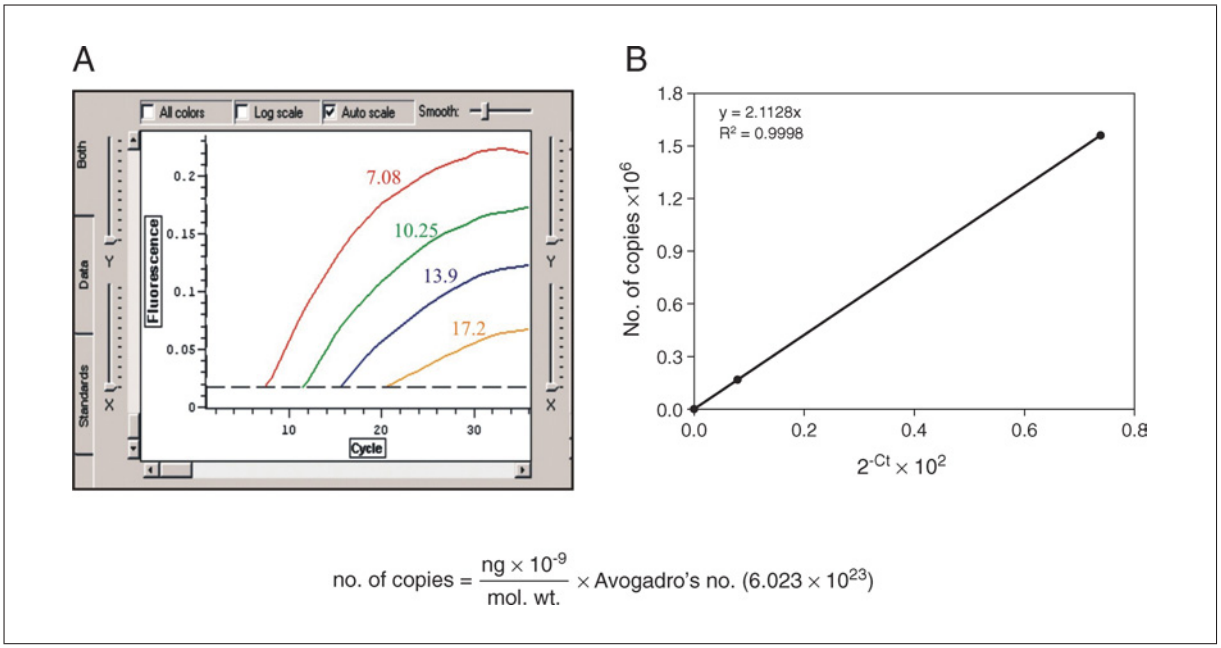

Figure 14E.2.6 (**A**) Typical graph showing Ct value of DNA template used for amplification. Ct value for all four samples is shown in their respective color. (**B**) Standard curve plotted based on the number of copies calculated based on the Ct value.

BASIC PROTOCOL 10

Freezing EBV-Infected Cells

Cells have been shown to be more viable if temperature is lowered gradually by storing at –80°C for 1 week before placing at –140°C for long-term storage.

Materials

EBV-infected or lymphoblastoid cells (see Basic Protocols 7 and 8)
Fetal bovine serum (FBS; *APPENDIX 2A*)
EBV-infected-cell freezing medium (see recipe)

Cryotubes
Liquid nitrogen or ultracold freezer at –140°C

1. Pellet 10^7 exponentially growing EBV-infected or lymphoblastoid cells and resuspend them in 1.5 ml FBS in a cryotube.
2. Add 0.5 ml of EBV-infected-cell freezing medium containing glucose and DMSO to the cryotube. Mix by inverting a few times.
3. Place cryotube with cells in –80°C for 1 week, then transfer to liquid nitrogen or ultracold freezer at –140°C.

BASIC PROTOCOL 11

Thawing EBV-Infected Cells

When the cells are needed, they can be thawed rapidly in a 37°C water bath.

Materials

Frozen EBV-infected cells in cryotubes (Basic Protocol 10)
Growth medium appropriate for cells used (see Reagents and Solutions)

Circular floating tube rack (Bioexpress, *http://www.bioexpress.com*; cat. no. R-8011-2)
15-ml conical polypropylene centrifuge tubes
Centrifuge
25-cm^2 tissue culture flasks

1. Place the cryotubes in a bubble rack. Shake the rack to help thaw the cells, usually for 1 to 2 min.
2. Transfer the sample to a 15-ml conical centrifuge tubes with 10 ml growth medium using a 1-ml disposable pipet.
3. Centrifuge the cells for 5 min at 1200 × *g*, room temperature. Aspirate the medium to just above the cell pellet, wash the pellet again with 10 ml medium, then centrifuge as before. Repeat the wash a total of three times.
4. Transfer the cell suspension to a 25-cm^2 flask and incubate in a 37°C, 5% CO_2 incubator.

   It is recommended that 1.0 × 10^7 suspension cells be incubated in at least 3 ml complete medium.

5. Increase the volume once cells clearly start to survive. Check daily for the first 5 days.

   Supernatant containing EBV virus particles can be stored without adding DMSO. Unlike the cells, supernatant containing the EBV virion does not require rapid thawing.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete DMEM medium containing 10% FBS

DMEM medium with L-glutamine (e.g., Invitrogen) supplemented with:
25 U/ml penicillin
25 μg/ml streptomycin
2 mM L-glutamine (in addition to that in the basal DMEM)
10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)

Complete RPMI medium containing 10% FBS

RPMI 1640 medium with L-glutamine (e.g., Invitrogen) supplemented with:
25 U/ml penicillin
25 μg/ml streptomycin
2 mM L-glutamine (in addition to that in the basal RPMI 1640)
10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)

EBV-A9 selection medium A

RPMI 1640 medium with L-glutamine (e.g., Invitrogen) supplemented with:
10% FBS (*APPENDIX 2A*)
25 U/ml penicillin
25 μg/ml streptomycin
2 mM L-glutamine (in addition to that in the basal RPMI 1640)
250 μg/ml hygromycin B (for cells transfected with EBV-hyg) or 800 μg/ml G418 for cells transfected with EBV-neo)

EBV-A9 selection medium B

DMEM medium with L-glutamine (e.g., Invitrogen) supplemented with:
10% FBS (*APPENDIX 2A*)
25 U/ml penicillin
25 μg/ml streptomycin
2 mM L-glutamine (in addition to that in the basal DMEM)
250 μg/ml hygromycin B (for cells transfected with EBV-hyg) or 800 μg/ml G418 for cells transfected with EBV-neo)

EBV-infected-cell freezing medium

Dissolve 12 g glucose in 35 to 50 ml serum-free RPMI 1640 medium (e.g., Invitrogen). Once glucose is dissolved, make the volume up to 60 ml with the RPMI 1640 and filter sterilize using a 0.2-μm filtration system. Add 40 ml of sterile dimethylsulfoxide (DMSO) solution. Store at 4°C.

Lysis buffer

10 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*)
1 mM EDTA, pH 8.0 (*APPENDIX 2A*)
1% (w/v) *N*-laurylsarcosine (Sarcosyl)
0.1 mg/ml proteinase K

Percoll microcell gradient mixture

46.7% (v/v) DMEM (e.g., Invitrogen)
10% (v/v) fetal bovine serum (FBS; *APPENDIX 2A*)
46.7% (v/v) Percoll (Sigma)
70 mM NaCl

continued

24 mM HEPES pH 7.2
19 μg/ml cytochalasin B
1 μg/ml Colcemid

COMMENTARY

Background Information

Epstein-Barr virus (EBV) was the first characterized human tumor virus (Epstein et al., 1964). EBV is a ubiquitous human γ-herpesvirus associated with numerous human malignancies. EBV is predominantly associated with the infection of two target tissues in vivo: B lymphocytes, where the infection is predominantly latent, and oropharyngeal epithelial tissue, in which the infection is strictly lytic, with the production of viral progeny (Epstein et al., 1964; Chan et al., 2002; Chan et al., 2004). Both target tissues are susceptible to EBV-associated malignant change, leading to tumors either of B cell origin, such as Burkitt's lymphoma (BL), Hodgkin's disease, and AIDS/transplant–associated immunoblastic lymphomas, or of epithelial cell origin, such as nasopharyngeal carcinoma (NPC; Epstein et al., 1964; Chan et al., 2002; Chan et al., 2004; Hammerschmidt and Sugden, 2004).

EBV is a large, double-stranded DNA virus that shares common structural features with other herpesviruses. Following primary lytic infection, this virus typically establishes lifelong latent infection in the host (Miller, 1989). Once EBV establishes latency in cells, it stays in the cells as a circular episome with limited latent gene expressed (Miller, 1989). To date, numerous cell lines have been generated which are stably infected with EBV. These cell lines can be induced with either chemical inducers or the EBV intermediate early gene *BZLF1* to produce viral progeny. They greatly facilitate studies on EBV both in vitro and in vivo.

Critical Parameters and Troubleshooting

A number of important variables can determine the extent to which the induction of EBV will be successful. Careful monitoring of the cell culture, the length of time in culture, and the number of passages of the cultures all play a significant role in the propagation of EBV for storage. EBV has been successfully induced using Ig cross-linking (Basic Protocol 1), expression of the intermediate early transactivator *BZFL1* from a heterologous promoter, or chemical inducers, which include the phorbol ester TPA or a combination of TPA and sodium butyrate (SB). These strategies have been consistently used over time, and the effective concentrations are usually arrived at by titration for the different cell types. The authors of this unit routinely use Invitrogen-Gibco's glutamine-containing RPMI 1640 medium with an additional 2 mM final concentration of glutamine added to the medium for culture of these cell lines. In addition, the serum used for maintenance of cultures both of primary and established lines should be routinely tested. This is critical, as it has been observed that serum lots from different companies vary in their ability to support growth of EBV-positive and -negative lymphocytes. At times, lots from the same company can have a dramatic difference in their ability to support growth of different B cell lines. Additionally, cell concentration is very critical for B cell culture since it is very easy for growth arrest to occur if the cells are diluted to too low a concentration. Only the very healthy EBV-infected B cells will yield a high titer of infectious virions. Based on the authors' observations, the ideal concentration for maintenance of long-term growth of B cells ranges from 300,000 to 800,000 cells/ml. With these suggestions, it should not be difficult to culture B cells harboring EBV.

For induction of EBV-infected cells using chemical inducers, a general working concentration is indicated in the protocols above. However, the concentration may vary between different cell lines. For example, 3 mM sodium butyrate has been found to be very toxic to EBV-positive Akata cells. At this concentration, cells die quickly and there are almost no viral progeny produced. It would be advisable for a lab obtaining a new EBV-infected cell to monitor these cells very closely. Some pretesting to titrate the optimal working concentration of chemical inducers for induction of cells might be required.

Since EBV-infected cells cannot form plaques, viral titers cannot be determined in the traditional way. However, the infection efficiency for certain cell types can be determined using an immunofluorescence assay. Once the viral stock is made, a small amount should be used for determining infection efficiency. Usually, 24 hr post infection, the virus can establish latency. At this time point, cells can be fixed and subjected to immunostaining for

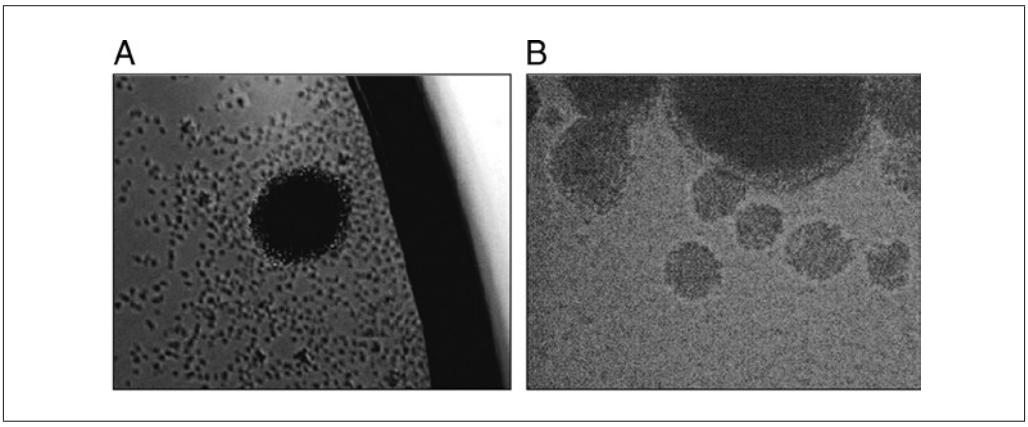

Figure 14E.2.7 Lymphoblastoid cell line, (**A**) 4 weeks post EBV infection and (**B**) 8 weeks post EBV infection.

a number of the latent antigens (EBNA1 or LMP1); thus, the percentage of EBV-infected cells can be calculated. The authors suggest that the conditions for preparing viral stock and infection should be standardized and kept consistent to achieve reproducibility of experiments.

Infection of primary B cells with EBV to produce lymphoblastoid cell lines (LCL) is a time-consuming process. Researchers need to be very patient during this process. Usually LCLs can be formed within 4 weeks post infection. However, sometimes it does take longer. When this happens, researchers will need to change the medium routinely every 7 days up to 8 to 12 weeks post infection. Typically, one cannot determine with any certainty that LCLs are produced by 2 weeks as B cells may clump during those initial weeks but lose that ability after 4 weeks. By 4 weeks, LCLs should be macroscopically visible.

Anticipated Results

The protocols provided above to induce EBV are all very straightforward. In general, virus produced by induction of 1.0×10^7 EBV-infected cells using chemical inducers or Ig cross-linking can yield more than 50% infection efficiency when used for infection of 1.0×10^7 permissive cells. Sometimes, induction of EBV by transfecting EBV-infected cells with a *BZLF1* expression construct may not be as efficient as use of chemical inducers or Ig cross-linking, depending on the transfection efficiency. However *BZLF1* transfection can mimic natural viral reactivation. Retroviral technique can greatly increase the transduction of *BZLF1*.

To produce LCLs, 4 to 8 weeks are necessary (Fig. 14E.2.7). Usually, more than 50% of wells will eventually form LCLs.

Time Considerations

As described in the protocol, induction of EBV will take 3 to 5 days. Concentration, on the other hand, will take 3 to 4 hr. Usually, a viral pellet is visible at the bottom of the tube after concentration of EBV by ultracentrifuging the supernatant. Infection of cells also requires 3 to 4 hr, as described above, and the infection efficiency varies depending on the permissiveness of the cell type and the amount of virus added to the cells.

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Lymphocytic Choriomeningitis Virus (LCMV): Propagation, Quantitation, and Storage

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ABSTRACT

Lymphocytic choriomeningitis virus (LCMV) is an enveloped, ambisense RNA virus and the prototypic virus of the arenavirus group. It can cause viral meningitis and other ailments in humans, but its natural host is the mouse. The LCMV/mouse model has been useful for examining mechanisms of viral persistence and the basic concepts of virus-induced immunity and immunopathology. This unit discusses strain differences and biosafety containment issues for LCMV. Recommendations are made for techniques for propagating LCMV to high titers to quantify it by plaque assay and PCR techniques and to preserve its infectivity by appropriate storage. *Curr. Protoc. Microbiol.* 8:15A.1.1-15A.1.11. © 2008 by John Wiley & Sons, Inc.

Keywords: BHK cell • defective-interfering (DI) virus • lymphocytic choriomeningitis virus • PCR • plaque • plaque-forming unit (pfu) • Vero cell

INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) is the prototype virus of the arenavirus group. It is an enveloped, ambisense RNA virus containing two RNA segments: L, which encodes an RNA-dependent RNA polymerase and a zinc finger binding Z protein; and S, which encodes a nucleoprotein (NP) and a glycoprotein precursor (GP0) that is cleaved into two subunits, GP1 and GP2 (Welsh, 2000). LCMV causes a persistent infection in its natural host, the mouse, but it is capable of infecting a wide range of animals, including humans. LCMV is easy to isolate from the wild, and many strains have been isolated.

Most studies in scientific laboratories have focused on derivatives of three isolates originating in the early 1930s: the Armstrong strain, isolated from a monkey undergoing a lymphocytic choriomeningitis (hence the name); the Traub strain, isolated from a laboratory colony of persistently infected mice; and the WE strain, isolated from a human after exposure to persistently infected mice. The Armstrong strain is sometimes referred to as "neurotropic," whereas the Traub and WE strains are sometimes referred to as "viscerotropic." The neurotropic designation is a confusing misnomer because each of these strains can grow well in the brain. However, high levels of viral replication in the viscera seem to either distract or clonally exhaust T cells, preventing a strong T cell–dependent meningitis and encephalitis from occurring. LCMV has been an important model for studying T cell–dependent pathology in the brain.

Many variants of these strains also exist. Notably, the clone 13 derivative of the Armstrong strain and the "docile" derivative of the WE strain seem to replicate better in mice than their respective parent strains and are more likely to cause the clonal exhaustion of T cells by high antigen load (Moskophidis et al., 1993; Zajac et al., 1998). Escape variants bearing mutations in T–cell or antibody epitopes have been generated (Lewicki et al., 1995; Ciurea et al., 2000). Recently, reverse genetic techniques for LCMV have been developed, making it possible to do sophisticated molecular studies and to generate recombinants between LCMV and other viruses (Lee and de la Torre, 2002).

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LCMV is considered an Old World arenavirus and may have originated in Africa. It is closely related to Lassa virus, which causes severe and potentially lethal infections of humans in West Africa. LCMV has disseminated throughout the world in its *Mus musculus* host.

CAUTION: Prior to undertaking any experiments outlined in this unit, the researcher should read and understand the information presented in the Safety Considerations section.

CAUTION: Established strains of LCMV are considered Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: New human or field isolates of LCMV are considered Biosafety Level 3 (BSL-3) pathogens. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

SAFETY CONSIDERATIONS

LCMV can cause persistent or acute infections in animal colonies and is a threat to rodents and primates in veterinary facilities. Therefore, it is best to keep LCMV-infected animals well separated from other animals. Most unexpected infections in animal facilities with LCMV have come when the source of LCMV was unknown, e.g., from an unknowingly contaminated cell line or animal that entered the facility. Usually an LCMV-infected animal colony can be safely maintained with appropriate containment, e.g., a biocontainment suite under negative pressure with a two-door autoclave, allowing one to autoclave the bedding material on its way out of the isolated area. It is important to have a facility with two or more exit doors so that animals can never escape.

LCMV is also a human pathogen, and in some areas ~5% of the human population is seropositive (Welsh, 2000). The agent can cause a variety of syndromes, from malaise to meningitis or encephalitis. Death from LCMV infection is exceedingly rare, and patients nearly always recover without sequellae. The recommended biosafety level for LCMV strains has been ambiguously listed by the NIH/CDC as BSL-2 or BSL-3. At one time the neurotropic strains were considered BSL-3, presumably because they were thought to be a greater hazard to humans. That, however, was an unfortunate characterization because the neurotropic strains are no more neurotropic to humans and certainly no more infectious in humans than the viscerotropic strains. They simply cause meningitis and encephalitis more easily in the mouse because they grow relatively poorly throughout the host, and the T cells are more free to attack the brain. The neurotropic Armstrong strain is now considered a BSL-2 agent, along with the viscerotropic agents.

There are no reliable quantitative data on the relative threats of the different strains to humans. Anecdotally, the authors have heard of more infections in laboratory personnel of strain WE origin than of strain Armstrong origin, although it is possible that the clone 13 variant of Armstrong may be more virulent, as it has caused laboratory infections. The NIH/CDC manual Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th edition now recommends BSL-2 for most procedures with most strains, but it recommends BSL-3 practices for new human or field isolates or for procedures that would result in high-titer aerosols.

PROPAGATION OF LCMV

The LCMV α-dystroglycan receptor is a ubiquitous protein expressed in many cell types and in different species, and the virus can consequently grow in a wide variety of cell types from many species, including mouse, hamster, monkey, and human (Cao et al., 1998). The best yields are from fibroblast or epithelial cell lines because it grows poorly in lymphocytes. Very good yields, 2–3 $\times$ 10^8 plaque forming units (pfu) per ml, can be obtained in cultures of baby hamster kidney cells, specifically BHK21, which do not shed any endogenous retrovirus (like many mouse cell lines) that may contaminate the end product. Good titers can be obtained at 48 hr by inoculating monolayers (or suspension cultures, an option with BHK21/13s cells) with a multiplicity of infection (MOI) of 0.03 to 0.1 pfu/cell or at 72 hr with an MOI of 0.003 to 0.01 pfu/cell. The advantage of a low-multiplicity infection is that it reduces the problem of viral interference and gives rise to high-titer stocks. For optimal yields these cells should be in a highly active metabolic state, and the monolayer should be ~50% to 75% confluent at the time of infection.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Baby hamster kidney (BHK) cells, lines 21 or 21/13s
BHK propagation medium
Lymphocytic choriomeningitis virus (LCMV; MOI of 0.03 to 0.1 pfu/cell for harvesting after 48 hr *or* MOI of 0.003 to 0.01 pfu/cell for harvesting after 72 hr)
75-cm^2 (T-75) or 150-cm^2 (T-150) tissue culture flasks, or tissue culture roller bottles
Plastic centrifuge tubes of appropriate size
Refrigerated centrifuge, 4°C

Procedure

1. Select an appropriate size plastic tissue culture vessel for propagation, depending on the volume to be harvested.

   These may be T75 or T150 flasks, or roller bottles for larger scale propagation.

2. Seed flasks with BHK cells in BHK propagation medium. Use 30 to 40 ml/T-75 flask, 60 ml/T-150 flask, or 150 ml/roller bottle and incubate.

   BHK cells divide quickly and can undergo up to two divisions a day at 37°C, 5% CO_2.

   The specific cell density to initiate the cultures should probably be empirically derived, due to differences in the rates of divisions of different cell preparations.

3. When vessels are ~50% confluent, decant the culture fluid and infect with virus in a limited volume (3 ml/T-75, 6 ml/T-150, 25 ml/roller bottle). Occasionally tilt flasks during the infection period and have roller bottle turning at about 1.5 rpm.

4. After 1 to 1.5 hr, add BHK propagation medium to previous levels and incubate 2 to 3 days (depending on MOI).

   LCMV does not cause substantial lysis of cells visible as cytopathic effect in these cultures, but there will likely be a reduction in cell density compared to uninfected control cultures.

5. Harvest cells after 48 hr (MOI of 0.03 to 0.1 pfu/cell) or 72 hr (MOI of 0.003 to 0.01 pfu/cell) by decanting the culture fluid into plastic centrifuge tubes and centrifuging the cells away from the virus-containing culture fluid 10 min at 350 $\times$ g, 4°C.

   Although a substantial amount of virus can be cell-associated, the intentional disruption of cells is not needed and, in fact, may impair purification efforts.

There may be many BHK cells in the culture fluid, and a second and similar centrifugation might be required to clear the culture fluid.

6. Keeping the virus cold at all times after the harvest, dispense the culture fluid into aliquots and store indefinitely at −70°C.

BASIC PROTOCOL 2

QUANTITATION OF LCMV INFECTIOUS UNITS BY PLAQUE ASSAY

LCMV has been assayed by many different techniques. Originally it was measured in a lethal dose assay in mice inoculated intracerebrally with dilutions of virus. This assay was very sensitive, as less than one pfu of virus could kill an intracerebrally inoculated mouse, but the assay is very expensive, time consuming (6 to 8 days), and causes needless suffering of mice. The most commonly used technique of the past 30 years has been a plaque assay on Vero cell monolayers.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Vero cells (African green monkey kidney cells; ATCC)
Eagle's MEM with 10% (v/v) heat-inactivated (56°C for 30 min) fetal bovine serum (FBS)
Lymphocytic choriomeningitis virus (LCMV) sample to be tested
Vero cell propagation medium (see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
2× LCMV plaque assay medium (see recipe)
1% (w/v) Seakem agarose-ME (Lonza), recently boiled (in a microwave)
1% (w/v) neutral red (aqueous solution); store up to several months at 4°C

6-well petri plates
96-well microtiter plates
Platform rocker

Prepare cells and virus

1. Seed ~5×10^5 Vero cells onto six-well petri plates in 4 ml/well Eagle's MEM with 10% FBS and incubate.
2. When the monolayers are ~80% confluent in 1 to 2 days (depending on how fast the cells are growing), decant the medium and replace with 1 ml fresh medium
3. Prepare a series of 10-fold dilutions of the test virus sample in Vero cell propagation medium

   For large scale titrations this can be done in 96-well microtiter plates by serially transferring 20 μl of inoculum into wells containing 180 μl medium, changing pipet tips with each transfer.

Infect cells

4. When dilutions are complete, add 100 μl to the Vero cell monolayers, starting with the most dilute sample and using the same pipet for increasing concentrations.
5. Incubate plates 60 to 90 min, with gentle rocking every 20 to 30 min.
6. *Optional:* For best accuracy, after this adsorption/penetration period, remove the medium and wash the monolayers with PBS.

   This will synchronize the infection and will result in more homogenous plaque sizes. However, this is an additional step that increases the possibility of contamination and may not be necessary for most experiments.

Visualize plaques

7. Prepare an agarose overlay by combining equal volumes of 2× LCMV plaque assay medium with recently boiled 1% agarose-ME solution in water that has cooled in a 42°C water bath.

   This will be about 10 min after the boiling step. The agarose solution can also just be left on the bench top and judged satisfactory to use by it not being uncomfortable to touch by one's inner wrist.

8. To each monolayer add ~4 ml agarose overlay. Allow medium to gel for 15 min.

9. Incubate the plates 4 days.

10. Stain the plates with 1.5 ml of a 1:10,000 dilution of neutral red (from a 1% aqueous solution) made up in 1:1 2× LCMV plaque assay medium/1% agarose and incubate overnight.

    This is the same medium as that used for the overlay (step 7), but with neutral red added.

    Neutral red is self-sterilizing, and a 1% solution can be made up in double-distilled water and be satisfactorily kept in the refrigerator for several months.

    If the neutral red starts crystallizing into clumps, avoid adding any clumps onto the plates because they may kill the cells.

    Plaques should be visible the next day.

Analyze results

11. To calculate a titer in plaque-forming units (pfu)/ml, select a petri well with a sufficient number of plaques to give a reliable count and not too many plaques (where they would be superimposed on each other).

    For example, choose plates with ~20 to 70 plaques.

12. Calculate the titer in pfu/ml using the following formula:

    titer (pfu/ml) = (plaque number)/(volume plated in ml × dilution factor of the virus preparation).

    For example, if 50 plaques were found at a 10^5 dilution of an inoculum of 0.1 ml, then the pfu/ml = $50/(0.1 \times 10^{-5}) = 5 \times 10^7$ pfu/ml.

QUANTITATION OF LCMV mRNA BY QUANTITATIVE POLYMERASE CHAIN REACTION (qRT-PCR)

BASIC PROTOCOL 3

The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) technique can be used to accurately measure relative levels of an RNA product. In the case of LCMV, qRT-PCR can be used to measure the amount of LCMV mRNA present in a tissue or cell sample. This can be useful for detecting residual gene expression in vivo when pfu can no longer be found or for detecting viral message from samples treated to remove infectivity. This first requires isolation of RNA from a target cell population followed by reverse transcriptase cDNA synthesis. The cDNA generated is then amplified using a quantitative PCR protocol that allows relative measurement of product generated during the reaction.

This protocol describes two methods by which cDNA may be quantified. The first requires addition of a fluorescent nucleic acid gel stain to the reaction mixture (SYBR Green I). SYBR Green I is a nucleic acid stain that binds to the minor groove of double-stranded DNA. The fluorescence emission of SYBR Green I is increased when bound to double-stranded DNA. After each cycle, a camera captures the amount of fluorescence emitted, and, as the amount of DNA product increases, so does the fluorescence emission. At

the end of the reaction, the qPCR machine brings the mixture to a temperature that is suitably below the expected T_m of the resultant product generated. The machine then increases the temperature of the reaction by half-degree intervals with a fluorescence capture after each half-degree increase. This will result in a melt curve that can then be used to confirm the T_m of the resulting product. This final step helps to ensure that a single expected product is generated during the reaction.

The other method used to quantify DNA product takes advantage of the 5′-exonuclease activity of *Taq* polymerase and fluorescent resonant energy transfer (FRET). A single-strand oligo is designed to anneal to a sequence within the product generated during the PCR reaction. This oligo has been labeled with two fluorochromes, typically a higher-energy fluorochrome designated the "reporter" at the 5′ end, and a lower-energy fluorochrome designated the "quencher" at the 3′ end. The oligo is designed to have a higher T_m than the primers, as the oligo must be 100% hybridized to the PCR product for the assay to be accurate. While the fluorochromes are within close proximity, no fluorescence is observed, as the fluorescence of the reporter is quenched by the 3′ quencher; but as *Taq* DNA polymerase degrades the oligo, the reporter and quencher are separated, and fluorescence emission is observed.

In this protocol two quantitative PCR reactions are performed, one using LCMV primers and an LCMV-specific oligo, and one using beta-actin (normally produced by these cells) and SYBR Green I. The beta-actin qRT-PCR is done to control for variations that may occur during the RNA isolation or reverse transcription steps. Both reactions are performed using the same cDNA sample. Quantification during the qRT-PCR reaction is only possible with use of a dilution series of a standard to compare the relative amount of product generated during the reaction. In other words, qRT-PCR cannot be used to directly quantify RNA levels, but is only useful as a method of comparison by way of quantification against a standard. In the following protocol, a qRT-PCR is described that measures relative LCMV RNA levels using the oligo method for LCMV, as initially reported by Roberts et al. (2004). This is then compared to a beta-actin qRT-PCR using primers initially reported by Miller et al. (2004) using SYBR Green.

Materials

Test sample (cells infected with LCMV)
SuperScript First-Strand Synthesis for RT-PCR (Invitrogen)
Double-distilled (dd) H_2O
500 mM Tris buffer
5 μg/μl bovine serum albumin (BSA)
30 mM $MgCl_2$
2.5 mM (each) dNTPs
SYBR Green I nucleic acid stain, 10,000× concentration (Molecular Probes)
10 μM LCMV GP forward primer: 5′-TGC CTG ACC AAA TGG ATG ATT-3′
10 μM LCMV GP reverse primer: 5′-CTG CTG TGT TCC CGA AAC ACT-3′
10 μM beta-actin forward primer: 5′-CGA GGC CCA GAG CAA GAG AG-3′
10 μM beta-actin reverse primer: 5′-CGG TTG GCC TTA GGG TTC AG-3′
1 μM fluorescein (Bio-Rad)
10 μM LCMV *Taq* Man MGB oligo: 6FAM-TTG CTG CAG AGC TT MGBNFQ (Applied Biosystems)
5 U/μl *Taq* DNA polymerase (Promega)

2-ml Phase Lock Gel tubes, heavy (Eppendorf)
iCycler iQ PCR plates, 96 well (Bio-Rad)
iCycler iQ Optical Tape (Bio-Rad)
iCycler iQ real-time PCR detection system (Bio-Rad)

1. Extract RNA from the test sample using 2-ml Eppendorf Phase Lock Gel tubes according to current manufacturer's protocol.
2. Convert RNA to cDNA using the Invitrogen SuperScript First-Strand Synthesis System for RT-PCR (also see *UNITS 16D.3 & 16F.1*).
3. On ice, prepare the following two PCR reaction mixes (one for LCMV and one for beta-actin):

   54 µl dd H_2O (for LCMV qPCR) *or* 42 µl dd H_2O (for beta-actin qPCR)
   12 µl of 500 mM Tris buffer
   12 µl of 5 µg/µl BSA
   12 µl of 30 mM $MgCl_2$
   12 µl of 2.5 mM dNTPs
   12 µl SYBR Green I, use at 1:1500 dilution (for beta-actin qPCR)
   6 µl of 10 µM forward primer (appropriate for LCMV qPCR *or* beta-actin qPCR)
   6 µl of 10 µM reverse primer (appropriate for LCMV qPCR *or* beta-actin qPCR)
   1.2 µl of 1 µm fluorescein (for beta-actin qPCR; background control)
   10 µl sample cDNA (from step 2)
   1.2 µl of 10 µM LCMV *Taq* Man MGB oligo (for LCMV qPCR)
   1.2 µl of 5 U/µl *Taq* DNA polymerase (added last)

   Where indicated in parentheses, use the appropriate component for each PCR mix. The other components are common to the two mixes.
4. Place two 50-µl aliquots (i.e., duplicates) of the reaction mixture into an iCycler iQ PCR plate and seal with iCycler iQ Optical Tape.
5. Carry out the PCR reactions.

   a. *To measure beta-actin cDNA:* Carry out the following program for beta-actin real-time PCR protocol in an iCycler iQ real-time PCR detection system:

Initial step:	2 min 30 sec	95°C (denaturation)
40 cycles:	30 sec	95°C (denaturation)
	25 sec	62°C (annealing)
	25 sec	72°C (extension)
Final step:	indefinite	72°C (hold).

   b. *To measure LCMV GP cDNA:* Carry out the following program for LCMV real-time PCR protocol in an iCycler iQ real-time PCR detection system:

Initial step:	2 min	50°C
40 cycles:	15 sec	95°C (denaturation)
	1 min	60°C (annealing and extension)
Final step:	indefinite	60°C (hold).

Analyses of data obtained are provided in the Commentary section (e.g., see Critical Parameters, qRT-PCR).

STORAGE OF LCMV

LCMV is a heat-labile, enveloped virus that needs to be kept cold, or it will rapidly lose infectivity. Storage should be at −70°C, preferably in the presence of some protein, such as 10% fetal bovine serum, which will enhance stability. Even with that, there will be an ∼50% loss in titer with each freeze-thaw cycle. Virus purified away from protein contaminants will be extremely unstable with a freeze-thaw, unless the purified virus is at a sufficiently high protein concentration to stabilize itself. When thawing the virus, do so quickly at 37°C and immediately put on ice.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BHK cell and virus propagation medium

500 ml Dulbecco's high glucose minimal essential medium (DMEM)
10 ml 200 mM glutamine
50 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
25 ml tryptose phosphate broth (5% final)
5 ml pen/strep solution (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate)
Filter sterilize and store up to 2 weeks at 4°C.

Vero cell propagation medium

500 ml Eagle MEM or MEM-Earles salts
5 ml of 200 mM glutamine
50 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
5 ml pen/strep solution (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate)
Filter sterilize and store up to 2 weeks at 4°C.

LCMV plaque assay medium, 2×

250 ml 2× Eagle MEM without phenol red (EMEM, Cambrex Bioscience)
5 ml of 200 mM glutamine
25 ml fetal bovine serum (FBS; 10% final), heat-inactivated (56°C for 30 min)
5 ml pen/strep solution (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate)
2.5 ml Fungizone (250 μg/ml amphotericin B)
Filter sterilize and store up to 2 weeks at 4°C.

COMMENTARY

Background Information

Quantifying LCMV

Many techniques for quantifying RNA levels using PCR approaches are currently available, and two such workable techniques are suggested in this unit. It is also possible to use probe sets that allow quantification of two cDNA products within the same tube (different fluorescent outputs, i.e., multiplexing), but this protocol has not been tested within our laboratory at the time of this writing. The primers shown in this unit for the qRT-PCR assay are designed to quantify LCMV GP1-specific mRNA (Roberts et al., 2004). The initial reverse transcriptase reaction uses an oligo(dT) primer that hybridizes to poly-A on the 3′ end of mRNA. This assay is unlikely to detect LCMV virion RNA, because the virion RNA, even though of similar positive polarity to mRNA, is not polyadenylated. An alternative assay could be designed to detect mRNA encoding other LCMV proteins, notably for NP, which is well expressed and not synthesized in synchrony with the GP RNA. If the purpose of the technique is to detect low levels of virus and viral gene expression, the analysis of the NP mRNA may be the most sensitive.

Critical Parameters

Autointerference

Of major importance in the generation of high-titer virus stocks is avoidance of the problem of autointerference (Welsh and Oldstone, 1978). LCMV very rapidly generates defective interfering (DI) viruses, especially late in its infecting cycle, where the ratio of DI to standard viruses increases. These DI viruses will greatly interfere (as much as 1000-fold) with the propagation of standard virus if they are present in the inoculum.

To prevent the accumulation of DI virus in seed stocks, these seed stocks should be initiated at a low multiplicity of infection and harvested just prior to the peak of standard virus synthesis. Serial passage of virus should always be with a diluted inoculum, because otherwise the DI virus proportion will increase. Similarly, for the propagation of high titer virus for purification, the carefully prepared seed stock should also be diluted, to ensure that cells are not initially co-infected with DI and standard virus. High-multiplicity infections (e.g., MOI = 1 to 10) rarely produce high-titer stocks, unless the seed stock is virtually devoid of DI virus, and that is a rarity.

The presence of DI viruses in stocks can often be detected in plaque assays, where, at high concentrations of virus the monolayers may look normal, without detectable cytopathic effect. Under those conditions plaques will be seen on monolayers receiving a more diluted inoculum, where there is a lower likelihood that a cell will be co-infected with a standard and a DI virus. LCMV, under optimal conditions, should grow to titers of $1–2 \times 10^8$ pfu/ml.

High titers of virus can also be obtained by freeze-thawing or sonically disrupting cells because more than half of the infectivity in a culture may be cell-associated, but this virus will be heavily contaminated with cell debris and may not be useful in some types of experiments.

Host cells

BHK cells. For optimal LCMV proliferation and plaque assays, cells must be in a suitable and healthy condition. Our subjective (though not quantitatively assessed) observation is that viral yields are poor if BHK cells are heavily confluent prior to seeding vessels with cells for the production of viral stocks, and that seeding the vessels with cells previously growing in log phase is more suitable. Also, waiting for BHK cell monolayers to be confluent before infection seems to reduce viral yield. BHK cells metabolize media very quickly, so limiting the amount of medium in order to increase the viral concentration may not be wise because it is likely to reduce the numbers of healthy BHK cells and the subsequent production of virus.

Vero cells. For plaque assays it is important to use Vero cells that have been carefully maintained in culture and have not been allowed to overgrow vessels during confluence. Vero cells sometimes alter their growth characteristics and become less useful in plaque assays after continuous passage in culture. Avoid mycoplasma contamination (see *APPENDIX 3B*).

qRT-PCR

The RNA levels of each of the tissue samples in each of the qPCR's are quantified by use of a standard. It is critical that quantification of RNA levels is performed at early cycles during the PCR reaction. At earlier cycles, all PCR reagents are in excess and the reaction occurs in an exponential manner, with a doubling of PCR product occurring after each cycle. As the reaction progresses, reagents become limiting, and different samples will generate different amounts of product at each cycle. The amount of relative LCMV RNA calculated is then divided by the amount of relative beta-actin RNA calculated, and this number will be a relative measure of how much LCMV RNA is present in each sample.

Troubleshooting

Propagation of LCMV

BHK cells do not stick to roller bottles. Slow down the RPM, especially during the initial seeding of the vessels. BHK cells slough off from monolayers into culture fluid. This is common for the BHK21/13s cell line, which can be adapted to suspension culture. Usually this does not pose any problems for generating high viral titers, but they should be cleared from the culture fluid by centrifugation before aliquoting virus.

Low viral titers. Viral titers of $<3 \times 10^7$ pfu are suboptimal; this could be caused by not harvesting the culture fluid at the peak of viral production, which may need to be empirically determined by titrating virus at different times after infection. It should be noted that viral titers sharply decline within 12 hr of the peak titer, even though the cells remain with only mild cytopathic effects, such as reduced cell growth. Low viral titers can also be caused by inoculating monolayers with too high a dose

of virus stock containing DI virus (see Critical Parameters, Autointerference). This can be overcome by generating seed stocks from very low dose inocula (MOI = 0.001 to 0.01 pfu/cell) and harvesting just before the peak in titer, because thereafter, DI virus will accumulate. Dilutions of this stock could then be used as inocula for the production of high-titer viral stocks.

Quantitation of LCMV infectious units by plaque assay

Plaques show abnormal morphology. Sometimes LCMV plaques develop concentric rings with a bull's eye effect. These are more readily apparent when incubator CO_2 levels are 5% or higher and less apparent at lower CO_2 concentrations.

Plaques are hard to read. This can happen when cell monolayers or media are suboptimal; sometimes plaques become clearer if the stained plates are left in the incubator for an extra day or two. The contrast in the plaque assay can also be enhanced by adding a 0.5 ml concentrated acetic acid on top of the gel in the plaque assay plate. In 5 to 10 min the staining will be briefly enhanced, and the plaques will need to be counted immediately because the cells will soon die thereafter.

Vero cells slough off monolayer. This can be due to contamination with mycoplasma or to a drift in the nature of Vero cells with continuous passage: use new or earlier passage Vero cells. The effect could also be due to problems with the medium or incubator. If the agarose is repeatedly boiled and used, it will become more concentrated due to evaporation of water, and the more concentrated agarose can be toxic for cells.

qRT-PCR

It is suggested that all reagents purchased for the qRT- PCR are of molecular biology grade, i.e., DNase and RNase free. All molecular biology work should be done using sterile aerosol filter tips because small amounts of a contaminating product are quickly amplified during a PCR. The $MgCl_2$ concentration within the PCR may need to be empirically adjusted. It is known that small variations within the amount of $MgCl_2$ may drastically alter the efficiency of the PCR. The parameters for PCR amplification listed (times and temperatures) may be further optimized because these may vary from machine to machine.

Anticipated Results

Time kinetic studies during the preparation of virus stocks should show a log-phase growth period followed by a peak in viral titer that may plateau for about 12 hr, after which there will be a rapid decline in viral titer, despite the fact that the BHK cell monolayer will remain intact. The peak in titer should be 5–30 $\times$ 10^7 pfu/ml, as measured in the Vero cell plaque assay. When titrating, the plaques should be readily readable on day 5 after infection, which is one day after staining with neutral red. The plaque number should be linear with dilution of inoculum, until the concentration becomes so high that plaques overlap with each other. It will not be uncommon to see little indication of plaques or cytopathic effect on plaque assay monolayers infected with undiluted or 10-fold diluted inocula because there often will be an interference phenomenon (see Critical Parameters, Autointerference).

The qPCR assay should parallel the pfu assay in assessment of viral load by quantifying mRNA for the GP gene. GP mRNA synthesis will slightly precede the increase in viral pfu because it encodes the GP necessary for the virion envelop and infectivity. GP mRNA is rapidly shut down late in the LCMV infection and is associated with an inhibition in viral GP expression on the plasma membrane and reduced release of infectious virus. Its shut down in virus-infected cells will likely be a few hours before the loss of viral titer in the culture fluid, because released virus, which is relatively labile at 37°C, will still take several hours to inactivate.

Time Considerations

Infection of cells with virus can be accomplished within 2 hr. Harvest of virus from culture fluid as well as dispensing into aliquots can be done within 1 hr, including centrifugation steps. Infection of plaque assay plates, incubation, and overlay with medium/agarose can be completed within 2 hr. RNA isolation, if done according to the manufacturer's protocol, takes about 3 hr. Conversion to cDNA takes ~2 to 3 hr, and setup for the PCR takes ~2 to 3 hr, with the actual reaction time varying depending on the setup of the machine (typically 2 to 4 hr).

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Sindbis Virus: Propagation, Quantification, and Storage

Sindbis virus is able to infect a wide variety of vertebrate and invertebrate cells. In nature, this virus demonstrates a complex life cycle, moving from the mosquito vector into the mammalian host, which in turn reinfects the mosquito vector. In cell culture, Sindbis virus produces lytic infections in mammalian cells while establishing persistent infections in cultured mosquito cells, as in the infected mosquitoes. The mechanism for the establishment of persistence in mosquito cells is not well understood, making this virus system a good model for the study of viral persistence. The tool of reverse genetics (in which an RNA genome is reverse-transcribed into cDNA) has become an invaluable tool for the study of this virus by producing many types of mutations in the viral cDNA. Careful genetic analysis of mutants produced by this or any other mutagenic method should be done using both mammalian and insect cell systems to assure that the installed mutation is not a host-range-specific mutation (Hernandez et al., 2003). In general, both host systems can be used with relative ease. The specific choice of cell line for the propagation of virus is determined by the type of study required. Mammalian cells lend themselves to metabolic labeling with radiolabeled isotopes while mosquito cells do not. Both host cell types can be used as indicator cells for virus titration by plaque formation. Insect cells have been used for the production of host-range mutants that do not grow abundantly in mammalian cells (Hernandez et al., 2003). These host range mutants were titered on both mammalian (baby hamster kidney, BHK) and insect (C7-10) cell lines to determine any change in infectivity of the mutant on one cell line or another. Mammalian and mosquito cells are genetically and biochemically distinct. These divergent hosts of Sindbis virus respond distinctly to drug treatments (Scheefers-Borchel et al., 1981). In one particular study, the block to Sindbis virus production by the drug chloroquine could only be demonstrated in mosquito cells (Hernandez et al., 2001).

Cell propagation methods will be described for BHK (see Basic Protocol 3), chicken embryo fibroblast (CEF; see Support Protocol 2), and mosquito cell cultures (see Support Protocol 4). While many different vertebrate cell types are used for the propagation of Sindbis virus, infection and transfection of baby hamster kidney (BHK) cells will be described in detail (see Basic Protocol 1 and Alternate Protocol 1). Primary cultures of chicken embryos (CEF) are also widely used for the cultivation of the virus and will also be discussed. The BHK protocols are easily adaptable to the infection of other mammalian cells. The most common insect cells in use include mosquito *Aedes albopictus* isolates originating from the virus-free cell line originally prepared by Singh (Singh, 1967; Brown et al., 1976). Cells provided by Sonya Buckley (Yale Arbovirus Research Unit) from the original Singh line have been used in the authors' laboratory for the clonal selection of a subline U4.4. Additional clonal isolates derived from the Singh cultures include the C6/36 and C7-10 cell lines. The C6/36 isolate was derived by Igarashi (1978) and provided by Kenneth Ekels (Walter Reed Army Institute). C7-10 cells were derived from LT-C-7 cells (Sarver and Stollar, 1977) and provided by Victor Stollar (Rutgers Medical School). The appropriate mosquito cell line to use for specific applications is determined by the investigator, because each of these cell lines responds differently to infection by Sindbis virus. The U4.4 and C6/36 cultures do not display cytopathic effect, while the C7-10 cultures will display significant cytopathology prior to establishment of a persistent infection. Both mammalian and insect cell cultures will produce wild-type Sindbis virus titers in the range of 10^9 to 10^{10} pfu/ml. The cell lines maintained in this

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laboratory have not been deposited with the ATCC but are available from the authors upon request (*rhernan2@unity.ncsu.edu*).

All methods employing mosquito cell propagation (see Support Protocol 4), infection (see Basic Protocol 4 and Alternate Protocol 2), and transfection (see Alternate Protocol 3) are identical for each of the different insect cell lines, except where noted. Virus freezing and storage enables the production of large quantities of virus stock, which remains viable for over 10 years when kept frozen. The stability of frozen virus facilitates the collection of samples from multiple experiments, which can subsequently be evaluated for virus titer by plaque assay. Plaque assays using mammalian (see Basic Protocol 3) or insect cell monolayers (see Basic Protocol 5) are routinely used for quantitation of infectious Sindbis virus and will be discussed in detail. Plaque purification of Sindbis virus is described in Basic Protocol 6.

General note on tissue culture media

Two culture media are repeatedly used in this unit: minimal essential medium with Earle's salts (EMEM, for BHK cells) and Mitsuhashi and Maramorosch (M & M) medium (for mosquito cells). EMEM is buffered by the equilibrium between $NaHCO_3$ and the 5% CO_2 in the humidified incubator. When flasks are employed with EMEM, the caps must be kept loose to allow for gas exchange, or vented flasks may be used. This holds for all temperatures used for the individual cultures. M & M medium is not buffered with $NaHCO_3$ and a CO_2 atmosphere is not required.

CAUTION: Sindbis virus is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

BASIC PROTOCOL 1

INFECTION OF BHK CELLS

The infection of BHK and CEF cells is essentially identical. If CEF cells are employed, follow protocols for BHK cells as described below (see Support Protocol 2 for preparation of the CEF culture). If other cell lines are to be used, infection of the cells is done in a manner identical to that described below with the exception of substituting the virus diluent appropriate for that cell culture. If the appropriate virus diluent is not known, the appropriate cell culture medium is substituted. Use the specific cell culture media and conditions suggested by the supplier for culturing the cells prior to infection.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Baby hamster kidney (BHK) cells (ATCC #CCL10), growing in culture (see Support Protocol 1)
1× complete EMEM medium (see recipe)
Sindbis virus (SVHR) stock, titered (see Basic Protocol 3)
1× PBS-D (see recipe for 10×) containing 3% FBS (heat-inactivated at 56°C; see *APPENDIX 2A*)
Glycerol, sterile
Liquid nitrogen

Platform rocker
25- or 75-cm^2 culture flasks (the authors have found that vented flasks are less susceptible to contamination with mold than the standard flasks)
15- or 50-ml conical polypropylene centrifuge tubes, sterile

NOTE: It is standard practice to grow a stock of virus from which additional virus stocks will be grown prior to any additional work with the virus. This practice avoids the production of defective interfering particles which will accumulate upon successive serial passage of high concentrations of virus. Generally, an MOI of 0.01 is required for production of this stock virus, referred to as the "stock stock," i.e., the primary source from which stock virus is grown. This "stock stock" does not need to be gradient purified. In the event that it is suspected that the virus source is contaminated by other virus strains or by mutants, plaque purification may be required. Generally, infection at an MOI of 0.01, which is the method used to replenish the "stock stock," is sufficient to suppress the production of defective interfering particles. Stock virus may be produced from any cell line of choice; stock virus grown in BHK cells yields a high titer and has the advantage that the progress of infection can be followed by evaluating the cytopathic effect.

NOTE: Sindbis virus is very sticky and will bind to glass and plastic surfaces. The authors have found that Corning polypropylene tubes (microcentrifuge tubes, self-standing, and conical) bind less virus than other brands of plastic.

1. Subculture BHK cells in 1× complete EMEM medium the day prior to infection in 75- or 25-cm^2 flasks such that the monolayer will be ~90% confluent at the time of infection.

   The subculture ratio will differ with individual cell lines, depending on the level of metabolism. The subculture dilution should produce a 90% confluent monolayer within 24 hr. The approximate number of mammalian cells should be calculated as in step 2, below.

   Determination of the degree of confluence is highly subjective and the assessment will differ from person to person. In general, the cells should cover 90% of the culture surface area to be described as being 90% confluent.

2. Calculate the amount of virus needed for the desired multiplicity of infection (MOI) according to the following formula.

   no. cells × MOI = pfu needed

   This requires knowledge of the number of cells in the culture vessel to be infected. For example, when calculating the MOI for BHK cells in a 90% confluent 75-cm^2 flask, the number of cells is ~2 × 10^7cells. A 90% confluent 25-cm^2 flask would contain ~6 × 10^6 cells. An experiment requiring the infection of a 25-cm^2 flask at an MOI of 50 would proceed as follows. The number of cells (6 × 10^6) is multiplied by the desired MOI (50), which equals 3 × 10^8 total pfu. This amount of virus should be diluted in the smallest volume that will cover the cells, so as not to dilute the virus excessively. If flasks are used, a rule of thumb is ~1 ml/75 cm^2 of surface area.

   An MOI of 50 to 100 is used for experiments that require the infection of all the cells in the culture, or for a single growth-cycle experiment.

   If suspension cells are used, follow the above guidelines as to the number of cells to be infected and the MOI to be used. Centrifuge the cells out of the culture medium at low speed (200 × g) and proceed with infection in a Corning conical tube as described below.

3. Remove the appropriate amount of virus from −80°C freezer and thaw the vial on ice. Once virus has thawed, dilute to the desired concentration in 1× PBS-D/3% FBS. Add appropriate amount of virus suspension to the monolayer.

A total volume of at least 1 ml is required for infection of 75-cm^2 monolayer, and a minimum of 200 μl is required for a 25-cm^2 monolayer.

With suspension cells, follow the volume guidelines appropriate for the number of cells, e.g., for 2 × 10^7 cells, use 1 ml diluted virus to resuspend the cells; for 6 × 10^6 cells, use 200 μl. SVHR is heat-stable; however, many other Sindbis strains and mutants are not, and thus must be thawed on ice to retain infectivity.

4. Place flask on rocking platform for 1 hr at room temperature, with cap closed tightly.

   Caps on flasks should be tightened to help retain the CO_2 dissolved in the remaining EMEM medium and maintain neutral pH. Alternatively, the PBS-D may be additionally buffered by the addition of 10 to 20 mM HEPES, pH 7.2 (see recipe for 1 M stock solution).

5. Remove inoculum and add 5 to 7 ml fresh 1 × complete EMEM medium for a 75-cm^2 monolayer or 3 ml of the same medium for a 25-cm^2 monolayer. Resume incubation with the caps loosened to allow for CO_2 exchange.

   If more concentrated virus is desired, add 3 ml medium to a 75-cm^2 monolayer.

   Suspension cells should be removed from the tube and placed in a new flask, again following the cell concentration guidelines described above.

6. Examine monolayers periodically for cytopathic effect (CPE), which generally becomes evident between 18 and 24 hr post-infection.

   Cell lines will differ in the time period in which CPE is demonstrated. Some indicators of CPE in BHK cells include the cells becoming long and thin, the cytoplasm assuming a "foamy" appearance, aggregations of cell nuclei, and, in advanced stages, cell lysis.

7. When CPE is observed, transfer medium from flasks to 15- or 50-ml conical polypropylene centrifuge tubes, pooling like samples if desired.

8. Centrifuge 10 min at 1000 × *g* to remove cell debris. Decant virus-containing supernatant into a new tube.

   A separate, dedicated centrifuge should be reserved for infected cells and viruses.

   If purification on tartrate gradient is to be performed (see Basic Protocol 2), use the supernatant from this step as the starting material for that protocol (do not add glycerol).

9. Add glycerol to a final concentration of 10% (v/v) as a cryoprotectant. Divide virus supernatant into aliquots in cryotubes and flash freeze in liquid N_2. Store virus at −80°C.

   Membrane-containing virus preparations will thaw at −65°C due to a phase transition in the ice. Thus, a freezer failure resulting in warming to −65°C should be considered a thaw. Sindbis virus SVHR will lose some titer (∼0.5 logs) upon freeze-thawing.

BASIC PROTOCOL 2

SINDBIS VIRUS PURIFICATION

Many protocols require the concentration or purification of the virus. Although clarification of the virus supernatant (see Basic Protocol 1, step 8) removes much of the larger cell debris, the virus remains associated with cell debris as well as unassembled viral proteins until further purified. The method of choice is ultracentrifugation through potassium tartrate gradients. Other gradient media may be suitable, but the authors have found that the virus retains viability in potassium tartrate longer than in other media. If less purity is required, or only a concentration step is needed, an alternative method is precipitation using polyethylene glycol (Sefton and Keegstra, 1974).

Materials

Virus supernatant (see Basic Protocol 1, step 8)
15% and 35% (w/v) potassium tartrate in PBS-D (see recipe for PBS-D), 4°C
1× PBS-D (see recipe for 10×), 4°C
Beckman UltraClear centrifuge tubes (25 × 89–mm)
Beckman ultracentrifuge with SW28 rotor and buckets (or equivalent), prechilled to 4°C
Ring stands
Small tube clamps
Hand-held low-intensity (e.g., 20-W) lamp
30- to 100-ml gradient former (e.g., Bio-Rad model 385)

1. Measure the volume of viral supernatant and determine the number of gradients that will be needed.

   Because manipulations will be done at the benchtop, the samples from this step forward should not be considered sterile for critical applications. If a sterile purified preparation is required, samples should be filter sterilized (see step 8).

   The SW28 rotor used to centrifuge the gradient will only hold six gradients; fewer are possible but more tubes will require extra runs.

2. For each gradient to be run, carefully layer 12 ml of 15% potassium tartrate onto a layer of 6 ml of 35% potassium tartrate (see Fig. 15B.1.1) in a Beckman UltraClear centrifuge tube. Place the tubes into the SW28 rotor buckets and weigh them upright (the authors use a small beaker cushioned with tissue for this purpose). Add 20 ml (typically) of virus. Add 1× PBS-D dropwise to balance the tubes.

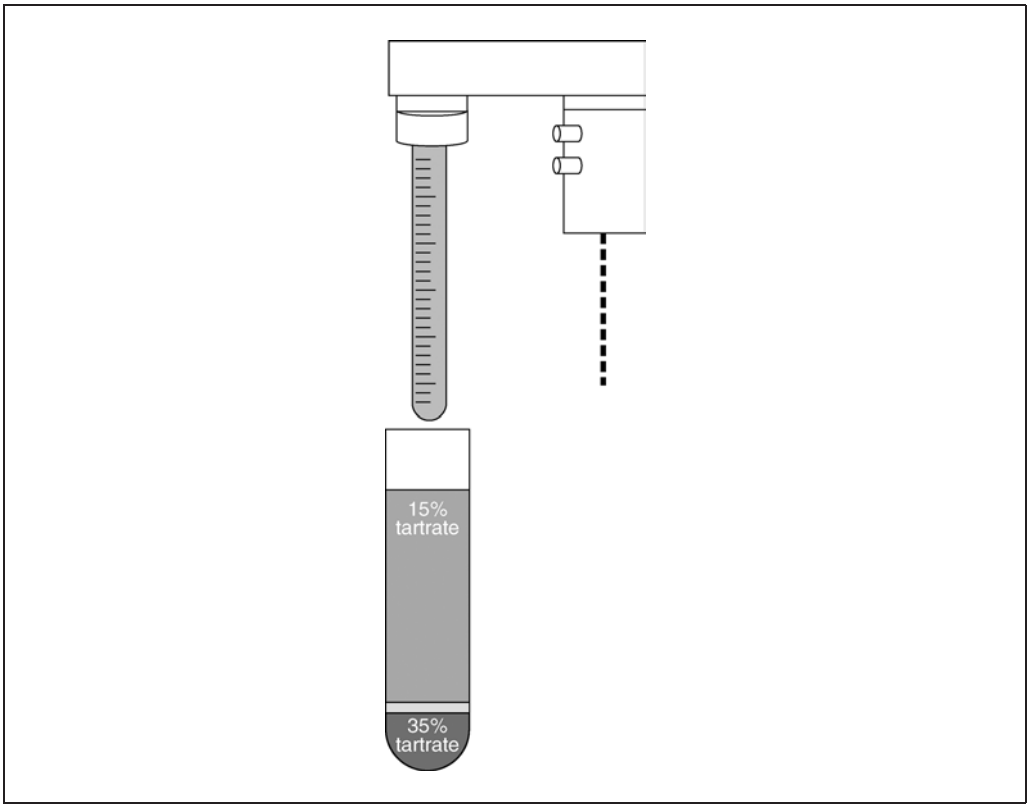

Figure 15B.1.1 Schematic of the step gradient used for virus purification. The 35% potassium tartrate step is shown at the bottom of the tube. 15% potassium tartrate is overlaid onto this "step" to form the second layer. The culture supernatant containing the virus forms the final layer on the gradient. After the centrifugation, a virus band (shown as light gray) will be visible at the interface between the 15% and 35% steps.

Take special care to weigh the buckets and tubes, and load the centrifuge rotor according to the manufacturer's tolerances and specifications as found in the manual. Failure to do so can result in severe damage to the rotor and the instrument.

A visible layer should be seen between the 35% tartrate, the 15% tartrate and the virus supernatant layers. Take care that the tube is full but not overflowing.

The rotor, buckets, and solutions should be prechilled to 4°C. If an odd number of gradients is required, prepare a "blank" gradient and overlay with buffer. Use this tube to balance the rotor.

3. Centrifuge the gradient overnight (this is purely for convenience) at 100,000 × *g* in an SW28 rotor, 4°C.

4. After the run is completed, remove a tube carefully from the bucket and attach to a ring stand using a small tube clamp. Illuminate from the side with a hand-held low-intensity lamp and locate the virus band, which will be iridescent blue and should be visible at the interface between the 15% and 35% potassium tartrate solutions. Repeat for each of the gradients.

5. Collect the band by puncturing the bottom of the tube and letting the tartrate solution flow into a waste beaker, while collecting the virus band into a 15-ml conical tube. Discard the remaining solution.

6. If highly purified samples are required, pool the virus-containing bands and dilute with 1.5 volumes of ice-cold 1× PBS-D.

The resultant solution should be less dense than 15% potassium tartrate. If concentration is correct, an aliquot of the diluted virus solution should not sink into an aliquot of 15% potassium tartrate.

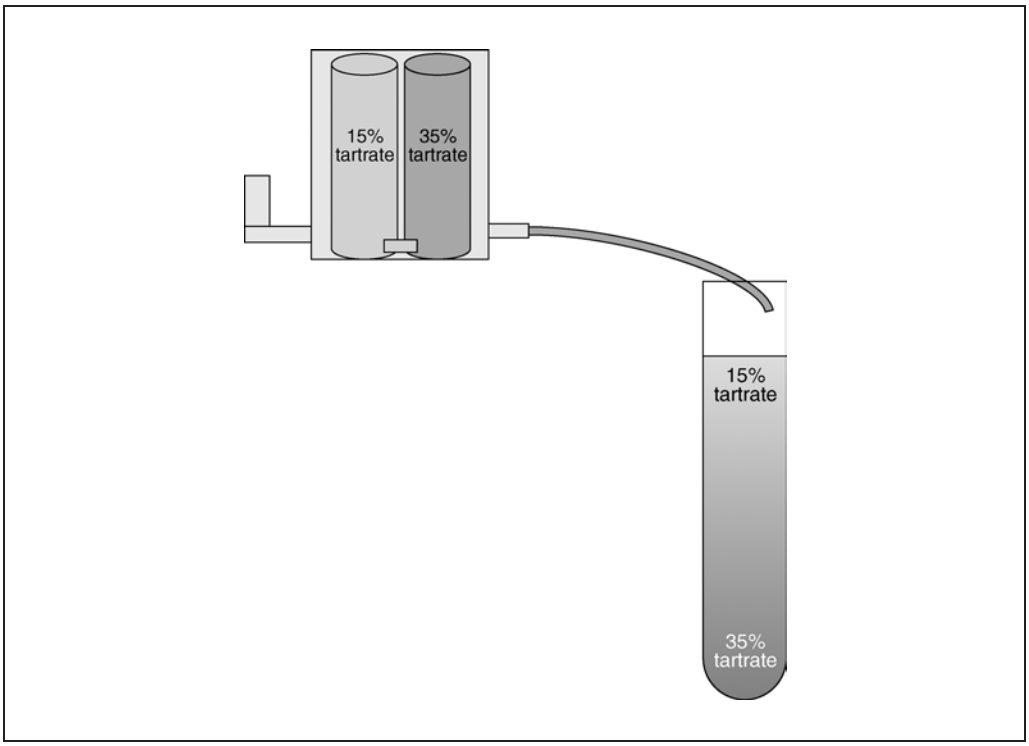

Figure 15B.1.2 Schematic of a linear gradient. A linear gradient is used as the second purification step. As with the step gradient, 35% potassium tartrate will be at the bottom of the tube and in the first chamber of the gradient former. The gradient former will mix the 35% and 15% solutions together to form a linear gradient. The diluted virus band from the first step gradient (Fig. 15B.1.1) is pipetted onto the top of the linear gradient.

7. Using a gradient former, prepare a continuous gradient from 15% to 35% potassium tartrate at a volume not less than three-quarters the virus solution to be added. Layer the diluted virus over the continuous gradient (see Fig. 15B.1.2).

   Read the manufacturers manual for instructions on use of the gradient former. As with the step gradient described above, the tube should be full without overflowing after addition of the diluted virus sample.

8. Balance tubes as described in step 2. Run the continuous gradients 2 hr at 120,000 × *g*, 4°C. Collect the virus band, which should appear about one-third of the way down the length of the tube, as described in step 5.

   If a sterile preparation is required, filter sterilize by passing through a 0.2-μm syringe filter.

BHK CELL TRANSFECTION

ALTERNATE PROTOCOL 1

BHK cells can be transfected with infectious Sindbis virus RNA, rather than infected with Sindbis virus. This procedure is crucial when expressing site-directed mutations constructed using the full-length cDNA clone. After the desired mutation is installed into the cDNA, an infectious transcript of the mutant is synthesized (see Support Protocol 3) and introduced into the cells by electroporation. Cells translate the RNA and an infection is initiated. Following verification by RT-PCR that the mutation has been retained, the virus produced from the transfections is kept as a stock from which other stocks can be made. If the mutations are found to revert easily, or if stringent conditions for analysis of mutant phenotypes are required, virus is only produced directly from transfections instead of being propagated in cells. This precaution reduces the probability that the mutant virus will revert, or that contamination with wild-type virus will occur.

Successful transfection of cells with infectious RNA requires the investigator to handle the cells and transcripts using RNase-free sterile technique. For a detailed description of the synthesis of infectious transcripts from Sindbis virus cDNA using SP6 polymerase, refer to Rice et al. (1987) and Liu et al. (1996). After the cells are trypsinized and pelleted, they are washed with RNase-free buffer to remove contaminating RNase found naturally in all cells. For a description of the preparations necessary for solutions and supplies (glassware and plasticware) to be used for RNase-free protocols, see *APPENDIX 2A* in this manual, Maniatis et al. (1982), and Gilman (2002).

The following protocol has been modified from Lilijestrom and Garoff (1991).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Baby hamster kidney (BHK) cells (ATCC #CCL-10), growing in culture (see Support Protocol 1)
1× complete EMEM medium (see recipe) without gentamicin
1× PBS-D (see recipe for 10×; use RNase-free PBS-D as indicated in protocol)
0.25% trypsin (see recipe)
Versene solution (see recipe)
RNA transcript(s) from cDNA(s) of interest (see Support Protocol 3)
75- and 25-cm^2 tissue culture flasks
Siliconized, low-retention RNase-free microcentrifuge tubes (Fisherbrand cat. no. 02-681-331)

Aerosol-barrier pipet tips
Electroporator
Electroporation cuvettes, 2-cm gap width

Additional reagents and equipment for counting cells using a hemacytometer (Strober, 1997) and harvesting of virus (see Basic Protocol 1)

Subculture and trypsinize cells

1. Subculture BHK cells in 1× complete EMEM medium without gentamicin the day prior to transfection in 75-cm^2 flasks such that the monolayer will be 90% confluent at the time of transfection.

   The cells must be in log phase when transfected, so it is important that the cells be split the day before and that they be not more than 90% confluent. Approximately two to three 75-cm^2 flasks of cells will be required for each RNA sample.

   Gentamicin kills electroporated cells (R. Hernandez, unpub. observ.).

   The authors have found that vented flasks are les susceptible to contamination with mold than the standard flasks.

2. The next day, decant medium from flasks and wash the monolayer once with 5 ml of 1× PBS-D.

3. Mix 25 ml of thawed 0.25% trypsin and 75 ml of versene solution, and prewarm to 37°C. Add this to the monolayer (2 ml for 25-cm^2 flask; 5 ml for 75-cm^2 flask). Incubate at room temperature until cells begin to detach from flask, then resuspend cells and disrupt cell clumps by pipetting up and down with a 5- to 10-ml pipet. Add an equal volume of 1× complete EMEM medium without gentamicin to stop the reaction.

   Mix the trypsin and versene solutions just prior to the trypsinization step. Be careful not to leave cells in the trypsin longer than it takes for the cells to begin to detach from the flasks. Cell trypsinized for too long do not recover well from electroporation. Pipet the cells up and down with a small-bore pipet (5 to 10 ml) to break cell clumps. The FBS in the completed medium will neutralize the trypsin.

4. Centrifuge cell suspension 5 min at 500 × *g*, room temperature, in a tabletop centrifuge, and discard supernatant. Combine all pellets into one tube, if multiple tubes have been used.

Prepare cells for transfection under RNase-free conditions

At this point it becomes important to use only RNase-free pipets, solutions, and pipet tips. Do not insert the barrel of the digital pipettor into any sterile solution or vessel; digital pipettors are a considerable source of contamination. All pipet tips must be plugged with aerosol barriers. See Gilman (2002) for preparing RNase-free solutions and equipment and for guidelines on working under RNase-free conditions. Also see *APPENDIX 2A*.

5. Add 25 ml ice cold 1× PBS-D, centrifuge as in step 4, then discard the supernatant. Repeat this wash step, then resuspend cell pellet in 1 ml ice-cold 1× PBS-D.

   Each transfection will require 400 μl cell suspension.

6. Dilute a small portion of the cell pellet 1:10 or 1:50 in 1× PBS-D and count on a hemacytometer (Strober, 1997). Keep remaining cells on ice.

7. Adjust the volume of the cell suspension so that the concentration is ~1 to 2 × 10^7 cells/ml.

8. For every RNA sample to be transfected, place a 400-μl aliquot of the cell suspension into an siliconized, low-retention RNase-free microcentrifuge tube.

9. Add RNA to the cells (in a volume of 20 μl containing not more than ~10 μg total RNA) and transfer the mixture into a 2-cm gap-length electroporation cuvette.
10. Pulse the cell/RNA mixture once at 1.5 kV, 25 μF, and infinite resistance.

    The time constant after pulsing one time should be ~0.7 sec; if the time constant is low, e.g., 0.2 sec, repeat the pulse.

11. Allow mixture to incubate in cuvette 10 min at room temperature.
12. Transfer the mixture to a 25-cm^2 flask containing 10 ml complete 1× EMEM medium without gentamicin.
13. Incubate 18 to 24 hr, or until CPE is evident (see Basic Protocol 1), before harvesting.
14. Harvest virus as described in Basic Protocol 1.

ASSAY VIRUS BY PLAQUE FORMATION ON BHK CELLS

BASIC PROTOCOL 3

The assay of virus titer by plaque formation is the most accurate method for measuring the amount of infectious virus. Although the assay requires several repeated evaluations of the same sample to establish a reliable titer, this is the long-standing method of choice for determining virus infectivity. This assay is used to determine the titer, in plaque-forming units (pfu) per milliliter virus, by infecting a standardized monolayer of cells with a known volume of a known dilution of a virus-containing solution. Virus from a single initially infected cell infects adjacent cells while the infection is localized to the original site of infection by an overlay of 1% agarose in 1× EMEM. This protocol allows for the formation of a localized clearing, or plaque, consisting of lysed cells. After staining, plaques of SVHR are visible to the naked eye within 2 to 3 days of incubation at 37°C. If there is an estimate of the titer of the virus being used based on previous experiments, only three dilution flasks are required. For instance, if the titer is estimated to be ~10^8 pfu/ml, a flask infected with a dilution of 10^{-6} would show 20 to 200 plaques; in this case, infecting flasks with dilutions of 10^{-5}, 10^{-6}, and 10^{-7} pfu/ml should give adequate data to make a relatively accurate calculation. If the titer of the virus is completely unknown, it may be necessary to infect flasks with a wide range of dilutions (10^{-1} to 10^{-8}). The number and quality of the plaques seen in a given assay can be influenced by a number of factors, including the pH and/or temperature of the medium, dilution buffer, and agarose overlay, or the condition of the cell monolayer. Due to the sensitivity, it is important to include both positive and negative controls with each assay. The negative control is a flask that is infected with diluent only, and the positive control consists of three dilutions of SVHR stock virus of known titer. The authors have found that some Sindbis virus strains are more "sticky" than the wild-type virus and do not give accurate numbers of plaques upon serial dilution. This was found to be a particularly intractable problem, for example, with some mutants of Sindbis virus, such as the transmembrane domain (TM) mutants described in Hernandez et al. (2003). Virus aggregation proved to be a problem for accuracy of the plaque assay. This problem was solved, however, by using the Sindbis virus transmembrane domain mutant (TM) plaque assay diluent, which the authors have found useful for other mutant strains of Sindbis virus. For wild-type virus assays, SVHR diluent is sufficient.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Baby hamster kidney (BHK) cells (ATCC #CCL-10), growing in culture (see Support Protocol 1)
1× and 2× complete EMEM medium (see recipe)
SVHR diluent (see recipe) *or* TM diluent for BHK cell assay (see recipe)
Virus stock to be titered (e.g., Basic Protocol 1, Basic Protocol 2, or Alternate Protocol)
2% agarose (see recipe)
2× PBS-D (see recipe for 10×)
2% neutral red stock solution (see recipe)
20 mM HEPES, pH 7.2 to 7.4 (see recipe; for less stable virus)
25-cm^2 tissue culture flasks (the authors have found that vented flasks are les susceptible to contamination with mold than the standard flasks)
Dilution tubes: 13 × 100–mm borosilicate glass test tubes
Dilution tube rack
Platform rocker
Light box

Prepare cells and virus dilutions

1. Determine the number of flasks required, with one flask per dilution of virus, plus a few control flasks. The day before the plaque assay, split BHK cells into 25-cm^2 flasks containing ~6×10^6 cell/flask, using 1× complete EMEM medium such that they will be ~80% to 90% confluent on the next day.

   If the cells are ready in the morning and the assay is to be done in the afternoon, the cells can be stored at room temperature with the caps closed tightly, or moved to a 28°C incubator to slow growth.

2. Remove the virus to be titered from the freezer and thaw on ice.

3. Fill the required number of dilution tubes with 900 µl SVHR diluent (for wild-type virus) or TM diluent (for unstable virus).

4. Prepare serial dilutions of the virus by adding 100 µl virus to the first tube containing 900 µl of diluent (10^{-1}), vortexing that dilution at maximum speed, removing 100 µl, adding it to the next dilution tube (10^{-2}), and continuing this process until the desired dilutions have been made. Change pipet tips between dilutions.

   Dilutions should be made immediately prior to use. Virus and dilutions should be kept on ice at all times.

Infect cells

5. Pour growth medium out of flasks (step 1) and infect each flask with 200 µl of the appropriate virus dilution.

   Do not let monolayers dry out while adding virus—i.e., do not pour medium off of too many flasks at one time.

6. Tighten the caps on the flasks and place them on a platform rocker at room temperature for 1 hr.

   The caps on the flask should be tightened while they are rocking, since they are not in the appropriate CO_2 environment.

Assay for plaques

7. Prepare a 1:1 mixture of 2% agarose and 2× complete EMEM as described below. Remove the inoculum from each flask and overlay the monolayer with 7 ml of this agarose/medium mixture.

Melt the agarose by autoclaving for 20 min or by microwaving for ∼1 min (for sterile agarose stock, the medium must be autoclaved; microwaved agarose is not sterile). Allow the agarose to cool but remain liquid by incubating in a 60°C water bath until needed. Once the 60°C agarose is combined with the 37°C 2× medium, the temperature of the mixture will be ∼40°C, and it will remain liquid for ∼5 min.

The agarose should be hot enough that it will not solidify too quickly, but allowed to cool enough to allow one to touch the bottle before mixing with the medium (∼60°C to 70°C). Bottles of medium at the correct temperature should not feel excessively hot to the touch. Do not try to move flasks before the overlay has solidified; the cell monolayer will tear.

Not all preparations of agarose are tolerated by cells in culture. In general, agarose formulated for gel electrophoresis or chromatography is not suitable for tissue culture. Use Sigma agarose as described in Reagents and Solutions.

8. Incubate flasks for 2 days, periodically holding the flasks up to the light to see if plaques are visible.

   The plaques will appear more opaque than the rest of the monolayer. Visible plaques are an indication that the assay has worked up to this step.

9. For the stain overlay, prepare a 1:1 mixture of 2% agarose and 2× PBS-D (or a 1:1 mixture of 2% agarose and 2× EMEM/20 mM HEPES, pH 7.2 to 7.4, for less stable virus). Add ∼2 ml of 2% neutral red stain stock solution per 100 ml for a final concentration of ∼0.06 % neutral red.

   Final concentrations will vary with different neutral red preparations.

10. Remove the medium from each flask and add 5 ml of the staining mixture prepared in step 9 to the monolayer. Cover the flasks to protect cells from light and incubate 4 hr at 37°C.

    Cells become light sensitive after they take up neutral red.

11. If necessary, return flasks to 37°C for 4 hr or 28°C overnight prior to counting plaques.

    This extended incubation is performed, e.g., if plaques appear faint (see flask 2 in Fig. 15B.1.3) or plaques are small and difficult to read. One should expect to see clear plaques surrounded by red, living cells. The number of plaques per flask should roughly follow the

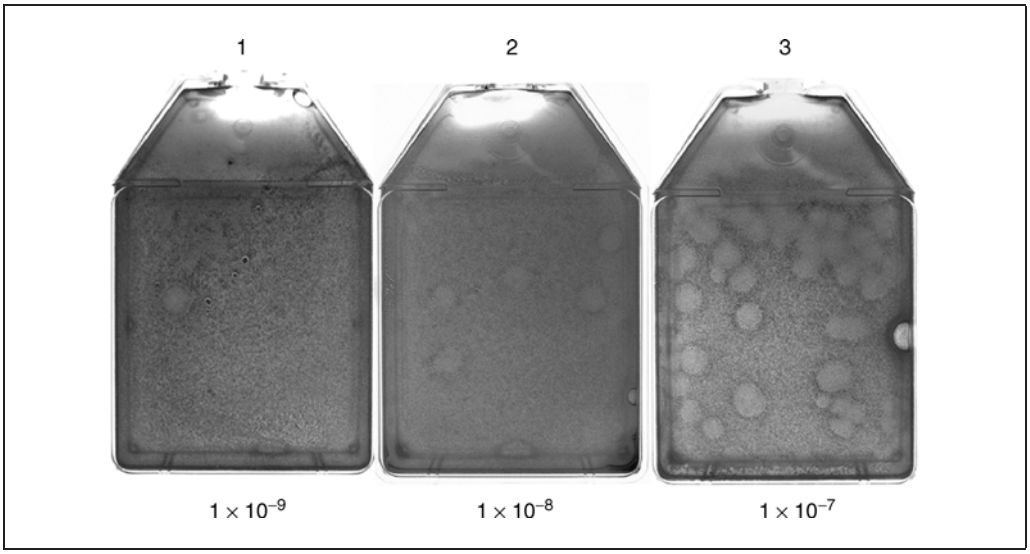

Figure 15B.1.3 Plaque assay flasks. Note the appearance of SVHR plaques at 10^{-7} (flask 3), 10^{-8} (flask 2), and 10^{-9} dilutions (flask 1). Most plaques are large; however, medium-sized plaques are also visible. Sindbis virus forms symmetrical plaques. At the lower dilutions, (10^{-7}), plaques are seen overlapping. Some monolayers, such as flask 2, may have monolayers that display faint less well defined plaques.

Table 15B.1.1 Sample Data for Plaque Assay (also see Fig. 15B.1.3)

	Flask 1	Flask 2	Flask 3
Dilution	10^{-9}	10^{-8}	10^{-7}
No. of plaques	1	6	>50

dilutions made (e.g., 1 plaque on 10^{-6} flask, 10 plaques on 10^{-5} flask, and 100 plaques on 10^{-4}).

12. Count plaques using a light box. Calculate titer using the following equation, and Table 15B.1.1 and Figure 15B.1.3 as examples, assuming that the 1×10^{-8} dilution flask has six plaques.

$$6 \text{ plaques}/(0.2 \text{ ml}/1 \times 10^{-8} \text{ dilution}) = 3.0 \times 10^{9} \text{ pfu/ml}.$$

Ideally the number of plaques in each dilution would differ by a factor of 10, but in practice this number will vary. It is best to pick a dilution with an intermediate number of plaques, e.g., 10 to 100, to calculate the virus titer. The titers are most reliable when more than one dilution closely follows the dilution trend. Unusually high- or low-titer data should be discarded.

SUPPORT PROTOCOL 1

PROPAGATION OF BHK CELLS

BHK cells are available from ATCC, and many laboratories maintain their own individual cell lines. As such, culture requirements and passage schedules for individual cultures vary greatly and protocols for BHK cell propagation should be obtained from the source from which the cells are obtained. Once the cells have become established as healthy cultures, they may be infected. It may be necessary to test several BHK cultures from different sources for virus production if maximum virus titers are desired.

The BHK cells used in the authors' laboratory are routinely passaged at a ratio of 1:3 every day. The passage number of the laboratory's BHK cell frozen stock is not known but may be passaged as required approximately an additional 20 times prior to loss of viability. Individual stocks of BHK cells may require different passage schedules and may have different levels of viable passages. It is good practice to keep track of the number of passages through which an individual lot of cells can be subcultured, so that a schedule of cell thawing and storage can be established.

This protocol ensures that the cell monolayers will be uniform and viable, that they will maintain the same passage schedule, and that they will contain approximately the same number of cells.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Baby hamster kidney (BHK) cells (ATCC #CCL-10), growing in culture
1× PBS-D (see recipe)
0.25% trypsin (see recipe)
Versene solution (see recipe)
1× complete EMEM medium
25- or 75-cm^2 tissue culture flasks
Conical tubes

1. Wash a confluent BHK cell monolayer once with 1× PBS-D, using 5 ml for a 25-cm^2 flask or 15 ml for a 75-cm^2 flask.

   The authors have found that vented flasks are les susceptible to contamination with mold than the standard flasks.

2. Mix 25 ml of thawed 0.25% trypsin and 75 ml of versene solution and prewarm to 37°C. Decant PBS-D and add the trypsin solution to the monolayer (2 ml for a 25-cm^2 flask or 5 ml for a 75-cm^2 flask). Incubate at room temperature until the cells begin to detach from the flask.

3. Resuspend cells in the trypsin solution and disrupt cell clumps by pipetting up and down using a 5- to 10-ml pipet. Pipet cell solution into a conical tube of the appropriate size.

4. Centrifuge 5 min at 500 × *g* (low speed on a tabletop centrifuge), room temperature, to form a firm pellet. Decant supernatant and replace with the appropriate amount of medium. Resuspend cells by pipetting up and down.

   The subculture ratio for these cells is 1:3. Hence, 3 ml medium/25-cm^2 flask of cells should be added to the conical tube and the cells resuspended using a pipet.

5a. *For preparation of a single 25-cm^2 flask:* Pipet a 1-ml aliquot of the cell suspension into 10 ml of 1× complete EMEM in the new 25-cm^2 flask.

   Up to three flasks can be prepared from one initial 25-cm^2 flask.

5b. *For preparation of a large number of 25-cm^2 flasks:* In a sterile bottle, pipet 10 ml of 1× complete EMEM for each flask to be prepared. Add 1 ml cell suspension to this medium per each flask to be prepared. Aliquot 10 ml cell/medium mixture to each new 25-cm^2 flasks, swirling continuously.

   One 75-cm^2 flask can be subcultured into nine 25-cm^2 flasks.

5c. *For preparation of a single 75-cm^2 flask:* Resuspend cells from a single 25-cm^2 flask in 1 ml medium and transfer to a 75-cm^2 flask containing 30 ml EMEM. Alternatively, subculture a 75-cm^2 flask into three 75-cm^2 flasks or nine 25-cm^2 flasks.

   The optimum split ratio remains 1:3.

6. Carefully place flasks in incubator with loosened caps and incubate 24 hr or until confluent.

   Care should be taken when placing the flasks in the incubator so as not to disturb the cell suspension.

7. Store cells as in Support Protocol 5.

PREPARATION OF PRIMARY CHICK EMBRYO FIBROBLAST (CEF) CULTURE

SUPPORT PROTOCOL 2

The use of CEF cultures for the cultivation of many animal viruses is still the "gold standard" for propagation of Alphaviruses and is described here in detail. The high titers of Sindbis virus obtained from BHK cells, however, have made the use of CEF cultures less frequent.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

Fertile chicken eggs, 10 days old (Spafas; *http://www.criver.com/RM/spf/egg_chickens.html*)
70% ethanol
1× phosphate-buffered saline (PBS) with calcium and magnesium (see recipe)
0.25% trypsin
Versene solution (see recipe)
1× complete EMEM (see recipe)

Strong light source (75 to 100 W)
Small sterile beaker
Sharp scissors and forceps, sterile
50-ml conical tubes
37°C water bath
75-cm^2 tissue culture flasks

Additional reagents and equipment for counting cells (Strober, 1997), subculturing BHK cells (see Support Protocol 1), and freezing cells (see Support Protocol 5)

Remove embryo from egg

1. "Candle" a 10-day-old egg by holding the egg in front of a bright light. Mark the position of the air sac with a pencil.

   Purchase the eggs so that they arrive at the 10-day-old stage. The embryo is seen as a dark mass on the yolk surface. Discard eggs without embryos or with dead embryos.

2. Wash the egg with 70% ethanol and place it pointed-end-down in a small sterile beaker.

3. Cut a large circular hole at the blunt end of the egg with a pair of sharp sterile scissors. Remove the shell above the air sac with sterile scissors or forceps, taking care not to touch the underlying membrane.

   It is helpful to carefully poke the eggshell, thereby creating a small hole in the egg from which to start cutting. Small scissors with thin blades are best for this purpose. Try to cut the shell away as a large circular piece that can easily be pulled away. Avoid creating shards of eggshell, which can fall into the egg and contaminate the embryo.

4. Using sterile forceps, remove the membrane, exposing the embryo.

5. Using fresh sterile forceps to avoid cross-contamination, gently remove the embryo slowly so that it will free itself from the yolk. Place in a sterile petri dish. Be careful not to touch the egg shell while removing the embryo.

Prepare single-cell suspension from embryo

6. Using a fresh pair of sterile scissors, dissect the head, wings, and body cavity contents from the embryo, and place in a 50-ml conical tube.

7. Remove contaminating blood/yolk by the addition and removal of 10 ml of 1× PBS with calcium and magnesium.

8. Macerate the embryo with dissecting scissors.

9. Add 50 ml 1× PBS with calcium and magnesium to the tube and invert to wash the embryo. Allow the large pieces to settle to the bottom of the tube.

10. Mix 25 ml of thawed 0.25% trypsin and 75 ml of versene solution and prewarm to 37°C. Remove buffer from the macerated embryo using a pipet and add 20 ml of this prewarmed solution. Shake tube gently at 37°C for 15 to 20 min.

11. Allow the large pieces to settle to the bottom of the tube. Using a pipet, transfer the supernatant, containing the cell suspension, to a tube containing 25 ml of 1× complete EMEM.

    The serum in the medium will inactivate the trypsin.

12. Centrifuge 10 min at 500 × *g*, room temperature, and remove the supernatant. Resuspend cell pellet in 20 ml 1× complete EMEM.

    The pellet will be loose, so care must be taken when removing the supernatant.

13. Take an aliquot of cell suspension and dilute 1:5 or 1:10 in PBS containing calcium and magnesium. Count cells using a hemacytometer (Strober, 1997).

    There should be ~10^6 to 10^7 cells/ml.

    A single embryo will yield ~1.0×10^8 viable cells, enough, conservatively, to inoculate ten 75-cm^2 flasks. The number of embryos needed must be determined based on this number.

Culture CEF

14. Prepare each 75-cm^2 flask required by adding 1×10^6 cells and 30 ml 1× complete EMEM. Begin incubation.

    Do not seed flasks with less that 10^6 cells, the monolayers will not grow.

    The authors have found that vented flasks are less susceptible to contamination with mold than the standard flasks.

15. Subculture at a ratio of 1:3 up to three passages using the same technique described for BHK cells (see Support Protocol 1).

    The cells will remain viable for more than three passages. However, bear in mind that there is variability in the cells from embryo to embryo. There are also changes in the cell characteristics with increasing passage number.

16. Freeze cells in cryotubes at ~10^6 cells/tube in 1 ml of freezing medium (see Support Protocol 5).

MOSQUITO CELL MONOLAYER INFECTION

BASIC PROTOCOL 4

Mosquito cells are used when a comparative analysis of virus titers or mutant virus phenotypes is required. To infect mosquito cells, a confluent monolayer of single cells (no clumps) must be formed. Mosquito cells do not adhere well to the substrate, and tend to lift from the flasks regardless of the medium used. To temporarily circumvent this problem, mosquito cultures can be serum-starved for 1 hr, which causes the cells to adhere to the substrate more tightly. Mosquito cultures in the authors' laboratory have also been adapted for use in EMEM; however, all other treatments are consistent with cells in M & M medium.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

Materials

Mosquito cells (e.g., U4.4 cells; laboratory isolate; contact authors at *rhernan2@unity.ncsu.edu*), growing in tissue culture in 75-cm^2 flasks
1× PBS-D (see recipe)
Serum-free M & M or EMEM medium (e.g., Invitrogen; also see recipes)
Sindbis virus (SVHR) stock, titered (see Basic Protocol 3 or 5)
Fresh 1× complete Mitsuhashi and Maramorosch (M & M) medium (see recipe) *or* 1× complete EMEM medium (see recipe)

Glycerol

Neutral-pH buffer (PBS-D or culture medium buffered to pH 7.2 to 7.4 using 10 to 20 mM HEPES)

28°C humidified 5% CO_2 incubator (CO_2 required only for use with EMEM)

75-cm^2 tissue culture flask

Additional reagents and equipment for counting cells (Storber, 1999) and infecting cell cultures with Sindbis virus and harvesting and storing the virus (see Basic Protocol 1)

Prepare cells

1. For every 75-cm^2 flask to be infected, transfer the contents of approximately one and one-half 75-cm^2 flasks to a centrifuge tube. Centrifuge 5 min at 200 to 500 × *g*, room temperature. Remove the supernatant.

   Mosquito cells grow as semisuspension cultures and are easily removed from the flask by tapping the flask against a hard surface, or alternatively by pipetting up and down with a 5- to 10-ml pipet.

2. Resuspend pellet in 10 ml 1× PBS-D, centrifuge as in step 1, and remove the supernatant.

   This wash serves to remove any remaining medium.

3. Resuspend the final cell pellet in 1 ml of serum-free M & M or EMEM medium for every flask to be infected. Count cells using a hemacytometer (Strober, 1997).

4. Pipet an aliquot of cell suspension containing ~6×10^7 cells into each 75-cm^2 flask to be infected, with enough serum-free M & M or EMEM medium to cover the monolayer (~10 ml).

5. Incubate the flask of cells at 28°C for 1 hr, or until cells are well attached to the surface of the flask.

   CO_2 is required only for use with EMEM.

Infect cells

6. Once cells are attached, remove medium and add the desired amount of virus to the monolayer (see Basic Protocol 1, steps 2 to 3). Close caps tightly.

7. Place flask on a platform rocker and rock slowly at room temperature for 1 hr.

   Slow rocking is needed to keep a minimum number of cells from lifting from the flask. Most of the cells should remain attached to the flask after rocking. If cells have lifted from the monolayer they should be removed with the inoculum.

8. Remove inoculum and add at least 3 ml fresh, 1× complete M & M or EMEM medium to each flask using the technique described in Basic Protocol 1, step 4.

   To increase the concentration of virus, minimize the volume of fresh medium added to the flasks. A minimum of 3 ml medium/75 cm^2 is required to cover the cells and support metabolism. Take care that the flasks are level to ensure complete coverage of the monolayers.

9. Incubate in a humidified 28°C, 5% CO_2 incubator.

   CO_2 is required only for use with EMEM.

   Virus may be harvested at between 24 and 72 hr post-infection (hpi) depending on the multiplicity of infection (MOI) used. Cytopathic effect (CPE) will not be evident in mosquito cells, and therefore virus will be harvested based on time post-infection rather than CPE. MOIs above 10 require 24 hr of growth. For an MOI of 1, flasks would be allowed to incubate 48 hr. To amplify virus from plaque (6000 pfu for SVHR; see Basic Protocol 6), 72 hr of incubation would be allowed.

Harvest and store virus

10. Centrifuge 10 min at 1000 × *g*, room temperature, to remove cell debris. Decant virus-containing supernatant into a new tube.

11a. *For normal storage:* Add glycerol to a final concentration of 10% (v/v) as a cryoprotectant. Divide virus supernatant into aliquots in cryotubes and flash freeze in liquid N_2. Store virus at −80°C.

Virus will lose 1/3 to 1/5 log of titer per freeze-thaw cycle. This is not a significant loss for use in infecting cells. It is possible to maintain virus stock for >10 years in glycerol without a significant loss in titer. However for applications requiring preservation of the highest virus titer store virus as in step 11b, below.

11b. *For preservation of highest infectivity level and virus particle/pfu ratio:* Store virus up to 5 days at 4°C in neutral-pH buffer.

This method preserves the highest level of infectivity. It is used for virus to be analyzed in structural studies (Phinney et al., 2000; Phinney and Brown, 2000) and for the calculation of particle-to-pfu ratios (Hernandez et al., 2003). Although titer is retained, this method is wasteful in that unused virus is discarded.

MOSQUITO CELL INFECTION IN SUSPENSION

ALTERNATE PROTOCOL 2

Mosquito cells can also be infected in suspension if required. The volumes in these protocols are arbitrary and should be kept low to keep the virus concentration high.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

Additional Materials *(also see Basic Protocol 4)*

Mosquito cells (e.g., U4.4 cells; laboratory isolate; contact authors at *rhernan2@unity.ncsu.edu*), growing in tissue culture in 75-cm^2 flasks at ∼6 × 10^7 cells/flask
Fresh 1× complete Mitsuhashi and Maramorosch (M & M) medium (see recipe) *or* 1× complete EMEM medium (see recipe)
Sindbis virus (SVHR) stock, titered (see Basic Protocol 3)
Liquid nitrogen
75- and 25-cm^2 tissue culture flasks
Humidified 28°C, 5% CO_2 incubator (CO_2 required only for use with EMEM)
Additional reagents and equipment for counting cells (Strober, 1997)

1. Two days before cells are to be infected, split a confluent monolayer of mosquito cells 1:3 into three 75-cm^2 flasks in 30 ml 1× complete M & M or EMEM medium, to be confluent at the time of infection. Incubate at 28°C in a humidified 5% CO_2 incubator.

Mosquito cells grow as semisuspension cultures and are easily removed from the flask by tapping the flask against a hard surface, or alternatively by pipetting up and down with a 5- to 10-ml pipet.

CO_2 is required only for use with EMEM.

2. Count cells (Strober, 1997) and calculate the amount of virus needed for the desired multiplicity (MOI).

A 1:3 split of a confluent monolayer should give ∼6 × 10^7 cells per 75-cm^2 flask in 2 days.

3. Pool all cells to be infected and centrifuge 5 min at 500 × *g*, room temperature. Remove supernatant.

4. Resuspend the pellet in the required volume of virus stock.

   A further dilution of the virus stock may not be necessary if the volume of the virus suspension is sufficient to resuspend the pellet.

5. Incubate 1 hr at room temperature, gently resuspending the pellet every 10 to 15 min.

6. Centrifuge cells 5 min at 500 × *g*, room temperature, and remove the supernatant.

7. Resuspend the cells in 5 to 7 ml of 1× complete M & M or EMEM medium to a density of 2×10^7 cells and plate in 25-cm^2 flasks. Incubate at 28°C for 24 to 72 hr (depending on the initial MOI) post-infection prior to harvesting.

8. Harvest virus by centrifuging 10 min at 1000 × *g*, room temperature, to remove cell debris. Decant virus-containing supernatant into a new tube.

9. Add glycerol to a final concentration of 10% (v/v) as a cryoprotectant. Divide virus supernatant into aliquots in cryotubes and flash freeze in liquid N_2. Store virus at −80°C.

ALTERNATE PROTOCOL 3

MOSQUITO CELL TRANSFECTION

As with the mosquito cell infections, mosquito cell transfections are performed to evaluate differences between the phenotypes of mutant virus in the alternate host cell. Successful transfections of these cells are slightly trickier because they contain much more endogenous RNase than BHK cells. Cultures in log phase with few dead cells will produce the best results. This analysis has been instrumental in identifying host-range mutants that grow in mosquito cells but not BHK cells (Hernandez et al., 2000, 2003). Transcripts are made as described in Support Protocol 3.

Materials

Mosquito cells (e.g., U4.4 cells; laboratory isolate; contact authors at *rhernan2@unity.ncsu.edu*), growing in tissue culture in 75-cm^2 flasks
1× PBS-D (see recipe for 10×)
HBS buffer (see recipe), RNase-free, ice-cold
RNA transcript(s) from cDNA(s) of interest (see Support Protocol 3)
1× complete Mitsuhashi and Maramorosch (M & M) medium (see recipe)
1 M MOPS buffer (see recipe; for use with mosquito cell cultures and mutant viruses requiring additional buffering)
50-ml conical polypropylene centrifuge tubes
Aerosol-barrier pipet tips, RNase free
RNase-free microcentrifuge tubes
Electroporation cuvettes 2-cm gap width
Electroporator
Additional reagents and equipment for counting cells (Strober, 1997) and harvesting virus (see Basic Protocol 1)

Prepare cells

1. For every RNA sample to be transfected, transfer the contents of two 75-cm^2 flasks of U4.4 cells (or other mosquito cell cultures) to a 50-ml conical polypropylene centrifuge tube at 40 ml per tube (do not fill beyond 40-ml mark, or cells will become contaminated). Hit side of flask sharply several times to loosen cells stuck to surface. Centrifuge 5 min at 500 × *g*, room temperature. Remove supernatants.

   5×10^7 cells/RNA sample will be required.

2. Pool all cells, centrifuge 5 min at 500 × *g*, room temperature, remove supernatant, then add 5 ml of 1× PBS-D, centrifuge again as before, and remove the supernatant.

   From this point it is important to use only RNase-free pipets, solutions, and aresol-barrier pipet tips.

3. Add 10 ml RNase-free, ice-cold HBS buffer, centrifuge 5 min at 500 × *g*, room temperature, and remove supernatant, then repeat for a second wash.

   The HEPES-containing HBS buffer is tolerated by mosquito cells for short periods of time (up to 1 hr) but is not used during long incubations.

4. Resuspend cell pellet in 0.5 ml cold HBS buffer per RNA sample to be transfected.

5. Dilute a small aliquot of the cell pellet 1:10 or 1:50 in HBS buffer and count cells in a hemacytometer (Strober, 1997). Keep remaining cells on ice.

6. Adjust the volume of the cell suspension so that the concentration is ~5 × 10^7 cells/ml.

Transfect cells

7. For each RNA sample to be transfected, prepare an RNase-free microcentrifuge tube containing 400 μl cell suspension.

8. Add RNA to the cells and transfer the mixture into a 2-cm gap-width electroporation cuvette.

9. Pulse the cell/RNA mixture once in an electroporate set at 2.0 kV, 25 μF, and infinite resistance

   The time constant after pulsing one time should be ~0.7 sec.

10. Let the mixture sit in the cuvette 10 min at room temperature.

11. Transfer the mixture to a 25-cm^2 flask containing 10 ml complete 1× complete M & M medium. For unstable mutants, add 1 M MOPS buffer to a final concentration of 10 mM in the medium.

12. Incubate flasks 48 hr in a humidfied 28°C, 5% CO_2 incubator.

    CO_2 is required only for use with EMEM.

13. Harvest virus as described in Basic Protocol 1.

    Cytopathic effect (CPE) will not be evident in mosquito cells, so virus will be harvested based on time post-transfection rather than CPE.

TRANSCRIPTION REACTION FOR THE PRODUCTION OF INFECTIOUS SINDBIS VIRUS RNA

SUPPORT PROTOCOL 3

Here, an infectious RNA transcript of cDNA containing a mutation of interest is prepared. The transcripts are then used for transfection of BHK or mosquito cells, in lieu of infecting them with the intact virus (see Alternate Protocol 1).

Materials

RNase-free H_2O (Gilman, 2002; aslso see *APPENDIX 2A*)
Linearized cDNA of interest: do not treat with RNase
10× transcription buffer (see recipe)
dNTPs: ATP, UTP, GTP, and CTP (*APPENDIX 2A*)
m^7GpppG (New England Biolabs)
RNase inhibitor (Ambion)
SP6 RNA polymerase (New England Biolabs)

Ethidium bromide staining solution (*APPENDIX 2A*)
RNase-free DNase

RNase-free tubes and pipets
40°C water bath

1. Prepare reaction mixture containing the following quantities or concentrations of reagents in a total volume of 20 μl (adjust volume if necessary with RNase-free water).

   1 μg linearized cDNA
   1× transcription buffer
   1 mM each ATP, UTP, and CTP
   0.5 mM GTP
   2 mM m^7GpppG
   10 U 2 RNAse inhibitor
   20 U SP6 polymerase

   Incubate reactions at 40°C for 1 hr.

   Also see Struhl (1999).

2. Check that the transcription reaction has worked by running 2 μl of the reaction on a 1% agarose gel for 1 hr at 80 mA. Stain with ethidium bromide (see Fig. 15B.1.4).

   CAUTION: *Ethidium bromide is a carcinogen and should be handled with gloves. See manufacturers enclosed MSDS sheet and UNIT 1A.3 for appropriate handling conditions.*

   Agarose gel electrophoresis and ethidium bromide staining are detailed in Maniatis (1982) and Voytas (1991).

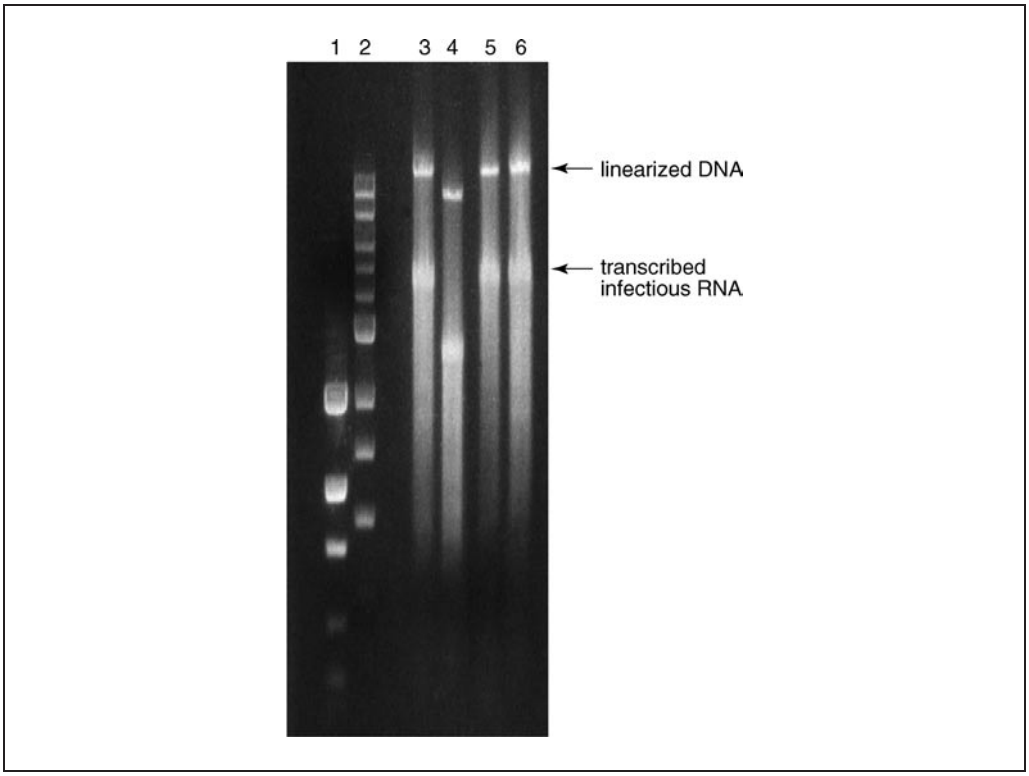

Figure 15B.1.4 1% agarose gel of transcripts from the transcription reaction. Lane 1, mass ladder; ~200 ng are present in the upper band. Lane 2, kb ladder; Sindbis virus cDNA (top bands in lanes 3 to 6) runs just slightly above the uppermost 10 kb band. Lanes 3 to 6, transcription reactions run prior to DNAse digestion. Note the nice tight bands of RNA above a smear of product. This quality and quantity ~500 μg per 20-μl reaction of RNA will produce a good transfection. The smaller transcript in lane 4 is used as a positive control for the transcription reaction. The resulting RNA is noninfectious and serves as a negative control for the cell transfection (see Alternate Protocols 1 and 3).

3. After evaluating the reaction, add 4 U of RNase-free DNase and incubate reaction 15 min at 37°C.

It is imperative to remove the DNA. Excess nucleic acid will interfere with the transfection and too much will kill the cells.

ASSAY OF VIRUS BY PLAQUE FORMATION ON C7-10 MOSQUITO CELLS

BASIC PROTOCOL 5

Plaque assay of Sindbis virus on cells from the alternate mosquito host has been found to provide valuable information. SVHR will give an equivalent virus titer when assayed on BHK cells or mosquito C7-10 cells adapted for growth in EMEM. Mutant virus, which is defective in the ability to attach to or penetrate either of these host cells, will give comparatively differential titers when assayed on both these cells. Some mutants that form small plaques may require many more days of incubation to carry out the assay. The titration is more easily accomplished on mosquito cells, which are viable up to 6 days under agarose, than on BHK cell monolayers, which begin to deteriorate after 3 days of incubation. To assay plaque formation on mosquito cells, it is necessary to use the C7-10 cell line; U4.4 cells do not display any cytopathic effect and C6/36 cells display limited cytopathic effect. C7-10 cells display cytopathology prior to becoming persistently infected and have been previously shown to produce the same number of plaques as those generated on BHK cell monolayers (Hernandez et al., 2000). The plaques from these cells will not be as clear as those formed by BHK cells, because some of the cells within the plaque will go into persistent infection and not lyse. This, however, will not affect the titer.

Materials

C7-10 mosquito cells (laboratory isolate; contact authors at *rhernan2@unity.ncsu.edu*) growing in 75-cm^2 tissue culture flasks
1× and 2× serum-free EMEM medium (see recipe)
1× and 2× serum-free MEM medium with Hanks' salts (see recipe)
TM diluent for C7-10 plaque assay (see recipe)
SVHR sample to be titered (any cell source)
2× complete EMEM (see recipe)
2× complete MEM with Hanks' salts (see recipe)
2% agarose (see recipe)
1× PBS-D (see recipe) containing 20 mM MOPS (prepare using 1 M MOPS buffer; see recipe)
2% neutral red stock solution (see recipe)
25-cm^2 tissue culture flasks
Humidified 28°C 5% CO_2 incubator (CO_2 required only for use with EMEM)
Dilution tubes: 13 × 100–mm borosilicate glass test tubes
Dilution tube rack
Platform rocker
Additional materials for counting cells (Strober, 1997)

Prepare cells

1. For every two 25-cm^2 flasks to be used in the plaque assay, transfer the contents of one 75-cm^2 flask of C7-10 mosquito cells to a 50-ml conical polypropylene centrifuge tube at 40 ml per tube (do not fill beyond 40-ml mark, or cells will become contaminated). Hit side of flask sharply several times to loosen cells stuck to surface. Centrifuge 5 min at 500 × *g*, room temperature. Remove supernatants.

2. Prepare a 1:1 mixture of 37°C serum-free 1× EMEM medium and 37°C serum-free 1× MEM medium with Hanks' salts (no supplements). Resuspend all of the pelleted cells in 1 ml of this medium for every four 75-cm^2 flasks used, and combine cells into one tube.

3. Using a small aliquot of the cell suspension, make a 1:10 or 1:100 dilution and count cells using a hemacytometer (Strober, 1997).

4. Seed 25-cm^2 flasks with ~2×10^7 cells in 5 to 10 ml of the 1:1 mixture of serum-free EMEM medium and serum-free MEM medium with Hanks' salts. Incubate flasks 1 hr at 28°C to allow cells to attach to flasks.

Prepare virus dilutions

5. Fill the required number of dilution tubes with 900 µl TM diluent for C7-10 plaque assay.

6. Prepare serial dilutions of the virus by adding 100 µl virus to the first tube containing 900 µl of diluent (10^{-1}), vortexing that dilution at maximum speed, removing 100 µl, adding it to the next dilution tube (10^{-2}), and continuing this process until the desired dilutions have been made. Change pipet tips between dilutions.

   Dilutions should be made immediately prior to use. Virus and dilutions should be kept on ice at all times. Alternatively, SVHR can be diluted in PBS-D containing 3% FBS.

Infect cells

7. Pour off medium from flasks and add 200 µl of the appropriate virus dilution.

8. Tighten the caps on the flasks and place them on a platform rocker at room temperature for 1 hr with very slow rocking to avoid disrupting the fragile cell layer.

9. Prepare a 1:1 mixture of 37°C 2× complete EMEM medium and 37°C 2× complete MEM medium with Hanks' salts, then, in turn, prepare a 1:1 mixture of this combined 2× medium and 2% agarose. Remove inoculum from flasks and overlay the cells in each of the flasks with 7 ml of this mixture.

   Melt the agarose by autoclaving for 20 min or by microwaving for ~1 min (for sterile agarose stock, the medium must be autoclaved; microwaved agarose is not sterile). Allow the agarose to cool but remain liquid by incubating in a 60°C water bath until needed. Once the 60°C agarose is combined with the 37°C 2× medium, the temperature of the mixture will be ~40°C, and it will remain liquid for ~5 min.

   To this point, the C7-10 plaque assay protocol will take ~3 hr depending on the number of virus samples to be titered. This technique takes about twice as long as the plaque assay on BHK cells.

10. Incubate flasks 3 to 6 days in a humidified 28°C, 5% CO_2 incubator.

    Incubation for 3 days is sufficient for most virus samples.

Assay plaques

11. Prepare a 1:1 mixture of 2% agarose and 2× PBS-D/20 mM MOPS. Add 2 to 3 ml of 2% neutral red stock solution per 100 ml of 1× agarose to a final concentration of 0.04% to 0.06%.

12. Remove the medium from each flask and add 5 ml of the staining mixture prepared in step 11 to the cells. Cover the flasks to protect cells from light. Return flasks to humidified 28°C, 5% CO_2 incubator overnight.

    Cells become light-sensitive after they take up neutral red

13. Count plaques.

    Plaques are easily seen using a light box. One should expect to see semi-clear to clear plaques surrounded by red, living cells. These plaques differ from those developed from BHK cells in that they will have a three-dimensional appearance due to the semisuspension nature of the cells and their propensity not to form single monolayers, but monolayer stacks, under agarose. The number of plaques per flask should roughly follow the dilutions made—e.g., 1 plaque on the 10^{-6} flask, 10 plaques on the 10^{-5} flask, and 100 plaques on the 10^{-4} flask, or 5×10^6 pfu/ml. Refer to the formula in Basic Protocol 3, step 12.

14. Calculate virus titers as described above (see Basic Protocol 3, step 12).

PROPAGATION OF MOSQUITO CELLS

SUPPORT PROTOCOL 4

Mosquito cells can be cultured in Mitsuhashi and Maramorosch (M & M) medium or in EMEM medium after adaptation. If EMEM is used, the cells should be incubated in a humidified 5% CO_2 atmosphere. M & M medium can be used in the presence or absence of a CO_2 atmosphere. Mosquito cells grow well at 28°C and will tolerate temperatures up to 34°C. Higher temperatures, however, will induce heat shock in these cultures. In the authors' laboratory, U4.4 cells are routinely maintained in M & M medium. C6/36 and C7-10 cell lines are maintained in complete 1× minimal essential medium with Earle's salts (EMEM). The authors also maintain U4.4 cells that have been adapted to be grown in 1× EMEM for some applications. All mosquito cell cultures are extremely sensitive to the serum component of the medium. A change in the serum lot being used may require the slow adaptation of the culture to the new serum. This is accomplished by initially substituting 2% to 5% of the new serum for the old serum and slowly substituting the amount of new serum over a period of several days. The medium should not be changed until the cultures are heavy and the cells begin to clump. Clumping cells are an indication that the culture should be split.

Materials

Mosquito cells growing in semisuspension
Medium: 1× complete Mitsuhashi and Maramorosch (M & M) medium (see recipe) *or* 1× complete EMEM medium (see recipe)

Tissue culture flasks
Humidified 28°C, 5% CO_2 incubator (CO_2 required only for use with EMEM)

1. Maintain mosquito cell lines in semisuspension in tissue culture flasks at 28°C in a 5% CO_2 humidified incubator. Subculture every other day as described in the following steps.

   Confluent monolayers contain ~6×10^7 cells per 75-cm^2 flask. These cultures may be subcultured every other day. Older or heavier cultures remain viable and are subcultured after counting cells by adding ~2×10^7 cells to each 75-cm^2 flask.

   Semisuspension cells adhere loosely to the substrate initially, and then begin to float as they age.

2. Hit the side sharply on any soft surface several times to loosen any cells that are stuck to the surface of the flask. If the cells are aggregated into clumps, try to break up the clumps by pipetting up and down several times using a 5- to 10-ml pipet before transferring the cells to a new flask.

3. Transfer one-third of the volume of the cell suspension to each of three flasks. To each of these flasks, add fresh medium to increase the volume to the original volume.

SUPPORT PROTOCOL 5

BHK AND MOSQUITO CELL STORAGE

Cell cultures are stored frozen under liquid nitrogen when not in use. Many cell cultures have been successfully preserved in this manner for over 20 years. Although cell viability declines with time, with care, cultures can be thawed, grown, and refrozen to ensure a continuous source of cells.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Materials

BHK cells (ATCC #CCL-10)
0.25% trypsin (see recipe)
Versene solution (see recipe)
1× complete EMEM medium
Freezing medium (see recipe) made with EMEM for BHK cells or with either M & M or EMEM for mosquito cells, depending on optimal growth medium
Mosquito cells: U4.4 and C7-10 (contact authors at *rhernan2@unity.ncsu.edu*) and C6/36 cells (ATCC #CRL-1660)
Cryotubes
Nalgene Cryo 1°C freezing container
Liquid nitrogen freezer

For BHK cells

1a. Decant medium from a culture flask that is in log phase and not more than 90% confluent.

The size of the flask or the quantity of cells will depend on the number of vials to be stored. For example, a 75-cm^2 flask of cells containing ~2 × 10^7 cells will yield three 1-ml vials.

It is crucial that the cells to be frozen not be confluent but actively growing in log phase for the cells to remain viable after the freezing procedure.

2a. Mix equal volumes of 0.25% trypsin and versene solution and prewarm to 37°C. Wash cells once with PBS-D, then add a large enough volume of the trypsin/versene solution to cover the monolayer. Allow digestion to proceed at room temperature until the cells begin to detach from the flask.

3a. Stop the trypsinization reaction by adding an equal volume of 1× complete EMEM.

4a. Centrifuge 5 min at 500 × *g*, room temperature, and decant off the trypsin solution.

5a. Resuspend cells in 1 ml of freezing medium per ~6 × 10^6 cells.

6a. Aliquot cells into cryotubes and freeze slowly to –80°C at a rate of 1°C/min using a Nalgene Cryo 1°C freezing container.

7a. After a minimum of 12 hr but not more than 24 hr, transfer the vials to a liquid nitrogen freezer.

Failure to transfer the cells at the appropriate time will kill them.

8a. To thaw cells, remove the appropriate vial from the liquid nitrogen freezer. Place in a 37°C water bath until the medium is thawed. Pipet cell solution from vial into a 25-cm^2 flask and slowly add 10 ml of prewarmed 1× complete EMEM dropwise to the cells.

CAUTION: *Gloves and goggles should be worn when removing vials from the liquid nitrogen.*

If there is a hissing sound emanating from the vial, N_2 has leaked into the vial and the cells will be contaminated. Discard a hissing vial. Likewise, discard cells that appear "foamy," as they contain dissolved N_2.

It is critical to add the warmed completed medium dropwise and slowly to allow the cells sufficient time to equilibrate from the 5% DMSO/5% glycerol medium, which is not of the same osmolarity. Cells that have been equilibrated poorly will appear blebbed and will eventually lyse. These cells should be discarded.

9a. After the cells have attached to the flask (1 to 2 hr), discard medium in the flasks, which contains dead cells, DMSO, and glycerol that could retard growth of the monolayer. Replace with 10 ml fresh 37°C, 1× complete EMEM medium and replace in the incubator.

For freezing mosquito cells

1b. Transfer the cells from a culture flask that is in log phase to a centrifuge tube. Centrifuge 5 min at 500 × *g*, room temperature. Decant off the medium.

Mosquito cells are also stored frozen under liquid nitrogen. The appropriate freezing medium is determined by the culture medium in which the cells are cultured, either EMEM or Mitsuhashi and Maramorosch (M & M).

Again as with BHK cells, mosquito cells should be in log phase.

2b. Resuspend cells in 1 ml of freezing medium per 2×10^7 cells.

Each milliliter of the resulting suspension, after thawing, will be sufficient to inoculate one 25-cm^2 flask.

3b. Divide cells into 1-ml aliquots in cryotubes and freeze as described in steps 6a and 7a.

4b. Thaw and replate cells as described in steps 8a and 9a.

Mosquito cells are much less adherent than BHK; therefore, proceed as in step 9a, but exercise additional care not to disturb the monolayer.

PURIFICATION OF VIRUS FROM PLAQUES

BASIC PROTOCOL 6

To ensure that virus stock or virus samples are free of contaminating virus, virus is eluted from individual plaques. This procedure is also used when isolating virus revertants or virus mutants. When working with mutant virus, it is important that wild-type SVHR not be present during manipulations of the sample in this plaque-purification protocol. It is very easy to cross-contaminate medium, agarose, and dilutions with small amounts of wild-type virus.

Materials

Virus stock to be purified (see above protocols)
Virus diluent: 1× PBS-D (for wild-type SVHR; see recipe) *or* TM diluent for BHK or C7-10 plaque assay as appropriate (for mutants; see recipes)
Soldering iron
Sterile Pasteur pipets and tubes
Additional reagents and equipment for plaque assay (see Basic Protocol 3 for BHK cells or Basic Protocol 5 for mosquito cells)

NOTE: All solutions and equipment that comes into contact with living cells must be sterile and aseptic technique must be used.

1. Prepare a plaque assay as in Basic Protocol 3 (for BHK cells) or Basic Protocol 5 (for mosquito cells), infecting with dilutions that will give 10 to 20 plaques in a single flask.

   Multiple flasks will ensure that sufficient plaques are produced.

2. Choose plaques that are well formed and sufficiently separated from one another to ensure that only a single plaque is isolated. Circle the plaques to be "picked."
3. If using flasks, burn through the top of the flask with the hot soldering iron just above the plaque to be "picked."
4. Wet a sterile Pasteur pipet by pipetting up and down in the virus diluent solution.

   This will reduce the tendency of the agarose to stick to the pipet.

5. Aspirate the chosen plaque with the Pasteur pipet, taking care to include all the agarose above the plaque.
6. Pipet the agarose into 1 ml PBS-D or TM diluent (for unstable mutants) in a sterile tube.
7. Elute overnight at 4°C for (for unstable mutants) or for 2 to 3 days at 4°C (for wild-type SVHR).
8. Remove all pieces of agarose prior to using or storing the plaque-purified virus.
9. *Optional:* To ensure purity, repeat the above steps with the plaque-purified virus.

REAGENTS AND SOLUTIONS

Use tissue-culture-grade water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose, 2%

Dilute agarose type I (Sigma, cat. no. A-6013) to 2% with tissue culture-grade water. Autoclave in 50- to 100-ml aliquots to sterilize.
Before use, microwave ~1 min to liquefy. Keep molten at 60°C until ready to dilute with 2× complete EMEM (see recipe).
Store at room temperature up to 1 year.

Complete EMEM, 1× and 2×

For 1× medium, prepare 1× minimal essential medium with Earle's salts (EMEM; see recipe) with the following additives:
10% heat-inactivated fetal bovine serum (FBS; Hyclone; also see *APPENDIX 2A*)
5% tryptose phosphate broth (TPB; see recipe)
0.02% L-glutamine (see recipe for 100×)
1× gentamicin sulfate (see recipe for 100×)
Store up to 2 weeks at 4°C
For 2× medium, prepare 536 ml medium by mixing the following:
400 ml 2× EMEM (incomplete, serum-free; see recipe)
80 ml FBS
40 ml TPB (see recipe)
8 ml 100× L-glutamine (see recipe)
8 ml 100× gentamicin sulfate (see recipe)
Store up to 6 months.

continued

IMPORTANT NOTE: *In this unit, complete media with supplements are not true percent solutions. The percentage indicated represents the percentage of the original volume of medium. This also holds for any other supplements added to the medium. For example, if the initial volume of the medium is 400 ml, 10% FBS is 40 ml. Addition of other supplements, such as 5% TPB would be 20 ml TPB, bringing the total volume to 460 ml.*

If problems arise with the cell cultures, growth of the virus, or plaque formation, discard old solutions and make fresh solutions.

Gentamicin is added to medium for BHK cells only. C6/36 and C7-10 cells do not require gentamicin for general maintenance.

Complete MEM with Hanks' salts, 2×

Prepare 536 ml medium by mixing the following:
400 ml 2× MEM with Hanks' salts (incomplete, serum-free; see recipe)
80 ml FBS
40 ml TPB (see recipe)
8 ml 100× L-glutamine (see recipe)
8 ml 100× gentamicin sulfate (see recipe)

EDTA, 0.1 M

Combine 3.36 g disodium EDTA and 100 ml tissue-culture-grade H_2O (pH will drop below 5.3 and the solution will remain cloudy until it is adjusted to pH ~7.0). Adjust pH to 8.0 with NaOH. Autoclave 20 min to sterilize. Store up to 1 year at room temperature.

EMEM, incomplete, serum-free, 1× and 2×

Minimal essential medium with Earle's salts (EMEM) is purchased in powder form from Invitrogen, with L-glutamine and without sodium bicarbonate. Prepare according to the manufacturers instructions and filter sterilize. This medium is prepared as a 2× solution and diluted to 1× with sterile tissue-culture-grade water to prepare the 1× complete medium. Incomplete medium can be stored at 4°C for ~6 months. Bottle caps should be sealed with Parafilm to maintain the proper pH in the medium and avoid precipitation of the salts.

Freezing medium, M & M amd EMEM

1× complete M & M medium (see recipe) or 1× complete EMEM medium (see recipe)
5% (v/v) glycerol (autoclaved 20 min in 50-to 100-ml aliquots; store up to 1 year at room temperature)
5% (v/v) DMSO
Filter sterilize
Store up to 1 year at −20°C

Be sure to add the DMSO to the medium to a concentration of 5% prior to filter sterilization. 100% DMSO cannot be autoclaved or filter sterilized (it will dissolve the filter membrane).

Gentamicin sulfate, 100×

Dilute 0.5 g gentamicin sulfate up to 100 ml in tissue-culture-grade water. Store up to 6 months at 4°C.

Dilution to 1× will give a final concentration of 50 μg/ml. If necessary, 100 μg/ml may be used.

CAUTION: *Antibiotics can become contaminated. If persistent problems are experienced with contaminated cultures, test all solutions in the absence of antibiotics.*

L-Glutamine, 100×

Weigh out 2.92 g L-glutamine and dissolve in a total volume of 100 ml 1× PBS-D (see recipe for 10×). Filter sterilize and divide into 50-ml aliquots in 100-ml bottles. Store frozen up to 6 months; once thawed, discard after 2 weeks.

Glutamine is essential and very labile. It is thus added to already complete media as a precaution.

HBS buffer

4.76 g HEPES
8.0 g NaCl
0.4 g KCl
0.1 g Na_2HPO_4
1.1 g D-glucose
Tissue-culture-grade H_2O to 1 liter
Store up to 1 year at room temperature

HEPES (pH 7.2 to 7.4), 1 M

Weigh out 238.3 g HEPES and dissolve in a total volume of 1 liter tissue-culture grade water. Adjust pH to 7.2 to 7.4 with NaOH. Autoclave 30 min to sterilize. Store up to 1 year at room temperature.

MEM with Hanks' salts, serum-free, 2×

MEM supplemented with Hanks' salts, with glutamine and without sodium bicarbonate is purchased in powder form from Invitrogen. Prepare this medium as described in the manufacturer's instructions, using half the recommended amount of water for the 2× solution of MEM. Filter sterilize. Store up to ∼6 months at 4°C with containers sealed using Parafilm.

Mitsuhashi and Maramorosch (M & M) medium, 1× serum-free and complete

Incomplete, serum-free medium:
0.2 g $CaCl_2 \cdot 2H_2O$
0.2 g KCl
0.1 g $MgCl_2 \cdot 6H_2O$
7.0 g NaCl
0.12 g $NaHCO_3$ (dissolve separately and then add to mixture)
0.2 g $NaH_2PO_4 \cdot H_2O$
5.0 g Yeastolate
4.0 g D-glucose
6.5 g lactalbumin hydrolysate
Tissue-culture-grade water up to 1 liter
Filter sterilize
Store incomplete medium up to ∼1 year at 4°C
Complete medium: Add 20% (v/v) heat inactivated fetal bovine serum (see note below on calculating percentages) and an additional 0.15% $NaHCO_3$. Store complete medium ∼3 to 4 weeks at 4°C.

IMPORTANT NOTE: *In this unit, complete media with supplements are not true percent solutions. The percentage indicated represents the percentage of the original volume of medium. This also holds for any other supplements added to the medium. For example, if the initial volume of the medium is 400 ml, 10% FBS corresponds to 40 ml. Other supplements, such as 5% TPB, corresponding to 20 ml TPB, bring the total volume to 460 ml.*

continued

The bicarbonate added to the incomplete, serum-free medium is used as a carbon source, while the bicarbonate added to the complete medium is used to adjust the pH. M & M medium is not buffered by bicarbonate and will not be affected by a CO_2 environment, nor is a CO_2 incubator required. For additional buffering (e.g., if needed for mutant viruses), add 20 mM MOPS, pH 7.2 to 7.4.

This medium has a high propensity for contamination with mold. Care should be taken to keep the incubators clean. If problems arise with the cell cultures, growth of the virus, or plaque formation, discard old solutions and make fresh ones.

MOPS buffer (pH 7.2), 1 M

Weigh out 231.2 g MOPS and dissolve in a total volume of 1 liter tissue-culture-grade water. Store at room temperature; discard solution when it turns yellow.

MOPS buffer is used during long incubations with mosquito cells when additional buffering capacity is required.

Neutral red stock solution, 2%

Dissolve 2 g neutral red and adjust volume to 100 ml with tissue-culture-grade water. Filter sterilize. Store up to 1 year at room temperature.

Some stain will be lost in the filtration process and each batch may differ. This stain is quite viscous and may require more than one filter to sterilize the entire quantity.

Alternatively, neutral red stock solution may be purchased ready made from many suppliers.

PBS-D, 10×

2.0 g KCl
2.0 g KH_2PO_4
80.0 g NaCl
11.3 g Na_2HPO_4 or 21.6 g $Na_2HPO_4 \cdot 7H_2O$
Tissue-culture-grade H_2O to 1 liter
Autoclave 20 min
Dilute to 1× with sterile tissue-culture-grade water
Store up to 1 year at room temperature

Also see annotations to the recipe for PBS with calcium and magnesium; check that the pH of the 1× solution is ~7.4.

PBS with calcium and magnesium, 1×

Prepare solutions 1 and 2 (which are 10× stock solutions) and autoclave separately for 20 min.
Solution 1:
1.0 g $CaCl_2$
1.3 g $CaCl_2 \cdot 2H_2O$
2.0 g KH_2PO_4 (dissolve separately, then add to mixture)
1.0 g $MgCl_2 \cdot 6H_2O$
80.0 g NaCl
Adjust volume to 1 liter with tissue-culture-grade H_2O

Solution 2:
21.6 g $Na_2HPO_4 \cdot 7H_2O$ or 11.3 g anhydrous Na_2HPO_4
Adjust volume to 1 liter with tissue-culture-grade H_2O

For 1× solution: Add 50 ml solution 1 to 400 ml tissue-culture-grade water. Mix, then add 50 ml solution 2. Do not directly mix solutions 1 and 2 together: salts will precipitate. Store at room temperature up to 1 year. Check that the final pH of the 1× solution is ~7.2 to 7.4.

continued

IMPORTANT NOTE: *Do not adjust the pH of the 10× solutions 1 and 2, because this will result in the wrong pH for the 1× solution. It is critical that all solutions that Sindbis virus comes in contact with are neutral pH. Acidic pH quickly inactivates the virus.*

Phenol red, 0.5%

Dissolve 1 g phenol red in 200 ml tissue-culture-grade water. Filter sterilize and store up to 1 year at room temperature.

SVHR diluent

Supplement 1× PBS-D (see recipe for 10×) to 3% with heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*). Store up to 2 months at 4°C.

TM diluent for BHK cell assay

1× PBS-D (see recipe for 10×)
10% heat-inactivated fetal bovine serum (*APPENDIX 2A*)
10% (v/v) glycerol
10 mM HEPES, pH 7.4
0.3% phenol red (add from 0.5% stock; see recipe)
Store up to 2 months at 4°C

TM diluent for C7-10 plaque assay

1× PBS-D (see recipe for 10×)
10% heat-inactivated fetal bovine serum (*APPENDIX 2A*)
10% (v/v) glycerol
10 mM MOPS, pH 7.4
0.3% phenol red (add from 0.5% stock; see recipe)
Store up to 2 months at 4°C

Transcription buffer, 10×

400 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
60 mM $MgCl_2$
20 mM DTT
2 mm spermidine

Trypsin stock, 0.25%

0.5 g trypsin
0.2 g disodium EDTA
0.6 g phenol red
Adjust volume to 200 ml with 1× PBS-D (see recipe for 10×)
Adjust pH with 1 N NaOH until a cherry red color is achieved
Divide into 25-ml aliquots
Store frozen up to 6 months at –20°C

Prior to use, thaw in 37°C water bath and dilute to 100 ml with versene solution (see recipe). Do not heat trypsin at 37°C for >20 min, as autodigestion of the enzyme will occur.

Tryptose phosphate broth (TPB)

Weigh out 29.5 g tryptose phosphate broth (TPB; Difco) and dissolve in a total volue of 1 liter tissue culture grade water. Autoclave 20 min in two separate 500-ml volumes to sterilize. Cool and store up to 6 months at 4°C.

Versene solution

500 ml 1× PBS-D (see recipe for 10×)
5 ml 0.1 M EDTA (see recipe)
1.5 ml 0.5% phenol red (see recipe)
Adjust pH to 7.4 with NaOH
Filter sterilize (if sterile stock solutions were not used)
Store up to 6 months at room temperature

COMMENTARY

Background Information

Alphaviruses are arthropod-borne viruses that are transmitted to mammals when the infected insect takes a blood meal. The Alphaviruses are found throughout the world and cause many illnesses of clinical significance. While Sindbis virus primarily infects birds and mammals, humans generally represent a dead-end host, responding to infection with limited viremia. Among the members of the Alphavirus genus, Chickungunya and O'Nyong-nyong viruses (Africa) produce rash, arthralgia, and febrile illness in humans. In the Western hemisphere, the Eastern (EEE), Western (WEE), and Venezuelan encephalitis viruses (VEE) are causes of encephalitis in humans, which can be fatal. Encephalitis, or inflammation of the brain, occurs when the viral infection crosses the blood-brain barrier and infects the central nervous system (CNS). VEE is a significant veterinary and public health problem in Central and South America (Strauss and Strauss, 2002).

Sindbis virus, the prototype of the Alphaviruses, is an easily propagated virus which has displayed the ability to infect all cell types tested, whether of vertebrate or invertebrate origin. Most of the difficulties encountered with the propagation of Sindbis result from problems related to the cell cultures chosen for its propagation or titration. The virus plaque assay is the "gold standard" for the determination of virus infectivity. Not all viruses produce a cytopathic effect, making it possible to perform a plaque assay for infectivity. However, Sindbis virus is particularly amenable to this method and produces large to pinpoint plaques, depending on the virus strain or mutant. Although this assay requires several days before the flasks can be evaluated, the titers are reproducible. If care is not taken, however, the error for the plaque assay can be quite large, up to 0.5 logs. Repeating the assay three to four times will minimize the amount of error, and the accuracy can be improved to within a 2-fold error. Specifically, infectivity measured in pfu/ml is used to establish the relative infectivity of wild-type and mutant virus as a ratio of particle/pfu. This measurement is required in specific experiments evaluating infectivity of mutant viruses (TM viruses; Hernandez et al., 2003). It is particularly important when working with these or other mutants to monitor the titers of the different viruses, because revertants can arise or contamination of the sample with wild-type virus can occur. Infectious virus titers are part of the virus phenotype and should be reproducible. An increase in the virus titer of a mutant that normally generates low titers is an indication that a reversion has occurred or that the sample has become contaminated with wild type virus.

While different methods of transfection with infectious RNA have been described, the ease with which both mammalian and mosquito cells become transfected by electroporation has made this the method of choice for the introduction of infectious RNA. When growing mutant viruses, care must be taken to buffer the medium. BHK cells tolerate HEPES buffer well, while the mosquito cells will tolerate only MOPS buffer for an extended period of time. While HEPES and MOPS buffers are both "Good's buffers" and both have pH ranges in the neutral pH range (see *APPENDIX 2A*), mosquito cells die in the presence of other buffering systems tested.

Wild-type SVHR freezes well in the presence of 10% glycerol when flash frozen with liquid N_2. In the authors' laboratory there are stocks of SVHR and Sindbis ts mutants which are over 30 years old and are still used to grow virus stocks. Mutant viruses that are particularly unstable (e.g., TM virus) may not withstand a freeze-thaw and should be titered for infectivity prior to freezing. TM mutants, may need to be titered before freezing and again after thawing to determine the titer of frozen virus.

Critical Parameters

Cell cultures

Of highest importance when growing Sindbis is the health of the cell cultures used. If the BHK or mosquito cultures are growing well and in log phase, virus should grow within 12 to 24 hr, depending on the MOI and the cell line used. It is also of paramount importance that good sterile technique be followed to avoid contamination of the cultures with microorganisms or cross-contamination between mutant and wild-type virus.

For best results, virus should be thawed on ice and all solutions used for dilution of the virus and the plaque assays should be kept cold and on ice. Care should be taken to avoid fluctuations in the pH of the flasks during infection by closing the caps tightly and monitoring the pH of the medium. Sindbis virus and many of the mutants can be stored at −80°C for many years. It is a good practice, however, to also

Table 15B.1.2 Common Problems Associated with the Sindbis Virus Plaque Assay, Their Possible Causes, and Solutions

Problem	Possible cause	Solution
No plaques visible	Cells may be unhealthy	Be sure that cultures are in log phase when infected
	Overlay too hot	Check monolayers under the microscope; cells should look healthy and dotted with stain
	Infection failed	Repeat infection or transfection
	Plaques may be too small to pinpoint, especially with mutant virus	Check plaque and surrounding monolayer under microscope; a definite clearing should be visible surrounded by stained cells
	2× medium is too dilute and therefore hypertonic; cells died during incubation	Care should be taken that the medium does not contain too much water, which will lyse the cells
	Wrong dilutions were plated	Too much virus will not produce plaques. The entire monolayer will be lysed.
		Too little virus or no virus in the chosen dilutions will not generate plaques

maintain a stock of virus cDNA when available, to transfect cells if necessary.

Serum

When purchasing new lots of serum, the authors routinely test a new lot, selected as that which most closely matches the serum specifications and chemical analysis of the serum in current use (matched lot), for the ability to maintain growth of U4.4 and BHK cells. This is done by subculturing cells into medium containing the test lot. Plaque formation of SVHR stock on BHK cells is also tested. The titer of the SVHR stock virus tested on BHK cells grown in the new serum should match the previous titer. The serum supplier should have the lot analysis for any lot of serum. This test should be performed prior to purchase of large lots. The serum should be purchased heat-inactivated, or heat inactivated after receipt by heating the thawed serum to 56°C for 30 min. The inactivation of complement is important for the propagation of Alphaviruses, because all cattle contain antibodies directed against mosquitoes and many Alphaviruses. All mosquito cell lines are handled in the same manner. Each of the mosquito cultures, U4.4, C6/36, and C7-10 which originally were grown in Mitsuhashi and Maramorosch media (M & M), have also been adapted in the authors' laboratory to grow in the same EMEM medium described for BHK cells.

Troubleshooting

Failure of the plaque assay may be due to many reasons. The most frequently encountered problems are listed in Table 15B.1.2.

Anticipated Results

It may require a few trials to master the plaque assay. However, the protocols described above should generate plaques, especially if SVHR is being employed. The sensitivity of the plaque assay for SVHR is not due to the procedure itself, but rather to the low particle/pfu ratio of the virus, ~5 particles/pfu. Reproducible titers therefore result in good accuracy. The elution of a single plaque, in general, does not generate a large yield of virus, ~1000 pfu/plaque; however, this is sufficient for a second round of plaque purification or for amplification of the plaque by infection of the chosen cell line.

Once proper conditions are established, mosquito cell culture is relatively simple and can produce ~8-fold more cells/ml than a BHK culture of comparable size. An additional advantage to working with this cell line is the long viability of these cells. Mosquito U4.4 cells can be kept at 28°C for several months, and viable cells can be rescued for subculture. Differences in the levels of virus produced from each of the mosquito cell cultures have been observed, with C7-10 producing the

most virus (3×10^9 pfu/ml) followed by C6/36 (2×10^9 pfu/ml), and finally U4.4 cells (4×10^8 pfu/ml) producing the lowest titers (Miller and Brown, 1992). However, any of these cell lines can be used to produce virus stocks of $\sim 10^9$ pfu/ml.

Another important point is that infection of cells (mammalian or insect) always produces more virus/cell (higher titer) than transfection of cells. This may be due, in part, to the damaging effects to cells of the electroporation, or to the loss of full-length infectious RNA via exonuclease activity.

Time Considerations

Splitting mosquito cell cultures should only take 15 to 30 min, depending on the number of flasks being split. In general, all infection protocols require ~1.5 to 2 hr to complete, including rocking time. This also is the case for the transfections, which require 1.5 to 3 hr to complete, depending upon the number of samples.

The C7-10 plaque assay protocol will take ~3 hr depending on the number of virus sample to be titered.

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Newcastle Disease Virus: Propagation, Quantification, and Storage

Newcastle disease virus (NDV) is an avian pathogen. Some strains of the virus are important agricultural pathogens that cause often fatal systemic, respiratory, or neurological disease in poultry. NDV is also a prototype paramyxovirus that has been utilized in laboratories to elucidate many fundamental principles of infection by this family of viruses. Preparation and titration of purified virus stocks are basic procedures required in most studies of this important pathogen.

Although NDV is found only in birds in nature, NDV will infect a wide variety of cell types derived from certain mammals as well. Stocks of the virus can be prepared by growth in either embryonated chicken eggs or cells grown in tissue culture. Preparation of virus stocks by growth in embryonated eggs is an old and reliable method of producing stocks of many different types of viruses and is, by far, the superior method for producing high titer NDV stocks. This method has the added advantage of producing stocks of infectious virus regardless of the virus strain. The same cannot be said of stocks produced in tissue culture cells; while stocks of virulent NDV strains prepared in tissue culture cells are infectious, stocks of avirulent NDV prepared in tissue culture cells are not infectious. Both methods of NDV propagation are described in this unit.

Virus stocks grown in eggs are prepared by inoculating embryonated eggs with a zero stock (low titer stock) of virus, and then harvesting and purifying progeny virus from the infected eggs. Alternatively, virus can be purified from supernatants of infected tissue culture cells. The procedure for preparation of the zero stocks is described in Basic Protocol 2, followed by procedures for growing virus in embryonated eggs (Basic Protocol 3), as well as the purification of egg-derived virus (Basic Protocol 4). Growth and purification of virus derived from infection of tissue culture cells are described in the Alternate Protocol.

The titration of NDV (determination of virus concentration in a preparation) can be measured in several ways, but the two most frequently used are described in this unit. Infectivity of the stock (the number of infectious virus particles) in plaque-forming units (pfu)/ml is routinely determined by a plaque assay as described in Basic Protocol 1. A relative measure of the concentration of total virus particles is routinely accomplished by determining the hemagglutination titer and is described in Basic Protocol 5.

CAUTION: Virulent Newcastle disease viruses are Biosafety Level 3 (BSL-3) pathogens. Follow all appropriate guidelines for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Perform all work in a Class II biosafety cabinet (BSC).

CAUTION: Avirulent Newcastle disease viruses are Biosafety Level 2 (BSL-2) pathogens. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information. Perform all work in a Class II BSC.

CAUTION: Decontaminate all surfaces that have come in contact with virus using 70% ethanol, 1% sodium dodecyl sulfate, or Vesphene. Autoclave all waste products and autoclavable items. Nonautoclavable liquids may be brought to concentrations of 70% ethanol, 1% sodium dodecyl sulfate, or 0.78% Vesphene for decontamination. Viruses

Contributed by Lori W. McGinnes, Homer Pantua, Julie Reitter, and Trudy G. Morrison
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removed from the BSC for centrifugation steps must be in sealed tubes, and the outsides of the tubes must be cleaned with 70% ethanol. Centrifugation of BSL-3 virus preparations must be performed in a BSL-3 laboratory.

IMPORTANT NOTE: Virulent or velogenic NDV has been classified as a Select Agent by the United States Government. Refer to the CDC Select Agent Program for more information (*http://www.cdc.gov/od/sap*). Also refer to *UNIT 1A.1* and other pertinent resources (see *APPENDIX 1B*).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aeseptic technique should be used accordingly.

BASIC PROTOCOL 1

PLAQUE ASSAY FOR NEWCASTLE DISEASE VIRUS

A plaque assay measures the number of infectious virus particles in a preparation, the most fundamentally important property of a virus stock. A virus may also be biologically cloned in this process. When a virus preparation is sufficiently diluted, a plaque is derived from infection of a single cell in a monolayer culture with a single infectious virus, and the plaque, a clear area due to cell death and lysis in the monolayer, is the result of the infection spreading from the original infected cell to surrounding cells. A wide variety of cell types can be used for NDV plaque assays, e.g., the primate-derived COS-7 cell line, an avian cell line derived from ELL-0 chickens, and primary chicken embryo cells grown in tissue culture.

Materials

Avian tissue culture cells (e.g., ELL-O chicken cells; ATCC #UMNSAH/DF-1)
Virus stock (see Table 15F.2.1; Southeast Poultry Research Laboratory, Athens, GA)
Tissue culture medium (e.g., DMEM with supplements; see recipe)
NDV overlay medium with or without trypsin, 46°C (see recipes)
Methanol (optional)
Giemsa stain (Sigma; optional)

35- or 60-mm tissue culture plates
Pipets
46°C water bath

NOTE: All culture incubations are performed in a humidified 37.5°C, 5% CO_2 incubator unless otherwise specified.

1. Prepare confluent monolayers of tissue culture cells by adding 7.5×10^5 cells or 3×10^5 cells per 60-mm or 35-mm tissue culture plates, respectively. Incubate ~16 to 20 hr at 37°C or until cells are confluent.

   These cell numbers can be added in any volume under the standard 5 ml for 60-mm and 2 ml for 35-mm plates. The cell numbers added is the important thing. Medium is then added to bring the volumes up to the standard 5 ml or 2 ml. The surface area of the plates is more important than the volume of the media overlaying the monolayer.

2. Make 10-fold serial dilutions of virus stock in 1 ml tissue culture medium.

   CAUTION*: Perform all work in a Class II BSC (see UNIT 1A.1).*

   Dilutions of 10^{-3} to 10^{-9} are recommended.

3. Remove medium from the cell monolayer using a pipet.

4. Add 0.2 ml virus dilution to the plate of tissue culture cells. Spread the inoculum by rocking the plate.

   Each dilution is usually plated in duplicate.

5. Incubate tissue culture cell monolayer with added virus for 45 min at 37.5°C.

   During this time the virus is adsorbed onto the cells.

   This step may also be performed at room temperature or at 4°C. Temperatures lower than 15°C to 20°C arrest infection at attachment. Attachment occurs from 4°C to 20°C, but infection does not proceed until the cells are placed at the higher temperatures.

6. Cover cell monolayer with NDV overlay medium by adding down the side of the plate 5 ml for a 60-mm plate or 2 ml for a 35-mm plate.

   Use NDV overlay agar without trypsin for virulent strains of NDV. Use NDV overlay agar with trypsin added for avirulent strains.

   The original virus inoculum may or may not be removed with a pipet before adding the agar overlay. The virus inoculum may dilute the overlay agar, resulting in failure of the agar to solidify.

7. Allow plates to sit undisturbed at room temperature until the agar solidifies (~30 min).

8. Incubate plates until plaques appear.

   The time required for plaques to appear depends upon the NDV strain used. Plaques of virulent NDV can appear from 36 to 48 hr while plaques of avirulent NDV may require 40 hr or longer. Plaques can disappear after 48 to 72 hr.

9. Count plaques.

   Plaques often appear as clear areas in the monolayer when holding the monolayer up to a light. Alternatively, they may appear as whiter or opalescent areas in the monolayer.

   Titer = number of plaques × 5 × dilution factor.

10a. *To harvest virus from a single plaque:* Proceed to Basic Protocol 2.

10b. *To improve visualization:* Gently remove the agar overlay using a spatula. Fix the monolayer by adding 5 ml methanol and incubating 5 min at room temperature. Air dry thoroughly, then add 5 ml Giemsa satin diluted 1:20 in distilled water. Incubate 30 min at room temperature. Remove the staining solution, wash the plates with water, and air dry for long-term storage (Fig. 15F.2.1).

   Giemsa staining may only be employed if viruses are not to be harvested from plaques.

PREPARATION OF NEWCASTLE DISEASE VIRUS ZERO STOCKS

BASIC PROTOCOL 2

Zero stocks are low titer virus stocks used to inoculate embryonated chicken eggs for the preparation of high titer stocks of virus. Zero stocks are prepared in order to eliminate contaminating defective interfering particles and to decrease the genetic heterogeneity of the inoculating virus. To accomplish both goals, the virus is biologically cloned two times using the plaque assay procedure (see Basic Protocol 1). Virus derived from a single plaque is then injected into a single egg (see Basic Protocol 3). The viruses harvested from a single egg, the zero stock, can be stored up to 5 years at −80°C and used to prepare multiple high titer stocks.

Materials

Viral plaque (Basic Protocol 1)
Hanks' balanced salt solution (HBSS; *APPENDIX 2A* or Gibco 24020; contains calcium, magnesium, and phenol red)
Pasteur pipets
1.5-ml microcentrifuge tubes (polypropylene)
Microcentrifuge (Eppendorf or comparable)

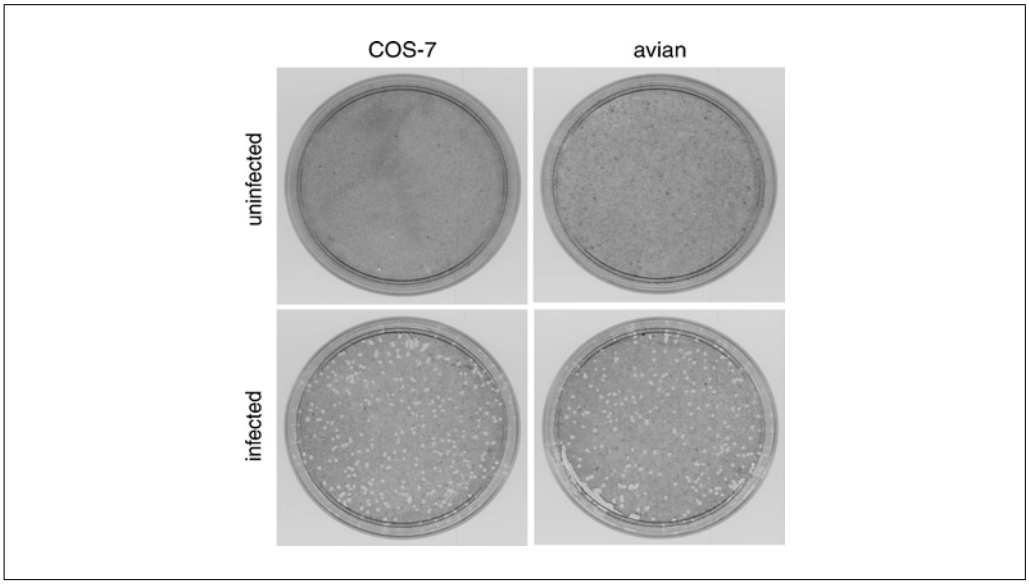

Figure 15F.2.1 Plates containing Newcastle disease virus plaques formed on monolayers of avian cells (right) or COS-7 cells (left). The agar overlay has been removed and the cells on the monolayers fixed with methanol and stained with Giemsa stain. Plaques are the circular areas with no stained cells.

15-ml polypropylene centrifuge tubes (Corning or comparable)
Sorvall centrifuge with an SS-34 rotor (or comparable)
Additional reagents and equipment for growth of virus in eggs (Basic Protocol 3, collection of allantoic fluid (Basic Protocol 4), and obtaining plaques (Basic Protocol 1)

Biologically clone NDV

1. Using a Pasteur pipette, remove the viral plaque from the plate by taking up the entire agar plug on top of an individual plaque into the hollow bore of the pipette.

   Choose a plaque, well isolated from surrounding plaques, from a plate containing 30 to 50 plaques.

2. Place the agar plug in 0.2 ml HBSS in a sterile 1.5-ml microcentrifuge tube, mix, and incubate 30 min on ice.

3. Remove the agar by centrifuging 5 min at 3000 × *g*, 4°C.

4. Plate several dilutions of the supernatant obtained from the single plaque as in Basic Protocol 1 in order to obtain plates with individual, isolated single plaques.

5. Remove a single plaque and collect virus as described in steps 1 to 3.

Grow viruses from a plaque in a single egg

6. Inoculate a single 10-day-old embryonated egg (i.e., virus derived from one plaque is used to infect one egg) as described in Basic Protocol 3 with the clarified supernatant from step 5.

   Note that in Basic Protocol 3, step 4, virus recovered from a single plaque is diluted to 10^3 pfu/0.1 ml before being injected. In contrast, undiluted virus is used in this protocol.

7. Grow the virus (see Basic Protocol 3) and collect the allantoic fluid in a 15-ml polypropylene centrifuge tube as for large virus preparations (see Basic Protocol 4, steps 1 and 2).

8. Centrifuge 10 min at 3000 × *g* (5000 rpm), 4°C to remove debris in the allantoic fluid.

   The supernatant is the zero stock.

9. Determine the virus titer of the supernatant (zero stock), using a plaque assay (Basic Protocol 1).

10. Dispense aliquots of the zero stock and freeze up to 5 years at −80°C.

    Aliquots of 0.5 ml are recommended.

GROWTH OF VIRUS IN EMBRYONATED CHICKEN EGGS

BASIC PROTOCOL 3

The cells in the allantoic membrane of embryonated chicken eggs are used to grow virus by injecting 10-day-old eggs with virus and then incubating the infected embryos to allow virus growth. High titers of virus can be recovered from the infected egg. Virus in the allantoic fluid of many eggs that have been inoculated with zero stocks can be purified and concentrated to make a high titer stock of virus as described in Basic Protocol 4. Virus in the allantoic fluid of a single egg, which has been injected with cloned virus (Basic Protocol 2), is used as the zero stock.

Materials

<10-day-old special pathogen free (SPF) eggs (e.g., Charles River/SPAFAS)
Zero stock (Basic Protocol 2)
Hanks' balanced salt solution (HBSS; *APPENDIX 2A* or Gibco 24020; contains calcium, magnesium, and phenol red) containing 2% (v/v) heat-inactivated FBS (*APPENDIX 2A*)
70% ethanol
Humidified egg incubator (e.g., Lyon Electric)
Egg candler (e.g., Lyon Electric)
16-G, 10-mm needle
1-ml tuberculin syringe with an attached 26-G, 10-mm needle (e.g., Becton Dickinson)
Nail polish or wax

1. Incubate eggs immediately upon arrival at 37.5°C in a humidified egg incubator.

   Order eggs 2 weeks before the actual starting date and specify that eggs are to be <10 days of age, preferably 8 days. If the eggs arrive cold, warm to room temperature prior to placing them at 37.5°C.

2. Candle eggs at 10 days of age. Hold each egg up to a light source (egg candler) to visualize the location of the embryo, membranes, and blood vessels. Discard eggs with dead embryos.

   Eggs with dead embryos will not have clear blood vessels and will have the embryo at the bottom of the egg.

3. Turn the egg so the embryo is in the back and the yolk is in front. Mark with a pencil, on the side of the egg opposite the embryo, a line where the membrane meets the air sac. Also place a dot ~5 mm above that line between blood vessels. The dot marks the injection point (see Fig. 15F.2.2).

4. Dilute the zero stock in HBSS containing 2% heat-inactivated FBS so that there are 10^3 pfu in 0.1 ml.

   Note that in Basic Protocol 2, step 6 for preparing a zero stock, virus recovered from a single plaque is injected into a single egg without dilution. In contrast, diluted virus is used in this protocol.

5. Rinse the eggs with 70% ethanol to sterilize outer surface.

6. Using a 16-G, 10-mm needle, puncture each egg at the pencil dot.

   Be careful to keep holes small.

7. Fill a 1-mm tuberculin syringe with attached 26-G, 10-mm needle with the diluted zero stock and inject 0.1 ml of the virus dilution per egg.

   Make sure needle is inserted such that it will enter the membrane below the line where the membrane meets the air sac but not deep enough to penetrate the embryo.

   Be careful not to push air into the eggs, particularly with the last 0.1 ml in the syringe.

8. Cover the puncture holes in the eggs with nail polish.

   The nail polish seal keeps the eggs from drying out. Use two coats of polish to make sure to cover holes completely. Alternatively, wax may be used.

9. Incubate eggs at 37.5°C in a humidified egg incubator.

10. Start checking eggs at 36 hr for embryo death since different strains of NDV induce embryo death with different kinetics. Check eggs every 6 hr.

    Embryo death is characterized by loss of blood vessels along the sides. In addition, the embryo falls to the bottom of the egg.

    Velogenic strains of NDV kill embryos at 40 to 60 hr. Mesogenic strains kill embryos from 60 to 90 hr while lentogenic strains results in death at >90 hr or not at all. For mesogenic and lentogenic strains, terminate incubation at 65 to 70 hr. Do not let eggs hatch since any chicks must be euthanized.

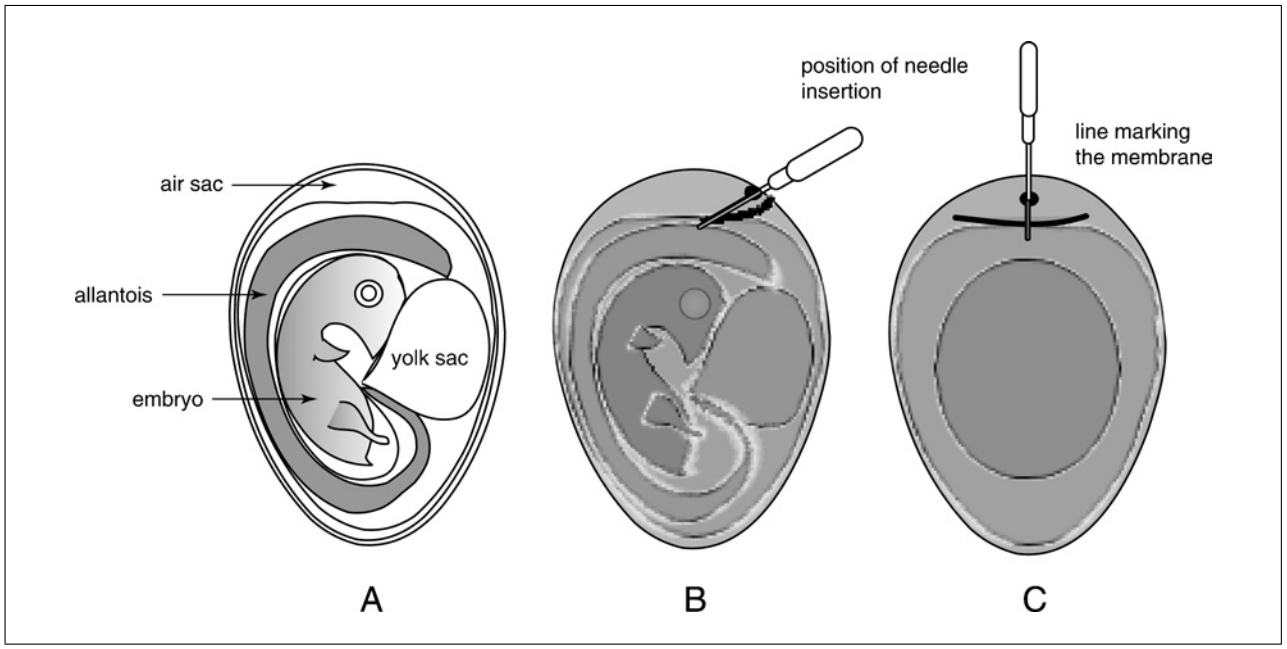

Figure 15F.2.2 Injection of a 10-day-old chick embryo. (**A**) Cross section of a 10-day-old chick embryo with the major structural components labeled. (**B**) Intact 10-day-old egg viewed with an egg candler. The top of the egg shows the position of the pencil marks that locate the membrane at the bottom of the air sac and the position at which the injection needle should be inserted. The diagram of the needle indicates the site of injection of virus. The needle is at a shallow angle to avoid the embryo and is aimed away from the yolk sac. (**C**) Intact egg turned with the embryo in back and the yolk sac in front.

11. As soon as the embryos are dead, place eggs at 4°C for at least 2 hr before collecting allantoic fluid.

Incubation at 4°C terminates infection.

PURIFICATION OF VIRUS FROM THE ALLANTOIC FLUID OF INFECTED EGGS

BASIC PROTOCOL 4

The allantoic fluid must be removed from each infected egg. The allantoic fluid contains a high titer of virus; however, virus must be purified from the contaminating egg components for most applications. Purification is accomplished by first concentrating the virus by centrifugation. The resuspended virus is then sedimented through a 20% sucrose solution onto a 65% sucrose pad by centrifugation, a step that further purifies the virus from contaminating egg material and concentrates the virus. The virus obtained at the 20% to 65% sucrose interface is satisfactory for most applications. If necessary, further purification can be attained by equilibrium centrifugation in a sucrose gradient.

Materials

Infected eggs (Basic Protocol 3)
70% ethanol
Hanks' balanced salt solution (HBSS; *APPENDIX 2A* or Gibco 24020; contains calcium, magnesium, and phenol red)
70% ethanol/1% (w/v) SDS
HBSS (*APPENDIX 2A* or Gibco 24020) containing 2% heat-inactivated FBS (*APPENDIX 2A*)
20%, 25%, 35%, 45%, 55%, and 65% (w/v) sucrose in standard buffer (see below for standard buffer)
Standard buffer: 0.1 M NaCl/0.01 M Tris·Cl, pH 7.4 (*APPENDIX 2A*)/2 mM EDTA

Forceps
10-ml syringe with a 16-G needle
Microspoon-end spatula (e.g., VWR)
250-ml bottles for GSA rotor (or comparable)
Sorvall centrifuge (or comparable) with GSA rotor
Beckman Type 19 bottles
Deldrin cap assemblies (or comparable): sterilize by rinsing with 70% ethanol and exposing to UV light for 20 hr
Beckman ultracentrifuge with type 19 rotor
10-ml glass Dounce homogenizer with a tight-fitting pestle
30-ml Sorvall centrifuge tube
Sorval SS-34 rotor
Beckman SW28.1 rotor with large buckets and small buckets
17- and 30-ml Beckman ultracentrifuge tubes (or comparable)
Ring stand with clamps
10-ml syringe with a 21-G needle
1- or 2-ml cryovials
−80°C freezer

NOTE: Precool all centrifuges and rotors and chill all solutions and glassware used for purification of virus.

NOTE: The following steps are for ten dozen infected eggs.

Collect virus from eggs

1. Wash infected eggs with 70% ethanol.

   This step reduces contamination of the virus stock with bacteria and fungi.

2. Open the wide end of the eggs above the membrane using sterile forceps. Clear away all shell fragments.

3. Break the milky-white air sac membrane covering the allantoic sac. Pull it to the edges of the egg. Remove the fluid underneath the membrane using a 10-ml syringe (16-G needle) and a microspoon-end spatula to push the membranes clear of needle bore.

   A single egg should yield between 3 and 10 ml fluid. The allantoic sac membrane, a clear membrane under the air sac membrane, can also be broken to gain access to more fluid. Care should be taken, however, not to break the yolk sac. If the yolk sack is broken, do not remove more fluid from the egg.

   After removal of fluid, eggs are autoclaved.

4. Place the harvested fluid directly into chilled, sterile 250-ml centrifuge bottles on ice.

5. Centrifuge the pooled fluid 10 min at 4000 × *g* (5000 rpm), 4°C in a precooled Sorvall centrifuge with GSA rotor.

   This will remove debris and erythrocytes.

Concentrate virus

6. Decant the supernatant into chilled, sterile Beckman Type 19 bottles.

7. Add HBSS to completely fill all bottles.

   HBSS should be precooled to 4°C.

   These bottles have a tendency to collapse if not completely filled.

8. Add the cap assembly to each bottle.

   Do not autoclave Deldrin cap assemblies. Sterilize with ethanol and UV light.

9. Centrifuge 4 hr to overnight at 48,000 × *g* (18,000 rpm), 4°C in a precooled Beckman ultracentrifuge with Type 19 rotor.

10. Pour off supernatant, autoclave to decontaminate, and discard. Decontaminate the rotor by rinsing inside and out with a solution of 70% ethanol and 1% SDS.

Purify virus

11. Resuspend the pellet in 5 ml HBSS containing 2% heat-inactivated FBS using 10 strokes in a Dounce homogenizer.

    The pellet is very sticky, thus, a dounce homogenizer is useful for resuspending the particulate material.

12. Pour the resuspended material into a 30-ml Sorvall centrifuge tube.

13. Centrifuge 10 min at 6000 × *g* (7000 rpm), 4°C using a SS-34 rotor.

    This step removes large particulate matter. The virus is in the supernatant.

    The pellet should be washed (steps 14 to 15) to release residual virus from the pellet.

14. Pour the supernatant into another 30-ml Sorvall centrifuge tube and keep on ice.

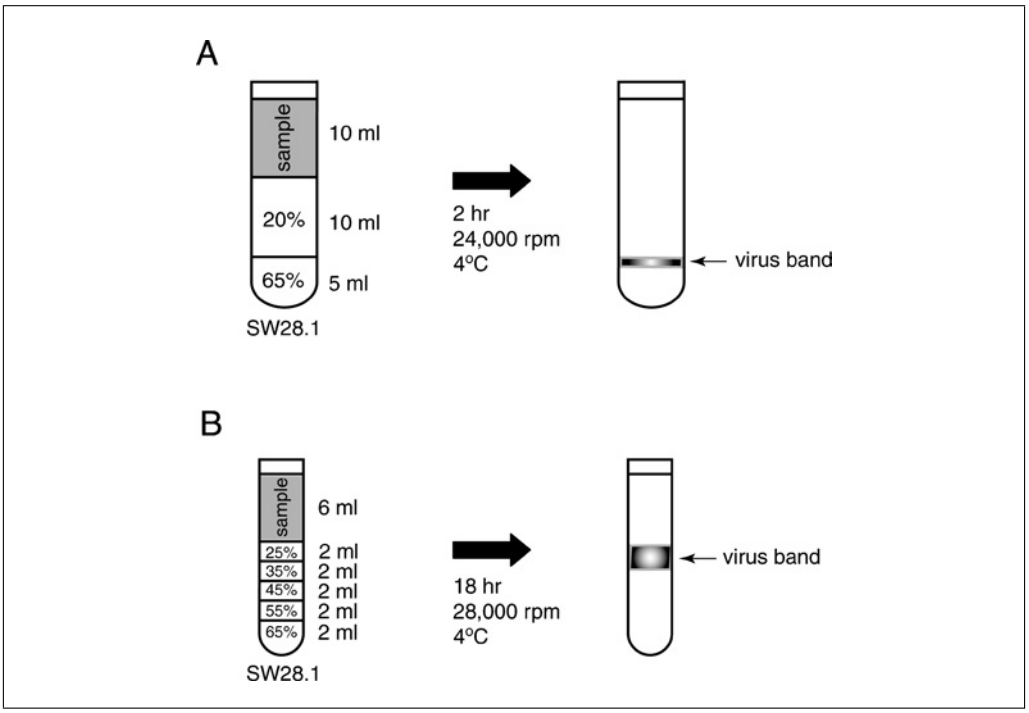

Figure 15F.2.3 Preparation of sucrose gradients used for virus concentration and purification of Newcastle disease virus. (**A**) Construction of the discontinuous gradient described in Basic Protocol 4, step 18. Resuspended virus is placed on top of the 20% sucrose layer. The position of virus is indicated after centrifugation. (**B**) Construction of the continuous gradient described in Basic Protocol 4, steps 22 to 24, used to further purify the virus preparation. The virus obtained from A is diluted in standard buffer and then placed on top of the sucrose gradient. The position of the virus is shown after centrifugation.

15. Add 5 ml HBSS containing 2% FBS to the pellet derived in step 13 and resuspend with a Dounce homogenizer to release any residual virus.

16. Pour the resuspended pellet into a 30-ml Sorvall centrifuge tube and centrifuge 10 min at 6000 × *g* (7000 rpm), 4°C using an SS-34 rotor.

17. Combine the supernatants from steps 13 and 16 (~10 ml) and centrifuge 10 min at 17,000 × *g* (12,000 rpm), 4°C using a SS-34 rotor using a SS-34 rotor.

    This step further clarifies the virus suspension. Virus remains in the supernatant.

18. Prepare a discontinuous sucrose gradient by slowly adding 5 ml of 65% sucrose in standard buffer and 10 ml of 20% sucrose in standard buffer, successively, to a 30-ml Beckman SW28.1 clear ultracentrifuge tube (see Fig. 15F.2.3A).

    Prepare one gradient for each 10 dozen eggs inoculated.

    Prior to forming the gradient, sterilize the tubes by washing the inside of the tubes with 70% ethanol and rinsing five times with sterile distilled water.

    Be sure the gradients all have the same weight prior to centrifugation.

19. Layer ~10 ml supernatant over the gradient (precooled to 4°C) and centrifuge 2 hr at 11,000 × *g* (24,000 rpm), 4°C, in a Beckman SW28.1 rotor, large buckets.

    The white "fluffy" layer between the sucrose layers is the purified virus.

20. Clamp the tube firmly on a ring stand in a Class II BSC. Collect the virus by puncturing the bottom of the tube with a 21-G needle. Let the 65% sucrose drain out. Collect the virus layer into a sterile tube as it drips from the bottom of the tube.

This working stock is pure enough for most applications such as infections of cell monolayers.

After harvesting the virus, autoclave the centrifuge tube and remaining sucrose.

21. Dilute the stock of virus by adding an equal volume of HBSS containing 2% FBS. Dispense aliquots into cryovials and store up to 10 years at −80°C or proceed with further purification.

    Virus is stable through multiple freeze-thaw cycles.

Further purify virus stocks

22. Make a continuous sucrose gradient by slowly adding 2 ml each of 65%, 55%, 45%, 35%, 25% sucrose solutions in standard buffer, successively, to a 30-ml Beckman SW28.1 clear ultracentrifuge tube (see Figure 15F.2.3B) Allow the sucrose solutions in the tube to diffuse by incubating the tubes overnight at 4°C.
23. Add standard buffer to the virus collected in step 19 above to make a total of 6 ml.
24. Layer the diluted virus on top of the gradient.
25. Centrifuge the gradient overnight (∼18 hr) at 15,000 × *g* (28,000 rpm), 4°C in a Beckman ultracentrifuge using SW28.1 small buckets.

    The virus will band near the middle of the gradient (density of 1.19 g/cc) and can be seen as a white layer.

26. Clamp the tube onto a ring stand. Carefully insert a 10-ml syringe with a 21-G needle into the side of the tube just below the virus band. Remove the virus band by gently pulling on the plunger of the syringe, sweeping the needle back and forth to remove the entire virus band.

    The virus can be entirely removed in 2 to 3 ml.

    If necessary, this stock of virus may be concentrated in a discontinuous sucrose gradient as described in steps 18 to 20.

27. Dispense aliquots into cryovials and store up to 10 years at −80°C.

ALTERNATE PROTOCOL

GROWTH AND PURIFICATION OF VIRUS DERIVED FROM INFECTION OF TISSUE CULTURE CELLS

Virus stocks may be prepared from infected tissue culture cells; however, to produce a stock with a titer comparable to that prepared in eggs, large numbers of tissue culture cells are required. Because of the cost of fetal bovine serum usually required for the growth of cells, this method of virus preparation is considerably more expensive than protocols using eggs. It is also difficult, if not impossible, to prepare stocks of virus at titers comparable to those prepared in eggs. Another disadvantage of tissue culture–derived virus is that avirulent strains of NDV produced in tissue culture cells are not infectious since the F protein in the released virus is not cleaved (see Background Information).

Materials

Avian tissue culture cells (e.g., ELL-O chicken cells; ATCC #UMNSAH/DF-1)
Tissue culture medium (e.g., DMEM with supplements; see recipe)
Virus zero stock (Basic Protocol 2), titered

100-mm tissue culture plates (avian cells)
37.5°C, 5% CO_2 tissue culture incubator, humidified
Additional reagents and equipment for virus concentration and sucrose gradient centrifugation (see Basic Protocol 4)

1. Plate avian cells by adding ~2 × 10^6 cell/100-mm plate and incubating 15 to 20 hr at 37°C until the monolayer is just confluent.

   For optimal yields, 100-mm plates are preferred.

   The cell type is important for these experiments since the efficiency of NDV assembly varies with cell type (unpub. observ.). Avian cells (primary chick embryo cells or an ELL-0 cell line obtained from ATCC) are very efficient in the production of virus particles (unpub. observ.) and are strongly recommended.

2. Infect cells with virus at a multiplicity of infection (MOI) of 10, i.e., add 10 plaque forming units (pfu) per cell. Calculate the required volume of virus stock as follows:

   no. cells to be infected × 10/titer of virus stock (pfu/ml) = amount (ml) virus stock to use.

   The MOI may vary from 10 to 25. There are usually 8 × 10^6 cells in a confluent 100-mm plate.

3. Dilute virus stock into tissue culture medium such that the appropriate number of pfu are in 0.5 ml for infection of a 100-mm plate of cells.

4. Remove medium from the monolayer.

5. Add the 0.5 ml diluted virus to the monolayer and spread the inoculum over the entire monolayer by rocking the plate.

6. Incubate tissue culture cell monolayer with added virus 45 min at 37.5°C.

   During this time the virus is adsorbed onto the cells.

7. Remove the medium, wash the monolayers two times with 10 ml tissue culture medium, and then add tissue culture medium to the cells.

   This step, which removes unbound virus, is optional unless it is important to remove residual virus in the inoculum.

8. Incubate infected cells at 37.5°C.

   Incubation times vary with the strain of NDV used. Virulent NDV can be incubated 9 to 12 hr at which time cells begin to lyse. Supernatants should be removed before there is extensive cell lysis in order to reduce contaminating cell debris in virus stocks. Avirulent NDV infections can be incubated >24 hr.

9. Collect supernatants from monolayers using a pipet and centrifuge 10 min at 4000 × *g* (5,000 rpm) in a Sorvall centrifuge with GSA rotor using 30-ml tubes to remove cell debri.

10. Concentrate the virus as described in Basic Protocol 4, steps 6 to 10.

    For small-scale experiments, it is possible to skip to step 18 in Basic Protocol 4, placing the supernatants directly onto discontinuous sucrose gradients.

11. Purify the virus by centrifugation through a 20% sucrose gradient onto a 65% sucrose pad as described in Basic Protocol 4, steps 18 to 21.

TITRATION OF VIRUS STOCKS

BASIC PROTOCOL 5

Titration of NDV stocks may be accomplished in a number of different ways. The measure of the number of infectious virus present is accomplished by a plaque assay described in Basic Protocol 1. Alternatively, total virus protein may be measured using standard methods for determining protein concentration (*APPENDIX 3A*). Another common method for determining the relative concentration of virus particles in a stock is a

hemagglutination assay (HA). Hemagglutination is a measure of the ability of virus particles to bind to sialic acid containing molecules on the surfaces of red blood cells (RBCs). When virus is present and bound to RBCs, the RBCs form a lattice-like structure, which coats the bottom and sides of a tube. In contrast, RBCs that are not bound to virus fall to the bottom of a tube forming a very tight round button. The difference between RBCs agglutinated by virus particles and those that are not agglutinated is readily visualized without the aid of a microscope (Figure 15F.2.4). Hemagglutinin assays are performed by mixing increasing dilutions of a virus stock with a constant number of RBCs. The reciprocal of the last or most dilute concentration of the virus that agglutinates RBCs is the hemagglutinin titer (HA titer). The relative concentrations of virus in different stocks can be compared in this way.

Materials

Red blood cells (avian alsever or guinea pig alsever; BioLink)
PBS, ice cold (*APPENDIX 2A*)

1.5 ml microcentrifuge tubes
Microcentrifuge
Hemacytometer
Microscope
Microtiter plates (96-well, round bottom)

NOTE: Maintain all solutions on ice.

Wash red blood cells

1. Pipet 0.5 ml red blood cells (RBCs) into a microcentrifuge tube.
2. Add 0.5 ml PBS and mix.
3. Microcentrifuge the RBCs 1 min at 3000 × *g* (4000 rpm) and pour off the supernatant.

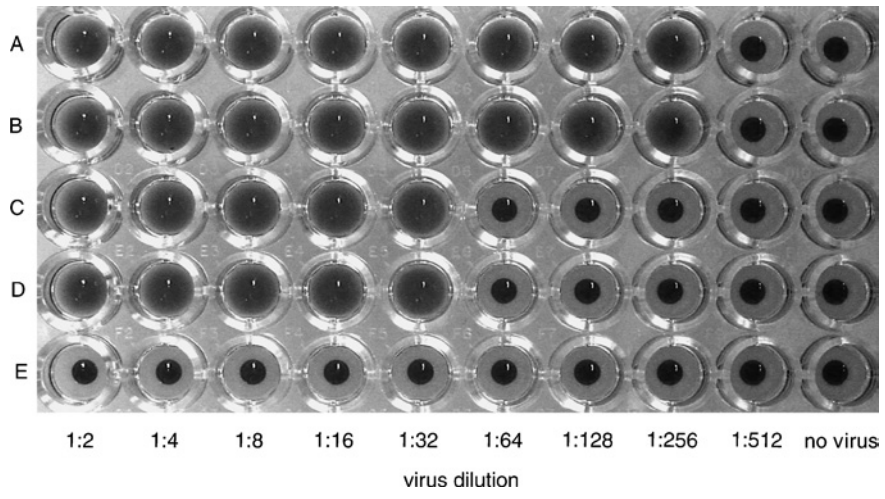

Figure 15F.2.4 A microtiter plate representative of a hemagglutination assay (HA) for two different preparations of virus (in duplicate). Rows A and B contain dilutions of stock 1, and rows C and D, contain dilutions of stock 2. Row E wells show red blood cells (RBCs) without added virus, and no hemagglutination is visible. Virus in each stock was diluted as indicated at the bottom of the plate. Stock 1 hemagglutinated RBCs at dilutions up to 1:256 (RBCs formed a lattice), but not at further dilutions; thus, the HA titer was 256. Stock 2 hemagglutinated RBCs up to dilutions of 1:32 but not at higher dilutions; thus, the HA titer was 32.

4. Resuspend RBCs in 1 ml PBS.
5. Wash two more times as described in steps 3 and 4.
6. Determine the concentration of the washed, resuspended RBCs by diluting a small amount 1:1000 in PBS and counting with a hemacytometer (e.g., see *APPENDIX 4A*).
7. Resuspend the washed RBCs in PBS to a concentration of 5.3×10^7 cells/ml.

Make virus dilutions

8. Prepare serial, two-fold dilutions (1:2, 1:4. 1:8, 1:16, etc) of virus in PBS.

   If a stock of virus is very concentrated, it may be necessary to make a 1:10 or a 1:100 dilution first and then make serial two fold dilutions.

9. Into microtiter plate wells, dispense 10 µl of each virus dilution and 10 µl of PBS with no virus for a negative control.

   If possible, a positive control with a known amount of virus should also be included in the assay. Assays should be performed in duplicate.

Perform hemadsorption assay

10. Add 75 µl diluted RBC from step 9 to each microtiter well.

    Mix by tapping microtiter plate.

11. Incubate 1 hr at 4°C.
12. Score wells as negative hemagglutination by the appearance of RBC buttons.

    The HA titer is the reciprocal of the last dilution of virus that results in hemagglutination (lattice formation). The RBCs will settle out to form a "button" where there is insufficient virus to agglutinate the RBCs (See Figure 15F.2.4).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

DMEM with supplements

Dissolve 4.5 g DMEM (Sigma 5648) in 1 liter water and sterilize by passing through a 0.22-µm filter. Add:

10% (v/v) heat-inactivated FBS (*APPENDIX 2A*)
0.5× pen/strep (obtained as 100× or 10,000 U/ml penicillin and 10,000 µg/ml streptomycin; Gibco 15140-122)
1× vitamins (obtained as 100× stock; Gibco)
1× glutamine (obtained as 100× stock; Gibco)

Other additions to the media will depend upon the requirements of the cells used. For example, some cells require added amino acids (nonessential amino acids)

NDV overlay medium

20 ml 5× DMEM: prepare by dissolving 4.5 g DMEM (Sigma D5648) in 200 ml water and filter sterilizing (0.22-µm filter)
Additions to DMEM necessary for cell type (e.g., pen/strep, nonessential amino acids)
10 ml heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*)
Bring to 45 ml with sterile water
Heat to 46°C

Add 55 ml of 1.8% (w/v) agar (prepare by heating in sterile water and cooling to 46°C)

Maintain medium up to 1 hr at 46°C

Additions to the media will depend upon the requirements of the cells used. For example, some cells require added amino acids (essential amino acids).

This overlay is used for deriving plaques from virulent NDV strains.

NDV overlay medium with trypsin

Combine in order with mixing:

10 ml 10× Medium 199 (Gibco 11825)

2.5 ml tryptose phosphate broth (TPB; Gibco)

1 ml 10% (w/v) sodium bicarbonate

32.2 ml sterile water

0.1 ml pen/strep (obtained as 100× or 10,000 U/ml penicillin and 10,000 μg/ml streptomycin; Gibco 15140-122)

Heat to 46°C and add the following:

55 ml of 1.8% (w/v) agar (prepare by heating in sterile water and cooling to 46°C)

0.1 ml 10 mg/ml (w/v) trypsin (Sigma T-6763, acetylated) dissolved in PBS (*APPENDIX 2A*) just before use

Hold medium up to 1 hr at 46°C

The concentration of trypsin may need to be adjusted depending on the cells used.

Additions to the media will depend upon the requirements of the cells used. For example, some cells require added amino acids (essential amino acids).

Some cell lines will perform better using DMEM instead of Medium 199. This is determined experimentally.

This overlay is used for deriving plaques from avirulent NDV strains.

COMMENTARY

Background Information

NDV is a member of the family Paramyxoviridae, which are enveloped, negative-stranded, RNA viruses. The NDV genome encodes six genes, in the 3′ to 5′ gene order: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), hemagglutinin-neuraminidase glycoprotein (HN), and L protein (reviewed in Nagai et al., 1989; Lamb and Kolakofsky, 2001; Morrison, 2003). The NP, P, and L proteins are associated with the genome in the core of the virus particle. This core is surrounded by a membrane derived from host cell plasma membranes. The M protein lines the inner surface of the host-derived membrane, and F and HN are transmembrane glycoproteins, which form the surface spike structures of virions. The HN protein is the virus attachment protein and binds to sialic acid–containing receptors. The F protein is directly responsible for the fusion of the viral membrane and the cellular membrane during virus entry (reviewed in Nagai et al., 1989; Lamb and Kolakofsky, 2001; Morrison, 2003).

In nature, many different strains or variants of NDV exist (see Table 15F.2.1 for a list of the more commonly used strains). These strains can be classified into two categories, virulent and avirulent, reflecting their ability to form plaques on monolayers of tissue culture cells and their ability to spread in an infected animal. This classification is based on the sequence at the cleavage site of the F protein (Nagai and Klenk, 1977; Toyoda et al., 1987; Glickman et al., 1988). The F protein is synthesized as a precursor, F_0, which must be proteolytically cleaved into disulfide-linked heterodimer, F_1 and F_2, to function as a fusion protein (Nagai et al., 1976; Garten et al., 1980; Rott and Klenk, 1988). F protein in virus particles must be cleaved for virus particles to be infectious. The F protein found in virulent viruses has a furin recognition sequence at the cleavage site (Toyoda et al., 1987; Glickman et al., 1988). This sequence (RXKR or RXRR) is recognized and cleaved by a ubiquitous set of host cell proteases located in the trans-Golgi membranes of most cell types (reviewed in Gotoh et al., 1992; Nagai, 1993). Thus,

virulent NDV readily forms plaques on monolayers of tissue culture cells and can spread in many organs of birds. In contrast, the cleavage site of the F protein encoded by avirulent viruses lacks a furin recognition sequence but rather has single basic residues at the cleavage site (Nagai, 1993). Cleavage of these F proteins is carried out by trypsin-like host proteases, but these proteases are extracellular and are located only in respiratory and gastrointestinal tracts of birds or in the chorioallantoic membranes of embryonated chicken eggs (Gotoh et al., 1990; Nagai, 1993). Avirulent strains do not form plaques on monolayers of cells grown in tissue culture, nor do they spread in most tissues of birds. Virions released from infected tissue culture cells contain an uncleaved F protein and are, therefore, noninfectious. Infectious stocks of these viruses can be prepared in embryonated eggs since the chorioallantoic membranes contain an enzyme (Factor X) that will cleave the fusion protein of avirulent viruses. Avirulent NDV will form plaques on monolayers of tissue culture grown cells if an exogenous protease such as trypsin is added to monolayers in the overlay media.

Historically, NDV strains have also been classified into three categories depending upon the time required for the virus to kill chicken embryos, as well as virulence of the viruses in day-old chicks (Hanson and Brandly, 1955; Waterson et al., 1967; Beard and Hanson, 1984; see Table 15F.2.1). Velogenic strains are defined as viruses that kill embryos in 40 to 60 hr, mesogenic strains kill embryos in 60 to 90 hr, and lentogenic viruses kill embryos in >90 hr. Some lentogenic strains do not cause embryo death. In fact, this classification is somewhat arbitrary, and there is a continuum of virulence in embryos (Waterson et al., 1967). The classification will, however, give the investigator an idea of the time each strain can be grown in eggs. Velogenic and mesogenic strains encode F proteins with a furin recognition sequence while lentogenic strains encode F proteins with a single basic residue at the cleavage site. The mechanisms responsible for differences in virulence between velogenic and mesogenic strains are not entirely clear and are the subject of current investigation.

Critical Parameters and Troubleshooting

Table 15F.2.2 presents a list of some of the most commonly encountered problems with the techniques described in this unit, as well as their possible causes and suggested solutions.

Table 15F.2.1 Representative NDV Strains

NDV strain[a]	F protein cleavage site[b]	Egg virulence[c]
B1 Hitchner	avirulent	lentogenic
La Sota	avirulent	lentogenic
D26/76	avirulent	lentogenic
Queensland	avirulent	lentogenic
Ulster	avirulent	lentogenic
Roakin	virulent	mesogenic
L	virulent	mesogenic
Beaudette C	virulent	velogenic
Italien	virulent	velogenic
Texas G. B.	virulent	velogenic
Hertz	virulent	velogenic
Australia-Victoria	virulent	velogenic
Miyadera	virulent	velogenic

[a]See Pedersen, et al. (2004) for more recent isolates.

[b]Virulent: F protein cleavage site is RXKR or RXRR; avirulent, F protein cleavage site is RQGR (Glickman et al., 1988; Toyoda et al., 1987, 1989)

[c]Egg virulence classification based on approximate time required to kill chicken embryos: lentogenic, >90 hr; mesogenic, 60 to 90 hr; velogenic, 40 to 60 hr (Hanson and Brandly, 1955; Waterson et al., 1967; Toyoda et al., 1989; Alexander, 1997).

Table 15F.2.2 Troubleshooting Guide for Newcastle Disease Virus Growth, Purification, and Assay Procedures

Problem	Possible cause	Solution
Plaque assay		
Contamination	Improperly sterilized reagents	Autoclave or filter sterilize all solutions
		Use a biosafety hood
		Be sure all labware is sterile
No plaques	Incorrect NDV overlay medium pH	Add buffer to NDV overlay medium
	Virus solution too concentrated or too dilute	Use additional virus dilutions
	Dead monolayer cells	See below
	Nonpermissive cells	Use COS-7 cells or avian cells
Dead monolayer cells	Incorrect NDV overlay medium pH	Add buffer to NDV overlay medium
	NDV overlay medium too hot	Cool NDV overlay medium to 46°C
	NDV overlay medium lacking a necessary ingredient	Check growth requirements of cells
	NDV overlay medium trypsin concentration too high	Lower trypsin concentration in NDV overlay medium
Lumpy agar	NDV overlay medium too cool	Keep NDV overlay medium at 46°C
	Plates moved too soon	Allow agar to solidify prior to moving plates
Preparation of zero stock		
No virus in zero stock	No virus in agar plug	Titer solution derived from solubilized agar plug prior to injecting eggs
	Eggs improperly injected	Be sure injection needle penetrated membrane at bottom of air sac
	Embryo dead prior to infection	Check embryo before injecting virus
Growth of virus in eggs		
Embryos mostly dead at 10 days	Incorrect shipping conditions	Contact supplier of eggs for replacement
Embryos not dead 70 hr after incubation with virus	No virus in inoculum	Retiter zero stock
	NDV strain not lethal to embryos within 70 hr	Harvest allantoic fluid and titer by plaque assay or HA
Purification of Virus		
Very little fluid in eggs	Eggs dried out	Check nail polish seal
		Check for cracks in eggs

continued

Table 15F.2.2 Troubleshooting Guide for Newcastle Disease Virus Growth, Purification, and Assay Procedures, *continued*

Problem	Possible cause	Solution
Harvested fluid is yellow	Yolk sac disrupted	Do not use eggs with yellow fluid
Virus not recovered or low virus titer	Failure to pellet virus from allantoic fluid	Repellet supernatants
	Virus not well resuspended after pelleting	Use tighter Dounce homogenizer to resuspend pellet aggregates
Hemagglutination Assay		
No RBC in pellet after washes	RBCs expired	Use fresh RBCs
	Incorrect salt concentration in PBS	Remake PBS
No HA activity	Defective RBCs	Use freshly obtained RBCs
	Low virus titers	Determine by titer by plaque assay and correct
		Use more concentrated virus
	Temperature too warm	Keep RBC-virus mix cold
All dilutions hemagglutinate	Virus titer too high	Dilute virus further
Results inconclusive	Improper mixing	Mix RBCs and virus thoroughly

Anticipated Results

Plaque assays

Plaques will appear as clear or opalescent areas within the monolayers. These are areas of cell death that result from the spreading infection. Plaques may be directly seen through the agar overlay or after removing the agar overlay and staining the cell monolayers with Giemsa as shown in Figure 15F.2.1.

To determine the titer of the virus stock, count plaques on plates where the number of plaques are easy to see and easy to count (30 to 100). The titer of the stock (pfu/ml) is equal to the number of plaques multiplied by the dilution factor multiplied by five (assuming an inoculum of 0.2 ml). For example, if one counted 30 plaques on the 10^{-7} dilution plate, the titer is $30 \times 10^7 \times 5 = 1.5 \times 10^9$ pfu/ml.

Preparation of zero stocks

Virus isolated from a single plaque as described above should yield $\sim 10^3$ virus/ml. The virus isolated from a single egg should yield ~3 to 10 ml of virus with a titer of $\sim 10^7$ pfu/ml.

Preparation of high titer stocks in eggs

Upon arrival there will be a few dead embryos. Candling each egg will allow you to eliminate these eggs. Candling is most important for determining the location for injection of virus. Figure 15F.2.2A illustrates with a diagram of an embryonated egg how to determine the injection site. Figure 15F.2.2B shows how to inject the eggs. To obtain 1.5 to 2 ml of a stock of virus (10^9 to 10^{11} pfu/ml) start with 10 dozen eggs. Avirulent virus stocks will likely have a higher titer than virulent virus stocks.

Hemagglutinin assay

Expect 4–6 $\times$ 10^3 pfu/HA unit.

Time Considerations

Plaque assay

Plaques derived from virulent strains of NDV appear at 36 to 40 hr. Plaques derived from avirulent NDV in the presence of trypsin may take from 40 to 72 hr to appear.

Preparation of zero stocks

Each plaque assay will require 36 to 72 hr depending upon the strain of virus. Growth of the zero stock in eggs will require 40 to 150 hr, depending upon the strain of NDV.

Preparation of stocks in embryonated eggs

Candling and injection of eggs should require 3 hr/10 dozen eggs. Incubation of infected eggs will require 40 to 70 hr depending upon the strain of NDV. Harvesting the allantoic fluid of 10 dozen infected eggs should take 3 to 4 hr.

Purification of virus

Protocols should take 2 to 3 days, depending upon the degree of purity required.

Hemagglutination assay

Washing of RBCs should take 20 to 30 min. Setting up the virus dilutions and microtiter plates will take ~1 hr. The hemagglutinations should take an additional hour.

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Influenza: Propagation, Quantification, and Storage

This unit covers several techniques for propagating, quantifying, and storing human influenza A viruses from existing stocks (see Basic Protocols 1 and 2) or from primary clinical specimens (see Alternate Protocols 2 and 4). Virus isolation is a highly sensitive and useful technique for the identification of viral infections. An important advantage of virus isolation is the amplification of the virus from the original specimen, making it available for further antigenic and genetic characterization. Influenza viruses are quantified either by a "unit" of hemagglutination, which is not a measure of an absolute amount of virus but is an operational unit dependent on the method used for the hemagglutination assay titration (see Basic Protocol 3), or by determining infectious units using the 50% tissue culture infectious dose assay (see Basic Protocol 4), 50% egg infectious dose assay (see Basic Protocol 5), or plaque assay (see Basic Protocol 6). After isolating and quantifying human influenza, the product must be properly stored to maintain virus viability.

CAUTION: The protocols presented in this unit are for use with contemporary human influenza virus subtypes, which must be handled under Biosafety Level 2 (BSL-2) conditions. For biosafety levels recommended for noncontemporary or nonhuman influenza viruses, refer to the influenza agent summary statement in the *Biosafety in Microbiological and Biomedical Laboratories* manual published by the Centers for Disease Control and Prevention and National Institutes of Health (see interim guidelines at *http://www.cdc.gov/flu/pdf/h2n2bsl3.pdf*). All work with infectious influenza virus should be conducted in a class II biological safety cabinet. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for additional information.

IMPORTANT NOTE: Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

PROPAGATION OF INFLUENZA VIRUSES IN CELL CULTURE FROM VIRUS STOCKS

BASIC PROTOCOL 1

Madin Darby canine kidney (MDCK) cells (ATCC# CCL-34) are the preferred host for the isolation and characterization of influenza A and B viruses, but not influenza C viruses due to the incompatibility of sialic acid moieties on the cell surface with the viral receptor specificity. Influenza viruses are also able to replicate in a few primary, diploid, and continuous cell cultures. The propagation of influenza viruses in primary monkey kidney (PMK) cells (Viro-Med Laboratories or BioWhittaker) and R-mix fresh cells (Diagnostic Hybrids) is presented in Alternate Protocol 1. For preparation of a virus stock from a primary clinical specimen, refer to Alternate Protocol 2. The protocol described below is for the propagation of existing human influenza A and B virus stocks in MDCK cell culture.

Materials

Influenza virus stock
Madin Darby canine kidney (MDCK) cells confluent in a 75-cm^2 flask (see Support Protocol 1)
Phosphate-buffered saline (PBS) containing potassium (*APPENDIX 2A*)
cDMEM/7.5% BSA (see recipe)
Influenza virus growth medium (see recipe)

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10- and 25-ml pipets, sterile
33° to 37°C incubator
50-ml tubes, sterile (Falcon or Corning)
2-ml cryovials, sterile (Nunc)

NOTE: All equipment and solutions coming into contact with cells must be sterile and proper sterile technique should be used.

1. Thaw vial of influenza virus stock in cool water.

   To reduce loss of infectivity, maintain virus at 4°C once thawed.

2. Remove the MDCK growth medium using a sterile 25-ml pipet and wash the MDCK monolayer two times with 10 ml of room temperature PBS and once with cDMEM/7.5% BSA, removing washes with sterile 10-ml pipets.

   This cell line requires the cells to be confluent, i.e., completely covering the surface of the flask, before the virus is inoculated. If the monolayer is overgrown (i.e., cells overlapping), it is less sensitive to virus infection.

   Fetal bovine serum (FBS) inhibits viral entry and must be removed for efficient infection of cells.

3. Dilute virus samples in influenza virus growth medium.

   Use a 1:10 to 1:1000 dilution of virus to achieve optimal growth.

4. Inoculate flask with 1 ml virus and rotate to cover monolayer with inoculum. Take care not to add medium directly onto the monolayer as this may disrupt the cells.

5. Incubate inoculated flasks for a minimum of 30 min or up to 1 hr at 37°C.

   Incubation for >1 hr may cause the MDCK cell monolayer to dry out.

6. Add 20 ml influenza virus growth medium to the inoculated flasks.

7. Incubate flasks at 33°C (optimal for influenza B) to 37°C (optimal for influenza A), observing the MDCK monolayer for cytopathic effect (CPE) daily. Harvest cell culture supernatant when at least 75% of the cell monolayer is exhibiting CPE.

   Typical CPE by influenza viruses include rounding up of infected cells and detachment from culture flask.

8. Centrifuge supernatant 15 min at 300 × *g*, 4°C, to pellet cellular debris. Transfer clarified supernatant to a fresh 50-ml tube.

9. Dispose of tissue culture flask(s) in an appropriate biological waste container.

10. Dispense supernatant into 2-ml aliquots in sterile 2-ml cryovials and store up to 1 year at −70° to −80°C or in liquid nitrogen at −135° to −150°C for optimal viability after long-term storage (see Basic Protocol 7).

ALTERNATE PROTOCOL 1

PROPAGATION OF INFLUENZA VIRUSES IN OTHER CELL LINES

The Madin Darby canine kidney (MDCK) cell line is the preferred cell line for the isolation and propagation of influenza viruses (see Basic Protocol 1). If unable to maintain or purchase the MDCK cell line, other cell lines may be used to isolate and propagate influenza viruses. R-mix fresh cells (Diagnostic Hybrids) and primary monkey kidney (PMK) cells (Viro-Med Laboratories or BioWhittaker) are widely used by diagnostic laboratories for the isolation of many human respiratory viruses. R-mix fresh cells are a combination of the human adenocarcinoma (A549) and mink lung (Mv1Lu) cell lines, and are an alternate for MDCK cells in the growth and characterization of influenza viruses. PMK cells do not require the addition of tosyl phenylalanyl chloromethyl ketone

(TPCK)-trypsin for virus growth, but do require the addition of 0.5% FBS to medium instead of BSA. Limitations of PMK cells include lower viral titers, contamination by adventitious simian agents such as foamy viruses, and batch-to-batch variability.

PROPAGATION OF INFLUENZA VIRUS IN CELL CULTURE FROM PRIMARY CLINICAL SPECIMENS

ALTERNATE PROTOCOL 2

Specimens for virus isolation should be kept at 4°C immediately after collection and inoculated into susceptible cell cultures as soon as possible. If the specimen cannot be processed within 48 to 72 hr, the specimen should be frozen up to 1 year at or below −70°C. Primary influenza specimens require the addition of antibiotics prior to inoculating into cell culture (see Support Protocol 3). Due to the unknown amount of influenza virus in the clinical specimen, it may require multiple passages in cell culture for the virus to grow. Typically, two passages may follow the initial inoculation before a specimen is considered to be negative by virus isolation.

Additional Materials *(also see Basic Protocol 1)*

Primary clinical specimen (see Support Protocol 3)

NOTE: All equipment and solutions coming into contact with cells must be sterile and proper sterile technique should be used.

1. Thaw vial of primary clinical specimen in cool water.

   Keep virus at 4°C once thawed to reduce loss of infectivity.

2. Remove the MDCK growth medium using a sterile 25-ml pipet and wash the MDCK monolayer two times with 10 ml of room temperature PBS and one time with cDMEM/7.5% BSA, removing washes with sterile 10-ml pipets.

   Fetal bovine serum (FBS) inhibits viral entry and must be removed for efficient infection of cells.

3. Dilute virus samples in influenza virus growth medium.

   Use a 1:10 to 1:1000 dilution of virus to achieve optimal growth.

4. Inoculate flask with 200 µl virus and rotate to cover monolayer with inoculum. Take care not to add medium directly onto the monolayer as this may disrupt the cells.

5. Incubate inoculated flasks for 30 min to 1 hr at 37°C.

   After 1 hr the monolayer will begin to dry out.

6. Add 6 ml influenza virus growth medium to the inoculated flasks.

   Do not add medium directly onto the monolayer as this may disrupt the monolayer.

7. Incubate flasks at 33° to 37°C, observing the MDCK monolayer for cytopathic effects (CPE) daily.

   If type of influenza is unknown, incubate at 37°C.

8. Harvest cell culture supernatant into 50-ml tubes, when at least 75% of the cell monolayer is exhibiting CPE.

   Typical CPE by influenza viruses include rounding up of infected cells and detachment from culture flask. If no CPE is present, perform a hemagglutination assay (see Basic Protocol 3). If HA titer is <8 HAU, then 600 µl of undiluted supernatant should be passaged into a new MDCK flask. Primary clinical specimens should be passaged up to three times. If no HA titer is detected after three passages, the virus should be considered unrecoverable (i.e., influenza virus was not recovered from the specimen by this method).

9. Dispose of tissue culture flask(s) in an appropriate biological waste container.
10. Centrifuge supernatant 15 min at 300 × *g*, 4°C, to pellet cellular debris. Transfer clarified supernatant to a fresh 50-ml tube.
11. Dispense supernatant into 2-ml aliquots in sterile 2-ml cryovials and store <1 year at −70° to −80°C or at −135° to −150°C for long-term storage. (see Basic Protocol 7).

ALTERNATE PROTOCOL 3

PROPAGATION OF INFLUENZA VIRUS FROM CLINICAL SPECIMENS IN SHELL VIALS

MDCK shell vials (Diagnostic Hybrids) are an alternative for the isolation of human influenza viruses from primary clinical material. Using the shell vial assay significantly reduces the length of time for the detection of virus in primary specimens. With this technique, cell monolayers are grown on coverslips contained in flat-bottomed shell vials. This type of culture vessel allows for centrifugation of the cultures after inoculation to enhance viral infection of the cells, increase sensitivity to virus isolation and allow for a shortened turnaround time for specimen identification. Detection can be accomplished within 48 to 72 hr after inoculation, prior to the development of CPE, by the identification of viral antigens synthesized in the early stages of replication. Any negative result does not rule out viral etiology.

Commercially available MDCK shell vials should have a 70% to 90% confluent monolayer and be free of contamination. Slightly subconfluent monolayers of MDCK cells are preferred for virus isolation. Three shell vials should be inoculated per specimen and each run of shell vials should contain an uninoculated shell vial to serve as a negative control.

Additional Materials *(also see Basic Protocol 1)*

MDCK shell vials (Diagnostic Hybrids)
Primary clinical specimen (see Support Protocol 3)
15-ml tubes

1. Carefully remove MDCK growth medium with sterile pipet tips so as not to disturb monolayer, viewing it under a microscope.

   The monolayer may be viewed under a microscope by tilting the vial at a slight angle to determine if the cells are completely covering the bottom of the vial.

2. Inoculate each shell vial monolayer with ~0.2 ml primary clinical specimen.

   This volume is sufficient to cover the monolayer in a shell vial. Do not pipet inoculum directly onto the monolayer as this may disrupt the cells.

3. Centrifuge shell vials for 30 to 60 min at 700 × *g*, at room temperature (24° to 27°C).

   Centrifugation of shell vials allows for more efficient absorption of virus into the cell monolayer.

4. Add 1 ml influenza virus growth medium and incubate 72 hr at 33° to 35°C.

   These are the optimal temperatures for isolation of influenza viruses in shell vials.

5. Observe the shell vials during the 72-hr incubation and harvest supernatant into 15-ml tubes at the first signs of cytopathic effect (CPE). Dispose of tissue culture materials in an appropriate biological waste container.

   Typical CPE by influenza viruses include rounding up of infected cells and detachment from culture flask.

6. Centrifuge supernatant 15 min at 300 × *g*, 4°C, to pellet cellular debris. Transfer clarified supernatant to a fresh 15-ml tube. Dispense supernatant into 2-ml aliquots in 2-ml sterile cryovials and store <1 year at −70° to −80°C or at −135° to −150°C for long-term storage (see Basic Protocol 7).

The coverslips in the shell vials can be used for immunofluorescence staining.

PROPAGATION OF INFLUENZA VIRUSES IN EMBRYONATED CHICKEN EGGS FROM VIRUS STOCKS

BASIC PROTOCOL 2

In the past, influenza viruses were often isolated in embryonated eggs; however, today, the majority of laboratories use cell culture due to availability and ease of isolation of some contemporary human influenza strains in mammalian cells versus embryonated eggs. Nevertheless, many laboratory-adapted viruses and all vaccine strains are egg-grown and may be propagated readily in 10- to 11-day-old embryonated chicken eggs.

Materials

Influenza virus stock
Egg diluent: antibiotic-supplemented KPBS or tryptose phosphate broth (see recipes)
10- to 11-day-old embryonated chicken eggs (see Support Protocol 2)
70% ethanol
Glue (Elmers), nonsterile
22-G, 1 $^1/_2$-in. and 18-G, $^1/_2$-in. needles
1-ml syringe
33° to 35°C incubator
Forceps, sterile
10-ml pipets
50-ml plastic concical tubes
2-ml cryovials, sterile (Nunc)
Additional reagents and equipment for performing a hemagglutination test (see Basic Protocol 3)

NOTE: All equipment and solutions coming into contact with eggs must be sterile and proper sterile technique should be used accordingly.

Inoculate eggs

1. Dilute influenza virus stock in egg diluent.

   The concentration of the seed stock will determine how dilute the inoculum should be. Typically, a 1:100 to 1:1000 dilution of virus will yield optimal virus growth (~10^4 to 10^6 50% egg infectious dose (EID_{50}) or 0.01 to 0.1 HAU of virus).

   Either KPBS or tryptose phosphate broth–based diluent can be used in eggs.

2. Arrange three eggs with the airsac up. Spray tops of eggs with 70% ethanol.

   Three to five eggs are recommended to produce sufficient volume of allantoic fluid for future use. Also, eggs may die during the incubation time due to bacterial contamination, virulence of inoculated virus, etc., therefore, the more eggs inoculated, the better the chance to obtain a viable stock of virus. One egg produces 3 to 10 ml allantoic fluid and 0.1 to 1 ml amniotic fluid.

   Allow the alcohol to evaporate before proceeding to next step.

3. Punch a small hole in the shell over the air sac using an 18-G, $^1/_2$-in. needle and dispose of needle in an appropriate sharps container.

4. Aspirate 0.6 ml diluted virus sample into a 1-ml syringe with a 22-G, $1^1/_2$-in. needle.

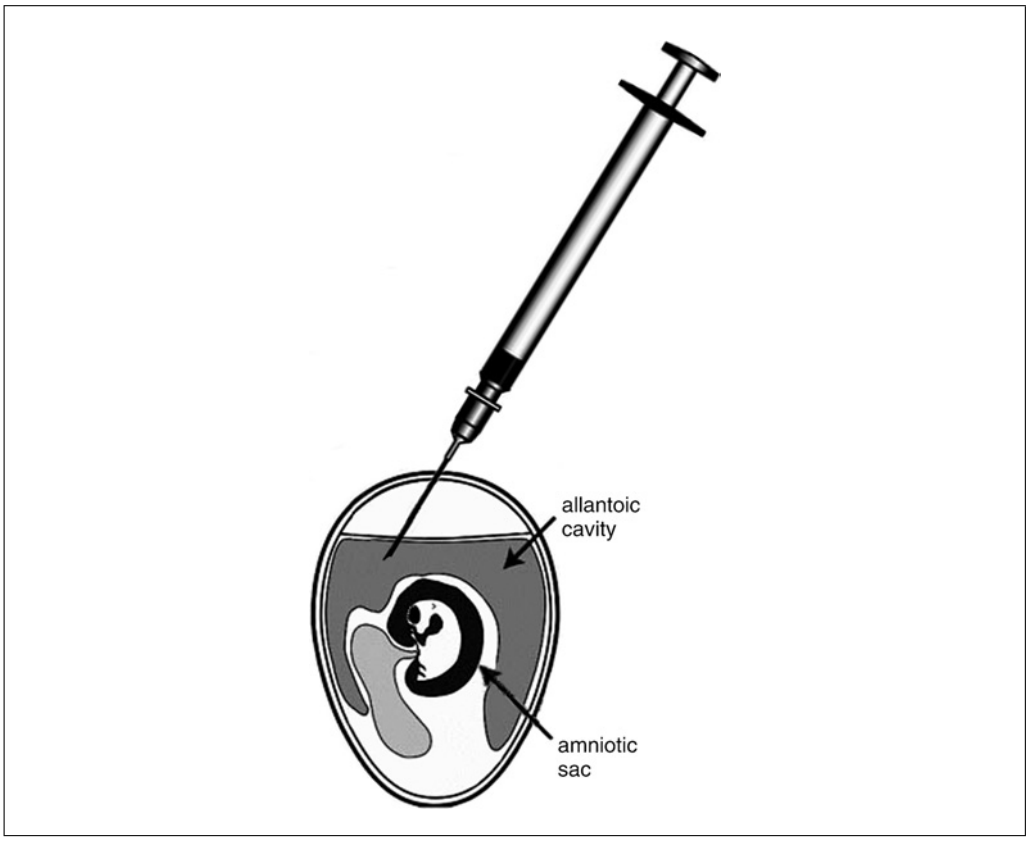

Figure 15G.1.1 Inoculation of a chicken egg with influenza virus.

5. Insert the needle at a 45° angle into the allantoic cavity and inoculate 0.2 ml influenza virus dilution; repeat with the other two eggs (Fig. 15G.1.1).
6. Discard syringe and needle into a sharps safety container.
7. Seal the hole punched in the eggshell with a drop of glue.

   Care should be taken not to contaminate glue in bottle with virus.

8. Incubate human influenza A virus for 48 hr at 33° to 35°C and influenza B viruses for 72 hr at 33° to 35°C.

Harvest virus from infected eggs

9. Chill eggs overnight (8 to 24 hr) at 4°C to halt embryo viability and minimize the flow of blood into the allantoic fluid during harvest.

   Eggs may be quick-chilled for 30 min in a −20°C freezer. However, this may cause the allantoic fluid to contain some blood.

10. With sterile forceps, break the shell over the air sac and push aside the allantoic membrane with the forceps, taking care not to break the yolk.
11. Using a 10-ml pipet, aspirate the allantoic fluid and place in a labeled 50-ml plastic conical tube. Dispose of eggs in an appropriate biological waste container.
12. Centrifuge tubes 5 min at 500 × *g*, 4°C, to pellet any blood cells and tissue fragments. Transfer clarified fluid into fresh 50-ml tube. Keep on ice (4°C).
13. Perform a hemagglutination test (see Basic Protocol 3).
14. Dispense allantoic fluid into 2-ml aliquots in 2-ml sterile cryovials and store <1 year at −70° to −80°C or at −135° to −150°C for long-term storage (see Basic Protocol 7).

PROPAGATION OF INFLUENZA VIRUS IN EMBRYONATED CHICKEN EGGS FROM PRIMARY CLINICAL SPECIMENS

With original clinical material, the amniotic sac is inoculated at the same time as the allantoic cavity. Virus inoculated into the amniotic sac that surrounds the embryo can replicate directly in the embryonic tissue and the resulting virus is released into the amniotic fluid. However, the amniotic sac will yield only a small volume of fluid and thus subsequent passage into the allantoic cavity may be required to produce sufficient volume for testing.

Additional Materials *(also see Basic Protocol 2)*

Primary clinical specimen (see Support Protocol 3)
Egg candler (KUHL)

1. Dilute primary clinical specimen in egg diluent.

   The concentration of the seed stock will determine how dilute the inoculum should be. Typically, an original specimen will be diluted 1:10 to achieve optimal virus growth.

2. Inoculate three eggs per specimen; label eggs to clearly identify original sample.
3. Punch a small hole in the shell over the air sac using an 18-G, $^1/_2$-in. needle and dispose of needle in appropriate sharps container.
4. Aspirate 0.6 ml clinical specimen into a 1-m syringe with a 22-G, $1^1/_2$-in. needle.
5. Using an egg candler apparatus, hold the egg up to light source and locate the embryo.
6. Insert the needle into the amniotic sac and inoculate 0.1 ml primary clinical specimen into the sac. Withdraw the needle about $^1/_2$-in. and inoculate 0.2 ml primary clinical specimen into the allantoic cavity (Fig. 15G.1.1).
7. Repeat with remaining clinical specimen and eggs.
8. Discard syringe and needle into a sharps safety container.
9. Seal the holes punched in the egg shells with a drop of glue.

   Care should be taken not to contaminate glue in bottle with virus.

10. Incubate the eggs for 48 to 72 hr at 33° to 37°C.

    The incubation temperature is dependent on the virus being grown (i.e., influenza A at 37°C and influenza B at 33°C).

11. Harvest the allantoic fluid as described in Basic Protocol 2, steps 9 through 11. Keep tubes on ice (4°C).
12. Invert the egg so that the embryo and amniotic sac hang down and are clearly visible and separate from the egg yolk. Using a 1-ml syringe and 22-G, 1 $^1/_2$-in. needle, pierce the air sac and remove as much amniotic fluid as possible from around the embryo. Dispose of eggs in an appropriate biological waste container.

    Keep allantoic and amniotic samples separate as the amniotic fluid may contain more virus than the allantoic fluid. The volume of allantoic fluid recovered may vary from 5 to 10 ml, while the volume of the amniotic fluid recovered may vary from 0.1 ml to 1 ml.

    The amniotic cavity provides a richer source of susceptible cells for virus replication and is used to help viruses from primary specimens grow more efficiently.

13. Centrifuge tubes 5 min at 500 × *g*, 4°C, to pellet any blood cells and tissue fragments. Transfer clarified fluid into fresh tube. Keep tubes on ice (4°C).

14. Perform a hemagglutination test on both the allantoic and amniotic fluids. (see Basic Protocol 3).

    If hemagglutination test is negative or titer is <8 HAU, passage the specimen two more times before reporting inability to recover virus from the specimen. If HA test results in titers >8 HAU, specimens can be dispensed into aliquots and stored.

15. Dispense allantoic and amniotic fluid into 2-ml aliquots in 2-ml sterile cryovials and store <1 year at −70° to −80°C or at −135° to −150°C for long-term storage. (see Basic Protocol 7).

BASIC PROTOCOL 3

QUANTIFICATION OF INFLUENZA VIRUSES BY HEMAGGLUTINATION ASSAY

Hemagglutination, or the ability to bind red blood cells, is a property of all influenza viruses that can be utilized as a rapid assay for determining the presence of virus in samples. Because the hemagglutination (HA) assay is dependent on the amount of hemagglutinin on the surface of influenza viruses and not the ability of the virus to replicate, this assay quantifies viral particles regardless of their infectivity. The highest dilution of virus that causes complete hemagglutination is considered the HA titration end point. The HA titer is the reciprocal of the dilution of virus in the last well with complete hemagglutination. A "unit" of hemagglutination is not a measure of an absolute amount of virus, but is an operational unit dependent on the method used for HA titration. An HA unit (HAU) is defined as the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension. This protocol uses turkey red blood cells as an example as these cells can be agglutinated by recent human influenza viruses as well as laboratory-adapted strains of human origin. Refer to Support Protocol 4 for a discussion of other types of red blood cells that can be used for hemagglutination assays. Hemagglutination units can be utilized when using inactivated influenza in experiments. For determining infectious units, use the 50% tissue culture infectious dose assay (see Basic Protocol 4), 50% egg infectious dose assay (see Basic Protocol 5), or the plaque assay (see Basic Protocol 6).

Materials

Phosphate-buffered saline (PBS) containing potassium (*APPENDIX 2A*)
Influenza virus stock (e.g., allantoic or amniotic fluid)
Standardized turkey red blood cells (see Support Protocol 4)
96-well V-bottom microtiter plate (Nunc)

NOTE: Keep virus stock on ice (4°C) during the HA test to maintain virus infectivity.

1. Pipet 50 µl PBS into wells 2 through 12 across a 96-well V-bottom plate (Fig. 15G.1.2).
2. Pipet 100 µl influenza virus stock into the first column of the 96-well plate.
3. Perform a two-fold dilution series across the 96-well plate by transferring 50 µl between wells, disposing of the final 50 µl from the last well.
4. Add 50 µl standardized turkey red blood cells to all wells. Tap the plate gently to mix.

   Approximately 5 ml standardized turkey red blood cells will be needed per 96-well plate used in this assay.

5. Incubate 96-well plate for 30 min at room temperature (24° to 27°C).

   Avian red blood cells in V-well microtiter plates require a 30-min incubation at room temperature, whereas mammalian red blood cells require a U-well microtiter plate with a 60-min incubation at room temperature.

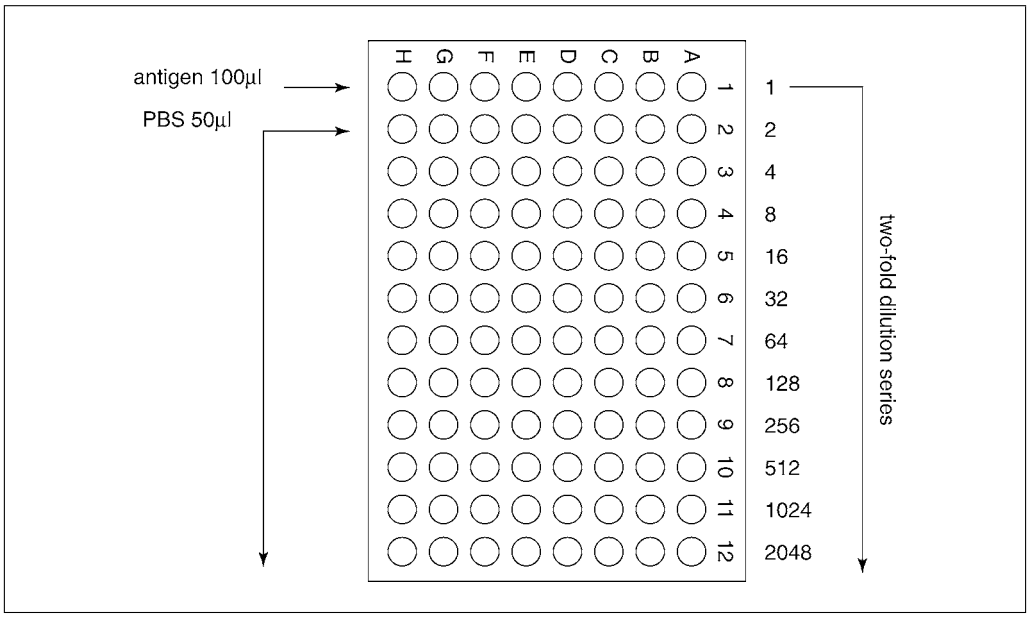

Figure 15G.1.2 Plate layout for hemagglutination titration assay.

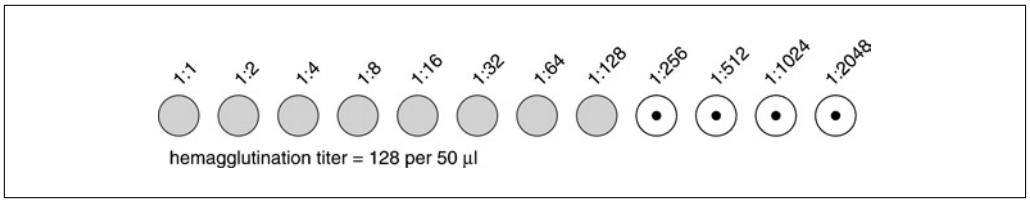

Figure 15G.1.3 Reading a hemagglutination titration assay plate. Positive samples will look pink as the red blood cells are held in solution by the virus. Negative samples will look clear with a red dot, since the red blood cells settle to the bottom of the V-bottom plate.

6. Observe endpoint of agglutination and record titer per 50 µl of sample (Fig. 15G.1.3).

   Red blood cells will settle to the bottom of the V-bottom well in negative samples, while red blood cells will agglutinate in positive samples. The endpoint should be read as the last well showing complete agglutination.

7. Store virus stocks with desired HAU titers (see Alternate Protocol 4, step 14). Dispose of materials in an appropriate biological waste container.

QUANTIFICATION OF INFLUENZA VIRUSES BY 50% TISSUE CULTURE INFECTIOUS DOSE ASSAY

BASIC PROTOCOL 4

The 50% tissue culture infectious dose ($TCID_{50}$) assay is a method to measure the amount of infectious virus in a sample by determining the highest dilution of the sample that can infect 50% of cells in culture. In this procedure, the virus sample is diluted across a 96-well tissue culture plate containing MDCK cells. The titration should be performed in quadruplicate.

Materials

MDCK cells in 96-well tissue culture plate (see Support Protocol 1)
Influenza virus growth medium (see recipe)
Influenza virus stock

96-well tissue culture plate (Costar)
Inverted microscope

NOTE: All equipment and solutions coming into contact with cells must be sterile and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a 37°C, 5% CO_2 humidified incubator.

1. Remove MDCK growth medium from plate and wash cells by adding 350 µl influenza virus growth medium to all wells. Aspirate the medium and repeat washing step. Care should be taken not to disrupt the cell monolayer.
2. Add 100 µl influenza virus growth medium to all wells, except the first column, of a 96-well tissue culture plate.
3. Thaw influenza virus stock and dilute 1:100 in influenza virus growth medium.
4. Add 146 µl diluted virus to first column of 96-well tissue culture plate and perform a $^1/_2$ $\log_{10}$ dilution series by transferring 46 µl between wells, disposing of final 46 µl after the eleventh column. Do not add virus to the final column as this is the cell control for the assay.
5. Incubate plates 2 hr at 37°C.
6. Remove inoculum and gently wash once with 250 µl influenza virus growth medium.
7. Add 200 µl influenza virus growth medium to all wells and incubate up to 72 hr at 37°C, observing for endpoints in cytopathic effect (CPE).

   Typical CPE by influenza viruses include rounding up of infected cells and detachment from culture flask.

8. Examine wells for presence or absence of CPE using an inverted microscope.

   To confirm results, 50 µl cell culture supernatant can be harvested into a 96-well V-bottom microtiter plate and tested for presence of virus by performing a spot HA assay (see Basic Protocol 5).

9. Record endpoint of CPE and determine $TCID_{50}$ titer per 100 µl using the Reed-Muench method (see Support Protocol 5).

BASIC PROTOCOL 5

QUANTIFICATION OF INFLUENZA VIRUSES BY 50% EGG INFECTIOUS DOSE ASSAY

The 50% egg infectious dose (EID_{50}) assay is a method to measure the amount of infectious virus in a sample by determining the highest dilution of the sample that can infect 50% of eggs. In this procedure, the virus sample is serially diluted prior to inoculation of eggs and a hemagglutination test is performed on undiluted allantoic fluid to determine the presence of virus in the eggs.

Materials

Egg diluent (see recipe)
Influenza virus stock
10- to 11-day-old embryonated chicken eggs (see Support Protocol 2)
Standardized turkey red blood cells (see Support Protocol 4)
1.5-ml microcentrifuge tubes, sterile
Forceps, sterile
96-well V-bottom plate (Nunc)
Additional reagents and equipment for inoculating eggs (see Basic Protocol 2)

NOTE: All equipment and solutions coming into contact with embryonated eggs must be sterile and proper sterile technique should be used accordingly.

1. Label ten 1.5-ml microcentrifuge tubes 1 through 10 and fill each with 450 µl egg diluent.
2. Pipet 50 µl influenza virus stock into 450 µl egg diluent in the first tube and perform a ten-fold dilution down to 10^{-10} by transferring 50 µl between tubes.

   Changing tips between tubes will decrease the chance of sample carryover and will result in a more accurate titration of the virus.

3. Inoculate three eggs with 100 µl per dilution as described in Basic Protocol 2, steps 1 to 8. Incubate eggs 48 hr at 35°C (optimal for influenza A) or 72 hr at 33°C (optimal for influenza B).

   If the type of virus is unknown, incubate eggs at 35°C.

4. Chill eggs overnight at 4°C or for 30 min at −20°C before harvesting.
5. With sterile forceps, break the shell over the air sac and push aside the allantoic membrane with the forceps.
6. Pipet 50 µl allantoic fluid from each egg to a corresponding well in a 96-well V-bottom plate.

   V-bottom or U-bottom plates are used to allow for better settling of the red blood cells.

7. Add 50 µl standardized turkey red blood cells to all wells and incubate 30 min at room temperature (24° to 27°C).

   Approximately 5 ml standardized turkey red blood cells will be needed per 96-well plate used in this assay.

8. Observe endpoint of agglutination and record.

   Red blood cells will settle to the bottom of the V-bottom well in negative samples, while red blood cells will agglutinate in positive samples.

9. Determine the EID_{50} titer per 100 µl by the Reed-Muench method (see Support Protocol 5).

QUANTIFICATION OF INFLUENZA VIRUSES BY PLAQUE ASSAY

BASIC PROTOCOL 6

Because influenza viruses cause cytopathic effect (CPE) and death of the infected cells, they may form plaques or circular zones of lysed cells on a monolayer. The plaque assay is a method to measure the amount of infectious virus in a sample by determining the number of plaque forming units on a MDCK cell monolayer. At a high dilution of virus stock, each plaque represents the zone of cells infected by a single virus particle. Therefore, the titer of a virus stock can be calculated in plaque forming units per milliliter. The titration should be carried out in duplicate.

Materials

2× plaque assay medium (see recipe)
1.6% (w/v) agarose solution
Influenza virus stock
Madin-Darby canine kidney (MDCK) cells confluent in 6-well tissue culture plates (see Support Protocol 1)
Plaque assay wash medium (see recipe)
2 mg/ml TPCK-trypsin working stock (see recipe)
70% ethanol
0.3% crystal violet solution
37° and 56°C water baths
Forceps, sterile
Inverted microscope

NOTE: All equipment and solutions coming into contact with cells must be sterile and proper sterile technique should be used accordingly.

1. Before beginning the plaque assay, warm 2× plaque assay medium in a 37°C water bath and place 1.6% agarose solution in a 56°C water bath.

   The 2× plaque assay medium should be warm enough so as not to solidify the 1.6% agarose solution when mixed together; also it should not be too hot, which will kill the MDCK cell monolayer when the media solution is added. The 1.6% agarose solution should be kept at 56°C to keep the solution from solidifying before use. When both solutions are mixed together the temperature of the resulting solution should be suitable for immediate addition to the MDCK cell monolayer while maintaining cell viability.

2. Thaw vial of influenza virus stock in cool water.

   Keep virus at 4°C once thawed to reduce loss of infectivity.

3. Remove the MDCK growth medium from the 6-well tissue culture plates and wash the MDCK monolayer three times with room temperature plaque assay wash medium.

   For wash steps, either a sterile 10-ml pipet or 1000-µl pipettor may be used to remove and add the medium. Do not pipet medium directly onto the monolayer as this may disrupt the cells.

   Fetal bovine serum (FBS) inhibits viral entry and must be removed for efficient infection of cells.

4. Perform a ten-fold dilution series starting at 10^{-1} and diluting virus samples down to 10^{-10} in plaque assay wash medium. Change disposable pipet or pipet tips between tubes to decrease the chance of sample carryover.

5. Inoculate 6-well tissue culture wells in duplicate with 100 µl diluted virus sample and rotate tissue culture plate to cover monolayer with inoculum. Take care not to add medium directly onto the monolayer as this may disrupt the cells.

6. Incubate inoculated plates for a minimum of 30 min or up to 1 hr at 37°C.

   Incubation for >1 hr may cause the MDCK cell monolayer to dry out and result in reduced cell viability.

7. Wash wells two times with room temperature plaque assay wash medium.

   For wash steps, either a sterile 10-ml pipet or 1000-µl pipettor may be used to remove and add the medium. Do not pipet medium directly onto the monolayer as this may disrupt the cells.

   Inoculum does not need to be removed before washing MDCK cell monolayer.

8. Add 1 µl of 2 mg/ml TPCK-trypsin working stock to 2× plaque assay medium before proceeding to the next step.

   TPCK-trypsin should not be added to the 2× plaque assay medium prior to this step to ensure optimal enzymatic activity.

9. Mix 1:1 2× plaque assay medium with 1.6% agarose solution and immediately add 2 ml of agarose medium solution to each inoculated well. Let solidify at room temperature (24° to 27°C).

   For one 6-well tissue culture plate, mix together 7 ml of 2× plaque assay medium with 7 ml of 1.6% agarose solution.

10. Incubate 6-well tissue culture plates at 37°C. Use an inverted microscope to observe the MDCK monolayer for plaque formation daily.

    Plaques will appear as small clear areas in the monolayer.

11. After 72 hr, carefully remove the agar plug from each well using sterile forceps and disposing of the agar in a biological waste container. Take care to avoid scratching the MDCK cell monolayer or letting the agar plugs rotate during removal, as this will make it difficult to accurately count plaques.
12. Pipet 2 ml of 70% ethanol into each well and incubate 20 min at room temperature (24° to 27°C) to fix the MDCK cell monolayer.

    Do not pipet 70% ethanol directly onto the monolayer as this may disrupt the cells.
13. Remove ethanol and add 1 ml crystal violet solution to each well. Incubate 10 min at room temperature (24° to 27°C) to stain MDCK cell monolayer.
14. Remove crystal violet solution and wash wells with water to rinse away excess stain solution.
15. Let plates dry overnight at room temperature (24° to 27°C) before counting plaques.
16. Count plaques in each well and determine the plaque forming units (pfu) per milliliter using the following formula: pfu/ml = (no. of plaques × dilution factor × 10).

    Plaques suitable for counting will appear as discrete clear circular zones on the background of the purple stained MDCK cell monolayer. At low dilution of virus, the monolayer may be completely destroyed, resulting in no or minimal purple staining in the wells. The pfu/ml calculation should be based on the dilution of virus sample that gives >10 plaques per well but is still countable (i.e., <150 plaques).

STORAGE OF INFLUENZA VIRUSES

BASIC PROTOCOL 7

Clinical specimens for viral isolation should be placed at 4°C and transported to the laboratory promptly. If clinical specimens are to be transported to the laboratory within 2 days, the specimens may be kept at 4°C; otherwise they should be frozen at or below −70°C until transported to the laboratory. Influenza virus stocks should be stored at −70° to −80°C and thawed just before use. For long-term storage, virus stocks should be kept at −135°C or in liquid nitrogen (−150°C). Once thawed, virus stocks should be kept on ice/4°C and used that day. To prevent loss of infectivity, repeated freezing and thawing must be avoided.

LYOPHILIZATION OF INFLUENZA VIRUSES

ALTERNATE PROTOCOL 5

Influenza viruses can be lyophilized for stable long-term storage at 4°C. Stabilizers need to be added to viruses grown in tissue culture, whereas viruses grown in eggs do not require an additional stabilizer. Make a 1:1 solution of tissue culture–grown virus stock with a 5% milk solution (5 g powdered dairy milk in 100 ml PBS, filter sterilize using a 0.45-μm filtration unit). Once virus stocks are properly stabilized, follow the manufacturer's instructions for lyophilization procedures specific to the equipment available. The lyophilization process may cause a loss of infectivity of the virus stock. Therefore, lyophilization should only be used if lower viral titers will not adversely affect assays or no other long-term storage option is available.

GROWTH AND MAINTENANCE OF MDCK CELL LINE

SUPPORT PROTOCOL 1

Madin-Darby canine kidney (MDCK) cells (ATCC# CCL-34) are the preferred cell line for the isolation and characterization of influenza A and B viruses, but not influenza C viruses due to the incompatibility of sialic acid moieties on the cell surface with the viral receptor specificity. This protocol describes how to maintain the MDCK cell line for use in the cell-based protocols presented in this unit.

Materials

Madin-Darby canine kidney (MDCK) cells (ATCC# CCL-34) grown confluent in a 75-cm^2 flask (see Support Protocol 1)
Trypsin/EDTA (GIBCO)
Heat-inactivated fetal bovine serum (FBS; *APPENDIX 2A*)
MDCK growth medium (see recipe)
75- or 25-cm^2 tissue culture flasks; or 6- or 96-well tissue culture plates (Corning)
Additional reagents and equipment for counting cells using a hemacytometer (*APPENDIX 4A*)

NOTE: All equipment and solutions coming into contact with cells must be sterile and proper sterile technique should be used.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator.

1. Decant growth medium from a confluent 75-cm^2 flask of MDCK cells, and add 5 ml trypsin/EDTA prewarmed to 37°C.
2. Gently rock the flask to distribute the trypsin/EDTA over the entire MDCK cell monolayer. Remove the trypsin/EDTA with a pipet.
3. Repeat one time with an additional 5 ml of trypsin/EDTA.
4. Add 1 ml prewarmed trypsin/EDTA, ensuring that entire cell monolayer is bathed in trypsin/EDTA and incubate flask at 37°C until all cells detach from the plastic surface (~5 to 10 min).

   Gentle shaking or tapping of the flask may help cells detach.

5. Add 1 ml FBS to inactive trypsin/EDTA.
6. Add 8 ml MDCK growth medium to flask, pipetting gently to break up clumps of cells.
7. Remove cell suspension and centrifuge 10 min at 200 × *g*, 4°C.
8. Resuspend cell pellet in 2 ml MDCK growth medium and count cells with a hemacytometer (*APPENDIX 4A*). Adjust the concentration accordingly.

   For 75-cm^2 flasks, 5 ml of a 1 × 10^5 cells/ml suspension added to 20 ml of MDCK growth medium will result in a confluent monolayer in 2 to 3 days. For 96-well tissue culture plates, 100 µl of 1.5 × 10^5 cells/ml suspension per well will result in a confluent monolayer after an overnight (18- to 22-hr) incubation.

9. Incubate flasks in a 37°C incubator.

   MDCK cell lines should not be passaged indefinitely. Relatively low passage number (e.g., 20 to 30 passages) after establishing the line from frozen stocks will ensure that cells retain their susceptibility to respiratory viruses. As such, a working stock of low-passage cells should be kept in liquid nitrogen as a source for renewing the cell line. For optimum results, the cells should be in log growth phase.

SUPPORT PROTOCOL 2

CANDLING 10-DAY EMBRYONATED CHICKEN EGGS

Candling chicken eggs is a standard procedure performed to ensure that only healthy embryonated eggs are used for the egg-based protocols presented in this unit. Use of dead, broken, or nonembryonated eggs will result in little to no virus growth and as such should be detected and removed from the supply of eggs to be used in experiments. Eggs should be used at an age of 9 to 11 days, because the 10-day-old chick embryo is old enough to support efficient virus growth and optimal volumes of fluid containing virus may be recovered. In addition, embryonated eggs <12 days of age have not yet developed adequate innate immunity competency, which can inhibit virus growth.

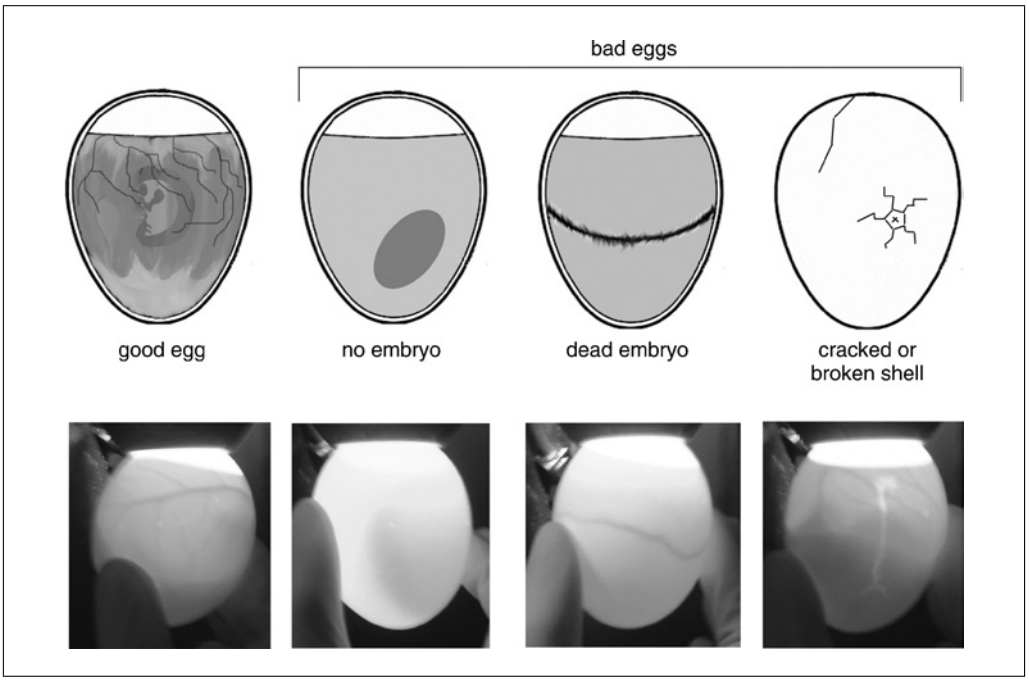

Figure 15G.1.4 Viable and nonviable chicken eggs as apparent from candling. This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.currentprotocols.com*

In a darkened room, hold the airsac end of the egg to the candler light source and gently rotate it while observing the morphology of the chick embryo. A healthy embryo has a well-defined airsac, free-flowing membranes, uniform vein development and reacts to the light of the candler. It is very important to screen out broken, unhealthy, very porous, or dead embryos, as these eggs will not support efficient influenza virus growth. Figure 15G.1.4 depicts viable and non-viable chicken eggs as viewed by a KUHL candler. Alternatively, refer to the instruction manual of the egg candler for more instructions.

NOTE: Egg incubations are performed in a 34°C, 50% humidity incubator with rotation for eggs <9 days old. Cell culture incubators can be used for eggs >9 days old, but the CO_2 must be turned off or the embryos will suffocate.

PREPARATION OF PRIMARY INFLUENZA VIRUS SPECIMENS

SUPPORT PROTOCOL 3

The success of virus diagnosis largely depends on the quality of the specimen and the conditions for transport and storage of the specimen before it is processed in the laboratory. Specimens for isolation of respiratory viruses in cell cultures and for the direct detection of viral antigens or nucleic acids should generally be taken during the first 3 days after onset of clinical symptoms.

Materials

Specimen in collection vials (e.g., from nasal, throat, or combined nasal/throat swabs; or nasopharyngeal, nasal, or throat aspirates or washings)
10 mg/ml gentamicin (GIBCO)
2- to 4-mm glass beads (VWR)
Polypropylene microcentrifuge tubes, sterile
Cryovials (Nunc)

NOTE: Clarified supernatants can be used to directly inoculate cell culture flasks or eggs.

From nasal, throat, or combined nasal/throat swabs:

1a. Vortex collection vial and express the fluid in the swab.

2a. Remove swab from the collection vial and add 0.2 ml of 10 mg/ml gentamicin.

3a. Incubate 15 min at room temperature (24° to 27°C).

4a. Centrifuge collection vial 5 min at 400 × *g*, 4°C.

5a. Remove the clarified supernatant, dispense into 0.5- to 2-ml aliquots, and store <1 year at −70° to −80°C or at −135° to −150°C for long-term storage (see Basic Protocol 7).

From nasopharyngeal, nasal, or throat aspirates or washings:

1b. Break clumps of mucus by adding 2- to 4-mm glass beads to the specimen and vortexing.

2b. Add 0.1 ml of 10 mg/ml gentamicin/ml of specimen.

3b. Transfer the specimen to a clean tube and centrifuge 10 min at 400 × *g*, 4°C.

4b. Remove the clarified supernatant, dispense into 0.5- to 2-ml aliquots, and store <1 year at −70° to −80°C or at −135° to −150°C for long-term storage (see Basic Protocol 7).

SUPPORT PROTOCOL 4

STANDARDIZATION OF RED BLOOD CELLS FOR HEMAGGLUTINATION-BASED ASSAYS

Human influenza A, B, and C viruses agglutinate red blood cells (RBC) of several avian and animal species as they bind sialic acid on the surface of red blood cells. Natural variation of circulating human influenza viruses and repeated passaging of specimens in the laboratory can result in the alteration in the ability of the virus to agglutinate certain species' RBC. As such, samples may need to be tested in hemagglutination-based assays using RBCs from different species of animals before determining that no virus is present. Table 15G1.1 outlines the species-specific concentration of RBC, type of microtiter plate, incubation time, and appearance of control cells when used to detect influenza A and B viruses in these assays. Accurate determination of RBC concentration is necessary as low concentration will overestimate and high concentration will underestimate the concentration of virus. Also, standardization of RBC concentration is critical for obtaining reproducible results between assays. This support protocol is commonly used for standardization of RBC regardless of species origin.

Materials

Whole blood mixed 1:1 in Alsever's solution (*APPENDIX 2A*)
Phosphate-buffered saline (PBS) containing potassium (*APPENDIX 2A*)
Cotton gauze, sterile
50-ml conical centrifuge tube

Table 15G.1.1 Properties of RBC from Different Species

	Chicken	Turkey	Guinea pig	Human type O
Concentration	0.5%	0.5%	0.75%	0.75%
Microtiter plate well shape	V	V	U	U
Incubation time at 25°C	30 min	30 min	1 hr	1 hr
Appearance of control cells	Button	Button	Halo	Halo

Additional reagents and equipment for counting cells using a hemacytometer (*APPENDIX 4A*)

NOTE: All equipment and solutions coming into contact with cells must be sterile and proper sterile technique should be used.

1. Filter ~5 ml whole blood through a piece of sterile cotton gauze into a 50-ml conical centrifuge tube. Centrifuge tube 10 min at 200 × *g*, 4°C.
2. Aspirate the plasma and buffy layer. Add 50 ml PBS and mix gently.
3. Centrifuge 5 min at 200 × *g*, 4°C, and aspirate supernatant. Repeat PBS washes two additional times.
4. Resuspend RBC to final volume of 12 ml with PBS. Centrifuge 10 min at 200 × *g*, 4°C.
5. Estimate volume of packed RBC and dilute to approximate concentration with PBS.
6. Determine actual concentration of RBC with a hemacytometer (*APPENDIX 4A*) and adjust the concentration accordingly.

The final concentration of chicken and turkey RBC is 0.5%. Guinea pig and human "O" RBC is 0.75%. A higher concentration of guinea pig and human type O RBC is desirable for complete settling of RBC and hence optimal discrimination between agglutination and non-agglutination.

CALCULATION OF INFECTIOUS DOSE 50 TITERS BY THE REED-MUENCH METHOD

SUPPORT PROTOCOL 5

The Reed-Muench method (Reed and Muench, 1938) can be utilized to determine the dilution of virus sample required to yield a 50% positive result. The advantage to using this method rather than a 100% endpoint is that the 50% endpoint is less affected by variations within the titration experiment. It is important to note that the unit of infectivity measured by this endpoint method may require more than one infectious particle. With the Reed-Muench method, it will be necessary to use a large number of small groups of test samples at different dilutions (see Basic Protocols 4 and 5 for set up of titration experiments). At the end of the titration experiment, determine the number of positive and negative samples at each dilution. Using these numbers, a percentage of infected samples can be calculated for each dilution. The dilution that would infect 50% of test samples is estimated using the following formulas:

$$\text{proportional distance formula} = [(\%\ \text{positive value} > 50\%) - 50\%]/[(\%\ \text{positive value} > 50\%) - (\%\ \text{positive value} < 50\%)]$$

Knowing the proportional distance between dilutions, the 50%-endpoint can be calculated using the exact dilutions used:

$$\text{log infectious dose } 50 = (\text{log dilution} > 50\%) + (\text{proportional distance} \times \text{log dilution factor})$$

The reciprocal of this number is used to express the titer in infectious units per unit volume.

An example an ID_{50} calculation using the Reed-Muench method is shown in Figure 15G.1.5.

Log of virus dilution	Infected samples	Cumulative positive (A)	Cumulative negative (B)	Ratio of A/(A+B)	Percent infected
–5	3/3	6	0	6/6	100%
–6	2/3	3	1	3/4	75%
–7	1/3	1	3	1/4	25%
–8	0/3	0	6	0/6	0%

proportional distance: $\frac{(75\%) - 50\%}{(75\%) - (25\%)} = 0.5$

log infectious dose$_{50}$: $(-6) + (0.5 \times 1.0) = -6.5$

infectious dose$_{50}$ titer: $10^{6.5}$ ID_{50}/0.1ml or $10^{7.5}$ ID_{50}/ml

Figure 15G.1.5 Example ID_{50} calculation using the Reed-Muench method.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Antibiotic-supplemented phosphate-buffered saline containing potassium

Supplement 100 ml PBS containing potassium (KPBS; *APPENDIX 2A*) with:
1 ml penicillin-streptomycin stock (100 U/ml penicillin G and 100 μg/ml streptomycin)
0.2 ml 50 mg/ml gentamicin stock (10 μg/ml)
Filter sterilize with a 0.2-μm membrane
Store up to 2 months at 4°C

Complete Dulbecco's modified Eagle medium (cDMEM)/7.5% BSA

Supplement 465 ml DMEM with:
5 ml penicillin-streptomycin stock (100 U/ml penicillin G and 100 μg/ml streptomycin)
5 ml L-glutamine (2 mM)
12.5 ml 7.5% bovine serum albumin solution (0.2% BSA)
12.5 ml HEPES buffer (25 mM)
Filter sterilize with a 0.2-μm membrane
Store <2 months at 4°C

Influenza virus growth medium

Supplement 500 ml cDMEM/7.5% BSA with 0.5 ml TPCK-trypsin working stock for a final concentration of 2 μg/ml. Store up to 2 months at 4°C.

This medium is for use with MDCK cells only.

Influenza virus plaque assay medium, 2×

Supplement 455 ml 2× DMEM with:
10 ml penicillin-streptomycin stock (200 U/ml penicillin G and 200 μg/ml streptomycin)
10 ml L-glutamine (4 mM)
25 ml HEPES buffer (50 mM)
Filter sterilize with a 0.2-μm membrane
Store <2 months at 4°C

Influenza virus plaque assay wash medium

Supplement 490 ml DMEM with 5 ml of penicillin-streptomycin stock (100 U/ml penicillin G and 100 μg/ml streptomycin). Filter sterilize with a 0.2-μm membrane. Store up to 2 months at 4°C.

L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin working stock, 2 mg/ml

10 ml DMEM
20 mg trypsin, TPCK-treated, type XIII from bovine pancreas (Sigma)
Filter sterilize for up to 0.2-μm membrane
Dispense into aliquots
Store aliquots at −70°C until reagents expire

Dispose of unused thawed TPCK-trypsin, do not refreeze, as the TPCK-trypsin is not stable after thawing.

MDCK growth medium

Supplement 440 ml DMEM with:
5 ml penicillin-streptomycin stock (100 U/ml penicillin G and 100 μg/ml streptomycin)
5 ml L-glutamine (2 mM)
50 ml heat-inactivated (inactivated 30 min at 56°C) fetal bovine serum (10% FBS)
Filter sterilize with a 0.2-μm membrane
Store for up to 2 months at 4°C

Tryptose phosphate broth

Dissolve 29.5 g tryptose phosphate broth in 1 liter ddH_2O
Sterilize by autoclave
Store up to 3 months at 4°C
Just prior to use, add 2% gentamicin 50 mg/ml to broth

COMMENTARY

Background Information

Influenza A and B virus genomes consist of eight gene segments encoding at least ten proteins (Palese, 1977; Lamb and Krug, 2001). The major surface glycoproteins are the hemagglutinin (HA) and the neuraminidase (NA), which form the basis of multiple serologically distinct influenza A virus subtypes. There are 16 HA and 9 NA subtypes of influenza A viruses known to currently circulate in nature (Kilbourne, 1975; Webster et al., 1992; Rohm et al., 1996; Fouchier et al., 2005). Wild water birds are the natural reservoir for all influenza A viruses (Webster et al., 1992). In the last century, influenza A viruses bearing one of three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes underwent sustained circulation and caused widespread disease in humans (Wright and Webster, 2001). Since 1977, influenza viruses of the H1 and H3 and N1 and N2 subtypes as well as influenza B viruses have caused widespread, seasonal disease in humans.

The influenza A virus undergoes two kinds of antigenic variation, antigenic drift, and antigenic shift. Antigenic drift is the result of accumulation of point mutations in the HA and/or NA genes caused by transcriptional errors of the viral RNA polymerases that lack proofreading mechanisms (Scholtissek et al., 1993). Influenza variants with amino acid substitutions in antigenic sites are able to escape neutralization by existing host antibodies and emerge from this selective pressure to become the predominant viral population (Wright and Webster, 2001). Antigenic drift variants are responsible for annual epidemics of influenza A viruses (Wright and Webster, 2001). Alternatively, influenza A viruses can undergo antigenic shift, which occurs when there is reassortment of gene segments between viruses, resulting in the appearance of a new subtype of influenza A virus containing a novel HA with or without a novel NA (Wright and Webster, 2001). Such a novel strain can circulate in an immunologically naïve human population

and can potentially result in an influenza pandemic if able to spread efficiently (Wright and Webster, 2001).

Human influenza A viruses are transmitted directly between individuals via aerosolized droplets generated by coughing and sneezing or indirectly through contact with fomites on contaminated surfaces (Alford et al., 1966; Lidwell, 1974; Bean et al., 1982). Infection begins in the nasal and tracheal passageways, and rapidly spreads throughout the upper and lower respiratory tract. Apical shedding of virus from respiratory epithelial cells generally limits viral infection to the respiratory tract. Clinical symptoms of an acute influenza A virus infection can range from mild to severe and typically include fever, cough, headache, malaise, and anorexia. Each year in the United States, ~36,000 people, on average, die from complications of influenza virus infection, with 90% of these deaths being in the elderly population aged >65 years (Thompson et al., 2003).

Virus isolation is a highly sensitive and useful technique for the diagnosis of influenza virus infection when used with clinical specimens of good quality. In fact, isolation of a virus in cell culture along with subsequent identification by immunologic or genetic techniques or by electron microscopy are standard methods for virus diagnosis. One important advantage of virus isolation is that this method amplifies the virus from the original specimen and makes it available for further antigenic and genetic characterization, and also for drug-susceptibility testing if required. The propagation or use of laboratory-adapted strains or influenza strains of animal origin in laboratories also attempting isolation of viruses from human clinical specimens for diagnostic or research purposes is not recommended, since there is a risk of contaminating the diagnostic specimens with laboratory or animal strains with high growth phenotypes.

In the past, human influenza viruses often were isolated in embryonated eggs; however, today the majority of laboratories use cell culture. Since vaccine candidate viruses must be isolated in eggs, the trend to use cell cultures has decreased the availability of suitable vaccine viruses. For this reason, laboratories that have the capability to isolate influenza in eggs are encouraged to continue. However, some strains of influenza, and in particular, recent human field isolates, are difficult to isolate and grow in eggs, even after amniotic inoculation. For this and other reasons, MDCK cells are the preferred cell line for isolation of human influenza viruses from clinical specimens.

Critical Parameters and Troubleshooting

Viruses must be kept on ice/4°C at all times after thawing. Do not freeze-thaw viruses more than once, a substantial reduction in virus infectivity will result. Specimens for virus isolation should be refrigerated immediately after collection and inoculated into susceptible cell cultures as soon as possible. If the specimen cannot be processed within 48 to 72 hr, the specimen should be kept frozen at or below −70°C. If a working stock with a defined infectivity is desired, virus should be harvested, aliquoted, and frozen and the EID_{50} or $TCID_{50}$ should be determined on a vial that has been frozen and then thawed once. For routine passage of laboratory stocks, virus inoculum should be diluted in the range of at least 1:100, since inoculation of large amounts of virus may lead to formation of defective interfering (D.I.) virus particles, which can lower the overall infectivity of a virus stock (Azzi et al., 1993). D.I. particles are virus particles that are lacking most of their genome. Because of these deletions in their genome, D.I. particles cannot sustain an infection by themselves. Instead, they depend on co-infection with a suitable helper virus, which provides the gene functions that are absent from the D.I. particles.

The cell-based assays described herein are very dependent on the quality of MDCK cells. Over a number of passages, MDCK cells might lose their susceptibility to influenza viruses. For this reason, the laboratory should keep a stock of frozen cells at a low passage level and return to this stock to refresh the working laboratory stock at regular intervals, e.g., after 20 to 30 passages. Cell lines should be free of mycoplasma contamination (*APPENDIX 3B*) and should be routinely tested. Optimal conditions for virus growth require the MDCK cells to be confluent and in exponential growth phase. If the monolayer is overgrown, it is less sensitive to virus infection. In the plaque assay, if MDCK cells are not confluent, the resulting plaques will be diffuse and difficult to count. In contrast, if the MDCK cell monolayer is overgrown, patches of the monolayer may be removed with the agarose plug after the 72-hr incubation, resulting in the inability to visualize and count plaques.

Although many mammalian cell types may be infected with human influenza viruses, few support productive infection, i.e., the release of infectious progeny virus into the culture supernatant. This is due, in part, to the requirement for proteolytic cleavage of the HA molecule into two subunits, HA1 and HA2. This enables the HA molecule to undergo

a conformational change essential for membrane fusion in the endosome and the release of the nucleic acid allowing for replication to proceed. Although MDCK cells lack such an endogenous protease, addition of exogenous trypsin to the medium at a typical concentration of 1 to 2 μg/ml is sufficient for proteolytic cleavage of the HA and the generation of infectious progeny. The trypsin used to propagate the virus is heat stable L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. This should not be confused with the trypsin-EDTA solution used to remove cell monolayer from flasks during cell passage, which is not suitable as a source of trypsin for virus propagation. Each new working stock of TPCK-trypsin should be titered for optimal activity and lack of toxicity to the cell monolayer. The optimal concentration of trypsin will produce maximal amounts of virus in infected culture supernatants but will not result in destruction of uninfected monolayers over the period of incubation. If the trypsin concentration is too high, cells will round up and detach from the plastic, i.e., present with morphology similar to the cytopathic effect observed in virus-infected cultures. Fetal bovine serum reduces the virus infectivity and may also inhibit the activity of trypsin. Therefore, removal of all traces of serum contained in growth medium by gently rinsing monolayers multiple times is an important prerequisite for virus inoculation.

Ten- to eleven-day-old embryonated eggs are optimal for growth of influenza A and B viruses. It is generally convenient to purchase freshly fertilized eggs from a supplier and to incubate them in the laboratory for the required time. The use of an egg incubator that automatically turns the eggs several times a day, and maintains constant humidity and temperature is optimal, but a humidified CO_2 incubator that has the CO_2 turned off to avoid asphyxiating the embryos may be used successfully if eggs are turned manually several times a day. From 4 to 5 days post-fertilization, the embryos should be clearly visible. A low percentage of eggs should be expected to be nonviable and should not be used for inoculation. The percent of nonviable eggs may increase in the summer months in warmer climates. Viability can be determined prior to inoculation by candling eggs.

The methods described here provide a source of a quantified infectious influenza virus stock for use in many laboratory procedures. However, if large quantities of viral antigens are required, e.g., for use in ELISA or in vivo vaccine studies, virus will need to be concentrated and purified from a large volume (typically, ≥1 liter) of infected allantoic fluid or cell culture supernatant. General methods for concentration and purification of influenza virus have been described elsewhere (Arora et al., 1985).

Avian RBCs are generally used to detect influenza virus agglutination. Turkey RBCs are optimal for agglutination of recent human influenza viruses and can also be used for detection of common laboratory adapted strains, but mutations in or around the receptor-binding site of a virus may influence the efficiency with which a given virus binds to a given species of RBC. In some cases, guinea pig RBC or human type "O" cells can be more sensitive than avian RBC for detecting human strains of influenza. Upon passage in tissue culture, most strains will adapt to avian RBC agglutination. Blood should be received in Alsever's solution and can only be stored for 5 to 7 days, after which hemolysis will occur and substantially reduce the accuracy of the hemagglutination assay. Accurate determination of the RBC concentration is necessary as low concentrations will overestimate and high concentrations will underestimate the concentration of virus. The hemagglutination assay must be read promptly as the viral NA will eventually cleave the sialic acid from the RBC and release the HA from the cell. Some viruses may have heightened NA activity. In such cases, the hemagglutination assay should be carried out at 4°C.

Anticipated Results

The extent of influenza virus growth in either MDCK cells or embryonated eggs is strain-dependent. Human viruses isolated from primary clinical specimens may grow poorly in either eggs or tissue culture (titers of 4 to 16 HAU per 50 μl and titers of 10^7 ID_{50}/ml or 10^6 pfu/ml) until the virus has been adapted to grow in vitro. In contrast, many laboratory strains that have been passaged numerous times in eggs may achieve titers of 256 to >1024 HAU per 50 μl or 10^8 to 10^9 ID_{50}/ml or 10^7 to 10^8 pfu/ml. For antigenic characterization, hemagglutination units should be ≥8 HAU. In tissue culture, virus release into the supernatant may be readily monitored at regular intervals by removing a small amount to detect rising hemagglutination activity.

Adaptation of human influenza viruses to grow in eggs may select for variants that possess amino acid substitutions in the HA molecule that can also affect biological

properties (Katz et al., 1990). The acquisition of amino acid substitutions in the HA in viruses repeatedly passaged in MDCK cells has also been reported (Mochalova et al. 2003). The potential for such changes should be considered when repeatedly passaging influenza viruses in eggs or tissue culture and may be identified by sequence analysis.

Time Considerations

Human influenza A viruses must be incubated for 48 hr, whereas influenza B viruses require a 72-hr incubation period. Since clinical material should be blind-passaged at least twice, it may take up to 14 days before the results of virus diagnosis can be stated to be "virus not recovered." Optimal infectivity of a virus stock will be achieved when the virus is kept on ice/4°C during harvest, clarification of fluid and estimation of virus HAU, and then frozen immediately at or below −70°C. Viruses may be stored for several years at −70°C, but prolonged storage at this temperature will cause a reduction in virus viability. Incubation times for the hemagglutination assay must be followed to produce accurate results; 30 min for avian RBC and 1 hr for mammalian RBC at room temperature is recommended. The plaque assay requires 4 days; a half day to set up, a 72-hr incubation, and a half day to process and read plates. During the plaque assay setup, work quickly after mixing the warm 2× plaque assay medium with the 1.6% agarose solution, to ensure that the media mixture does not solidify prior to applying to 6-well tissue culture plates, but is not too hot for the MDCK cell monolayer. The optimal temperature of the overlay media should be in the range of 37°C to 42°C.

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Human Immunodeficiency Viruses: Propagation, Quantification, and Storage

Of the retroviruses, human immunodeficiency viruses (HIVs) have the largest human health impact. The two types of HIV, HIV-1 and -2, are the causative agents of acquired immune deficiency syndrome (AIDS). Since the epidemic began over 20 years ago, 20 million people have died from AIDS worldwide. In December 2004, nearly 40 million people were estimated to be infected with HIV (WHO, 2004).

HIV-1 is divided into three groups, M (major), N (new), and O (outlier). The vast majority of infections worldwide are caused by M-group viruses, which are subdivided into subtypes or clades depending on sequence diversity. Clades are termed A, B, C, D, F, G, H, and J. There are also recombinant viruses (circulating recombinant forms or CRFs) that are epidemic in particular regions, e.g., AE in Thailand, AB in Russia and the Ukraine, and BF in Argentina and Uruguay. Note that there are no clades E or I, since these subtypes were found to be recombinants with other clades. Protocols provided here are appropriate for all HIV-1 clades; however, as noted below, not all of these techniques can be applied to HIV-2.

HIV-1 requires interactions with CD4 and either CCR5 or CXCR4 coreceptors on the cell surface in order to infect cells. CXCR4-using (X4) variants can be isolated from ~50% of AIDS patients and are associated with a more rapid loss of $CD4^+$ T cells and faster disease progression (Asjo et al., 1986; Tersmette et al., 1989; Connor and Ho, 1994; Scarlatti et al., 1997). CXCR4 is more widely expressed on different $CD4^+$ T cell populations compared to CCR5, and X4 viruses thus have a broader T cell tropism (Ostrowski et al., 1999; Blaak et al., 2000). Nevertheless, CD4 depletion and AIDS occur in patients from whom only CCR5-using viruses can be isolated (de Roda Husman et al., 1999; Cecilia et al., 2000). CCR5-using (R5) viruses are the predominantly transmitted form, and individuals homozygous for a defective CCR5 gene (Δ32 CCR5) are substantially protected from infection (Carrington et al., 1999).

The protocols described in the following pages represent the basic tools for HIV research. Basic Protocols 1 to 5 describe methods to obtain and propagate the virus for various applications, while Basic Protocols 6 to 10 describe methods for quantitation of viral preparations. More specific or adapted protocols are beyond the scope of this unit, although relevant procedures are discussed briefly.

CAUTION: Regulations regarding HIV research vary around the world. Consultation with local Institutional Biosafety Committees or equivalent oversight body is strongly advised. Institutional Biosafety Committee review will usually be required prior to commencing work with HIV. In the laboratory, human immunodeficiency virus is most likely to be transmitted via cuts and skin abrasions. Removal of all sharp objects from the laboratory is essential. Disposable gowns and double gloves must be worn. HIV should be manipulated in a class II biosafety cabinet or a cabinet that provides equivalent or greater protection to the operator. In the United States, HIV is designated as a Biosafety Level 2 (BSL-2) pathogen provided that additional practices and containment equipment recommended for BSL-3 are used. However, for procedures that may generate high-infectivity titers of HIV, BSL-3 conditions are required. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

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CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

STRATEGIC PLANNING

Many HIV-1 primary isolates, replication-competent molecular clones, and HIV plasmid DNA constructs, as well as other reagents, are available via the NIH AIDS Research and Reference Reagent Program (*http://www.aidsreagent.org*) or from the European Centre for AIDS Reagents (*http://www.nibsc.ac.uk/catalog/aids-reagent*).

A number of protocols described in this unit require preparation of peripheral blood mononuclear cell (PBMC) or macrophage cultures from blood. Protocols for both of these procedures can be found in Kanof et al. (1996).

Uninfected $CD4^+$ cell lines can become contaminated with HIV. Contamination will result in cytopathic effects that include the formation of multinucleated giant cells (syncytia). However, the extent of cytopathicity depends on the cell line infected and the HIV strain involved. Chronically infected cell lines may not be readily distinguished from their uninfected counterparts by light microscopy. Uninfected cells should be cultured in a laboratory separate from the HIV laboratory. If this is not possible, uninfected cells should be cultured separately from infected cells and stored in separate incubators. Uninfected cells that have been cultured in an HIV laboratory should not be supplied to other laboratories.

PROPAGATION OF HUMAN IMMUNODEFICIENCY VIRUS

Infectious, replication-competent viruses are widely used for HIV research. Several types of infectious virus are used, including primary virus isolates, T cell line–adapted strains (TCLA), and viruses derived from molecularly cloned proviral DNA. Primary isolates are viruses derived from infected individuals by coculturing infected and uninfected PBMC together. Primary isolates can be further propagated by reinfecting PBMCs. $CD4^+$ T cell lines are used to culture laboratory-adapted HIV-1. The human embryonic kidney cell line (293T) is often used to produce virus or reporter viruses from molecularly cloned HIV constructs encoded by plasmid DNA.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 1

Isolation of HIV from Blood

Typically, HIV-1 is isolated by coculturing PBMC from an HIV^+ individual with uninfected donor PBMC. However, coculture of HIV^+ PBMC with primary macrophage cultures is an alternative that will select for macrophage-tropic HIV-1 strains.

Materials

Blood from infected individual
Complete RPMI medium/20% FBS (see recipe) containing 0.5 μg/ml phytohemagglutinin (PHA; Sigma; store in aliquots at −20°C and add to medium just before use)
Complete RPMI medium/20% FBS (see recipe) containing 20 U/ml interleukin 2 (IL-2; Roche Applied Science; store in aliquots at −20°C and add to medium just before use)
Blood from uninfected individual

Tabletop centrifuge (e.g., Sorvall Legend RT)
Plastic hemacytometer (preferred for safety reasons)
25- or 75-cm^2 tissue culture flasks
0.45-μm low-protein-binding filters (Millipore; optional)
Cryovials
Dry ice/ethanol bath
−80° or −152°C freezer

Additional reagents and equipment for preparing PBMC (Kanof et al., 1996), counting cells with a hemacytometer (*APPENDIX 4A*), and RT activity assay (Basic Protocol 9) or p24 ELISA (Basic Protocol 10)

Prepare infected PBMC

1. Isolate PBMC from infected individual's blood (Kanof et al., 1996). Dilute to 2–2.5 × 10^6 cells/ml in RPMI 1640 medium containing 20% FBS and 0.5 μg/ml PHA, to induce T cells to grow. Incubate for 2 days.

2. Centrifuge cells 5 min at 300 × *g* (1000 rpm), room temperature, without braking. Remove medium. Resuspend cells in RPMI 1640 medium containing 20% FBS and 20 U/ml IL-2. Incubate 2 days.

   The IL-2 also encourages T cells to grow.

Prepare coculture of infected and uninfected PBMC

3. Prepare PBMC from the blood of an uninfected individual (Kanof et al., 1996). Culture the cells under PHA stimulation for 2 days as described in step 1. Count uninfected PBMC in this preparation and infected PHA/IL-2-stimulated PBMC from the culture prepared in step 2 with a plastic hemacytometer (*APPENDIX 4A*).

4. Mix infected and uninfected PBMC at a 1:3 ratio to a final density of 5 × 10^6 cells. Centrifuge cells 5 min at 300 × *g* (1000 rpm), room temperature, without braking. Resuspend cells in 1 to 3 ml RPMI 1640 medium containing 20% FBS and 20 U/ml IL-2. Incubate for 3 hr.

5. Transfer cells into 25- or 75-cm^2 tissue culture flasks at a density of 1 × 10^6 cells/ml. Continue incubation.

   The size flask used will depend on the total volume of the culture.

Obtain viral supernatants

6. Monitor cultures daily for production of virus in the cell supernatant by reverse transcriptase (RT) activity assay (see Basic Protocol 9) or p24 ELISA (see Basic Protocol 10).

7. Every other day, count cells (*APPENDIX 4A*) and readjust the cell concentration to 1 × 10^6 cells/ml by adding uninfected PBMC (prepared from blood of uninfected individual as in Kanof et al., 1996) or fresh medium.

8. Incubate cells until peak p24 antigen or RT production is detected. At the peak of RT or p24 production, centrifuge cultures 5 min at 600 × *g* (1500 rpm), room temperature, to remove cellular debris.

   Peak viral replication will likely be at ~14 days.

9. *Optional:* For more efficient removal of cellular debris, filter viral supernatants through 0.45-μm low-protein-binding filters after the centrifugation in step 8.

10. Divide supernatants into 0.5- to 1-ml aliquots in cryovials. Snap-freeze in a dry-ice ethanol bath, then transfer to a −80° or −152°C freezer for long-term storage.

Repeated freeze-thaw cycles will reduce infectivity. Storage at −152°C may preserve infectivity better than −80°C, particularly if the −80°C freezer is frequently accessed, undergoing cycles of elevated temperature.

BASIC PROTOCOL 2

Propagation of Primary HIV Isolates

Typically, primary isolates are expanded only once in donor PBMC. However, many laboratories use isolates that have been further expanded in PBMCs but which still represent minimally passaged strains.

Materials

Blood from uninfected individual
Complete RPMI medium/20% FBS (see recipe) containing 0.5 μg/ml phytohemagglutinin (PHA; Sigma; store in aliquots at −20°C and add to medium just before use)
Complete RPMI medium/20% FBS (see recipe) containing 20 U/ml interleukin 2 (IL-2; Roche Applied Science; store in aliquots at −20°C and add to medium just before use)
Cell-free virus isolate seed stock (viral supernatant; Basic Protocol 1)
RPMI 1640 medium containing 20% FBS

Tabletop centrifuge (e.g., Sorvall Legend RT)
Plastic hemacytometer (preferred for safety reasons)
25- or 75-cm^2 tissue culture flasks
0.45-μm low-protein-binding filters (Millipore; optional)
Cryovials
Dry ice/ethanol bath
−80° or −152°C freezer

Additional reagents and equipment for preparing PBMC (Kanof et al., 1996), counting cells with a hemacytometer (*APPENDIX 4A*) and p24 ELISA (Basic Protocol 10) or RT activity assay (Basic Protocol 9)

Prepare uninfected PBMC

1. Isolate PBMC from uninfected individual's blood (Kanof et al., 1996). Dilute to 2–2.5 × 10^6 cells/ml in RPMI 1640 medium containing 20% FBS and 0.5 μg/ml PHA to induce T cells to grow. Incubate for 2 days.
2. Centrifuge cells 5 min at 300 × *g* (1000 rpm), room temperature, without braking. Remove medium. Resuspend cells in RPMI 1640 medium containing 20% FBS and 20 U/ml IL-2. Incubate 2 days.
3. Count cells using a plastic hemacytometer (*APPENDIX 4A*).

Mix uninfected cells and viral inoculum

4. Place virus stocks in a 37°C water bath until just thawed, then keep on ice until used. Centrifuge cells 5 min at 300 × *g* (1000 rpm), room temperature, without braking. Resuspend 5 × 10^6 stimulated PBMC, without washing, in 0.5 to 1 ml cell-free virus isolate stock. Incubate tube 3 hr in a 37°C water bath.
5. Centrifuge cells 5 min at 300 × *g* (1000 rpm), room temperature without braking. Remove supernatant. Resuspend cells in 10 ml RPMI 1640 medium containing 20% FBS.
6. Centrifuge cells 5 min at 300 × *g* (1000 rpm), room temperature, without braking. Remove supernatant and resuspend cells in 5 ml RPMI 1640 containing 20% FBS and 20 U/ml IL-2. Transfer cells to 25-cm^2 tissue culture flasks and continue incubation.

Obtain viral supernatants

7. Monitor cultures daily for production of virus in the cell supernatant by reverse transcriptase (RT) activity assay (see Basic Protocol 9) or p24 ELISA (see Basic Protocol 10).

8. Every other day, count cells (*APPENDIX 4A*) and readjust cell concentration to 1×10^6 cells/ml by adding uninfected PBMC (prepared as in Kanof et al., 1996) or fresh medium.

9. Incubate cells until peak RT or p24 antigen production is detected. At the peak of p24 or RT production, centrifuge cultures 5 min at $600 \times g$ (1500 rpm), room temperature, to remove cellular debris.

   Peak viral replication will vary from isolate to isolate; however, most peak at 7 to 10 days.

10. *Optional:* For more efficient removal of cellular debris, filter viral supernatants through 0.45-μm low-protein-binding filters after the centrifugation in step 9.

11. Divide supernatants into 0.5- to 1-ml aliquots in cryovials. Snap-freeze in a dry ice/ethanol bath, then transfer to a −80° or −152°C freezer for long-term storage.

    Repeated freeze-thaw cycles will reduce infectivity. Storage at −152°C may preserve infectivity better than −80°C, particularly if the −80°C freezer is frequently accessed, undergoing cycles of elevated temperature.

Propagation of T Cell Line–Adapted (TCLA) HIV

BASIC PROTOCOL 3

TCLA strains are patient-derived viruses that have been adapted for replication in $CD4^+$ T cell lines, e.g., H9 (Popovic et al., 1984). Culture of HIV in vitro, in the absence of host immunity, is likely to select for variants that may not represent HIV in vivo. This issue needs to be considered when interpreting data from primary isolates and particularly with TCLA strains.

Materials

$CD4^+$ T cell line, e.g., H9 (Popovic et al., 1984) or MOLT-4 cl.8 (Kikukawa et al., 1986) or C8166 (Salahuddin et al., 1983)
Seed stocks of T cell line–adapted viruses (available from the NIH AIDS Research and Reference Reagent Program; *http://www.aidsreagent.org*)
RPMI medium/10% FBS (see recipe)
25- or 75-cm^2 tissue culture flasks
Cryovials
Tabletop centrifuge (e.g., Sorvall Legend RT)
Plastic hemacytometer (preferred for safety reasons)
Dry ice/ethanol bath
−80° or −152°C freezer
Additional reagents and equipment for counting cells with a hemacytometer (*APPENDIX 4A*), quantification by $TCID_{50}$ (Basic Protocol 6), and p24 ELISA (Basic Protocol 10) or RT activity assay (Basic Protocol 9)

1. In a 25-cm^2 tissue culture flask, infect 5×10^6 cells from the $CD4^+$ T cell line with 0.5 to 1 ml seed stock of virus at a multiplicity of infection of 0.01 $TCID_{50}$ per cell (see Basic Protocol 6 for quantification by $TCID_{50}$). Incubate for 3 hr.

2. Centrifuge culture 5 min at $300 \times g$ (1000 rpm), room temperature, without braking. Remove supernatant, and resuspend cells in 5 ml of RPMI 1640 medium containing 10% FBS.

   Note that T cell lines require 10% FBS for growth.

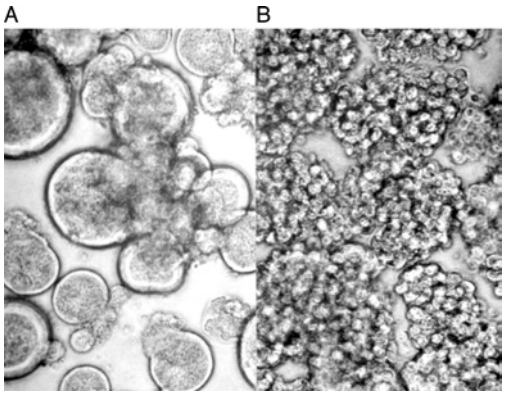

Figure 15J.1.1 Typical syncytia and cytopathic effect in C8166 T-cells infected with a TCLA HIV-1 strain. (**A**) Infected C8166 cells. (**B**) uninfected C8166 cells.

3. Incubate cultures, monitoring for cytopathic effect (Fig. 15J.1.1) at daily intervals.

   HIV usually causes cell death and the formation of syncytia in cultures of CD4$^+$ T cell lines which can be distinguished from smaller single cells.

4. Maintain cell concentration at 1×10^6 cells/ml by counting cells (*APPENDIX 4A*) and adding growth medium to appropriate volumes every 3 to 4 days.

5. When cultures show extensive cytopathic effects, mix infected cells with uninfected cells at a 1:4 ratio.

6. Harvest viral supernatant 48 to 72 hr after adding cells, or at peak RT or p24 values (see Basic Protocols 9 and 10), by centrifuging cells 5 min at $600 \times g$ (1500 rpm), room temperature, without braking, and transferring the supernatant in 0.5- to 1-ml aliquots into cryovials.

7. Snap-freeze in dry ice/ethanol bath, then transfer to a -80° or -152°C freezer for long-term storage.

   Repeated freeze-thaw cycles will reduce infectivity. Storage at -152°C may preserve infectivity better than -80°C, particularly if the -80°C freezer is frequently accessed, undergoing cycles of elevated temperature.

BASIC PROTOCOL 4

Production of Replication-Competent HIV from Molecular Clones

Viruses derived from transfected, molecularly cloned HIV-1 are useful for examining the function of particular genes, as well as the effects of mutations on gene function, without the complication of working with a mixture of quasispecies (see Background Information). Replication-competent molecular clones of HIV-1 that carry a GFP fluorescent protein fused to the matrix protein of gag were reported by Neumann et al. (2005). These constructs are useful tools for studying HIV interactions in live cells.

NOTE: Transfection of 293T cells by the calcium chloride–mediated procedure is described. Solutions can be prepared in house; however, a calcium chloride transfection kit (Promega) is available commercially. Alternative transfection procedures, e.g., using lipid-based reagents, can also be used.

Materials

293T cells (human embryonic kidney cell line transformed by sheared adenovirus 5 DNA and expressing SV40 T antigen; Pear et al., 1993)
DMEM medium (e.g., Invitrogen) containing 10% FBS (prepare with nuclease-free H_2O)
HIV cDNA: plasmid DNA containing HIV proviral cDNA (pNL4.3, pYU-2, or pJRCSF (available from the NIH AIDS Research and Reference Reagent Program; *http://www.aidsreagent.org/*)
2 M $CaCl_2$ (prepare with nuclease-free H_2O)
Nuclease-free (DEPC-treated) H_2O (*APPENDIX 2A*)
2× HEPES-buffered saline (HBS), pH 7.05 (see recipe)

6-well tissue culture dishes or 60- or 100-mm tissue culture plates
Clear plastic tubes
15- or 50-ml centrifuge tubes
Cryovials
Dry ice/ethanol bath
−80° or −152°C freezer

1. Plate 293T cells in 6-well tissue culture plates or 60-mm or 100-mm tissue culture dishes at 1×10^5 cells/ml the day before transfection using DMEM medium containing 10% FBS. On the day of transfection, change medium on cells 3 hr before beginning transfection.

2. Referring to Table 15J.1.1 for volumes of reagents needed, mix plasmid DNA with water in a clear plastic tube and mix by gently vortexing on a low setting.

   Clear plastic tubes are used in order to more easily see the precipitate described in step 4.

3. Again referring to Table 15J.1.1 for the appropriate volumes, add 2 M calcium chloride to the DNA/water mixture and mix by gently vortexing on a low setting. In another tube, pipet the appropriate volume of 2× HBS.

4. While gently vortexing the tube containing the 2× HBS, add the DNA/water/$CaCl_2$ mixture dropwise. Incubate tube at room temperature for 30 min.

   The mixture may appear slightly opaque after 30 min because of the formation of DNA/calcium phosphate precipitate.

5. Gently vortex the mixture, then add dropwise directly to the medium on the cells (see step 1). Gently swirl the plate of cells while adding the mixture. Incubate cells overnight.

6. At 12 to 16 hr post-transfection, change the medium on the cells.

Table 15J.1.1 Appropriate Reagent Volumes for Calcium Chloride Transfection

Reagent	6-well plate	60-mm dish	100-mm dish
HIV cDNA	2.5 μg	6-12 μg	10-20 μg
2 M $CaCl_2$	10.34 μl	37 μl	62 μl
Nuclease-free water	To 83.4 μl	To 300 μl	To 500 μl
2 × HBS, pH 7.05	83.4 μl	300 μl	500 μl
Total	166.8 μl	600 μl	1000 μl

7. At 48 hr post-transfection, harvest the virus by transferring the medium from the cells to a 15- or 50-ml centrifuge tube and centrifuging 5 min at 600 × *g* (1500 rpm), room temperature.

   The size tube used will depend on the amount of supernatant harvested.

8. Divide supernatants into 0.5- to 1-ml aliquots in cryovials. Snap-freeze in dry ice/ethanol bath, then transfer to a −80°or −152°C freezer for long-term storage.

   Repeated freeze-thaw cycles will reduce infectivity. Storage at −152°C may preserve infectivity better than −80°C, particularly if the −80°C freezer is frequently accessed, undergoing cycles of elevated temperatures.

   In the authors' experience, this protocol routinely yields >10^6 focus-forming units per milliliter of HIV-1 when measured on coreceptor$^+$ GHOST cells (see Basic Protocol 7).

BASIC PROTOCOL 5

Propagation of Reporter Viruses

Many HIV laboratories exploit single-round reporter viruses to measure infectivity. These viruses are capable of a single round of replication after infection, and infected cells fail to release infectious progeny virus. Single-round assays have been used to evaluate envelope coreceptor use (Peters et al., 2004) and sensitivity to neutralizing antibodies or therapeutic drugs (Spenlehauer et al., 2001).

The most commonly used reporter viruses carry green fluorescent protein (*GFP*) or luciferase (*luc*) as reporter genes. These reporters are usually cloned into the *nef* gene. Upon infection, the reporter gene is expressed as spliced mRNAs under viral LTR control. Figure 15J.1.2 shows typical constructs used to prepare reporter viruses.

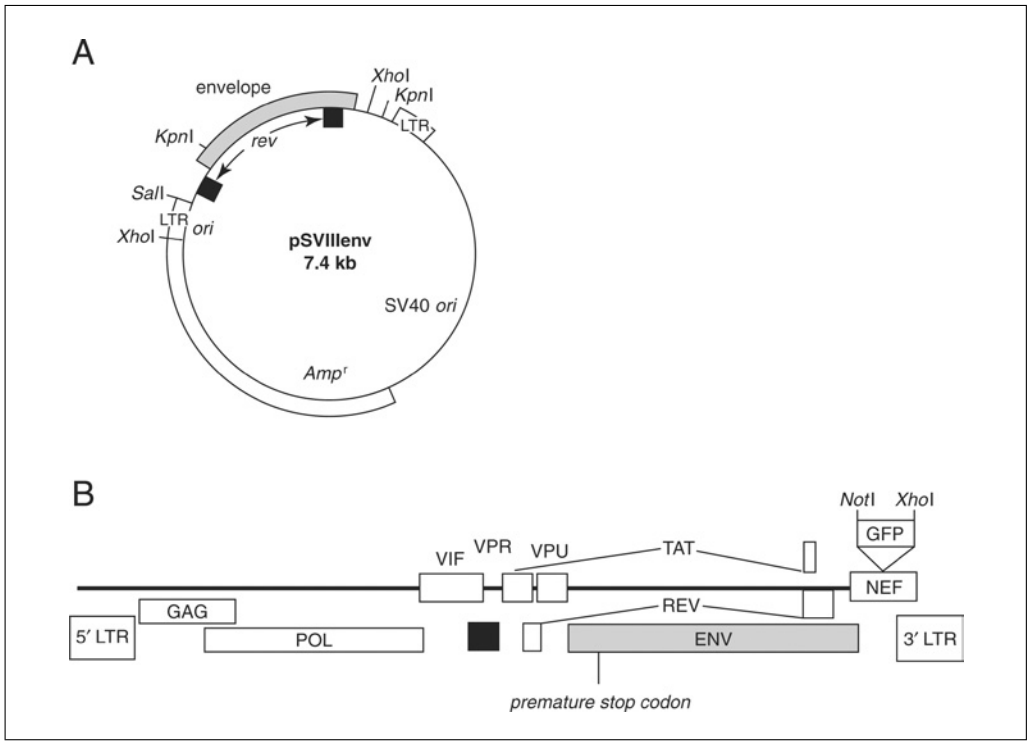

Figure 15J.1.2 Vectors used to produce envelope$^+$ reporter viruses. (**A**) pSVIIIenv expression vector encodes envelope and rev proteins. (**B**) env$^-$ pNL4.3 is a full-length clone of HIV-1 which contains a premature stop codon in the envelope gene. Reporter genes are often incorporated into the *nef* gene. Thus, env$^-$ pNL4.3 encodes all the viral genes required for particle production except for envelope. Cotransfection of 293T cells with env$^-$ pNL4.3 and pSVIIIenv carrying the envelope of choice results in the production of high-titer envelope$^+$ pseudotype virions.

pNL4.3env$^-$ contains a full-length proviral genome with a premature stop codon in the envelope gene (*env*) and a reporter gene in *nef*. Therefore, pNL4.3env$^-$ expresses all viral genes except for *nef* and envelope. The envelope gene, in an appropriate expression vector, is provided in *trans* via cotransfection. Envelope expression vectors vary in their capacity to generate high-titer reporter viruses. In the authors' experience, envelopes expressed from the pSVIIIenv vector consistently yield reporter viruses with infectivity titers of $>10^4$ per ml on indicator cell lines (GHOST/CCR5; see Basic Protocol 7 and Peters et al., 2004). The envelope glycoprotein of vesicular stomatitis virus (VSV-G) can be used in place of an HIV envelope when investigating post-entry events. VSV-G can also be used as a control if neutralizing antibodies or potential therapeutic drugs that target viral entry are being investigated. VSV-G confers efficient infectivity to HIV reporter viruses and also allows the study of cells that lack HIV receptors.

CAUTION: Single-round reporter viruses are not considered replication competent beyond the first round of infection. However, the potential for replication-competent recombinants to form in cotransfected 293T cells exists. This should be taken into account when considering the appropriate level of containment.

CAUTION: The receptor for VSV-G is present on many different cell types of diverse species. VSV-G thus confers infection of human cells without CD4 or CCR5. Use great care when preparing and handling HIV particles that carry VSV-G.

Materials

293T cells (human embryonic kidney cell line transformed by sheared Adenovirus 5 DNA and expressing SV40 T antigen; Pear et al., 1993)
DMEM medium (e.g., Invitrogen) containing 10% FBS (prepare with nuclease-free H_2O)
Reporter construct DNA (i.e., pNL4.3env$^-$, pNL4.3env$^-$ nef$^-$ GFP$^+$, pNL4.3env$^-$ nef$^-$ luc$^+$(available from the NIH Reagent Program; *http://www.aidsreagent.org/*)
pSVIIIenv (available with various primary isolate envelopes from the NIH Reagent Program; *http://www.aidsreagent.org/*), or pVSV-G
Calcium phosphate transfection kit (optional; Promega)
6-well tissue culture plates
15-ml centrifuge tubes
Tabletop centrifuge (e.g., Sorvall Legend RT)
0.45-μm low-protein-binding filters (Millipore; optional)
Cryovials
Dry ice/ethanol bath
−80° or −152°C freezer
Additional reagents and equipment for transfection of cells (see Basic Protocol 4) and quantification of virus (Basic Protocol 7)

1. Plate 293T cells at 1×10^5 cells/ml in DMEM containing 10% FBS, at a volume of 2 ml per well in a 6-well plate.

   Other appropriate cell densities and volumes can be used for larger plates or flasks.

2. Wash cells with DMEM containing 10% FBS before transfection.

3. Cotransfect pNL4.3env$^-$ and pSVIIIenv DNA at a 1:1 stoichiometric ration into 293T cells using the $CaCl_2$ method (see Basic Protocol 4 or use kit from Promega). Incubate for 12 to 16 hr, then remove medium and replace with fresh DMEM containing 10% FBS.

4. Harvest the cell culture supernatant 48 hr post-transfection by transferring it to a 15-ml centrifuge tube.
5. Centrifuge supernatant 5 min at 600 × *g* (1500 rpm), room temperature, to remove cell debris.
6. *Optional:* After centrifugation in previous step filter supernatant through a 0.45-μm low-protein-binding filter to remove smaller cell debris.
7. Divide supernatants into 0.5- to 1-ml aliquots in cryovials. Snap-freeze in liquid nitrogen, then transfer to a −80° or −152°C freezer for long-term storage.

   Repeated freeze-thaw cycles will reduce infectivity. Storage at −152°C may preserve infectivity better than −80°C, particularly if the −80°C freezer is frequently accessed, undergoing cycles of elevated temperature.
8. Quantify the infectivity of reporter viruses as described in Basic Protocol 7.

QUANTIFICATION OF HIV

There are many different methods for quantifying the infectivity of HIV particles. The infectivity assay of choice depends on the purpose of the experiment, as well as the cell type used. Tissue culture infectious dose 50 ($TCID_{50}$) assays can be readily applied to almost any cell type including cells that grow in suspension (e.g., PBMC, lymphocyte cell lines). Focus-forming unit (FFU) assays, where foci are identified by immunostaining for viral antigens, are a useful tool for evaluating infectivity of HIV, which does not readily form plaques (see Commentary for explanation). FFU assays are best applied to adherent cell types including cell lines and primary cell cultures. For instance, HIV infection of primary macrophages can easily be evaluated by estimation of FFUs following in situ immunostaining. Advantages of FFU assays include an evaluation of the number of cells infected and sensitivity that allows identification of individual HIV^+cells. FFU assays that detect very few infected cells can be used to evaluate infection of cell types that are less permissive to HIV-1 (e.g., primary astrocytes; Willey et al., 2003).

ELISAs that quantify p24 antigen or RT activity provide an estimate of the number of physical virus particles, but do not evaluate viral infectivity. The viral load (number of virus particles) in patient plasma is routinely quantified by assays that measure viral RNA present in virions.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 6

Tissue Culture Infectious Dose 50 Quantification of HIV

$TCID_{50}$ assays can be applied to evaluate infection of various cell types. Here, a method used to measure infectivity of PBMC is described.

Materials

Viral stock to be assayed
Growth medium for cells
5×10^5 cells/ml suspension of PHA and IL-2 stimulated PBMC (Basic Protocol 1)
96-well flat bottom plate

Virus replication detected at the following $\log_{10}$ dilutions								
−1.0	−1.5	−2.0	−2.5	−3.0	−3.5	−4.0	−4.5	−5.0
+	+	+	+	+	+	−	−	−
+	+	+	+	−	+	+	−	−
+	+	+	+	+	+	−	−	−
+	+	+	+	+	−	−	+	−
+	+	+	+	+	+	+	−	−
+	+	+	+	+	+	+	−	−

$TCID_{50} = L - d(S - 0.5)$
L is the highest dilution to give 100% of positives. $L = -2.5$.
d is the log of the dilution factor. For half log dilutions, this is 0.5. For tenfold dilutions, d is 1.0.
S is the sum of the number of positive wells divided by the total number of wells for each of the dilutions, starting from L and going up to the highest dilution to contain positives.
at −2.5 dilution (i.e., L), this fraction = 1
at −3.0 dilution, this fraction = 5/6 or 0.833
at −3.5 dilution, this fraction = 5/6 or 0.833
at −4.0 dilution, this fraction = 3/6 or 0.5
at −4.5 dilution, this fraction = 1/6 or 0.167

Thus, $S = 1 + 0.833 + 0.833 + 0.5 + 0.167 = 3.33$

$TCID_{50} = -2.5 - 0.5(3.33 - 0.5) = -3.9$ = dilution that will result the infection of 50% of wells.
Usually, $TCID_{50}$s are expressed as a positive value, e.g., $10^{3.9}$ or 8×10^3 $TCID_{50}$s.

Figure 15J.1.3 $TCID_{50}$ calculation. The table shows the virus dilutions that resulted in viral replication. Viral replication may be detected by RT or p24 estimation of virus particles produced in the cell supernatant (see Basic Protocols 9 and 10).

1. In a 96-well flat-bottom plate, make 10-fold serial dilutions of virus to 10^{-6} in growth medium. Place 50 µl of each dilution into appropriate wells. Add 100 µl of a 5×10^5 cells/ml cell suspension to each well.

   Each virus dilution should be tested in replicates of at least four.

2. Incubate plate. Examine or test cultures for infection at an appropriate time that allows for a single infection event to be sufficiently amplified by subsequent rounds of replication for detection (e.g., up to 18 days for PBMC).

   Infection of PBMC can be determined by estimation of RT or p24 antigen in supernatants (see Basic Protocols 9 and 10). Infection of $CD4^+$ T cell lines by TCLA strains can be detected by direct observation for cytopathic effects (e.g., syncytia; Fig. 15J.1.1).

3. Calculate the dilution where 50% of wells are positive (i.e., the tissue culture infectious dose 50 or $TCID_{50}$) using the Karber method (McKnight et al., 1995):

$$TCID_{50} = L - d(S - 0.5)$$

where L is the highest dilution to give 100% of positives; d is the log of the dilution factor (for half-log dilutions this is 0.5; for tenfold dilutions, d is 1.0); and S is the sum of the number of positive wells divided by the total number of wells for all the dilutions to give any positives starting from L and going up to the highest dilution to contain positives.

An example is given in Fig. 15J.1.3.

BASIC PROTOCOL 7

Focus-Forming Unit (FFU) Quantification of HIV

The FFU assay described below is suitable for estimating infection of adherent cell types (e.g., primary macrophage cultures and various adherent cell lines). See Figure 15J.1.4 for an example of staining.

Materials

$CD4^+$, $coreceptor^+$ cells (e.g., GHOST/CCR5; available from NIH AIDS Research and Reference Reagent Program; *http://www.aidsreagent.org*)
Complete DMEM medium/10% FBS (see recipe)
Virus stock to be assayed (see Basic Protocols 1 to 4)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
1:1 methanol:acetone, –20°C
PBS (*APPENDIX 2A*) containing 1% (v/v) FBS and 0.05% (w/v) sodium azide
Monoclonal antibody specific for HIV-1 p24 antigen (available from UK Centralized Facility for AIDS Reagents (CFAR, *http://www.nibsc.ac.uk/spotlight/aidsreagent/index.html*): ARP#365 and CFAR ARP#366 for HIV-1 *or* $HIV\text{-}2^+$ human sera (WHO Panel C for HIV-2; also available from CFAR)
Secondary antibody: goat anti-mouse β-galactosidase conjugate for HIV-1 *or* goat anti-human β-galactosidase conjugate for HIV-2 (Southern Biotechnology)
0.5 mg/ml Xgal in *N*,*N*-dimethylformamide
Yellow PBS (see recipe)
PBS (*APPENDIX 2A*) containing 0.05% (w/v) sodium azide
48-well tissue culture plate

Infect cells with virus

1. Plate GHOST/CCR5 cells at $4–6 \times 10^4$ cells/ml in a 48-well tissue culture plate in complete DMEM/10% FBS the day before infection, placing 500 µl of cell suspension in each well. Incubate cells overnight.
2. Thaw virus stocks quickly in a 37°C water bath until just thawed. Serially dilute virus stocks 10-fold in complete DMEM/10% FBS, taking into account the expected infectivity titer when deciding on the range of dilutions.
3. Remove medium from cells. Place 100 µl virus or virus dilution into each well. Incubate 3 hr.
4. Remove virus from cells. Wash cells once by adding 500 µl complete DMEM/10% FBS to each well and then aspirating, then place 500 µl fresh DMEM containing 10% FBS onto cells. Incubate 72 hr.
5. Remove medium and wash cells once with 500 µl/well PBS using the technique described in the previous step.

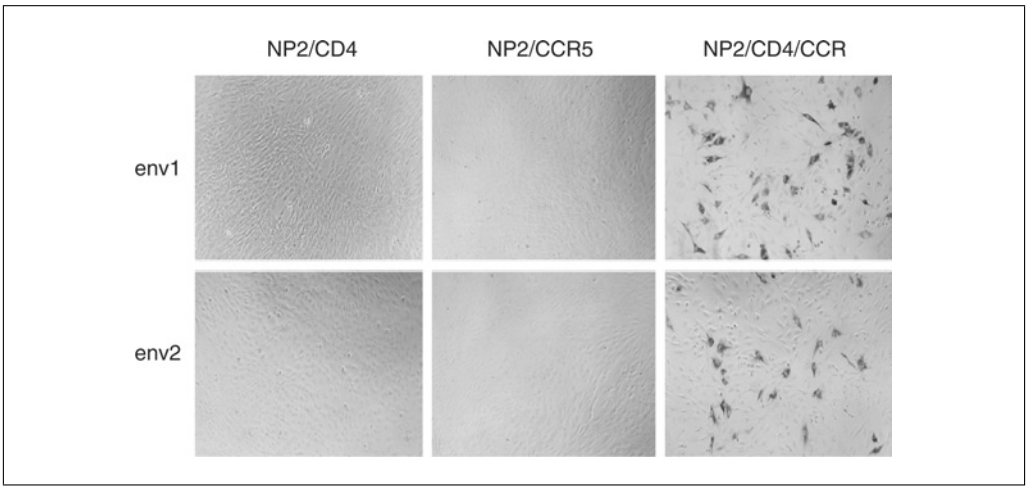

Figure 15J.1.4 Infectivity of HIV-1 R5 viruses detected as foci. HIV-1 reporter viruses were used to infect NP2 cells with and without CD4 and the coreceptor CCR5. Reporter viruses were prepared as described in Basic Protocol 5. In this assay, infection was detected (in this case without using the reporter gene) by immunostaining for intracellular p24 antigen as described in Basic Protocol 7.

Fix cells and label with antibodies

6. Place 500 µl cold (−20°C) 1:1 methanol:acetone onto cells. Incubate at room temperature for 10 min to fix cells.
7. Wash cells gently once by adding 500 µl/well PBS and then aspirating. Wash once more using the same technique with PBS containing 1% FBS and 0.05% sodium azide.
8. Dilute p24 antibody 1:40 in PBS containing 1% FBS and 0.05% sodium azide. Remove PBS solution from fixed cells and place 100 µl diluted p24 antibody into each well of cells.
9. Incubate at room temperature for 1 hr.
10. Remove p24 antibody. Wash cells once with 500 µl/well PBS containing 1% FBS and 0.05% sodium azide.
11. Dilute secondary antibody 1:400 in PBS containing 1% FBS and 0.05% sodium azide. Remove PBS from cells.
12. Add 100 µl of the diluted secondary antibody to each well. Incubate at room temperature for 1 hr.
13. Remove secondary antibody. Wash cells once with 500 µl/well PBS containing 1% FBS and 0.05% sodium azide, then twice with 500 µl/well PBS, using the technique described in the preceding steps.

Develop color reaction and count cells

14. Dilute 0.5 mg/ml Xgal stock 1:80 in yellow PBS.
15. Add 500 µl of the Xgal/yellow PBS to each well. Incubate at 37°C for 3 hr or until blue color develops.
16. After blue color develops, remove Xgal/yellow PBS from cells. Wash cells once with PBS containing 0.05% sodium azide. Cover cells with PBS containing 0.05% azide.
17. Count blue-stained cells by light microscopy. Multiply this number by the dilution factor and then by 10 (taking in account the volume of virus used to infect the cells).

This final number represents the number of FFU per ml of virus stock.

BASIC PROTOCOL 8

Quantification of HIV Using Reporter Genes

As described in Basic Protocol 5, single-round HIV viruses that carry a reporter gene are frequently exploited to study HIV infectivity. In the plasmid pNL4.3 luc, the luciferase gene has been cloned into the HIV-1 genome in place of the *nef* gene, and is expressed following infection. Alternatively, HIV-1 without a reporter gene (e.g., primary isolates) can be assayed on cell lines that carry a stably integrated reporter gene controlled by an upstream HIV LTR promoter region. After infection, newly produced HIV-1 tat protein will transactivate the reporter gene. HeLa TZM-bl cells (see Background Information) carry a luciferase gene and a β-galactosidase gene, both of which are controlled by HIV-1 LTR sequences. These genes are switched on following infection and synthesis of the tat protein. Here assays are described that exploit luciferase as a reporter gene.

Materials

Target cells for titrating virus with a luciferase reporter gene: PBMC, macrophages, or other target cells that carry a luciferase reporter gene, e.g., HeLa TZM-bl ($CD4^+$, $CXCR4^+$, $CCR5^+$; Wei et al., 2002); available from the NIH AIDS Reagent Program at *http://www.aidsreagent.org*

Complete DMEM medium/10% FBS (see recipe)

Reporter virus (see Basic Protocol 5) carrying a luciferase reporter cloned into the *nef* gene of pNL4.3 env^- construct (available from the NIH AIDS Reagent Program at *http://www.aidsreagent.org*) *or* HIV virus stock (Basic Protocols 1 to 4)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Bright-Glo substrate (Promega)

96-well luminometer culture plate (Corning)

Luminometer (e.g., Clarity from Bio-Tek)

1. Set up target cells at 4–6 × 10^4 cells/ml in DMEM containing 10% FBS. Add 100 µl of cell suspension per well to a 96 well-luminometer plate the day before titration.

   Use HeLa/CD4/CCR5 TZM-bl cells for titrating HIV without a reporter gene. Use other cell types for titrating HIVs that carry a luciferase reporter.

2. Thaw virus stocks quickly in a 37°C water bath until just thawed. Serially dilute virus stocks 10-fold in complete DMEM/10% FBS, taking into account the expected infectivity titer when deciding on the range of dilutions.

3. Remove medium from cells. Place 100 µl virus or virus dilution into each well. Incubate 3 hr.

4. Remove virus from cells. Wash cells twice with DMEM containing 10% FBS, then place 100 µl fresh DMEM containing 10% FBS onto cells. Incubate 72 hr.

5. Aspirate medium, then place 50 µl PBS into each well.

6. Add 50 µl Bright-Glo substrate to each well. Read plate with luminometer.

BASIC PROTOCOL 9

Radioactive Reverse Transcriptase Assay

HIV particles can also be quantified using assays that estimate the gag p24 antigen or activity of the reverse transcriptase (RT) enzyme contained in virions. These assays provide values related to the number of physical virus particles, but not on their infectivity (unless applied to $TCID_{50}$ assays). Here, an in-house radioactive RT assay that is applicable for both HIV-1 and HIV-2 is described.

RT activity is quantified as counts per minute (cpm). Usually a sample is considered positive if counts are at least twice as high as those in supernatant from a control uninfected cell culture. The Radioactive Reverse Transcriptase (RRT) assay can be used

to score wells as positive or negative in a $TCID_{50}$ estimation (see Basic Protocol 6), or can be used to compare the production of viral particles released from different cultures. To compare RT levels between assays done at different times, a standard control supernatant needs to be included. The standard should be a single virus-containing supernatant that is stored in aliquots at −80°C. Each time an RRT assay is done, a freshly thawed control supernatant aliquot can be tested alongside experimental samples.

CAUTION: Radioactive materials require special handling. Follow the guidelines provided by your local radiation safety adviser. See *UNIT 1A.4* for more information.

Materials

RRT assay solution A (see recipe)
Cell culture supernatants (infected and uninfected) for testing RT activity (Basic Protocols 1 to 4)
10μCi/ml [^{3}H]TTP (sp. act. 10 to 25 Ci/mmol)
RRT assay solution B (see recipe)
RRT stop solution (see recipe)
5% (w/v) Na_2HPO_4
70% and 100% ethanol
Scintillation fluid (Emulsifier Safe from Perkin-Elmer)
Plastic flexible 96-well plate (Falcon)
Cell harvester (e.g., Skatron)
DE-81 chromatography paper
Fan for air drying filters
Polystyrene board

1. Pipet 10 μl RRT assay solution A into each well of a plastic 96-well flexible plate.
2. Add 2 μl cell culture supernatant from infected cells to the appropriate wells and add 2 μl supernatant from uninfected cells to appropriate wells as controls.
3. Incubate at 37°C for 15 min.
4. Add 12 μl [^{3}H]TTP to 3 ml RRT assay solution B. Add 25 μl of this mixture to each well. Mix gently, then incubate 6 hr at 37°C.
5. Add 20 μl of RRT stop solution to each well. Mix by swirling.
6. Filter reaction mix on to DE-81 chromatography paper using a cell harvester.
7. Wash filters five times, each time with 2 ml of 5% Na_2HPO_4, then once with 2 ml of distilled water, followed by 2 ml of 70% ethanol, and then 2 ml of 100% ethanol. Dry filters by pinning them to a polystyrene board and placing in front of a fan.
8. Transfer filters to scintillation vials and add 5 ml scintillation fluid to each. Quantify incorporation of [^{3}H]TTP by RT onto the p(dT)12-18 primer by counting ^{3}H using a scintillation counter.

Quantification of HIV by P24 ELISA

BASIC PROTOCOL 10

The p24 ELISA estimates the amount of gag p24 antigen present in a viral sample. The following protocol is for an economical in-house ELISA. Several commercial p24 ELISA kits are also available. The assay described here, and most commercially available ELISAs, are designed to detect HIV-1 and are inefficient or ineffective in detecting HIV-2 (see Commentary).

Materials

Coating antibody: sheep anti–HIV p24-I/II/III mixture (Cliniqa)
Coating buffer: 0.1 M $NaHCO_3$ (pH 8.5)/0.15 M NaCl
Supernatants to be assayed for HIV (Basic Protocols 1 to 4)
5% (w/v) benzalkonium chloride
Tris-buffered saline (TBS), pH 7.4 (*APPENDIX 2A*)
TBS, pH 7.4 (*APPENDIX 2A*), containing 0.05% (w/v) benzalkonium chloride
TBS, pH 7.4 (*APPENDIX 2A*), containing 2% (w/v) nonfat dry milk
10 μg/ml p24 standard (National Institute of Biological Standards and Controls; *http://www.nibsc.ac.uk/*)
Nonfat dry milk
Sheep serum (Sigma)
10% (v/v) Tween-20
Mouse anti-p24 alkaline phosphatase (AP)–conjugated antibody (Cliniqa)
TBS (*APPENDIX 2A*) containing 0.05% Tween-20
AMPAK enzyme amplification kit (Dako)
  AMPAK wash buffer
  Substrate
  Amplifier
  Stop solution
Maxi-Sorp 96-well plate (Nunc) with lid
Automated ELISA plate washer (optional)
Spectrophotometer with microtiter plate reader

Prepare coated plates and viral supernatants for testing

1. Dilute coating antibody 1:200 in coating buffer. Add 100 μl to each well of a Maxi-Sorp 96-well ELISA plate. Incubate at room temperature overnight or at 4°C for 2 days.

2. Inactivate virus in the supernatants to be tested by adding 80 μl to 20 μl of 5% benzalkonium chloride. Prepare a 1:20 dilution by adding 10 μl of the inactivated sample to 190 μl TBS. Add 20 μl of the 1:20 dilution to 180 μl TBS containing 0.05% benzalkonium chloride for a 1:200 dilution.

   Only the 1:20 and 1:200 dilutions need to be assayed. All samples should be assayed in triplicate.

3. After coating, wash plate twice, each time by filling the wells with TBS and then hitting the inverted plate over paper towels. Alternatively, if available, use an automated ELISA plate washer. Block plate by adding 200 μl fresh TBS containing 2% nonfat dry milk per well and incubating plate 5 to 60 min at room temperature.

Prepare p24 standards

4. Dilute standard to 400 ng/ml by adding 18 μl of concentrated (10 μg/ml) standard to 432 μl TBS containing 0.05% benzalkonium chloride. Perform 2-fold serial dilutions to prepare eight separate standards, adding 225 μl of the previous dilution to 225 μl TBS containing 0.05% benzalkonium chloride.

Perform ELISA

5. Wash plate twice with TBS using the technique described in step 3. Add 100 μl samples and standards to the designated wells of the plate in duplicate. Use TBS containing 0.05% benzalkonium chloride as a blank. Cover the plate.

6. Incubate at room temperature for 4 hr.

7. Wash plate twice with TBS using the technique described in step 3.

8. Mix the following to prepare the 1:2000 anti-p24–AP conjugate solution:

   7.5 ml TBS
   0.4 g nonfat dry milk
   2 ml sheep serum
   500 µl 10% Tween-20
   5 µl mouse anti-p24–AP conjugate.

9. Add 100 µl of this solution to each well. Cover the plate. Incubate at room temperature for 1 hr.
10. Wash plate twice with TBS containing 0.05% Tween-20.
11. Wash plate four times with AMPAK wash buffer according to the manufacturer's instructions for the AMPAK kit.
12. Add 50 µl per well of the reconstituted substrate from the AMPAK kit. Incubate at room temperature for 30 to 75 min (incubation time will depend on the age of the AMPAK kit).
13. Add 50 µl per well of reconstituted amplifier from the AMPAK kit. Incubate at room temperature for 30 min, or until color develops.
14. Add 25 µl per well of stop solution from the AMPAK kit. Read plate at 492 nm in a spectrophotometer.

REAGENTS AND SOLUTIONS

Use deionized, distilled water for all recipes and protocol steps unless otherwise specified. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete DMEM medium/10% FBS

Dulbecco's Modified Eagle Medium (DMEM; e.g., Invitrogen) containing:
100 U/ml penicillin
100 µg/ml streptomycin
10% or 20% (v/v) fetal bovine serum (FBS)

Complete RPMI medium/10% or 20% FBS

RPMI 1640 medium (e.g., Invitrogen) containing:
100 U/ml penicillin
100 µg/ml streptomycin
10% or 20% (v/v) fetal bovine serum (FBS)

HEPES-buffered saline (HBS), pH 7.05, 2×

16.4 g NaCl (0.28 M final)
11.9 g HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 0.05 M final)
0.21 g Na_2HPO_4 (1.5 mM final)
800 ml nuclease-free H_2O
Titrate to pH 7.05 with 5 N NaOH
Add H_2O to 1 liter
Filter sterilize through a 0.22-µm nitrocellulose filter
Test for transfection efficiency
Store at −20°C in 50-ml aliquots

RRT assay solution A

100 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
300 mM KCl
10 mM DTT
0.1% (v/v) NP-40
Prepare fresh

RRT assay solution B

50 mM Tris·Cl, pH 7.9 (*APPENDIX 2A*)
150 mM KCl
5 mM DTT
15 mM $MgCl_2$
0.05% (v/v) NP-40
10 μg/ml poly(A) (GE Healthcare)
0.25 U/ml oligo d(T)12-1 (GE Healthcare)
Prepare fresh

RRT assay stop solution

18 ml 100% trichloroacetic acid (TCA)
7.2 ml 0.5 M $Na_4P_2O_7 \cdot 10H_2O$
4.8 ml H_2O
Store at 4°C in the dark

Yellow PBS

Phosphate-buffered saline (PBS; *APPENDIX 2A*) containing:
3 mM potassium ferricyanide
3 mM potassium ferrocyanide
1 mM magnesium chloride
Store in aliquots in 50-ml tubes up to 6 months at −20°C

COMMENTARY

Background Information

Primary isolates versus TCLA strains

Primary isolates and strains of HIV that have been cultured for a limited number of passages are used extensively to study various aspects of HIV infectivity. This includes viral tropism and sensitivity to neutralizing antibodies and viral inhibitors. However, the error-prone nature of HIV reverse transcriptase means that viral isolates consist of a mixture of closely related genotypes (and potentially phenotypes), known as quasispecies. TCLA viruses are often utilized for proof-of-principle experiments; however these strains are more easily neutralized than primary isolates, and have evolved distinct properties from viruses found in vivo (Dejucq et al., 2000, Moore and Ho, 1995).

Plaque assays

Plaque assays can be used to quantify a variety of cytopathic viruses, e.g., adenoviruses (*UNIT 14C.1*), herpesviruses (*UNIT 14E.1*), and rhabdoviruses. Retroviruses are generally less cytopathic and do not readily form plaques. HIV is cytopathic for some cell types. For instance, T cell line–adapted strains are cytopathic for several $CD4^+$ T cell lines. Nevertheless, few quantitative HIV assays based on plaque formation have been reported. Harada et al. (1985) described one of the few HIV plaque assays (also see McKeating et al., 1989). This assay uses the suspension T cell lines MT-2 and MT-4 that have been made to adhere to plates by poly-L-lysine. However, the protocol is technically difficult, and, although it is suitable for CXCR4-using T cell line–adapted variants, it is not readily adapted for primary isolates or CCR5-using viruses.

Focus-forming unit (FFU) assays and reporter genes

Several human cell lines that express recombinant CD4 and an appropriate coreceptor are suitable for use in focus-forming assays. These include several derived from HeLa (Platt et al., 1998, Wei et al., 2002), HOS (Cecilia et al., 1998), U87 (Bjorndal et al.,

1997, Deng et al., 1997), and NP2 (Soda et al., 1999). The GHOST cell line, a $CD4^+$ modified version of HOS cells, is commonly used in FFU assays. Different GHOST cell clones are available that express a variety of potential coreceptors—e.g., CCR5, CXCR6, and GPR15. GHOST cells carry GFP under an LTR promoter; these cells will express GFP and fluoresce after infection with HIV. Infection can thus be evaluated by fluorescence microscopy or flow cytometry. $CD4^+$ U87 cell lines expressing a variety of potential coreceptors are also available, and, along with the GHOST cell lines, are useful reagents for investigating coreceptor usage by different HIV-1 strains. However, GHOST cells naturally express CXCR4, and are therefore not useful for studying additional coreceptors used by CXCR4-tropic HIV variants. U87 cells do not express CCR5 or CXCR4, and are thus appropriate for studying HIV variants that use one or both of these coreceptors.

Infectivity assays that exploit reporter genes have been widely used. Replication-competent HIV can be assayed on cells carrying a gene that will express a detectable protein following infection. Reporter genes are usually controlled by an HIV long terminal repeat (LTR) promoter that is switched on following infection.

HeLa TZM-bl cells (Wei et al., 2002; see Basic Protocol 8) carry luciferase and *lacZ* genes under LTR promoters. These cells can be used to evaluate HIV infectivity and for assays to measure HIV inhibitors. Luciferase activity can be assessed by following the procedure described in Basic Protocol 8. Alternatively, infected HeLa TZM-bl cells may be identified by fixing in 1% glutaraldehyde in PBS and adding Xgal solution (as described in Basic Protocol 7). Infected cells will stain blue; there is no requirement for detection by specific antibody staining.

For single-round viruses, the reporter gene is incorporated into the HIV genome. HIV-1 env^- constructs for single-round assays are available that encode luciferase or other reporter genes. These include green fluorescent protein (GFP), which allows infection to be evaluated by fluorescent microscopy or flow cytometry; heat-stable antigens (HSA) that are expressed on the cell surface and which can also be evaluated by flow cytometry; placental alkaline phosphatase (PLAP); and chloramphenicol acetyl transferase (CAT).

Unlike the quasispecies that form primary isolates, reporter viruses are derived from molecular clones, and results obtained have high reproducibility. Reporter viruses are readily adapted to different experimental systems. For example, reporter viruses carrying VSV-G envelopes have been exploited to investigate the post-entry TRIM-5α restriction (Ikeda et al., 2004). The authors of this unit have used reporter viruses carrying different envelopes derived from HIV-1 in brain and immune tissue to investigate viral tropism and receptor use (Peters, 2004). Another reporter virus system that has been adapted to evaluate the first events in HIV entry utilizes a β-lactamase-vpr fusion protein (Cavrois et al., 2002). This fusion protein is coexpressed with an envelope expression vector and an env^- construct encoding the other viral proteins (e.g., pNL4.3env^-). The virions produced contain the β-lactamase-vpr fusion protein, which is delivered into the cytoplasm of cells upon virus entry. Target cells can be prelabeled with CCF2, a cytoplasmic green fluorescent dye. Upon infection and delivery of β-lactamase-vpr, the lactamase enzyme cleaves CCF2, changing its fluorescence from green (520 nm) to blue (447 nm). The β-lactamase-vpr fusion protein system is useful for confirming HIV entry and distinguishing entry from attachment of virions to the cell surface and their endocytosis.

Plasma viral load

The amount of virus or viral load present in the plasma of HIV-infected individuals is routinely estimated for HIV^+ individuals. The most widely used method quantifies HIV RNA present in virus particles in plasma to provide an estimate of plasma viral load (PVL). The specific protocol is beyond the scope of this chapter. Briefly, HIV-specific primers are used to amplify viral RNA by RT-PCR. Several kits are available commercially with specificities for different subtypes and with different sensitivities.

Critical Parameters and Troubleshooting

The efficiency of virus isolation and the infectivity titers of isolates varies considerably. Isolation efficiency can be enhanced by selective removal of CD8 T cells from the HIV^+ PBMCs and the donor PBMC. $CD8^+$ T cells produce inhibitory β-chemokines (Cocchi et al., 1995) and other inhibitory factors (Levy, 2003). Their selective removal thus enhances the quantity of virus produced from PBMC. Optimal virus production from PBMC cultures also requires careful maintenance of pH and cell density of cultures. Peaks of virus

production are sometimes transient and may either be missed or harvested when virus quality has deteriorated, if cultures are not tested daily.

Single-round viruses are prepared by cotransfecting two plasmids into 293T cells. The first plasmid contains a full-length proviral genome with a premature stop codon in the envelope gene and usually with a reporter gene in the *nef* coding region. The second is an envelope expression vector. Different vectors result in different infectivity titers of reporter viruses. The authors have found that inclusion of the envelope expression vector pSVIIIenv results in reporter viruses with infectivity titers of between 10^4 and 10^5 FFU/ml for HeLa/CD4 TZM-BL cells, sufficient to investigate infectivity for primary macrophage cultures. In contrast, envelope expression from the pCR3.1uni vector resulted in infectivity titers that were at least 20-fold lower. Unfortunately, a survey of different envelope expression vectors in reporter systems has not yet been described. However, if reporter virus titers are insufficient for the intended purpose, then alternative envelope expression plasmids should be tested. Low yield of virus from 293T cells may also result from inefficient transfection procedures. 293T cells need to be trypsinized and passaged twice weekly and kept subconfluent and adherent to maintain transfection sensitivity.

Important considerations for ELISAs and RT assays include the pH of the buffers used, the age of the substrate reagents, and the age of the viral samples. Substrate reagents should be used as soon as possible after reconstitution. Viral samples for p24 or RT evaluation should be stored at −80°C and assayed as soon after collection as possible. ELISAs for p24 are usually specific for HIV-1, although kits adapted for SIV will work for HIV-2 and are available commercially or via the NIH AIDS Research and Reference Reagent Program. An advantage of RT assays is that they detect HIV-1, HIV-2, or SIV. Extremely sensitive, nonradioactive RT ELISA kits can also be purchased from Cavidi Tech Inc. (*http://www.cavidi.com*; Porstmann et al., 1991, Sano et al., 1995).

Anticipated Results

Isolation of HIV from PBMC is a skill, and efficiency of isolation improves with practice. In contrast, the production of virus following transfection of 293T cells is much more consistent and good titers (10^9 to 10^6 FFU/ml as assayed on TZM-bl cells) can reliably be expected.

Time Considerations

Isolation of HIV into PBMC cultures usually takes 2 to 3 weeks, with daily monitoring, before virus is detected in the cell supernatant. Similarly, the production of new viral stocks from PBMC involves up to 2 weeks of culture. In contrast, replication-competent virus or reporter viruses are harvested just 48 hr after transfection of 293T cells with appropriate plasmids.

Infectivity titrations vary in length considerably. $TCID_{50}$ assays on PBMCs take up to 18 days to allow virus at endpoint dilutions to expand sufficiently for detection. FFU assays on cell lines are complete within 4 days, while FFU assays on macrophages can be completed within 7 days. Reporter viruses with a luciferase readout can usually be read 48 hr after infection.

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Aphid Transmission of Plant Viruses

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ABSTRACT

A majority of plant viruses are transmitted between hosts by insect vectors, and it is often important to use insect transmission in the laboratory to maintain virus isolates or to study virus-vector-plant interactions. Although many of these viruses can also be mechanically transmitted in the laboratory using infected sap, maintenance by mechanical transmission can often lead to changes in the virus, either minor changes in gene sequences or, in some cases, major deletions of genome sequences. These can affect both virus-vector and virus-host interactions. This unit describes some simple and practical methods for conducting virus transmission experiments using sap-sucking insects. *Curr. Protoc. Microbiol.* 10:16B.1.1-16B.1.10. © 2008 by John Wiley & Sons, Inc.

Keywords: plant virus • transmission

INTRODUCTION

This unit describes how to use aphids, or other sap-sucking insects, to transmit viruses either from infected plant material (Basic Protocol) or by using an in vitro membrane system that allows insects to feed on purified virus alone or in combination with other purified proteins (Alternate Protocol). Transmission methods are provided for both the short duration transmission of nonpersistent viruses and the longer duration transmission of semipersistent or persistent viruses. Additionally, general information is provided on rearing aphids for use in virus transmission experiments (Support Protocol).

STRATEGIC PLANNING

Choosing the Host Plant

The host range of most species of aphids is described in the literature (Blackman and Eastop, 2000, 2006; Dixon, 2005). Ideally, it is best to rear the aphids on a plant that is not susceptible to the virus of interest in order to avoid virus infection in the plants used to rear aphids. However, it is more important to determine whether the aphids will transfer easily from the maintenance host to the host used for virus transmission studies. Keep in mind that not all varieties of a host plant are equally palatable to an aphid. Aphids reared on one host may not readily feed on another. Reduced or absent virus transmission may be due to the lack of proper feeding on the virus recipient host, rather than an inability to transmit the virus. Check the survival and reproduction on both hosts prior to the start of the virus transmission experiments. In addition, virus infection may alter the suitability of a host.

Confining the Aphids on Test Plants

Cages may enclose an entire plant or group of plants, or they may confine aphids to a leaf or area of a single plant. Adequate ventilation is required to reduce the relative humidity and free moisture within the cage that can lead to disease development and aphid entrapment. Cages must also be able to prevent escape of the aphids from the cage or intrusion of foreign pests into it. A mesh aperture size of 210 μm or less will prevent ingress or egress of most first instar aphids. Certain plastics, glues, and sealers can have

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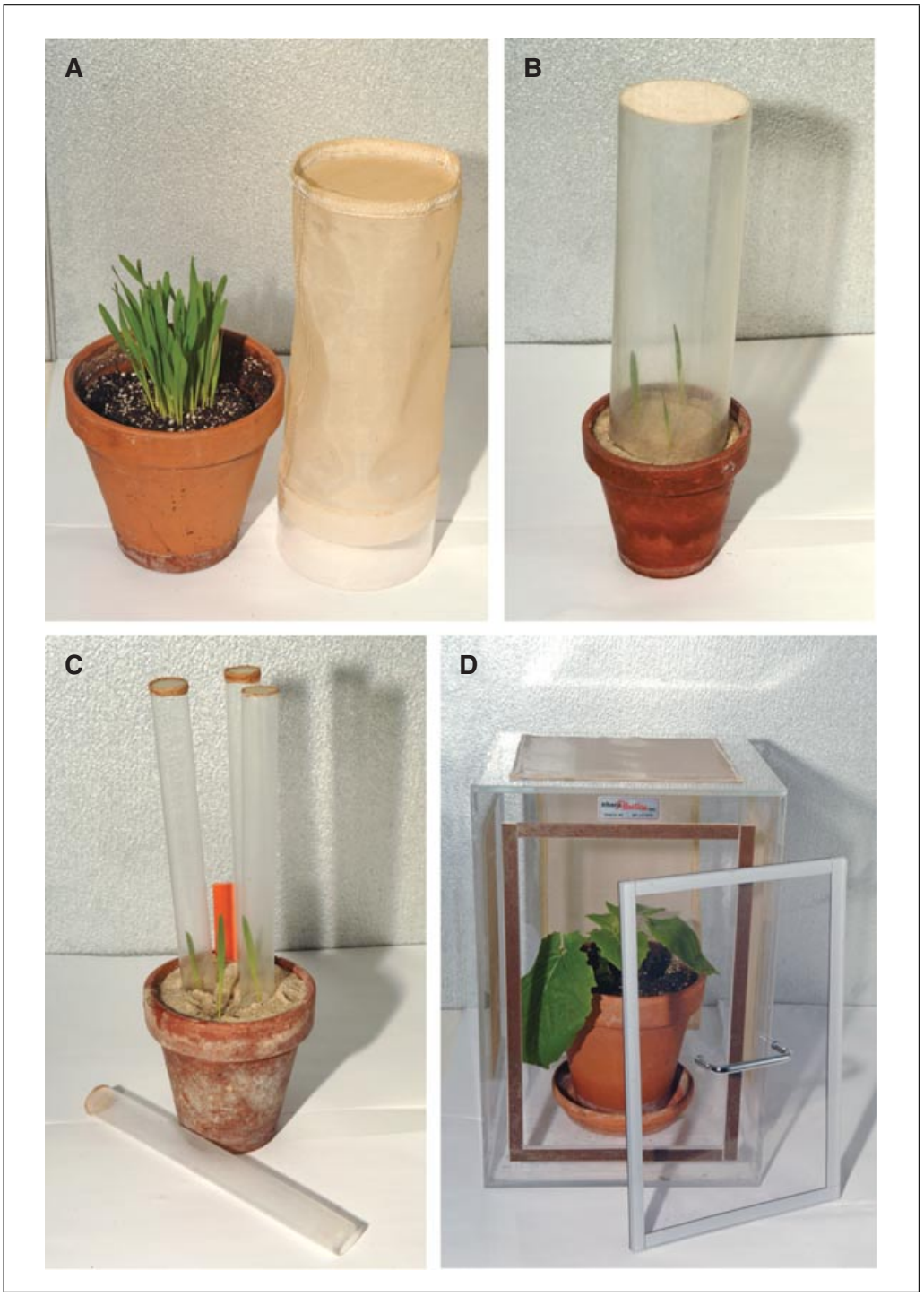

Figure 16B.1.1 Examples of insect cages appropriate for small insects. Cages are used to retain aphids on healthy plants during inoculation. They may enclose an entire plant or group of plants, or they may confine aphids to a leaf or area of a single plant.

toxic effects on the aphids and/or the host plant. Thus, any new material should be tested for toxic/adverse effects to both the plant and the insect prior to use. Commercially available insect cages are rarely appropriate for working with small insects (e.g., aphids, thrips, whiteflies), and most researchers construct their own cages. Cages can be as simple as a plastic container covered with muslin cloth or more elaborate plastic and mesh containers. Some examples are shown in Figure 16B.1.1.

Facilities

Design

If you are fortunate enough to be able to design aphid rearing and working facilities, there are several features to consider. The physical layout should minimize the possibility of contamination, both viral and insect. If possible, rearing facilities should be planned so that there are separate areas for working with virus and virus-free materials, as well as a third space for a plant nursery. All work areas should be free of other insect pests, pathogens, or insecticide contamination.

Minimizing contamination

It is best to grow all plants used for rearing aphids and for use in the transmission studies in an isolated greenhouse or growth room that is not treated with any pesticides and with limited access by nonessential personnel, to minimize the introduction of insects and diseases.

Colony maintenance activities or collection of aphids for use in virus transmission tests are conducted in a "no virus" work area. Caged colonies are removed from the rearing chambers, aphids collected, and plants (and extra aphids) disposed of within the workroom, without contaminating the rearing chambers or outside areas with any aphids. A freezer is an effective and environmentally friendly way to dispose of the unwanted aphids remaining on the plants and in the pots.

Recaging a colony once the cage is removed often leads to the escape of individual aphids, which can contaminate other colonies. Additionally, once an aphid colony is removed from a cage or the cage is removed from the plant, there is the possibility of introducing either contaminating aphids or pests. A "one use only" colony is the best insurance against contamination problems.

Aphid transfer

The transfer of aphids exposed to virus is conducted in a separate facility consisting of an isolated workroom directly connected to plant growth rooms. This arrangement allows viruliferous aphids to be transferred from virus source plants or tissues to recipient plants and held in isolation during the inoculation access period. All plants in the work room and growth rooms are caged at all times except during the brief periods that aphids are placed on the plants.

TRANSMISSION FROM INFECTED PLANT MATERIAL

BASIC PROTOCOL

Insect transmission of plant viruses from infected plant material to healthy plants is useful for determining the identity of insect vectors and the transmission efficiency of individual vectors. Additionally, this technique is useful for generating additional infected plant material if the virus is not transmitted by mechanical, bombardment (*UNIT 16B.3*), or agroinfiltration (*UNIT 16B.2*) methods. Insects are allowed to feed on infected material for an appropriate length of time (acquisition access period), then moved to a healthy host for an appropriate inoculation access period. This seemingly simple process can present many challenges if the proper protocols are not followed. For example, the use of improper feeding times could prevent or reduce the probability of transmission, or damaging insect mouthparts during improper handling could prevent virus transmission. This protocol describes methods involving aphid vectors, but the general techniques are useful for other insects with piercing sucking mouthparts (e.g., whiteflies, leafhoppers).

NOTE: If the virus or vector is not found in your state or geographical area, regulations may apply. If uncertain, check with the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS; *http://www.aphis.usda.gov*) plant

protection and quarantine (PPQ) manuals for information about permits for insects and plant pests.

Materials

Aphids (Support Protocol) or other insects
Infected plant material
1.5-ml tubes, optional
Parafilm, optional
Healthy plants
Appropriate insecticide: insecticidal soaps, contact insecticides (e.g., pyrethroids), *or* systemic insecticides (e.g., organophosphates and carbamates)

Fine-tip paint brush (e.g., round camel hair #2)
Shallow plastic or glass dishes with tight-fitting lids, ~10 cm in diameter and 2 cm deep
Cages to retain aphids on healthy plants during inoculation (e.g., see Fig. 16B.1.1)
Fine white sand (sandbox sand, available in garden supply stores), optional

1. Collect healthy aphids that have not been exposed to virus by gently tapping the leaf with the handle of a paint brush and letting the aphids fall onto a clean sheet of paper. Alternatively, remove individual aphids with a moistened paint brush by gently prodding the aphid from behind until it begins to move and then gently picking it up with the brush. Transfer the aphids to the collection dish.

   Handle the aphids gently. Pulling them off a leaf while they are feeding may damage their stylets (a small feeding tube that is inserted into the leaf tissue). Aphids adopt a characteristic posture while feeding. The antennae are laid back over the body and the posterior end is raised. Tapping a feeding aphid with a paintbrush usually causes it to withdraw its stylet; the antennae will be raised and moved forward, and the insect may begin to walk.

2. Transfer the aphids to a dish with a tight-fitting lid.

3. Starve the aphids in the dish for 30 to 60 min prior to use in the experiments.

   This may cause them to settle down and begin feeding when they are introduced to virus infected leaf tissue. Starvation can improve the efficiency of virus transmission.

4a. *For stylet-borne, nonpersistently transmitted viruses:* Introduce five to ten aphids onto the infected tissue and observe for feeding. If the insects walk off the leaf, discard them (see step 5, second annotation, or step 8 below, for disposal methods). Use only those aphids that actually probe the leaf tissue for 15 to 90 sec for transfer to healthy plants (step 5).

   Transmission experiments involving stylet-borne, nonpersistently transmitted viruses require short acquisition feeding periods (<2 min). Nonpersistently transmitted viruses are carried on the very distal tips of the insect mouthparts (stylets). These viruses are acquired during very brief probes of epidermal or mesophyll cells, usually 15 to 60 sec in duration. Transfer of the aphids should take place after 30 to 90 sec of feeding activity because longer feeding times actually decrease the probability that an insect will transmit these viruses.

4b. *For circulative persistently or semipersistently transmitted viruses:* Add whole leaves or leaf sections from infected plant material to the dishes containing starved aphids. Allow the aphids a 24 to 48 hr acquisition access period. Keep the dish at 18°C to 22°C.

   If the infected plant material is from herbaceous plants prone to wilting, the petiole of the leaf can be immersed in water in 1.5-ml tube sealed with Parafilm and placed in a dish with a tight-fitting lid. It is best if the leaf material does not wilt during the acquisition access period.

Younger symptomatic tissue is usually a good source of virus, but some aphid-virus combinations may require the use of older tissue.

Persistently or semipersistently transmitted viruses require longer acquisition feeding periods (minutes to hours; actual feeding time during the acquisition access period); generally, increased feeding times means increased transmission efficiency. Semipersistently transmitted viruses are carried in the foregut of the insects and are regurgitated back into the plant during prolonged feeding, i.e., not during brief probes.

Persistently transmitted viruses actually circulate through the insect body, being taken up through gut tissues and eventually winding up in salivary tissues where they are secreted back into the plant during the salivary phase of feeding. These viruses will require one to several days to complete the circulative pathway that starts with ingestion and ends with salivation.

See Background Information for a discussion of mechanisms of virus infection (e.g., circulatively, semipersistently, and nonpersistently transmitted viruses).

5. Carefully dislodge the aphids that were observed to be feeding (nonpersistent) or that were actively feeding on the tissue at the end of the acquisition feeding period (persistent, semipersistent) and transfer one or more to healthy plants in appropriate cages.

   The number of aphids transferred to each healthy recipient plant depends on the purpose of the experiment. Single aphids will provide the most accurate assessment of transmission efficiency, but this is often cumbersome, and efficiency levels may be very low, requiring hundreds of plants. Transfer of three, five, or ten aphids per plant is more common.

   If using whole-plant cages, you can put a 1- to 2-cm layer of fine white sand on top of the soil, wet the sand and then push the cage into the sand (see Fig. 16B.1.1). This firmly anchors the cage and helps to locate the insects should they fall while you are putting multiple insects on the plants.

   Leftover aphids and infested source material can be discarded by placing them in the freezer for several hours.

6. Allow a suitable inoculation access period.

   Hours (usually overnight) are convenient for nonpersistent viruses. Two to four days are appropriate for persistent and semipersistent viruses.

7. Observe the aphids at the end of the inoculation feeding period to be sure they are on the plant and alive.

8. Kill all the aphids (including newly produced aphids) by treating the plants with an appropriate insecticide.

   If adult aphids are used in this transmission experiment, they will produce young aphids during the inoculation access period; Observing young aphids is a good indication that the adults were feeding well.

   Contact insecticides such as pyrethroids work well for many insects and do not remain active on or in the plant for more than a few days, so the infected plants can be used as source plants for subsequent transmission experiments.

   Systemic insecticides (e.g., organophosphates and carbamates) are slower acting, but longer lasting, so the plants will not be useful for future transmission experiments.

   Insecticidal soaps will also work to kill many sap-sucking insects, and they are relatively safe to use.

9. Allow the plants to grow in the greenhouse and observe for symptoms.

ALTERNATE PROTOCOL

IN VITRO–TRANSMISSION ASSAY

In vitro techniques to test for insect transmission of plant viruses were first developed in the 1920s when it was discovered that insects with piercing sucking mouthparts would feed on solutions through an animal membrane. This technique later evolved to use synthetic membrane-like materials, most notably Parafilm. In vitro techniques are useful if the vectors will not feed on the natural hosts of the virus. They are especially useful for investigations using purified preparations of virus. For example, these techniques have enabled the testing of purified (or semipurified) virus preparations from plants or from cloned virus inoculated into cell culture. They also allow the mixing of virus with other proteins to determine the need for and identification of transmission helper factors. This protocol describes methods involving aphid vectors, but the general techniques are useful for other insects with piercing, sucking mouthparts, e.g., whiteflies, leafhoppers. A dated but useful reference is Kunkel (1977).

Additional Materials *(also see Basic Protocol)*

Test solution of virus or test proteins: prepare in an appropriate buffer or water (vehicle determined by virus or protein used and tested alone to determine any effect on insect feeding or survival) containing 15% (w/v) sucrose and sterilize by passing through a 0.2-μm filter

Feeding chamber (see Fig. 16B.1.2) consisting of :
Parafilm: sterilized with UV light (from several hours to overnight)
1- to 2-cm diameter plastic or glass tubes ~2 to 3 cm in length
Rigid foam block
Screening material that prevents the smallest life stage of aphid from escaping but allows air movement; muslin may be used
Contact cement or other adhesive
Yellow light, optional

1. Collect and starve aphids in dishes as in the Basic Protocol, steps 1 to 3.
2. Construct feeding chambers as shown in Fig. 16B.1.2.
   a. Stretch a single sheet of Parafilm across one end of the cylinder.
   b. Press on the Parafilm with a gloved finger to make a depression.
   c. Pipet the virus solution onto the surface of the Parafilm.

      The amount of liquid used will depend on the diameter of the cylinder. For an ~1-cm diameter cylinder, we use 25 to 50 μl of solution.

   d. Stretch a second sheet of Parafilm and carefully lower it over the liquid. Wrap the Parafilm onto the sides of the cylinder, trapping the liquid between the two sheets.

      It is best to stretch the second sheet of Parafilm and lower it onto the top of the liquid and then slowly relax the edges of the Parafilm and allow contact with the edges of the cylinder.

      Kunkel (1977) provides excellent schematics for preparing these chambers.

   e. Attach the screening to the foam, using contact cement or similar glue.
3. Place the aphids on the screen covering the hole in the bottom of the foam block. Quickly lower the cylinder into the foam block before the aphids climb out.

   Add the aphids as a clump rather than individually.

   Do not crush any of the aphids and do not overcrowd the membrane.

   See Video 1 at http://www.currentprotocols.com for instructions on how to assemble the feeding chamber shown in Figure 16B.1.2. See Video 2 at http://www.currentprotocols.com for assembly of an alternate type of feeding chamber.

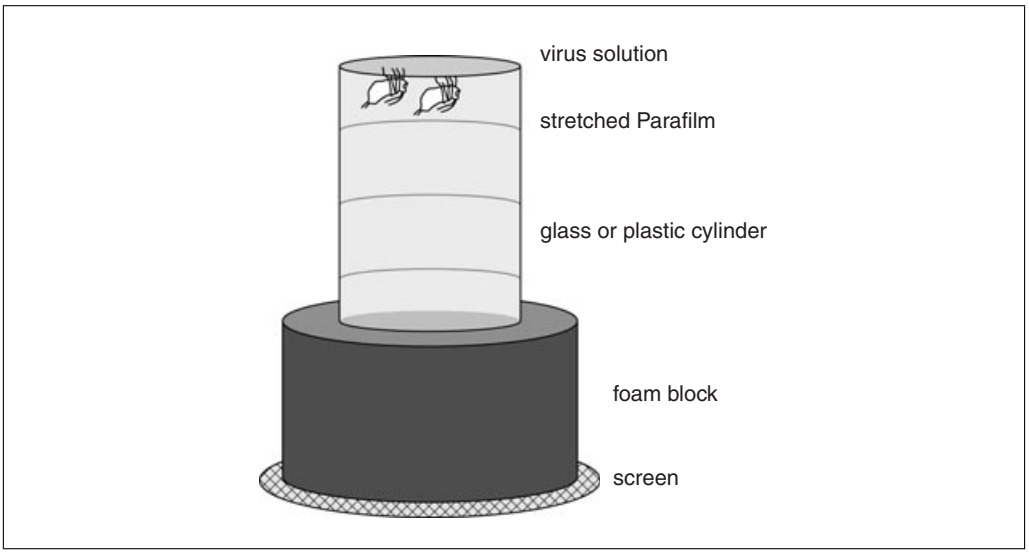

Figure 16B.1.2 Device for feeding aphids on solutions through a Parafilm membrane.

4a. *For stylet-borne, nonpersistently transmitted viruses:* Allow the aphids a <2 min acquisition.

In the case of the short acquisition feeding periods involving stylet-borne, nonpersistently transmitted viruses, it is best to actually observe the aphids feeding and transfer only those that actually probe the membrane for 30 sec or more.

See Background Information for a discussion of mechanisms of virus infection (e.g., circulatively, semipersistently, and nonpersistently transmitted viruses).

4b. *For circulative persistently transmitted viruses or semipersistently transmitted viruses:* Allow aphids a 24 to 48 hr acquisition access period. Keep the feeding chamber at 18°C to 25°C and elevate on a rack to allow air movement through the screen.

Placing a yellow light above the chamber may improve the aphid movement onto the membrane, but do not allow the light to heat the solution.

5. Carefully dislodge (see Basic Protocol) the aphids that were observed to be feeding (nonpersistent viruses) or that were actively feeding on the membrane at the end of the acquisition feeding period (persistent, semipersistent viruses) and transfer to healthy plants. Cage aphids on the plant using either a whole plant cage or some type of cage that clips onto a leaf without damaging the tissue.

6. Allow a suitable inoculation access period.

Hours (usually overnight) are convenient for nonpersistent viruses; 2 to 4 days is appropriate for persistent and semipersistent viruses.

7. Observe the aphids at the end of the inoculation feeding period to be sure they are on the plant and alive.

8. Kill all the aphids, including the newly produced aphids, by treating the plants with an appropriate insecticide (see Basic Protocol, step 8 annotation).

9. Allow plants to grow in the greenhouse and observe for symptoms.

SUPPORT PROTOCOL

REARING APHIDS

Aphids are among the easiest of insects to maintain in the laboratory; however, certain rules need to be strictly followed in order to generate physiologically normal insects that will behave in a normal manner. The following are some specifics of one system that has been in use for over 40 years to maintain multiple aphids used in virus transmission studies (see Katsar and Gray, 1999; also contains chapters about rearing other types of sap-sucking insect vectors of plant viruses), although there are numerous systems described that are equally effective. Details on facilities, maintenance schedules, handling, and potential pitfalls are provided in the Strategic Planning section. In addition, general information on starting new colonies and selecting appropriate rearing conditions are provided. The system outlined here is easily adaptable for use with many aphid species and host plants. In general, pathogens and parasites are not transovarially transmitted to the progeny, although this rule does not apply to viruses that replicate in aphids. To minimize the chance of parasite or pathogen contamination, start all colonies with first instar nymphs. Most aphids reproduce via parthenogeneisis and give birth to live young. There are four instar (immature) developmental stages prior to the adult stage. The first instars are the live-borne young; they will usually molt to second instars within 2 to 3 days.

Materials

Aphids, either collected from the wild or obtained from other researchers' colonies
Leaves from healthy plants (or moist filter papers)
Healthy plants

Fine-tip paint brush (e.g., round camel hair #2)
Small containers with tight-fitting lids
Plant cages (see Fig. 16B.1.1)

1. Using a fine-tip paint brush, place apterous, parthenogenic, female adults in small containers with tight-fitting lids containing moist filter paper or leaves from healthy host plants.
2. Allow the adult aphids to produce progeny over a 12 to 24 hr period at a temperature appropriate for the aphid and plant species used.

   A temperature of 18°C to 22°C is a good place to start.

3. Transfer (using a paint brush) five to ten newly emerged first instar nymphs to healthy plants in new cages to initiate the colony. Dispose of the remaining adults and nymphs (see Basic Protocol).
4. Allow colonies to develop for 2 to 3 weeks.

   Depending on the aphid species, the plant, and environmental conditions, the plant will usually begin to decline in 3 to 4 weeks, and aphid colonies are not as robust on declining plants. In addition, more winged forms of the aphids will develop.

5. Collect aphids (see Basic Protocol, step 1 annotation) for experimental use or to start new colonies.

COMMENTARY

Background Information

The transmission of plant viruses by insect vectors is routine in many laboratories and can be accomplished by most laboratories if the vectors and general mechanisms of transmission are known. Transmission studies become more difficult if there is limited information about efficient vectors and if the viruses are not easily maintained by mechanical transmission. Fortunately, the vectors of most plant viruses or virus families are known, and taxonomic and mechanistic specificity exists at the

genus and often the family level. Taxonomic specificity means that the same family of insect transmits all members of a virus genus. This will help narrow the potential vectors for a newly described virus species although a number of different species of vector would need to be tested to determine which is the most efficient. Additionally, there can be considerable variation in vector efficiency within populations of an insect species for a given virus.

Mechanistic specificity means that all members of a virus genus are transmitted in the same manner. Nonpersistent viruses are carried on the cuticle lining inside the insect stylets (food and salivary canals) and are acquired and inoculated within seconds to minutes. Examples include members of the potyviruses and cucomoviruses. Semipersistent viruses are associated with the cuticle lining along the foregut. These viruses can be acquired and inoculated within minutes, although longer feeding times usually improve transmission efficiency. Examples include members of the closteroviruses and caulimoviruses. Persistent viruses are internalized by their insect vectors, and the viruses are transported across multiple insect membranes, usually in the gut and salivary glands, but other tissues may be involved. The viruses may replicate in the vectors (propagative, e.g., rhabdoviruses, reoviruses), or they may just circulate through the vector (nonpropagative, e.g., luteoviruses and geminiviruses). Persistent viruses require acquisition and inoculation times generally measured in hours, and longer feeding times will generally increase transmission efficiency.

Critical Parameters

It should be noted that viruses maintained for extended periods by mechanical transmission or vegetative propagation may loose their ability to be transmitted by their natural vector. This can occur because of selection for mutations or deletions in genes for critical components of the virus required for vector transmission, but dispensable for host plant infection. Additional information on the mechanics of plant virus transmission by insects is reviewed in Ammar and Nault (2002), Brown and Czosnek (2002), Gray and Gildow (2003), Hogenhout et al. (2003), Ng and Perry (2004), and Ullman et al. (2005).

Troubleshooting

Insects die or do not feed on virus-infected tissue

Be sure that plant material has not been treated with any type of pesticide that may act as an antifeedant or is toxic to the insects.

Insects may not feed well on infected plant material; try another host or another cultivar of the same host.

Be sure the dishes do not contain any detergent or chemical residues.

Insects die or do not feed on membranes

Insects will vary in their ability to feed and preference for feeding on membranes. Try to feed them on a 15% sucrose solution. It may be necessary to increase or decrease the sucrose content (10% to 20% range). Be sure to use fresh solutions and filter sterilize.

If the insects feed on sucrose, but not the virus solution, it may be the choice of buffer, concentration of virus, or impurities in the solution. Filter sterilize the solution, change the concentrations of the virus and/or buffer, or use a different buffer (or water).

Insects feed well on tissue/membranes but not on healthy plants

Many insects do not acclimate well to different hosts. It may be best if the healthy plants are of the same host and variety as the virus source material and/or the same host on which the aphids are reared.

Insects do not transmit virus

The insect may not be a vector, or it may be an inefficient vector. Try increasing the number of aphids placed on each healthy plant. Alternatively, try another source of the same insect species or a different species.

Anticipated Results

Virus symptoms should develop on inoculated plants within 2 to 3 weeks. Transmission efficiency can range from 0% to 100%, depending on the vector species and population, source and recipient plants, and number of vectors used to inoculate the host. If an ideal system is developed, it is rare that 100% of the individual insects will transmit virus, and in many cases, the transmission efficiency is low enough that transmission tests using individual insects will yield negative results. Therefore, it is best to start the transmission experiments by

inoculating each plant with multiple insects. If 100% of the plants are routinely infected, begin decreasing the number of insects used to inoculate each plant.

Time Considerations

Transmission experiments can take considerable time, much of which is related to rearing insects. For example, it will generally take 3 to 4 weeks to establish a sizable colony of most aphid species on a suitable host, longer if they are reared on a marginal host. Other insects will take longer or shorter times to establish colonies, depending on generation times.

The actual transmission experiments are relatively quick to set up and conduct, but this depends on the acquisition and inoculation times required, as well as the number of treatments and plants used in each experiment. Then, there is the time required for the inoculated virus to infect and spread in the plant until it can be detected either by symptoms or some other diagnostic method. In short, a transmission experiment can take months if it includes establishing insect colonies and determining all the suitable parameters.

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Delivery and Expression of Functional Viral RNA Genomes In Planta by Agroinfiltration

The majority of plant viruses, if not all, are experimentally introduced into epidermal cells of intact plant leaves by simple mechanical inoculation. However, abrasives commonly used to create surface injury to facilitate virus entry often cause extensive cell damage, exposing RNA inoculum to nucleases. This constraint, however, does not apply to inoculation with infectious virions purified from symptomatic leaves. Despite the fact that mechanical inoculation of whole plants is the simplest approach, it suffers from the difficulty of attaining a synchronous and uniform delivery of the inoculum to the majority of plant cells. Attainment of a synchronized infection is not applicable to viruses having monopartite genomes (e.g., tobacco mosaic tobamovirus; TMV); whereas, replication and gene expression studies for viruses having multipartite genomes (e.g., brome mosaic bromovirus; BMV) requiring the presence of all genome components in the same cell are hindered by this approach.

The advent of recombinant DNA technology has greatly enhanced approaches in handling RNA-based viral genomes as well as for delivering one or multiple foreign genes into a wide range of plant cells for gene expression studies. The literature is replete with a variety of delivery approaches, such as electroporation, microinjection, or particle bombardment (*UNIT 16B.3*), that successfully deliver nucleic acids to plant cells. However, these approaches often require highly sophisticated equipment and skilled personnel. Research conducted over the years has demonstrated that plant pathogens could serve as potential tools for effective delivery of genes into plant cells. One such pathogen that has been exploited for gene delivery is the bacterial pathogen *Agrobacterium tumefaciens*. Delivery of cultures of *Agrobacterium* transformants into whole leaves by infiltration, referred to as agroinfiltration (Bendahmane et al., 2000; Voinnet et al., 2003), is a simple, user-friendly, highly reproducible approach to transient gene expression. Agroinfiltration results in transfer of transgenes from the transfer DNA (T-DNA) region of the bacterial tumor-inducing (Ti) plasmid into plant cells. The nonintegrated T-DNA copies remaining transiently present in the nucleus can be transcribed, leading to transient expression of T-DNA genes (Fig. 16B.2.1A). *Agrobacterium*-mediated transient gene expression has been widely used in plant biology for identification of disease-resistance genes (Bendahmane et al., 2000) and induction and suppression of post-transcriptional gene silencing (PTGS; Johansen and Carrington, 2001; Voinnet et al., 2003). Most importantly, agroinfiltration facilitates delivery and coexpression of multiple genes to the same cell. Although this technique was developed originally for use with DNA plant viruses (Grimsley et al., 1987), it subsequently proved to be useful for initiating infection with RNA viruses (Leiser et al., 1992).

To facilitate agroinfiltration, the complementary DNA (cDNA) copy of a given viral RNA genome initially has to be placed under the control of a cauliflower mosaic virus 35S promoter (CaMV 35S) by subcloning into a binary vector, i.e., a two-plasmid system in *Agrobacterium* for carrying the virulence genes on one plasmid and the engineered T-DNA region on the other. Creation of the binary vector is followed by transformation into a suitable *Agrobacterium* strain. The presence of a natural 5′ viral sequence is an essential prerequisite for achieving wild type–level biological activity (Boyer and Haenni, 1994). Therefore, cloning of cDNA copies into a suitable binary vector should be designed such that the transcription by the CaMV 35S promoter should initiate precisely at the authentic viral 5′ end. Unlike the 5′ end, the effect of 3′ nonviral nucleotide extensions on the biological activity of viral RNAs is varied (Boyer and Haenni, 1994; Ishikawa et al.,

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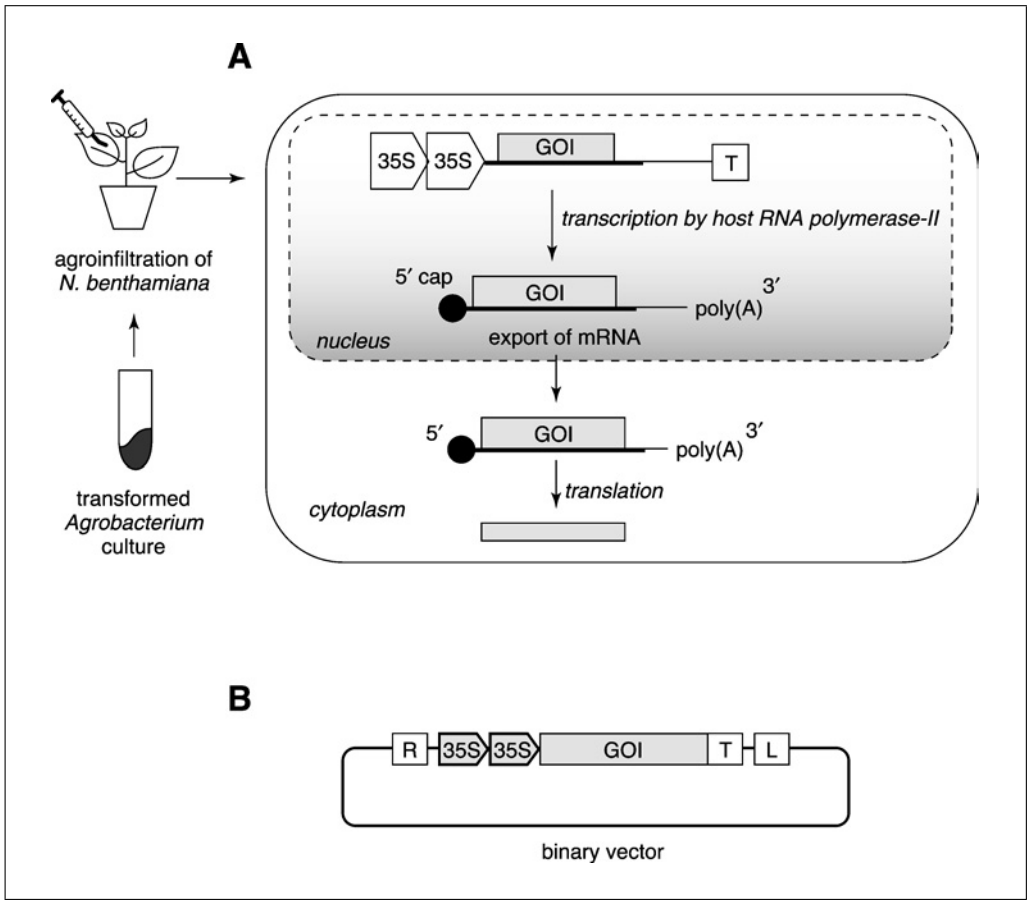

Figure 16B.2.1 (**A**) Events leading to *Agrobacterium*-mediated transient gene expression in plant cells. Cauliflower mosaic virus 35S promoter (35S) initiates transcription of the gene of interest (GOI) which terminates at the *Nos* terminator (T). (**B**) Representative example of a typical binary vector suitable for agroinfiltration. The left (L) and right (R) borders indicate the sequences of 25-bp imperfect repeats that flank the T-DNA and are required for its transfer.

1997; Annamalai and Rao, 2005). However, if viral mRNAs with correctly terminated 3′ ends are required, a self-cleaving ribozyme cassette from either the satellite tobacco ringspot virus (Bruening et al., 1988) or hepatitis δ virus (Been, 1994) can also be engineered. A variety of binary vectors suitable for agroinfiltration have been developed (Hellens et al., 2000; Annamalai and Rao, 2005): some common features of a typical binary vector are shown schematically in Figure 16B.2.1B.

Several T-DNA vectors suitable for agroinfiltration are now available and include the following: pBIN19, pBINPLUS, pC22, pCAMBIA series, pCGN series, pPZP series, and pGREEN series. The choice of vector depends on the application and considerations of convenience (Hellens et al., 2000). Additional information concerning the characteristics of various binary vectors can be obtained from the following web sites: *http://www.cambia.org* or *http://www.sainsbury-laboratory.ac.uk*.

Numerous *Agrobacterium* strains suitable for transformation of binary plasmids carrying the gene of interest are also available and the choice of an *Agrobacterium* strain depends on the host plant to be infiltrated. Some commonly used strains include LBA4404, C58C1, GV3100, GV2260, GV3101(pMP90), GV3101(pMP90RK), EHA101, or EHA 105 (Hellens et al., 2000; Weigel and Glazebrook, 2002). Most *Agrobacterium* strains (except C58C1 and GV3100) carry a rifampicin resistance gene for selection. Strain GV3101(pMP90) carries gentamycin resistance on the Ti plasmid, whereas GV3101(pMP90RK) carry gentamycin and kanamycin resistance. Earlier techniques used strain LBA 4404, but this strain often appeared to be avirulent.

Alternatively, more virulent strains derived from C58 such as GV3101(pMP90) and GV 3101(pMP90RK) or EHA105 are also available (Hellens et al., 2000).

In the methods described here, transformed *Agrobacterium* culture is grown to log phase, collected by low-speed centrifugation, and resuspended in an infiltration solution. The suspension is then infiltrated to the abaxial (back) surface of a leaf using a syringe (without the needle). Expression of the gene of interest can be analyzed 2 to 6 days postinfiltration. Given the fact that agroinfiltration facilitates transformation of a single cell with multiple genes, the approach is ideal for not only delivering DNA-based genomes of monopartite RNA viruses but also for coexpression of individual viral RNA components comprising either bipartite or tripartite (i.e., the genome divided among two or three distinct RNA species, respectively) viruses for a synchronized high-level gene expression within the same cell (Vlot et al., 2001; Liu and Lomonossoff, 2002; Annamalai and Rao, 2005). This inherent trait associated with agroinfiltration is also suitable for co-delivery of either defective interfering RNAs (DI RNAs) or satellite RNAs along with their respective helper viral genomes.

This unit describes procedures for the preparation of competent cells of *Agrobacterium* (Basic Protocol 1) and their transformation using a freeze-thaw approach (Basic Protocol 2), preparation of competent cells (Basic Protocol 3 and Alternate Protocol 1) and their transformation using electroporation (Basic Protocol 4), and finally, preparation of transformed cultures of *Agrobacterium* for infiltration of a plant species (Basic Protocol 6). In addition, Basic Protocol 5 and Alternate Protocol 2 provide methods for verifying the presence of the gene of interest in the transformed cells. Both transformation procedures are effective, and the decision for choosing one largely depends on convenience and availability of required laboratory equipment. The basic approaches described in this unit are generally applicable not only to analyzing transient gene expression but also to the study of high-level expression of biologically active viral genomes in virus replication, movement, and assembly.

CAUTION: Agrobacterium is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

PREPARATION OF *AGROBACTERIUM* COMPETENT CELLS FOR TRANSFORMATION BY FREEZE-THAW METHOD

BASIC PROTOCOL 1

Since DNA is a very hydrophilic molecule, it would not normally pass through a bacterial cell's membrane. Bacteria that are able to take up DNA are called "competent." Competency can be induced by treatment with calcium chloride in the early log phase of growth. The bacterial cell membrane is permeable to chloride ions but is nonpermeable to calcium ions. As the chloride ions enter the cells, water molecules accompany the charged particles. This influx of water causes the cells to swell and is necessary for uptake of DNA. Competent cells must always be maintained in a cold environment, during storage and use. Competent cells cannot be defrosted more than once. This protocol describes procedures for preparing competent *Agrobacterium* cells for transformation with a binary plasmid carrying the gene of interest using a freeze-thaw approach (see Basic Protocol 2).

Materials

Agrobacterium strain (e.g., LBA 4404, C58C1, GV2260, or EHA 105; see Hellens et al., 2000)
LB plates (see recipe)

LB medium (see recipe)
150 mM NaCl: prepare from autoclaved 1 M NaCl with sterile H_2O and store up to 3 months at 4°C
20 mM $CaCl_2$: prepare from filter-sterilized (22-μm filter) 1 M $CaCl_2$ and sterile H_2O; store up to 3 months at 4°C
Glycerol, sterile

28°C incubator
28°C orbital shaker
250-ml flask, sterile
50-ml polypropylene centrifuge tubes with tops, sterile (e.g., Fisher)
1.5-ml microcentrifuge tubes, sterile

1. Streak a loopful of the desired strain of *Agrobacterium* from a glycerol stock onto an LB plate supplemented with the appropriate antibiotic. Incubate at 28°C for 2 days.

   Agrobacterium cultures can be stored as glycerol stocks prepared according to standard procedures (e.g., Sambrook and Russell, 2001). The authors prepare Agrobacterium glycerol stocks by adding 300 μl of sterile 30% glycerol to 600 μl Agrobacterium culture in a 1.5-ml microcentrifuge tube, vortexing, and storing indefinitely at −80°C.

2. Pick a discrete single colony and inoculate a tube containing 5 ml LB medium.
3. Incubate the tube on an orbital shaker overnight at 28°C, 200 to 300 rpm.
4. Inoculate 100 ml LB medium in a 250-ml flask with 1 ml overnight culture. Incubate ~6 hr at 28°C or until the culture reaches an optical density (OD_{600}) of 0.5 to 0.6.
5. Transfer the flask containing the grown bacterial cells to a bucket of ice and incubate 30 min.
6. Transfer bacterial cells to 50-ml polypropylene centrifuge tubes and centrifuge 5 min at 2000 × *g*, 4°C. Discard supernatant.

   All solutions in subsequent steps must be prechilled on ice or kept at 4°C.

7. Resuspend pellet in 10 ml of 150 mM NaCl. Centrifuge 5 min at 2000 × *g*, 4°C. Discard supernatant.
8. Resuspend final pellet in 1 ml of 20 mM $CaCl_2$. Maintain on ice 1 to 2 hr.

9a. *To freeze:* Aliquot 50 μl competent cells into sterile microcentrifuge tubes, immediately freeze in liquid nitrogen, and store up to 3 months at −80°C.

9b. *To freeze with glycerol:* Add 10% glycerol to the competent cells, dispense 50-μl aliquots into sterile microcentrifuge tubes, and store up to 3 months at −80°C.

   Both methods work very well.

BASIC PROTOCOL 2

TRANSFORMATION OF COMPETENT *AGROBACTERIUM* BY FREEZE-THAW METHOD

The efficient introduction of plasmid DNA into bacterial cells (transformation) is critical to subsequent molecular characterization of gene products. Once a desired plasmid is constructed, it can be transformed into *Agrobacterium* using a variety of techniques. The freeze-thaw method is one of the commonly used approaches for bacterial transformation. The method is simple, rapid, and does not require specialized equipment, although transformation efficiency is low, yielding ~10^3 transformants/μg DNA.

Materials

Competent *Agrobacterium* cells (Basic Protocol 1), −80°C
Plasmid DNA: binary vector containing the gene of interest (e.g., see Sambrook and Russell, 2001)
LB medium (see recipe)
LB plates with 50 μg/ml kanamycin and 50 μg/ml rifampicin (see recipe) or other appropriate antibiotic
28°C orbital shaker
28°C incubator

1. Thaw one of the microcentrifuge tubes containing competent cells on ice.
2. Add 2 to 5 μg plasmid DNA (binary vector containing the gene of interest).
3. Mix gently and incubate 45 min on ice.
4. Freeze contents of the microcentrifuge tube by placing in liquid nitrogen for 1 min; immediately transfer to 37°C. Incubate 3 min.
5. Add 0.9 ml LB medium and incubate on an orbital shaker 3 hr at 28°C, 200 to 300 rpm.

   This will allow recovery of cells from frozen state and expression of the marker gene.

6. Centrifuge 5 min at 500 × *g*, room temperature. Discard most of the supernatant, leaving ~100 μl for resuspension.
7. Gently mix pellet by tapping with a finger.
8. Spread the transformed mixture onto an LB plate containing 50 μg/ml kanamycin and 50 μg/ml rifampicin or other appropriate antibiotic.

   Generally, the contents of one tube are spread onto one plate.

9. Incubate plates 48 hr at 28°C.

   After 48 hr, significant numbers of colonies are visible on the plate. Generally these colonies appear compact compared to normal bacterial colonies.

10. Store plates wrapped in Parafilm or plastic bags up to 3 weeks at 4°C.

PREPARATION OF *AGROBACTERIUM* COMPETENT CELLS FOR TRANSFORMATION BY ELECTROPORATION

BASIC PROTOCOL 3

Since DNA is a very hydrophilic molecule, it would not normally pass through a bacterial cell's membrane. The bacterial cells are competent when they are amenable to uptake of the plasmid DNA. Competent cells prepared for the freeze-thaw method of transformation (Basic Protocols 1 and 2) are not suitable for electroporation because the methods are different. In transformation by electroporation, DNA is forced into cells by initially incubating on ice the reaction mixture containing the competent cells and the DNA, followed by electroporation.

Materials

Agrobacterium strain (e.g., LBA 4404, C58C1, GV2260, or EHA 105; see Hellens et al., 2000)
LB plates (see recipe)
LB medium (see recipe)
1 mM 4-(2-Hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES), pH 7: pass through a 0.45-μm filter to sterilize; store up to 3 months at room temperature
Glycerol, sterile

28°C incubator
28°C orbital shaker
250-ml flask
50-ml polypropylene centrifuge tubes with tops, sterile (e.g., Fisher)
1.5-ml microcentrifuge tubes, sterile

1. Streak desired strain of *Agrobacterium* from a glycerol stock onto an LB plate supplemented with the appropriate antibiotic. Incubate at 28°C for 2 days.
2. Pick a discrete single colony and inoculate a tube containing 5 ml LB medium.
3. Incubate the tube on an orbital shaker overnight at 28°C, 200 to 300 rpm.
4. In a 250-ml flask, inoculate 100 ml LB medium with 1 ml of overnight culture. Incubate ~6 hr at 28°C or until the culture reaches an optical density (OD_{600}) of 0.5 to 0.6.
5. Transfer the flask containing the grown bacterial cells to a bucket of ice and incubate 30 min.
6. Transfer bacterial cells to 50 ml-polypropylene centrifuge tubes and centrifuge 5 min at 2000 × *g* (5000 rpm), 4°C. Discard supernatant.
7. Resuspend pellet in 20 ml prechilled 1 mM HEPES, pH 7.0. Centrifuge 5 min at 2000 × *g*, 4°C. Discard supernatant.
8. Wash pellet with 20 ml prechilled 1 mM HEPES, pH 7.0, and repeat centrifugation. Discard supernatant.
9. Resuspend the final pellet in 2 ml prechilled 1 mM HEPES, pH 7.0, and add sterile glycerol to a final concentration of 10% (v/v).
10. Dispense 50-µl aliquots of the electrocompetent cells into sterile 1.5-ml microcentrifuge tubes.
11. Use cells immediately or store up to 3 months at −80°C.

ALTERNATE PROTOCOL 1

GLYCEROL METHOD FOR PREPARATION OF *AGROBACTERIUM* ELECTROCOMPETENT CELLS

Competent cells suitable for electroporation can also prepared using glycerol. This protocol is simple and ideal for rapid preparation of cells for large-scale transformation.

Additional Materials *(also see Basic Protocol 3)*

SOB medium (see recipe)
10% (v/v) glycerol, ice cold: prepare using autoclaved glycerol and sterile H_2O
1.5-ml microcentrifuge tubes, sterile

1. Grow bacterial cells as in Basic Protocol 3, steps 1 through 6, except substituting SOB medium for LB medium in steps 2 and 4.

   SOB is a richer medium than LB and results in faster bacterial growth. Not only can transformants be observed earlier in SOB, but transformation efficiency is much higher (ten to thirty times) than in LB.

2. Add 10 ml of 10% ice-cold glycerol to the pellet and mix gently.
3. Centrifuge 5 min at 2000 × *g*, 4°C, and discard supernatant.
4. Repeat steps 2 and 3 three more times.

5. Resuspend final pellet in 2 ml of 10% ice-cold glycerol.
6. Dispense 50-μl aliquots into sterile microcentrifuge tubes and store up to 3 months at −80°C.

TRANSFORMATION OF COMPETENT *AGROBACTERIUM* BY ELECTROPORATION

BASIC PROTOCOL 4

The electroporation method of transforming DNA into *Agrobacterium* can produce efficiencies greater than those achieved with other methods. The underlying principle of electroporation involves generation of electric shock stimulus to open pores in host cells. By opening these pores in the cell wall, plasmid DNA is able to enter into the cell. This method is much more efficient than the freeze-thaw method described in Basic Protocol 2, approaching a transformation efficiency of 10^9 to 10^{10} transformants/μg DNA. However, unlike the freeze-thaw method, electroporation requires specialized equipment for generating the desired high-voltage electric pulse.

Electrocompetent *Agrobacterium* cells (Basic Protocol 3 or Alternate Protocol 1)
Plasmid DNA: binary vector containing gene of interest (e.g., see Sambrook and Russell, 2001)
YEP or SOC medium (see recipes)
YEP plates supplemented with 50 μg/ml kanamycin and 50 μg/ml rifampicin (see recipe) or other appropriate antibiotic
Electroporation cuvettes with 0.1- or 0.2-cm path length (e.g., BioRad), ice cold
Electroporator (e.g., BioRad)
1.5-ml microcentrifuge tubes or 5-ml plastic tubes, sterile
28°C orbital shaker
28°C incubator
Microcentrifuge

1. To the frozen 50-μl aliquot of electrocompetent cells, add 2 to 5 μg plasmid DNA.
2. Incubate 5 min on ice.
3. Dispense the mixture to an ice-cold 0.1- or 0.2-cm path length electroporation cuvette.
4. Electroporate cells with 25 μF (capacitance), 400 Ω (resistance) for a 1.25 KV (0.1 cm) or 2.5 KV (0.2 cm) pulse followed by 8 to 9 milliseconds delay.

   Generally any electroporator can be used. For efficient electroporation, follow the conditions specified by the manufacturer.
5. Immediately add 0.9 ml YEP or SOC medium.
6. Transfer solution to a sterile 1.5-ml microcentrifuge tube and incubate 2 to 3 hr with shaking at 28°C.

   This would allow recovery of cells from frozen state and expression of marker gene.
7. Centrifuge cells 5 min at 500 × *g*, room temperature. Discard most of the supernatant, leaving ~100 μl for resuspension.
8. Gently mix pellet by tapping with a finger.
9. Spread the transformed mixture onto YEP plates supplemented with 50 μg/ml kanamycin and 50 μg/ml rifampicin.

   Generally, the contents of one tube are spread onto one plate.
10. Incubate plates 48 hr at 28°C.

After 48 hr, significant numbers of colonies are visible on the plate. Generally, these colonies appear compact compared to normal bacterial colonies.

11. Store plates up to 3 weeks at 4°C.

BASIC PROTOCOL 5

VERIFICATION OF *AGROBACTERIUM* TRANSFORMATION BY AGAROSE GEL ELECTROPHORESIS

Prior to the preparation of cultures for infiltration, it is imperative to verify the transformed colonies for the presence of the gene of interest. This can be done utilizing either agarose gel electrophoresis, as described in this protocol, or via PCR (Alternate Protocol 2).

Materials

Transformed *Agrobacterium* cultures (Basic Protocol 2 or Basic Protocol 4)
LB medium with appropriate antibiotics (see recipe)
Plasmid DNA extraction kit (e.g., QIAprep Spin miniprep kit; Qiagen; optional)
28°C orbital shaker
Additional reagents and equipment for isolation (Engebrecht et al., 1991) and digestion (Sambrook and Russell, 2001) of plasmid DNA, and agarose gel electrophoresis (Voytas, 2000)

1. Pick a single discrete colony of transformed *Agrobacterium* and inoculate a tube containing 5 ml LB medium with appropriate antibiotics.

   Generally ten to fifteen individual colonies should be randomly picked for screening.

2. Incubate on an orbital shaker 48 hr at 28°C, 200 to 300 rpm.
3. Extract plasmid DNA using standard protocols (e.g., see Engebrecht et al., 1991) or commercially available kits.
4. Digest plasmid DNA with appropriate restrictions enzymes (e.g., see Sambrook and Russell, 2001).
5. Resolve the digested DNA samples by agarose gel electrophoresis (e.g., see Voytas, 2000).

ALTERNATE PROTOCOL 2

VERIFICATION OF *AGROBACTERIUM* TRANSFORMATION BY PCR

The procedure described in Basic Protocol 5 for identifying the gene of interest is a time consuming and lengthy process. This can be circumvented by using PCR, which is a fast and reliable method for screening several transformants. The gene of interest can be amplified in <3 hr in a PCR reaction, using appropriate 5′ forward and 3′ reverse primers for the gene of interest, and the amplified product can be verified by agarose gel electrophoresis.

Materials

Transformed *Agrobacterium* cultures (Basic Protocol 2 or Basic Protocol 4)
H_2O, sterile
10× reaction buffer (NEB or Promega)
20 μM primer 1 (5′ forward primer of gene of interest)
20 μM primer 2 (3′ reverse primer of gene of interest)
2.0 mM dNTPs (Amersham or Promega)
100 mM $MgCl_2$ (Promega)
Taq polymerase (Promega)
Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000)

1. Pick fully grown colonies from the plates containing the transformed *Agrobacteria* and suspend in 10 μl distilled water.

   Normally 10 to 20 such colonies should be selected for analysis.

2. Incubate each suspension for 3 min at 100°C in a thermocycler.

3. While the sample is incubating, prepare the following cocktail mix:

   2.0 μl 10× reaction buffer
   1.0 μl 20 μM primer 1
   1.0 μl 20 μM primer 2
   3.0 μl 2.0 mM dNTPs
   0.5 μl 100 mM $MgCl_2$
   0.3 μl *Taq* polymerase
   2.2 μl H_2O.

4. Add 10 μl cocktail mix to each suspension and mix gently.

5. Place each reaction mixture in a thermocycler. Carry out PCR using the following amplification cycles:

25 to 30 cycles:	1 min	94°C	(denaturation)
	1 min	55°C	(annealing)
	1 min	72°C	(extension)
	10 min	72°C	(final extension).

6. Analyze the PCR products on a 1% agarose gel (Voytas, 2000) to verify presence or absence of the gene of interest.

   The size of the PCR product can be used to confirm the presence of the gene of interest in the transformant.

   Once a positively transformed bacterial colony has been identified, it can be stored permanently as a glycerol stock for future agroinfiltration experiments.

PREPARATION AND INFILTRATION OF TRANSFORMED *AGROBACTERIUM* CULTURE

BASIC PROTOCOL 6

Generally young seedlings of *Nicotiana benthamiana* having two to three well expanded leaves (five-leaf stage, three- to four-week-old plants) are ideal for infiltration.

Materials

Positively identified, transformed *Agrobacterium* cultures (Basic Protocol 5 or Alternate Protocol 2)
LB plates supplemented with 50 μg/ml kanamycin and 50 μg/ml rifampicin (see recipe) or other appropriate antibiotic
LB medium supplemented with 50 μg/ml kanamycin and 50 μg/ml rifampicin (see recipe) or other appropriate antibiotic
1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.6: pass through a 0.45-μm filter to sterilize and store up to 3 months at room temperature in a brown bottle
100 mM 5′dimethoxy-4′-hydroxyacetophenone (acetosyringone) in DMSO
10 mM $MgCl_2$: prepare from filter-sterilized (0.45-μm filter) 1 M $MgCl_2$ and sterile H_2O
Nicotiana benthamiana seedlings: five-leaf stage, i.e., three- to four-week-old plants having two to three well expanded leaves, or other appropriate seedling
28°C incubator
28°C orbital shaker

50-ml plastic tubes, sterile (e.g., Fisher or Oak Ridge)
1-ml syringe without needle
Greenhouse, growth room, or 20°C to 23°C growth chamber with adjustable lighting conditions

CAUTION: Some virulent strains of *Agrobacterium* are classified as plant pests and should only be used only after obtaining all relevant state and federal permits. Plants infiltrated with such virulent strains must be held in a restricted containment area and destroyed or disposed of according to USDA guidelines (*www.aphis.usda.gov/ppq/permits*).

CAUTION: DMSO is hazardous. Use caution when handling solutions made with DMSO. See *UNIT 1A.3* for more information.

1. Streak a positively identified, appropriately transformed bacterial colony on LB plate supplemented with appropriate antibiotics. Incubate 48 hr at 28°C.
2. Pick a single discrete colony to inoculate 5 ml LB medium supplemented with 50 μg/ml kanamycin and 50 μg/ml rifampicin (or other appropriate antibiotic).
3. Incubate on an orbital shaker 48 hr at 28°C, 200 to 300 rpm.

   Grown cultures can be stored up to 1 month at 4°C and can be used to restart a 5-ml fresh culture by inoculating 0.2 ml stored culture into 5 ml fresh LB medium supplemented with antibiotics.
4. Inoculate 1 ml culture from step 3 into 50 ml LB medium supplemented with (final concentrations) 50 μg/ml kanamycin, 50 μg/ml rifampicin, or other appropriate antibiotic. Add 1 M MES, pH 5.6, and 100 mM acetosyringone to final concentrations of 10 mM and 100 μM, respectively.

   Either 100-ml or 250-ml sterile Erlenmeyer flasks are ideal for starting a fresh culture. The volume of fresh cultures is scalable depending on the number of samples and plants to be infiltrated.
5. Incubate on an orbital shaker 16 hr at 28°C, 200 to 300 rpm.
6. Measure OD_{600} of freshly grown culture, which must be 1.0.

   At this stage, the culture should emit an odor resembling sweet corn. This indicates that the virulence gene of the Ti-plasmid has been activated.
7. Transfer the fresh culture to a sterile tube.
8. Centrifuge 10 min at 2000 × *g*, room temperature.
9. Discard supernatant and resuspend pellet in 50 ml of 10 mM $MgCl_2$.

   Alternatively, bacterial cells can also be suspended in 10 mM MES and 10 mM $MgCl_2$. Addition of 10 mM MES (pH 5.6) to the suspension solution has been shown to slightly enhance transient expression.
10. Centrifuge 10 min at 5000 rpm, room temperature. Discard supernatant.
11. Resuspend pellet in 50 ml of 10 mM $MgCl_2$, and add 100 mM acetosyringone (from 100 mM acetosyringone stock) to a final concentration of 100 μM. Mix gently to aid resuspension.
12. Keep the *Agrobacterium* cultures to be used for infiltration at room temperature for at least 3 hr without shaking.
13. Using a 1-ml syringe, gently press the syringe onto one half of the abaxial surface of a fully expanded leaf of a *Nicotiana benthamiana* (or other appropriate plant) seedling. Repeat the process on the opposite side of the main vein.

There is not a specific volume for each infiltration. Depending on the type of assay, either the entire leaf or a 1- to 2- cm spot can be infiltrated with liquid culture.

A fully infiltrated part of the leaf gives a water-soaked appearance.

Generally infiltration is very efficient in N. benthamiana, requiring only one infiltration site. However, depending on the age of the leaf, more than one infiltration may be required. Depending on the nature of experiment, the extent of infiltrated area can be controlled.

14a. *For immediate transfer:* After agroinfiltration, transfer plants to greenhouse, growth room, or large growth chamber.

14b. *For delayed transfer:* Hold agroinfiltrated plants in a growth chamber 1 day under mild light conditions (1,000 lux) with 16 hr light and 8 hr dark photoperiod cycle, 20°C to 23°C, and then transfer plants to a greenhouse.

Incubating infiltrated plants at higher temperatures can inhibit T-DNA transfer.

15. Collect leaf samples from 2 days post-infiltration onwards to screen for transient expression of the gene of interest.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Kanamycin, 50 mg/ml

Dissolve 50 mg kanamycin in 1 ml water. Sterilize by passing through a 0.22-μm filter. Dispense 100-μl aliquots into sterile 1.5-ml microcentrifuge tubes and store up to 3 months at −20°C.

LB medium with and without 50 μg/ml kanamycin and 50 μg/ml rifampicin

Dissolve the following in 1 liter H_2O with mixing:
5 g yeast extract
10 g bacto-tryptone
10 g sodium chloride

Dispense 100 ml medium into 250-ml Erlenmeyer flasks and autoclave 20 min at 15 psi on liquid cycle. Store up to 1 month at room temperature or up to 4 months at 4°C. If antibiotics are required, before use add 50 μl of 100 mg/ml rifampicin (see recipe) and 100 μl of 50 mg/ml kanamycin (see recipe) or other appropriate antibiotics to each 100 ml medium.

LB plates with and without 50 μg/ml kanamycin and 50 μg/ml rifampicin

Dissolve the following in 1 liter H_2O with mixing:
5 g yeast extract
10 g bacto-tryptone
10 g sodium chloride
15 g agar powder

Dispense 100 ml medium into 250-ml Erlenmeyer flasks and autoclave 20 min at 15 psi on liquid cycle. Cool the medium to 55°C in a water bath and, if required, add 50 μl of 100 mg/ml rifampicin (see recipe) and 100 μl of 50 mg/ml kanamycin (see recipe) or other appropriate antibiotics to each 100 ml medium. Dispense 25 to 30 ml into sterile 15 × 100–mm disposable petri dishes. When the medium has completely solidified, store plates in a covered plastic box or wrapped with Parafilm up to 6 weeks at 4°C.

Rifampicin, 100 mg/ml

Dissolve 100 mg rifampicin in 1 ml of 100% methanol. Store solutions in tightly closed microcentrifuge tubes for up to 3 months at −20°C.

Antibiotics dissolved in methanol need not be sterilized.

SOB medium

Dissolve the following with shaking in 950 ml H_2O:
20 g tryptone
5 g yeast extract
0.5 g sodium chloride

Add 10 ml 250 mM $CaCl_2$. Adjust pH to 7.0 with 5 N NaOH. Adjust final volume to 1 liter with water. Autoclave 20 min at 15 psi. Dispense 100 ml medium into 250-ml Erlenmeyer flasks and autoclave 20 min at 15 psi on liquid cycle. Store up to 1 month at room temperature or up to 4 months at 4°C. Just before use add 0.5 ml sterile 2 M $MgCl_2$ to 100 ml medium.

SOC medium

Dissolve the following with shaking in 950 ml H_2O:
20 g tryptone
5 g yeast extract
0.5 g sodium chloride

Add 10 ml of 250 mM $CaCl_2$. Adjust pH to 7.0 with 5 N NaOH. Adjust final volume to 1 liter with water. Autoclave 20 min at 15 psi. Add 20 ml sterile 1 M glucose solution. Dispense 100 ml medium into 250-ml Erlenmeyer flasks and autoclave 20 min at 15 psi on liquid cycle. Store up to 1 month at room temperature or up to 4 months at 4°C. Just before use add 0.5 ml sterile 2 M $MgCl_2$ to 100 ml medium.

YEP plates with and without 50 μg/ml kanamycin and 50 μg/ml rifampicin

Dissolve the following in 1 liter H_2O with mixing:
10 g Bacto peptone
5 g yeast extract
15 g NaCl
15 g agar powder

Dispense 100 ml medium into 250-ml Erlenmeyer flasks and autoclave 20 min at 15 psi on liquid cycle. Cool the medium to 55°C in a water bath and add 50 μl of 100 mg/ml rifampicin (see recipe) and 100 μl of 50 mg/ml kanamycin (see recipe) or other appropriate antibiotics to each 100 ml medium. Dispense 25 to 30 ml into sterile 15 × 100–mm disposable petri dishes. When the medium has completely solidified, store plates in a covered plastic box or wrapped with Parafilm up to 6 weeks at 4°C.

COMMENTARY

Background information

Agroinfiltration is simple, efficient, and robust compared to conventional mechanical inoculation or other delivery methods. Most importantly, it results in synchronous delivery of *Agrobacterium* transformants to a majority of the cells encompassing the infiltrated area. Furthermore, agroinfiltration is ideal for delivering multiple *Agrobacterium* transformants to a single cell. Because of the high transformation rate and efficient accumulation of mRNAs, the method is ideal for analyzing biological activities of viral genomes with defective replication traits. One caveat with this approach is that not all host plants are suitable for infiltration. Among several tobacco species tested, *Nicotiana benthamiana* was found to be the easiest to infiltrate. Likewise,

the authors have also observed that leaves of *Chenopodium quinoa* are readily infiltratable and susceptible to *Agrobacterium* transformation. Other hosts that have been successfully used for agroinfiltration include *Arabidopsis thaliana*, cucumber (Daros and Flores, 2004), and cowpea (Liu and Lomonossoff, 2002). However, it should be noted that infiltration does not always guarantee transformation of cells by *Agrobacterium* because of incompatibility between the host and *Agrobacterium* strain used. It is also possible that the intercellular spaces of a given host may allow the liquid to infiltrate but not the bacterium. Therefore, it is imperative to verify the level of compatibility between a given host and the *Agrobacterium* strain to be used. This can be done by initially testing a binary construct competent to express a reporter gene such as GFP (Li et al., 1999; Voinnet et al., 2003).

Agroinfiltration has been widely used in plant molecular biology and related studies such as analysis of gene silencing (Balucombe, 1999), foreign gene expression (Kapila et al., 1997), hypersensitive reaction (Palanichelvam et al., 2000), promoter activity (Yang et al. 2000), identification of new disease resistance genes (Bendahmane et al., 2000), propagation of a virus (Liu and Lomonossoff, 2002), and more recently, for analyzing assembly and packaging of viral RNA independent of replication (Annamalai and Rao, 2005). Apart from agroinfiltration, depending on the plant species and type of experiments, other approaches to inoculating plants with *Agrobacterium* have been used, e.g., picking the *Agrobacterium* colony with a toothpick and stabbing the toothpick into seedling leaf of *N. benthamiana* (Lu et al., 2003). This is an efficient way of inoculating a large number of plants and is also suitable for high-throughput screens. However, this approach does not work well with other plant species and requires screening of compatibility between *Agrobacterium* strain and the host.

Critical Parameters

A fresh culture must be grown for 48 hr from the initial stock each time an infiltration is to be performed. Furthermore, a 16-hr culture should be grown using a 48-hr-old primary culture. This will eliminate the presence of dead cells in the inoculum. Verify the final optical density at OD_{600} of the culture, which must be 0.8 to 1.0. When two or more *Agrobacterium* transformants are to be co-infiltrated, grow each culture independently as described above. When two *Agrobacterium* transformants are to be co-infiltrated, the OD_{600} of each culture must be at least 1.0. Routinely, equal volumes of each culture are mixed prior to infiltration. After mixing in equal volumes, the final OD_{600} of the mixture containing two cultures should be 1.0. Likewise, when three *Agrobacterium* transformants are to be co-infiltrated, the OD_{600} of each culture must be at least 1.0 and the final OD_{600} of the mixture containing all three cultures should be 1.0.

The level of transient gene expression resulting from agroinfiltration usually peaks 60 to 72 hr postinfiltration and declines rapidly thereafter (Voinnet et al., 2003). Initially, the reason for this decline in transient expression was attributed to the bacterial/host combination, but recently it was proposed that PTGS is another limiting factor (Johansen and Carrington, 2001). This, however, can be alleviated by coexpression of viral suppressors of PTGS (Voinnet et al., 2003). Commonly used suppressors of RNA silencing include HC-Pro of tobacco etch potyvirus HC-Pro (Brigneti et al., 1998); tomato aspermy cucumovirus-2b (TAV-2b; Li et al., 1999) and tomato bushy stunt tombusvirus p-19 (TBSV; Voinnet et al., 2003).

Troubleshooting

Possible causes of problems related to agroinfiltration and potential solutions are listed in Table 16B.2.1.

Anticipated Results

The high level of *Agrobacterium*-mediated transformation generally results in the production of an abundant amount of mRNA from each transformed cell. As a result, mRNA levels and the expression of respective genes can be verified with ease using routine detection assays such as northern blots, immunoblots, and confocal laser scanning microscopy (*UNIT 2C.1*), if reporter genes such as GFP are used.

Time Considerations

Approximately 3 days are required to prepare the competent cells of *Agrobacterium* from glycerol stock. Transformation generally requires 4 to 5 hr. Following transformation and plating, appearance of transformed colonies require 48 hr. Growing cultures of transformants requires 48 hr, followed by an additional 12 to 16 hr for growth of fresh culture for infiltration. Finally, preparation of cultures for infiltration requires 4 to 5 hr. Collectively, the entire experimental procedure can be successfully completed in a week. These

Table 16B.2.1 Troubleshooting for Problems Related to Agroinfiltration

Problem	Possible Cause	Solution
Agrobacterium cells did not grow	Concentration of rifampicin too high	Some strains are sensitive to rifampicin; do not use more than 10–50 μg/ml
Overnight culture did not reach OD_{600} of 0.5	Temperature too high	Set the incubating temperature to exactly 28°C
No transformants	Voltage too high during electroporation	Reduce voltage
No transformants	Reduced quality or quantity of plasmid DNA	Verify the quality of DNA by gel electrophoresis; use higher concentration of plasmid DNA
No transformants	No insert	Verify the plasmid for desired insert
No transformants	Use of inappropriate antibiotic	Verify the selection marker of the Ti-plasmid used
No sweet corn odor	No activation of virulence genes	Add sufficient concentration of acetosyringone
No transient expression	No insert	Verify the plasmid for desired insert
No transient expression	No activation of virulence genes	Add sufficient concentration of acetosyringone
No transient expression	Activation of RNA silencing	Co-infiltrate RNA silencing suppressors

time considerations are in addition to cloning of the gene of interest into a binary plasmid.

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Inoculation of Plants Using Bombardment

This unit addresses particle bombardment, a method for the acceleration of microscopic particles of metal coated with viral material into plant tissue by the use of pressurized gas. It is primarily used for inoculating intact plants with full-length infectious cDNA or transcripts of RNA viruses. Particle bombardment has been used to insert DNA into living plant cells for both transient and permanent gene expression since the pioneering work of Klein et al. (1987). Additionally, Klein et al. were the first to show the expression of tobacco mosaic virus (tobamovirus) in onion epidermal cells following bombardment with RNA. Inoculating RNA viruses by bombardment requires full-length, infectious, viral cDNA which has been cloned or PCR-amplified and has appropriate promoters and termination signals. Bombardment with RNA transcripts refers here to in vitro–produced transcripts.

Bombardment does not replace the use of other mechanical or natural vector inoculation methods described in this section; however, it permits cDNA inoculation of plants without the production of viral transcripts, resulting in lower costs, less work, improved stability of transcripts, and greater efficiency (for potyviruses). Inoculation of cDNA can also be accomplished by infiltration with *Agrobacterium tumefaciens* (*UNIT 16B.2*), although this lengthier method inserts another pathogen into the plant-virus system.

There are many types of particle bombardment equipment, including the particle inflow gun (PIG; Finer et al., 1992), the plastic PIG (Grey et al., 1994), and the Bio-Rad PBS (Klein et al., 1987), all of which may require technical assistance from outside the laboratory to construct a vacuum chamber for their use (Finer et al., 1992; Gray et al., 1994). Here, the authors discuss two types of bombardment equipment—the Blowpipe and the HandGun—neither of which require a vacuum chamber and both of which can be assembled in the laboratory with little assistance, using commonplace, commercially available parts. The more complex apparatus, the HandGun, should cost $200 to $300 in parts, excluding the cost of a gas cylinder. Consumables, e.g., gas, metal particles, and chemicals, are also low cost with this bombardment method. Other advantages of homemade equipment are ease of maintenance and the ability to alter the device, according to need, without concern for a manufacturer's warranty.

In Basic Protocol 1, the authors describe the parts required for the Blowpipe, a simple but useful instrument—effectively the same as the device described by Takeuchi et al. (1992)—and the HandGun, a partially automated version of the Blowpipe, which is easier to handle and therefore generally preferable but which requires more parts, cost, and construction effort (Gal-On et al., 1997). Both of these devices share a common component, the discharge assembly.

In addition, instructions for the use of the equipment are provided (Basic Protocol 2), along with information about preparation of cDNA or RNA transcripts (Support Protocol 1) and the metal mixtures for bombardment (see Reagents and Solutions). Bombardment with plant sap, virus preparation, or viral RNA extract is also discussed (Support Protocol 2).

The technology described here is very efficient. Particle bombardment without a vacuum is 10^5 more efficient than mechanical inoculation (*UNIT 16B.1*) with a plasmid encoding a full-length clone driven by a promoter, and particle bombardment in a vacuum is 10-fold

Contributed by Victor Gaba and Amit Gal-On

Current Protocols in Microbiology (2005) 16B.3.1-16B.3.14

Table 16B.3.1 Materials Used for Plant RNA-Virus Inoculation by Particle Bombardment

Bombardment material	Reference
Full-length infectious cDNA clone	Gal-On et al., 1995, 1997
cDNA products produced by PCR	Fakhfakh et al., 1996
Virus from plant sap	Gal-On et al., 1997
Total RNA from infected plant	Gal-On and Gaba, unpub. observ.
RNA-virus preparation	Gal-On et al., 1997
Transcripts (capped and uncapped)	Gal-On et al., 1995
Phloem-limited RNA viruses	Yang et al., 1997

Table 16B.3.2 Techniques and Investigations Enabled by the Blowpipe and HandGun Bombardment Technology

Technique	Reference
Inoculating large numbers of plants	Shiboleth et al., 2001
Inoculating plants with thin, soft leaves (e.g., *Chenopodium quinoa*, *Nicotiana bentamiana*)	Gal-On et al., 1997
Inoculating plants in greenhouse	Gal-On and Gaba, unpublished
Studying viral recombination in planta	Gal-On et al., 1998
Rapid plant or viral promoter analysis	Wang et al., 2000
Monitoring virus movement using mutants of infectious clone bearing a reporter gene	Kimalov et al., 2004

more efficient than without a vacuum (Gal-On et al., 1995, 1997). The materials described in Table 16B.3.1. can be used for plant RNA virus inoculation by particle bombardment. This technology allows for the techniques and investigations detailed in Table 16B.3.2, among them the convenience of mass bombardment methods (Alternate Protocol) and particle bombardment in the greenhouse or the field.

BASIC PROTOCOL 1

ASSEMBLY OF BOMBARDMENT APPARATUS

For the construction of the Blowpipe and Handgun, the authors have used parts available in Israel, all of which have threads following British Standard Pipe (BSP) or British Standard Pipe Parallel (BSPP), the local piping standard; however, the use of other piping standards is also acceptable. Take care that all parts have compatible diameters and pipe threads: do not mix piping of different standards. The materials list in this protocol includes some catalog numbers for parts from specific suppliers, e.g., Cole-Parmer, but parts from other manufacturers can be easily used (e.g., see Finer et al., 1992; Gray et al., 1994) and are commercially available. Parts are also commercially available at different voltages (e.g., 110V). To date, the authors have used only brass adapters for the discharge assembly. The pipe fittings are all either stainless steel or brass. The quick release gas fittings, while convenient, can be replaced by barbed fittings threaded at one end for a simple connection to a rubber gas hose.

It is possible to move the gas cylinder and the associated equipment on a trolley into a greenhouse to inoculate more mature plants. Even greater field mobility can be attained by using a miniature gas cylinder with a battery-operated timer.

The assembled HandGun is shown in Figure 16B.3.1.

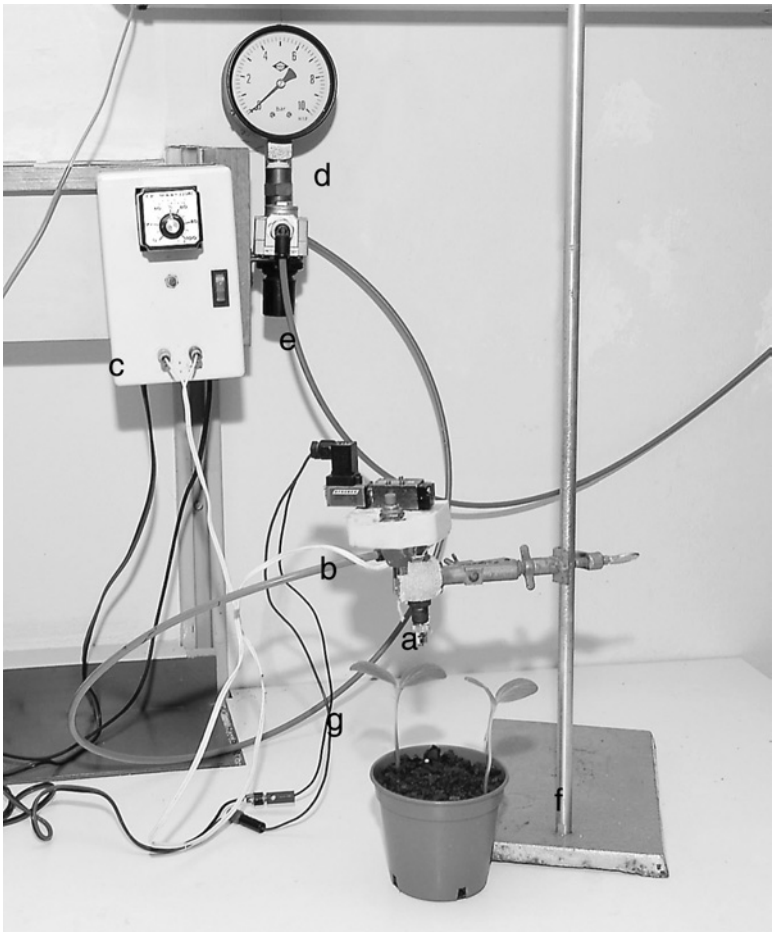

Figure 16B.3.1 The assembled HandGun. (a) HandGun. (b) The trigger switch taped in position. (c) Wall-mounted timer. (d) Secondary wall-mounted gas pressure gauge. (e) Secondary gas regulator. (f) Clamp stand to hold the HandGun. (g) Plant to be bombarded. The tubing on the right hand side of the picture connects to the He gas cylinder. The filter holder is not attached.

Materials

Materials for construction of the discharge assembly (Fig. 16B.3.2):

Luer adapter, male luer lock to $^1/_4$-in. (6.35-mm) unified fine threads (UNF; e.g., Cole-Parmer cat. no. 31507-73; a)

Luer adapter, male (M) $^1/_4$-in. (6.35-mm) fitting attached to a machined UNF female thread to accept the luer lock adapter (b)

13-mm-diameter plastic filter holder (Pall-Gelman cat. no. 4312; c)

Materials for construction of the Blowpipe apparatus (Fig. 16B.3.3):

7-mm rubber vacuum hose, ~5 m (a)

Plastic two-way tap, 8 to 10 mm (b)

Barbed fitting to connect rubber tubing to a metal fitting (c)

$^1/_4$-in. (6.35-mm) coupling adapter female piping thread (FPT)/FPT, if required

Worm-driven hose clips for fixing rubber tubing onto metal pipes (d)

Materials for construction of the HandGun apparatus:

Materials for assembling the HandGun device:

Electrically operated miniature solenoid valve (Dynamco cat. no. D3533KL0; Fig. 16B.3.4a)

Base plate (Dynamco cat. no. B03B2B) with $^1/_4$-in. (6.35-mm) apertures (Fig. 16B.3.4b).

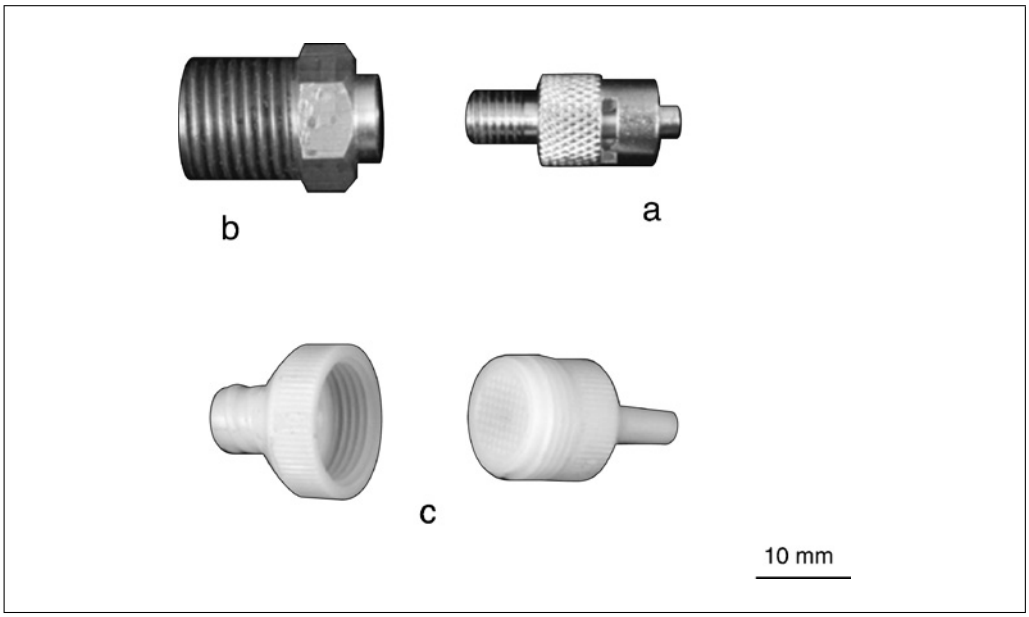

Figure 16B.3.2 Components of the discharge assembly. (a) Leur adapter male, luer lock to UNF thread. (b) Leur adapter, male $^1/_4$-in. BSP fitting with a machined UNF female thread. (c) Filter holder (open): top on left, lower part on right, showing the grid.

$^1/_4$-in. (6.35-mm) plugs, nonprotruding (Fig. 16B.3.5)
Pipe-tubing elbow for connecting the pneumatic hose (Fig. 16B.3.4c)
$^1/_4$-in. (6.35-mm) adapter(s) FPT/M: either one ~40 mm length or two short ones (Fig. 16B.3.4d)
Clamp stand (Fig. 16B.3.1f)
Miniature DIN plug (Fig. 16B.3.4e)

Timer components (Fig. 16B.3.6):
Electrical wire, flexible, two-conductor, ~6 m
220 V, variable (0 to 100 msec) timer with suitable base (Megatron Electronics and Controls cat. no. MSST-700-CPT; a)
Electrical push-button switch (b)
Plastic box for mounting the timer with electrical connections (c)

Secondary gas regulator components:
Pipe-tubing elbows (Fig. 16B.3.5c)
Pressure regulator (~0.05 to 0.85 MPa), with female $^1/_4$-in. (6.35-mm) fittings (Fig. 16B.3.7a)
T-adapter, MFM, $^1/_4$, $^1/_2$, $^1/_4$ in. (6.35, 12.7, 6.35 mm) (Fig. 16B.3.7b)
0- to 10-bar (0- to 1000-kPa) gas pressure gauge, 100 mm diameter, $^1/_2$-in. (12.7-mm) male fitting (Fig. 16B.3.7c)
$^1/_4$-in. (6.35-mm) adapter M/M (Fig. 16B.3.5b)
Two-way tap; $^1/_4$-in. (6.35-mm) female fittings (optional; Fig. 16B.3.7d)
20-µm gas line filter with female $^1/_4$-in. (6.35-mm) fittings (optional)
Wall-mounting bracket

Gas cylinder and accessories:
Pipe tubing elbow (Fig. 16B.3.5c) or fitting to adapt to gas line or a pipe-tubing elbow fitted directly to the cylinder regulator
Gas cylinder (helium or alternate, e.g., nitrogen) with regulator
6-mm nylon pneumatic hose, ~6 m

NOTE: Wind each male joint with Teflon tape prior to closing.

Construct the discharge assembly for both Blowpipe and HandGun (Fig. 16B.3.2)

1. Screw the UNF thread of the luer adapter into the UNF female thread of the adapter machined to accept it and having a $^1/_4$-in. fitting on the other end.

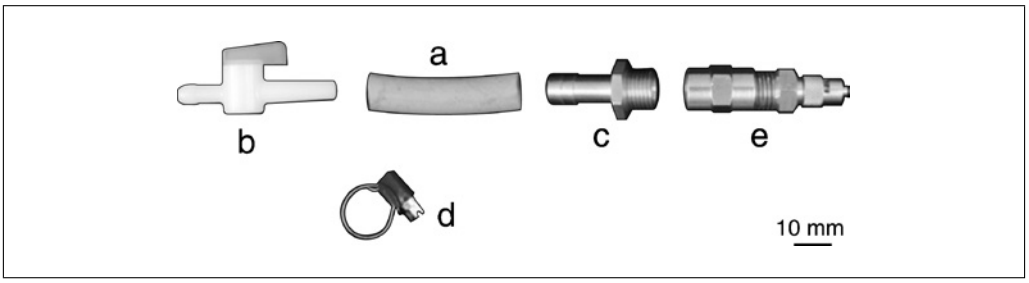

Figure 16B.3.3 Materials for constructing the Blowpipe apparatus. (a) Rubber vacuum hose, ~7-mm diameter. (b) Two-way plastic tap. (c) Barbed fitting to connect rubber hose to a pip-efitting. (d) Worm-drive hose clips. Four are required. (e) Assembled discharge assembly (from Fig. 16B.3.2). Filter holder is not attached.

2. Twist the 13-mm-diameter plastic filter holder into the luer fitting at the end of the assembly.

   The filter holders have an internal grid on which to place the drop of nucleic acid–coated metal, a screw fitting to attach to the bombardment device, and an exit nozzle shape well suited to the bombardment process. It is advisable to purchase at least ten units.

Construct the apparatus

To construct the Blowpipe

3. Connect one end of the 7-mm rubber vacuum hose to the building's compressed air supply, securing with a worm-driven hose clip. Remove a few centimeters of rubber hose with a sharp knife.

   Appropriate building air supply should be in the range of 2.5 to 4 bars (250 to 400 kPa).

4. Insert the plastic two-way tap between the lengths of tubing beside the discharge assembly. Connect the barbed fitting to the discharge assembly (see steps 1 and 2), with a $^1/_4$-in. coupling adapter FPT/FPT, if required. Secure all joints with worm-driven hose clips.

   The assembled discharge assembly and coupling adapter are shown in Figure 16B.3.3.

To construct the HandGun

5. Mount the electrically operated miniature solenoid valve on its base plate with the bolts provided. Close the holes in the base plate marked 2, 3, and 5 with blank, nonprotuding, $^1/_4$-in. plugs.

6. Screw the pipe-tubing elbow into the gas entry port on the base plate marked 1.

7. Attach $^1/_4$-in. BSP adapter(s) FPT/M to a total length of ~40 to 50 mm to the gas exit port number 4 (in the base plate, Fig. 16B.3.4b), forming the barrel of the gun.

8. Screw the discharge assembly (steps 1 and 2) into the end of the barrel. Mount the HandGun in the clamp stand with the nozzle down, ~15 cm from the bench (Fig. 16B.3.1).

Assemble the timer

9. Following the instructions given by the manufacturer of the timer, connect a flexible, two-conductor, electrical wire (2 to 3 m) from the 220 V variable (0 to 100 msec) timer to the solenoid valve by the miniature DIN plug (Fig. 16B.3.6).

10. Connect a second two-conductor wire (2 to 3 m) to the timer at one end, and at the other to the electrical push button switch.

    Pushing on the button will activate the timer for the period set. (Generally, the authors use 50 msec).

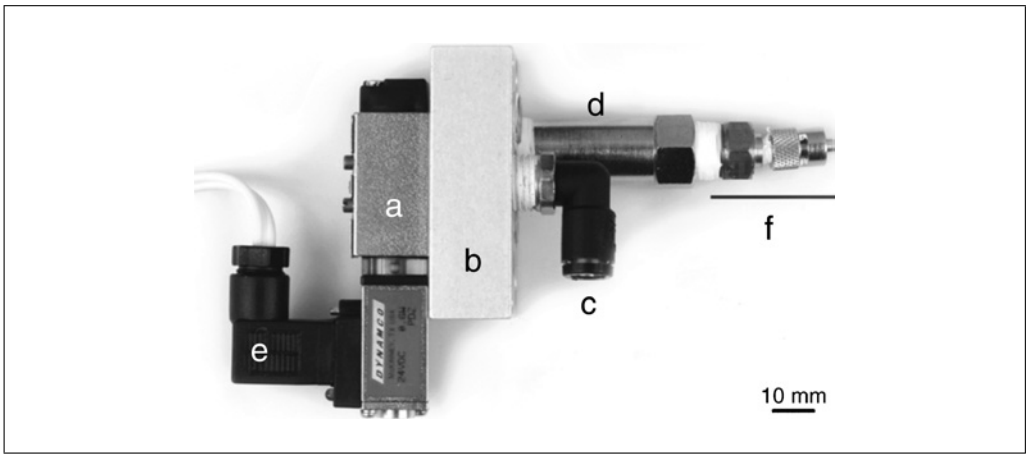

Figure 16B.3.4 The HandGun apparatus. (a) Electrically operated miniature solenoid valve. (b) Base plate. (c) Pipe-tubing elbow (also see Fig. 16B.3.5c). (d) $^1/_4$-in. BSP adapter FPT/M (~40 mm). (e) A miniature DIN plug. (f) Discharge assembly (from Fig. 16B.3.2). Filter holder is not attached.

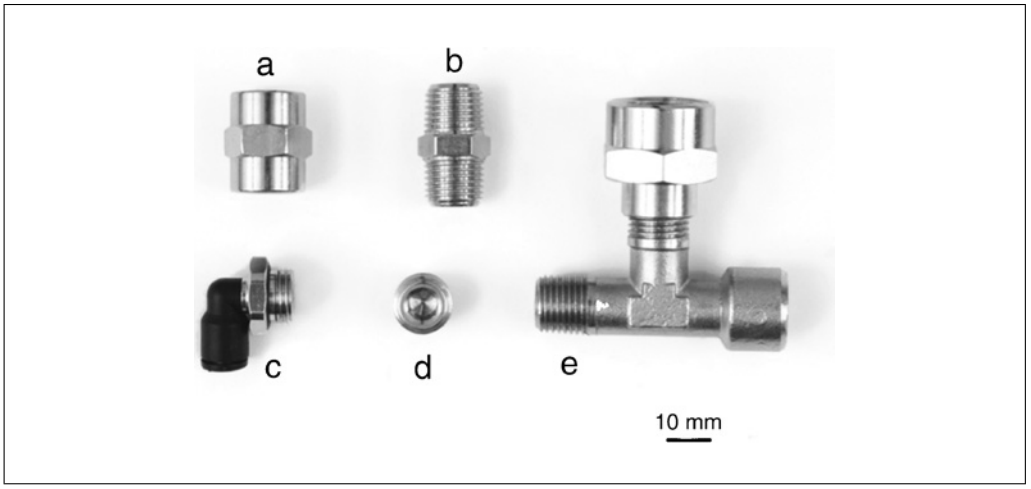

Figure 16B.3.5 Assorted components required for constructing the bombardment devices. (a) $^1/_4$-in. BSP coupling adapter FPT/FPT. (b) $^1/_4$-in. BSP nipple adapter M/M. (c) Pipe-tubing elbow (see Fig. 16B.3.4). (d) $^1/_4$-in. BSP plugs (non-protruding). (e) T-adapter, MFF, $^1/_4$, $^1/_2$, $^1/_4$ in. (see Fig. 16B.3.7b). If the HandGun is constructed with a secondary regulator, six BSP coupling adapters, six BSP nipple adapters, and four pipe-tubing elbows will be required.

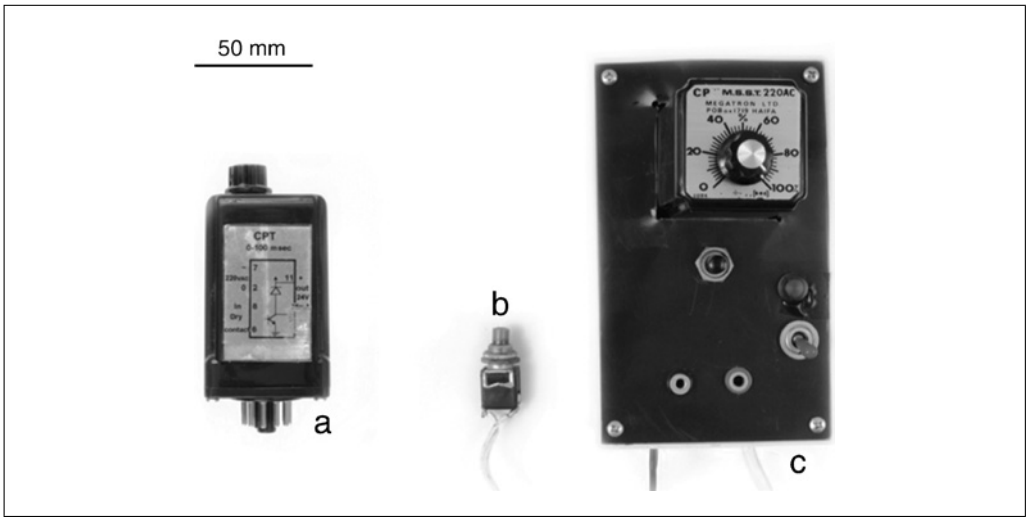

Figure 16B.3.6 Components for the timer assembly. (a) 220 V variable (0 to 100 msec) timer. (b) Push-button electrical switch. (c) Plastic box for mounting the timer with electrical connections.

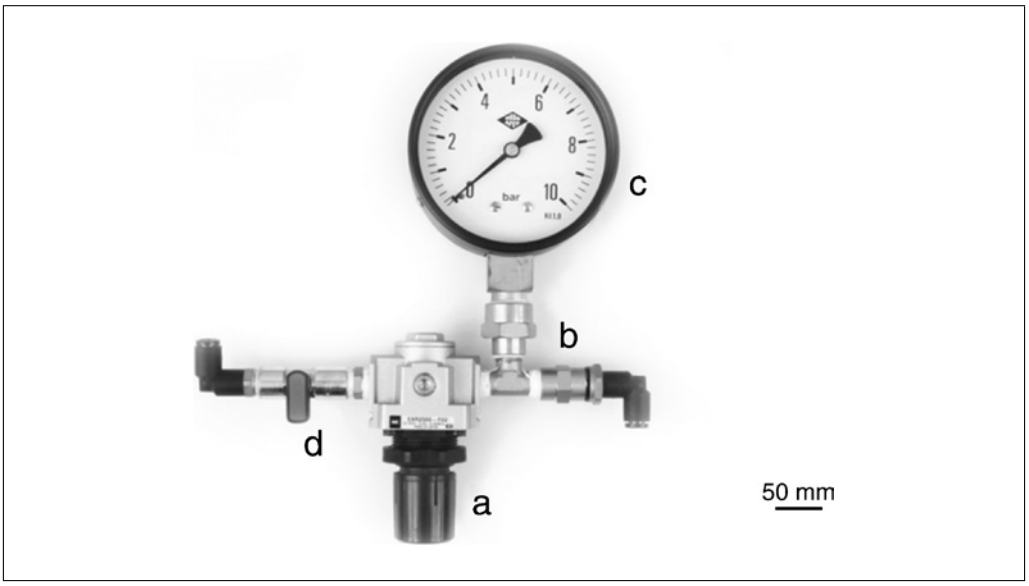

Figure 16B.3.7 Components for the secondary gas regulator. (a) Pressure regulator (~0.05 to 0.85 MPa), with female $^1/_4$-in. BSP fittings. (b) T-adapter, MFM, $^1/_4$, $^1/_2$, $^1/_4$ in. (see Fig. 16B.3.5e). (c) 0- to 10-bar gas pressure gauge, 100 mm diameter, $^1/_2$-in. BSP male fitting. Pipe-tubing elbows (Fig. 16B.3.5c) are at each end of the secondary gas regulator. Adapters (couplings and nipples; see Fig. 16B.3.5) connect the pieces when necessary. Direction of helium gas flow is indicated by arrows. (d) Two-way tap; $^1/_4$-in. female BSP fittings.

11. Place the timer in a plastic box, which may be wall mounted. Make sure that both of the wires are long enough to place the box with the timer where necessary, and that both wires reach the position where the HandGun will be operated (Fig. 16B.3.1).

Assemble the secondary gas regulator

This component (Fig. 16B.3.7) is optional but worthwhile, because it permits more accurate control of the gas pressure.

12. Screw a pipe-tubing elbow into the pressure regulator on the side of the regulator marked for entry of gas.

13. Screw the male entry of the T-adapter into the other side of the pressure regulator. Screw the 0- to 10-bar gas pressure gauge into the $^1/_2$-in. female entry of the T-adapter.

14. Connect a pipe-tubing elbow to the other port of the T-adapter, using a $^1/_4$-in. adapter M/M.

15. Add a two-way tap after the pipe-tubing elbow before the gas entry point in the pressure regulator and a 20-μm gas line filter after the pressure gauge.

    These are optional but useful additions. The two-way tap closes the system to prevent gas loss, and the gas line filter prevents dirt from damaging the miniature solenoid valve.

Assemble the major HandGun components

16. Attach a pipe-tubing elbow to the gas cylinder, or modify the entry to permit connection to 6-mm diameter nylon pneumatic tubing.

17. Plan where the components of the system (gas cylinder, HandGun, secondary gas regulator, timer) will be situated (Fig. 13B.3.1). Screw the wall-mounting bracket onto the secondary gas regulator and attach to a suitable position on a permanent surface.

18. Cut the 6-mm diameter nylon pneumatic hose, with a sharp knife into two pieces with square ends.

These hoses will run from the gas cylinder to the secondary gas regulator and from there to the HandGun.

19. Set the exit port from the gas cylinder to a maximum of ~5 bars (~500 kPa), so that the miniature solenoid valve will not be under too much pressure.

20. Tape the trigger button of the timer to the right-hand side of the HandGun (Fig. 16B.3.1).

    This allows the hand holding the HandGun to operate the switch and the other hand to hold the plant.

BASIC PROTOCOL 2

BOMBARDMENT WITH cDNA OR RNA

Transcripts or cDNA are precipitated onto heavy metal particles. Tungsten and gold particles are prepared using different methods and have different biological effects. Tungsten is toxic to plants (Russell et al., 1992), and the bombardment of cotyledons or leaves with tungsten generally produces a necrotic area. Most plants (e.g., squash and other cucurbits) survive the necrotic effect of tungsten bombardment, but it is lethal for softer plants such as *N. bethamiana*. These plants do, however, survive bombardment with gold particles. Additionally, as bombardment with gold causes less damage, it is probably more efficient, although this point has yet to be demonstrated. Cotyledons are generally stronger than leaves, and it is important to inoculate plants at an early stage. Particle and cDNA/RNA mixture preparation is the same for both Blowpipe and HandGun. Note that gold sticks to plasticware when suspended in water (Klein et al., 1987) but is much less sticky when suspended in 50% glycerol, as described in this protocol.

Particles coated with nucleic acids are deposited in a small volume on the grid of a filter holder, from which the particles are accelerated by a burst of gas under pressure. The authors prefer to use helium, as it is clean and cheap, although other gases (e.g., air, nitrogen) have also been used. Particle bombardment using air under pressure can be toxic in the case of plant transformation (Takeuchi et al., 1992), but the authors have not found this to occur with virus inoculation.

Materials

1.25 M $Ca(NO_3)_2 \cdot 4H_2O$, pH 10.5 or 7.8: adjust pH slowly with HCl, autoclave, aliquot, and store up to 2 years at −20°C
Tungsten or gold particle stock (see recipes)
cDNA or RNA transcripts in water (Support Protocol 1) *or*
Plant sap, purified virus, viral RNA extract, or total RNA (Support Protocol 2)
Target plants at correct stage, e.g., cotyledon
Microcentrifuge tubes, sterile
Blowpipe or HandGun (Basic Protocol 1)
Disposable gloves (*UNIT 1A.3*)
10-ml syringe

NOTE: Use normal precautions for working with nucleic acids, including sterile solutions and autoclaved utensils.

Prepare the tungsten or gold bombardment mixture with cDNA or RNA

1. Prepare sufficient bombardment mixture by combining the following in a sterile microcentrifuge tube at 1:1:1 proportions, adding the nucleic acid last:

   1.25 M $Ca(NO_3)_2$, pH 10.5 or pH 7.8
   Tungsten or gold stock
   50 to 250 ng/μl cDNA or RNA.

Vary the total amount according to need, calculating for 2.5 µl per shot, preparing only enough mixture for bombardment of five to ten plants.

The unadjusted pH of the $Ca(NO_3)_2$ solution is 9 to 11, appropriate for precipitation of cDNA onto the metal particles. However, RNA should be precipitated at pH 7 to 8. Lower the pH with careful addition of HCl, since the solution is not buffered.

Use of Eppendorf-brand microcentrifuge tubes and tips can reduce the clumping on addition of DNA. Additionally, the use of siliconized microcentrifuge tubes and tips, while optional, can help avoid loss of tungsten- or gold/DNA on vessel walls. See APPENDIX 2A for silanization procedure.

The authors use a tungsten particle size of 1.6 µm, but other groups have used other sizes successfully.

2. At the beginning, middle, and end of a 5- to 10- min period, mix by tapping.

Bombard using the Blowpipe

3a. Charge the Blowpipe by fully opening both the gas tap to the building's compressed air supply and the tap beside the discharge assembly, raising the airflow to maximum.

4a. Close the taps, first the one beside the discharge assembly, then the tap to the air supply.

The purpose of steps 3a and 4a is to load the Blowpipe's tubing with high pressure air or gas. The order of tap opening and closing is important for correct functioning. The method will not work properly if the wrong order of steps is used.

5a. Open the tap to the building's compressed air supply.

6a. Open the Blowpipe filter holder. Mix bombardment mixture by pipetting up and down. Pipet 2.5 µl onto the center of the grid of the open filter holder.

Before each shot, mix tube contents by pipetting up and down prior to loading onto the grid of the filter holder.

The grid will hold the liquid.

7a. Screw the filter holder closed and attach it to the luer-lock adapter at the end of the Blowpipe.

8a. Support the cotyledon or leaf of the target plant with a gloved hand.

CAUTION: *Use gloves so that the metal is not bombarded into the experimenter's hand. Tungsten and gold are considered toxic metals.*

9a. Hold the discharge assembly close to the target plant, and sharply open and close the tap beside it, discharging the Blowpipe onto the plant.

10a. Repeat steps 3a to 9a until no bombardment mixture is left. Discard the microcentrifuge tube.

11a. Make a fresh bombardment mixture. Use a fresh, autoclaved filter and holder every time a different cDNA or RNA transcript is used (steps 1 to 10a).

Bombard using the HandGun

3b. Set gas pressure at the secondary regulator or cylinder and set the timer.

Generally, the authors use 2.5 to 3.5 bars (250 to 350 kPa) of gas pressure, depending on the target species. The correct pressure depends on the plant material and must be determined experimentally. Do not exceed 6 bars (600 kPa) of gas pressure with this apparatus, as this is the limit for the solenoid valve.

Timer settings of 50 or 100 msec were found to be effective in the authors' experience. Periods of less than 50 msec may be problematic because the response of the solenoid valve may be adversely affected.

4b. Fire HandGun twice to fill tube with gas.

5b. Open the filter holder. Mix bombardment mixture by pipetting up and down. Pipet 2.5 μl onto the center of the grid of the open filter holder.

Before each shot, mix tube contents by pipetting up and down prior to loading on the grid of the filter holder.

The grid will hold the liquid.

6b. Screw the filter holder closed and attach it to the luer-lock adapter at the end of the HandGun.

7b. Adjust the clamp stand so that the end of the filter holder is ~2 to 3 cm away from the target leaf or cotyledon of the target plant.

8b. Support the cotyledon or leaf to be bombarded with a gloved hand and press the button to fire.

CAUTION: *Use gloves so that the metal is not bombarded into the experimenter's hand. Tungsten and gold are considered toxic metals.*

9b. Fire the HandGun again at a second cotyledon or leaf on the same plant to discharge entirely.

10b. Repeat steps 3b to 9b until no bombardment mixture remains.

11b. Discard the microcentrifuge tube and make a new bombardment mixture, using a clean, autoclaved, filter holder for each different cDNA or mixture of cDNAs (steps 1 and 2, and 3b to 10b).

Clean filters and holders

12. At the conclusion of a bombardment experiment, close the gas valve on the gas cylinder or compressed air supply.

13. Put the filter holder on the end of a 10-ml syringe. Draw distilled water through the filter holder into the syringe and discharge to waste. Repeat four to five times.

14. Open the filter holder and allow to dry on absorbent toweling.

Take care not to lose the grid from the filter holder, as it may come loose and fall out. Sometimes a small residue of tungsten or gold remains on the grid after washing, but it is of no consequence.

15. When dry, reassemble and autoclave.

Washing the filters is very important to prevent contamination since a picogram of cDNA is enough for infectivity.

ALTERNATE PROTOCOL

MASS BOMBARDMENT USING A HANDGUN

This method describes use of the HandGun to bombard many plants with a construct of known efficiency. The limit to the speed of this procedure is the clumping of the bombardment mixture and the organization of the plants.

Additional Materials *(also see Basic Protocol 2)*

Drill with 2-mm bit
Blocked micropipet tip: prepare by burning the end

1. Drill a small hole in the top of a filter holder (Fig. 16B.3.2.c, left) with a 2-mm bit.

A pipet tip can be pushed through the drilled hole to touch the grid, and release the bombardment mixture.

2. Prepare a volume of bombardment mixture adequate for bombardment of 50 to 100 plants (see Basic Protocol 2, steps 1 and 2). Mix by swirling.
3. Pipet 2.5 μl bombardment mixture through the hole instead of unscrewing the filter holder.

   It is best to use a volume of 2 to 4 μl.
4. Close the hole with a blocked pipet tip.
5. Proceed as in Basic Protocol 2, steps 3b to 15.

   Be sure to mix the bombardment mixture between each shot to avoid precipitation. Discard the bombardment mixture when it has clumped seriously, making pipetting difficult.

   By this method, two operators have inoculated 500 plants in 3 hr (Shiboleth et al., 2001).

PURIFICATION AND PREPARATION OF cDNA OR RNA TRANSCRIPT FOR BOMBARDMENT

SUPPORT PROTOCOL 1

In this unit bombardment with cDNA refers only to the use of infectious cloned DNA of a plant RNA virus under control of any constitutive plant-active promoter (e.g., the CaMV or SVBV promoter; Wang et al., 2000). The authors have been successful in using a plant terminator at the end of the viral sequence. Alternatively, the viral cDNA (Gal-On et al., 1995) or PCR-produced fragments (Fakhfakh et al., 1996) can be linearized at the 3′ end, resulting in effective termination. The efficiency of a viral poly(A) signal as a terminator is not yet clear. Bombardment with RNA transcripts here refers to in vitro–produced transcripts.

Prepare plasmids for cDNA bombardment, preferably using a commercial column (e.g., Qiagen; also see Heilig et al., 1998) or any miniprep method (Engebrecht et al., 1991). The plasmid preparation can be bombarded directly onto plants from the column eluate or from the miniprep. However, sometimes dilution of the plasmid preparation is necessary to obtain better results. It is also possible to bombard with cDNA amplified by PCR without cloning or purification (Fakhfakh et al., 1996).

For RNA transcripts (both naturally capped and naturally uncapped) produced using cDNA cloned under the control of a nonplant promoter (e.g., phage promoter T7), which allows in vitro transcription, 5′-capping is advisable, although uncapped transcripts are infectious but less efficient (Gal-On et al., 1997). DNase treatment and phenol/chloroform extraction are not necessary prior to bombardment.

Dilute the cDNA or transcripts to a range of 50 to 250 ng/μl. High inoculation efficiency can also be obtained with 10–50 ng per/μl. The best results are obtained with 50 ng/ml of nucleic acids. Excess cDNA could reduce efficiency due to aggregation (clumping) in the bombardment mixture.

BOMBARDMENT WITH PLANT SAP, VIRUS PREPARATION, OR VIRAL RNA EXTRACT

SUPPORT PROTOCOL 2

Plant RNA viruses (nonphloem limited) are usually inoculated mechanically, using infected plant sap or natural vectors. Using bombardment methods with sap extracted from plant tissue can save plant material but has an efficiency similar to mechanical inoculation. However, using bombardment methods with purified virus and viral RNA is more efficient than mechanical inoculation, as demonstrated by potyvirus inoculation (Gal-On et al., 1995). Extra preparation is unnecessary with plant sap, purified virus, RNA extract, or total RNA from an infected plant, except to adjust the calcium nitrate to a pH of 7 to 8 in these cases (Basic Protocol 2).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Gold particle stock

Wash 50 mg gold particles (spherical APS 1.5-3.0 μm, 99.96+% metals basis; Alfa Aesar or Bio-Rad) in 1 ml fresh 70% (v/v) ethanol. Sonicate 5 min in an ultrasonic bath. Vortex, then centrifuge briefly. Wash three times with 1 ml sterile 50% glycerol. Resuspend in 0.5 ml of 50% glycerol. Aliquot 40 μl into sterile microcentrifuge tubes and store up to 2 years at −20°C.

Sterile glycerol is prepared by autoclaving in a glass container, as glycerol leaches from plastic containers during autoclaving. Store up to 1 year at room temperature.

Also see Acknowledgements.

Tungsten particle stock

In a 1.5-ml microcentrifuge tube, wash 50 mg tungsten particles (M17 particles; size 1.6 μm; Bio-Rad) for 1 hr in 1 ml absolute ethanol. Vortex, then microcentrifuge briefly to precipitate. Wash three times with 1 ml sterile water. Resuspend washed tungsten particles in 1 ml sterile 50% glycerol. Sonicate 2 to 3 min in an ultrasonic bath to better separate the particles (optional). Store indefinitely at −20°C.

Sterile glycerol is prepared by autoclaving in a glass container. Glycerol leaches from plastic containers during autoclaving. Store up to 1 year at room temperature.

COMMENTARY

Background Information

The procedures described in this unit require nucleic acids (cDNA or RNA) to be precipitated onto heavy metal (tungsten, gold) particles. The coated particles are deposited in a small volume on the grid of a filter holder and accelerated into plant tissue by a blast of pressurized gas. The apparatus enables inoculation with full-length infectious clone cDNA, PCR products, virus from sap or virus preparation, cloned viral transcripts, and some phloem-limited RNA viruses. Additionally, this technology allows the inoculation of large numbers of plants, the inoculation of soft plants that do not survive bombardment inoculation by other means, inoculation in the greenhouse, the study of viral recombination in planta, rapid promoter analysis, and the monitoring of virus movement using an infectious clone bearing a reporter gene.

Critical Parameters

Following the parameters listed here is critical to the success of the procedure:

1. Secure the gas cylinder according to local safety rules.
2. Ensure that the electrical output of the timer (current and voltage) is suitable to run the solenoid gas valve.
3. Ensure that the gas pressure at the solenoid gas valve is within its performance limits.
4. All solutions and equipment used should autoclaved.
5. Treat the toxic tungsten and gold powders with care.
6. Mix the tungsten- or gold-DNA mixture well by pipetting up and down before each shot.
7. Keep the plants in an isolated area after bombardment to prevent chance inoculation from another source, or inoculation of other host plants by accident.
8. The volume of bombardment mixture should be kept low (2-3 μl). The liquid does not interfere with the bombardment process.
9. Wash and autoclave the filters as recommended to prevent contamination.

Troubleshooting

Table 16B.3.3 provides a list of possible problems, their potential cause, and how to solve them.

Anticipated Results

Visible leaf damage due to bombardment with tungsten particles is commonly observed a day or two after bombardment but does not

Table 16B.3.3 Troubleshooting Guide for Bombardment

Problem	Possible Cause	Solution
cDNA not infectious	cDNA quality poor, subsequent contamination with DNase, or old preparation	Prepare fresh cDNA
	Inhibitory material in cDNA preparation	Dilute cDNA preparation
cDNA or transcript not infectious	cDNA or transcript is genuinely not infectious	Bombard many plants (~50-100) to prove lack of effectiveness and then improve construct
Construct not infectious	Tissue specificity	Bombard other organs
Transcript not infectious	Improperly sterilized reagents and equipment	Prepare all reagents fresh (including tungsten or gold), and ensure solutions and filter holders are clean and autoclaved
Heavy damage to plant	Tungsten clumping	Dilute cDNA, reduce firing pressure, and/or increase distance between HandGun and target plant
Particle clumping	Protein contamination of cDNA preparation	Dilute cDNA preparation

mean that the plants are not infected. Score the plants for visible symptoms several days after inoculation, and use molecular techniques (e.g., ELISA or RT-PCR) to detect systemic infection 10 to 14 days after inoculation. As these bombardment inoculation techniques are very efficient, previously inactive full-length clone constructs can become active (Gal-On et al., 1995). However, mutant viruses may be slower to cause symptoms, or the symptoms may be less marked, taking longer to become apparent, and may require molecular analysis rather than symptomatic diagnosis. Results should be scored as number infected/number inoculated. An experiment should always include uninoculated controls, because of the possibility of natural infection, and a positive control (i.e., cDNA of wild type virus) for screening mutant clones.

Time Considerations

The longest period of the procedure is waiting for the ordered components, and this will depend entirely on the suppliers. Assembling the Blowpipe will take a few minutes, assembly of the HandGun will take 20 min, and wiring up the timer and inserting into a suitable box may take 1.5 to 2 hr. Bombardment will be at the pace of ~20 to 30 plants/hr for a single worker making a metal-DNA preparation for five shots, bombarding five plants, and then making the next bombardment mixture. This process can be accelerated by two operators working together, one making preparations, and the other bombarding. The mass bombardment method permits more rapid inoculation.

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Gal-On, et al., 1997. See above.

Particle bombardment as an inoculation method using handheld devices.

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Reverse Transcription-Polymerase Chain Reaction-Based Detection of Plant Viruses

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ABSTRACT

A one-step reverse transcription–polymerase chain reaction (RT-PCR) is used to detect two cherry flexiviruses, Cherry green ring mottle virus (CGRMV) and Cherry necrotic rusty mottle virus (CNRMV), in *Prunus* species. This unit presents procedures for collection of plant samples, preparation of total nucleic acids, viral RNA-rich or total RNA extracts from plant tissues, and subsequent amplification of the viral targets by one-step RT-PCR using a pair of consensus primers. The PCR amplicons are visualized by electrophoresis in a 1% agarose gel containing ethidium bromide in TAE buffer and viewed under ultraviolet light. This procedure is rapid, sensitive, reliable, and cost-effective and is generally useful on a wide variety of plant/virus systems. The use of a semi-automatic homogenizer for sample preparation and one-tube RT-PCR for virus detection makes this approach ideal for screening large numbers of samples. *Curr. Protoc. Microbiol.* 6:16C.1.1-16C.1.9. © 2007 by John Wiley & Sons, Inc.

Keywords: RT-PCR • plant viruses • Cherry green ring mottle virus (CGRMV) • Cherry necrotic rusty mottle virus (CNRMV)

INTRODUCTION

Advances in genomic characterization of plant viruses have made reverse transcription–polymerase chain reaction (RT-PCR) a common technique for the detection of plant RNA viruses, due to its high sensitivity and specificity. Viral nucleotide sequence is a prerequisite for the RT-PCR-based detection method. If the nucleotide sequence is not available for a particular virus, the viral genome will first have to be cloned and sequenced. Nucleotide sequences of Cherry green ring mottle virus (CGRMV, AF017780, AJ291761) and Cherry necrotic rusty mottle virus (CNRMV, AF237816), two closely related flexiviruses infecting *Prunus* species, are available, and are used as the basis of this protocol which illustrates a general procedure that may be useful for other RNA viruses. The sequence data are used to design primers that anneal to highly conserved regions in the genomes to enable RT-PCR-based detection of a wide range of different isolates of these viruses (Zhang et al., 1998; Rott and Jelkmann, 2001b; Gentit et al., 2002). The availability of commercial one-step RT-PCR kits makes the RT-PCR more efficient and convenient. Suitable RT-PCR templates can be prepared (see Template preparation) from different plant tissues year round by using the basic protocols, or the alternate protocols can be used to prepare template from young leaves in the spring. The amplified PCR products are electrophoresed through 1% agarose gels containing ethidium bromide (0.1 μg/ml) in TAE buffer and visualized under UV light. When a large number of samples must be tested, a 96-well PCR plate and a 96-well E-Gel system (Invitrogen) may be used to replace PCR strips and standard agarose gels.

NOTE: Information about commercial reagents and equipment is provided solely to assist the identification of suitable sources of such items. No specific endorsement or approval is intended and equivalents from other manufacturers may be equally suited.

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STRATEGIC PLANNING

The time of year and the growth stage of the host are critically important for virus testing. In general, virus titer typically falls in response to high ambient temperature. Therefore, detection of viruses including CGRMV and CNRMV in *Prunus* species is best done in early spring when plant tissues are succulent and temperatures are mild. The low concentration of phenolic compounds and polysaccharides in young tissues and high concentration of the viruses at this time make the isolation of the viral nucleic acids easier (Demeke and Adams, 1992; Rott and Jelkmann, 2001a). Samples, usually young leaves, are collected from five different shoots of a plant and pooled because the distribution of these viruses in plants, especially field trees, is not uniform (Parker et al., 1976; Li and Mock, 2005). Flowers, bark, and root tips can also be used. The extraction can also be done in early fall from leaves, and in winter from the buds and bark of dormant budwood, if either the Basic Protocol or the QIAGEN RNeasy plant mini kit is used (Li and Mock, 2005).

BASIC PROTOCOL 1

EXTRACTION OF TOTAL NUCLEIC ACIDS FROM MATURE PLANT TISSUES USING CTAB BUFFER

Cetyltrimethylammonium bromide (CTAB), also known as hexadecyltrimethylammonium bromide, is a detergent. Such surfactants are used in extractions of biological materials to facilitate the separation of proteins and nucleic acids. This particular buffer system was first popularized by Doyle and colleagues (Doyle and Dixon, 1987), based on the buffer system used earlier to isolate DNA from barley seedlings (Saghai-Maroof et al., 1987). This protocol has been used to prepare templates suitable for the RT-PCR from different tissues (e.g., leaves, petioles, bark, and dormant buds) of virus-infected plants, including *Prunus* species at different seasons. In the protocol, disruption of plant tissues in the buffer is performed in sealed plastic tubes using steel shot, obtained at an economical price from a sporting goods dealer, and a bead mill homogenizer. This modification speeds the process and reduces cross contamination between samples. It is easily scalable to large numbers of samples in a virus-indexing or certification program.

CTAB is a cationic detergent and will lyse cells and simultaneously precipitate and inactivate proteins, including RNase. It will bind and precipitate polysaccharides (Jones and Walker, 1963), which are present in high concentrations in plant extracts, and this property of CTAB is crucial to the success of the protocol. The presence of 1 M NaCl in the extraction buffer prevents the precipitation of nucleic acids by CTAB and keeps them in the aqueous phase during the subsequent solvent extraction step, which removes the proteins and polysaccharides (Murray and Thompson, 1980).

Materials

Plant sample
CTAB buffer (extraction buffer; see recipe)
24:1 (v/v) chloroform/isoamyl alcohol
Isopropanol, room temperature
70% (v/v) ethanol
20 mM Tris·Cl, pH 8.0

1.5- and 2.0-ml microcentrifuge tubes (Labsource or Fisher Scientific)
1/4-in. steel shots (Slingshot AMMO, Crosman; *http://www.crosman.com/*)
Razor blades
Wooden coffee stirrer
Screw caps (Labsource or Fisher Scientific)
FastPrep instrument (bead-mill homogenizer, Savant)Water bath, dry-bath incubator or hybridization oven
Vortex
1.7-ml microcentrifuge tubes

Prepare plant material

1. Label a 2.0-ml microcentrifuge tube and add two steel shots to it.
2. Tear five leaves or petals into small pieces by hand, or slice buds, bark, or root tips into small pieces with a razor blade.
3. Weigh 0.1 g pooled plant tissue and transfer to the labeled tube using a wooden coffee stirrer. Change gloves, razor blade (if used), weighing paper, and wooden coffee stirrer between samples.

Extract total nucleic acids

4. Add 1 ml CTAB buffer to each tube, close it with a screw cap, and label the cap.

   More than 1 ml of the extraction buffer may be needed if buds, bark, and mature leaves are used, due to high concentration of polysaccharides in these tissues. Add the 2-mecaptoethanol under a chemical exhaust hood.

5. Cool the tube at −20°C for 15 min until the tubes are partially frozen.

   Cooling time may vary. Make sure the buffer in the tubes is half frozen. This step is critical to reduce RNA degradation during homogenization.

6. Process the cooled tube with a FastPrep bead-mill homogenizer two times, for 30 sec at speed setting 4.5.

   If a FastPrep bead-mill homogenizer or similar bead mill is not available, a mortar and pestle cooled to −20°C can be used to grind the sample. However, this method is not as efficient as using the FastPrep homogenizer.

7. Incubate the tube in a water bath, a dry-bath incubator, or a hybridization oven for 15 to 60 min at 65°C.
8. Microcentrifuge the tube 5 min at maximum speed, room temperature.
9. Transfer 650 µl supernatant to a labeled 1.5-ml microcentrifuge tube.

   The supernatant may contain pieces of plant debris.

10. Add an equal vol (650 µl) of 24:1 chloroform/isoamyl alcohol to the tube and vortex vigorously for 30 sec.
11. Microcentrifuge 10 min at maximum speed, room temperature.
12. Carefully remove 500 µl of the upper (aqueous) phase to a labeled 1.5-ml microcentrifuge tube.
13. Add 70% vol (350 µl) of isopropanol to the tube.
14. Incubate the tube 10 min on ice.
15. To pellet the nucleic acid, microcentrifuge 10 min at maximum speed, room temperature.
16. Note the position of the pellet and carefully remove the supernatant by pipet. Be careful not to disturb the pellet.
17. Add 0.5 ml of 70% ethanol, and invert several times.
18. Microcentrifuge 5 min at maximum speed, room temperature.
19. Note the position of the pellet and carefully remove the ethanol using a pipet. Centrifuge again to collect remaining ethanol.
20. Carefully remove the remaining ethanol by pipet and air dry the pellet on the bench for 10 to 15 min.

21. Add 100 µl of 20 mM Tris·Cl, pH 8.0, and put the tube on ice for 10 to 15 min.

    This will soften the pellet.

22. Pipet up and down to dissolve the pellet. Use immediately or store at −20°C for future use (up to 3 months).

ALTERNATE PROTOCOL 1

ISOLATION OF VIRAL RNA-RICH EXTRACT FROM YOUNG LEAVES BY PLATE-TRAPPING

Plate trapping of viral coat proteins and virus particles from plant extracts was first documented by Clark and Adams (1977), and their ELISA methods were rapidly and widely adopted (Clark, 1981). In the original technique, antibodies specific for the virus were used to coat the wells of a microtiter plate. These antibodies selectively bound to virus particles from a complex mixture such as plant extracts, and a subsequent rinse of the plate removed unbound host compounds. However, the capturing antibody is not always required, and virus particles will stick to an uncoated microtiter plate as well (Jones and McGavin, 2002). This nonspecific adhesion is sufficient for our purpose of trapping the virus particles from solution prior to extraction of the nucleic acids. The specificity of virus detection is provided by the subsequent RT-PCR assay. This protocol is simple and avoids use of toxic chemicals like phenol, chloroform, and 2-mecaptoethanol needed for other procedures and has been used to prepare templates suitable for the RT-PCR from flowers, leaves, and bark of infected *Prunus* plants. The disadvantage of this method is that the extracts can not be stored for longer than two months at −20°C because some RNase activity remains in these preparations.

Materials

Prepared tissue (Basic Protocol 1, steps 1 to 6)
Extraction buffer (see recipe)
Sterile distilled water
3% (v/v) Triton X-100 (Sigma-Aldrich)
ELISA microplate (Dynatech Laboratories)

Additional reagents and solutions for preparing plant tissue (Basic Protocol 1)

1. Prepare tissue as described in Basic Protocol 1, steps 1 to 6.
2. Microcentrifuge tube 5 min at 12,000 to 15,000 × *g* (depending on the microcentrifuge used), 4°C.
3. Load 175 µl of the supernatant containing virions into wells of an ELISA microplate.

   To avoid contamination, it may be necessary for the novice to include empty wells between samples.

4. Incubate the plate overnight at 4°C.
5. Carefully remove the solution using a pipet or multichannel pipet.
6. Carefully rinse the wells two times, each time with 200 µl of the extraction buffer using a pipet, and then one time with sterile distilled water. Remove remaining solutions by pipet.
7. Incubate the plate on ice for 10 min.
8. Add 12 µl of 3% Triton X-100, and pipet up and down to disrupt virus particles.

   Dilute Triton X-100 with sterile distilled water.

9. Use the viral RNA extract immediately for RT-PCR assays or store at −20°C or −80°C until use.

   The viral RNA extract cannot be stored at −20°C for more than two months.

EXTRACTION OF TOTAL RNA FROM YOUNG TISSUES BY QIAGEN RNEASY PLANT MINI KIT

ALTERNATE PROTOCOL 2

The RNeasy Plant Mini Kit (QIAGEN) has been found suitable to extract total RNA from young leaf tissues. The manufacturer's instructions should be followed for this purpose, and pure template is obtained by this method. However, three factors limit its use when large numbers of samples must be tested. First, the high concentration of polysaccharides in mature leaves clogs the flow of extract through the columns, making sample recovery difficult. Second, plant tissues are ground in liquid nitrogen with a mortar and pestle, which does not easily scale to large numbers of samples. Finally, it is more expensive than other methods.

RNA samples should be stored at −20°C following extraction until RT-PCR is carried out.

RT-PCR ASSAY FOR IDENTIFICATION OF VIRAL PATHOGENS

BASIC PROTOCOL 2

RT-PCR (reverse transcription–polymerase chain reaction) is a rapid, sensitive, and specific method for the detection of viral RNA. Complementary DNA (cDNA) is first generated from viral genomic RNA using a primer that is reverse complementary to the 3′-end of a known nucleotide sequence by a reverse transcriptase, and then used as template for subsequent PCR amplification using a pair of primers complementary to the two ends of the target sequence. The PCR is based on repeated cycles of template denaturation (94° to 96°C), primer annealing (40° to 60°C), and primer extension (72°C) by a thermostable *Taq* polymerase, resulting in many copies of the target fragment (Mullis et al., 1986).

Primers determine the specificity of the RT-PCR, and primers used in virus detection are usually based on conserved regions in their genomic sequences to be able to detect a wide range of different strains or isolates. In our example using CGRMV and CNRMV, the high nucleotide sequence identity among two viruses and their isolates (>67%) results in many pairs of potential primers. Primer pairs may be selected using the Oligonucleotide Properties Calculator available at (*http://www.basic.northwestern.edu/biotools/oligocalc.html*). Primers should have an annealing temperature (T_m) of ~60°C and lack sequences with self complementarity. Primer pairs that meet these criteria may be compared using RT-PCR to detect the viruses of interest (Li and Mock, 2005). The consensus primers used in the protocol are based on two highly conserved regions adjacent to the coat protein (CP) genes and have been used successfully to detect all CGRMV and CNRMV isolates tested. Another advantage of this primer pair is that the amplicon can be used to obtain the complete nucleotide sequence of the CP gene, which is very useful in determining the taxonomic position of the target virus or isolate.

Materials

QIAGEN OneStep RT-PCR Kit (QIAGEN)
Sense primer: 5′- CCTCATTCACATAGCTTAGGTTT-3′
Antisense primer: 5′-ACTTTAGCTTCGCCCCGTG-3′
Total nucleic acid extract (Basic Protocol 1 or Alternate Protocols 1 or 2)
40 U/μl RNase OUT (Invitrogen)
1% agarose gel
0.1 μg/ml ethidium bromide (*APPENDIX 2A*)

PCR tube, PCR strip, or PCR plate
Thermal cycler
Gel imaging system

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000)

Table 16C.1.1 Components of Reaction Mixtures that Included Either Total Nucleic Acids or Viral RNA-rich Extracts of Plant Tissues as Templates for RT-PCR

	RT-PCR template	
Reagents	Total nucleic acids or total RNA	Viral RNA-rich extract
RNase-free water[a]	8.88 µl	4.88 µl
5× reaction buffer[a]	5.0 µl	5.0 µl
Q solution[a]	5.0 µl	5.0 µl
10 mM dNTPs[a]	1.0 µl	1.0 µl
10 µM sense primer	1.5 µl	1.5 µl
10 µM antisense primer	1.5 µl	1.5 µl
RNase inhibitor (40 U/µl)[b]	0.12 µl	0.12 µl
Enzyme mix[c]	1.0 µl	1.0 µl

[a]Included in the QIAGEN Onestep RT-PCR kit.
[b]RNase OUT (Invitrogen).
[c]Contains Omniscript reverse transcriptase, Sensiscript Reverse Transcriptase, and HotStar *Taq* DNA polymerase.

1. Prepare RT-PCR master mix according to the recipes given in Table 16C.1.1.
2. Add 1 µl of total nucleic acid extract or 5 µl of viral RNA−rich extract to a PCR tube, PCR strip, or well of a PCR plate.
3. To the tube, strip, or well, add 24 µl PCR master mix if using total RNA, or 20 µl if using viral RNA−rich extract, and mix thoroughly by pipetting.

   Avoid generation of air bubbles during mixing. A brief centrifugation of the PCR strip or PCR plate at low speed can remove air bubbles.

4. Close the tube or PCR strip tightly, or seal the plate well.

   This step is important in preventing evaporation of the PCR solution during PCR cycling.

5a. *For most commercially available thermal cyclers:* Run the PCR reaction with the following thermal cycling conditions:

1 cycle:	45 min	50°C	(reverse transcription)
	5 min	95°C	(activation of HotStar *Taq* DNA polymerase)
13 cycles:	1 min	94°C	(denaturation)
	1 min	(72-1)°C	(touch-down annealing temperature)
	90 sec	72°C	(extension)
22 cycles	30 sec	94°C	(denaturation)
	30 sec	60°C	(annealing)
	60 sec	72°C	(extension)
1 cycle	10 min	72°C	(final extension).

These conditions are appropriate for most commercially available thermal cyclers. A touch-down thermal cycling program that includes a series of sequentially lower annealing temperatures as used here ensures optimal amplification of target sequences. The calculated optimal annealing temperature, based on the nucleotide content of the oligonucleotides, can be used to replace those in the touch-down cycles if the thermal cycler used does not have this function. Amplification of the viral target has been

obtained in the short thermal cycling conditions listed below using a Peltier thermal cycler (PTC-200, MJ Research). However, optimization of the conditions is recommended for different thermal cycles.

5b. *For Peltier thermal cycler:* Run the PCR reaction with the following thermal cycling conditions:

1 cycle:	45 min	50°C	(reverse transcription)
	5 min	95°C	(activation of HotStar *Taq* DNA polymerase)
13 cycles:	30 sec	94°C	(denaturation)
	30 sec	(72-1)°C	(touch-down annealing temperature)
	60 sec	72°C	(extension)
22 cycles	20 sec	94°C	(denaturation)
	20 sec	60°C	(annealing)
	30 sec	72°C	(extension)
1 cycle	5 min	72°C	(final extension).

6. Electrophorese 10 µl of the amplified PCR product through a 1% agarose gel (Voytas, 2000) containing 0.1 µg/ml ethidium bromide.

   If ethidium bromide is not added in the agarose gel, stain it with ethidium bromide after electrophoresis. If a PCR plate is used for thermal cycling, the 96-well E-Gel (Invitrogen) can be used for electrophoresis.

7. View the gel under UV light to determine if the expected amplicon (~959 bp) is present.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CTAB buffer

Dissolve 2% cetyltrimethylammonium bromide (CTAB) in distilled water with stirring. Use gentle heat (60°C), if necessary. Add 2% polyvinylpyrollidone 40,000 (PVP 40,000), 1.4 M NaCl, 20 mM EDTA (from 0.5 M solution, pH 8.0; *APPENDIX 2A*) and 100 mM Tris·Cl (from 1 M solution, pH 8.0; *APPENDIX 2A*) to the solution. Stir until dissolved. Add water to make final volume. Store 2 to 3 months at room temperature (CTAB will precipitate at 4°C). Add 2-mecaptoethanol to 0.2% immediately before use.

Extraction buffer

Dissolve 20% polyvinylpyrollidone 40,000 (PVP 40,000), 10% sucrose, and 0.15% chicken egg albumin in distilled water with stirring. Then add 100 mM potassium phosphate (from 1 M solution, pH 7.6; *APPENDIX 2A*), to the solution. Stir to mix. Add water to make final volume. Store 2 to 3 months at 4°C. Add ascorbic acid to 30 mM immediately before use.

COMMENTARY

Background Information

Cherry green ring mottle virus (CGRMV) and Cherry necrotic rusty mottle virus (CNRMV), unassigned members of the family *Flexiviridae* (Fauquet et al., 2005) are flexuous filamentous plant viruses with a

single-stranded, positive-sense RNA genome of ~8.4 kb (Zhang et al., 1998; Rott and Jelkmann, 2001b; Gentit et al., 2002).

Despite symptomless infection in most *Prunus* species, CGRMV and CNRMV have potential to cause disease in certain sour, flowering, and sweet cherry varieties. Testing for these and other viruses is required for *Prunus* germplasm entering into the United States from other countries.

CGRMV and CNRMV can be detected by graft inoculation of bud chips onto susceptible hosts for symptom expression and by reverse transcription-polymerase chain reaction (RT-PCR) using virus-specific (Zhang et al., 2000), consensus (Li and Mock, 2005) or degenerate primers (Rott and Jelkmann, 2001a; Foissac et al., 2002).

This unit presents basic procedures for preparation of total nucleic acids or viral RNA-rich extracts from plant tissues and subsequent amplification of the viral targets by one-step RT-PCR using a pair of consensus primers designed to anneal to the highly conserved regions adjacent to the coat proteins (CP) of both CGRMV (AF017780, AJ291761) and CNRMV (AF237816). Both Basic Protocol 1 and Alternate Protocol 1, presented here for template preparation, are simple, fast, and inexpensive, and can be used at most times of the year for the detection of viruses in different plant tissues. Use of one-step RT-PCR is less laborious, faster, and minimizes sample contamination between reverse transcription and PCR amplification as compared with two-step RT-PCR. The authors have used this RT-PCR assay to detect eleven CGRMV isolates, three CNRMV isolates, and several uncharacterized but closely related viruses.

Critical Parameters and Troubleshooting

To increase the likelihood of a successful application of the RT-PCR for detection of CGRMV and CNRMV, it is better to prepare template extracts in seasons when temperatures are not high (see Strategic Planning). High concentrations of phenolic compounds and polysaccharides and low concentration of the viruses in plant tissues in summer make the isolation of the nucleic acids difficult.

Caution should be taken to avoid contamination at every step. Changing gloves, razor blades, wooden coffee stirrers, and tips between samples is important during sample collection and template isolation. The use of tips with filter barriers is also important to avoid contamination.

Isolation of template that is free of compounds inhibitory to PCR is critical. To prevent oxidation during tissue grinding, precool the tube containing the sample and buffer in a freezer and work fast. Nucleic acids or virus particles are protected once they are released into buffer. A translucent white pellet should be obtained from Basic Protocol 1 if the conditions are right; the quality of the extract is poor if a brown pellet is obtained. The disruption of some plant cells may release reactive phenolic compounds which oxidize rapidly to form oxidized phenolics that undergo irreversible electrophilic substitution reactions with proteins and nucleic acids. Basic Protocol 1 and the accompanying alternative protocols emphasize simplicity, low cost, and broad options. However, the extracts obtained by these methods contain host proteins including nucleases, which slowly degrade the nucleic acids even when frozen; therefore, the template preparations cannot be stored for more than 2 months. Further purification of the extracts is necessary if long-term storage is required.

There are several one-step RT-PCR kits commercially available. The protocol presented here has been optimized for the QIAGEN OneStep RT-PCR kit. The RT-PCR reaction volume is reduced to minimize the cost per reaction. However, the reduction of the recommended reaction volumes may affect the efficiency of RT-PCR. Therefore, reaction conditions should be optimized if other kits are used. The dNTP mixtures provided in the RT-PCR kit should be distributed in aliquots and stored frozen. Frequent freeze-thaw cycles can degrade dNTP mixtures and reduce the efficiency of RT-PCR.

Anticipated Results

A strong band at 959 bp (958 bp for CNRMV) is produced if the source plant is infected. No amplicons are detected from uninfected plants. Amplification of weak band(s) sometimes occurs from infected samples, but not from uninfected samples.

Time Considerations

Basic Protocol 1 can be completed in 3 hr if twelve samples are processed. Forty-eight samples can be processed easily in a single day. The alternative protocol for template preparation is simpler and faster in term of hands-on time, usually 2 hr per twelve samples, but overnight incubation is required. RT-PCR can be completed in 3.5 hr.

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Callus Cultures of *Arabidopsis*

Protoplast inoculations, unlike infection of whole plants, can provide a quantitative assay for measuring viral RNA accumulation. Protoplasts are generated by enzymatically treating plant tissue to produce cells lacking cell walls. Infectious viral RNA can be inoculated into a calculated number of protoplasts by permeabilizing the cell membrane and then culturing the cells in a medium that supports their growth. There are three main sources of tissue for the preparation of protoplasts: (1) fresh tissue such as excised leaves or cotyledons, (2) tissue that is maintained in long-term suspension culture, and (3) undifferentiated callus that is cultured on solid media. The use of fresh tissue requires time-consuming peeling of the epidermal layer, and protoplasts produced in different experiments can vary in their ability to be infected by viruses. Suspension cultures are a more common choice, but initiating and maintaining the cultures can be problematic. The authors have found that callus cultures prepared from sterilized seeds are advantageous because they require little effort to initiate and maintain, and upon enzymatic treatment they produce uniform protoplasts that are nearly always competent for virus infection.

This unit describes how to generate protoplasts prepared from callus and inoculate them with infectious viral RNA. Basic Protocol 1 outlines generation of callus from seeds of *Arabidopsis thaliana* ecotype Col-0. Basic Protocol 2 describes the production of protoplasts from the callus using cellulase and pectinase and the inoculation of protoplasts using polyethylene glycol. Basic Protocol 3 addresses the extraction of total RNA from infected protoplasts.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aeseptic technique should be used accordingly.

CULTURING OF *ARABIDOPSIS* CALLUS

BASIC PROTOCOL 1

In this protocol, *Arabidopsis thaliana* seeds are sterilized and plated onto callus maintenance medium (CM), which supports the dedifferentiation of embryonic cells into callus. For optimal growth, the callus is grown at 20°C using a photoperiod of 16 hr light and 8 hr dark, and an illuminance of 35 μmol/m^2S. To maintain healthy cultures, callus is passaged every 3 to 4 weeks for up to six passages by mechanically breaking up the callus and transferring the smaller clumps to new CM plates. Callus that has been passaged between three and six times is competent for viral inoculation.

Materials

Arabidopsis thaliana (ecotype Col-0) seeds
70% ethanol
Bleach containing 4% to 6% sodium hypochlorite
10% (w/v) sodium dodecyl sulfate (SDS; *APPENDIX 2A*)
H_2O, sterile
Callus maintenance medium (CM) 1% agar plates (see recipe)

1.5-ml microcentrifuge tubes, sterile
Forceps, sterile
20°C incubator with light control (e.g., Percival Scientific I-36LL; *http://www.percival-scientific.com*)

Contributed by John C. McCormack and Anne E. Simon
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1. Aliquot ~300 *Arabidopsis thaliana* seeds into a 1.5-ml microcentrifuge tube and wash twice with 1 ml of 70% ethanol by vortexing vigorously. Pulse centrifuge and remove the alcohol by pipetting.
2. Add 1 ml bleach containing 4% to 6% sodium hypochlorite followed by 50 µl of 10% SDS. Vortex vigorously. Incubate 5 to 10 min at room temperature. Pulse centrifuge and remove supernatant.
3. Wash the seeds five times by suspending them in 1 ml sterile water, vortexing, and pulse centrifuging. After the final centrifugation, resuspend the seeds in sterile water.

   For steps 3 and 4, all work should be conducted in a sterile environment. Surface-sterilized seeds should be placed immediately onto CM plates.

4a. *To create new callus:* Spread ~100 surface-sterilized seeds onto each callus maintenance CM agar plate. Seal lids of individual plates with Parafilm

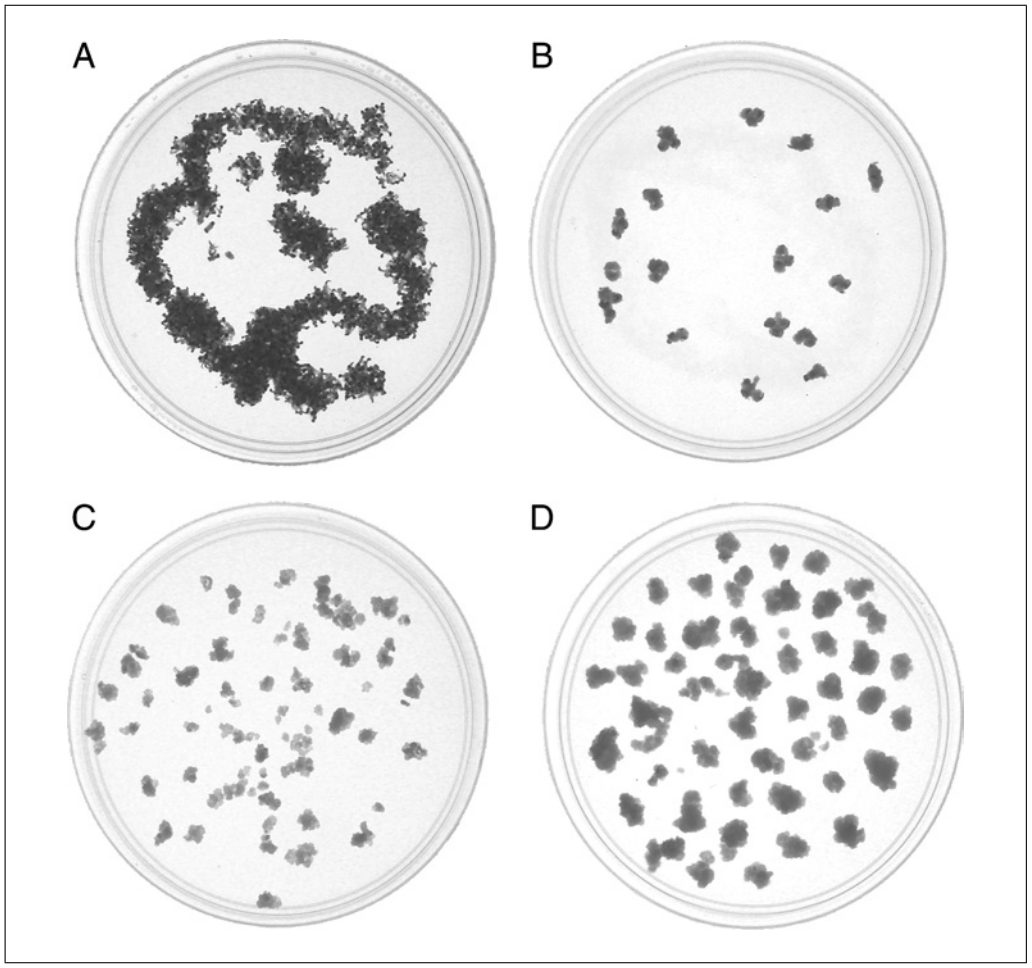

Figure 16D.1.1 Appearance of callus cultures in sterile 100 × 15–mm petri dishes at several stages of growth. (**A**) Plant tissue appears green and has not undergone dedifferentiation at 2 weeks into the first passage. (**B**) At 1 week into the second passage, callus appears green with tinges of yellow and becomes undifferentiated. (**C**) Callus appears mostly yellow and friable at $1\frac{1}{2}$ weeks into the third passage. This plate contains ~1 g callus and is the earliest time point for digestion of callus into protoplasts. (**D**) Callus clumps become larger as tissue grows within each passage. This plate is 3 weeks into the third passage and is ready to be split to begin the next passage. Appearance of callus in the fourth through sixth stages should not change. For the color version of this figure go to *http://www.currentprotocols.com*.

4b. *To passage existing callus:* Use sterilized forceps to mechanically break up the callus and transfer onto new CM plates. Seal individual plates with Parafilm.

Callus should be passaged every 3 to 4 weeks for up to six passages. During passages one through three, the color of the callus should change from green to yellow. For passages three through six, tissue should be light yellow and friable (Fig. 16D.1.1). Browning or softening of callus indicates dead or dying tissue and should be discarded. When passaging, break up the intact callus into smaller clumps of 0.2 to 0.4 cm in diameter; do not simply move intact callus from old to new CM plates. One plate of developing callus can be passaged onto three to four new plates.

5. Incubate callus at 20°C using a photoperiod of 16 hr light and 8 hr dark, and an illuminance of 35 μmol/m^2S.

PREPARATION AND INOCULATION OF CALLUS CULTURE PROTOPLASTS WITH INFECTIOUS VIRAL RNA USING POLYETHYLENE GLYCOL

BASIC PROTOCOL 2

In this protocol, protoplasts are generated by treating callus with pectinase and cellulase to separate cells from one another and to degrade the cell wall. Protoplasts are then washed with 0.6 M mannitol to remove the enzymes and cells are enumerated using a hemacytometer. Protoplasts (5×10^6) are then inoculated with in vitro transcripts of infectious viral RNA using polyethylene glycol (PEG) and calcium chloride, which make cell membranes permeable to RNA. Finally, inoculated protoplasts are washed with 0.6 M mannitol containing 1 mM calcium chloride to remove the PEG and then suspended in protoplast culture medium (PCM), a medium that supports cell growth. Protoplasts can be incubated for a finite time, generally <50 hr before regeneration of the cell wall begins.

Materials

0.6 M mannitol, room temperature and 4°C
Arabidopsis callus (in the third to sixth passage; Basic Protocol 1)
Protoplast inoculation medium (PIM; see recipe)
Cellulase, *Trichoderma viride* (10 KU/g dry weight; Calbiochem)
Pectinase, *Rhizopus* sp. (3 KU/g dry weight; Calbiochem)
RNA of interest: transcribe in vitro (see *UNIT 16D.4*)
1.0 M $CaCl_2$
50% polyethylene glycol (PEG; see recipe)
0.6 M mannitol containing 1 mM $CaCl_2$, 4°C
Protoplast culture medium (PCM; see recipe)

14.6-cm Pasteur pipet: melt into L-shape
Rotating shaker
50-ml polypropylene centrifuge tubes, sterile
125-ml glass bottles: sterilize by autoclaving
Refrigerated centrifuge with bucket rotor appropriate for 50-ml tubes
Funnel: sterilze by autoclaving
53-μm nylon mesh (Small Parts): sterilize by autoclaving

Digest the callus

1. Add 15 ml of 0.6 M mannitol (room temperature) to each plate of callus in a sterile environment. Break the callus into smaller pieces using a Pasteur pipet that has been melted into an L-shape.

Callus is friable; very little manipulation is necessary to break it up without breaking the agar matrix.

2. Pour callus and mannitol from plates into a sterile 50-ml polypropylene centrifuge tube. Shake on a rotating shaker 20 min at 100 rpm, room temperature.

3. For each plate of callus being processed, dispense 50 ml PIM into a sterile 125-ml glass bottle along with 0.5 g cellulase and 0.1 g pectinase. Incubate mixture on a rotating shaker at 100 rpm, room temperature, until completely dissolved.

   Cellulase and pectinase dissolve more quickly if PIM is added to the bottle first, followed by the powdered enzymes.

4. Using a refrigerated centrifuge with a bucket rotor, centrifuge the callus from step 1 for 4 min at 900 × *g* (brake off), room temperature, and pour off supernatant.

5. For each plate of callus being processed, pour 25 ml PIM containing dissolved enzymes into the polypropylene centrifuge tube containing the callus and transfer to a sterile 125-ml glass bottle.

6. Add another 25 ml PIM containing dissolved enzymes to the polypropylene tube to collect any remaining callus and transfer to the glass bottle. Wrap bottle completely in aluminum foil.

7. Incubate glass bottle containing callus and PIM with dissolved enzymes 3.5 hr on a rotating shaker at 100 rpm, room temperature.

   Solution should become turbid after 3.5 hr as the callus is digested and cells are released.

8. Filter the turbid solution into a 50-ml polypropylene tube using a sterile funnel and 53-μm nylon mesh.

Prepare the protoplasts

9. Collect the filtered liquid containing the protoplast suspension and centrifuge 4 min at 228 × *g* (brake off), 4°C. Decant the supernatant, leaving ~10 ml solution. Resuspend protoplasts by gently shaking the tube to disperse the pellet.

   For washes in steps 9 through 18, decant supernatant with one slow deliberate movement leaving ~10 ml liquid in the tube. Attempting to pour out supernatant twice or pipetting out the supernatant instead of pouring may result in excessive loss of cells. To resuspend the pellet, always add 10 to 20 ml cold 0.6 M mannitol (depending on the wash); increasing the volume will result in a less compact pellet that is more easily lost during decanting of the supernatant. Keep the protoplasts on ice and the 0.6 M mannitol chilled between washing.

10. Add 20 ml cold (4°C) 0.6 M mannitol and gently invert the centrifuge tube several times to wash the protoplasts. Centrifuge as in step 9. Repeat for a total of three washes.

11. After the final wash, centrifuge cells, resuspend protoplasts in cold 0.6 M mannitol up to a volume of 20 ml, and keep on ice.

12. Enumerate protoplasts using a hemacytometer (*APPENDIX 4A*).

    Intact protoplasts should appear round with a large vacuole under a light microscope. Each plate of callus (~1 g) after digestion should yield at least 3 × 10^7 protoplasts.

13. Aliquot 5 × 10^6 cells into 50-ml polypropylene centrifuge tubes, one tube for each inoculation. Collect protoplasts by centrifuging 5 min at 228 × *g* (brake off), 4°C. Pour off supernatant leaving ~100 μl. Keep protoplasts on ice until inoculation (up to 1 hr).

Infect the protoplasts

14. For each inoculation, combine in vitro–transcribed viral RNA, 8 μl of 1.0 M $CaCl_2$, and sterile water up to a volume of 430 μl. Keep inoculation mix on ice.

    For turnip crinkle virus (TCV), the authors routinely add 15 pmol of in vitro–transcribed RNA to each inoculation mix; the amount of inoculated RNA may differ for other viral

systems. Subviral RNAs can be co-inoculated with the helper virus; for the TCV system, an equimolar amount of viral and subviral RNA is added to the inoculation mix. All inoculation mixes should be prepared at the same time and within an hour of inoculating protoplasts; failing to do so may result in uneven or poor inoculations.

15. Add the inoculation mix to the tube containing the protoplasts and pipet gently up and down twice. Add 2.2 ml of 50% PEG, mix well by gently shaking the tube 15 sec, and incubate at room temperature for 20 sec.

16. Add cold 0.6 M mannitol containing 1 mM $CaCl_2$ to a volume of 30 ml and incubate 15 min on ice.

17. Centrifuge protoplasts 5 min at 228 × *g* (brake off), 4°C. Decant the supernatant leaving 10 ml residual supernatant.

18. Add 10 ml cold 0.6 M mannitol containing 1 mM $CaCl_2$. Centrifuge protoplasts 5 min at 228 × *g* (brake off), 4°C. Decant the supernatant. Repeat wash and centrifugation two more times. After the final centrifugation completely decant the supernatant.

19. Resuspend each tube of protoplasts in 3 ml PCM and pour into a 60 × 15–mm petri dish.

20. Cover petri dishes in aluminum foil and incubate at room temperature.

    For the turnip crinkle virus system, protoplasts are generally incubated between 24 and 40 hr.

EXTRACTION OF TOTAL RNA FROM *ARABIDOPSIS* PROTOPLASTS

BASIC PROTOCOL 3

In this protocol, protoplasts are collected at 24 to 40 hr postinoculation (hpi) by centrifugation and cells are lysed using RNA extraction buffer. RNA is then purified by 1:1 phenol/chloroform extraction and ethanol precipitation. Both single- and double-stranded RNA are isolated using this procedure. Total RNA (2 µg) can be loaded onto a polyacrylamide or agarose gel for northern blot analysis of viral accumulation.

Materials

Inoculated protoplasts (Basic Protocol 2)
1:1 phenol/chloroform (Tris-buffered phenol; *APPENDIX 2A*)
RNA extraction buffer (see recipe)
3 M sodium acetate, pH 5.2 (*APPENDIX 2A*)
100% and 70% ethanol
H_2O, sterile
Microcentrifuge, refrigerated

1. Check the condition of the inoculated protoplasts by examining plates under a light microscope. Tilt plates to evenly distribute cells prior to removal.

   Tilting the plates prior to pipetting allows for an equal number of cells to be dispensed when the protoplast-containing medium is divided into two tubes (step 3).

   Intact protoplasts should appear round with a large vacuole under a light microscope.

2. At 24 to 40 hpi, split each plate of infected cells into two microcentrifuge tubes. Centrifuge 3 min at 1500 × *g* (brake off), room temperature.

3. Remove one-half of the supernatant using a pipet. Centrifuge 3 min at 1500 × *g* (brake off), room temperature. Remove the remainder of the supernatant, leaving 100 µl of medium. Place one tube at −80°C as a backup for each transformation. Proceed with total RNA extraction using the second tube.

   Do not pipet off all the supernatant after the second centrifugation; leave ~100 µl in each tube to avoid excessive loss of cells.

4. Add 200 µl of 1:1 phenol/chloroform and 200 µl RNA extraction buffer to each tube and vortex vigorously for 10 sec. Microcentrifuge 2 min at 10,400 × *g*, 4°C. Transfer the upper layer to a new tube.

   To phenol, add 8-hydroxyquinoline to a final concentration of 0.1% for partial inhibition of RNases. Avoid removing any of the lower layer to minimize RNA degradation.

5. Add 25 µl of 3 M sodium acetate, pH 5.2, and 575 µl of 100% ethanol. Vortex vigorously and incubate 5 min at −80°C. Microcentrifuge 10 min at 10,400 × *g*, 4°C. Discard the supernatant.

6. Add 500 µl of 70% ethanol, invert tube several times to wash the pellet, and microcentrifuge 5 min at 10,400 × *g*, 4°C. Discard supernatant, dry pellet, and resuspend RNA in 20 to 30 µl sterile water.

7. Determine RNA concentration at an optical density of 260 using a spectrophotometer (Gallagher, 2004).

   Amount of total extracted RNA for each tube should be at least 10 µg.

   RNA may be analyzed by loading 2 µg total RNA onto a PAGE or agarose gel for northern blot procedures (Brown et al., 2004).

REAGENTS AND SOLUTIONS

Use deionized, distilled water that has been sterilized in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS INDEX****.*

2,4-D, 2 mg/ml

Fully dissolve 0.02 g of 2,4-dichlorophenoxyacetic acid (2,4-D) in 5 ml of 100% ethanol. Adjust volume to 10 ml with sterile water. Store up to 6 months in the dark at −20°C.

Do not autoclave solution.

Callus maintenance medium 1% agar plates

60 g sucrose
8.8 g Murashige and Skoog basal salt mixture (MS Salts; Sigma-Aldrich)
20 ml vitamins/glycine stock, 100× (see recipe)
Adjust volume to 2 liters with sterile H_2O
Adjust pH to 5.8 with NaOH
Dispense 400-ml aliquots in each of five 500-ml bottles
Add 4 g bacteriological agar to each bottle
Autoclave 20 min at 121°C and cool to 50° to 60°C

In a sterile environment, add 100 µl of 2,4-D (see recipe) and 100 µl of 2 mg/ml kinetin (see recipe) to each 400 ml callus maintenance medium (CM) agar and mix. Pour ∼30 ml into sterile petri dishes (100 × 15 mm). Allow plates to solidify and dry up to 1 day at room temperature. Wrap plates in aluminum foil and store up to 3 months at 4°C.

To reduce the risk of contamination, avoid drying CM plates for longer than 1 day at room temperature.

CM agar base (before the addition of 2,4-D and kinetin) can also be stored up to 3 months at 4°C.

Hormone stock, 2000×

0.004 g 2,4-dichlorophenoxyacetic acid (2,4-D)
0.004 g kinetin
0.5 ml 1 N KOH

Adjust volume to 10 ml with sterile H_2O
Divide into 1-ml aliquots
Store up to 1 year at −20°C

Do not autoclave solution.

Kinetin, 2 mg/ml

0.02 g kinetin
667 µl 1 N NaOH
Fully dissolve kinetin in 1 N NaOH
Adjust volume to 10 ml with sterile H_2O
Store up to 6 months in the dark at −20°C

Do not autoclave solution.

Polyethlyene glycol (PEG), 50% (w/v)

25 g PEG (Polysciences)
Adjust volume to 50 ml with 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
Completely dissolve PEG by shaking
Store up to 1 month at 4°C

Do not autoclave solution.

Protoplast culture medium

1 ml 1000× vitamin stock (see recipe)
0.5 ml 2000× hormone stock (see recipe)
4.4 g MS salts (Sigma-Aldrich)
34.2 g sucrose
0.585 g MES
72.8 g mannitol
Adjust volume to 1 liter with sterile H_2O
Adjust pH to 5.8 with 1 N KOH
Autoclave 20 min at 121°C
Store up to 2 months at 4°C

Protoplast inoculation medium

1 ml vitamin stock, 1000× (see recipe)
0.5 ml hormone stock, 2000× (see recipe)
4.4 g MS salts (Sigma-Aldrich)
34.2 g sucrose
0.585 g MES
91 g mannitol
0.555 g $CaCl_2$
Adjust volume to 1 liter with sterile H_2O
Adjust pH to 5.8 with 1 N KOH
Autoclave 20 min at 121°C
Store up to 2 months at 4°C

RNA extraction buffer

2.5 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)
0.50 ml 0.5 M EDTA, pH 8.0
1 ml 5 M NaCl
5 ml 10% SDS
41 ml sterile H_2O
Store up to 1 year at room temperature

Do not autoclave solution.

Vitamins stock, 1000×

0.02 g thiamine HCl
0.01 g pyridoxine HCl
0.01 g nicotinic acid
2 g myo-inositol
Adjust volume to 20 ml with sterile H_2O
Divide into 1-ml aliquots
Store up to 1 year at −20°C

Do not autoclave solution.

Vitamins/glycine stock, 100×

0.025 g nicotinic acid
0.25 thiamine HCl
0.025 g pyridoxine HCl
2.5 g myo-inositol
0.1 g glycine
Bring volume up to 250 ml with sterile H_2O
Store up to 1 year at 4°C

Do not autoclave solution.

COMMENTARY

Background Information

Protoplasts were first isolated in the early 1960s by using an enzyme preparation to degrade the cell walls of tomato root tips (Cocking, 1960); however, the use of these cells in research did not become widespread until a procedure was developed eight years later that facilitated the large-scale production of active intact protoplasts (Takebe et al., 1968). Due to the simplicity of this procedure, the isolation of protoplasts from different tissues of various plants species was made possible, resulting in numerous ways to study and manipulate plant cells.

Within the authors' laboratory, the inoculation of callus-derived protoplasts with infectious viral RNA has proven indispensable in providing a quantitative assay for measuring accumulation of turnip crinkle virus (TCV) in vivo (Wang & Simon, 1997). Unlike infection of whole plants, a specified number of cells can be synchronously infected and results obtained as quickly as two days after inoculation. RNA stability assays have also been performed in protoplasts by inoculating RNA that is unable to replicate and plotting a time course of RNA levels beginning at the time of inoculation. Since mutations introduced into RNA may be destabilizing, these studies are useful in differentiating whether a reduction in accumulation is due to instability of the inoculated RNA or a replication defect (Nagy et al., 1999).

Other applications using callus cultures include the development of an RNA tracking system using protoplasts by co-inoculation of TCV with an expression vector encoding green fluorescent protein (GFP) fused to a nuclear localization signal (NLS) and the coat protein (CP) of MS2 bacteriophage. The TCV used is engineered to contain a 19-base MS2 CP–binding hairpin. Viral RNA is tracked within the cell by binding to the GFP-NLS-MS2 fusion protein, which can then be visualized by confocal microscopy (Zhang and Simon, 2003). Seedlings grown on callus plates have also been used to study the effects of temperature on the RNA expression patterns of protein genes exhibiting circadian rhythm (Kreps and Simon, 1997).

Critical Parameters and Troubleshooting

Callus culture contamination

Fungal or bacterial contamination of callus may appear one to two weeks after passaging. Contamination may arise from improper preparation of CM plates, inadequate surface-sterilization of *Arabidopsis thaliana* seeds, or poor aseptic technique during passaging.

Dead or dying callus cultures

Healthy callus is mostly light yellow and friable, whereas dying tissue appears brown and should not be used for passaging. Excess condensation on callus culture plates may contribute to tissue death. To reduce condensation, CM plates should be dried at room temperature for one day before use. Plates containing excess condensation can still be used

for passaging of callus; however, care must be taken to only passage tissue that appears healthy.

Poor or uneven accumulation of viral RNA

Once experimental parameters have been optimized for the viral system of interest, poor RNA accumulation in protoplasts may be due to incorrect determination of RNA transcript concentration, poor RNA quality, inadequate shaking of the mixture containing protoplasts and in vitro–transcript RNA, or old calcium chloride. Uneven accumulation of viral RNA in protoplasts may be the result of inadequate resuspension of in vitro synthesized RNA transcripts after precipitation and prior to inclusion in the inoculation mix. Furthermore, it is important that all inoculation mixes are prepared at the same time and no more than 1 hr prior to inoculation.

Excessive loss of protoplasts

Each plate of callus should yield at least 3×10^7 protoplasts and each tube of cells during RNA extraction should contain at least 10 μg total RNA. Amounts less than these may indicate an excessive loss of cells due to inadequate centrifugation or incorrect decanting of the supernatant.

Degradation of extracted total RNA from protoplasts

Total RNA extracted from protoplasts is very sensitive to degradation by RNases and usually occurs because of poor technique. The following general measures should be taken during total RNA extraction. (1) Use only autoclaved tips, microcentrifuge tubes, and solutions that have been designated solely for work involving RNA. (2) Keep RNA extracts on ice and chill solutions prior to use. (3) If subjecting RNA to electrophoresis, take care to avoid RNase contamination of the electrophoresis gel box, gel bed, and comb. Thoroughly rinse gel box, gel bed, and comb with sterile water and avoid touching these items with ungloved hands after rinsing. Treat equipment with RNase decontamination solution, if necessary.

Anticipated Results

Each plate of callus culture should yield at least 3×10^7 protoplasts upon treatment with cellulase and pectinase. For the turnip crinkle virus (TCV) system, the efficiency of protoplast infection has been found to be ~30% using the polyethylene glycol method. Extraction of total RNA from 5×10^6 inoculated protoplasts incubated for 40 hr yields at least 20 μg of total RNA. Twenty percent of this RNA is estimated to be TCV genomic and subgenomic RNAs.

Time Considerations

Preparation of CM plates and surface-sterilization of seeds can be accomplished within 2 days. Once the seeds are plated, callus should be ready for enzymatic digestion in the middle of the third passage, which takes ~8 weeks. Digestion of callus into protoplasts and inoculations of protoplasts with viral RNA can be accomplished in ~9 hr. At the desired time point, extraction of total RNA from protoplasts can be performed in ~ 2 to 3 hr.

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Preparation and Inoculation of Mesophyll Protoplasts from Monocotyledenous and Dicotyledenous Hosts

Studies leading to an increased understanding of various aspects of the plant-virus infection cycle in whole plants are hindered by two major limitations. First, it is often difficult to achieve infection of substantial numbers of primary cells during mechanical inoculation of a leaf; consequently, the inability to attain synchronous infection makes it difficult to characterize viral-specific events, most importantly during early stages of infection. Second, the infection cycle in initially infected cells is randomized because of cell-to-cell movement of the replicating virus. These limitations were, however, circumvented when Takabe and Otsuki (1969) discovered that single plant cells, namely protoplasts, can be isolated by enzymatic treatment of intact leaves and synchronously infected with either purified virus or viral RNA. The protoplast system has been regarded as a hallmark in plant virology since it broke barriers that had prevented plant virus replication studies at the level attainable with bacterial and animal viruses. Merits of the protoplast system include (1) establishment of synchronous infection, (2) delivery of a known concentration of the inoculum to a defined number of cells, (3) flexible control of experimental conditions, (4) uniform sampling, as well as facile isolation and characterization of virus-related products, and (5) the ability to study replication of viruses that cannot be transmitted mechanically. The only disadvantage of this system is that cell-to-cell movement functions cannot be analyzed. Nevertheless, over the past decade experiments performed with plant protoplasts have significantly advanced our understanding of virus replication. A majority of earlier studies involved infection of protoplasts with purified virus preparations. However, the advent of recombinant DNA technology and our ability to manipulate RNA viral genomes at the DNA level provided new directions. The following sections describe isolation and infection of protoplasts obtained from leaves of monocotyledonous and dicotyledonous hosts. Note that the basic protocol for isolating protoplasts from either monocotyledonous or dicotyledonous hosts is similar except the enzyme solutions used are different.

NOTE: A complimentary procedure for preparing protoplasts using callus cultures is presented in *UNIT 16D.1*.

ISOLATION OF MESOPHYLL PROTOPLASTS FROM MONOCOTYLEDENOUS HOSTS

BASIC PROTOCOL 1

This section describes a basic procedure, adapted from Loesch-Fries and Hall (1980), for the preparation and inoculation of mesophyll protoplasts from barley plants (*Hordium vulgare*; Rao et al., 1994). This procedure can be used for isolating mesophyll protoplasts from other dicotyledonous hosts such as rice. Various steps required for successful isolation of viable protoplasts are presented in the first half of the protocol, while the second half describes inoculation of protoplasts with viral RNA using polyethylene glycol.

Materials

5- to 6-day-old barley leaves
Enzyme solution for barley (see recipe)
0.55 M sucrose (see recipe)
0.55 M mannitol, pH 5.9 (see recipe)
5 mg/ml fluorescein diacetate: dissolve 5 mg flourescein diacetate (Sigma) in 1 ml acetone; store in an opaque container for up to 6 months at 4°C

Contributed by A.L.N. Rao
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28°C incubator
100-ml beakers
300- to 350-μm gauze
15- and 50-ml polypropylene tubes
Beckman Model J6B centrifuge (preferred) or clinical tabletop centrifuge
Large-bore pipet
Microscope equipped with UV light source (also see *UNIT 2A.1*)

Additional reagents and solutions for counting cells in a hemacytometer (*APPENDIX 4A*)

Prepare leaves for protoplast extraction

1. Harvest 0.5 g of 5- to 6-day-old barley leaves.

   Avoid harvesting dry or yellow leaves.

2. With a sharp razor, slice leaves lengthwise and then crosswise into 1-mm^2 sections.

3. Transfer sliced material into 25 ml enzyme solution for barley and incubate 3 to 3.5 hr at 28°C, without shaking.

   Completely digested leaf material will have a transparent appearance.

4. Decant the solution containing the enzyme-digested leaf material into a 100-ml sterile beaker.

   At this stage, a gentle swirling will facilitate release of protoplasts from the digested leaf material.

5. Filter the solution through gauze into another 100-ml sterile beaker and transfer the filtrate into a sterile 50-ml polypropylene tube.

6. Add 12.5 ml of the enzyme solution containing the digested material into each 50-ml polypropylene tube containing enzyme-digested leaf material.

7. Underlay 10 ml of 0.55 M sucrose beneath the digested material in each tube.

Remove the protoplasts

8. Centrifuge for 7 min, at 1000 × *g*, room temperature

   Either a Beckman Model J6B centrifuge or a clinical tabletop centrifuge is ideal for all specified centrifugations. Unless otherwise mentioned, all centrifugations steps must be performed at room temperature. Although centrifugation at 4°C will not harm protoplasts, when the protoplasts are viewed under a microscope they appear transparent making it difficult to get an accurate estimation of live cells.

9. Using a large-bore pipet tip, carefully remove protoplasts.

   Protoplasts constitute a dark green interface forming between the light-brown-colored enzyme and the sucrose solutions.

10. Transfer protoplasts into a 50-ml tube containing 10 to 15 ml of 0.55 M mannitol, pH 5.9. Mix gently.

11. Centrifuge 4 min, at 1000× *g*, room temperature.

12. Remove most of the supernatant and gently resuspend protoplasts in the remaining liquid.

13. Add 10 ml of 0.55 M mannitol, pH 5.9, and repeat wash as described above.

Count the protoplasts

14. Suspend protoplasts carefully in 2 ml of 0.55 M mannitol, pH 5.9.

15. Determine the number of viable protoplasts by adding 1 to 2 µl of 5 mg/ml fluorescein diacetate to a 20- to 30-µl aliquot of protoplasts (prepared in step 14). Incubate 1 to 2 min at room temperature.

16. Apply the stained protoplast solution to a hemacytometer (*APPENDIX 4A*) and view under a microscope equipped with UV light source.

17. Count live protoplasts, which fluoresce bright green.

    Avoid counting dead cells, which appear red.

18. Determine the number of viable protoplasts per cubic centimeter (10 cells/mm^2 = 10^5 cells/cm^3) and estimate the number of viable protoplasts per milliliter.

19. Dispense desired number of protoplasts ($\sim 1 \times 10^5$) to be inoculated into a 15-ml polypropylene tube and add 1 ml of 0.55 M mannitol, pH 5.9.

    From this stage, absence of enzyme solution enhances cell wall formation that will significantly affect uptake of the inoculum. Therefore, speed and accuracy are essential to achieve high infection rate.

20. Proceed to Basic Protocol 3 immediately.

ISOLATION OF MESOPHYLL PROTOPLASTS FROM TOBACCO (*NICOTIANA BENTHAMIANA*) LEAVES

BASIC PROTOCOL 2

A wide range of plant viruses infect dicotyledonous hosts such as tobacco species (e.g., *Nicotiana benthamiana*). Even some viruses that are restricted to monocotyledonous hosts, such as Brome mosaic virus, can also infect *Nicotiana benthamiana*. There is little host restriction at the protoplasts level. Nevertheless, a comparison of the replication profiles of a given virus accumulating in transfected protoplasts from natural and nonhost systems will be useful in assessing the basic events that regulate the infection pattern.

NOTE: The procedure used for isolating protoplasts from intact tobacco leaves is the same as that described above for barley protoplasts with some modifications.

Materials

3-week-old *N. benthamiana* leaves
0.55 M mannitol, pH 5.9 (see recipe)
Carborundum powder (Fisher)
Enzyme solution for tobacco (see recipe)

100 × 15-mm petri dish
Cotton swabs
28°C incubator

Additional reagents and equipment for preparing the leaves for protoplasts extraction and removing and counting the protoplasts (Basic Protocol 1, steps 4 to 20)

1. Harvest 1 g of 3-week-old *N. benthamiana* leaves.

   Select only well expanded leaves, avoiding dry or yellow ones.

2. Rinse harvested leaves in 0.55 M mannitol solution by floating in a petri dish.

3. Gently dry the leaves using paper towels.

4. Turn the leaf up-side-down and sprinkle with carborundum powder evenly, covering the entire leaf.

5. Using a cotton swab, gently rub in the carborundum.

   Avoid excessive rubbing to minimize mechanical damage.

   Gentle abrasion caused by carborundum helps enzymes to enter for increased digestion of the leaf tissue.

6. Transfer leaf to a petri dish containing 0.55 M mannitol, pH 5.9, and gently wash off carborundum powder.

7. With a sharp razor, slice the leaf on either side of the midrib portion into two halves. Then slice crosswise into 1-mm^2 sections.

8. Float the sliced materials in enzyme solution for tobacco such that the bottom (abrasive side) of the sliced leaf is touching the enzyme solution. Incubate 3 to 3.5 hr at 28°C.

   After this step, follow Basic Protocol 1, steps 4 to 20.

BASIC PROTOCOL 3

POLYETHYLENE GLYCOL–MEDIATED INOCULATION OF PROTOPLASTS WITH VIRAL RNA

Two of the most commonly used techniques to deliver viral RNA into protoplasts are electroporation and polyethylene glycol. Although electroporation works well, it suffers from the disadvantage that the electric field can damage RNA as well as protoplasts. This limitation can be circumvented by using polyethylene glycol, making this procedure amenable for transfecting protoplasts isolated from a variety of host plants. The authors have not encountered any protoplasts system or RNA sources that are not compatible with the procedure described here.

Materials

Protoplasts in 15-ml polypropylene tubes (Basic Protocol 1 or 2)
Viral RNA or transcripts (*UNIT 16K.2*)
40% polyethylene glycol (PEG; see recipe)
0.55 M mannitol, pH 5.9 (see recipe)
Culture medium for mesophyll protoplasts (see recipe)

24-well plate (Corning)
Fluorescent lamp

1. Centrifuge tubes containing desired number of protoplasts (from Basic Protocol 1) for 3 min at 1000 × *g*, room temperature.

2. Remove most of the supernatant and add desired concentration of either viral RNA or transcripts, keeping the volume small (5 to 10 μl; 1 to 5 μg of RNA).

3. Gently mix the protoplast pellet to resuspend in the inoculum, then add 150 μl of 40% PEG and gently shake the contents for 10 sec.

4. Add 2 drops of 0.55 M mannitol, pH 5.9, to the side of the tube and mix gently.

5. Continue to add mannitol dropwise over the next 5 to 10 min until the volume has reached 1.5 ml.

6. Incubate the sample on ice for 15 min.

7. Pellet protoplasts by centrifuging for 3 min at 1000 × *g*, room temperature.

8. Wash once with 1 ml of 0.55 M mannitol, pH 5.9.

9. Suspend 1×10^5 protoplasts in 1 ml of culture medium for mesophyll protoplasts.

10. Place inoculated protoplasts in a culture plate (24-well plate from Corning) and keep under a fluorescent lamp for 20 to 24 hr or desired length of time.

An alternative procedure over PEG mediated transfection is electroporation (UNIT 16D.4). Since application of an electric pulse is likely to damage the majority of protoplasts, it is advisable to use significantly higher number of cells (a minimum of 10^6 per sample).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Ca solution, 100×

Weigh 14.70 g $CaCl_2$ and dissolve in 100 ml of 0.55 M mannitol, pH 5.9 (see recipe). The pH of this solution should be around 6.5. Sterilize the solution by autoclaving. Store up to 1 to 3 months at room temperature.

CKM solution, 1000×

Weigh 0.0025 g $CuSO_4$, 0.017 g KI, and 24.65 g $MgSO_4$, and dissolve in 100 ml of 0.55 M mannitol, pH 5.9 (see recipe). The final pH of the solution should be around 5.8. Sterilize by autoclaving and store up to 1 to 3 months at room temperature.

Culture medium for mesophyll protoplasts

Dilute the following to 1× in 0.55 M mannitol, pH 5.9 (see recipe):
1000× CKM solution (see recipe)
1000× KK solution (see recipe)
100× Ca solution (see recipe)
Add 10 μg gentamicin from a 10 mg/ml stock in sterilie water (store stock up to 1 to 3 months at 4°C)
Add cephaloridine (Sigma) to a final concentration of 0.3 mg/ml, diluted from a 30 mg/ml stock in sterile water (store stock up to 1 to 3 months at 4°C)
Prepare the culture medium and use immediately

Enzyme solution for barley

In a sterile beaker, weigh 0.25 g of cellulase (commercially available from Calbiochem as cellulysin, 100,000 U), 12.5 mg of BSA (Sigma), and 12.5 mg of macerozyme (Yakult Pharmaceutical). Add 12.5 ml 0.55 M mannitol, pH 5.9 (see recipe), and stir at room temperature until the contents are dissolved. Adjust pH of the solution to 5.9 with 0.1 M KOH and sterilize by filtration using a 0.2-μm pore size filter. Store up to 6 months at −20°C.

The quantities given in this recipe are for 0.5 g leaf tissue. Generally 0.5 g material will yield no more than 1 to 4 × 10^5 viable protoplasts. Depending on the number of samples to be inoculated, the amount of leaf material used can be scaled accordingly.

Enzyme solution for tobacco (N. benthamiana)

In a sterile beaker, weigh 125 mg cellulase (1% final concentration; commercially available from Calbiochem as Cellulysin, 100,000 U), 6.25 mg BSA (Sigma), 6.25 mg macerozyme (0.5% final concentration; Yakult Pharmaceutical), and 125 mg Driselase (1% final concentration; Sigma). Add 12.5 ml of 0.55 M mannitol, pH 5.9 (see recipe), and stir at room temperature until the contents are dissolved. Adjust the pH of the solution to 5.9 with 0.1 M KOH and sterilize the solution by filtration through a 0.2-μm pore size filter into a sterile petri dish.

The quantities given above are for 1 g leaf tissue. Depending on the number of samples to be inoculated, the amount of leaf material used can be scaled accordingly.

KK solution, 1000×

Dissolve 2.72 g KH_2PO_4 and 10.11 g KNO_3 in 100 ml sterile water and adjust the pH to 6.5 with 1 N KOH. Sterilize the solution by autoclaving. Store up to 1 to 3 months at room temperature.

Mannitol, 0.55 M (pH 5.9)

Weigh 100.2 g mannitol and dissolve in 800 ml double-distilled water. After adjusting to pH 5.9 with 0.1 M KOH, bring the volume to 1 liter. Sterilize the solution by autoclaving and store up to 1 to 3 months at room temperature.

Polyethylene glycol (PEG), 40%

Weigh 40 g PEG-1540 and add 50 ml sterile distilled water, 10 ml of 5% morpholine ethanesulfonic acid (MES) buffer (pH 5.9), and 1 ml of 0.3 M $CaCl_2$. Briefly (5- to 10-sec intervals) warm the mixture in a microwave oven and stir at room temperature until all PEG is completely dissolved. The pH of this solution will be around 5.8; if necessary, adjust the pH with 0.1 M KOH. Finally, adjust the volume to 100 ml with sterile water. Filter the PEG solution through 0.45-μm pore size filter and then through 0.2-μm pore size filter. Store up to 2 to 3 weeks at room temperature.

CAUTION: *Avoid boiling of PEG while heating in microwave oven. This will increase the toxicity of PEG. Because PEG solutions turn acidic with age, it is not advisable to use solutions stored beyond 3 weeks at room temperature.*

Sucrose, 0.55 M

Weigh 18.82 g sucrose and dissolve in 80 ml sterile distilled water. Adjust volume to 100 ml and sterilize by filtration. Store up to 1 to 3 months at 4°C.

COMMENTARY

Background Information

The protoplast system is a powerful tool for studying viral replication. Generally, protoplasts are easy to infect with either purified virus or viral RNA, or in vitro–synthesized transcripts. Detection of viral progeny in whole plants requires efficient virus-controlled cell-to-cell spread. Therefore, whole plant infections are not ideal for analyzing replication characteristics of viral mutants defective in replication and/or cell-to-cell movement. The protoplast system circumvents this limitation. Furthermore, synchronous infection and uniform sampling are well suited for the analysis of temporally regulated accumulation of virus-related gene products. Most importantly, the protoplast system is ideal for phloem-limited viruses because these viruses are not mechanically transmissible. Although preparation and infection of protoplast from a variety of tobacco species is widely used, conditions for synchronous infection of protoplast, prepared from a wide range of host plants such as cowpea (Hibi et al., 1975), cucumber (Maule et al., 1980), turnip (Howell and Hull, 1978), and tomato (Motoyoshi and Oshima, 1979) have also been established for many plant viruses. An alternative approach to the preparation of stationary protoplasts from the mesophyll layer of intact leaves is the preparation of single cells from callus tissue (Hall, 1991). When callus tissue is introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a cell suspension culture (*UNIT 16D.1*). Thus, cell suspension offers advantages when rapid cell division or many cell generations are required or when uniform treatment application is required.

Critical Parameters

The kind of leaf material used will have a profound influence on the quality of protoplasts and subsequent infection to a given inoculum source. Therefore, the following criteria will help in isolating protoplasts that yield consistent results: (1) grow plants under optimum light and humidity conditions; (2) avoid using leaves that are dry, yellow, or damaged; (3) while slicing leaf material, always use a sharp blade and do not exert pressure minimizing release of leaf extract; (4) during initial stages of protoplast preparations, be gentle and

Table 16D.2.1 Troubleshooting Guide for Isolation of Protoplasts

Problem	Possible Cause	Solution
No digestion of leaf slices	Enzyme quality is poor	Use freshly prepared enzyme solution with an enzyme having >10,000 U/g; also check incubation temperature
No dark interface containing protoplasts	Bad leaf material	Select healthy green leaves; avoid using dry or damaged leaves
	Centrifugation	Optimize centrifugation speed
Not many viable protoplasts	Incubation temperature is too high	Try to maintain optimal temperature
	Rough handling procedures	Be gentle while handling protoplasts; avoid vigorous mixing or shaking
Poor infectivity	Inoculation too slow	After removing protoplasts from the enzyme solution, inoculation must be completed within 10 min
	Quality of protoplasts	Verify that majority of protoplasts are viable (appearing as bright fluorescent green) after treating with fluorescein diacetate
	Quality of RNA	Verify the quality of RNA gel electrophoresis
	PEG	PEG is known to be toxic; avoid over heating or autoclaving while making the PEG solution
	Culture medium	Verify that all solutions used to prepare culture medium are properly sterilized

avoid vigorous shaking or vortexing practices; (5) presence of dead cells, appearing red when stained with fluoresceine diacetate constitute a major source of ribonuclease that rapidly degrades viral RNA or transcripts resulting in poor infectivity. Therefore exercise caution to minimize the number of dead cells by gentle handling and other suggested precautions; (6) avoid adding buffer solutions directly to protoplast preparations to minimize damage; always dispense buffer to the side of the tube; (7) always use pipet tips or pipets having wider openings; (8) to avoid sticking of protoplasts to the glass, always use sterile pipets made of polypropylene; (9) to avoid contamination, it is advisable to perform all operations under a sterile hood or create a sterile environment by working close to a flame. The obvious negative and positive should be mock (infect with water only) and wild-type viral RNA, respectively. To verify whether transfection has worked or not, viral progeny (either coat protein or replicated RNA) in positive control should be verified.

Troubleshooting

Commonly encountered problems and their solutions are found in Table 16D.2.1.

Anticipated Results

From the specified isolation procedure, generally 1 g leaf tissue should yield ~1 to 2×10^6 protoplasts. Infection of high quality and viable protoplasts generally results in a high level of infectivity. This would offer advantage of characterizing viral progeny at various time post-inoculation with ease.

Time Considerations

Incubation of protoplasts in the enzyme solution requires 2.5 to 3 hr. Alternatively, overnight enzyme digestion at room temperature can be performed by reducing the concentration of the enzyme by 5- to 10-folds. This however should be normalized depending on the experimental conditions. The time required for isolation and inoculation with RNA samples require a nonstop operation of 2 hr. So, the entire process takes about 5 hr.

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Preparation and Electroporation of Oat Protoplasts from Cell Suspension Culture

Plant protoplasts provide a rapid means of introducing nucleic acids into cells. Thus, the use of protoplasts is a valuable tool to investigate gene expression control or virus replication in vivo, in a very short time frame (<48 hr).

In the plant, each cell is surrounded by a wall consisting of a thick matrix of polysaccharides, proteins, and polyphenolic compounds. These walls are impervious to macromolecules. To allow rapid uptake of nucleic acids, the walls must be removed using cellulases. This yields delicate, spherical cells (protoplasts) held intact only by the plasma membrane. Protoplasts are free floating, and do not attach to surfaces like most cultured animal cells. They are highly vulnerable to changes in osmotic pressure, so the medium in which they are obtained must be iso-osmotic with the cell interior. Protoplasts live only a few days. Upon longer incubation they either die or regenerate cell walls. In fact, whole plants can be regenerated from some protoplast types. Therefore, protoplasts must be prepared fresh for each experiment.

Protoplasts can be derived from whole plants, stationary undifferentiated tissue (callus cultures; refer to *UNIT 16D.1*), or from undifferentiated tissue grown in solution (suspension culture), in which it is suspended by constant shaking. The authors find that large quantities of uniform protoplasts can be obtained more rapidly and reliably from suspension cultures than from plants. This unit describes a simple, rapid, and reliable method to generate large amounts of protoplasts from an oat (*Avena sativa*) suspension culture. Basic Protocol 1 is divided into sections that describe: (1) isolation of protoplasts by digestion with cellulases, (2) preparation of protoplasts for electroporation, (3) electroporation of protoplasts with RNA to assay gene expression or RNA replication, and (4) culture of cells post-electroporation and collection for reporter gene assays or RNA extraction. A Support Protocol is provided for subculturing oat suspension cultures.

Oat protoplasts have been used commonly for viruses that infect monocots. However, it is noteworthy that the host range of viruses is greatly expanded at the protoplast level. Basic Protocol 2 describes methods for maintaining and storing the oat suspension culture cells on solid medium. More details on electroporation are described in *UNIT 16D.4*. Methods for extraction of RNA from protoplasts are described in *UNIT 16E.2*.

PREPARATION AND ELECTROPORATION OF OAT PROTOPLASTS FROM CELL SUSPENSION CULTURE

BASIC PROTOCOL 1

The oat cell suspension culture system described herein allows one to follow the activity of both stably and transiently transformed cells. Plant protoplasts are prepared by overnight digestion of plant cell walls, followed by washing of the cells. An electrical pulse is applied to cells in the presence of DNA or RNA, and the cells are transferred to growth medium. This unit describes the steps for preparation of cells through their harvest. Basic Protocol 1 outlines digestion and handling of protoplasts for viral replication studies. Minor differences in the handling of the cells for reporter gene expression assays are described at specific steps in the protocol and discussed in the Commentary. Methods for generating and maintaining suspension cultures are described in the Support Protocol.

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Materials

7-day-old oat suspension culture (*Avena sativa* cv. Stout; see Support Protocol)
Oat protoplast enzyme solution (see recipe)
Artificial sea water (ASW)/0.6 M mannitol (1:1 ratio; see recipe)
Oat protoplast electroporation buffer (see recipe)
1 M spermidine: filter sterilize using a 0.2-μm filter and divide into 1-ml aliquots (store up to 1 year at –20°C)
Murashige and Skoog (MS) medium with 0.4 M mannitol (see recipe)
RNA sample

50-ml conical centrifuge tubes
145 × 20– *or* 100 × 15–mm sterile plastic petri dishes
Gyrotory shaker (e.g., New Brunswick Scientific Model G2)
Motorized pipet filler/dispenser (e.g., Eppendorf EasyPet)
10-ml serological pipets
Centrifuge (e.g., Sorvall RC-5C Plus) with SH-3000 or comparable swinging-bucket rotor with inserts for 15-ml conical tubes
4-mm electroporation cuvettes
1-ml wide-bore polypropylene pipet tips
6-well cell culture plates
Electroporator with square wave pulse (e.g., BTX T820 ElectroSquare Porator or Bio-Rad GenePulser Xcell with CE module)

Additional reagents and equipment for counting cells with a hemacytometer (*APPENDIX 4A*)

NOTE: All work should be carried out under sterile conditions. Unless otherwise noted, all solutions should be autoclaved and then stored at 4°C. Equipment which will come in direct contact with the protoplasts should be sterilized before use. Keep solutions sterile at all times.

Digest oat cell suspension culture

1. Transfer a 7-day-old oat cell suspension subculture (~40 to 50 ml volume), into a sterile 50-ml conical tube and let cells settle in the tube for 5 min.

   Oat cell suspension culture is available from the authors (wamiller@iastate.edu).

   For best results, use cell suspension cultures that have been subcultured within the previous 5 to 7 days. This assures (1) an adequate amount of healthy cell growth (expect to obtain ~10 ml packed cells) and (2) efficient cell wall digestion (the thick cell walls of older cultures greatly reduce digestion efficiency; see Critical Parameters).

   A satisfactory suspension culture consists of finely growing cells with some small clumps that are <1-mm in diameter.

2. Remove supernatant and gently resuspend cells in 20 ml freshly prepared oat protoplast enzyme solution.

   Always use a freshly prepared enzyme solution.

   Protoplasts are very fragile to mechanical manipulation. Resuspend cells by gently inverting the tube or by pipetting up and down slowly.

   50 ml of the enzyme solution (also see step 3) is sufficient for the digestion of up to 10 ml packed cells. If the amount of packed cells exceeds 10 ml, adjust volume of cells to 10 ml or increase the total amount of enzyme solution to maintain a 5:1 ratio of enzyme solution to packed cells.

3. Transfer cell/enzyme mix into a large petri dish (145 × 20–mm) and add the remaining 30 ml enzyme solution.

   If using a small petri dish (100 × 15–mm), transfer the cell/enzyme mix into three sterile petri dishes and divide the remaining enzyme solution into each petri dish in equal aliquots. Final volume in each dish should not exceed 20 ml.

4. Seal the petri dish with Parafilm and cover it with aluminum foil to protect it from light.

   At this point, the cell/enzyme suspension has a granulated appearance.

5. Shake cell/enzyme suspension at 42 rpm on a gyrotory shaker overnight at room temperature.

   To avoid overdigestion, do not incubate longer than 16 to 18 hr. If digestion time exceeds 16 to 18 hr, cells will become too fragile and will not survive downstream treatments.

   After digestion, cell clumps should no longer be visible with the naked eye, giving the digest a smooth homogenous appearance.

6. Inspect a drop of digested cells under a light microscope to observe isolated protoplasts.

   Freely floating protoplasts should have a uniform spherical shape.

   Proceed to preparing cells for electroporation only if a large number of healthy protoplasts are observed. A digest that has a few partially digested clumps may be used for replication studies but is not ideal for gene expression assays.

   Refer to UNIT 2A.1 for information on light microscopy.

Wash protoplasts to remove debris

It is recommended that protoplasts be inspected under a light microscope after each wash step. Handle the protoplasts very carefully during the following steps to minimize cell rupture. A motorized pipet filler/dispenser (e.g., Eppendorf EasyPet) works best for collecting protoplasts and dispensing medium. Protoplasts and all added solution should be released very slowly against the wall of the tube during the wash step. Avoid introducing air bubbles.

7. Carefully collect the protoplasts with a sterile 10-ml serological pipet and gently dispense the cells into two sterile 50-ml conical tubes.

8. Harvest protoplasts by centrifuging 5 min at 100 × *g* (700 rpm in a Sorvall SH-3000 swinging bucket rotor), 4°C.

9. Remove supernatant by aspiration. In each tube, resuspend protoplasts in 10 ml ASW/0.6 M mannitol.

10. Wash cells by centrifuging as in step 8.

    Healthy protoplasts should give a yellow pellet in a clear supernatant.

11. Aspirate off supernatant and resuspend cells again in 10 ml of ASW/0.6 M mannitol.

12. Repeat wash by centrifuging as in step 8.

    If cell debris is still observed under the light microscope, one additional wash is recommended. Protoplasts are very sensitive to osmotic pressure. Osmotic differences due to improperly prepared buffer can contribute to the constant presence of broken cells. Such a protoplast preparation should not be used for electroporation.

Prepare the cells for electroporation

13. Remove supernatant by aspiration. Use 10 ml oat protoplast electroporation buffer containing 0.2 mM spermidine to resuspend and combine protoplasts into one tube.

    Add spermidine (2 μl of 1 M stock per 10 ml of oat protoplast electroporation buffer) to the electroporation buffer prior to use.

14. Repeat wash by centrifuging as in step 8.

15. Remove supernatant. Resuspend the protoplasts in electroporation buffer containing 0.2 mM spermidine to a final density of $\sim 6 \times 10^6$ cells/ml/sample. Use a hemacytometer (*APPENDIX 4A*) to count cells.

    A volume of 10 ml packed cells yields $\sim 60 \times 10^6$ protoplasts. The final volume of electroporation buffer used to resuspend protoplasts varies with the type of assay. For replication assays, resuspend protoplasts in up to 10 ml electroporation buffer (and use 1 ml suspended protoplast per sample). For transient expression, protoplasts can be resuspended in up to 25 ml of buffer for $\sim 2.5 \times 10^6$ cells/ml/sample.

16. Inspect a drop of the protoplast suspension under a light microscope. Proceed to step 17 only if protoplast suspension is largely free of cell debris.

Add RNA and electroporate protoplasts

17. Using 1-ml wide-bore pipet tips, divide the protoplast suspension into 1-ml aliquots and transfer the aliquots into each sterile 4-mm electroporation cuvette. Place on ice for a minimum of 5 min (up to 30 min).

18. According to the total number of samples, aliquot 5 ml MS medium with 0.4 M mannitol into the appropriate number of wells on a 6-well plate.

    This plate will be needed in the final step of electroporation (step 22) and can be prepared ahead of time at any of the previous steps.

19. When ready to electroporate, add RNA to the cells and mix by gently inverting the cuvette.

    To minimize degradation of the RNA sample, electroporate sample as soon as RNA is added.

    The amount of RNA to be added depends on the type of assay. For replication studies, it is recommended that as much as 10 μg RNA per sample be used. Higher amounts can be used for virion purification. For transient expression, 1 μg or less of RNA is sufficient.

    Efficient co-inoculation of more than one RNA simultaneously can be performed simply by mixing more than one RNA sample prior to electroporation. Alternatively, a two-step electroporation approach can be used if RNAs are to be introduced into the same cells at different times (see Commentary).

20. After adding RNA to the cells, place cuvette immediately into the holder of the electroporator device and electroporate cells at a defined voltage and capacitance setting, depending on the electroporator available.

    If using the BTX Electro Square Porator T820, the optimal setting for electroporating oat protoplasts is one pulse for 6 msec at 300 V.

    If using the Bio-Rad GenePulser Xcell, the optimal setting is 300 V/500 μF.

    Both electroporators perform with similar efficiencies at the above settings.

21. After electroporation, place cuvette back on ice until the remaining samples have been electroporated (step 18).

22. Under a sterile hood, transfer each sample into one well on the 6-well cell culture plate containing 5 ml MS medium with 0.4 M mannitol.

For maximum recovery of cells, remove 1 ml MS medium from the well prior to transferring the electroporated sample into the well. Use this 1-ml aliquot of medium to rinse the cuvette.

White threads of debris resulting from cell lysis after electroporation are commonly observed in the sample.

For transient expression assays, it is not critical to work under sterile conditions. The sample can be poured into the MS medium immediately after electroporation.

23. Seal the plates with Parafilm and cover with aluminum foil. Incubate the plates 24 to 48 hr at room temperature.

The quality of the protoplast preparation determines the viability of protoplasts for at least 48 hr after electroporation.

For transient expression, cells can be harvested 30 min post-transfection. Optimal expression is observed after 4 hr, although individual experiments may require some optimization.

Harvest cells

24. Inspect electroporated samples in culture plates under a light microscope.

A mixture of intact protoplasts and cell debris clumps is commonly observed.

25. Carefully collect cells from the culture plate and gently transfer each sample into a separate 15-ml centrifuge tube.

26. Pellet cells by centrifuging as in step 8.

For virion purification, centrifuge cells 5 min at 700 × g, 4°C.

27. Aspirate off the supernatant.

For replication assays, it is recommended to perform at least one additional wash with 1:1 ASW/0.6 M mannitol to remove cell debris before proceeding to isolation of total RNA.

At this stage, the cell pellet can be stored in an appropriate buffer at −80°C until needed for downstream applications (Basic Protocol 2). However, this is not recommended for recovery of RNA for replication analysis. It is best to isolate total RNA from a fresh sample.

SUBCULTURE OAT SUSPENSION CULTURE

SUPPORT PROTOCOL

It is important to maintain a healthy cell suspension culture. This is accomplished through weekly transfers of subcultures into fresh MS medium in a 1:4 ratio of suspension:medium. Cell cultures can then be used indefinitely if transferred to fresh medium on a weekly basis. Transfers should be carried out under aseptic conditions to avoid contamination. It is recommended to work under a laminar-flow hood.

***Additional Materials** (also see Basic Protocol 1)*

Oat suspension culture (available from the authors)
40 ml Murashige and Skoog (MS) medium (see recipe) in 150-ml Erlenmeyer flask sealed with cotton and wrapped in aluminum foil

Platform shaker (e.g., New Brunswick Scientific Classic Series C24 incubator/shaker)

1. To subculture cells, transfer 10 ml of a 7-day-old oat suspension culture into 40 ml fresh MS medium in a 150-ml Erlenmeyer flask.

2. Seal the flask by plugging the top with sterile cotton wrapped in aluminum foil.

3. Incubate at 20° to 25°C with shaking between 160 to 220 rpm on a platform shaker.

4. Subculture cells every 7 days.

Oat suspension cultures can be used for protoplast preparation 3 days after subculturing. To obtain the appropriate cell growth/volume for digestion in such a short period of time, transfer 25 ml of 7-day-old culture into 40 ml of fresh MS medium.

BASIC PROTOCOL 2

LONG-TERM STORAGE AND SHIPPING OF CELL CULTURE

For shipping cells, it is recommended that they be supported on solid medium. Prepare fresh Murashige and Skoog medium containing a solidifying agent such as phytagel or phytablend (see recipe for Murashige and Skoog medium containing phytagel). Under sterile conditions, pour cooled medium into sterile 50-ml conical tubes or any sterile test tube. Tilt the tubes so that the medium has an angled surface after it solidifies. Cut several pieces of oval-shaped Whatman 3MM paper disks to a diameter that allows them to fit inside the 50-ml conical tube. Wrap precut disks in foil and sterilize by autoclaving. Once the medium solidifies, place the sterile oval paper disk onto the angled medium surface using sterile forceps. While optional, the paper disk helps to hold cells in place and prevents them from sliding during transport.

Collect cells as described in Basic Protocol 1, step 8, by centrifuging and aspirating off liquid medium. Using a sterile spatula, scoop a sample of cells from the pellet and carefully place the sample onto the paper disk in the 50-ml conical tube or directly on the medium. Gently spread cells over the paper disk. Replace the cap and seal with Parafilm. Cells are now ready for shipping. The callus cultures that grow on this medium are stable for several weeks at room temperature. To re-establish a suspension culture, scrape cells from the paper disk and transfer them into 40 ml fresh MS medium. Alternatively, remove the entire paper disk and place it into the flask containing the MS medium. Allow the cell suspension to grow and proceed with subculturing into fresh medium.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Artificial sea water (ASW)/0.6 M mannitol (1:1 ratio)

Prepare artificial sea water:
311 mM NaCl (18.18 g/liter)
18.8 mM $MgSO_4$ (2.26 g/liter)
6.8 mM $CaCl_2$ (1.0 g/liter $CaCl_2 \cdot 2H_2O$)
10 mM MES (2.13 g/liter)
6.9 mM KCl (0.514 g/liter)
16.7 mM $MgCl_2$ (3.39 g/liter $MgCl_2 \cdot 6H_2O$)
1.75 mM $NaHCO_3$ (0.148 g/liter)
Adjust volume to 1 liter with H_2O
Adjust to pH 6.0 using NaOH

Prepare 1 liter of 0.6 M mannitol and add to ASW solution at a 1:1 ratio. Autoclave for 25 min and cool to room temperature. Store up to 6 months at 4°C.

MS vitamin solution, 100×

For 1000 ml:
50 mg pyridoxine·HCl
50 mg thiamine·HCl
50 mg nicotinic acid
10 g myoinositol
Adjust volume to 1 liter with H_2O
Store up to 1 year at –20°C

Murashige and Skoog (MS) medium

1 packet of MS plant salt mixture *without* agar (MP Biomedicals, no. 2633020)
87.6 mM sucrose (30 g/liter)
10 ml of 100× MS vitamin solution (see recipe)
Adjust volume to 1 liter with H_2O
Adjust pH to 5.7 using NaOH
Autoclave
Cool to room temperature and store up to 6 months at 4°C

Murashige and Skoog medium with 0.4 M mannitol

1 packet of MS plant salt mixture *without* agar (MP Biomedicals, no. 2633020)
0.4 M mannitol (72.8 g/liter)
87.6 mM sucrose (30 g/liter)
10 ml 100× MS vitamin solution (see recipe)
Adjust volume to 1 liter with H_2O
Adjust pH to 5.7 using NaOH
Autoclave
Cool to room temperature and store up to 6 months at 4°C

Murashige and Skoog medium with phytagel

1 packet of MS plant salt mixture *without* agar (MP Biomedicals, no. 2633020)
87.6 mM sucrose (30 g/liter)
10 ml 100× MS vitamin solution (see recipe)
2.5 g phytagel (Sigma no. P 8169)
Adjust volume to 1 liter with H_2O
Adjust pH to 5.7 using NaOH
Autoclave for 25 min
Store up to 6 at 4°C

Other gelling agents such as agar, phytagar, etc., may also be used.

Oat protoplast electroporation buffer

10 mg KH_2PO_4
57.5 mg $Na_2HPO_4 \cdot 7H_2O$
3.75 g NaCl (130 mM final)
18.2 g mannitol (0.2 M final)
Adjust the volume to 495 ml with ddH_2O
Adjust the pH to 7.2 using NaOH
Cool solution to room temperature
Add 4 ml of 400 mM filter-sterilized $CaCl_2$
Autoclave, cool to room temperature, and store up to 6 months at 4°C
Just before use add spermidine (See Basic Protocol 1)

Oat protoplast enzyme solution

Prepare the following in ASW/0.6 M mannitol (see recipe):
0.1% (w/v) Driselase (Sigma)
0.175% (w/v) Cellulase (Onozuka RS, Yakult Pharmaceuticals)
0.8% (w/v) hemicellulase (Sigma)
Stir for at least 30 min to allow all the enzymes to solubilize
Adjust the pH to 5.6 to 5.7 using NaOH
Filter through a 0.2-μm filter
Prepare fresh

continued

50 ml of the oat protoplast enzyme solution is sufficient for the digestion of up to 10 ml of packed cells.

Avoid vigorous stirring of the enzyme solution to prevent excessive foaming, which can result in protein denaturation.

COMMENTARY

Background Information

Plant protoplasts are valuable tools for the study of plant gene expression and plant virus replication. Transient expression of genes in protoplasts provides a rapid means of measuring expression of cloned genes in a matter of hours. In contrast, stable transformation of plants takes weeks, and shows considerable plant-to-plant variation. Each transgenic plant is derived from a single transformation event, whereas a typical protoplast assay represents the average of thousands of separate transformation events. Protoplasts allow studies of gene expression directly from RNA, avoiding the need for the nuclear transcription, processing, and export that takes place in DNA-mediated transformation. This is valuable for direct assessment of post-transcriptional control of gene expression at the levels of mRNA stability and translation.

Protoplasts can be generated from whole plant parts, such as leaf mesophyll cells, or they can be generated from undifferentiated suspension culture cells. The authors find suspension cells to be more convenient for production of protoplasts. Suspension cells are immortal, more uniform than plant-derived cells that come in many types, and the walls are less refractory to cellulolytic degradation than cells from whole plants. Different cell cultures have different properties, and of the different kinds that were tested, oat suspension cultures proved to be easy to maintain over many years, and produced reliable and high yields of protoplasts. However, other cell cultures such as tobacco BY2 cells and maize BMS cultures are widely used.

The first isolation of protoplasts was from tomato fruit, and the cells were inoculated with tobacco mosaic virus particles (Cocking, 1960; Cocking and Pojnar, 1969). Subsequent generalization of the technique for different starting materials (reviewed in Sander and Mertes, 1984), including cell suspension cultures, opened new avenues to study viruses at the cellular level. The use of this technique in viral research offers unique advantages. First, the protoplast system allows one to synchronously infect a large number of cells with one or more viral RNAs. Generally, a much higher percentage of cells is infected than in whole plant infections. Secondly, it provides an experimental system for transient assays that circumvents some limitations of working in whole plants such as tissue specificity, or a requirement for vector transmission. Also, host range of viruses is greatly expended at the protoplast level. Thirdly, such a system helps to distinguish events that affect viral accumulation at the cellular level from events that affect cell-to-cell and/or systemic movement and infection of plants.

The use of cell suspension culture for protoplast isolation instead of plant tissues offers several advantages: (1) reproducibility of protoplast preparations with constant yield as the quality of cell suspension culture is less variable, (2) greater viability of the protoplasts, (3) reduced labor, and (4) more sterile conditions and thus less contamination by fungi or bacteria (reviewed in Sander and Mertes, 1984).

Oat protoplasts have been used for studying viruses such as barley yellow dwarf luteovirus (BYDV), barley stripe hordeivirus, and brome mosaic virus (Furusawa and Okuno, 1978; Barnett et al., 1981; Zheng and Edwards, 1990). The protocol described herein was developed in the authors' laboratory using oat cell suspension culture for studying BYDV and is based largely on the work of Dinesh-Kumar et al. (1992), whose protocols were derived from Young et al. (1989). The oat cell suspension culture (*Avena sativa* cv. Stout) used originated from Howard Rines, University of Minnesota (Dinesh-Kumar et al., 1992). Oat cell suspension culture can be generated from callus culture. Please refer to Birsin et al., (2001) and Chen et al., (1994) for further details.

Viral RNA or DNA can be readily introduced into cells after the removal of the cell wall. Several methods including use of electroporation, polyethylene glycol (PEG), or polyanions such as poly-L-ornithine (Owens, 1979; Samac et al., 1983; Young et al., 1989) can be used to introduce macromolecules into protoplasts. Electroporation and PEG, when optimized, can give comparable efficiencies of transformation. PEG is less damaging to cells and it is inexpensive because it does not

require any special equipment. Electroporation, as described in this unit, is generally the most efficient, rapid, and reproducible technique to introduce nucleic acids into plant and mammalian cells (See *UNIT 16D.4*). It involves passing an electric pulse through a sample of cells which momentarily opens pores within the plasma membrane to allow macromolecules to enter (or exit) the cell (Fromm et al., 1985, 1987). Although it is a harsh procedure, a sufficient number of cells recover to be used for downstream applications. Viability and successful inoculation of the protoplasts by electroporation depend on the nucleic acid concentration, the ionic strength of the buffer, the voltage, and the duration of the electrical pulse applied (see Critical Parameters; Fromm et al., 1985, 1987). The same conditions can be used for electroporation of RNA or DNA.

If appropriate care is taken, it is possible to electroporate the same batch of protoplasts more than once, for example to introduce different macromolecules at 24 hr intervals (Shen and Miller, 2004). To do this, critical changes in the procedure include the use of higher concentrations of protoplasts per sample and a decrease of voltage during electroporation from 300 to 280 V (or lower) to minimize cell death. Twenty-four hours after the first electroporation, cells are harvested, washed, and resuspended in electroporation buffer. After addition of the second RNA (or DNA) sample, the cells are electroporated at 280 V. Satisfactory cell recovery was observed under such conditions.

Critical Parameters

The use of cell suspension cultures instead of plants as starting material for making protoplasts, offers the advantage of having a source material that is already aseptic. For the protocols described above, it is important that these sterile conditions be maintained throughout the course of the experiment. This is especially true for replication assays, which require long incubation periods in sucrose-containing culture medium that is an ideal breeding ground for fungal and bacterial contaminants. All media should be prepared, autoclaved, and opened only under a laminar-flow hood.

Before preparing protoplasts from oat cell suspension cultures, it is vitally important that the protoplasts be generated from a cell suspension culture that has been maintained properly. The age of the cell suspension culture is critical when using it as a source for isolating protoplasts. Well-maintained cultures ensure reproducible results. To minimize the presence of old and dying cells in the culture, oat cell suspension cultures should be subcultured weekly. For preparation of protoplasts, it is recommended to use suspension cultures 7 days after subculturing. If enough cells are present, younger subcultures can be used. The yield of protoplasts is reduced greatly if older cultures are used. The authors found that cultures older than 7 to 8 days do not digest properly.

Another parameter critical for the success of performing an inoculation assay is the quality of protoplasts generated from the overnight enzyme digestion. This is probably the most important factor for any experiment requiring the use of protoplasts. Using the proper buffer along with the appropriate concentration of enzyme is crucial to the success of the digest. The authors recommend the RS cellulase from Onozuka, Yakult. For oat cells, the enzymes are dissolved in artificial sea water (ASW)/0.6 M mannitol with the pH adjusted to 5.6 to 5.7. Each enzyme lot varies slightly and differs in activity, so it is important to compare each new batch of enzyme with the older lot. If necessary, the amount of enzyme added may be adjusted to optimize the digestion.

Medium quality is another important critical parameter that should be addressed when preparing and inoculating protoplasts. Once protoplasts are isolated, maintaining them in the appropriate medium ensures that the cells survive and do not rupture during downstream applications. Because protoplasts lack a cell wall, their plasma membranes are extremely sensitive to osmotic differences introduced by the medium in which they are maintained. Therefore, correct osmotic conditions must be maintained in the medium throughout the entire procedure. Osmotic changes introduced through improperly prepared medium can interfere greatly with the success of the experiment. For example, poor quality protoplasts enhance RNA degradation and result in poor quality northern blots from extracted RNA. Thus, inert substances such as mannitol are added to the medium to equalize the osmotic pressure inside and outside of the cell. Protoplasts should be handled carefully due to their fragile nature and should be siphoned and dispensed slowly during transfers. It is recommended to slowly release the protoplasts and added medium against the wall of the tube during pipetting. Avoid sudden jarring or violent shaking of the sample.

Table 16D.3.1 Troubleshooting for Electroporation of Oat Protoplasts

Problem	Possible cause(s)	Solution(s)
Poor growth of suspension cells	Poor quality medium	Prepare fresh medium
	Improper growth temperature	Set growth temperature between 20° and 25°C
	Cells not shaken properly	Set platform shaker to 160-220 rpm
Contamination of suspension culture/buffers	Medium/buffer opened in nonsterile conditions	Work under sterile conditions; always open medium/buffers under laminar-flow hood
	Poorly autoclaved medium/ buffer	Autoclave solutions properly and store them at 4°C
Poor digestion of suspension culture	Cell suspension culture was too old	Make sure that the cells are 7 days old or younger (relative to the day of previous subculture)
	Digestion time too short	Increase digestion time
	Improper ratio of enzyme solution/packed cells	Increase the amount of enzyme solution accordingly (50 ml enzyme solution for ~10 ml packed cells)
	Poor-quality enzyme solution	Use only freshly prepared enzyme solution. Verify the pH of the enzyme solution. It is recommended to use cellulase from Onuzaka, RS, Yakult Pharmaceuticals for best results.
Too many cells burst during protoplast preparation	Poor-quality medium/buffer	Prepare medium/buffer properly
	Harsh handling of the cells	Avoid sudden jarring, violent shaking, rapid pipetting of cells; dispense suspended protoplasts against the wall of the tube
Low efficiency of transformation	Poor digestion of the cells	Increase digestion time
	Not enough RNA inoculum	Increase RNA amount, check integrity of the RNA used
	Degraded RNA inoculum	Minimize exposure of the RNA to RNAses; keep samples on ice; once inoculum is added to the cells, electroporate immediately
Poor viability of the cells after electroporation	Poor quality protoplast preparation	Electroporate only good preparation of protoplast
	Overdigestion of the cells	Shorten digestion time
	Wrong voltage setting	Excessive voltage will increase cell mortality
	Poor-quality electroporation buffer	Prepare new electroporation buffer
	Poor quality of culture medium	Prepare new culture medium

Once a healthy batch of protoplasts has been isolated and washed, it is ready for electroporation. It is necessary to resuspend protoplasts in the appropriate electroporation buffer and wash at least once in the buffer before electroporating the sample. Buffers used for electroporation should be made with double-deionized water to ensure that that the medium is free of any contaminating electrolytes that can affect the electric conductivity of the buffer (Hibi, 1989). The length of the electric pulse and the voltage setting vary for different cell types and are critical parameters for the success of the experiment. Too long a pulse at a given voltage can damage protoplasts due to the dramatic effects on the cell wall (Fromm et al., 1987). For oat protoplasts, good results have consistently been obtained using the BTX Electro Square Porator T820 with one 6 msec pulse at 300 V, or using the Bio-Rad GenePulser Xcell (with CE module) at 300 V/500 μF. All samples are electroporated in sterile 4-mm-gapped electroporation cuvettes. If electroporating protoplasts from cells other than oat, electroporation settings may have to be optimized before a successful transfection can be achieved. Refer to Fromm et al., (1985 and 1987) and Hibi (1989) for more details on how to optimize conditions for successful transfections of protoplasts.

Broken cells within a sample can release nucleases that can degrade the nucleic acid sample. Therefore, samples should always be kept on ice during the electroporation procedure to reduce their activity and electroporated immediately after adding RNA to minimize exposure of the RNA to RNases (Hibi, 1989). The lower temperature also allows the pores that were generated in the membrane from the DC pulse to remain open longer to allow for a more efficient uptake of the nucleic acid (Hibi, 1989). For best results, use freshly prepared transcripts for inoculation experiments. However, properly stored samples can be used as inoculum as long as the integrity of the RNA is maintained and monitored. Routinely, commercial kits are used to prepare transcribed RNA for the inoculation assay experiments and good results have been achieved with Ambion's Megascript kits. It is recommended not to pause longer than the times indicated at any stage of the protoplast preparation procedure. All steps should be followed to completion to ensure a successful and reproducible experiment.

Troubleshooting

Table 16D.3.1 provides some commonly encountered problems as well as their solutions and possible causes.

Anticipated Results

Seven-day-old suspension cultures should appear as fine clumps. Inspection of the cells under a light microscope following digestion should show that the majority of cells are spherical, freely floating individual cells that are uniform in size. The washing procedure should remove most of the debris. Following electroporation, viability of the protoplasts is high (~80%), with a small amount of debris resulting from cell lysis observed in the medium. At 24 to 48 hr post-electroporation, there will be a mixture of intact cells and clumps of debris visible under the light microscope.

From one 50-ml culture of oat suspension culture, it is possible to electroporate up to 10 samples for viral replication studies, and up to 25 samples for transient expression assays. The washing and electroporation steps are carried out routinely with one to two 50-ml cultures at a time.

Time Considerations

The process of digestion of plant cells requires 30 min to 1 hr for set up, followed by overnight digestion. Washing and electroporation takes <2 hr, depending on the number of samples handled. For translation assays, 4 hr incubation of electroporated cells is optimal, although reporter gene activity is above background within 15 to 30 min following electroporation. To study viral replication, a 24 to 48 hr incubation is usually allowed before harvesting of cells, but this may vary depending on the replication of the virus.

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Transfecting Protoplasts by Electroporation to Study Viroid Replication

This unit presents protocols for inoculating protoplasts prepared from tobacco (*Nicotiana tabacum*) and the related species *Nicotiana benthamiana* with viroid RNA by electroporation in order to study viroid replication. The unit covers maintenance of cultured cells, preparation of protoplasts, electroporation parameters, and culturing of electroporated protoplasts. It also highlights critical aspects of these steps and discusses common problems and their solutions.

TRANSFECTING PROTOPLASTS WITH VIROIDS BY ELECTROPORATION

BASIC PROTOCOL

This protocol describes materials as well as procedural and technical details for transfecting protoplasts with viroid RNA by electroporation. The protocol has been developed based on experiments that characterize replication of potato spindle tuber viroid (PSTVd). Although the procedures are straightforward, it is important to stress that a great deal of patience and practice may be required to master the techniques in order to succeed in a transfection experiment. Each step should be performed with gentle treatment of cells. The vendors for specific chemicals and equipment used by the authors are listed. Obviously, many of the chemicals and equipment are available from multiple vendors. The electroporation parameters presented here have proven to work reproducibly for the specific model of electroporator used by the authors. Trial-and-error experiments may be necessary to work out the optimal settings for a different electroporator.

Materials

Tobacco BY-2 (*N. tabacum* L. var Bright Yellow 2) cultured cells; available from many sources including authors' laboratory (*ding.35@osu.edu*)

N. benthamiana cultured cells (Qi and Ding, 2002; Sunter and Bisaro, 2003); available from authors' laboratory (*ding.35@osu.edu*)

Murashige & Skoog (MS) medium (see recipe)

Digestion and washing buffer (see recipe) containing 1.5% (w/v) cellulase ("Onozuka" RS; Yakult) and 0.2% (w/v) macerase (Calbiochem-Novabiochem)

Digestion and washing buffer (see recipe), prechilled to 4°C

Electroporation buffer (see recipe), prechilled to 4°C

RNA prepared from plasmid containing PSTVd cDNA by in vitro transcription (see Support Protocol 1)

Protoplast culturing solution (see recipe)

Protoplast culturing solid medium (see recipe) in 6-well culture plates

RNeasy Plant Mini Kit (Qiagen; also see Support Protocol 2)

250-ml flasks

50-ml sterile polypropylene conical tubes (e.g., Fisher)

Swinging-bucket centrifuge (e.g., Marathon 21000R, Fisher)

Platform shaker (New Brunswick Scientific)

100- and 40-μm nylon mesh (Spectrum)

400-ml beakers

Electroporator Plus and 4-mm-gap electroporation cuvettes (all available from BTX; also see Internet Resources)

Disposable sterile 1.5-ml microcentrifuge tubes (Fisher)

UV spectrophotometer (e.g., Thermo Spectronic)

Additional reagents and equipment for RNA quantitation (Gallagher, 2004) and northern blot analysis (Brown et al., 2004)

Contributed by Xuehua Zhong, Asuka Itaya, and Biao Ding

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NOTE: Autoclave all glassware and plasticware prior to beginning this protocol, unless they are purchased sterile.

NOTE: Use large-orifice pipet tips to handle protoplasts. Prepare large-orifice 200- and 1000-μl pipet tips by cutting tips and autoclave them. Large-orifice tips are commercially available as well. The wider opening is necessary to avoid damaging the protoplasts.

NOTE: When testing replication of PSTVd mutants or a different type of viroid, include wild-type PSTVd as a positive control. In all experiments, use mock- (i.e., water-) inoculated cells as a negative control.

Prepare protoplasts

1. Culture tobacco BY-2 and *N. benthamiana* suspension cells in 50 ml MS medium in a 250-ml flask by shaking at 250 rpm under constant ambient light at 24°C. Subculture weekly by making a 1:25 (for tobacco) and a 1:10 dilution (for *N. benthamiana*).

   The dilution ratio can vary based on cell growth rate and density. One useful criterion for optimal growth rate is that, after dilution for subculturing, the cells reach the original density within 7 days. The ratios suggested above have worked best in the authors' experiments.

2. Collect 50 ml of 7- to 10-day cultured cells in a 50-ml conical tube. Centrifuge cells in a swinging-bucket centrifuge 6 min at 150 × *g*, room temperature. Discard the supernatant.

   The volume of the centrifuged cell pellet must be adjusted to <10 ml for efficient digestion of cell walls and production of viable protoplasts.

3. Resuspend the cells in 40 ml digestion and washing buffer with 1.5% cellulase and 0.2% macerase. Incubate the cells for 2 to 4 hr at room temperature in the dark with slow shaking at 70 rpm.

   It is good practice to microscopically examine a fraction of cells (~10 μl) every 30 min after 2 hr digestion to monitor progress of digestion. Prolonged digestion will not improve yield of protoplasts and may cause damage to the cells.

   The authors have found that BY-2 cells need more time (3 to 4 hr) for digestion than N. benthamiana cells (2 to 3 hr). Some procedures have used macerozyme as an alternative to macerase. It is reported that macerase has nearly three-fold higher activity than macerozyme (Sinha et al., 2003). The authors also found macerase to be much more potent than macerozyme.

4. Examine the cells under a light microscope to estimate the percentage of cells with walls digested (i.e., protoplasts).

   These cells are round-shaped with a smooth surface (see Commentary). A good yield should be ~70% to 90%.

5. Filter the protoplasts through 100-μm nylon mesh over a 400-ml beaker. Refilter the protoplasts through a 40-μm nylon mesh over a 400-ml beaker.

   The two-step filtration yields better quality protoplasts than a single-step filtration through the 40-μm nylon mesh. In practice, the authors place the nylon mesh over the beaker, wrap the edge around the beaker, and fasten with a rubber band. Downward pressure is then gently applied to the mesh to create a well to hold protoplasts. The cells will flow through the mesh very slowly. Gentle tapping of the mesh will accelerate cell flow.

6. Transfer the protoplast suspension to a 50-ml conical tube and centrifuge 5 to 15 min at 150 × *g*, 4°C. Discard the supernatant and resuspend the protoplasts in 25 ml prechilled (4°C) digestion and washing buffer.

   Avoid exceeding a total volume of 30 ml (solution plus protoplasts) in the 50-ml tube; otherwise, it will be difficult to centrifuge down the good protoplasts. The authors have

found that a 5-min centrifugation is sufficient to collect the majority of protoplasts for infection studies, while avoiding potential damage to the protoplasts. Extending the centrifugation time to 15 min helped collect more protoplasts; however, the authors do not recommend centrifugation beyond 15 min because longer times may not improve the yield and may cause damage to some of the protoplasts.

7. Repeat step 6 for a second wash with prechilled (4°C) digestion and washing buffer. Discard the supernatant and resuspend the protoplasts in prechilled (4°C) electroporation buffer to a density of 2×10^6 protoplasts/ml. Incubate the protoplasts 1 to 2 hr on ice before electroporation.

   The authors have found that it is better to resuspend the protoplasts first in ~5 to 10 ml (i.e., ~1/4 of the starting volume) of electroporation buffer, and then calculate the density of the protoplasts by performing a 1/10 dilution on an aliquot using a hemacytometer. Add more of electroporation buffer if the density is higher than 2×10^6 protoplasts/ml. Be sure that 70% to 90% of the protoplasts are intact before proceeding to electroporation.

Perform electroporation

8. Set electroporator at 0.2 to 0.3 kV.

   Make sure that all connections are in good order before electroporation. For different electroporators, trial and error may be necessary to determine the optimal settings.

9. Place 5 to 10 μg RNA transcribed from PSTVd cDNA into an RNase-free 1.5-ml microcentrifuge tube. Add 1×10^6 protoplasts (in 0.5 ml electroporation buffer) to the RNA sample, mix, and transfer the mixture into a 4-mm-gap electroporation cuvette that has been precooled on ice.

10. Wipe off the water from the outside of the the cuvette and place it in the electroporator. Electroporate the protoplasts according to the manufacturer's instructions.

Harvest protoplasts and isolate RNA

11. After electroporation, immediately take out the cuvette and add 1 ml precooled (4°C) electroporation buffer. Mix gently, transfer to a microcentrifuge tube, and keep on ice until step 12. Proceed to step 12 within 30 min.

    For N. benthamiana protoplasts, the authors found that keeping electroporated cells longer than 30 min can significantly reduce cell viability and transfection efficiency.

    The tube used to mix RNA and protoplasts in step 9 can be used here.

12. Microcentrifuge 3 to 6 min at $150 \times g$, room temperature. Remove the supernatant and resuspend the transfected protoplasts in 1 ml of protoplast culturing solution.

13. Using large-orifice pipet tips, transfer protoplasts into the 6-well culture plate containing protoplast culturing solid medium. Incubate the protoplasts in the dark at room temperature for 2 to 4 days without shaking.

14. Harvest protoplasts in a microcentrifuge tube by centrifuging in a microcentrifuge 3 to 6 min at $150 \times g$, room temperature. Wash the protoplasts twice, each time with 1 ml of protoplast culturing solution.

    Washing must be thorough; otherwise the leftover inoculum can produce high background signals on northern blots.

15. Isolate total RNA from the protoplasts by using RNeasy Plant Mini Kit according to manufacturer's instructions, or by the standard procedure described in Support Protocol 2.

16. Quantify RNA by UV spectrometry at 260 nm (Gallagher, 2004) and reserve a 10-μg aliquot of RNA for northern blot analysis (Brown et al., 2004).

SUPPORT PROTOCOL 1

SYNTHESIS OF RNA INOCULUM FOR TRANSFECTING PROTOPLASTS WITH POTATO SPINDLE TUBER VIROID

In the authors' experiments, the plasmid used for in vitro transcription has cDNA from the potato spindle tuber viroid (PSTVd) inserted into the double-ribozyme expression cassette pRZ6-2 (Hu et al., 1997; Feldstein et al., 1998). The plasmid is linearized with *Hin*dIII and used as template to generate in vitro transcripts. Self-cleavage of the ribozymes produces precisely unit-length PSTVd RNA transcripts that have high infectivity. SP6 transcription systems without ribozymes (which produce PSTVd RNA containing extra nucleotides) have been commonly used for plant inoculation (Owens et al., 1986). However, the infectivity is 10,000-fold less than transcripts derived from the pRZ6-2 (Hu et al., 1997; Feldstein et al., 1998). Plamids containing PSTVd cDNA have also been used directly as inoculum to infect a plant (Tabler and Sänger, 1984; Owens et al., 1986). The authors have not tested the infectivity of PSTVd cDNAs or transcripts derived from the SP6 transcription systems in the protoplasts.

Materials

Plasmid containing PSTVd cDNA with T7, T3, or SP6 promotor, suitable for in vitro transcription (e.g., pRZ6-2; available from the authors; *ding.35@osu.edu*)
Restriction endonuclease (e.g., *Hin*dIII for pRZ6-2) and buffer (e.g., Invitrogen)
MEGAscript kit (Ambion)
MEGAclear kit (Ambion)
UV spectrophotometer (e.g., Thermo Spectronic)
Additional reagents and equipment for restriction digestion (Bloch and Grossmann, 1995), in vitro transcription (Tabor, 1987), and RNA quantitation (Gallagher, 2004)

1. Linearize the plasmid containing PSTVd cDNA with an appropriate restriction enzyme for 2 to 3 hr at 37°C. Terminate reaction and purify linearized plasmid according to the manufacturer's instructions for the MEGAscript kit.
2. Use linearized plasmid to synthesize PSTVd RNA by in vitro transcription using the MEGAscript kit according to the manufacturer's instructions or using a standard procedure (e.g., Tabor, 1987).

   The authors recommend using the MEGAscript kit if the laboratory's budget permits.
3. Purify RNA transcripts by using the MEGAclear kit according to the manufacturer's instructions.
4. Measure the concentration of RNA by UV spectrometry at 260 nm (Gallagher, 2004).

SUPPORT PROTOCOL 2

EXTRACTION OF TOTAL RNA FROM TRANSFECTED PROTOPLASTS

The RNeasy Plant Mini Kit comes with a complete protocol for total RNA extraction. This kit yields RNA of very high purity. The total RNA can also be extracted using the following protocol, which is less expensive. It yields total RNA that is of lower purity but is sufficient for northern blot analysis of PSTVd accumulation.

Materials

TRIzol Reagent (Invitrogen)
Transfected protoplasts, harvested (see Basic Protocol, steps 1 to 14)
25:24:1 phenol:chloroform:isoamyl alcohol (*APPENDIX 2A*)
100% and 75% (v/v) ethanol
5 M ammonium acetate (see *APPENDIX 2A* for 10 M)
DEPC-treated H_2O (*APPENDIX 2A*)
UV spectrophotometer (e.g., Thermo Spectronic)

Additional reagents and equipment for RNA quantitation (Gallagher, 2004) and northern blot analysis (Brown et al., 2004)

1. Add 10 vol of TRIzol Reagent (Invitrogen) to the harvested protoplasts (e.g., 500 µl Trizol Reagent to 50 µl harvested protoplasts) and mix vigorously by vortexing for 1 min.
2. Add 1 vol (i.e., 550 µl) of 25:24:1 phenol:chloroform:isoamyl alcohol (25:24:1) to the above tube and vortex thoroughly.
3. Microcentrifuge 5 min at 13,000 rpm. Carefully transfer the upper aqueous layer to a new microcentrifuge tube and add 3 vol of 100% ethanol and 1/10 vol of 5 M ammonium acetate to the tube. Incubate 20 min to overnight (depending on the working schedule) at −20°C.
4. Microcentrifuge 30 min at 13,000 rpm at 4°C. Discard the supernatant and add 1 ml of 75% ethanol. Microcentrifuge 10 min at 13,000 rpm, 4°C, discard the supernatant, and dry the pellet at room temperature for ∼5 min.
5. Add 30 to 50 µl DEPC-treated water and measure the RNA concentration by UV spectrometry (Gallagher, 2004). Reserve a 10-µg aliquot of RNA for northern blot analysis (Brown et al., 2004).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps, except where DEPC-treated water is specified. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Digestion and washing buffer

82 g mannitol
0.7 g 2-(*N*-morpholino)ethanesulfonic acid (MES)
H_2O to 1 liter
Adjust pH to 5.5 with 1 M KOH
Autoclave 20 min and remove from autoclave as soon as possible to avoid evaporation
Store up to 1 month at 4°C

Electroporation buffer

82 g mannitol
0.7 g 2-(*N*-morpholino)ethanesulfonic acid (MES)
11.1 mg $CaCl_2$ [add 1 ml of 1000× (11.1 g/liter) stock solution]
H_2O to 1 liter
Adjust pH to 5.5 with 1 M KOH
Autoclave 20 min and remove from autoclave as soon as possible to avoid evaporation
Store up to 1 month at 4°C

Murashige & Skoog (MS) medium

4.3 g Murashige & Skoog (MS) basal salt mixture (Caisson Laboratories or Sigma)
30 g sucrose
100 mg myo-inositol (Sigma cat. no. I-5125)
10 mg KH_2PO_4 [add 1 ml of 1000× (10 g/liter) stock solution]
Adjust volume to 1 liter with H_2O
Adjust pH to 5.5 with 1 M KOH

continued

Autoclave 20 min and remove from autoclave as soon as possible to avoid evaporation of H_2O and caramelization of sucrose

If not to be used immediately, store autoclaved medium up to 1 month at 4°C

Before use, add the following supplements (filter-sterilized through 0.22-μm cellulose filter):

1 ml 1000× Gamborg's vitamin stock (B5 vitamins; Caisson Laboratories or Sigma; store up to 2 years at −20°C)

100 μl 10 mg/ml 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma; store up to 2 years at −20°C)

Protoplast culturing solid medium

Supplement protoplast culturing solution (see recipe) with 1% (w/v) agar, autoclave 20 min, allow to cool to ∼50°C, then pour 5-mm-thick layers (∼4 ml) in wells of 6-well Costar tissue culture plates (Corning). Store plates up to 1 month at 4°C, wrapped in Parafilm.

Protoplast culturing solution

4.3 g Murashige & Skoog (MS) basal salt mixture (Caisson Laboratories or Sigma)

30 g sucrose

100 mg myo-inositol (Sigma cat. no. I-5125)

10 mg KH_2PO_4 [add 1 ml of 1000× (10 g/liter) stock solution]

82 g mannitol

H_2O to 1 liter

Adjust pH to 5.5 with 1 M KOH.

Autoclave 20 min and remove from autoclave as soon as possible to avoid evaporation of H_2O and caramelization of sucrose

If not to be used immediately, store autoclaved medium up to 1 month at 4°C

Before use, add the following supplements (filter-sterilized through 0.22-μm cellulose filter):

1 ml 1000× Gamborg's vitamin stock (B5 vitamins; Caisson Laboratories or Sigma; store up to 2 years at −20°C)

100 μl 10 mg/ml 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma; store up to 2 years at −20°C)

COMMENTARY

Background Information

Viroids are single-stranded, noncoding, and nonencapsidated RNAs that infect plants (Flores et al., 2000; Tabler and Tsagris, 2004). Without encoding proteins, a viroid genome must rely on direct recruitment of host factors to initiate and complete replication. Although the rolling-circle model for viroid replication has been in existence for over 20 years (Branch and Robertson, 1984), the viroid structures and host factors that interact to accomplish replication remain poorly understood. A large body of research on viroid replication or infection, including mutational analyses of viroid structures important for infection, was performed on mechanically inoculated plants (e.g., Owens et al., 1986, 1991, 1995, 1996; Hammond and Owens, 1987; Loss et al., 1991; Qu et al., 1993; Hu et al., 1996). Such assays, however, cannot easily separate defects in replication at the cellular level from defects in other functions (e.g., cell-to-cell and long-distance transport) that are required for systemic infection. Therefore, noninfection at the whole-plant level cannot be definitively interpreted as indication of nonreplication at the cellular level. Furthermore, the long infection process, up to several weeks, often results in new mutations or reversions of mutations to wild-type, which complicates interpretation of the role of specific nucleotide changes in replication.

Analysis of viroid replication in single cells is necessary to advance mechanistic understanding of replication. Protoplasts offer an ideal system for such analysis. Mühlbach and Sänger (1977) showed that protoplasts prepared from leaves of tomato (cultivar Hilda 72) supported efficient replication of Cucumber pale fruit viroid when inoculation was

accomplished by incubating the protoplasts in an inoculation medium of alkaline pH; however, other plant species and viroid combinations did not yield informative results. This approach has not gained widespread utilization. The barrier to its popularity is presumably the tedious procedure for isolating protoplasts from leaves, in combination with the low efficiency of protoplast transfection. Cultured cells provide an alternative, convenient, and economical source and constant supply of cells for protoplast preparation. In early studies, cultured cells were developed from infected plants to analyze viroid replication (reviewed in Semancik and Conejero-Tomas, 1987). A pitfall is that viroids are present in the cells already at the start of protoplast preparation, making experiments to dissect specific steps of the replication circle technically challenging. A cultured cell system that can be readily inoculated with viroid RNA is therefore highly desirable. An outstanding advantage of inoculating protoplasts is that this procedure may offer a synchronous system to study viroid replication. Protoplasts prepared from potato suspension cells supported replication of PSTVd inoculated via liposomes (Faustmann et al., 1986). With this method, viroid inoculum was packaged in liposomes, which, upon application, fuse with cell membranes to deliver the inoculum into the cells. The cumbersome procedure of preparing viroid-containing liposomes has presumably hindered acceptance of liposome transfection as a routine practice.

In exploring alternative strategies to inoculate protoplasts for viroid replication studies, Qi and Ding (2002) demonstrated that electroporation was a simple, effective, and reproducible method. The method was developed based on the use of PSTVd and protoplasts prepared from cultured cells of tobacco and *N. benthamiana*. It has allowed studies of several aspects of the PSTVd biology, including mutational analysis of PSTVd structures important for replication (Qi and Ding, 2002, 2003a), intranuclear trafficking of PSTVd replication intermediates (Qi and Ding, 2003b), and separation of PSTVd mutations that affect cell-to-cell trafficking and replication (Qi et al., 2004; X. Zhong, A. Itaya, Y. Qi, and B. Ding, unpub. observ.). These examples suggest that further utilization of the protoplast system should allow in-depth investigation of the structural elements of viroids essential for replication, as well as potential identification of host factors for replication. Thus far, electroporation is the main method that proves to be highly effective and reproducible for mechanical inoculation of protoplasts to study PSTVd replication. Whether alternative methods can achieve the same remains to be seen.

Critical Parameters

Make sure the cultured cells are maintained under good conditions. One useful measure is the observation that after dilution for subculturing, the cells will grow to reach the original density within 1 week. Cells with lower growth rates will usually not grow well after inoculation and thereby support active viroid replication. Avoid using cells >10 days old, because the protoplasts derived from them tend to lose competence in supporting viroid replication. Whether this is attributable to their recalcitrance to inoculation or to their reduced metabolic rate is unclear.

Do not overdigest the cells. Digesting cells over an extended period of time will not improve the yield of protoplasts and may cause damage to the protoplasts. It is therefore important to use the shortest time possible to achieve optimal digestion, as judged by the percentage of protoplasts obtained (70% to 90%).

Use large-orifice pipet tips to transfer protoplasts at all steps. This is critical. The protoplasts, without cell walls, are very fragile. Use of regular (i.e., small-orifice) tips will squeeze and break the protoplasts. The large-orifice tips will allow easy passage of the protoplasts without physical damage.

All pipet tips and tubes should be RNase-free. Because viroid RNA is used as the inoculum, presence of RNase in the tips and tubes may cause degradation of the inoculum. Furthermore, presence of RNase may also degrade the quality of RNAs extracted from the protoplasts.

Do not use any antibiotics to maintain or subculture cells. Because the cells are constantly maintained, use of antibiotics poses the risk of selecting for antibiotic-resistant bacteria. If the culture cells are contaminated, simply discard them and obtain clean cell cultures. A useful practice is to maintain several flasks of culture cells, ideally in separate places, so that the risk of contaminating all of them can be minimized.

All utensils should be clean and free from contamination by any detergents. Presence of even trace amounts of detergents may inhibit cell growth and damage protoplasts during or after electroporation. One method of detecting the presence of detergent residues on glassware

Table 16D.4.1 Troubleshooting Guide for Transfection of Protoplasts

Problem	Possible cause(s)	Solution(s)
Cultured cells do not grow well	B5 vitamins have degraded	Prepare fresh solution
Cultured cells grow very slowly	Improper temperature	Adjust temperature
	Low subculture ratio	Increase subculture ratio to 1:5
More than 70% of the cells are broken during digestion	Improper ratio of cultured cells to digestion solution	Reduce proportion of culture cells, trying not to reach over 1:4 ratio
	Improper concentration of mannitol	Adjust the mannitol concentration; after autoclaving, remove solution from autoclave to minimize water evaporation
	Utensils contaminated with detergents	Clean all utensils carefully
	Overdigestion	Shorten digestion time
	Vigorous shaking during digestion	Reduce shaking speed
Many protoplasts cannot be centrifuged down	Volume of solution in 50-ml conical tube is too large	Put <30 ml solution in 50-ml tube
	Centrifugation time is too short	Increase centrifugation time
Low transfection efficiency	RNA amount is too low	Increase RNA amount
	Electroporated protoplasts are kept in electroporation buffer on ice for too long	Do not leave electroporated protoplasts in electroporation buffer on ice for >30 min
	Low quality of protoplasts	Improve quality of protoplasts

is to watch for the appearance of bubbles when water is applied.

Before electroporation, be sure that 70% to 90% of the protoplasts are intact. This is essential for high transfection efficiency. It is useful to keep in mind that not every protoplast will be transfected and that some cells may be damaged during electroporation. Therefore, starting with a high percentage of good protoplasts ensures that a decent population of protoplasts will survive electroporation and grow well to support viroid replication.

Troubleshooting

Some problems that may arise with the protocols in this unit, along with their possible causes and solutions, are listed in Table 16D.4.1.

Anticipated Results

Cultured cells that are growing well appear turgid and are often found in clusters (Fig. 16D.4.1A). Many cells are oblong-shaped. Healthy protoplasts are round-shaped and have a smooth surface (Fig. 16D.4.1B). As reported by Qi and Ding (2002), accumulation of PSTVd resulting from replication in both tobacco and *N. benthamiana* protoplasts can be detected as early as 6 hr post-inoculation by northern blotting (Fig. 16D.4.2). The more sensitive RT-PCR method may detect replication at an even earlier time point. If kinetic analysis of replication is not a priority, the authors suggest collection of transfected cells at 3 days post-electroporation for analysis. In situ hybridization may be used to estimate the percentage of protoplasts showing active viroid replication (Qi and Ding, 2002). As with other types of experiments, positive and negative controls should be included in each batch of electroporations.

Time Considerations

With the availability of plasmids for in vitro transcription to synthesize RNA inoculum and hybridization probes, along with healthy, growing cultured cells, the entire procedure from preparation of inoculum, probes, and protoplasts, to finishing the northern blot analyses, can be accomplished within 1 week.

Cultured cells need to be maintained constantly. Cultured cells 7 to 10 days old (starting from subculturing) are best for preparing protoplasts. The total time for protoplast preparation, starting from cell wall digestion to having protoplasts ready for electroporation, is ~4 to

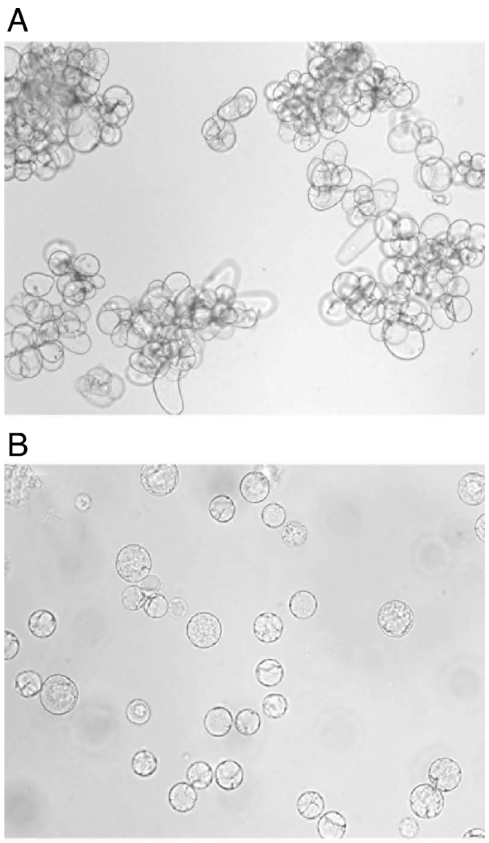

Figure 16D.4.1 Cultured cells and protoplasts of *N. benthamiana*. (**A**) Cultured cells. (**B**) Protoplasts prepared from cultured cells. Healthy protoplasts should account for ~70% to 90% of the total cells in a good preparation.

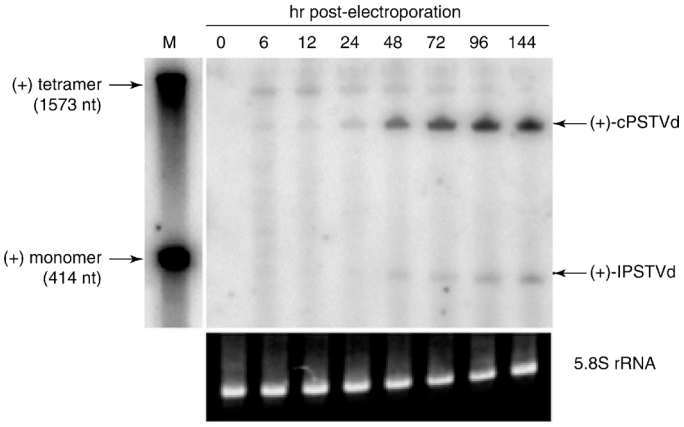

Figure 16D.4.2 Northern blot showing replication of PSTVd in tobacco BY2 protoplasts at increasing hours post-electroporation. (+)-cPSTVd and (+)-lPSTVd indicate monomeric circular and monomeric linear PSTVd, respectively, produced during rolling-circle replication. 5.8S rRNA serves as a loading control. Lane M shows in vitro RNA transcripts containing tetrameric and monomeric PSTVd sequences and some vector sequences as size markers. See Qi and Ding (2002) for further information. Reprinted from Qi and Ding (2002), with permission from Elsevier Publishers.

8 hr. In vitro transcription time varies depending on the methods. If the Ambion MEGAscript kit is used for in vitro transcription, it takes ~3 to 4 hr, starting with assembly of the reaction, to complete purification of RNA transcripts using the MEGAclear kit and measure the concentration of RNA via UV spectrometry (see Support Protocol 1). The time for RNA extraction from the transfected protoplasts for northern blots also varies with the chosen method. Using the RNeasy Plant Mini Kit or a non-kit method, it takes ~2 hr.

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Key References

Qi and Ding, 2002. See above.

Establishes electroporation protocol to inoculate tobacco and N. benthamiana protoplasts for studying PSTVd replication.

Internet Resources

http://www.btxonline.com/products/

Provides product information about BTX electroporator and cuvettes

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Extracting Viral RNAs from Plant Protoplasts

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ABSTRACT

The analysis of viral RNA is a fundamental aspect of plant RNA virus research. Studies that focus on viral RNAs often involve virus infections of plant protoplasts (see *UNITS 16D.1-16D.4*). Protoplast offer the advantage of simultaneous initiation of infections, which allows for superior temporal and quantitative analyses of viral RNAs. The efficient isolation of intact viral RNA is key to any such investigations. This unit describes two basic protocols for extracting viral RNAs from plant protoplasts. An approach for preparing double-stranded viral RNA from total RNA pools is also provided. The viral RNA prepared by using these techniques can be used for further analyses such as primer extension, reverse transcription–PCR, and northern blotting. *Curr. Protoc. Microbiol.* 6:16E.1.1-16E.1.6. © 2007 by John Wiley & Sons, Inc.

Keywords: RNA • protoplast • isolation

INTRODUCTION

Viral RNAs can be extracted from virus-infected plant protoplasts (see *UNITS 16D.1-16D-4*) using a number of different methods. In general, these protocols involve isolating and lysing the protoplasts, removing the protein components from the lysate, precipitating the total RNA, and resuspending the purified RNAs in an appropriate buffer. Several variations of these basic protocols are currently used to isolate RNA from plus-strand RNA virus-infected protoplasts. Total nucleic acids, including viral RNAs, can be extracted from protoplasts by using an EDTA-detergent lysis buffer and phenol-chloroform extraction method (see Basic Protocol 1). By including an additional step to this protocol (i.e., lithium chloride precipitation), double-stranded (ds) nucleic acids (including ds forms of viral RNAs) can be separated from single-stranded (ss) nucleic acids (including ss forms of viral RNAs). Viral RNAs can also be extracted from protoplasts by using a guanidinium-based solution (see Basic Protocol 2). Additional methods include commercially available kits that also use guanidinium-containing solutions. These include TRIzol Reagent (Invitrogen) and RNeasy (Qiagen). The former is similar to Basic Protocol 2, described below, while the latter utilizes a proprietary RNA binding membrane that eliminates the phenol extraction step. The reagents and protocols for these kits are provided by the respective suppliers and will not be discussed here.

It should be noted that the methods mentioned are largely interchangeable and most will be effective with the types of plant protoplasts used commonly in virus research (e.g., tobacco BY-2, *Arabidopsis thaliana*, *Nicotiana benthamiana*, barley, cucumber, etc.). The method selected for a particular application will therefore depend largely upon personal preference and other factors such as time and cost.

NOTE: Use sterile and RNase-free vessels and deionized, DEPC-treated H_2O (see *APPENDIX 2A*) in all recipes and at all protocol steps.

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BASIC PROTOCOL 1

ISOLATION OF TOTAL NUCLEIC ACIDS USING EDTA-DETERGENT LYSIS AND PHENOL/CHLOROFORM EXTRACTION

This procedure offers a relatively inexpensive and simple method for extracting viral RNAs from protoplasts. The method has been adapted from a protocol used in the laboratory of Dr. T. J. Morris, University of Nebraska-Lincoln. In this protocol, cellular RNases, as well as other cellular proteins, are inactivated and removed by treatment with EDTA, SDS, and phenol-chloroform. It is important to note that endogenous cellular nucleic acids (DNA and RNA) will also co-purify with the viral RNAs. The cellular DNA can be removed by DNase treatment (as described in steps 20 to 26), though its presence does not interfere with most downstream applications.

Materials

Infected protoplasts (see *UNITS 16.D.1-16.D.4*)
Nucleic acid extraction buffer (see recipe)
25:24:1 (v/v/v) phenol:chloroform:isoamyl alcohol (PCI; *APPENDIX 2A*)
1 M and 3M sodium acetate, pH 5.2
95% and 70% ethanol, ice-cold
Diethylpyrocarbonate (DEPC)-treated H_2O (*APPENDIX 2A*)
7 M lithium chloride (LiCl)
2× DNase buffer (see recipe)
RNase-free DNase I (10 U/μl; bovine pancreas, Amersham Biosciences)

1.5-ml microcentrifuge tubes
Standard microcentrifuge
Tube vortexer

Additional reagents and equipment for performing agarose gel electrophoresis and ethidium-bromide staining (Voytas, 2000) and northern blot analysis (Brown et al., 2004)

NOTE: All reagents and vessels used should be sterile and RNase-free.

Extract total nucleic acids from virus-infected protoplasts

1. Resuspend infected protoplasts (500,000 to 1,000,000; see *UNITS 16.D.1-16.D.4*) in their incubation medium by gentle pipeting up and down, then transfer them to an empty 1.5-ml microcentrifuge tube.
2. Spin the microcentrifuge tube 5 min at 800 × *g*, at ambient temperature to sediment protoplasts.
3. Carefully remove and discard the supernatant using a pipet. Lyse protoplast pellet by adding 300 μl nucleic acid extraction buffer and pipetting rapidly up and down five times.
4. Immediately add an equal volume of PCI (300 μl) to the cell lysate and vortex vigorously (highest setting) for 30 sec.
5. Centrifuge the emulsion in a microcentrifuge 2 min at 12,000 × *g*, 4°C to separate the phases.
6. Remove the upper aqueous phase (which contains the nucleic acids) and transfer to a new microcentrifuge tube containing 300 μl of PCI. Vortex and then centrifuge as before.
7. Remove the upper aqueous layer and transfer it to a microcentrifuge tube containing 100 μl of 1 M sodium acetate and 1000 μl of 95% ethanol, ice-cold.

8. Mix tube by vortexing and place it for 1 hr at −80°C, or overnight at −20°C, to allow the nucleic acids to precipitate. Alternatively, incubate the tube in a dry ice/ethanol bath for 5 min.
9. Collect the nucleic acid precipitate by centrifugation for 15 min, at 12,000 × *g*, 4°C.
10. Remove the supernatant, and gently cover the nucleic acid pellet with 1 ml of ice-cold 70% ethanol, then centrifuge 15 min at 12,000 × *g*, 4°C.
11. Remove the 70% ethanol supernatant completely and let the pellet air-dry for 10 min.
12. Resuspend the dried pellet in an appropriate volume of DEPC-treated water and store at −80°C. Analyze a 5 µl aliquot by ethidium bromide gel electrophoresis, northern blotting, or RT–PCR.

    The volume used for resuspension (generally 10 to 50 µl) dictates the viral RNA concentration and will depend on the accumulation efficiency of the virus under study.

Isolate double-stranded nucleic acids

An extension to the procedure above allows for the isolation of double-stranded (ds) nucleic acids (adapted from Morris and Dodds, 1979). This type of preparation can be useful as it eliminates most of the cellular RNAs and greatly enriches for the viral replicative intermediate (i.e., ds viral RNA). Co-purifying dsDNA is removed by DNase treatment. The ds viral RNA can be detected by ethidium bromide gel electrophoresis, northern blotting, or used for cloning purposes after denaturation.

13. In a microcentrifuge tube, to a given volume of resuspended nucleic acids (from step 12), add a half-volume of 7 M LiCl and mix by gently vortexing.
14. Incubate the tube containing the mixture overnight (minimum of 6 hr) on ice at 4°C (i.e., in a refrigerator).
15. After incubation, spin the sample in a microcentrifuge 15 min at 12,000 × *g*, 4°C.

    The pellet will contain the lithium-insoluble ssRNAs, whereas the supernatant will contain lithium-soluble DNA and dsRNAs.

16. To isolate the ds nucleic acids, carefully remove the supernatant by pipetting it into a new microcentrifuge tube, add three volumes of 95% ethanol, and precipitate overnight at −20°C .
17. Spin the sample in a microcentrifuge 15 min at 12,000 × *g*, 4°C.
18. Remove the supernatant, and gently cover the nucleic acid pellet with 1 ml of ice-cold 75% ethanol and then centrifuge 15 min at 12,000 × *g*, 4°C.
19. Remove the 70% ethanol supernatant completely and let the pellet air-dry for 10 min and resuspend pellet in 50 µl of DEPC-treated water.
20. To eliminate the DNA, add 50 µl of 2× DNase buffer and 20 U DNase I (RNase-free).
21. Incubate for 30 min at 37°C, add 20 µl of 3 M sodium acetate, 80 µl DEPC-treated water, and 200 µl PCI.
22. Vortex on high setting for 30 sec and then centrifuge 2 min 12,000 × *g*, ambient temperature.
23. Remove the upper aqueous phase and transfer to a new tube, add 600 µl of ice-cold 95% ethanol, vortex, and incubate 1 hr at −20°C.
24. Collect the precipitated nucleic acids by centrifuging 15 min at 12,000 × *g*, 4°C.

25. Remove the supernatant and gently cover the nucleic acid pellet with 1 ml of ice-cold 70% ethanol. Centrifuge 15 min at 12,000 × *g*, 4°C.

26. Remove the 70% ethanol supernatant completely and let the pellet air-dry for 10 min.

27. Resuspend the dsRNA pellet in 10 to 20 µl of DEPC-treated water.

BASIC PROTOCOL 2

ISOLATION OF TOTAL RNA USING GUANIDINIUM ISOTHIOCYANATE SOLUTION

Total RNA may be isolated from plant protoplasts using a guanidinium solution method (Chomczynski and Sacchi, 1987). In this procedure, guanidinium isothiocyanate (a strong protein denaturant) in combination with phenol and chloroform, under acidic conditions, is used to efficiently inactivate endogenous cellular RNases and preferentially partition RNA into the aqueous phase. It is particularly useful in cases where RNA integrity is paramount and contaminating DNA is undesirable. This method is also used for isolation of RNA from homogenized plant and animal tissues.

Materials

Infected protoplasts (see *UNITS 16.D.1-16.D.4*)
Guanidinium solution (see recipe)
25:24:1 (v/v/v) phenol:chloroform:isoamyl alcohol (PCI; *APPENDIX 2A*)
2 M sodium acetate, pH 4 (*APPENDIX 2A*)
DEPC-treated water (*APPENDIX 2A*)
Isopropanol, ice-cold
70% ethanol, ice-cold

1.5-ml microcentrifuge tubes
Tube vortexer

Additional reagents and equipment for performing agarose gel electrophoresis and ethidium-bromide staining (Voytas, 2000) and northern blot analysis (Brown et al., 2004)

NOTE: Use sterile, RNase-free reagents and vessels.

Extract total RNA from virus-infected protoplasts

1. Resuspend infected protoplasts (500,000 to 1,000,000; see *UNITS 16.D.1-16.D.4*) in their incubation medium by gentle pipeting up and down, then transfer them to an empty microcentrifuge tube.

2. Spin the microcentrifuge tube for 5 min at 800 × *g*, 4°C to sediment protoplasts.

3. Remove the supernatant and add to the pelleted protoplasts 400 µl of guanidinium solution, 400 µl of 25:24:1 phenol:chloroform:isoamyl alcohol, and 40 µl 2M sodium acetate, pH 4.

4. Vortex the mixture on high for 30 sec and centrifuge for 10 min at 12,000 × *g*, 4°C.

5. Carefully remove the upper aqueous layer without disturbing the interface and transfer into a new microcentrifuge tube containing 400 µl of 25:24:1 phenol:chloroform:isoamyl alcohol.

6. Vortex the mixture on high for 30 sec and centrifuge 10 min at 12,000 × *g*, 4°C. Remove the aqueous layer and place in a new microcentrifuge.

7. Precipitate the RNA by adding 1 volume (800 µl) of ice-cold isopropanol and placing for 1 hr at −80°C or overnight at −20°C.

8. Centrifuge for 10 min at 12,000 × *g*, 4°C.

9. Remove the supernatant and carefully cover the pellet with 1 ml of ice-cold 70% ethanol.
10. Remove the 70% ethanol supernatant and let the pellet air dry for 10 min.
11. Once complete, resuspend RNA pellet in 20 to 50 µl of DEPC-treated water.

Analyze total RNA

12. Analyze a 5-µl aliquot by ethidium bromide gel electrophoresis, northern blotting, or RT-PCR.

REAGENTS AND SOLUTIONS

Use sterile and RNase-free vessels and deionized, DEPC-treated H_2O in all recipes and at all protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

2× DNase buffer

0.2 M Tris·Cl, pH 7 (*APPENDIX 2A*)
0.2 M NaCl
0.1 M $MgCl_2$
Store up to 6 months at 4°C

Guanidinium solution

4 M guanidinium thiocyanate
25 mM sodium acetate, pH 7
20 mM EDTA (*APPENDIX 2A*)
50 mM 2-mercaptoethanol (*APPENDIX 2A*)
Store up to 1 week in dark bottle at 4°C

Nucleic acid extraction buffer

0.1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
0.2 M sodium chloride
2 mM EDTA (*APPENDIX 2A*)
1% SDS
Store up to 1 week at 4°C

COMMENTARY

Background Information

These methods describe the isolation of viral RNAs associated with plus-strand RNA plant virus infections of plant protoplasts (Section 16D). The protocols allow for the isolation of either total RNA or partially purified RNA that also include viral RNAs. Thus, it is important to remember that these methods do not generate pure viral genomic RNAs, that generally requires the prior isolation of virus particles (from which viral genomes can be isolated) and a customized purification scheme for each individual virus. Instead, the protocols described are useful for obtaining RNA preparations that contain genomic RNAs, subgenomic mRNAs, defective viral RNAs, complementary viral RNAs, and/or double-stranded viral RNAs in association with cellular RNAs. Though crude by some standards, these RNA preparations can be used directly in a variety of downstream applications, including (i) agarose/acrylamide gel electrophoresis and viral RNA detection by ethidium bromide staining, (ii) plus- or minus-strand-specific northern blotting, (iii) primer-extension analysis, (iv) RT-PCR, (v) 5′- or 3′-RACE, and (vi) passaging or infectivity assays. These types of analyses can provide important information on the structure and accumulation levels of viral RNAs.

Critical Parameters

Due to the unstable nature of RNA, it is important that extra care is taken when performing the steps in these protocols. For example, it is often better to be conservative when removing the aqueous phases from organic phases and to sacrifice some sample at

the interface in order to ensure purity. One may also want to maintain separate aliquots of working solutions (e.g., isopropanol, ethanol, etc.) that will be used only for RNA isolation procedures. Using sterile and RNase-free disposable vessels and DEPC-treated water will also reduce sample exposure to RNases. Finally, wearing sterile disposable gloves, working quickly, and keeping samples on ice whenever possible will help to reduce RNA degradation.

Anticipated Results

Total nucleic acids isolation (Basic Protocol 1) provides good quality viral RNA; however, these preparations also contain cellular RNA and DNA. Further processing of total nucleic acids (via lithium chloride precipitation) can yield a preparation that is highly enriched for viral dsRNA. Contaminating DNA can be removed from either the crude dsRNA or total nucleic acids preparations by DNase treatment. The isolation of total RNA by the guanidinium isothiocynate method (Basic Protocol 2) is primarily devoid of DNA but contains both viral and cellular RNAs. In either case, these protocols yield RNA samples that will be stable for several years when stored at −80°C and can be used successfully in the many applications listed above.

Time Consideration

The protocols described are moderately time consuming but, with the exception of the dsRNA preparation, can be completed in about 2 hr. This estimate will increase if large numbers of samples are processed simultaneously.

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Morris, T.J. and Dodds, J.A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytopathology* 69:854-858.

Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

Northern Analysis of Viral Plus- and Minus-Strand RNAs

An essential feature of viral infection is replication of the viral genome. RNA viruses are broadly categorized into single-stranded, positive- and negative-sense RNA viruses, and double-stranded (ds)RNA viruses. Many plant and animal viruses have plus-strand (i.e., messenger-sense) RNA genomes in their virions. RNA genomes of positive-strand RNA viruses share similar replication strategies despite vast differences in genome organization, primary sequence, and secondary and tertiary structure. After release of the genomic RNA(s) from the viral capsid, translation proceeds using host ribosomes, leading to expression of the RNA-dependent RNA polymerase (RdRp). The RdRp, together with known or hypothetical host proteins and additional viral proteins, forms an active replicase that recognizes specific cis-acting elements, leading to initiation of minus-strand synthesis (Buck, 1996; Ahlquist, 2002). Minus strands, which either complex with the plus-strand template (Khromykh and Westaway, 1997) or remain single-stranded (Garnier et al., 1980; Axelrod et al., 1991), are transcribed into full-length plus strands such that an asymmetric ratio of plus- and minus-strands accumulates, ranging from 10:1 to 1000:1 (Buck, 1996). A negative-sense RNA genome and dsRNA, upon entering into a suitable host cell, must be copied into positive-sense mRNA before translation. To study the replication of RNA viruses in tissues, cells, or protoplasts, total RNA is extracted, the type of viral transcripts is detected, and the abundance is determined by northern blot analysis using strand-specific probes. Though different investigators follow northern analysis protocol with modifications based on their experimental requirements, this unit describes the general strategies for northern analysis.

STRATEGIC PLANNING

Northern Blotting

RNA

Total RNA is extracted from tissues (see Support Protocol 1), cells, or protoplasts (Kong et al., 1995; Wang and Simon, 1999; Qi et al., 2004; Grdzelishvili et al., 2005; Hema et al., 2005; Herranz et al., 2005; Picard et al., 2005) and used for northern analysis. In the total cellular RNA, ribosomal RNAs (rRNAs) are the dominant components and may contribute >75%. Among the rRNAs, 25S (3.5 kb) and 18S (1.9 kb) are highly abundant.

Quality of RNA

Integrity of rRNA is a critical factor for successful northern blot analysis and can be assessed by agarose gel electrophoresis and ethidium bromide staining (Fig. 16E.3.1).

Concentration of RNA

If comparing the abundance of viral RNA transcripts in different samples, it is extremely important to load equal amounts of RNA into each lane of a gel. If equal amounts of RNA are not loaded into each lane, it is not possible to compare and, therefore, results in misinterpretations. Before loading the RNA onto a gel, quantitate RNA.

To quantitate RNA, dissolve the salt-free RNA in DEPC-treated water by briefly vortexing at 4°C or gently pipetting up and down several times. Incubate 15 min on ice. Dilute 1 μl RNA with 499 μl DEPC-treated water and measure the absorbance at 260 nm. A_{260}/A_{280} ratio of the extracted RNA is within the range of 1.6 to 2.0. Calculate the RNA concentration (μg/μl) = O.D. × 500 × 0.04 μg/μl. The yield of total RNA depends on

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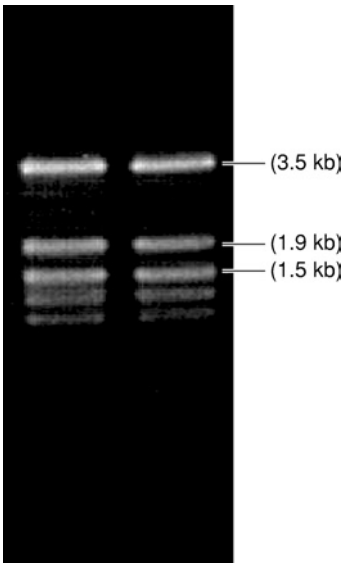

Figure 16E.3.1 Integrity and size distribution of total RNA isolated from *Nicotiana benthamiana* is checked by agarose gel electrophoresis and ethidium bromide staining. A concentration of 1 μg RNA was loaded per lane. The rRNA bands should appear as sharp bands on the stained gel. The size of 25S, 18S, and 16S rRNA is 3.5, 1.9, and 1.5 kb respectively. The 25S and 18S rRNAs are cytoplasmic and the 16S rRNA is from the chloroplast. The large chloroplast rRNA is always cleaved into two smaller RNAs during RNA extraction from plant tissue. The 25S ribosomal RNA band should be present with an intensity approximately twice that of the 18S rRNA. If the rRNA bands are not sharp, but appear as a smear of smaller sized RNAs, it is likely that the RNA was degraded during preparation.

the tissue, cell, or protoplast source. For instance, the yield of total RNA is between 0.25 and 0.5 μg/mg for tobacco, tomato, or potato leaf tissue (Verwoerd et al., 1989).

CAUTION: DEPC (diethylpyrocarbonate) is a potent protein denaturant and a suspected carcinogen, and should be handled with care.

Equalizing RNA in gels

To compare the rRNAs in different samples, load equal concentrations of total RNA on an agarose gel and detect by ethidium bromide staining (Voytas, 2000). For northern analysis, load 1 to 20 μg total RNA into each lane.

Abundance of viral RNA in the total RNA can be normalized based on the content of mRNA of an endogenous, constitutively expressed housekeeping gene such as actin, RUBISCO (ribulose-1,5-biphosphate carboxylase/oxygenase), or ubiquitin since they are abundantly expressed in plants. Briefly, mRNA of any one of the housekeeping genes in the total RNA is detected by northern analysis using a gene-specific probe. Based on the intensity of the hybridization signal of the housekeeping gene, abundance of viral RNA can be normalized.

RNA prepared from polyadenylated virus–infected samples contains viral mRNA with poly(A) tails located at the 3′ termini. In contrast, RNA prepared from nonpolyadenylated virus–infected samples does not contain viral mRNA with poly(A) tails at the 3′ termini. The poly(A) content of the mRNA in total RNA can be compared by dot blot or slot blot hybridization using a poly(dT) probe (Harley, 1987). Based on the signal intensities, equivalent amounts of RNA can be loaded into lanes of a northern gel.

IMPORTANT NOTE: This method is applicable only when polyadenylated RNA is being detected; it is not applicable for nonpolyadenylated RNAs.

A known amount of RNA transcript synthesized in vitro (positive control) from the recombinant plasmid with the gene of interest is used in dot blot or slot blot hybridization and the relative intensity of the hybridization signal is used to calibrate the expression of the RNA.

Gels

Nondenaturing agarose gels (see Support Protocol 3 and 5) or formaldehyde agarose gels (see Support Protocol 4) are commonly used for the separation of RNA. Nondenaturing agarose gels are preferred because formaldehyde gels have fumes that are unpleasant and highly toxic, are more fragile, and great care is necessary to handle them. Also, formaldehyde diffuses from the gel into the buffer during electrophoresis. RNA appear as distinct bands in agarose gels without any denaturant, whereas RNA bands in formaldehyde gels appear fuzzy. For resolving 0.5- to 0.8-kb RNAs, 1.5% (w/v) agarose gels are suitable and for larger RNAs, 1% to 1.2% (w/v) agarose gels can be used.

CAUTION: Formaldehyde is highly volatile, toxic, and carcinogenic. It irritates skin, eyes, mucous membranes, and the respiratory tract. Avoid breathing in vapors and use in a fume hood.

Materials for Northern Blotting

RNA

Total RNA extracted from virus-infected samples (see Support protocol 1) or poly(A) RNA separated from total RNA (see Support Protocol 2) is used to detect viral RNA.

To increase the specificity of detecting low-abundant plus- or minus-strand RNAs in the total RNA, respective RNA species can be enriched before detection (see Support Protocol 10).

IMPORTANT NOTE: In this unit, total RNA or poly(A) RNA used for investigation is referred to as sample RNA.

Standards

To obtain quantitative results, it is essential to include positive and negative controls during northern analysis. Positive control RNAs have structural properties similar to that of the sample RNA and complementary to that of the probe. A positive control RNA transcript is synthesized in vitro from DNA templates that have been engineered into a plasmid where two different bacteriophage promoters, with opposite orientations, flank the cloning site. A sense-strand positive control is synthesized and used for hybridization when an antisense-strand is used as a probe. An antisense-strand positive control is synthesized and used for hybridization when a sense-strand is used as a probe. The negative control should consist of RNA from tissue, cells, or protoplasts that do not express the target viral RNA.

To prepare standards, mix an in vitro transcript of sense-strand RNA with the negative control RNA to approximately equal the mass of the sample RNA. For electrophoresis, positive and negative control RNAs are prepared similar to that of the sample RNA. These manipulations are necessary to reduce the presence of impurities in total RNA that affect the intensity of the hybridization signal.

Markers

The size of the viral RNA can be measured accurately by using RNA ladders or DNA markers which can be commercially purchased (e.g., Ambion, Life Technologies, Promega). Markers are also used to detect RNase contamination or to check for problems that may have occurred during sample preparation or electrophoresis. Glyoxylated denatured RNAs and DNAs of equal length migrate to equivalent locations on agarose gels. Therefore, small DNAs of known size can also be used as markers in this system. Do not use DNA standards on gels containing formaldehyde since the migration of RNA is faster than that of the DNA of equivalent size (Wicks, 1986). Occasionally, the contaminating vector sequence in the probe may hybridize to the RNA or DNA standards, and may generate either a smear or discrete band where none should be.

Highly abundant rRNAs, i.e., 25S (3.5 kb) and 18S (1.9 kb), in total RNA preparations, visualized while checking the quality of RNA in ethidium bromide agarose gels, can also be used as markers.

In denaturing agarose gels, commonly used tracking dyes, bromophenol blue, which migrates faster than 5S rRNA, or xylene cyanol, which migrates slower than 18S rRNA, are also used as indicators of migration.

Gel

Prepare nondenaturing agarose gels (see Support Protocol 3 or 5) or formaldehyde-agarose gels (see Support Protocol 4).

Membranes

A solid support is required to immobilize the RNA. Nitrocellulose membranes have been the choice for several years (Thomas, 1980). The disadvantage of using the nitrocellulose membrane is that it becomes brittle when baked under vacuum at 80°C to fix the RNA. Also, such membranes cannot be used for reprobing. Nylon membranes (available from Amersham Biosciences, Ambion, and PerkinElmer Life Sciences) are more durable than nitrocellulose membranes (Reed and Mann, 1985) and therefore commonly used for nucleic acid hybridization. A good nylon northern blot can be kept for years and reprobed a dozen or more times with good results. Two types of nylon membranes are available: neutral/uncharged and positively charged.

Neutral/uncharged nylon membranes

When using a neutral/uncharged nylon membrane, use of a neutral or nonalkaline buffer over a wide pH range is preferred for the transfer of RNA. Nucleic acid retention capacity of a neutral/uncharged nylon membrane is ~200 to 300 μg/cm^2 and nucleic acids of >50 bp bind efficiently. Nucleic acids are immobilized either by baking or UV irradiation at 254 nm. Commonly used neutral nylon membranes are Hybond-N (Amersham Biosciences) and Gene-screen (PerkinElmer Life Sciences). The disadvantage of an uncharged membrane is that it has a lesser capacity to bind nucleic acids compared to a charged membrane.

Positively charged nylon membranes

Though nonalkaline systems can be used for transferring RNA to the charged membrane, charged membranes have a greater capacity to retain RNA in alkaline solutions (Reed and Mann, 1985). Under this condition, RNA is partially hydrolyzed, thereby increasing the efficiency and speed of transfer of RNAs >2.3 kb. The retention capacity of a charged nylon membrane is 400 to 500 μg nucleic acid/cm^2. The disadvantage of using a charged membrane is that an alkaline transfer may generate a high level of background during hybridization. This problem can be solved by increasing the amount of blocking

agents in the prehybridization and hybridization solutions, and also by decreasing the transfer time of RNA from gel to membrane. Nucleic acids in charged membranes are immobilized by either baking or UV cross-linking. Commonly used, charged membranes include Hybond-N^+ (Amersham Biosciences) and Gene-screen Plus (PerkinElmer Life Sciences). For both neutral and charged nylon membranes, buffers of low ionic strength over a wide pH range can be used (Sambrook and Russel, 2001).

DNA/RNA probes

DNA or RNA probes are prepared (see Support Protocols 8 and 9) and used for hybridization. The working probe must be single-stranded. RNA or DNA polymerase is used to produce labeled RNA or DNA, respectively, copies from the starting DNA. Probes are labeled by an in vitro synthesis reaction or by PCR. One of the four nucleotide precursors carries a labeled group and is incorporated into the newly synthesized RNA or DNA. Double-stranded probes have secondary structure and are not ideal for use, therefore, to improve the efficiency of hybridization, denature the double-stranded probe by heating 5 min at 100°C. Double-stranded DNA probes are about three times less sensitive than single-stranded probes. High sensitivity in northern blotting is obtained from single-stranded DNA or RNA probes radiolabeled in vitro by ^{32}P. When preparing the radiolabeled probe, consider the half-life, emission type, energy, and specific activity of the radioactive material. The probe concentration in the hybridization solution should be 10 ng/ml if the specific activity is 10^8 dpm/μg, or 2 ng/ml if the specific activity is 10^9 dpm/μg.

CAUTION: When using radiolabeled dNTPs, order only the required amount of isotope for probe preparations. Always wear a laboratory coat and protective shield when preparing and purifying probes. Prepare the probe as soon as the isotope arrives. Follow the general safety guidelines for use and disposal of radioactive materials. See *UNIT 1A.4* for more information.

NORTHERN ANALYSIS OF PLUS- AND MINUS-STRAND

BASIC PROTOCOL

Northern blot analysis is generally performed to detect and quantify the abundance of positive- and negative-sense strand viral RNA transcripts in infected samples. Although there are modifications in northern hybridization protocols adapted by different researchers, the basic steps remain unchanged. It is impossible to follow a universal protocol to detect all types of positive- and negative-sense strand RNA. Therefore, the most common protocol for northern blot analysis for the detection of plus- and minus-strand RNAs is described. RNA is first denatured and fractionated on an agarose gel. Methylmercuric chloride and guanidine thiocyanate are not commonly used for denaturing RNA.

The following methods are most commonly used to separate RNA: (1) electrophoresis of glyoxal/DMSO-denatured RNA on a nondenaturing agarose gel (see Support Protocol 3; McMaster and Carmichael, 1977; Cheng and Tsai, 1999; Cheng et al., 2002; Annamalai et al., 2003; Yeh et al., 2004; Hema et al., 2005; Hsu et al., 2006); (2) electrophoresis of formamide/formaldehyde-denatured RNA on an agarose gel containing formaldehyde (see Support Protocol 4; Boedtker, 1971; Lehrach et al., 1977; Seeley et al., 1992); and (3) electrophoresis of formamide-denatured RNA on a nondenaturing agarose gel and post-treatment of the gel with formaldehyde (see Support Protocol 5). This method is preferred to overcome the problems with formaldehyde during electrophoresis (Kong et al., 1997; Guan et al., 2000; Sun et al., 2005).

CAUTION: Formamide and formaldehyde are toxic, carcinogenic, and can irritate skin, eyes, mucous membranes, and the respiratory tract. Avoid inhalation or direct contact.

Materials

Sample RNA (Support Protocol 1 or 2)
Methylene blue solution (see recipe)
0.2× SSC (*APPENDIX 2A*)
1% (w/v) SDS
Prehybridization solution (see recipe)
Low-stringency wash buffer (see recipe)
High-stringency wash buffer (see recipe)
Strip buffer (see recipe), 70° to 75°C

Blotting paper
UV crosslinker (Stratagene), optional
80°C vacuum oven, optional
Microwave oven, optional
Glass tray
Camera with yellow filter, optional
Hybridization tube
65°C hybridization oven with rotation
Plastic wrap
X-ray film (e.g., Kodak Biomax MS film) and cassette (e.g., Hypercasette, Amersham Biosciences), optional
Densitometer (e.g., GS-700, BioRad), optional
PhosphorImager (Molecular Dynamics, Amersham Pharmacia Biotech)

Additional reagents and equipment for electrophoresis (see Support Protocols 3 to 5); membrane transfer (see Support Protocols 6 and 7); DNA/RNA probe preparation (see Support Protocols 8 and 9)

Electrophorese and transfer RNA

1. Perform electrophoresis of sample RNA as described in Support Protocols 3 to 5.

2. Cut and prepare the membrane and subsequently transfer the RNA from the gel to the membrane by the upward or downward capillary system (see Support Protocols 6 and 7).

   IMPORTANT NOTE: *Always use gloves when handling the membrane.*

   Transfer of denatured RNA from the agarose gel to the surface of a membrane is the critical step in northern blot analysis. Capillary transfer by the upward (see Support Protocol 6, Fig. 16E.3.2) or downward (see Support Protocol 7, Fig. 16E.3.3) system is usually the method of transfer.

Fix RNA to membrane

Fixing RNA to the membrane is not essential for charged membranes since there is a covalent attachment of RNA to the membrane. However, either of the following methods does not affect fixing of RNA in the charged nylon membrane. In contrast, if using neutral or uncharged nylon membranes for transferring RNA, it is necessary to fix the RNA by any of the following methods.

3a. *UV crosslinking:* Place damp membrane on a piece of blotting paper and irradiate at 254 nm for 2 min at 1.5 J/cm^2.

   CAUTION: *UV radiation is dangerous and can damage the retina of the eyes. It is also mutagenic and carcinogenic. Use safety glasses or a UV protection shield.*

   Crosslinking by UV irradiation is highly preferred since fixing results are best and quickly accomplished in a commercial UV crosslinker.

3b. *Baking under vacuum:* Air dry the membrane, place between two pieces of blotting paper, and bake 2 hr under vacuum in a 80°C vacuum oven.

3c. *Baking in a microwave oven:* Place the damp membrane on a dry piece of blotting paper and heat for 2 to 3 min at full-power (750 to 900 W) in a microwave oven.

Any membrane that is not immediately used for hybridization should be dried thoroughly, wrapped in blotting paper, placed in a sealable plastic bag, and can be stored for several months at room temperature, preferably under vacuum.

Check efficiency of transfer (optional)

RNA that has been transferred to the membrane can be visualized by staining with methylene blue (Herrin and Schmidt, 1988) as follows.

4. *Optional:* Transfer the membrane to a glass tray containing methylene blue solution, stain for ~5 min, and photograph under visible light using a yellow filter.
5. Before proceeding to hybridization, destain the membrane by washing for 15 min in 0.2× SSC and 1% SDS at room temperature.

Hybridize membrane

6. Prehybridize the membrane by placing the membrane in a hybridization tube with the RNA-side facing in and add 0.2 ml prehybridization solution/cm^2 membrane. Incubate 1 hr in a 65°C hybridization oven with rotation (about six to eight rotations/min).

   Alternatively, resealable plastic bags can be used on a shaker or rocker in a suitable incubator.

7. Prepare DNA or RNA probe (see Support Protocols 8 and 9). If the probe is already prepared and stored at −70°C, thaw the probe on ice before use.

   Strand-specific oligonucleotide probes are commonly used for hybridization of non-polyadenylated viral RNA (for preparation see Support Protocol 8).

   Double-stranded probes have secondary structure, therefore, to improve the efficiency of hybridization, denature the double-stranded probe by heating 5 min at 100°C. Chill the probe in ice immediately and use it.

   Single-stranded probes usually do not require denaturation.

8. Pipet the ^{32}P-labeled probe directly into the prehybridization solution and continue to incubate with rotation (about six to eight rotations/min) overnight at 42°C (DNA probe) or 65°C (RNA probe).

   Use 10 ng/ml probe if the specific activity is 10^8 dpm/μg or 2 ng/ml if the specific activity is 10^9 dpm/μg.

   If resealable bags are used, the probe can be added by inserting a syringe to the opened plastic bag and the bag should be resealed with great care.

9. Pour off the hybridization solution into a radioactive waste container.

   If resealable bags have been used, the membrane can be transferred to a plastic dish for the following washes.

10. Remove the probe not homologous to the target mRNA by the following stringent washes:

    a. Low-stringency wash: Wash the membrane in a large volume (~50 ml) of low stringency wash buffer for 1 to 2 hr at 37°C with rotation (six to eight rotations/min).
    b. High-stringency wash: Discard the buffer and continue washing two times with ~50 ml high-stringency wash buffer for 1 hr at 65°C.
    c. Remove the membrane from the wash solution. Blot excess liquid and cover the membrane in plastic wrap as quickly as possible.

       If the blot is to be reused, do not allow membrane to dry until the blot has been stripped.

Aquire signal and visualize

11a. *Using X-ray film:* Place the blot into a cassette with intensifying screen and expose to X-ray film at −70°C until a good signal is achieved. To quantify the intensity of the signals, scan the auotoradiogram with GS-700 densitometer.

Tungstate-based intensifying screens are more effective than the rare-earth screens.

To detect minus-strand RNAs, X-rays are exposed for a longer period of time since the abundance of minus-strand RNAs is always lower than that of the positive strands.

11b. *Using a PhosphorImager:* Expose the membrane to a phosphorimager cassette screen at room temperature until a good signal is achieved. Analyze the image by scanning into a PhosphorImager.

Strip probe from membrane

12. If the membrane is to be reused, remove the radiolabeled probes by washing the membrane in 50 ml preheated (70° to 75°C) strip buffer for 1 to 2 hr with two to three buffer changes or until no radioactivity is detected on the membrane.

Biotin- and DIG-labeled probes can also be stripped off from the membrane and the membrane can be used for reprobing.

13. Air dry the membrane and store at room temperature.

A good nylon membrane with RNAs can be stripped and rehybridized at least a dozen times.

SUPPORT PROTOCOL 1

EXTRACTION OF RNA FROM PLANT TISSUE

The success of intact RNA extraction from tissues, cells, or protoplasts depends on the speed of inactivation of cellular RNases. This protocol describes how to obtain total RNA from virus-infected plant tissues (Verwoerd et al., 1989), which is then used as sample RNA for northern analysis.

Materials

Infected plant tissues
Liquid nitrogen
RNA extraction buffer (see recipe), 80°C
24:1 (v/v) chloroform/isoamyl alcohol mixture, 4°C
4 M lithium chloride (LiCl)
DEPC-treated water (*APPENDIX 2A*)
3 M sodium acetate, pH 5.2
Isopropanol/100% ethanol, ice cold
70% ethanol
10 U/µl RNase-free DNase I (Roche)
0.1% to 0.5% SDS in TE buffer, pH 7.6 *or* 0.1 mM EDTA, pH 7.5
Deionized formamide
Sterile mortar and pestle
15-ml propylene snap-cap tube
Centrifuge
Micropipettor

Extract RNA

1. Isolate infected plant tissues by dissection, cut into small pieces, and place immediately in liquid nitrogen.

Frozen tissues can be used immediately or stored at −70°C until use.

2. Transfer 0.5 g frozen tissue to a mortar containing liquid nitrogen and pulverize tissue to a fine powder using a pestle.

3. Immediately transfer the powdered tissue to a 15-ml polypropylene snap-cap tube with a precooled spatula, add 2.5 ml hot (80°C) RNA extraction buffer, and mix the contents vigorously by inverting 30 sec.

4. Add 0.5 ml of 24:1 chloroform/isoamyl alcohol mixture and mix thoroughly. Centrifuge 5 min at 12,000 × *g*, 4°C.

Precipitate RNA

5. Collect upper aqueous phase and add an equal volume of 4 M LiCl. Allow the RNA to precipitate overnight at –20°C or 2 hr at –70°C.

6. After incubation, vortex or thaw the RNA precipitate on ice and centrifuge 15 min at 12,000 × *g*, 4°C.

7. Remove and discard the supernatant with a micropipettor. Dissolve the pellet in 700 µl DEPC-treated water and vortex. Briefly spin down to remove large debris.

8. Add 1/10 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol ice-cold 100% ethanol or 1 vol ice-cold isopropanol. Mix well and precipitate RNA overnight at −20°C or 2 hr at –70°C.

9. Collect the precipitated RNA by centrifuging 15 min at 12,000 × *g*, 4°C. Carefully remove and discared the ethanol/isopropanol with a micropipettor.

   Do not decant the supernatant as the pellet may be lost.

10. Wash the pellet with 1 ml of 70% ethanol, centrifuge 5 min at 12,000 × *g*, 4°C, and remove any remaining ethanol with a micropipettor.

11. Keep the tube open for 10 to 20 min on ice to allow ethanol to evaporate. Do not overdry the pellet as it affects solubilization.

12. Resuspend the RNA pellet in 250 µl DEPC-treated water and store the RNA solution at −70°C until use.

Purify total RNA

13. To avoid DNA contamination, treat the RNA preparation with 1 µl RNase-free DNase I 15 min at 37°C. Purify the RNA by phenol/chloroform extraction and ethanol precipitation. Check the purity and recovery of RNA on an agarose gel containing ethidium bromide.

    RNA preparations may have small amounts of genomic DNA contamination and this may alter the relative migration rate of the RNA in an agarose gel. DNase I treatment is necessary if cellular transcripts are being detected.

Store RNA

14a. Suspend RNA precipitates in 100% ethanol and store indefinitely at –20°C.

14b. Dissolve RNA precipitates in aqueous buffers (0.1% to 0.5% SDS in TE buffer, pH 7.6 or 0.1 mM EDTA, pH 7.5) and store indefinitely at –20°C. Remove SDS by chloroform extraction and ethanol precipitation.

14c. Dissolve the RNA precipitate in deionized formamide and store indefinitely at –20°C.

    Formamide provides stability and protection against degradation by RNases. Formamide can be removed by ethanol precipitation.

SEPARATION OF POLY(A) RNA FROM TOTAL RNA

Poly(A) RNA usually yields better results than total RNA with dot or slot blot analysis. When the abundance of viral mRNA is very low in the infected sample, it is always better to enrich the poly(A) RNA and use it for northern analysis. Poly(A) RNA is separated from total RNA and enriched by affinity chromatography using oligo(dT) cellulose. In general, 1% to 10% of the total RNA is recovered as poly(A) RNA.

Materials

Oligo(dT) cellulose (Ambion, Invitrogen)
10 N sodium hydroxide (NaOH)
40 U/μl RNasin (Promega)
DEPC-treated water (*APPENDIX 2A*)
1× column loading buffer (see recipe)
Total RNA (Support Protocol 1)
Elution buffer (see recipe)
100% ethanol

Platform rocker or rotator
Disposable poly-prep columns (Bio-Rad)

1. Suspend 0.5 to 1.0 g of oligo(dT) cellulose in 0.5 to 1 ml of 0.1 N NaOH in a 1.5-ml microcentrifuge tube and allow to swell for 2 min. Add 1 μl RNasin in column buffer and elution buffer before use.

   The oligo(dT) cellulose should disperse readily (∼1 μg poly(A) RNA binds to 1 mg oligo(dT) cellulose).

2. Wash the oligo(dT) cellulose three times with DEPC-treated water by centrifuging 15 sec at 3000 × *g*, room temperature. Remove the supernatant with a micropipettor after centrifugation.

3. Wash the oligo(dT) cellulose with an equal volume of 1× column loading buffer and centrifuge 15 sec or until the pH is >8.0 (use pH paper for measurement) at 3000 × *g*, room temperature.

4. Denature the total RNA 5 min at 65°C. Cool the solution to room temperature and add an equal volume of 2× column loading buffer.

5. Add the total RNA solution to the oligo(dT) cellulose and rock the tube gently for 30 to 60 min at room temperature.

   Rocking or rotating increases the efficiency of mRNA binding to the oligo(dT) cellulose.

6. Pack the poly(A) RNA–bound oligo(dT) cellulose into a disposable poly-prep column and collect the flow through.

   Store all the flow-through until the eluted fractions are tested for poly(A) RNA.

7. To remove poly(A)$^-$ RNA from the column, wash the column with 5 column volumes of 1× column loading buffer or until the OD_{260} of the final wash fraction (diluted to 1:20) is <0.05 or 0 absorbance.

8. Elute poly(A) RNA from the column with 2 column volumes of elution buffer. Collect the fractions, dilute each fraction 100-fold, and measure A_{260}. Pool the fractions having an absorbance >0.05 and precipitate with 100% ethanol.

   After the removal of poly(A)$^-$ from the matrix, low salt buffer is used to destabilize the double-stranded structure of poly(A) RNA and to elute the poly(A) RNA from the matrix.

9. Dissolve the poly(A) RNA precipitate in 25 to 50 μl DEPC-treated water and store indefinitely at −70°C until use.

NORTHERN HYBRIDIZATION OF GLYOXALATED RNA FRACTIONATED ON A NONDENATURING AGAROSE GEL

Glyoxal is a commonly used denaturant that eliminates the secondary structure of single-stranded RNA. The two aldehyde groups of glyoxal react slightly under acid conditions with the imino groups of guanosine to form a cyclic compound that prevents the formation of intrastrand Watson-Crick bonds (Shapiro and Hachmann, 1966; Nakaya et al., 1968). Glyoxalated RNA is unable to form stable secondary structures and it migrates on agarose gels at a rate that is approximately proportional to the $\log_{10}$ of its size (McMaster and Carmichael, 1977). Integrity of the sample RNA should be checked before adding glyoxal because glyoxal reacts with ethidium bromide. Agarose gel electrophoresis of glyoxalated RNA must be carried out at low ionic strength to prevent super renaturation of the RNA and the commonly used buffers are 10 mM sodium phosphate (pH 6.3) or 40 mM 3-*N*-morpholinopropanesulfonic acid (MOPS), pH 7.0.

Materials

Agarose (SeaKem)
1× sodium phosphate buffer, pH 6.3
Sample RNA, salt-free (Support Protocol 1 or 2)
DEPC-treated water (*APPENDIX 2A*)
Standards
Markers
Glyoxal reaction mix (see recipe)
Nondenaturing gel loading buffer (see recipe)
10× SSC (*APPENDIX 2A*)
55°C water bath
2-mm comb
Horizontal electrophoresis apparatus for 20 × 10–cm gels (built-in recirculation system, optional)
Peristaltic pump, optional
1.5-ml microcentrifuge tubes
Glass plates

Prepare gel

1. To prepare 150 ml of a 1% agarose gel (20 × 10–cm), add 1.5 g SeaKem agarose to 150 ml of 1× sodium phosphate buffer, pH 6.3, and microwave until agarose is completely dissolved.

2. Cool agarose to 55°C in a 55°C water bath and cast the gel using a 2-mm comb with a sufficient number of teeth for all samples to be analyzed, including a few extra wells for running standards and markers. Allow the gel to solidify for at least 1 hr at room temperature.

   The gel should be 3- to 10-mm thick. Thin gels (<3-mm thick) do not support efficient transfer of RNA to the membrane and also tend to deform during RNA transfer. When preparing low concentration (i.e., 0.5%) agarose gels, first prepare a supporting gel (i.e., 1%) without wells. After the gel hardens, pour the lower percentage gel directly on top of the supporting gel. Stacked gels avoid fracturing during handling. It is important that both gels be prepared from the same batch of 1× running buffer.

3. After the gel solidifies, remove the comb, place the gel in the electrophoresis tank, and add 1× sodium phosphate buffer to cover the gel to a depth of ~1 mm.

   Set up a peristaltic pump if the electrophoresis system does not have a built-in recirculation system.

Prepare RNA

4. Dissolve salt-free sample RNA in DEPC-treated water and prepare the denaturation mix in a 0.2-ml microcentrifuge tube as follows (prepare standards and markers in the same manner):

5 µl	RNA (1 to 10 µg)
10 µl	glyoxal reaction mix

Loading of >10 µg RNA per lane causes smearing of the RNA during electrophoresis.

5. Incubate RNA solutions 1 hr at 55°C. Cool the mixture 10 min on ice and briefly centrifuge at 7000 × *g*, 4°C.

Perform electrophoresis

6. To the glyoxylated RNA mix, add 3 µl nondenaturing gel loading buffer, mix well, and immediately load into the wells of the gel. Load standards and markers on the extreme wells of the gel to enable easy excision of marker lanes.

7. Perform electrophoresis at 4 to 5 V/cm until the bromophenol blue has migrated to ~8 cm, circulating the buffer during the run. As the gel is running, cut the membrane, blotting papers, and paper towels (see Support Protocols 6 and 7).

The major drawback of using glyoxal as a denaturing agent is the upward shift in pH of the electrophoresis buffer that occurs in the cathodic chamber during elecrophoresis. If this shift occurs, a steep pH gradient will form as small ions in the buffer migrate rapidly along the gel from the cathode, resulting in removal of the glyoxal from the RNA (Nakaya et al., 1968). Therefore, it is necessary to recirculate the buffer mechanically.

Check RNA integrity after electrophoresis (optional)

8. Cut the lanes containing marker and use for acridine orange staining. Visualize the RNA in a UV transilluminator. Align a UV-transparent ruler with the stained gel and photograph the gel. Measure the distance of each marker band from the loading well. Plot the $\log_{10}$ of the size fragments of RNA against the distance migrated. Using the resulting curve, calculate the size of the viral mRNA after hybridization.

CAUTION: *UV radiation is dangerous and can damage the retina of the eyes. It is also mutagenic and carcinogenic. Use safety glasses or a UV protection shield.*

Staining glyoxalated RNA with ethidium bromide is insensitive because ethidium bromide reacts with glyoxal and results in high background (McMaster and Carmichael, 1977). Therefore, acridine orange is a better choice to stain RNA (Richards et al., 1965) but requires extensive washing of the gel before transfer of RNA onto the membrane.

Prepare gel for RNA transfer

9. Move the gel to a glass plate and excise unnecessary areas with a scalpel. Cut along the wells and a small triangle at the bottom left-hand corner to ensure the orientation. Immerse the gel into 10 gel volumes of 10× SSC for 20 min.

SUPPORT PROTOCOL 4

NORTHERN HYBRIDIZATION OF RNA FRACTIONATED ON AN AGAROSE-FORMALDEHYDE GEL

Sample RNAs denatured with formamide are separated by agarose-formaldehyde gels. Formamide is an ionizing solvent in aqueous buffers, which is generally used to denature RNA. Formaldehyde forms unstable Schiff bases with a single imino group of guanine residues that maintain the denatured state of RNA. Schiff bases are unstable and easily removed by dilution with formaldehyde. The denatured state of RNA is maintained only in the presence of formaldehyde in the buffer. A disadvantage of using agarose-formaldehyde gels are that the gels are more fragile, therefore, great care is necessary when handling them. Formaldehyde diffuses from the gel into the buffer during

electrophoresis. However, diffusion can be overcome by running gels for shorter periods of time at a higher voltage (7 to 10 V/cm). Agarose-formaldehyde gels are preferred for northern analysis because formaldehyde can be easily removed from the gel after electrophoresis.

Materials

Agarose (SeaKem)
DEPC-treated water (*APPENDIX 2A*)
1× and 10× MOPS buffer, pH 8.0 (see recipe)
Formladehyde (deionized; see recipe)
Sample RNA, salt-free (Support Protocol 1 or 2)
Standards
Markers
Formamide, stored indefinitely at –20°C
Formaldehyde gel loading buffer (see reicipe)
55°C water bath
Horizontal electrophoresis apparatus with 20 × 10–cm gel capacity (built-in recirculation system, optional)
2-mm comb
Peristaltic pump, optional
1.5-ml microcentrifuge tubes
Glass plate

Prepare gel

1. Prepare 150 ml of 1% agarose gel (20 × 10–cm) containing 2.2 M formaldehyde by adding 1.5 g SeaKem agarose to 115 ml DEPC-treated water and microwaving until agarose completely dissolves. Cool to 55°C and add 15 ml of 10× MOPS buffer and 27 ml deionized formaldehyde.

   When preparing low concentration (0.5%) agarose gels, first prepare a supporting gel (1%) without wells. After the gel hardens, pour the lower percentage gel directly on top of the supporting gel. Stacked gels avoid fracturing during handling. It is important that both gels be prepared from the same batch of 1× running buffer.

2. Cool to 55°C and cast the gel using a 2-mm comb with a sufficient number of teeth for all samples to be analyzed, including a few extra wells for running standards and markers. Allow the gel to solidify for at least for 1 hr at room temperature.

3. Remove the comb, place the gel in the electrophoresis tank, and add 1× MOPS buffer to cover the gel to a depth of ~1 mm.

   Set up a peristaltic pump if the electrophoresis system does not have a built-in recirculation system.

Prepare RNA

4. Prepare denaturation mix as follows (prepare standards and markers in the same manner) in a 1.5-ml microcentrifuge tube:

1.0 µl	RNA (1 to 20 µg)
1.0 µl	10× MOPS buffer
2.0 µl	formaldehyde (deionized)
5.0 µl	formamide

   Loading of >20 µg RNA per lane causes smearing of the RNA during electrophoresis.

   CAUTION: *Formamide is an irritant and destroys skin, mucous membrane, and the respiratory tract. Avoid direct contact.*

5. Incubate the mixture 1 hr at 55°C. Cool the mixture on ice and briefly microcentrifuge to collect the mixture at the bottom of the tubes.

Perform electrophoresis

6. Add 1 µl formaldehyde gel loading buffer to each sample, mix well, and place tubes on ice. Load standards and markers in the extreme wells of the gel to enable easy excision of marker lane.

7. To prevent diffusion of formaldehyde from the gel into the buffer, electrophorese at 7 to 10 V/cm. To avoid change of pH in the electrophoresis buffer, circulate the buffer. As the gel is running, cut membrane, blotting paper, and paper towels (see Support Protocols 6 and 7).

Check RNA integrity after electrophoresis (optional)

8. Cut the lanes containing the marker and use for acridine orange staining. Visualize the RNA on a UV transilluminator. Align a transparent ruler with the stained gel and photograph the gel. Measure the distance of each marker band from the loading well. Plot the $\log_{10}$ of the size fragments of RNA against the distance migrated. Using the resulting curve, calculate the size of the viral mRNA after hybridization.

   CAUTION: UV radiation is dangerous and can damage the retina of the eyes. It is also mutagenic and carcinogenic. Use safety glasses or a UV protection shield.

   Some investigators use ethidium bromide in gels containing 0.6 M formaldehyde. Ethidium bromide should not be used when the concentration of formaldehyde is >0.6 M because the background signal is too high to detect very small amounts of RNA in the gel. Therefore, acridine orange is a better choice to stain RNA (Richards et al., 1965) but requires extensive washing of the gel before transfer of RNA onto the membrane.

Prepare gel for transfer

9. Remove formaldehyde in the gel by soaking the gel four times, 5 min each soak, in 10 gel volume of distilled water. Move the gel to a glass plate and excise unnecessary areas of the gel with a scalpel. Cut along the wells and a small triangle at the bottom left-hand corner to ensure the orientation.

SUPPORT PROTOCOL 5

NORTHERN HYBRIDIZATION OF FORMAMIDE-DENATURED RNA FRACTIONATED ON A NONDENATURING AGAROSE GEL

The disadvantages of using agarose-formaldehyde gels are that the fumes from the formaldehyde are unpleasant and highly toxic, the gels are more fragile and tend to distort during manipulations, and formaldehyde diffuses from the gel into the buffer during electrophoresis, which in turn may lead to renaturation of RNA. Therefore, some investigators prefer to denature the RNA with formamide and fractionate the RNA on a nondenaturing agarose gel, and then post-treat the electrophoresed gel in formaldehyde (Kong et al., 1995; Zhang et al., 2004; Sun et al., 2005). Formamide is an ionizing solvent in aqueous buffers, which is generally used to denature RNA. Formaldehyde forms unstable Schiff bases with a single imino group of guanine residues that maintain the denatured state of RNA. Schiff bases are unstable and easily removed by dilution with formaldehyde.

Materials

Agarose (SeaKem)
1× sodium phosphate buffer, pH 6.3 (*APPENDIX 2A*)
Sample RNA, salt-free (Support Protocol 1 or 2)
DEPC-treated water (*APPENDIX 2A*)
50% to 70% formamide, stored indefinitely at –20°C
Standards

Markers
Nondenaturing gel loading buffer (see recipe)
6% formladehyde (deionized; see recipe)
10× SSC (*APPENDIX 2A*)
55°C water bath
Horizontal electrophoresis apparatus with 20 × 10–cm gel capacity (built-in recirculation system, optional)
2-mm comb
Peristaltic pump, optional
1.5-ml microcentrifuge tubes
Glass plate

Prepare gel

1. To prepare 150 ml of a 1.5% agarose gel (20 × 10–cm), add 2.25 g SeaKem agarose to 150 ml of 1× sodium phosphate buffer, pH 6.3, and microwave until completely dissolved.

2. Cool to 55°C and cast the gel using a 2-mm comb with a sufficient number of teeth for all samples to be analyzed, including a few extra wells for running standards and markers. Allow the gel to solidify for at least 1 hr at room temperature.

   The gel should be 3- to 10-mm thick. Thin gels (<3-mm thick) do not support efficient transfer of RNA to the membrane and also tend to deform during RNA transfer. When preparing low concentration (0.5%) agarose gels, first prepare a supporting gel (1%) without wells. After the gel hardens, pour the lower percentage gel directly on top of the supporting gel. Stacked gels avoid fracturing during handling. It is important that both gels are prepared from the same batch of buffer.

3. Remove the comb, place the gel in the electrophoresis tank, and add 1× sodium phosphate buffer, pH 6.3, to cover the gel to a depth of ~1 mm.

   Set up a peristaltic pump if the electrophoresis system does not have a built-in recirculation system.

Prepare RNA

4. Dissolve salt-free RNA in appropriate volume of DEPC-treated water in a 1.5-ml microcentrifuge tube. Denature total RNA by heating 1 hr at 55°C in 50% to 70% formamide. Cool the RNA 10 min on ice and collect the mixture at the bottom of the tube by briefly centrifuging at 7000 × *g*, 4°C. Prepare standards and markers in the same manner.

   CAUTION: *Formamide is an irritant and destroys skin, the mucous membrane, and the respiratory tract. Avoid direct contact.*

Perform electrophoresis

5. To the denatured RNA mix, add 3 μl of nondenaturing gel loading buffer, mix well, and immediately load into the wells of the gel. Load standards and markers on the extreme wells of the gel to enable easy excision of marker lanes.

6. To avoid change of the electrophoresis buffer pH, recirculate the buffer. Run the gel at 4 to 5 V/cm until the bromophenol has migrated to ~8 cm. As the gel is running, cut membrane, blotting paper, and paper towels (see Support Protocols 6 and 7).

Check RNA integrity after electrophoresis (optional)

7. Cut the lanes containing the marker and use for acridine orange staining. Visualize the RNA on a UV transilluminator. Align a transparent ruler with the stained gel and photograph the gel. Measure the distance of each marker band from the loading

well. Plot the $\log_{10}$ of the size fragments of RNA against the distance migrated. Using the resulting curve, calculate the size of the viral mRNA after hybridization.

CAUTION: UV radiation is dangerous and can damage the retina of the eyes. It is also mutagenic and carcinogenic. Use safety glasses or a UV protection shield.

Prepare gel for transfer

8. After electrophoresis, rinse the gel with water and incubate the gel in 5 gel volumes of 6% formaldehyde for 1 hr at room temperature.
9. Soak the gel in 10 gel volumes of 10× SSC for 20 min at room temperature.
10. Move the gel to a glass plate and excise unnecessary areas of the gel with a scalpel. Cut along the wells and a small triangle at the bottom left-hand corner to ensure the orientation.

SUPPORT PROTOCOL 6

RNA TRANSFER USING AN UPWARD CAPILLARY SYSTEM

Set up the system as shown in Figure 16E.3.2. Upward capillary transfer is generally preferred for transferring short RNA species (<1 kb), however, the transfer time is longer, generally overnight. A major limitation of the upward capillary system is retention of large RNA molecules within the gel. However, it can be overcome by using thin gels, i.e., 3- to 10-mm thick, through buffer saturation of filter paper with immediate contact with the gel, or by partial hydrolysis of RNA by alkali treatment (Reed and Mann, 1985). Another serious problem is that the RNA may descend into the buffer, which can be avoided by buffer saturation of the filter paper beneath the gel. In a downward capillary system, transfer of RNA from agarose gel to membrane is by the downward flow of buffer and the transfer is faster and more efficient for longer RNA species (>15 kb).

Materials

2× and 20× SSC (*APPENDIX 2A*)
Gel (Support Protocols 3 to 5)
Nylon membrane
Glass plates
Plastic wrap
Blotting paper (Schleicher & Schuell GB002)
Thick blotting paper (Whatman 3 MM, Schleicher & Schuell GB004)
Glass rod or pipet
Paper towels and ~500-g weight

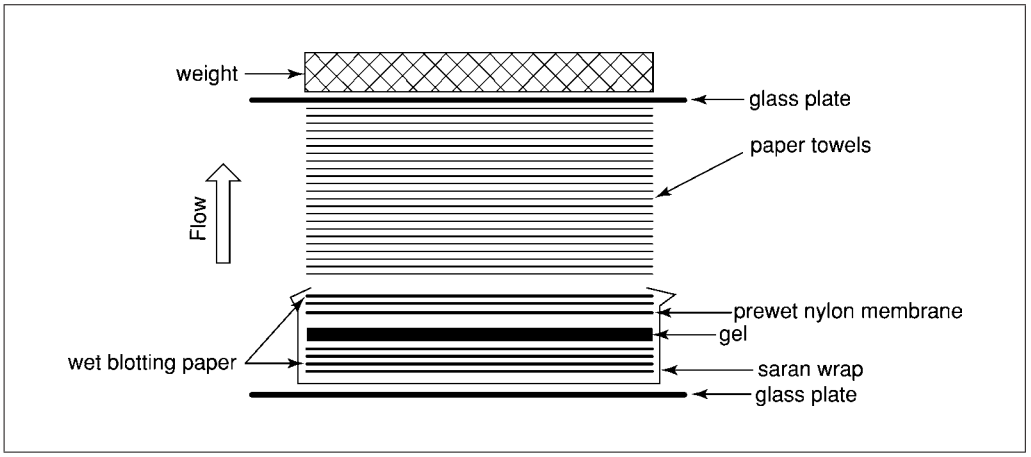

Figure 16E.3.2 Upward capillary transfer. Capillary transfer of RNAs from the agarose gel to the membrane is achieved by an upward flow of surrounding buffer through the gel to the stack of paper towels.

1. On a glass plate, spread out a piece of plastic wrap large enough to surround the gel.

   This barrier is necessary to prevent buffer flow directly from the reservoir to the paper towels stacked on top of the gel.

2. Cut a piece of blotting paper that is longer and wider than the gel, soak in 2× SSC, and place in the center of the plastic wrap.

3. Cut three thick pieces of blotting paper similar to the size of the gel, soak in 2× SSC, and place in the center of the presoaked larger blotting paper.

4. Place the gel on the wet thick blotting paper. Remove air bubbles between the gel and the blotting paper by rolling a glass rod/pipet over them.

5. Cut a piece of membrane ~5 mm larger than the gel, soak in 2× SSC for 2 min.

   Float the membrane on the surface of the buffer and then immerse the membrane into the buffer. If the membrane is not saturated after floating for 2 min, then use a new membrane as the unsaturated membrane affect the transfer of RNA.

6. Wet the top of the gel with 2× SSC and place the wet membrane on top of the gel so that the corners are aligned. Avoid trapping air bubbles under the membrane. Never move the membrane once it has been placed on top of the gel.

7. Cut three thick blotting papers similar to the size of the gel, soak in 2× SSC, and place on top of the membrane without any bubbles between the membrane and blotting paper.

   Do not disturb the gel once the membrane has been placed on top of it.

8. Pour sufficient 20× SSC (transfer buffer) on the plastic wrap. Surround the gel with the plastic wrap but do not cover the gel.

9. Cut a stack of paper towels (5- to 10-cm high), the size of the gel, and place the stack on top of the wet thick blotting papers.

   Do not soak the paper towels in buffer. Precisely pack the paper towels and avoid extra over the edge of the gel, which will affect the efficiency of RNA trasfer.

10. Place a thin glass plate on top of the paper towels to prevent evaporation.

11. Over the glass plate, place an ~500-g weight on top. Ensure that the setup is balanced. Transfer RNA overnight. Do not disturb the setup.

12. After the transfer, disassemble the capillary system. Mark the positions of the wells on the membrane in a corner with a pencil. Remove the membrane, drain the buffer, and place the membrane RNA-side up on a piece of dry blotting paper.

RNA TRANSFER USING A DOWNWARD CAPILLARY SYSTEM

SUPPORT PROTOCOL 7

Set up the downward capillary system as in Figure 16E.3.3.

RNA molecules of up to 8 kb are transferred with high efficiency within 1 to 4 hr in alkaline (e.g., 8 mM NaOH with 3M NaCl) or neutral (e.g., SSC) pH buffer (Chomczynski and Mackey, 1994). Alkaline transfer of RNA for >4 hr decreases the strength of the hybridization signal.

Materials

Nylon membrane
Gel (see Support Protocols 3 to 5)
20× SSC (*APPENDIX 2A*)

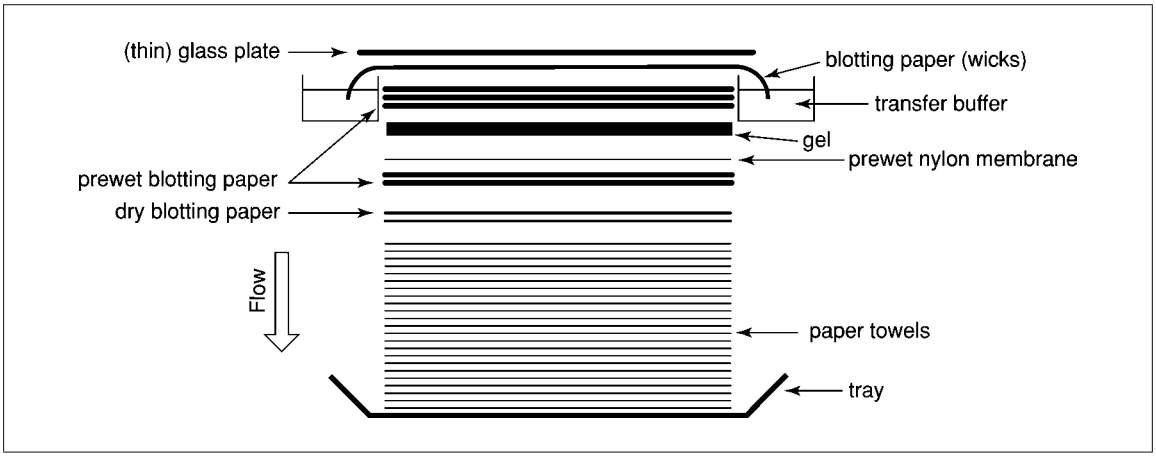

Figure 16E.3.3 Downward capillary transfer. Capillary transfer of RNAs from the agarose gel to the membrane is achieved by a downward flow of buffer from the reservoir through the gel into the stack of paper towels.

Glass tray (30 × 20–cm)
Thick blotting paper (Whatman 3 MM, Schleicher & Schuell GB004)
Blotting paper (Schleicher & Schuell GB002)
Glass rod or pipet
Resevoir troughs
Thin glass plate

1. Prepare a 3-cm stack of paper towels 1 to 2 cm larger than the gel. Place the stack of paper towels on the center of a glass tray.
2. On top of the stack, place four pieces of dry thick blotting paper 1 to 2 cm larger than the gel.
3. Over the dry thick blotting paper, place three pieces of thick blotting paper 1 to 2 cm larger than the gel and soaked in 20× SSC.
4. Place a presoaked nylon membrane (~5 mm larger than the gel) exactly on top of the wet blotting paper.

   To soak the membrane, float the membrane on the surface of 20× SSC and then immerse the membrane. If the membrane is not saturated after floating for 2 min, then use a new membrane, as unsaturated membranes affect the transfer of RNA.
5. Place the gel on top of the membrane so that the cut corners are aligned. Ensure that air bubbles are not present between the membrane and the gel. If air bubbles are present, remove by rolling a glass rod or pipet over the gel. Do not disturb the gel once it has been placed on top of the membrane.
6. Wet the top surface of the gel with 20× SSC and cover with three thick blotting papers the size of the gel and soaked in 20× SSC.
7. Connect the stack to the reservoir troughs containing 20× SSC with two large pieces of wet thick blotting papers larger than the gel as wicks.
8. Place a thin glass plate on top of the stack to prevent evaporation.
9. Allow downward transfer of RNA to occur for 4 hr.

   During transfer, check whether adequate buffer is present in the troughs. If necessary, gently add buffer directly to the troughs without disturbing the capillary system.

10. After the transfer, disassemble the capillary system. Mark the position of the wells in a corner on the membrane with a pencil. Remove the membrane, drain the buffer, and place membrane, RNA-side up, on a piece of dry blotting paper.

PREPARATION OF OLIGONUCLEOTIDE PROBE BY END-LABELING

SUPPORT PROTOCOL 8

Synthetic oligonucleotides lack phosphate groups at the 5′ termini. Therefore, the 5′ termini can be labeled by transferring γ-^{32}P from [γ-^{32}P]ATP in a reaction catalyzed by bacteriophage T4 polynucleotide kinase. Oligonucleotides with a cytosine residue at their 5′ termini are four-fold less efficiently labeled than oligonucleotides with an A or T at their 5′ termini, and six-fold less efficiently labeled than oligonucleotides with a G at their 5′ termini. Under standard conditions, >50% of the oligonucleotides are radiolabeled. This protocol describes how to radiolabel an oligonucleotide probe.

CAUTION: Perform the reaction and purify the oligonucleotide probe behind a plexiglass screen as the mixture contains [γ-^{32}P]ATP. See *UNIT 1A.4* for more information.

Materials

100 pmol/μl synthetic oligonucleotides
Bacteriophage T4 polynucleotide kinase and 10× bacteriophage T4 polynucleotide kinase buffer (New England BioLabs)
>5000 Ci/mmol [γ-^{32}P]ATP
1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
5 M ammonium acetate
70% and 100% ethanol, ice cold
TE buffer, pH 7.6 (*APPENDIX 2A*)
1.5-ml microcentrifuge tubes
37° and 68°C water baths
Additional reagents and equipment to measure specific activity (see Support Protocol 9)

1. Set up the following reaction mix in a 1.5-ml microcentrifuge tube:

1 μl	Synthetic oligonucleotide (100 pmol/μl)
2 μl	10× bacteriophage T4 polynucleotide kinase buffer
15 μl	[γ-^{32}P]ATP (specific activity >5000 Ci/mmol)
1.5 μl	H_2O

   Mix gently by tapping the bottom of the tube.

2. Remove 0.5 μl of the reaction mix and transfer to a new tube containing 10 μl of 1 M Tris·Cl, pH 8.0, and keep aside.

   This fraction can be used as a control while measuring the labeling efficiency of the probe.

3. Add 10 U bacteriophage T4 polynucleotide kinase to the reaction mix. Mix well and incubate 1 hr at 37°C.

4. At the end of the incubation period, heat the reaction mix 10 min at 68°C to inactivate the ploynucleotide kinase and store the tube on ice. Measure the specific activity of the oligonucleotides (see Support Protocol 9).

   The oligonucleotide probe is now radiolabeled.

5. Add 40 μl water to the tube containing the radiolabeled oligonucleotide and mix well.

6. Add 240 µl of 5 M ammonium acetate and mix well.
7. Add 750 µl of ice-cold 100% ethanol, mix well, and incubate 30 min on ice.
8. Centrifuge mixture 15 min at 12,000 × *g*, 4°C. Carefully remove the supernatant using a micropipettor.

   The supernatant contains most of the unincorporated [γ-^{32}P]ATP.
9. Wash the pellet with 0.5 ml of ice-cold 70% ethanol and centrifuge 5 min at 12,000 × *g*, 4°C.
10. Remove the ethanol using a micropipettor. Keep the tube open on ice until the ethanol evaporates.
11. Dissolve the radiolabeled oligonucleotide probe in 100 µl TE buffer, pH 7.6.

    The probe can be used immediately or stored at −70°C until the half-life of the radiolabeled compound.

SUPPORT PROTOCOL 9

PREPARATION OF RIBOPROBE BY IN VITRO TRANSCRIPTION

Strand-specific RNA probes are synthesized by in vitro transcription of linearized recombinant plasmids (Fig. 16E.3.4). To detect plus (sense)- or minus (antisense)-strand RNAs, radiolabeled transcripts complementary to the plus- or minus-strands are synthesized from the template DNA that is located immediately downstream of the bacteriophage promoter. Plasmids are linearized with respective restriction enzymes (see Background Information for selecting enzymes) and probes are prepared using respective DNA-dependent RNA polymerase.

Materials

Plasmid DNA
Restriction enzymes
DEPC-treated water (*APPENDIX 2A*)
5 mM ATP, GTP, and UTP (*APPENDIX 2A*)
100 µM CTP
10× transcription buffer
20 mCi/µl or 3000 Ci/mM [α-^{32}P] rCTP
40 U/µl RNasin (Promega)
15 U/µl T7/SP6 DNA-dependent RNA polymerase
10 U/µl RNase-free DNase I (Roche)
Scintillation fluid
37°C (for T7/T3 polymerase) or 40°C (for SP6 polymerase) water bath
Nuc-Trap push column (Stratagene), optional
Scintillation counter
Whatman filter paper disk (DE81 filter, Whatman)
Additional reagents and equipment for restriction digestion and purifying DNA (Moore and Dowhan, 2002)

1. Digest plasmid DNA with a restriction enzyme that cuts downstream of the desired probe sequence. Extract digested DNA with phenol/chloroform, precipitate with ethanol, and redissolve DNA in DEPC-treated water to a final concentration of 1 µg/µl (Moore and Dowhan, 2002).

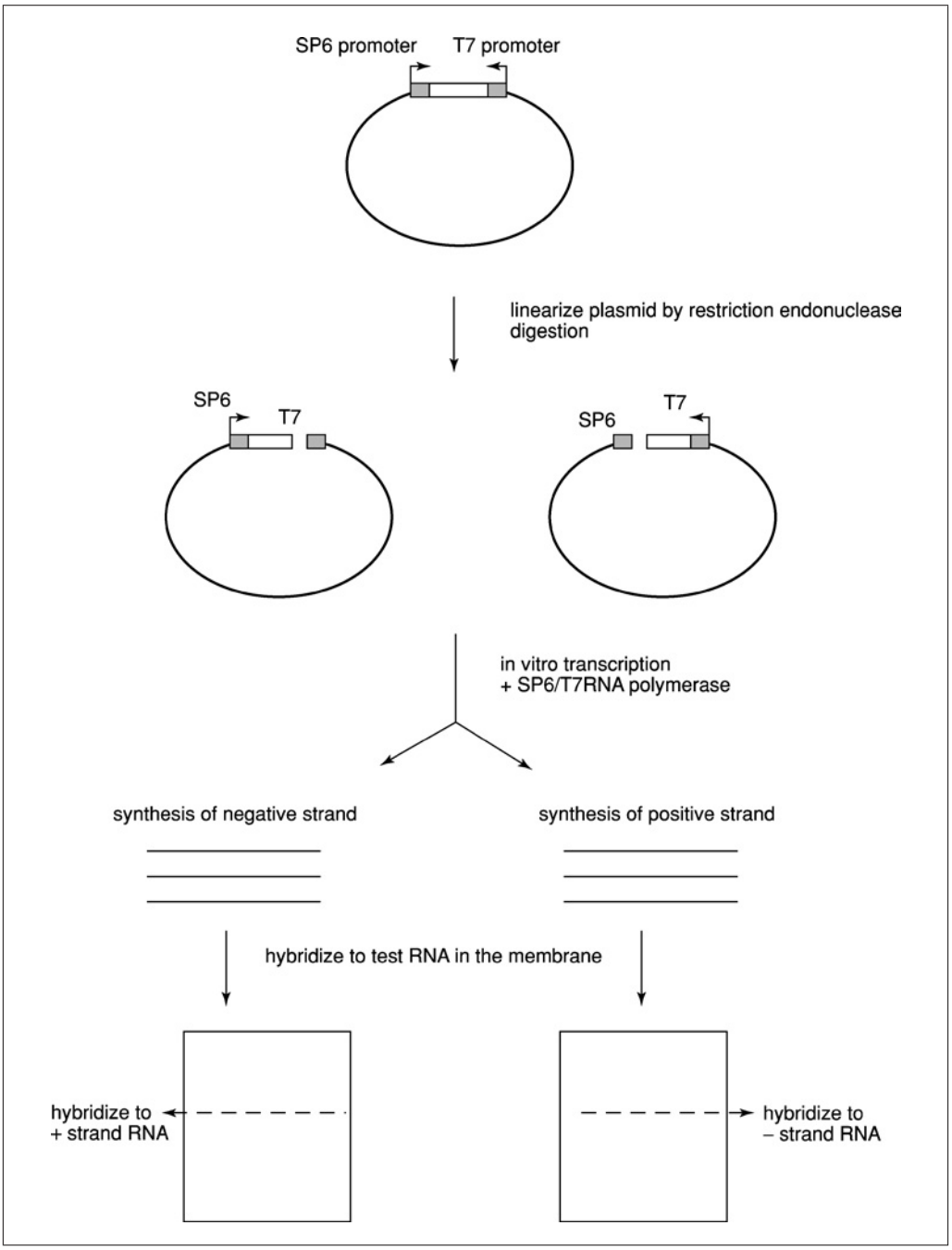

Figure 16E.3.4 Synthesis of riboprobes by in vitro transcription. Linearized plasmid encoding bacteriophage promoter (T7, T3, or SP6) at the 5′ terminus and defined restriction enzyme site at the 3′ terminus of the target DNA is used as template. Respective DNA-dependent RNA polymerases are used in the reaction mix, which recognizes and initiates the synthesis of RNA complementary to one strand of the template DNA that is located immediately downstream from the promoter. The reaction is performed in the presence of rNTPs, where one is radiolabeled. To detect the plus- and minus-strand RNAs in the membrane, radiolabeled complementary transcripts are generated and used as probes in the hybridization analysis.

2. Prepare the transcription reaction mix behind a plexiglass screen in the order given:

1.0 µl	Linearized DNA (1 µg)
10.0 µl	DEPC-treated H_2O
5.0 µl	5 mM ATP, GTP, and UTP + 100 µM CTP
2.5 µl	10× transcription buffer
5.0 µl	[α-^{32}P] rCTP (20 mCi/µl or 3000 Ci/mM)

Mix the components at room temperature by gently tapping the tube, then add:

0.5 µl	RNasin (40 U/µl)
1.0 µl	T7/SP6 DNA-dependent RNA polymerase (15 U/µl)

3. Briefly centrifuge to bring the contents to the bottom of the tube and incubate 1 hr at 37°C (T7/T3 DNA-dependent RNA polymerase) or 40°C (SP6 DNA-dependent RNA polymerase).

4. Terminate the reaction by adding 1 µl RNase-free DNase I to the reaction tube. Gently tap the tube and incubate 15 min at 37°C.

5. Purify the riboprobe by ethanol precipitation, column chromatography (e.g., Nuc-Trap push column), or elution after polyacrylamide gel electrophoresis.

   Purification by column chromatography is safer and facilitates the removal of unincorporated isotopes.

6. After purification of the riboprobe, place 1 µl of the riboprobe on a Whatman filter paper disk and allow to dry. Transfer the disk into the vial containing scintillation fluid and count the radioactivity using a scintillation counter.

   The Whatman filter paper disk is positively charged and strongly adsorbs and retains nucleic acids. Unincorportaed nucleotides adsorb less tightly and can be washed in 0.5 M Na_2HPO_4 (pH 7.0).

7. Calculate the specific activity of the radiolabeled probe (Sambrook and Russel, 2001) using the following equation:

$$\text{Specific activity of the probe} = \frac{L\ (2.2\times10^9)\ (PI)}{m + [(1.3\times10^3)\ (PI)\ (L/S)]}$$

where, L = input radioactive label (µCi), PI = proportion of the precursor that has been incorporated (cpm in washed filter/cpm in unwashed filter), m = mass of template DNA (ng), and S = specific activity of input label (µCi/nmol).

The numerator of this equation is the product of three terms: the total dpm in this reaction [L (2.2 × 10^6 cpm/(µCi)], the proportion of this dpm which was incorporated (PI), and a factor to convert the final value for specific activity from cpm/ng to cpm/µg (10^3).

The denominator represents: the total mass of RNA (ng) at the end of the reaction, equal to the starting mass of DNA (ng) synthesized during the reaction. The latter is calculated from the number of nanomoles of rNMP incorporated [(PI)(L/S)] multiplied by four times the average molecular mass of the four rNMPs (4× 325 ng/nmol = 1.3 × 10^3/ng/nmol).

8. Use the probe immediately for hybridization or store at –70°C until the half-life of [α-^{32}P] CTP.

SUPPORT PROTOCOL 10

PREPARATION OF TOTAL RNA TO DETECT LOW ABUNDANT RNA SPECIES

Generally, in virus-infected cells, an asymmetric ratio of plus- and minus-strands exists ranging from 10:1 to 1000:1. However, in some cases, accumulation of plus-strand RNA may also be as low as the minus-strand RNAs. Therefore, to increase the probing specificity, low abundant plus- or minus-strand RNAs can be protected and used to measure the abundance (Ishikawa et al., 1991; Guan et al., 2000). The purpose of this technique is to hybridize the sample RNAs to complementary RNA or riboprobe, followed by digestion of nonhybridized RNA with ribonuclease. After digestion, ribonuclease is

inactivated and the protected RNA is incubated with the riboprobe, if it has not been previously incubated with the riboprobe, and then used for gel analysis.

Materials

Sample RNA (Support Protocol 1)
^{32}P-labeled riboprobe (see Support Protocol 9)
Annealing buffer A (see recipe)
RNase A
Annealing buffer B (see recipe)
Proteinase K
SDS
Viral RNA
Annealing buffer C (see recipe)
30°, 37°, 45°, 60°, 80°, and 100°C water baths
Additional reagents and equipment for purifying DNA (Moore and Dowhan, 2002)

To prepare sample RNA for detecting low abundant plus-strand RNAs:

1a. Incubate the DNase I-treated sample RNA with 0.2 pmol of ^{32}P-labeled riboprobe in 20 μl of annealing buffer A 5 min at 100°C and then 20 min at 45°C.

2a. Remove the single-stranded RNAs (ssRNA) in the mixture by digesting with 20 μg RNase A in 200 μl annealing buffer B 30 min at 30°C.

3a. Terminate RNase digestion with 30 μg of proteinase K and SDS (0.4% final concentration) 10 min at 37°C.

4a. Recover the protected dsRNA by phenol-chloroform extraction and ethanol precipitation (Moore and Dowhan, 2002).

5a. Separate the RNA in an agarose gel, transfer the RNA to the membrane (see Support Protocols 6 and 7), and detect the plus-strand RNAs by autoradiography.

To prepare sample RNA for detecting low abundant minus-strand RNAs:

1b. Incubate DNase I-treated sample RNA with an excess amount (0.3 μg) of viral RNA in 30 μl annealing buffer C with the following sequential incubations: 5 min at 80°C, 10 min at 60°C, and 10 min at 37°C.

2b. Remove ssRNAs with 1 μg/ml RNase A for 30 min at 30°C.

3b. Terminate RNase digestion with 30 μg of proteinase K and SDS (0.4% final concentration) for 10 min at 37°C.

4b. Recover the protected dsRNAs by phenol-chloroform extraction and ethanol precipitation (Moore and Dowhan, 2002).

5b. Mix dsRNAs with 0.07 pmol of ^{32}P-labeled riboprobe in 20 μl of annealing buffer B and incubate the mixture 5 min at 100°C to denature the dsRNAs and then incubate 1 hr at 45°C to anneal the minus-strand RNA with the probe.

6b. Separate the RNA on an agarose gel, transfer the RNA to a membrane (see Support Protocols 6 and 7), and detect the minus-strand RNAs by autoradiography.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Annealing buffer A

40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.4
0.4 M NaCl
1 mM EDTA
80% formamide
Prepare fresh

Annealing buffer B

0.2 M NaCl
0.1 M LiCl
10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM EDTA
Prepare fresh

Annealing buffer C

0.3 M KCl
50 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM EDTA
Prepare fresh

Bakers yeast RNA, 20 mg/ml

1 g bakers yeast RNA (Sigma)
50 ml TE buffer, pH 8.0 (*APPENDIX 2A*)
Store in 25-ml aliquots indefinitely at −20°C

Column loading buffer, 2×

40 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
1 mM NaCl
2 mM EDTA
0.2% SDS
Prepare fresh stock solutions of Tris·Cl, NaCl, and EDTA, and autoclave. Dilute with sterile DEPC-treated water and cool to 65°C, then add SDS from 10% stock solution.

Denatured salmon DNA, 10 mg/ml

0.5 g salmon sperm DNA (Sigma)
50 ml TE buffer, pH 8.0 (*APPENDIX 2A*)
Shear in syringe with 18-G needle
Stir 2 to 4 hr at room temperature and store in 25-ml aliquots indefinitely at –20°C
Before use, boil 5 min and chill quickly

Elution buffer

10 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
1 mM EDTA
0.05% SDS
Prepare fresh stock solutions of Tris·Cl and EDTA, and autoclave. Dilute with sterile DEPC-treated water and cool to 65°C, then add SDS from 10% stock solution.

Formaldehyde

Purchase 37% to 40% (w/v) or 12.3 M formaldehyde solution (Bio-Rad). Formaldehyde readily oxidizes when exposed to air and becomes yellow (pH < 4.0). Use fresh stock solution.

Formaldehyde gel loading buffer, 10×

50% fresh glycerol
10 mM EDTA
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
Store in 0.1-ml aliquots indefinitely at −20°C

Glyoxal, 6 M, deionized

Glyoxal is purchased as a 40% or 6 M solution, which may contain various hydrated forms of glyoxal and oxidation products such as glyoxalic acid, formic acid, and other compounds, which may degrade RNA. Minimize exposure of glyoxal to air and use deionized glyoxal. Store in 1-ml aliquots indefinitely at −20°C.

Glyoxal reaction mix

100 µl 6 M deionized glyoxal (see recipe)
300 µl DMSO
12 µl 1 M phosphate buffer, pH 6.3 (see receipe)
Prepare fresh

CAUTION: *DMSO (dimethylsulfoxide) is a harmful inhalent, absorbs into the skin, and is combustible. Store in a tightly closed container.*

High-stringency wash buffer

10 ml 20× SSC (*APPENDIX 2A*)
20 ml 10% SDS
Make up to 1 liter with water
Store up to 3 months at room temperature

Hybridization buffer

^{32}P-labeled probe (10 ng/ml if the specific activity is 10^8 dpm/µg or 2 ng/ml if the specific activity is 10^9 dpm/µg)
10 ml prehybridization buffer (see recipe)
Prepare fresh

Low-stringency wash buffer

100 ml 20× SSC (*APPENDIX 2A*)
20 ml 10% SDS
Make up to 1 liter with water
Store up to 3 months at room temperature

Methylene blue solution

0.02% (w/v) methylene blue
0.3 M sodium acetate, pH 5.5
Store indefinitely at 4°C in the dark

MOPS buffer, 10×

0.2 M MOPS (free acid; adjust to pH 7.0 with 2 N NaOH)
20 mM sodium acetate
10 mM EDTA
Sterilize through a 0.45-μm Millipore filter and store until solution turns yellow at room temperature in the dark

Nondenaturing gel loading buffer, 6×

50% glycerol
10 mM sodium phosphate buffer, pH 6.3 (see recipe)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
Store in 0.1-ml aliquots indefinitely at −20°C

Prehybridization buffer

250 ml formamide
25 ml 1 M sodium phosphate buffer, pH 6.3 (see recipe)
80 ml 5 M NaCl
5 ml 0.1 M EDTA
50 ml 100× Denhardt's solution (*APPENDIX 2A*)
12.5 ml 10 mg/ml denatured salmon DNA (see recipe)
12.5 ml 20 mg/ml bakers yeast RNA (see recipe)
25 ml 10% SDS
40 ml H_2O
Store in 10-ml aliquots indefinitely at −20°C

RNA extraction buffer

51 ml DEPC-treated H_2O
5 ml 4 M lithium chloride
20 ml 1 M Tris·Cl, pH 8.0 (*APPENDIX 2A*)
4 ml 0.5 M EDTA
20 ml 10% SDS
Prepare fresh, mix with buffered phenol (*APPENDIX 2A*) in a 1:1 ratio, and heat at 80°C before use

Sodium phosphate buffer, 1 M (100×, pH 6.3)

31.95 g Na_2HPO_4
106.95 g $NaH_2PO_4 \cdot H_2O$
Make up to 1 liter with water
Store up to 3 months at room temperature

Strip buffer

10 mM Tris·Cl, pH 7.4 (*APPENDIX 2A*)
0.2% SDS
Prepare fresh stock solution of Tris·Cl and autoclave. Dilute with sterile DEPC-treated water and after cooling to 65°C, add SDS from 10% stock solution. Store up to 3 months at room temperature.

COMMENTARY

Background Information

Extraction of total RNA

For intact RNA isolation, guanidium hydrochloride and guanidium thiocyanate are two strong denaturants used to disrupt cells, solubilize their components, and denature endogenous RNases (Ullrich et al., 1977; Chirgwin et al., 1979; Han et al., 1987). However, these methods are unsuitable for handling numerous samples. Some investigators, when handling many samples, follow a single-step technique for RNA extraction, which generally does not yield high-purity RNA. For RNA extraction, commercial reagents, such as Trizol (Life Technologies), Isogen (Nippon Gene), and Tri reagent (Molecular Research Center), can also be used following the manufacturer's instructions.

Separation of poly(A) RNA from total RNA

Viral mRNA may or may not have a poly(A) tail at their 3′ termini. Based on the presence or absence of the poly(A) tail at their 3′ termini, viruses are classified as polyadenylated or nonpolyadenylated, respectively. If the RNA is from a polyadenylated virus-infected sample, then DNA contamination can be avoided by separating poly(A) RNA from total RNA by affinity chromatography or batch elution. Affinity chromatography is generally the preferred method to purify >25 μg poly(A) RNA from total RNA. In this method, mRNA form stable RNA-DNA hybrids with short chains of oligo(dT) (generally 18 to 30 nucleotides in length) linked to a supporting cellulose matrix. A percentage of 1% to 10% of total RNA is recovered as poly(A) RNA. Batch chromatography on oligo (dT)-cellulose is a technique of choice when working with a small amount of total RNA (<25 μg). Poly(A) RNA can be separated from total RNA by using commercial kits (Invitrogen and Ambion).

Transfer of RNA from gel to membrane

Many methods, including capillary blotting, vacuum blotting, electroblotting, and semi-dry blotting, have been used to transfer RNA from gel to membrane. The capillary system is the original simple technique devised by Southern (1975) for the transfer of nucleic acids from gel to membrane using an ascending flow of buffer (Fig. 16E.3.2). Capillary transfer is commonly used because it does not need any special equipment. This system has two types, the upward or downward capillary system. In an upward capillary system, the transfer of RNA from gel to membrane is by ascending flow of buffer and this system is more suitable for transferring small RNA species (<1 kb). In a downward capillary system, transfer of RNA from agarose gel to membrane is by descending flow of buffer (Fig. 16E.3.3). Downward capillary systems result in faster transfer and increased efficiency of transfer for longer RNA (Chomczynski and Mackey, 1994).

DNA or RNA probes

For northern analysis, a DNA or RNA probe is used. In vitro synthesis of a DNA probe is accomplished by the following methods.

Random-primed labeling

In random-primed labeling, template DNA is denatured and then cooled slowly in the presence of all possible combinations of hexanucleotides, which can bind to suitable complementary sequences within the DNA strands. The reaction mix contains four dNTPs, at least one of which has a labeled group, and a Klenow subunit of DNA polymerase I. Primer binds to the template in a random manner and the probe is of high specific activity.

Nick translation

In nick translation, a single-strand break is introduced resulting in a 3′-hydroxyl termini and 5′-phosphate termini using DNase I. The exposed nick becomes the starting point for introducing newly labeled nucleotide using DNA polymerase while the existing nucleotides are removed from the other side of the nick by exonuclease activity of the same enzyme. The synthesis allows for the incorporation of labeled nucleotides in place of the previously unlabeled ones.

End-labeling

For details on end-labeling, see Support Protocol 8.

Labeling by PCR

Double-stranded DNA or single-stranded DNA probes are labeled by PCR in the presence of two or one primer, respectively. Probe labeling by PCR eliminates the need of cloning a desired fragment of DNA into a vector containing bacteriophage promoters. A very small amount of DNA (1 to 10 ng) is required. The probe DNA fragment can be directly synthesized and labeled independently in the presence of an isotope or nonisotope such as biotinylated or DIG-labeled dNTPs by PCR.

Radiolabeling

For radiolabeling the riboprobe, [α-^{32}P]UTP is the best choice, as it is specific to RNA. Alternatively, [α-^{32}P]GTP, [γ-^{32}P]CTP, [α-^{32}P]CTP, or [γ-^{32}P]ATP can be used. ^{32}P-labeling of riboprobes afford a high sensitivity of detection (Koev and Miller, 2000; Cheng et al., 2002; Annamalai et al., 2003; Choi et al., 2004; Komoda et al., 2004; Grzelishvili et al., 2005). RNA can also be transcribed in vitro using commercial kits (Ambion and Promega).

Nonisotopic labeling

Nonisotopic labeling involves chemical coupling of a modified reporter molecule to a nucleotide precursor. Nonisotopic probes have become increasingly popular in recent years and have replaced radioactive probes in northern analysis. Advantages of these techniques are that the technologies are safe, probes can be stored for at least 1 year, and hybridization solutions can be reused several times. Two common nonisotopic systems include biotin-streptavidin and digoxigenin. Hybridization buffers and conditions vary in these systems. In both systems, the probe is detected with either chromogenic, fluorescent, or chemiluminescent substrate. Colorimetric detection uses the substrates NBT and BCIP to generate purple/brown precipitate directly on the membrane. Biotin- or digoxigenin-labeled probes, upon coupling to the alkaline phosphatase substrate (CSPD or CDP-Star), produces a light signal that can be detected by exposing the membrane to an X-ray film.

Biotin-streptavidin system

The biotin-streptavidin system utilizes extremely high-affinity ligands: biotin (a naturally occurring vitamin) and streptavidin (a bacterial protein). Biotin is incorporated in the probe by using biotinylated dNTPs during probe synthesis.

Digoxigenin system

Digoxigenin (DIG; a plant steroid) containing nucleotides is incorporated into the probe and detected using a DIG-specific antibody. DIG-probe synthesis incorporates DIG-11-dUTP by PCR, random primer labeling, T7- or SP6-mediated transcription, end-labeling, nick translation, etc. (Vlot et al., 2001; Haasnoot et al., 2003).

Preparation of riboprobes

Rioprobes are prepared by in vitro transcription using either linearized plasmid or PCR-amplified DNA fragments encoding a bacteriophage promoter (T7, T3, or SP6) at the 5′ termini. Advantages of using PCR product as templates are the need for cloning is avoided, probes do not contain vector sequence, and probes of high specificity and any size can be synthesized. DNA-dependent RNA polymerase is used to generate large quantities of RNA complementary to one strand of template DNA that is located immediately downstream from the promoter.

Critical Parameters

RNase contamination

RNase contamination is the most serious problem in northern analysis. RNase contamination cannot be removed by autoclaving, but it can be avoided by taking careful precautionary steps.

Gloves

Wear gloves at all times and use new gloves while handling equipment when contamination is suspected. Pipets and pipet tips are another source of RNase contamination. Therefore, use a special set of pipets and tips for RNA work. Glassware, spatulas, and stir bars should be decontaminated by baking 4 hr at 300°C. Reagents and plasticware should be from fresh packages set aside for RNA work. Plasticware can be treated with commercial RNase inactivators like RNasezap (Ambion). Prepare all solutions and reagents in RNase-free water and chemicals reserved for RNA work.

Dedicated reagents

Use separate stocks of reagents for RNA work. It is better to store solutions in small aliquots and to discard previously used solutions or those that are suspected to have contamination. The growth of microorganisms in buffers and reagents may cause RNase contamination. Therefore, simply discard the contaminated solutions and prepare fresh with RNase inhibitors.

DEPC treatment

DEPC, a highly reactive alkylating agent, is the most common and potent RNase inhibitor. Store small aliquots of DEPC in a dry environment. Glassware and plasticware should be washed with 0.1% DEPC-treated water and allow it to stand for 1 hr to overnight at room temperature, then rinse with DEPC-treated water several times and autoclave. In aqueous solutions, DEPC hydrolyzes rapidly to CO_2 and ethanol, and hydrolyzation is accelerated

by Tris and amines. Therefore, DEPC is incompatible with buffers like Tris.

Dedicated apparatus

Use a specific horizontal electrophoresis apparatus and combs for resolving RNA and never run DNA in the RNA unit. Wash gel box, electrophoresis tank, and combs with detergent, rinse with water, and fill with 3% H_2O_2 for 30 min at room temperature. Rinse the tank and comb thoroughly with DEPC-treated water before use.

Choice of membrane

Use of positively charged nylon membranes is always better than neutral or uncharged membranes since the former retain nucleic acids by covalent bonding.

Preparation of riboprobes

When in vitro transcription is performed, it is essential to consider the disposition of the restriction sites within the template DNA and those that are downstream from it. The 5′ terminus of the template DNA should have the bacteriophage promoter (T7, SP6, or T3) and the 3′ terminus should be defined by the restriction enzyme cleavage site. During the generation of template DNA, complete cleavage of plasmid DNA by restriction enzyme is essential. Presence of small amounts of circular plasmids significantly reduces the yield of transcripts. Enzymes, which generate blunt ends or 5′ protrusions, produce good templates. Use of templates with 3′ protrusions often results in double-stranded RNA. Therefore, avoid using restriction enzymes that generate 3′ protrusions.

Denaturation of the probe

Double-stranded probes have secondary structures. To increase the specificity of the probes during hybridization, it is essential

Table 16E.3.1 Troubleshooting Guide

Problem	Cause	Possible solution
Excessive background on the film	Incomplete blocking of the membrane	Prehybridize for longer periods
	Drying out of membrane during hybridization steps	Handle membrane carefully with sufficient buffer
	Paper towels become wet during transfer	Use larger stack of paper towels
	Use of impure formamide	Use purified formamide
	Improper washing	Use 1% SDS in wash solutions
	Unincorporated nucleotides in the probe and use of probes containing poly(T)	Use poly(A) at 1 μg/ml in hybridization solutions
Smears	RNA is degraded	Before blotting, confirm the integrity of the RNA by methylene blue staining
High background on the lanes	Improper denaturation of the DNA probe	Denature the double-stranded probe properly and do not allow annealing
Halos over the entire film	Presence of bubbles in solutions or failure of adequate agitation of the membrane during hybridization and washing	Prewarm the solutions and agitate membrane properly
Intense black spots on the film	Use of old radiolabeled probe	Do not use probes stored a long time and consider the half-life of the radiolabeled compound
No signal	Probe may not be denatured prior to hybridization or hybridization and washing conditions may be too stringent	Denature the double- or single-stranded probe properly and do not allow annealing; wash the membrane under appropriate stringent conditions

Table 16E.3.2 Type of Probes and Their Specific Activities

Type of probe	Method of synthesis	Size of probe	Specific activity (dpm/μg)
Double-stranded DNA	Random priming using DNA templates	~400–600 nt	$\sim 2 \times 10^9$
Single-stranded DNA	Random priming using RNA templates	~400–600 nt	$\sim 1 \times 10^9$
Double-stranded DNA	Nick translation	~400 nt	5×10^8–1×10^9
Single- or double-stranded DNA	PCR	Generates radiolabeled DNAs of defined length, depending on the spacing of primers	$\sim 1 \times 10^9$– 2×10^9
Single-stranded RNA	In vitro transcription of double-stranded DNA templates	Generates radiolabeled RNAs of defined length	$\sim 1 \times 10^9$

Table 16E.3.3 Timeline for Performing a Northern Blot

Time	Procedure
Day 1	Agarose gel electrophoresis and transfer of RNA to membrane overnight
Day 2	Prehybridization and hybridization overnight
Day 3	Blot washings and detection of hybridization signals

to denature probes by heating 5 min at 100°C.

Buffer system

Transfer of RNA to the nylon membrane can be performed with a variety of low-ionic buffers, SSC being the most frequently used.

Troubleshooting

Table 16E.3.1 presents some of the problems commonly encountered when performing these techniques, as well as their possible causes and solutions.

Anticipated Results

The specific activity of different probes varies (Table 16E.3.2). Riboprobes offer high sensitivity of detection than DNA probes and ^{32}P-labeled riboprobes are more sensitive.

Time Considerations

A northern blot can be completed in 3 days as shown in Table 16E.3.3.

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Cloning of Large Positive-Strand RNA Viruses

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ABSTRACT

Full-length, biologically active cDNA clones of the positive-strand RNA plant viruses are indispensable for investigating the functions of viral genes and control elements as well as generating virus-derived gene expression and silencing vectors. Even though engineering of such clones for 4- to 10-kb viral RNAs has become routine, it remains a challenging task for 15- to 20-kb RNA genomes of the monopartite viruses in a family *Closteroviridae*. This unit describes strategic considerations and techniques used to generate an infectious cDNA clone of a closterovirus. The use of agroinfection to improve specific infectivity of the resulting clone is also explained. *Curr. Protoc. Microbiol.* 7:16F.1.1-16F.1.26. © 2007 by John Wiley & Sons, Inc.

Keywords: RNA virus • full-length clone • cDNA • agroinfection

INTRODUCTION

Application of reverse genetics to RNA viruses requires generation of their infectious cDNA clones, which are amenable to genetic manipulations using a vast array of DNA-specific enzymes and established protocols for DNA amplification, cloning, and mutagenesis. For the viruses with large genomes, conversion of the RNAs into faithful cDNA clones is a challenging task. Such conversion involves putting together multiple cDNA fragments for which reliable consensus nucleotide sequences must be obtained. In addition, large RNA transcripts of the viral cDNA clones are prone to degradation and therefore exhibit relatively low specific infectivity. In this unit, the authors present strategic considerations and techniques designed to facilitate the generation of biologically active cDNA clones for large RNA viruses.

It is also important to develop a reliable procedure for conversion of the clones back to RNA that can be used to initiate infection. In plants this can be accomplished by using in vitro transcription followed by mechanical inoculation (e.g., see *UNIT 16B.3*) of the resulting RNA. However, mechanical inoculation is inefficient for many closteroviruses that are normally limited to phloem and hence are recalcitrant to this approach. This limitation can be overcome by using *Agrobacterium*-mediated delivery of viral cDNAs (agroinfection). The efficiency of agroinfection can be drastically increased by co-expression of the plant virus-derived suppressors of RNA silencing (Chiba et al., 2006; Basic Protocol 9).

This unit provides protocols that were used to engineer an infectious molecular clone of Beet yellow virus (BYV). This virus belongs to the genus *Closterovirus* (family *Closteroviridae*) and possesses a one-component, 15.5-kb, positive-strand, RNA genome (Dolja, 2003). Methods are provided for isolation of single-stranded (ss) and double-stranded (ds) genome-size viral RNAs (Basic Protocols 1 and 2) and for determining the exact 3′- and 5′-terminal sequences of these RNAs (Basic Protocol 3 and Alternate Protocol 1; Basic Protocol 4 and Alternate Protocol 2). Procedures are described for molecular cloning of these sequences and introducing plant transcriptional control elements upstream and downstream from the viral cDNAs (Strategic Planning and Basic

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Protocols 5 and 6). Procedures are also provided also for cloning long cDNA fragments using reverse transcription PCR (RT-PCR; Basic Protocol 7) as well as traditional cDNA synthesis (Basic Protocol 8) for the generation of a full-length DNA copy of the viral genome. Finally, testing of biological activity of the resulting clones using agroinfection (Basic Protocol 9 and Support Protocols 1 & 2; also see *UNIT 16B.2*) of host plants is explained.

CAUTION: Plant viruses are subject to regulation by USDA Animal and Plant Health Inspection Service (APHIS). An APHIS permit must be obtained for virus propagation in plants. Researchers also need to conform to local and institutional policies for working with infectious agents. See Chapter 1, Section A and other appropriate resources (e.g., *APPENDIX 1B*) for further information.

STRATEGIC PLANNING

Availability of high-quality viral RNA for which a nucleotide sequence is known is a prerequisite for obtaining a cDNA clone. As a starting point, this unit uses the published sequence of a European isolate of BYV (GenBank accession no. X73476). A restriction map of corresponding cDNA was generated using online NEBcutter program from New England Biolabs (*http://tools.neb.com/NEBcutter2/index.php*). ClustalW (*http://www.ebi.ac.uk/clustalw*) is a convenient tool for comparing nucleotide or amino acid sequences of the clones using multiple sequence alignments. The GenBank sequences are only a starting point for obtaining a cDNA clone because they often do not result in complete biological activity of an engineered virus.

Using low-copy plasmids for all crucial cloning steps (e.g., manipulations with very long cDNA fragments or final assembly of the resulting clone) is recommended. This helps avoid excessive accumulation of the foreign DNA and reduces potential toxicity of the viral cDNA to bacteria used for cloning. Such toxicity is a common problem with viruses having large genomes (Lai, 2000). For constructing a cDNA clone of BYV (Prokhnevsky et al., 2002) the authors used pCB301 mini-binary vector provided by Dr. D.J. Oliver (see Xiang et al., 1999). This plasmid has a relatively small size of ~3.5 kb, accumulates to a low level in *E. coli*, carries a convenient polylinker site (Fig. 16F.1.1, step 1), and can be used for agroinfection. A NOS terminator sequence (required for transcription termination; see Fig. 16F.1.2) should be first added to the pCB301 by regular cloning using unique sites S and K (Fig. 16F.1.1, step 3).

The first steps in generating a full-length cDNA clone are identification of exact terminal sequences of the viral RNA genome and cloning of the corresponding cDNA fragments (Fig. 16F.1.4, steps 4 and 5). Because authentic genome termini are critical for infectivity, at least two different methods should be used for determining these RNA regions. The 3′-terminal nucleotide sequences of the viral RNAs, which lack poly(A) tails, can be determined by using two simple techniques that yield equally good results. The authors recommend using both of them to ensure correct identification of the very terminal nucleotides that are critical for virus viability. Both nucleotide sequencing and cDNA cloning are performed using reverse transcription and either conventional synthesis of the dsDNA or PCR amplification. The deoxyoligonucleotide primers for these reactions should be of 24 to 30 residues in length with the GC content close to 50%. Designing primers that overlap naturally occurring restriction sites in the viral genome is suggested to simplify future manipulations.

The next steps include insertion of a ribozyme downstream from the 3′-terminal nucleotide of a viral cDNA (Fig. 16F.1.1, step 4) and RNA polymerase promoter upstream from the 5′-terminal nucleotide of viral cDNA (Fig. 16F.1.1, step 5). These control elements are needed to ensure transcription and processing of exact copies of viral RNAs upon agroinoculation.

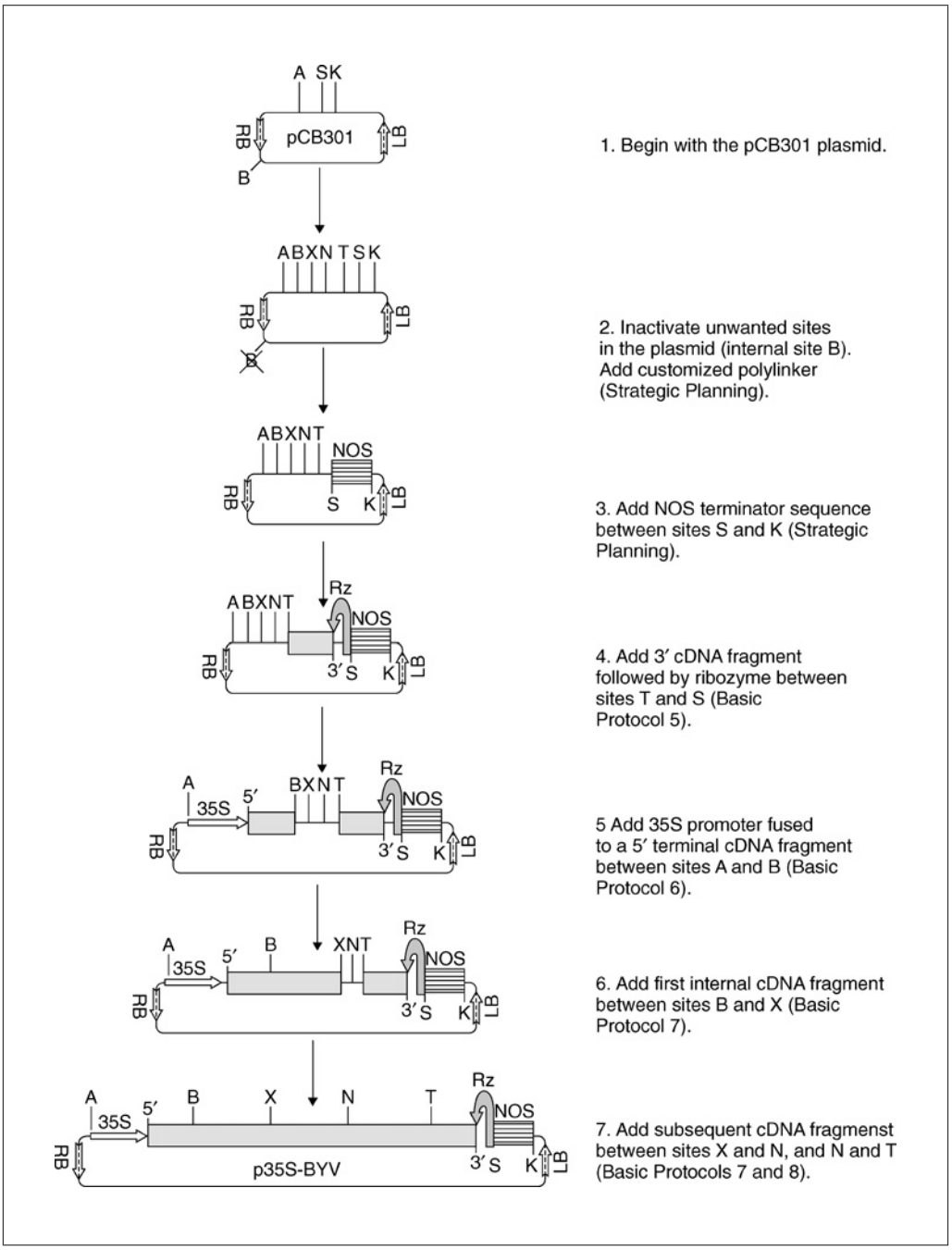

Figure 16F.1.1 Diagram showing consecutive steps (and their associated Protocols) for generating a full-length cDNA clone for beet yellow virus (BYV) in a mini-binary plasmid. Dotted boxes represent viral cDNA. Restriction sites used for cloning: A, *Sac*I; B, *Bgl*II; X, *Xba*I; N, *SnaB*I; T, *Bst*EII; S, *Sma*I; K, *Kpn*I. Other abbreviations: 5′ and 3′, 5′ and 3′ termini of BYV cDNA, respectively; 35S, cauliflower mosaic virus (CaMV) 35S promoter; NOS, nopaline synthase terminator; RB and LB, transferred DNA (T-DNA) right border and left border, respectively; Rz, ribozyme sequence.

```
5′-GATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATT
ATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTAT
GAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGC
GCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATC-3′
```

Figure 16F.1.2 NOS terminator sequence.

Finally, internal cDNA fragments are added to complete assembly of the full-length clone (Fig. 16F.1.1, steps 6 and 7; Basic Protocols 7 and 8). Each of the cDNA fragments must be sequenced in at least three independent clones to determine a consensus nucleotide sequence and exclude incorporation of incidental mutations that are often detrimental for virus infectivity. The final steps involve transformation of the *Agrobacterium* with the full-length cDNA clone and infectivity tests. For these purposes the authors use plants of *Nicotiana benthamiana*, which is an experimental host for many viruses including BYV and is amenable to infiltration with agrobacterial culture.

Note that parameters for the polymerase chain reactions described in this unit were optimized for the PTC-100 thermocycler (MJ Research, distributed by Bio-Rad); they have not been tested on other machines.

BASIC PROTOCOL 1

ISOLATION OF SINGLE-STRANDED VIRAL RNAs

Single-stranded RNA for the experimental procedures described in this unit can be isolated from virus particle preparations by routine phenol extraction (see below). For purification of double-stranded (ds) RNA from infected plants using uncharged cellulose chromatography in the presence of ethanol, see Basic Protocol 2 and Valverde (1990).

Materials

5 mg/ml virus suspension of interest
10% (w/v) SDS (DNase and RNase free; Bio-Rad)
Neutral saturated phenol (EMD Chemicals)
Neutral phenol (EMD Chemicals)/chloroform/isoamyl alcohol mixture (25:24:1)
Chloroform/isoamyl alcohol (24:1)
7.5 M ammonium acetate
100% and 70% (v/v) ethanol (cold)
Sterile nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)

1.5-ml microcentrifuge tube
Refrigerated microcentrifuge
Vacuum evaporator (e.g., SpeedVac concentrator, Thermo Scientific)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000) and spectrophotometrically quantifying nucleic acids (Gallagher, 2004)

Extract ssRNA from virus particles

1. To 0.7 ml of 5 mg/ml virus suspension, add 70µl of 10% (w/v) SDS and vortex briefly.
2. Immediately add an equal volume (0.77 ml) of neutral phenol and shake 1 minute.
3. Centrifuge 1 min at maximum speed, room temperature in a tabletop microcentrifuge.
4. Transfer upper aqueous phase (avoiding the interphase) to a fresh tube and extract twice with 0.8 ml of neutral phenol/chloroform/isoamyl alcohol mixture (25:24:1) by shaking 1 min and centrifuging as in step 3. After each centrifugation transfer the upper phase to a fresh tube.
5. Extract twice with 0.8 ml of chloroform/isoamyl alcohol (24:1) as in step 4, transferring the upper phase after each extraction to a fresh tube.
6. Adjust supernatant to 2.5 M with 7.5 M ammonium acetate and precipitate the RNA with 2.5 volumes 100% cold ethanol.
7. Incubate a few hours at –20°C.

8. Microcentrifuge 10 min at maximum speed, 4°C, to recover precipitated RNA. Discard the supernatant.
9. Wash pellet by adding 1 ml of cold 70% ethanol and microcentrifuge 1 min at maximum speed, 4°C. Dry under vacuum.

   A SpeedVac concentrator is recommended to prevent loss of the RNA pellet due to air flow.

Determine RNA concentration and size

10. Resuspend the pellet in 40 µl sterile nuclease-free water and measure optical density at 260 and 280 nm to determine concentration (see Gallagher, 2004).
11. Electophorese 2 µl of the RNA on a 1% nondenaturing agarose gel (see Voytas, 2000) to analyze the RNA preparation. Stain with ethidium bromide to visualize the RNA.
12. Examine the gel to determine if intact RNA of the expected size has been obtained.

ISOLATION OF DOUBLE-STRANDED RNA FROM VIRUS-INFECTED PLANTS

BASIC PROTOCOL 2

The dsRNA purification method described in (Valverde, 1990) recommends two rounds of chromatography on cellulose sorbent. The authors have found that one round is sufficient to obtain dsRNA preparation suitable for applications discussed in this unit. Precipitating the nucleic acids after the phenol extraction and before loading them onto cellulose sorbent improves the recovery and quality of dsRNA. This method can be easily scaled up if more dsRNA is needed for a particular protocol; it is also applicable to small RNA viruses.

Materials

1× STE buffer (see recipe)
SDS, DNase- and RNase-free (Bio-Rad)
Phenol equilibrated with 1× STE buffer (see recipe for buffer)
7.5 molar ammonium acetate
95% (v/v) ethanol
CF-11 cellulose (Whatman)
1× STE(see recipe)/15% ethanol
Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)

Mortar and pestle, chilled in ice
50-ml conical, polypropylene centrifuge tubes
5-ml polypropylene columns (Qiagen)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000) and spectrophotometrically quantifying nucleic acids (Gallagher, 2004)

Extract dsRNA from virus particles

1. Grind 5 g of plant tissue in 10 ml of ice-cold 1× STE buffer using mortar and pestle chilled in ice. Transfer the homogenate into a centrifuge tube.
2. Add 1.5 ml of 10% SDS and extract (by quickly inverting the tubes ~10 times) twice with equal volume (16.5 ml) of phenol equilibrated with 1× STE buffer. Centrifuge 5 min at 5000 × *g*, room temperature. Transfer supernatants into a fresh tube after each centrifugation.
3. Adjust the combined supernatants to 2.5 M ammonium acetate, using 7.5 M ammonium acetate. Pellet nucleic acids as described in Basic Protocol 1, steps 6 to 8.

4. Resuspend the pellet in 10 ml 1× STE buffer, add 2 ml of 95% ethanol, and mix well.
5. Add 1 g of dry CF-11 cellulose powder to the tube, vortex well, and incubate 30 min at room temperature, with occasional mixing.
6. Load the cellulose slurry into an empty 5-ml polypropylene column and allow to drain.
7. Wash column with 1× STE/15% ethanol and elute the retained dsRNA with 4 aliquots (1 ml each) of 1× STE without ethanol. Combine eluates and pellet the dsRNA as described in Basic Protocol 1, steps 6 to 8.
8. Resuspend pellet in 1 ml of nuclease-free water and clarify by microcentrifuging 1 min at maximum speed, room temperature, to remove residual cellulose. Transfer the supernatant into fresh tube and precipitate the dsRNA.

Determine RNA concentration and size

9. Resuspend pellet in 20 µl of water and measure optical density at 260 and 280 nm to determine concentration (see Gallagher, 2004).
10. Electophorese 2 µl of the RNA on a 1% nondenaturing agarose gel (see Voytas, 2000) to analyze the RNA preparation. Stain with ethidium bromide to visualize the RNA.
11. Examine the gel to determine if intact RNA of the expected size has been obtained.

BASIC PROTOCOL 3

PRIMER-LIGATION METHOD FOR MAPPING THE 3′ END OF THE VIRAL RNA

Both ss and dsRNA preparations can be used as a starting material, although the authors have found that ssRNA yields higher amounts of the resulting PCR product. The first mapping approach employs ligation of a phosphorylated deoxyoligonucleotide to the 3' end of the RNA followed by reverse transcription PCR (RT-PCR) to amplify the 3'-terminal fragment, with cloning and sequencing of the resulting product. A primer is used to add an artificial 3′-tail to viral RNA; its reverse complementary counterpart used as a primer for initiation of the cDNA synthesis should not anneal to the template RNA and should have T_m (melting temperature) suitable for the PCR amplification. These primers should include restriction sites (convenient for subsequent cloning) that are not expected to be present in the 3′-terminal part of the viral sequence. The same pair of primers can be used for many primer ligation–based applications.

Although primer ligation (this protocol) and polyadenylation methods (Alternate Protocol 1) each can produce reliable results, using both methods increases confidence in terminal sequence determinations. In addition, depending on their structural features, RNAs of different viruses may be better substrates for either T4 RNA ligase or yeast poly(A) polymerase.

Materials

100 pmol/µl oligonucleotide PCR primers, in sterile water: C-dG-PCR and dG-PCR, and 5-p20-*Nde* primer (see Table 16F.1.1)
10 mM ATP: prepared using 100 µl 100 mM ATP (Fermentas) and 900 µl sterile nuclease-free water; store in 50-µl aliquots up to a few months at −20°C
10× T4 polynucleotide kinase buffer (Fermentas)
10 U/µl T4 polynucleotide kinase (Fermentas)
Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)

7.5 M ammonium acetate
100% ethanol
1 μg/μl viral ssRNA (Basic Protocol 1)
10× T4 RNA ligase buffer (Fermentas)
10 U/μl T4 RNA ligase (Fermentas)
Neutral phenol (EMD Chemicals)/chloroform/isoamyl alcohol mixture (25:24:1)
Chloroform/isoamyl alcohol (24:1)
5× First-Strand synthesis buffer (Invitrogen)
4 mM (each) dNTP mixture: prepared using 8 μl of each of four 100 mM dNTPs (Promega) and 168 μl sterile RNase-free water; store up to a few months at −20°C
100 mM dithiothreitol (DTT)
200 U/μl SuperScript II reverse transcriptase
10× *Taq* extender buffer (Stratagene)
Taq extender PCR additive (Stratagene)
5 U/μl *Taq* DNA polymerase
Restriction endonucleases: e.g., *Bam*HI (for sites in dG-PCR and C-dG-PCR primers) and *Nde*I (for sites in 5-p20-*Nde* primer)
High-copy plasmid cloning vector (e.g., pGEM plasmid series; Promega)

1.5-ml microcentrifuge tubes
PCR tubes
Thermal cycler (e.g., PTC-100 thermocycler; Bio-Rad)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000), digestion of DNA with restriction endonucleases (Bloch and Grossmann, 1995), and cloning DNA

Table 16F.1.1 Primers Used in Cloning Large Positive-Strand RNA Plant Viruses

Primer	Sequence (5′ to 3′)
C-dG-PCR	GGATCCGAGCTCGAATTCGC
dG-PCR	GCGAATTCGAGCTCGGATCC
dT-*Sac*	GGTGAGCTCTTTTTTTTTTTTTTTT
5-p20-*Nde*	GCCATATGACTAGCTCTGTCGAAC
C-BYV-*Bgl*II	TACGAGAGATCTCGTAAGCTTCAT
5-35S	TAGAGCTCAACATGGTGGAGCAC
3-35S-vir	GGATGGTTAAAAACTGCTCTCCAAATGAAATGAACTTCC
5-vir-35S	CATTTCATTTGGAGAGCAGTTTTTAACCATCCTTC
3BYV-Rib	TACCCGGGCCGTTTCGTCCTCACGGACTCATCAGAAGACAT GTGAATCATGTCTTGACGGCCCTTATTTTTTCTTC
BYV-*Bst*EII	GTGACGGAGGTGACCTACCTGCC

Phosphorylate synthetic oligonucleotide primer

1. In a test tube, mix the following:

   5 μl C-dG-PCR primer
   5 μl 10 mM ATP
   5 μl 10× T4 polynucleotide kinase buffer
   2 μl T4 polynucleotide kinase
   33 μl nuclease-free water.

2. Incubate 30 min at 37°C.

   Incubations may be performed in the thermal cycler or other heating devices.

3. Add 25 µl of 7.5 M ammonium acetate and 400 µl of 100% ethanol. Microcentrifuge 10 min at maximum speed, 4°C.

Ligate phosphorylated primer to viral RNA

4. Discard the supernatant, resuspend the pellet in 10 µl of nuclease-free water, and add the following to the tube:

   4 µl viral ssRNA (~1 µg/µl)
   5 µl 10× T4 RNA ligase buffer
   2 µl T4 RNA ligase
   29 µl nuclease-free water.

   Double-stranded RNA may also be used, but preincubation of the dsRNA for 5 min at 70°C is recommended.

5. Incubate 2 hr at 30°C.

6. Extract once with neutral phenol/chloroform/isoamyl alcohol mixture and once with chlorophorm/isoamyl alcohol as described in Basic Protocol 1, steps 4 and 5.

7. Precipitate the product by adding 25 µl of 7.5 M ammonium acetate and 200 µl of ethanol. Microcentrifuge 10 min at maximum speed, 4°C.

Reverse transcribe viral RNA

8. Discard the supernatant and resuspend the pellet in 9.5 µl of nuclease-free water, add 1 µl of primer dG-PCR, heat at 70°C for 2 min, and cool on ice.

   Primer dG-PCR is complementary to C-dG-PCR primer ligated to RNA's 3′-end.

9. Add the following to the tube:

   4 µl 5× First-Strand synthesis buffer
   2.5 µl 4 mM dNTP mixture
   2 µl 100 mM DTT
   1 µl SuperScript II reverse transcriptase.

10. Incubate 1 hr at 42°C.

PCR amplify viral DNA

11. Transfer 10 µl of the First-Strand synthesis product (step 10) to a PCR tube and add the following:

    9 µl 10× *Taq* extender buffer
    5 µl 4 mM dNTP mixture
    1 µl dG-PCR primer
    1 µl *Taq* extender PCR additive
    1 µl *Taq* DNA polymerase
    72 µl nuclease-free water
    1 µl sequence-specific primer (5-p20-*Nde*).

    The primer should carry a convenient restriction site at its 5′-end, situated about 1,000 nucleotides upstream from the expected 3′ terminus. This is exemplified by the 5-p20-Nde primer that includes Nde I site and 16 nucleotides of the 3′-penultimate BYV ORF encoding p20.

12. Carry out PCR using the following amplification cycles:

26 to 30 cycles	1 min	93°C	(denaturation)
	1 min	56°C	(annealing)
	1 min	72°C	(extension).

13. Electophorese 2 μl of the DNA on a 1% nondenaturing agarose gel (see Voytas, 2000) to analyze the DNA preparation. Stain with ethidium bromide to visualize the DNA product of PCR.

    A successful PCR reaction should yield one major product of expected size that appears as a clearly visible band on the stained gel.

14. Digest the product with appropriate restriction endonucleases (see Bloch and Grossman, 1995).

    Appropriate restriction endonucleases are those with restriction sites that were included in the sequences of the PCR primers (in this case, BamHI present in dG-PCR and C-dG-PCR primers, and NdeI present in 5-p20-Nde primer).

15. Clone the released fragments into a suitable plasmid cloning vector.

    Any commonly available high-copy plasmid such as pGEM plasmid series (Promega) can be used at this step since products of the described RT-PCR reaction are of relatively small sizes and unlikely to cause any problem associated with the toxicity of viral cDNA in E. coli.

16. Analyze the clones by restriction digestion and select those that release restriction fragments of expected sizes or bigger. The bigger than expected products can result if the previous work underestimated viral RNA length or mismapped the 3′ terminus.

17. Select at least ten clones and sequence them to obtain a consensus sequence of a putative 3′ end of the viral cDNA.

POLYADENYLATION METHOD FOR MAPPING THE 3′ END OF THE VIRAL RNA

ALTERNATE PROTOCOL 1

This method is based on the ability of yeast poly(A) polymerase to add adenosine residues to the 3′ end of RNA. This enzyme works more efficiently than the *E. coli* poly(A) polymerase. The resulting polyadenylated viral RNA can be used as a template for cDNA synthesis using oligo-(dT) primer to initiate the reaction. A fragment that corresponds to the 3′ end of the viral RNA is then PCR amplified and cloned for sequencing and analysis. Both ssRNA and dsRNA preparations can be used as a starting material, although the authors have found that ssRNA yields higher amounts of the resulting PCR product.

This procedure is best suited for nonpolyadenylated viral RNAs. Presence of extensive stretches of adenosine residues near the 3′ end of RNA may lead to appearance of truncated RT-PCR products due to annealing of the oligo(dT) primer to those regions. This method does not discriminate between naturally occurring and artificially added 3′ terminal adenosine residues; correctly determining the identity of very terminal nucleotide(s) calls for an independent approach such as the primer-ligation method (Basic Protocol 3).

Although primer ligation (Basic Protocol 3) and polyadenylation methods (this protocol) each can produce reliable results, using both methods increases confidence in terminal sequence determinations. In addition, depending on their structural features, RNAs of different viruses may be better substrates for either T4 RNA ligase or yeast poly(A) polymerase used in the alternative methods.

Materials

1 μg/ml viral ssRNA (Basic Protocol 1)
5× yeast poly(A) polymerase buffer (USB)
10 mM ATP: prepared using 100 μl 100 mM ATP (Fermentas) and 900 μl sterile nuclease-free water; store in 50-μl aliquots up to a few months at −20°C
600 U/μl yeast poly(A) polymerase (USB)
Neutral phenol (EMD Chemicals)/chloroform/isoamyl alcohol mixture (25:24:1)

Chloroform/isoamyl alcohol mixture (24:1)
7.5 M ammonium acetate
100% ethanol
Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)
100 pmol/μl oligonucleotide primers in sterile water: dT-*Sac*, 5-p20-*Nde* sequence specific primer (see Table 16F.1.1)
4 mM (each) dNTP mixture: prepared using 8 μl of each of four 100 mM dNTPs (Promega) and 168 μl sterile RNase-free water; store up to a few months at −20°C
5× First-Strand synthesis buffer (Invitrogen)
100 mM dithiothreitol (DTT)
200 U/μl SuperScript II RT (Invitrogen)
10× *Taq* extender buffer (Stratagene)
Taq extender PCR additive (Stratagene)
Taq DNA polymerase
Restriction enzymes (e.g., *Sac*I and *Nde*I)
High-copy plasmid cloning vector (e.g., pGEM plasmid series; Promega)

0.6-ml microcentrifuge tubes
PCR tubes
Thermal cycler (e.g., PTC-100 thermocycler; Bio-Rad)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000), digestion of DNA with restriction endonucleases (Bloch and Grossman, 1995), and cloning DNA (Sambrook and Russell, 2001)

Polyadenylate RNA

1. In a 0.6-ml microcentrifuge tube, mix the following:

   5 μl ssRNA (1 μg/μl)
   2 μl 5× yeast poly(A) polymerase buffer
   1 μl 10 mM ATP
   2 μl yeast poly(A) polymerase.

   Double-stranded RNA may also be used, but preincubation of the dsRNA for 5 min at 70°C is recommended.

2. Incubate 20 min at 30°C.

   One should not exceed the recommended incubation time for the poly(A) tailing reaction because it can result in formation of needlessly long oligo(A) tails which may hamper future sequencing of the cloned fragments.

3. Extract once with neutral phenol/chloroform/isoamyl alcohol mixture, once with chloroform/isoamyl alcohol.

4. Precipitate the RNA by adding 5 μl of 7.5 M ammonium acetate and 40 μl of 100% ethanol. Microcentrifuge 10 min at maximum speed, 4°C.

Reverse transcribe the RNA

5. Discard the supernatant and dissolve RNA pellet in 9.5 μl of nuclease-free water, add 1 μl dT-*Sac* primer and heat 2 min at 70°C. Cool on ice.

   The dT-Sac primer contains the SacI restriction site for subsequent cloning of the PCR product.

6. Add the following to the tube:

   4 μl 5× First-Strand synthesis buffer
   2.5 μl 4 mM dNTP mixture
   2 μl 100 mM DTT
   1 μl SuperScript II RT.

7. Incubate 1 hr at 42°C.

PCR amplify the DNA

8. Transfer 10 μl of the First-Strand synthesis product (step 7) into a PCR tube and add the following:

   9 μl 10× *Taq* extender buffer
   5 μl 4 mM dNTP mixture
   1 μl dT-*Sac* primer
   1 μl sequence-specific primer
   1 μl *Taq* extender PCR additive
   1 μl *Taq* DNA polymerase
   72 μl nuclease-free water.

   The primer should carry a convenient restriction site at its 5′ end situated ∼1000 nucleotides upstream from the expected 3′ terminus (5-p20-Nde).

9. Carry out PCR using the following amplification cycles:

26 to 30 cycles	1 min	93°C	(denaturation)
	30 sec	46°C	(annealing)
	30 sec	52°C	(annealing)
	30 sec	56°C	(annealing)
	1 min	72°C	(extension).

10. Analyze the product on a 1% agarose gel (see Voytas, 2000).

11. Digest the product using restriction endonucleases with sites included in the sequences of the PCR primers (*Sac*I and *Nde*I in this case; see Bloch and Grossman, 1995).

12. Clone the fragments into a suitable vector.

    Any commonly available high-copy plasmid such as pGEM plasmid series (Promega) can be used at this step since products of the described RT-PCR reaction are of relatively small sizes and unlikely to cause any problem associated with the toxicity of viral cDNA in E. coli.

13. Analyze clones by restriction digestion and select those that release restriction fragments of expected sizes or bigger.

14. Select at least 10 clones and sequence them to obtain consensus sequence of a putative 3′ end of the viral cDNA.

RLM-RACE METHOD FOR MAPPING THE 5′ END OF THE VIRAL RNA

BASIC PROTOCOL 4

To map a 5′ end of a capped, positive-strand viral RNA, the authors suggest using the RLM-RACE kit (FirstChoice RLM-RACE kit, Ambion; or equivalent) with modifications described below. This kit has been used in the authors' laboratory for determining 5′ ends of both viral and cellular capped RNAs and has proven to be a reliable tool. Standard protocols for reverse transcription and PCR amplification used in the authors'

laboratory yield superior results compared to the protocols provided by the supplier of the kit. This maybe attributed to use of the SuperScript II RT enzyme, which lacks RNaseH activity, rather than the M-MLV reverse transcriptase recommended by the kit's manufacturer. In addition, a blend of a regular *Taq* polymerase and *Taq* extender PCR additive increased the amount of the PCR product. While an original RLM-RACE protocol suggests two consecutive rounds of PCR using two sets of nested primers, one round of PCR amplification yields enough PCR product for further cloning, due to a substantial concentration of the viral RNA. (The original RLM-RACE method was designed to amplify 5′ ends of low-abundance mRNAs.)

Materials

1 μg/μl viral ssRNA (Basic Protocol 1)
Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)
FirstChoice RLM-RACE kit (Ambion) including:
  10× calf intestine alkaline phosphatase buffer
  calf intestine alkaline phosphatase
  10× tobacco acid pyrophosphatase buffer
  tobacco acid pyrophosphatase
  5′ RACE adapter
  Random primer
  10× RNA ligation buffer
  T4 RNA ligase
  5′RACE outer primer
Neutral phenol (EMD Chemicals)/chloroform/isoamyl alcohol mixture (25:24:1)
Chloroform/isoamyl alcohol mixture (24:1)
7.5 M ammonium acetate
Isopropanol
10× *Taq* extender buffer (Stratagene)
4 mM (each) dNTP mixture: prepared using 8 μl of each of four 100 mM dNTPs (Promega) and 168 μl sterile RNase-free water; store up to a few months at −20°C
100 mM dithiothreitol (DTT)
200 U/μl SuperScript II RT (Invitrogen)
Sequence-specific primer carrying a convenient restriction site at its 5′ end (e.g., C-BYV-*Bgl*II; see Table 16F.1.1)
Taq extender PCR additive (Stratagene)
5 U/μl *Taq* DNA Polymerase
High-copy plasmid cloning vector (e.g., pGEM plasmid series; Promega)

0.6-ml microcentrifuge tubes
PCR tubes
Thermal cycler (e.g., PTC-100 thermocycler; Bio-Rad)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000), digestion of DNA with restriction endonucleases (Bloch and Grossman, 1995), and cloning DNA (Sambrook and Russell, 2001)

Treat RNA with alkaline phosphatase

1. Mix in a 0.6-ml microcentrifuge tube:

   4 μl 1 μg/μl viral RNA
   12 μl nuclease-free water
   2 μl 10× calf intestine alkaline phosphatase buffer
   2 μl calf intestine alkaline phosphatase.

2. Incubate 1 hr at 37°C.

   Incubations may be performed in the thermal cycler or other heating devices.

3. Extract once with neutral phenol/chloroform/isoamyl alcohol mixture and once with chloroform/isoamyl alcohol.
4. Add 10 μl of 7.5 M ammonium acetate and 30 μl of isopropanol to precipitate the RNA.
5. Microcentrifuge 10 min at maximum speed, 4°C.

Remove cap

6. Discard the supernatant and resuspend the RNA pellet in 7 μl nuclease-free water.
7. Add 1 μl of 10× tobacco acid pyrophosphatase buffer and 2 μl tobacco acid pyrophosphatase.
8. Incubate 1 hr at 37°C.

Ligate adaptor to viral RNA

9. Mix in a 0.6-ml microcentrifuge tube:

   4 μl of RNA from step 8
   1 μl of 5′ RACE adapter
   1 μl of 10× RNA ligation buffer
   2 μl of nuclease-free water
   2 μl of T4 RNA ligase.

10. Incubate 1 hr at 37°C.

Reverse transcribe the RNA

11. Mix in a 0.6-ml microcentrifuge tube:

    5 μl of RNA from step 10
    4 μl of 5× First-Strand synthesis buffer
    2 μl of 4 mM dNTP mixture
    2 μl of random primer
    2 μl of 100 mM DTT
    4 μl of nuclease-free water
    1 μl of SuperScript II RT.

12. Incubate 1 hr at 42°C.

PCR amplify the DNA

13. Transfer 10 μl of the product from step 12 to a PCR tube.
14. To the tube, add:

    9 μl of 10× *Taq* extender buffer
    5 μl of 4 mM dNTP mixture
    1 μl of 5′ RACE outer primer
    1 μl of the sequence-specific primer carrying a convenient restriction site at its 5′-end
    1 μl of *Taq* extender PCR additive
    1 μl *Taq* DNA Polymerase
    72 μl nuclease-free water.

15. Carry out PCR using the following amplification cycles:

32 cycles	30 sec	93°C	(denaturation)
	1 min	52°C	(annealing)
	2 min	72°C	(extension).

16. Analyze product on a 1% agarose gel (see Voytas, 2000).

17. Clone appropriate sized fragments into a suitable high-copy plasmid vector and sequence five to 10 positive clones to determine the consensus 5′-terminal nucleotide sequence of the viral RNA.

ALTERNATE PROTOCOL 2

METHOD USING POLYADENYLATION OF DOUBLE-STRANDED RNA FOR MAPPING THE 5′-END END OF VIRAL RNA

To confirm the mapping results obtained by the method described in Basic Protocol 5, one can use a viral dsRNA in combination with poly(A) tailing and RT-PCR amplification of the 5′-terminal fragment. As in the case of the 3′-end mapping, using both approaches is recommended, taking into account that the 3′ end of a negative strand of a dsRNA may have an additional nontemplate nucleotide (Dolja and Atabekov, 1987).

The steps for mapping the 5′ end of viral genomic RNA are the same as in the protocol for identification of the 3′-terminal sequence of viral RNA described above. They include: (1) poly(A) tailing of the dsRNA using yeast poly(A) polymerase; (2) cDNA first-strand synthesis using SuperScript II reverse transcriptase and the dT-*Sac* primer, which hybridizes to the oligo(A) tail; (3) PCR using the dT-*Sac* primer and a second DNA primer that anneals to a known internal region near the 5′ end of the RNA; and (4) cloning and sequencing of the PCR products. It is best to analyze multiple clones (at least 10) to determine the most conserved 5′ terminal viral sequence. For an exact protocol, one can follow the step by step procedure provided in Alternate Protocol 1.

As a starting material, use 5 μl of ~1 μg/μl dsRNA. As a BYV sequence-specific primer for the PCR step the authors used primer C-BYV-*Bgl*II which is a reverse complement of the BYV sequence approximately 850 nucleotides downstream from the putative 5′ terminus. Products of the RT-PCR reaction can be digested with *Sac*I and *Bgl*II and cloned into a suitable plasmid vector (e.g., pGEM; Promega) for sequencing. For some recalcitrant dsRNA preparations, increasing the denaturation temperature during the first-strand synthesis to 90°C is suggested.

BASIC PROTOCOL 5

ADDING RIBOZYME TO THE 3′ END OF THE VIRAL cDNA

Although the importance of generating the authentic 3′ end of the viral cDNA transcript for starting viral replication varies among the different viruses, in the authors' experience with closteroviruses, it appears to be critical. To generate a nearly authentic 3′ terminus of nascent RNA following transcription of viral cDNA in a plant nucleus, a hammerhead ribozyme sequence between the viral cDNA and NOS terminator is added. The ribozyme RNA is capable of *in cis* cleavage at a defined position. This self-cleavage occurs spontaneously and can be used to generate the desirable 3′ end of viral RNA in the cells. Design of a ribozyme is based on the minimal sequence described in (Haseloff and Gerlach, 1988). Each ribozyme should be individually designed and tailored to suit the target sequence. The 3′-terminal part of the ribozyme should fold back and form an antiparallel complementary structure with the 3′ portion of the transcript. A Web-based RNA secondary structure prediction program (*http://www.bioinfo.rpi.edu/applications/mfold/old/rna)* allows analysis of the secondary structure of a newly engineered ribozyme. The easiest way to add a ribozyme sequence

is by PCR. A megaprimer that has a virus-specific part complementary to the 3′ end of the viral cDNA and includes a ribozyme sequence followed by a convenient cloning site has a length of ~80 nucleotides. This megaprimer can be used in combination with a regular PCR primer without modifications to the cycle parameters.

The NOS terminator and polyadenylation signal sequences are routinely used as a natural transcriptional stop signal. This signal adds ~100 nontemplate nucleotides to the 3′ end of a transcript, followed by a poly(A) tail of variable length. These nonviral extensions may be detrimental to viral replication. A ribozyme sequence positioned in between the 3′ viral terminus and NOS terminator acts as a small site-specific endoribonuclease that cleaves off most of the nonviral part of the transcript (Fig. 16F.1.1, step 4). However, the conserved structure of a ribozyme itself adds two nucleotides (-*XXXXXUH*-3′, where *X*s are viral terminal nucleotides and *H* is any nucleotide but G) to the 3′ end of the BYV sequence, which can reduce the specific infectivity of the transcript. As a compromise one might consider modifying the sequence of a ribozyme to ensure cleavage at the exact 3′-terminal nucleotide of the viral sequence. This can impede the cleavage efficiency because of suboptimal ribozyme structure, but it increases the infectivity of the resulting transcript.

Materials

10× *Taq* extender buffer (Stratagene)

4 mM (each) dNTP mixture: commercially prepared using 8 μl of each of four 100 mM dNTPs (Promega) and 168 μl sterile RNase-free water; store up to a few months at −20°C

100 pmol/μl oligonucleotide PCR primers, in sterile water: 5′ sequence-specific primer BYV-*Bst*EII, ribozyme-encoding primer 3BYV-Rib (see Table 16F.1.1)

Taq extender PCR additive (Stratagene)

Taq DNA polymerase

Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)

Plasmid vector containing viral cDNA (see Basic Protocol 3, step 17 or Alternate Protocol 1, step 14)

Restriction endonucleases (*Sma*I or *Xma*I and *Bst*EII)

Modified plasmid vector (see Fig. 16F.1.1, steps 2 and 3): e.g., pCB301 mini-binary vector provided by Dr. D. J. Oliver (see Xiang et al., 1999) with added NOS terminator sequence and modified polylinker (see Strategic Planning)

PCR tubes

Thermal cycler (e.g., PTC-100 thermocycler; Bio-Rad)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000), digestion of DNA with restriction endonucleases (Bloch and Grossman, 1995), and cloning DNA (Sambrook and Russell, 2001)

1. In a PCR tube, mix the following:

   10 μl 10× *Taq* extender buffer
   5 μl 4 mM dNTP mixture
   1 μl 5′ sequence-specific primer BYV-*Bst*EII
   1 μl ribozyme-encoding primer 3BYV-Rib
   1 μl *Taq* extender PCR additive
   1 μl *Taq* DNA polymerase
   72 μl nuclease-free water
   1 μl plasmid vector contalning viral cDNA.

   Choose a plasmid that carries the correct consensus cDNA sequence of the 3′ terminus of viral genome.

2. Carry out PCR using the following amplification cycles:

26 cycles	1 min	93°C	(denaturation)
	1 min	56°C	(annealing)
	2 min	72°C	(extension).

3. Digest the PCR product with *Sma*I (or *Xma*I) and *Bst*EII and clone the fragments into a modified plasmid vector pCB301 (Fig. 16F.1.1, Step 4).

BASIC PROTOCOL 6

ADDING A SEQUENCE OF RNA POLYMERASE PROMOTER TO THE 5′ END OF THE VIRAL cDNA

The Cauliflower mosaic virus RNA polymerase promoter (CaMV 35S promoter) is routinely used to obtain high transcription levels in a variety of plants including *N. benthamiana*. To add this promoter upstream from the 5′ end of the viral cDNA and facilitate initiation of the transcription at the first nucleotide of the viral cDNA, the authors suggest using a PCR-mediated DNA splicing technique. As shown in Figure 16F.1.3, separate PCRs are used to amplify the 35S promoter (primers 5-35S and 3-35S-vir in Table 16F.1.1) and the 5′ end of the BYV cDNA (primers 5-vir-35S and

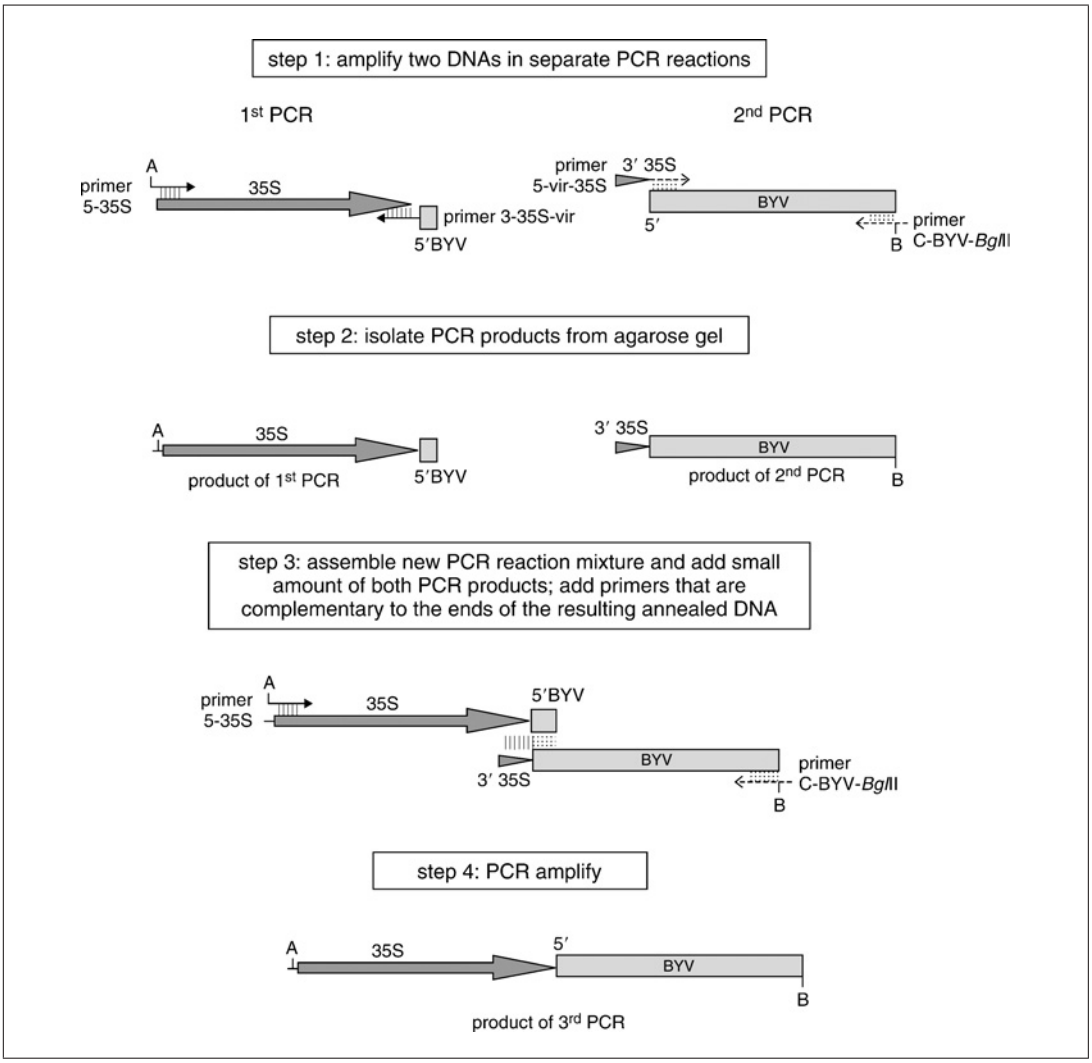

Figure 16F.1.3 Diagram showing consecutive steps of the PCR-assisted generation of the fusion between 35S RNA polymerase promoter and 5′-terminal region of the viral cDNA. The dotted boxes represent viral cDNA, while the cross-hatched arrows correspond to the 35S promoter.

C-BYV-*Bgl*II in Table 16F.1.1) and generate products with overlapping ends (3′-end of 35S promoter and 5′ end of viral cDNA). As a template for the latter PCR, one can use a clone carrying the correct sequence of BYV obtained in Alternate Protocol 2. This PCR amplifies the 5′ terminus of the BYV cDNA and adds a few anchoring nucleotides complementary to the 3′ end of CaMV 35S promoter. After the two first rounds of PCR, the fragments are joined together by mixing products of both reactions and performing a third PCR using primers complementary to the 5′ and 3′ ends of the desired full-length product (primers 5-35S and C-BYV-*Bgl*II; see Fig. 16F.1.3). Overlap in ~20 nucleotides is usually sufficient for annealing of the two fragments during the annealing step of a second PCR. Gel purification of the products of the first PCR reactions significantly improves the yield of a full-length product.

RT-PCR AMPLIFICATION AND CLONING OF THE INTERNAL PART OF VIRAL RNA

BASIC PROTOCOL 7

The procedures described up to this point generate a cassette that contains the 35S promoter, 5′- and 3′-terminal regions of the viral cDNA, ribozyme, and NOS terminator in a low-copy plasmid (Fig. 16F.1.1, step 5). This cassette is used for assembly of the full-length clone by stepwise addition of large internal cDNA fragments using appropriate restriction sites naturally present in viral cDNA. It is critical to ensure that viral cDNA fragments represent a consensus sequence. Due to the inherent low fidelity of PCR, amplification of large viral cDNAs results in accumulation of incidental mutations that render the full-length clone nonviable with virtual certainty. Use of high-fidelity enzymes (e.g., PfuTurbo DNA polymerase; Stratagene) may help alleviate the problem but in the authors' experience has never solved it entirely. One way to circumvent this problem is to screen several independent PCR-derived clones, and reassemble a full-length clone from mutation-free variants. Although this approach was successfully used for animal viruses (Yount et al., 2003; Lee et al., 2005), it requires extensive sequencing and subcloning efforts.

An alternative strategy is to use an RT-PCR-generated "draft" clone as a convenient structural platform for replacing defective fragments with those obtained by conventional cDNA synthesis. The protocol below details the generation of sequences by RT-PCR for cloning into an appropriate low-copy plasmid (the draft clone). Basic Protocol 8 describes conventional cDNA synthesis of viral sequences and their incorporation into the draft clone, where they replace the RT-PCR-generated sequences.

High-quality viral RNA in microgram quantities allows RT-PCR amplification of cDNA fragments up to 10 kb long. Generating fragments of 4 to 6 kb is recommended; they provide an optimal balance between the size of the product (the longer the PCR product the fewer cloning steps are needed) and the amount of the output DNA. A blend of *Taq* polymerase and *Taq* extender PCR additive significantly improves the yield and quality of the PCR product. It is also worth noting that the 10× *Taq* extender buffer supplied with the enzyme already has an optimal concentration of Mg^{2+} ions for the enzyme mixture and does not require additional fine tuning of the reaction conditions.

Materials

~1 µg/µl viral ssRNA (Basic Protocol 1)
Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)
100 pmol/µl oligonucleotide primers (see Table 16F.1.1): 3′ and 5′ sequence-specific primers
First-Strand synthesis buffer (Invitrogen)

4 mM (each) 4 mM dNTP mixture: prepared using 8 μl of each of four 100 mM dNTPs (Promega) and 168 μl sterile RNase-free water; store up to a few months at −20°C

100 mM dithiothreitol (DTT)

200 U/μl SuperScript II RT (Invitrogen)

10× *Taq* extender buffer

5 U/μl *Taq* extender PCR additive (Stratagene)

5 U/μl *Taq* DNA Polymerase

Restriction enzymes appropriate for sites near the ends of the PCR product

Low-copy plasmid vector (e.g., see pCB301 derivative shown in Fig. 16F.1.1, step 5; modified to carry the viral 5′ and 3′ fragments spliced to 35S promoter and NOS terminator regulatory elements; Basic Protocol 6)

0.6-ml microcentrifuge tubes

PCR tubes

Thermal cycler (e.g., PTC-100 thermocycler; Bio-Rad)

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000), digestion of DNA with restriction endonucleases (Bloch and Grossmann, 1995), cloning DNA (Sambrook and Russell, 2001), and purifying digested DNA from agarose gels (Moore et al., 2002)

Reverse transcribe viral RNA

1. To a 0.6-ml microcentrifuge tube, add the following:

   2 μl viral ssRNA (∼1 μg/μl)
   7.5 μl nuclease-free water
   1 μl 3′ sequence-specific primer.

2. Heat 2 min at 70°C. Cool in ice.

   Incubations may be performed in the thermal cycler or other heating devices.

3. Add the following:

   4 μl 5× First-Strand synthesis buffer
   2.5 μl 4 mM dNTP mixture
   2 μl 100 mM DTT
   1 μl SuperScript II RT.

4. Incubate 1 hr at 42°C.

Generate and clone PCR products

5. Transfer 10 μl of the first-strand synthesis product from step 2 to a PCR tube and add the following:

   9 μl 10× *Taq* extender buffer
   5 μl 4 mM dNTP mixture
   1 μl 5′ sequence-specific primer
   1 μl 3′ sequence-specific primer
   1 μl *Taq* extender PCR additive
   1 μl *Taq* DNA polymerase
   72 μl nuclease-free water.

6. Carry out PCR using the following amplification cycles:

26 cycles	1 min	93°C	(denaturation)
	1 min	56°C	(annealing)
	4 min	72°C	(extension).

7. Analyze the PCR product on a 1% agarose gel.
8. Carry out restriction endonuclease digestion using enzymes that recognize sites near the ends of the PCR product.
9. Purify the digested product from the agarose gel (Moore et al., 2002) and clone it into an appropriate low-copy plasmid (see Fig. 16F.1.1, steps 6 and 7).

CONVENTIONAL SYNTHESIS AND CLONING OF cDNAs: FINAL ASSEMBLY OF THE FULL-LENGTH CLONE

BASIC PROTOCOL 8

This protocol describes conventional cDNA synthesis of viral sequences and their incorporation into the draft clone (see Basic Protocol 7), where they replace the RT-PCR-generated sequences. It is recommended as an optimal way to generate a faithful, biologically active, full-length cDNA clone of viral genome.

Even the traditional cDNA synthesis using *E. coli* DNA Polymerase I may result in a population of fragments, some of which can carry unwanted mutations. This could be due to the natural variability of the virus population as well as the lack of proofreading activity of the M-MLV (e.g., SuperScript II) reverse transcriptase. However, this approach provides a dramatic improvement in the quality of the cDNA compared to RT-PCR. Sequencing of clones obtained by conventional cDNA cloning has revealed that virtually all of them have a consensus sequence. More specifically, a 12,800-nucleotide fragment of the BYV that was cloned and assembled using this method had only three nonlethal mutations, compared to on average one lethal mutation introduced by RT-PCR per 1000 bases of a product.

The process of conventional synthesis and cloning of the cDNA fragments is no more difficult than the RT-PCR approach. With the availability of viral RNA of sufficient quality one can expect to see a discrete band after digestion of a cDNA with the restriction enzymes that flank the desired region. Using microgram quantities of the input RNA, the authors have been able to generate cDNAs of up to 7 kb in concentrations sufficient for routine cloning manipulations. For practical purposes, using restriction endonuclease sites that produce fragments of ~4 kb is recommended.

Materials

Nuclease-free water (see *APPENDIX 2A* for DEPC-treated solutions or purchase commercially)
1 μg/μl viral ssRNA (Basic Protocol 1)
Sequence-specific primer
First-Strand synthesis buffer (Invitrogen)
4 mM (each) dNTP mixture: prepared using 8 μl of each of four 100 mM dNTPs (Promega) and 168 μl sterile RNase-free water; store up to a few months at −20°C
100 mM dithiothreitol DTT
200 U/μl SuperScript II RT (Invitrogen)
10× *E. coli* DNA ligase buffer (Invitrogen)
3 U/μl RNase H (Invitrogen)
10 U/μl *E. coli* DNA ligase (Invitrogen)
10 U/μl *E. coli* DNA polymerase (Invitrogen)
Neutral phenol (EMD Chemicals)/chloroform/isoamyl alcohol (25:24:1)
Chloroform/isoamyl alcohol (24:1)
7.5 M ammonium acetate
100% and 70% (v/v) ethanol

0.6-ml microcentrifuge tubes
Vacuum source

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000), digestion of DNA with restriction endonucleases (Bloch and Grossmann, 1995), and cloning DNA (Sambrook and Russell, 2001)

Reverse transcribe RNA

1. In a 0.6-ml microcentrifuge tube, mix the following:

   15 µl nuclease-free water
   6 µl viral ssRNA (1 µg/µl)
   1 µl sequence-specific primer.

   The primer should overlap a convenient cloning site in the viral genome

2. Heat 2 min at 70°C. Cool on ice.

   Incubations may be performed in the thermal cycler or other heating devices.

3. Add the following to the tube:

   8 µl 5× First-Strand synthesis buffer
   4 µl 4 mM dNTP mixture
   4 µl 100 mM DTT
   2 µl SuperScript II RT.

4. Incubate 2 hr at 42°C.

Second-strand synthesis

5. Add the following to the tube from the step 4:

   131 µl nuclease-free water
   16 µl 10× *E. coli* DNA ligase buffer
   6 µl 4 mM dNTP mixture
   0.7 µl RNAse H
   1.5 µl *E. coli* DNA ligase
   5 µl *E. coli* DNA polymerase I.

6. Incubate 8 hr at 16°C.
7. Extract once with neutral phenol/chloroform/isoamyl alcohol mixture and once with chloroform/isoamyl alcohol.
8. Precipitate by adding 100 µl of 7.5 M ammonium acetate and 750 µl of 100% ethanol. Microcentrifuge 10 min at maximum speed, 4°C.
9. Discard the supernatant and wash the pellet with 70% ethanol. Dry under vacuum.

Clone viral cDNA into "draft clone" plasmid

10. Digest the product from step 9 with appropriate restriction endonucleases.
11. Digest the plasmid containing the full-size RT-PCR-based version of the viral cDNA central sequences (e.g., p35S-BYV; Fig. 16F.1.1, step 7; Basic Protocol 7) with the same restriction endonucleases used to digest the product of conventional cDNA synthesis in step 10.

    At this step the vector is retained and the released "draft" insert is discarded because it originates from the error-prone RT-PCR and most likely carries multiple detrimental mutations.

12. Clone the cDNA fragment released in step 10 into the plasmid released in step 11. Repeat steps 10 to 12 as many times as necessary to replace all draft clone DNA with conventionally synthesized cDNA.

    As a result of this cloning step, the RT-PCR-derived cDNA region (which tends to contain multiple mutations introduced by PCR amplification of large DNA stretches) is replaced by a much more faithful cDNA obtained via conventional cDNA synthesis.

13. Sequence the entire cDNA to confirm that it does not carry any detrimental mutation.

14. Prepare a glycerol stock for each plasmid-carrying strain after the first passage in a liquid culture by mixing 850 µl of a liquid culture and 150 µl of sterile 100% glycerol in an microcentrifuge tube. Store indefinitely at –80°C.

AGROINOCULATION OF *N. BENTHAMIANA* PLANTS

BASIC PROTOCOL 9

Agrobacterium-mediated delivery of viral cDNA to plants is the method of choice for launching large RNA viruses. Its major advantage over mechanical inoculation with in vitro RNA transcripts is its very efficient delivery of the viral cDNA to cells, including phloem tissue that is preferentially colonized by closteroviruses and is recalcitrant to mechanical inoculation. Upon simple injection into leaves, *Agrobacterium* actively delivers part of a binary vector containing viral cDNA and control elements to nucleus, such that cells receive multiple copies of a viral expression cassette. A strong 35S promoter drives transcription of the viral cDNA; at least some of the resulting RNA molecules are able of exiting to cytoplasm, being correctly processed by ribozyme, and starting virus infection. In addition, the *Agrobacterium*-mediated inoculation method does not require expensive equipment or reagents. *UNIT 16B.2* can be used to obtain additional detailed information and protocols for agroinfiltration and agroinfection.

A. tumefaciens C58 GV2260 cultures carrying a full-size cDNA copy of a large virus may grow very slowly in the medium containing three antibiotics (kanamycin, rifampicin and streptomycin). If this rate of growth becomes impractical for research progression, one may consider propagating the bacterial culture in the presence of 50 µg/ml kanamycin alone. However, this can lead to gradual disappearance of the Ti plasmid and loss of virulence of the bacterial strain. Because of such potential events, it is imperative to make a glycerol stock of each plasmid-carrying strain after the first passage in a liquid culture (see Basic Protocol 8, step 12) to ensure preservation of the clone. *Agrobacterium* cultures carrying large binary plasmids should never be allowed to overgrow because this may lead to aberrant rearrangement of the plasmid. Each culture grows to its own maximum density, after which it begins to lose its infectivity. As a rough guideline, do not exceed a density of 1.0 at OD_{600}. For reliable and reproducible inoculation of plants, always start the experiment from a frozen glycerol stock cultures and grow for the shortest possible time required to attain the desired cell density. The efficiency of the viral infection in agroinoculated leaves can be increased to a few thousand-fold by using plant virus-derived suppressors of RNA silencing, such as BYV p21 or potyviral HC-Pro (Chiba et al., 2006) as described below.

Materials

A. tumefaciens C58 GV2260 carrying viral cDNA cultured on agar plates (Support Protocol 1)

LB medium (*APPENDIX 4A*) supplemented with 25 µg/ml kanamycin, 12.5 µg/ml rifampicin and 25 µg/ml streptomycin (see *APPENDIX 4A*)

LB medium (*APPENDIX 4A*) supplemented with 25 µg/ml kanamycin, 12.5 µg/ml rifampicin and 25 µg/ml streptomycin (see *APPENDIX 4A*), 10 mM MES, and 20 µM acetosyringone (see recipes for stock solutions)

Agrobacterium induction solution (see recipe)

3 to 4 week old (6 to 8 leaf stage) *N. benthamiana* plants (grown from seed)

28°C shaking incubator
Spectrophotometer
3-ml syringe without a needle

1. Inoculate a single colony of *A. tumefaciens* C58 GV2260 strain carrying a viral cDNA copy into a tube with 5 ml of LB medium supplemented with 25 μg/ml kanamycin, 12.5 μg/ml rifampicin and 25 μg/ml streptomycin. Incubate 24 hr at 28°C, with shaking.

   The efficiency of the viral infection in agroinoculated leaves can be increased by including selected plant virus-derived suppressors of RNA silencing. This is accomplished by transforming A. tumefaciens with a binary vector (the same type as used for cloning the viral DNA) engineered to express the suppressors of RNA silencing (e.g., BYV p21 or potyviral HC-Pro; Chiba et al., 2006) and adding this culture in a 1:10 ratio to the culture carrying the viral cDNA-containing vector in step 5.

2. Transfer the entire contents of the tube into 100 ml of LB medium containing the same antibiotics, 10 mM MES, and 20 μM acetosyringone. Incubate overnight at 28°C, with shaking at 250 rpm.

3. Centrifuge culture 10 min at 5000 × *g*, room temperature.

4. Decant the supernatant and resuspend the pellet in 10 ml *Agrobacterium* induction solution.

5. Adjust the culture to an OD_{600} of 1 with *Agrobacterium* induction solution. Incubate 2 hr at room temperature.

6. Infiltrate 3 to 4 week old (6 to 8 leaf stage) *N. benthamiana* plants with a 3-ml syringe without a needle as follows:

   a. Fill the syringe with 3 ml of the bacterial culture from step 4.
   b. Press the syringe gently against the lower surface of the leaf, placing the finger of the other hand underneath the infiltration point.
   c. Slowly inject the bacterial suspension into the leaf blade.

      The leaf blade darkens gradually in the area where the liquid fills intercellular space.

      Wearing lab coat, gloves, and face shield is strongly recommended to avoid contamination of the skin and clothing. Infiltrate the maximum number of young leaves.

      The first systemic symptoms of BYV infection can be detected 14 to 24 days after inoculation. These symptoms include leaf vein clearing and, later, deformation and curling of the leaves. In the next week or two the plants wilt and die.

SUPPORT PROTOCOL 1

TRANSFORMATION OF *A. TUMAFACIENS* BY ELECTROPORATION

Materials

Electrocompetent *A. tumefaciens* C58 GV2260, frozen culture (Support Protocol 2)
Binary vector carrying viral cDNA (Basic Protocol 8)
LB broth (*APPENDIX 4A*)
LB agar plates supplemented with 25 μg/ml kanamycin, 12.5 μg/ml rifampicin and 25 μg/ml streptomycin (see *APPENDIX 4A*)
Electroporation cuvette (standard 2-mm electrode gap; electroporator specific), ice cold
Electroporator (e.g., BTX ECM 630 electroporator; BTX Instrument Division, Harvard Apparatus)
28°C incubator
14-ml sterile, polypropylene tubes

1. Remove a tube with the electrocompetent *A. tumefaciens* C58 GV2260 cells from –80°C freezer and thaw on ice.
2. Add 1 μl of a binary vector carrying viral cDNA to the tube and mix by gently pipetting.
3. Transfer the suspension into an ice-cold, standard, 2 mm electrode gap, electroporation cuvette.
4. Set parameters of pulse as follows: 2,000 V, 500 Ohm, 50 μF.

   The authors use the BTX ECM 630 electroporator.
5. Place a cold cuvette into the cuvette compartment and electroporate.
6. Remove the cuvette and quickly add 1 ml of LB without antibiotics. Mix the cells by gently pipetting.
7. Transfer the cell suspension to a 14-ml sterile tube and incubate 2 hr at 28°C, without shaking to allow bacteria to recover.
8. Spread 100 μl of the cells onto an LB agar plate supplemented with 25 μg/ml kanamycin, 12.5 μg/ml rifampicin and 25 μg/ml streptomycin and incubate at 28°C for two days or until colonies appear.

PREPARATION OF ELECTROCOMPETENT *A. TUMEFACIENS* CELLS

SUPPORT PROTOCOL 2

Materials

A. tumefaciens C58 GV2260
LB agar plate supplemented with 25 μg/ml of rifampicin and 50 μg/ml of streptomycin (see *APPENDIX 4A*)
LB medium supplemented with 25 μg/ml rifampicin and 50 μg/ml streptomycin (see *APPENDIX 4A*)
Sterile, distilled water, ice cold
20% (v/v) glycerol, sterile and ice cold: autoclave and store up to 1 year at 4°C

28°C shaking incubator
Refrigerated centrifuge

1. Streak *A. tumefaciens* C58 GV2260 onto an LB agar plate containing 25 μg/ml of rifampicin and 50 μg/ml of streptomycin. Incubate at 28°C for two days or until colonies become clearly visible.
2. In the morning, inoculate a single bacterial colony into 5 ml of LB medium supplemented with 25 μg/ml rifampicin and 50 μg/ml streptomycin and incubate 24 hr at 28°C, with shaking at 250 rpm.
3. Inoculate 100 ml of LB medium containing 25 μg/ml rifampicin and 50 μg/ml streptomycin with 2 ml of the overnight culture and incubate 4 to 6 hr at 28°C, with shaking at 250 rpm.
4. Measure the OD_{600} of the culture.

   It should be between 0.5 and 1.0 (do not exceed the latter value).
5. Transfer cells into a sterile centrifuge bottle and centrifuge 10 min at 5000 g, 4°C.

   Carry out all procedures on ice from this point on.
6. Decant the supernatant and carefully resuspend the pellet in 100 ml of ice-cold, sterile distilled water. Centrifuge 10 min at 5000 g, 4°C.

7. Repeat step 6 two more times.
8. Discard supernatant and resuspend pellet gently by pipetting in 5 ml 20% sterile ice-cold glycerol. Dispense the cells into microcentrifuge tubes in 100-μl aliquots store indefinitely at –80°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps unless other solvents are specified. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetosyringone, 150 mM

Dissolve 300 mg of acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone) in 10 ml DMSO. Store up to 1 month at −20°C in 1 ml aliquots.

Agrobacterium induction solution

10 mM MES, pH 5.85 (see recipe)
10 mM $MgCl_2$
150 μM acetosyringone (see recipe)
Prepare fresh before each experiment

MES, 1 M

Dissolve 21.3 g of MES in 80 ml of water. Adjust pH to 5.85 with 1 M KOH. Adjust volume to 100 ml with water and sterilize by passing through a 0.2-μm filter. Store up to 1 month at room temperature.

STE buffer, 1×

50 mM Tris
100 mM NaCl
1 mM Na_2EDTA
Adjust pH to 6.8 with HCl
Autoclave
Store up to 1 month at 4°C

COMMENTARY

Background Information

The family *Closteroviridae* includes three genera and a growing number of important and emerging viruses (Karasev, 2000; Dolja et al., 2006). BYV is a prototype member of a genus *Closterovirus* for which a wealth of information on gene functions and mechanisms has been accumulated (Dolja, 2003; Chapman et al., 2004; Peremyslov et al., 2004a,b) after an infectious cDNA clone of this virus has been obtained (Peremyslov et al., 1998) and improved by tagging with reporter genes (Hagiwara et al., 1999; Peremyslov et al., 1999). Development of the agroinfection procedure for BYV further facilitated studies of its systemic spread (Prokhnevsky et al., 2002; Peng et al., 2003; Chiba et al., 2006). Another member of genus *Closterovirus* for which an infectious cDNA clone is available, is Citrus tristeza virus (CTV) (Satyanarayana et al., 1999). However, CTV has even larger genome and a host range restricted to citrus species. Infection of the citrus trees with cDNA clone-derived virus requires isolation of virus particles following multiple cycles of protoplast transfection (Satyanarayana et al., 2001). Despite these limitations, important progress in understanding CTV RNA synthesis and gene functions has been made (Satyanarayana et al., 2002, 2004; Ayllon et al., 2003).

Critical Parameters and Troubleshooting

Despite successful generation and utilization of the cDNA clones of BYV and CTV, making such clones for closteroviruses still remains a trial-and-error endeavor. The sheer sizes of closteroviral genomes make the process technologically challenging. A critical issue to consider is ability to generate RNA transcripts of the cDNA clone that should be as close to viral genome as possible. This task

depends on the ability to design junctions of the RNA polymerase promoter and ribozyme with 5′ and 3′ ends of viral cDNA to result in efficient RNA accumulation and a few if any nonviral nucleotides. Obviously, these designs are unique for each virus of interest. Another issue that is beyond the control of a researcher, is incidental toxicity of large foreign DNAs for *E. coli*. This results in a very slow growth of transformed cells and selection for bacterial strains that possess mutant, rearranged, or lost cDNA clone (Satyanarayana et al., 2003). As indicated above, we recommend using low-copy number binary vectors to mitigate this problem, and to further benefit from agroinoculation as the most efficient way to infect plants with cDNA clone-derived viral RNAs. If these approaches do not work, one should consider using bacterial artificial chromosomes (Almazan et al., 2000), or recede to a protocols that do not require cloning of full-size viral cDNA, but use alternatives such as *in vitro* ligation of partial cDNA clones (Lai, 2000).

Anticipated Results

Starting with 0.2 to 0.5 mg of the high-quality virion RNA and 0.05 to 0.1 mg of the viral dsRNA, it should be possible to identify terminal nucleotide sequences of the viral genome and generate enough RT/PCR products and cDNA fragments to engineer biologically active cDNA clone of a closterovirus. However, this task could be very challenging for viruses that accumulate to low levels and/or are restricted to inconvenient hosts such as woody plants.

Time Considerations

A realistic estimate for the completion of infectious cDNA clone of a closterovirus is one year. Availability of a consensus nucleotide sequence of the entire viral genome and presence of the useful restriction endonuclease sites could facilitate the cloning. On the other hand, lack of a convenient experimental host or incidental toxicity of viral cDNA could play a negative role and slow the progress down.

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Key References

Peremyslovet al., 1998. See above.

This paper describes generation of the fully biologically active cDNA clone of BYV and its tagging via insertion of the reporter gene encoding green fluorescent protein.

Prokhnevsky et al., 2002. See above.

This paper describes generation of the binary vector designed to launch infectious viral genome via agroinfection that dramatically improved systemic infectivity of the cDNA clone.

Chiba et al., 2006. et al., 2006. See above.

This paper describes the use of viral RNA silencing suppressors to drastically increase the infectivity of virus launched by agroinfection.

Rapid Full-Length Cloning of Nonpolyadenylated RNA Virus Genomes

An infectious clone is an indispensable tool to gain significant understanding of the life of a virus. It allows dissection of *cis*-acting signals, determination of gene function, and construction of virus expression vectors. Moreover, for most viruses, propagating the viral genome as a plasmid clone in *E. coli* is easier, cheaper, and safer than as a virus in a host organism or cell culture system. Full-length infectious clones can be made from nanogram amounts of viral RNA, using the reverse transcription–polymerase chain reaction (RT-PCR).

Most infectious clones have been generated by assembling less-than-full-length DNA fragments. However, the ability to clone the genome from a single full-length PCR product has distinct advantages. First of all, it is extremely rapid. Efficient cloning of many independent full-length clones allows rapid testing for the most infectious of the transcripts contained in the pool of slightly different genomes that occur in any virus preparation. A second advantage is that a transcript is more likely to be infectious if it arises from a single genomic RNA. Assembly of a clone from separately cloned fragments derived from different viable genomes may yield a chimera of different genome fragments that are incompatible when combined on a single RNA. The latter approach has often required time-consuming mixing and matching of genome fragments until an infectious combination is obtained (Boyer and Haenni, 1994; Lai, 2000).

Construction of cDNA clones requires synthesis of cDNA by reverse transcriptase primed by an oligonucleotide complementary to the 3′ end of the viral RNA. To construct a full-length clone of a nonpolyadenylated genomic RNA requires a primer complementary to the 3′ end of the genome. If the sequence of the 3′ end is unknown, then known sequences can be attached to the 3′ end to allow cDNA synthesis with a defined primer, in the widely used process of ligation-anchored PCR (LA-PCR). Optimized methods for first- and second-strand synthesis using virus end-specific primers, as well as 3′ and 5′ LA-PCR are described in this unit.

Genomes of nonpolyadenylated positive strand viruses can exceed 10 kb. Most reverse transcription reactions typically yield products that are only 2 or 3 kb long. Thus, in most cases, much effort must be devoted to amplify full-length genome-sized DNA in a single PCR reaction. Here the authors present methods for achieving PCR products of at least 6 kb, and describe approaches for optimization to achieve potentially much longer RT-PCR products.

This unit contains methods for rapid construction of full-length infectious clones of nonpolyadenylated, sense-stranded RNA viruses. The core process involves three steps. (1) Reverse transcription to create a single-stranded DNA (ssDNA) molecule complementary to the RNA genome. This is commonly referred to as the first strand. (2) Production of a DNA second strand complementary to the first and amplification of this double-stranded DNA (dsDNA) by the polymerase chain reaction (PCR). (3) Cloning of the PCR product into a plasmid vector and replication of this plasmid construct in a suitable bacterial host.

Many factors can slow or undermine the effort to generate a full-length infectious clone of an RNA virus. RNA secondary structure often prevents the reverse transcriptase from producing full-length first-strand ssDNA. Variables such as magnesium concentration,

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annealing temperature, and primer concentration require optimization in order to amplify adequate quantities of full-length dsDNA during PCR. Large DNA fragments are inefficient to clone, and some sequence motifs will be "toxic" to plasmid replication in the bacterial host. Furthermore, a cloned full-length viral construct can be rendered noninfectious by misincorporation of incorrect bases or by strand-switching of the enzyme during either reverse transcription or PCR amplification. For these reasons, the construction of a full-length infectious clone is often a difficult and lengthy process (Boyer and Haenni, 1994).

Numerous advances have been made over the last decade. Enzymes used at critical steps in the procedure are available from a wider range of commercial sources and often in more robust forms, allowing procedures to be more tightly optimized. Also, there have been advances in technique. Because of these developments, barriers that were thought to be insurmountable, such as cloning very long viral genomes, have been overcome (Lai, 2000) and processes that routinely took years for even average-sized viral genomes can be achieved in a matter of weeks.

This protocol is known to work with RNA virus genomes of small to average size (~6 kb). The Basic Protocol assumes that the user has knowledge of the 5′ and 3′ terminal sequences. Support protocols are suggested for cloning genomes when the terminal sequences are not known. The support protocols assume that some portion of internal sequence is known or that alignment of related viral genomes will reveal conserved regions from which internal primers can be designed.

CAUTION: Follow all biosafety requirements relevant to the microorganism under investigation. Refer to *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

NOTE: Use nuclease-free water in all solutions for all steps.

BASIC PROTOCOL

CLONING FULL-LENGTH VIRAL GENOMES OF KNOWN SEQUENCE

If the sequence of the viral genome or at least the genome terminal regions are known, then the following method can be used to amplify and clone the genome. Primers matching the 5′ end region and complementary to the 3′ end region are synthesized and used to prime the first-strand reaction and the following PCR amplification.

The oligonucleotide primers must be designed carefully. The downstream primer will be ~20 bases long and complementary to the 3′ end of the viral genome. The upstream primer will be ~40 bases in length and will contain a bacteriophage DNA-dependent RNA polymerase (DdRp) promoter sequence (either T7-TAATACGACTCACTATA, SP6-ATTTAGGTGACACTATA, or T3-AATTAACCCTCACTAAA). This promoter will be followed by a single G residue, needed for optimal transcription by the RNA polymerase. The G residue will be followed by at least 20 bases that are identical to the 5′ end sequence of the viral genome.

Materials

Downstream primer (25 pmol/μl in nuclease-free water)
Purified viral RNA
Nuclease-free water, sterile (Ambion)
10 mM dNTP mixture: 10 mM each of dATP, dCTP, dGTP, dTTP (also see *APPENDIX 2A*)
Zero Blunt TOPO PCR Cloning Kit (Invitrogen, no. K2800-20) containing:
  12.5 mM dNTP mixture
  Salt solution

TOPO Vector: pCR-Blunt II-TOPO
SOC medium (also see *APPENDIX 4A*)
One Shot TOP10 Chemically Competent *E. coli*

5× cDNA synthesis buffer (supplied by Invitrogen with Thermoscript reverse transcriptase)
0.1 M dithiothreitol (DTT; *APPENDIX 2A*)
RNase inhibitor: RNasin (Promega) or equivalent
Thermoscript reverse transcriptase (Invitrogen)
10× PCR buffer (supplied by Invitrogen with Platinum *Pfx* DNA polymerase)
50 mM $MgSO_4$
2.5 mM dNTP mixture: 2.5 mM each of dATP, dCTP, dGTP, dTTP (also see *APPENDIX 2A*)
Upstream primer (25 pmol/μl in nuclease-free water)
Thermostable DNA polymerase: Platinum *Pfx* (see Table 16F.3.1)
QIAquick Gel Extraction Kit (Qiagen)
LB plates containing selective antibiotics (*APPENDIX 4A*)

0.2-, 0.5-, or 1.5-ml microcentrifuge tubes, sterile and nuclease free
Water bath or thermal cycler
0.5-ml or 0.2-ml thin-walled PCR tubes
−80°C freezer
Thermal cycler

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2000) and isolating DNA from an agarose gel (Moore et al., 2002, optional)

Table 16F.3.1 Commercial Proof-Reading DNA Polymerases

Polymerase	Comments[a]	Supplier
Vent polymerase	Requires optimization for each template	New England Biolabs
Deep Vent polymerase	—	New England Biolabs
Pfu polymerase	—	Promega
Tli polymerase	—	Promega
Pfu polymerase	Very high fidelity, but low yield	Stratagene
Pfu Turbo polymerase	—	Stratagene
r*Tth* polymerase XL	Very high fidelity and yield; setup is complicated	Applied Biosystems
Pwo polymerase	—	Roche
Ex*Taq* polymerase	Not as high fidelity as others	Panvera
LA *Taq* polymerase	—	Panvera
KOD HiFi polymerase	—	Novagen
KOD XL polymerase	—	Novagen
Platinum *Pfx* polymerase	High fidelity and yield on diverse templates; built-in hot-start	Invitrogen
Platinum *Taq* polymerase High Fidelity	—	Invitrogen
Elongase Amplification System	Complicated to use	Invitrogen
Pfu polymerase	—	Fermentas

[a]Comments indicate the authors' experience with selected polymerases, under specific conditions. In some cases the authors did not attempt to optimize the reactions, thus the properties indicated may not apply in all conditions.

Produce ssDNA first strand

1. For each reaction, combine the following (13 µl final volume) in a nuclease-free, sterile microcentrifuge tube (0.2 ml, 0.5 ml, or 1.5 ml):

   1 µl (25 pmol) downstream primer (complementary to the 3′ end region of the viral genome)
   1 µl (1 µg) purified viral RNA
   9 µl sterile, nuclease-free water
   2 µl 10 mM dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP).

   The success of the protocol depends on the integrity of the viral RNA at this step. For best results, use recently purified viral RNA, and confirm by agarose gel electrophoresis that the RNA is full-length and not degraded. Store viral RNA at −80°C when not in use, and minimize the number of times you freeze/thaw the sample.

2. Incubate the sample 5 min at 65°C, then transfer the reaction tube quickly onto ice.

   This step reduces long-distance secondary structure that could interfere with the reverse transcription reaction.

3. Add the following to the reaction tube (20 µl final volume):

   4 µl 5× cDNA synthesis buffer
   1 µl 0.1 M dithiothreitol (DTT)
   1 µl RNase inhibitor
   1 µl Thermoscript reverse transcriptase.

   Areas of stable secondary structure within the RNA will cause the reverse transcriptase reaction to terminate prematurely. This will reduce the yield of full-length first strand which in turn will reduce the yield of PCR product. Thermoscript reverse transcriptase is used here because it is a thermostable enzyme that functions at temperatures (up to 65°C) where secondary structure is reduced. If secondary structure is not an issue, Superscript RT II or Superscript RT III (Invitrogen) may also be used.

4. In a thermal cycler, incubate for:

   12 min at 37°C
   12 min at 42°C
   12 min at 47°C
   12 min at 51°C
   12 min at 55°C.

   The initial low temperature favors annealing of the downstream primer to the RNA template. As the primer is extended by the reverse transcriptase the thermal stability of the RNA/ssDNA duplex rises. Then the incubation temperature can be raised to levels which melt secondary structure without disrupting binding of the nascent ssDNA strand with the RNA template. If a primer with a high T_m is used, it may be possible to incubate immediately at higher temperatures. Alternatively, an initial 10 min incubation at 37°C followed by incubations at higher temperatures for the remainder of the reaction may work well.

5. Heat to 85°C to terminate the reaction.

6. Store first-strand reaction at −80°C.

Amplify full-length viral genome

7. Set up the following PCR reaction (50 µl final volume):

   5 µl 10× PCR buffer
   1 µl 50 mM $MgSO_4$
   6 µl 2.5 mM dNTPs (2.5 mM each of dATP, dCTP, dGTP, dTTP)

1 µl (25 pmol) upstream primer
1 µl (25 pmol) downstream primer
1 µl first-strand ssDNA template
1 µl (2.5 U) thermostable DNA polymerase
34 µl sterile water.

Normally a portion of the first-strand reaction can be used without further purification. Alternatively, the first-strand sample can be applied to a size-exclusion purification column such as those provided by Amicon. This has the advantage of removing free primer and of exchanging the first-strand buffer for nuclease-free water. The disadvantage is that there may be a loss of yield. It is important at this step to use a high-fidelity proofreading DNA polymerase. This will minimize the introduction of potentially lethal errors into the viral genome prior to cloning. This is also necessary when, as in this protocol, the cloning strategy requires that the PCR product have true blunt ends. Many thermostable, high fidelity DNA polymerases are commercially available. The authors use Platinum Pfx DNA polymerase because it combines a low error rate with high yields on a variety of full-length viral templates. Platinum Pfx DNA polymerase also has the added feature of remaining inactive until denaturation temperature is reached during the first round of PCR. This eliminates the generation of nonspecific products during setup of the PCR reactions. (See Critical Parameters and Troubleshooting for a discussion of polymerases; see Table 16F.3.1 for suppliers of polymerases.)

8. Amplify in a thermal cycler as follows:

30 cycles:	1 min	94°C	(denaturation)
	1 min	52°C	(annealing)
	6 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

The extension time is determined by the length of the fragment being amplified. The rule of thumb is to allow 1 min/kilobase. Additionally the annealing temperature may need to be raised if there is nonspecific product generated, or it may need to be lowered if using oligonucleotide primers with a low T_m. An extension temperature of 72°C is standard for most thermostable DNA polymerases. Although the product literature for Platinum Pfx DNA polymerase calls for extension at 68°C, this laboratory has found 72°C to be adequate. See Kramer and Coen (2001), for complete optimization guidelines and resources for calculating the T_m.

9. Check PCR products by electrophoresis on a 0.8% agarose gel (Voytas, 2000).

Clone PCR product (full-length viral genome)

10. Gel purify the PCR fragment using the QIAquick Gel Extraction kit or equivalent procedure (e.g., Moore et al., 2002) if necessary.

If the PCR reaction yields a single band, this step is not necessary. However, if multiple products arise in the PCR reaction, this step will be necessary in order to avoid cloning small, less-than-full-length cDNA fragments. The QIAquick Gel Extraction Kit (Qiagen) provides reliable recovery of cDNA from standard agarose gels.

11. Set up the following reaction (6 µl final volume)

0.5 to 4 µl fresh or gel-purified PCR product
1 µl salt solution
to 5 µl sterile water
1 µl TOPO vector.

Here the protocol makes use of the TOPO cloning technology developed by Invitrogen. Their Zero Blunt TOPO PCR cloning system allows for very rapid cloning of blunt-ended PCR products. Other TOPO systems allow for T/A cloning or directional cloning. The salt solution described above is a proprietary solution provided with the cloning kit. The vector comes precut with topoisomerase I covalently bound to the 3′ end of each DNA strand. The activity of this enzyme is used to efficiently clone the PCR product. Blunt-end fragments clone with the highest efficiency. Therefore, the Zero Blunt TOPO PCR cloning system containing the vector pCR-Blunt II-TOPO is preferred in this laboratory.

12. Incubate 30 min at room temperature.

According to the product literature this reaction can be done in 30 sec to 30 min. The authors' laboratory prefers the longer incubation time because the PCR product being cloned is the size of a full-length viral genome.

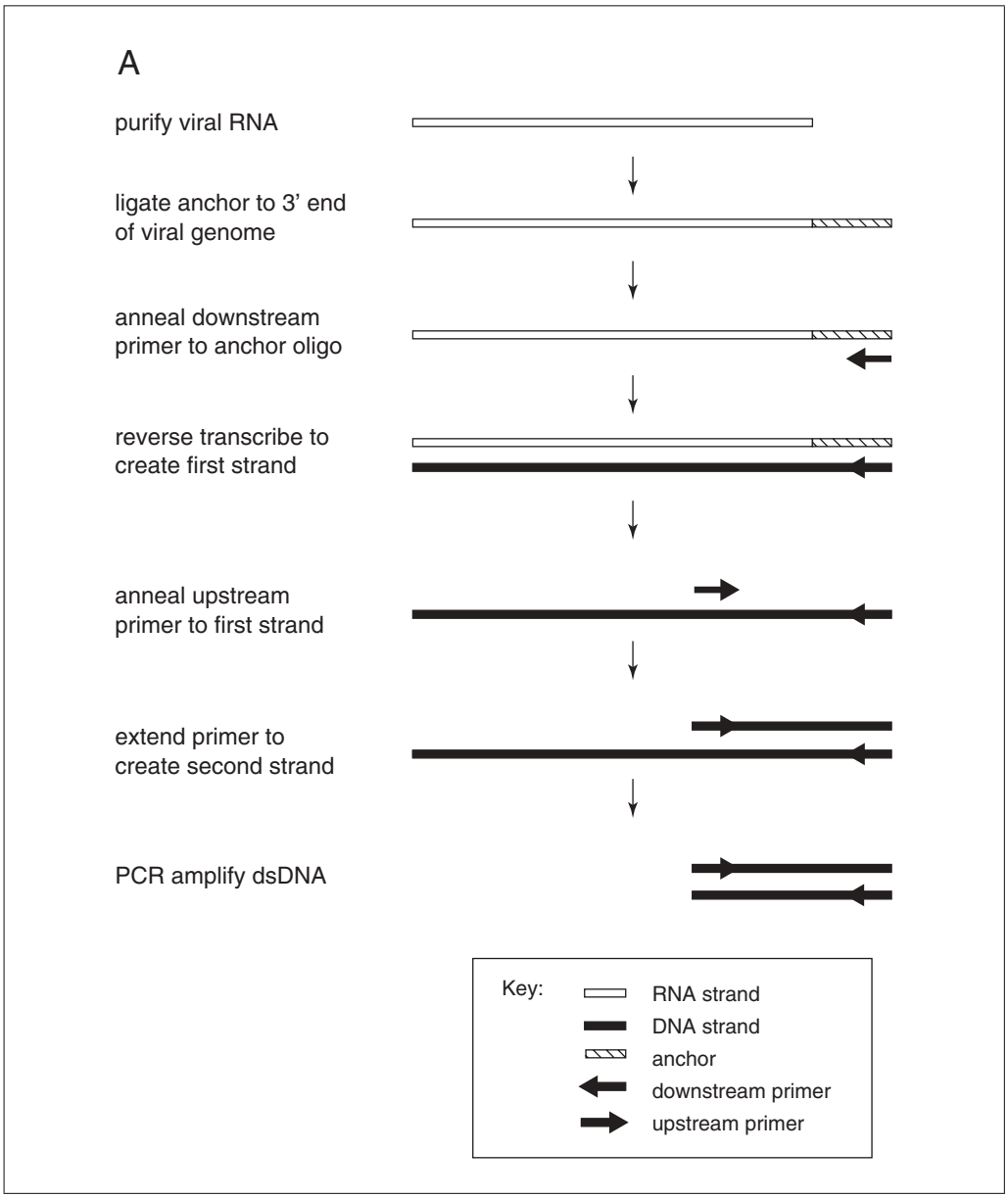

Figure 16F.3.1 Use of ligation-anchored PCR (LA-PCR) to determine the sequence of the 3′ end region of an unknown RNA virus (**A**).

13. Transfer 2 µl of the TOPO reaction to a vial of One Shot TOP10 Chemically Competent *E. coli* cells. Incubate 5 to 30 min on ice.
14. Heat shock 30 sec at 42°C, then place back on ice.
15. Add 250 µl room temperature SOC medium to a vial of transformed cells. Cap and incubate horizontally with shaking at 37°C for 1 hr.
16. Plate on LB containing selective antibiotic and incubate overnight at 37°C.
17. Pick 12 to 24 colonies for screening and analysis.

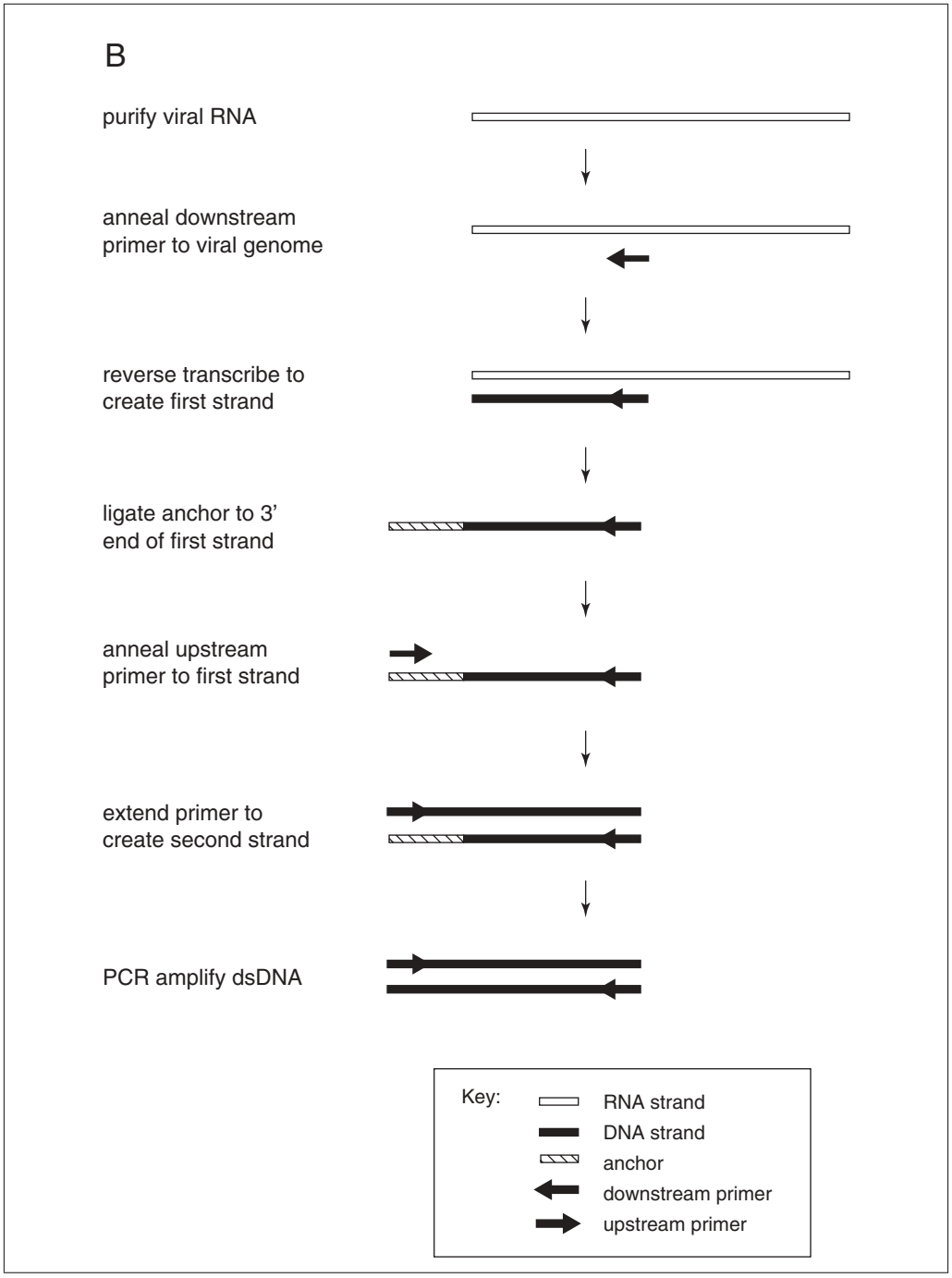

Figure 16F.3.1 *(continued)* Use of LA-PCR to determine the 5′ end region sequence (**B**).

DETERMINING THE 5′ AND 3′ TERMINAL SEQUENCES OF UNKNOWN OR PARTIALLY KNOWN VIRAL GENOMES

One must know the sequence of the 5′ and 3′ terminal regions of a genome in order to carry out the Basic Protocol. Often, however, there is a need to clone the genomes of unknown viruses, of partially sequenced viruses, or of variant isolates of a type virus. In these cases, the sequences of the terminal regions of the genome must be determined before appropriate primers can be designed for use in reverse transcription and PCR amplification. 5′ and 3′ RACE (rapid amplification of cDNA ends) has become perhaps the most popular technique for determining the end regions of sensed-stranded RNAs (Frohman et al. 1988). However, it assumes the presence of a poly(A) tail, and it leaves the identity of the 5′ and 3′ terminal base ambiguous. Here the authors describe the technique known as ligation-anchored PCR (LA-PCR). This technique is more technically demanding, but it works readily on nonpolyadenylated RNA viral genomes, and it clearly identifies the bases lying at the extreme termini of the genome. The method is depicted schematically in Figure 16F.3.1, panels A and B.

Several factors should be considered when designing the anchor oligomer: (1) The sequence of the anchor must not have homology with the viral genome. (2) The anchor oligo needs to be phosphorylated on the 5′ end and amino blocked on the 3′ end. This ensures that the only ligation event which can take place is ligation of the viral RNA or first-strand ssDNA 3′ end with the anchor 5′ end. Most commercial suppliers of synthesized DNA oligos will make these modifications. (3) The anchor must be long enough (~40 nucleotides) to allow for two separate priming sites of 15 to 20 bases each. If PCR amplification with the outside primer yields high background, then a second round of PCR with the internal second primer may be necessary to reduce background and increase the yield of the desired products.

SUPPORT PROTOCOL 1

Determination of 3′ End Sequence

Additional Materials *(see Basic Protocol 1)*

10× RNA ligation buffer (supplied by Ambion with T4 RNA Ligase)
Anchor oligo
T4 RNA Ligase (Ambion)
17°C incubator

Ligate anchor to viral RNA 3′ end

1. Set up the ligation reaction by combining the following in a nuclease-free, sterile microcentrifuge tube (0.2 ml, 0.5 ml, or 1.5 ml; 10 μl final volume):

   1 μl (0.25 μg to 1 μg) purified viral RNA
   1 μl 10× RNA ligation buffer
   1 μl (50 pmol) anchor oligo
   1 μl T4 RNA ligase
   1 μl RNase inhibitor
   5 μl nuclease-free water.

2. Incubate overnight at 17°C.

Produce ssDNA first strand

3. Combine the following in a nuclease-free, sterile microcentrifuge tube (0.2 ml, 0.5 ml, or 1.5 ml; 13 μl final volume):

   1 μl (25 pmol) downstream primer (complementary to the 3′ end region of the anchor oligo)

5 µl viral RNA-anchor molecule
5 µl sterile, nuclease-free water
2 µl 10 mM dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP).

The first-strand reaction can normally be performed by adding 5 µl of the ligation reaction directly, without any additional cleanup. This has the advantage of accelerating the procedure, and the RNA template is not lost during a cleanup step. However, unligated, free anchor will be carried into the reverse transcriptase reaction. This free anchor will compete with the ligated-anchor as a priming site for the primer. If the concentration of primer is kept high enough, the free anchor does not interfere with production of first strand ssDNA.

4. Incubate the mixture 5 min at 65°C. Then transfer the reaction tube quickly onto ice.

5. Add the following to the reaction tube (20 µl final volume):

   4 µl 5× cDNA synthesis buffer
   1 µl 0.1 M DTT
   1 µl RNasin
   1 µl Thermoscript reverse transcriptase.

6. In a thermal cycler, incubate for:

   12 min at 37°C
   12 min at 42°C
   12 min at 47°C
   12 min at 51°C
   12 min at 55°C.

7. Heat to 85°C to terminate the reaction.

8. Store first-strand reaction at −80°C.

Amplify the 3′ terminal region of the viral genome

9. Set up the following PCR reaction (50 µl final volume):

   5 µl 10× PCR buffer
   1 µl 50 mM $MgSO_4$
   6 µl 2.5 mM dNTPs (2.5 mM each of dATP, dCTP, dGTP, dTTP)
   1 µl upstream primer (homologous to an internal virus sequence)
   1 µl downstream primer (annealing to the ligated anchor)
   1 µl first-strand ssDNA template
   1 µl thermostable polymerase
   34 µl sterile water.

   The authors use 2.5 U Platinum Pfx DNA polymerase.

10. Amplify using a thermal cycler as follows:

30 cycles:	1 min	94°C	(denaturation)
	1 min	52°C	(annealing)
	6 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

11. Check PCR products by electrophoresis on a 0.8% agarose gel (Voytas, 2000).

Cloning of the 3′ end fragment is done just as in the Basic Protocol. Bear in mind that the goal of this Support Protocol is to determine the 3′ end sequence of an unknown genome. Once the sequence of the 3′ terminal region is known, a downstream primer complementary to this region needs to be designed as described in the Basic Protocol. Then the full-length genome can be amplified using this downstream primer.

SUPPORT PROTOCOL 2

Determination of 5′ End Sequence

Additional Materials *(see Basic Protocol 1)*

1.5 M sodium hydroxide
10× RNA ligation buffer (supplied by Ambion with T4 RNA Ligase)
T4 RNA Ligase (Ambion)

Boiling water bath
YM-30 column (Amicon)
17°C incubator

Produce ssDNA first strand

1. Combine the following in a nuclease-free, sterile microcentrifuge tube (0.2 ml, 0.5 ml, or 1.5 ml; 13 μl final volume):

   1 μl (25 pmol) downstream primer (complementary to an internal viral sequence)
   1 μl (1 μg) purified viral RNA
   9 μl sterile, nuclease-free water
   2 μl 10 mM dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP).

2. Incubate 5 min at 65°C. Transfer the reaction tube quickly to ice.

3. Add the following to the reaction tube (20 μl final volume):

   4 μl 5× cDNA synthesis buffer
   1 μl 0.1 M DTT
   1 μl RNase inhibitor
   1 μl Thermoscript reverse transcriptase.

4. In a thermal cycler, incubate for:

   12 min at 37°C
   12 min at 42°C
   12 min at 47°C
   12 min at 51°C
   12 min at 55°C.

5. Add 1 μl of 1.5 M sodium hydroxide. Mix and place in a boiling water bath for 5 min.

   Treatment with NaOH hydrolyzes the RNA, leaving the ssDNA first-strand free for amplification without interference from the original RNA template. An alternative procedure is to treat with the RNA/DNA hybrid with RNase H. However, this leaves RNA fragments with phosphorylated 3′ ends that will compete with the ssDNA for ligation to the anchor oligomer.

6. Increase volume to 100 μl with nuclease-free water. Exchange the buffer using a YM-30 column. Resuspend the ssDNA first strand in 10 μl nuclease-free water.

Ligation of ssDNA first strand and anchor oligo

7. Set up the following ligation reaction (10 µl final volume):

   6 µl ssDNA first strand
   1 µl 10× RNA ligation buffer
   1 µl (50 pmol) anchor oligomer
   1 µl T4 RNA ligase
   1 µl RNasin.

8. Incubate at 17°C overnight.

Amplify 3′ terminal region of the viral genome

9. Set up the following PCR reaction (50 µl final volume):

   5 µl 10× PCR buffer
   1 µl 50 mM $MgSO_4$
   6 µl 2.5 mM dNTPs (2.5mM each of dATP, dCTP, dGTP, dTTP)
   1 µl upstream primer (annealing to the ligated anchor)
   1 µl downstream primer (homologous to an internal virus sequence)
   1 µl first-strand ssDNA template
   1 µl thermostable DNA polymerase
   34 µl sterile water.

   The authors use 2.5 U Platinum Pfx DNA polymerase.

10. Amplify as follows:

30 cycles:	1 min	94°C	(denaturation)
	1 min	52°C	(annealing)
	6 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

11. Check PCR products by electrophoresis on a 0.8% agarose gel (Voytas, 2000).

Cloning of the 5′ end fragment is done just as in the Basic Protocol. Remember that the goal of this Support Protocol is to determine the 5′ end sequence of an unknown genome. Once the sequence of the 5′ terminal region is known, a upstream primer homologous to this region needs to be designed as described in the Basic Protocol. Then the full-length genome can be amplified using this upstream primer.

GRADIENT BLOCK PCR FOR OPTIMIZATION OF ANNEALING TEMPERATURES

SUPPORT PROTOCOL 3

When dealing with difficult or unknown templates, newly designed primer pairs, or variable first-strand concentrations, it is critical to use the optimal annealing temperature during PCR. Advances in thermocycler technology allow one to compare twelve different annealing temperatures at one time using a single gradient PCR block. The authors' laboratory uses the DNA Engine with gradient block from Bio-Rad. Most suppliers of thermocyclers sell a unit with this capability. Application of this simple protocol can save weeks of time when adjustment of the annealing parameters is the bottleneck in the cloning pipeline.

Additional Materials *(see Basic Protocol 1)*

0.2-ml tubes in a 12-tube strip (USA Scientific)
Gradient PCR block and compatible PCR base unit (e.g., Bio-Rad)

1. Prepare the following cocktail in a sterile 1.5 ml microcentrifuge tube (650 µl final volume):

   65 µl 10× PCR buffer
   13 µl 50 mM $MgSO_4$
   78 µl 2.5 mM dNTPs (2.5 mM each of dATP, dCTP, dGTP, dTTP)
   13 µl (325 pmol) upstream primer
   13 µl (325 pmol) downstream primer
   13 µl ssDNA first-strand template
   13 µl (32.5 U) Platinum *Pfx* DNA polymerase
   442 µl sterile water.

2. Dispense 50-µl aliquots into every tube of a 12-tube strip (0.2-ml tube volume)

3. PCR amplify as follows:

30 cycles:	1 min	94°C	(denaturation)
	1 min	40° to 60°C gradient	(annealing)
	6 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

4. Check PCR products by electrophoresis on a 0.8% agarose gel (Voytas, 2000).

   For speed, use a gel system where the teeth spacing on the combs used for preparing the gels allows for gel loading with a multichannel pipettor.

SUPPORT PROTOCOL 4

GEL PURIFICATION OF PCR PRODUCTS

When RT-PCR amplification results in multiple bands, it is necessary to gel purify the desired band before cloning. This laboratory prefers using the QIAquick Gel Extraction Kit (Qiagen) for this purpose. However, the following protocol will also provide reliable recovery of cDNA fragments from low melting point agarose gels.

Additional Materials *(see Basic Protocol 1)*

0.8% low-melting-point agarose gel (Bio-Rad; also see Voytas, 2000)
Buffered phenol (*APPENDIX 2A*)
25:24:1 (v:v:v) phenol/chloroform/isoamyl alcohol (*APPENDIX 2A*)
3 M sodium acetate, pH 5.5
70% and 95% ethanol, −20°C

1. Separate the multiple cDNA bands by electrophoresis on a 0.8% low-melting-point gel (Voytas, 2000).

   If the yield of PCR product is high, then only load a fraction of the PCR reaction on the gel. If the yield is low, prepare the gel using a preparative comb and load the entire reaction. Low-melting-point agarose from Bio-Rad is preferred by this laboratory.

2. Place the gel on a UV light box and use a clean razor blade to remove a gel plug containing the desired band.

   Keep the UV light on for minimal period of time possible, to limit UV radiation damage to the DNA and the user.

3. Incubate 5 min at 65°C to melt the gel plug.

4. Add 1 vol buffer-saturated phenol. Vortex briefly and microcentrifuge for 2 min at maximum speed, 4°C.
5. Remove the upper aqueous phase and transfer to a new tube.
6. Add 1 vol phenol/chloroform/isoamyl alcohol (25:24:1). Vortex briefly and centrifuge for 2 min at maximum speed, 4°C.
7. Remove the upper aqueous phase and transfer to a new tube.
8. Add 1/10 vol of 3 M sodium acetate (pH 5.5) and 2 vol of 95% ethanol at −20°C. Mix and place at −80°C for 1 hr.
9. Microcentrifuge 30 min at maximum speed, 4°C.
10. Wash the pellet with 1 ml of 70% ethanol at −20°C. Microcentrifuge 10 min at maximum speed, 4°C.
11. Dry the pellet under vacuum for 5 min.
12. Dissolve the pellet in 15 μl nuclease-free water.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

cDNA synthesis buffer, 5×

250 mM Tris·acetate, pH 8.4
375 mM potassium acetate
40 mM magnesium acetate

This buffer is supplied by Invitrogen with Thermoscript RNase H⁻ Reverse Transcriptase.

RNA ligase buffer, 10×

0.5 M Tris·Cl, pH 7.8 (*APPENDIX 2A*)
0.1 M $MgCl_2$
0.1 M DTT
10 mM ATP

This buffer is supplied by Ambion with T4 RNA Ligase.

COMMENTARY

Background Information

The study of RNA viruses was revolutionized in the 1980's by two major methodological breakthroughs. The first was construction of full-length infectious clones of viral genomes (Taniguchi et al., 1978; Racaniello and Baltimore, 1981; Ahlquist et al., 1987). This, combined with rapid developments in site-directed mutagenesis, allowed viral reverse genetics to become the norm. The second major breakthrough was the polymerase chain reaction. This allows very simple site-directed mutagenesis and the ability to work with extremely small amounts of starting genetic material (Kowalik et al., 1990). Perhaps the most extreme example of the application of PCR and in vitro transcription to characterize a nonpolyadenylated, positive-sense RNA virus is the study of hepatitis C virus. This virus accumulates to extremely low levels in its host, but its RNA structure and function are well-characterized owing to PCR amplification, cloning, and transcription of its genome (Lohmann et al., 2003).

RNA viruses exist as quasispecies, i.e., any single virus isolate consists of a population of slightly different copies of the viral genome (Domingo, 2003). Many individual RNA molecules may not be infectious on their own, but have acquired mutations that make them dependent on the viable genomes for replication. Efficient cloning of many independent full-length clones allows rapid testing for the most infectious transcripts. Individuals

in the quasispecies may contain sets of polymorphisms that must occur together on the same genome to allow replication. Hence, assembly of a clone from separately cloned fragments derived from different viable genomes may not produce an infectious transcript.

Nonpolyadenylated RNAs require special attention for cloning of the complete 3′ end. For host mRNAs and many viral genomes an oligo(dT) primer complementary to the poly(A) tail will suffice for first-strand synthesis. However, unlike mRNAs, the genomes of many positive-strand RNA viruses are not polyadenylated. Examples include the bromoviruses, tobamoviruses, tombusviruses, luteoviruses, cucumoviruses, and alfamoviruses of plants; nodaviruses of insects and fish; and the pestiviruses, flaviviruses, and hepaciviruses of mammals. The methods for full-length RT-PCR cloning detailed in this unit have been applied to produce seven independent full-length clones of cereal yellow dwarf virus-RPV (CYDV-RPV) in about one week. The clones differed in infectivity. Some were not infectious. This variation in infectivity reflects the slight variations in sequence between each RNA, even though all were cloned from a highly homogeneous virus isolate. Extensive previous attempts to assemble separately cloned fragments into an infectious clone were unsuccessful. Thus, this unit should be valuable to researchers attempting to construct one or many full-length infectious clones of any RNA virus whether or not it harbors a poly(A) tail.

Critical Parameters and Troubleshooting

Even with the technological advances made in recent years, the process of creating a full-length infectious clone of any RNA virus is far from routine. A sensitive multistep process is required, and each step may demand "tweaking" or intensive optimization. The comments below address the experimental parameters that may need optimizing at each step. Bear in mind that the various details all address one or more of the following broad issues:

1. *Physical construction*: What can hinder the step-by-step movement from an RNA genome to a cloned dsDNA copy of this genome?

2. *Preserving infectivity*: What factors might cause a successfully constructed full-length clone to still be noninfectious?

3. *Speed*: How can a full-length infectious clone be constructed quickly?

RNA purification

RNA purification methods vary from system to system and from virus to virus (see Section 16E). To obtain full-length clones it is essential to start with high-quality, full-length RNA. A number of standard precautions should be followed. Wear gloves to avoid contaminating the virus sample with fingertip nucleases. Use nuclease-free pipet tips and microcentrifuge tubes. Prepare reagents in baked glassware using nuclease-free water (Ambion or Sigma) and chemicals dedicated to RNA work. Ideally, use freshly extracted viral RNA which has been stored at −80°C and has gone through a minimum of freeze/thaw cycles. Before proceeding to subsequent steps, visually check the viral RNA by agarose gel electrophoresis. For electrophoresis, the E-gel precast agarose gel system (Invitrogen) works well for quick assessment of viral RNA quality. The E-gel system eliminates the need for laborious treatments of gel rigs and running buffers with diethylpyrocarbonate (DEPC) or other RNase deactivators. Presence of ribonuclease in a gel system can degrade RNA in the gel leading to the erroneous conclusion that the whole batch is degraded.

Design of primer pairs

If the 5′ and 3′ terminal sequences of an RNA virus are known, then an effective pair of upstream and downstream primers can be designed. The *downstream primer* will be used first and will come into direct contact with the RNA genome of the virus. Thus, ensure that this primer is nuclease free. Many commercial suppliers of oligomers offer RNase-free synthesis. At minimum, dissolve the newly synthesized primer in nuclease-free water (Ambion) and handle it as if working with RNA. The downstream primer must be complementary to the 3′ end region of the viral RNA genome. Design the 5′ prime extension of the primer with a unique restriction enzyme site that incorporates the terminal bases of the genome. Ideally, create a blunt-ended restriction site which is not found in the viral genome and which will leave a perfect viral 3′ end when the eventual full-length clone is cut at this site. For example, the genomes of barley yellow dwarf virus and the Tombusviridae terminate in CCC. By using the 5′ prime extension of the downstream primer to add GGG, a unique *Sma*I site is created. When the full-length clone is linearized with *Sma*I, the resulting transcript terminates with a perfect CCC 3′ end. If it is not possible to create a unique restriction site

that generates a perfect viral 3′ end, design the downstream primer with the goal of leaving a 3′ extension with as few nonviral bases as possible. Many positive strand RNA viruses tolerate extra bases at the 3′ end. Ideally, the 3′ primer should have a T_m of 65° to 80°C.

The 3′ end of the *upstream primer* must normally be identical to the 5′ end of the viral genome (unless mutations are being introduced intentionally). The primer should contain the promoter sequence immediately adjacent to (5′ of) the viral sequence in the primer. This ensures that transcription initiates precisely at the 5′ end of the viral genome. Because bacteriophage polymerases used for in vitro transcription require a G at the initiation site, if the viral 5′-terminal base is not G, one or two G's can be added in the primer at the initiation site. More than one G gives more efficient transcription initiation and thus higher yield of transcript but at the cost of reduced infectivity of the RNA. The RNAs of positive-strand RNA viruses generally require near-perfect 5′ ends to be infectious (Bujarski and Miller, 1992). This obviously creates a rather long upstream primer and a potential imbalance between the upstream and downstream primers during PCR. With proper optimization, however, this will not be a problem. In general, primers with longer sequences of homology to the viral genome give more specific and more efficient production of the desired PCR product, regardless of the primer pair compatibility predicted by primer design programs.

If the 5′ and 3′ terminal sequences of the viral genome are not known, then ligation-anchored PCR (LA-PCR) can be used to sequence the ends. As with the downstream primer, the *ssDNA anchor* needs to be handled using RNase-free technique. During synthesis of this oligomer a 5′ phosphate and a 3′ amino block must be added. The 5′ phosphate allows the anchor to be ligated to the 3′ hydroxyl group of an RNA or a ssDNA molecule. The 3′ amino group prevents the anchor from ligating to itself. The anchor needs to be 40 to 50 nucleotides long in order to allow for two distinct primer binding sites on the anchor molecule. During PCR amplification of either viral end, an "outside" primer annealing close to the 3′ end of the anchor should be used first. If PCR yields high background or nondistinct bands, then a second round of PCR should be done using a second primer annealing on the anchor internally to the first. As stated previously, the 5′ and 3′ RACE methods are used commonly for determining the terminal sequences of RNA molecules. (Frohman et al., 1988; Frohman, 1994). However, the identity of the 5′ and 3′ terminal base on the viral RNA is left ambiguous with the RACE methodologies. Because incorrect terminal bases have rendered full-length clones noninfectious, the LA-PCR protocol provides a better method for end sequencing than RACE (Troutt et al., 1992; Richman et al., 2002).

Anchor ligation to 3′ end of RNA

The first step of 3′ LA-PCR is to ligate the ssDNA anchor molecule to the 3′ end of the viral RNA genome. This ligation reaction is mediated by T4 RNA ligase, which can ligate ssDNA to RNA but does so inefficiently. The authors have tested several commercial sources of T4 RNA ligase and found the enzyme supplied by Ambion to be effective. The optimal concentration of anchor used in the ligation reaction may need to be determined empirically. Some protocols suggest adding as few as 6 pmol anchor to the ligation reaction. This laboratory has obtained higher yields of subsequent PCR product by increasing the level of anchor in the initial ligation reaction to as high as 50 pmol.

First-strand synthesis by reverse transcription

The crucial issue at this step is acquiring full-length first strand. RNase H activity, template secondary structure, and misincorporation of nucleotides can all cause reverse transcriptase to create truncated cDNA molecules. (Das et al., 2001; Hawkins et al., 2003). As the genome increases in length so does the likelihood that one of these factors will reduce the yield of full-length first strand. The inherent RNase H activity associated with reverse transcriptases can be avoided by using a commercially available RNase H^- enzyme such as Superscript II, Superscript III, or Thermoscript (Invitrogen). Secondary structure is reduced either with additives such as DMSO, glycerol, formamide, betaine, or trehalose (Chester and Marshak, 1993; Carninci et al., 1998; Spiess and Ivell, 2002; Hawkins et al., 2003) or by adjusting the temperature at which the first-strand reaction is carried out. The authors have used Thermoscript (Invitrogen) in optimization protocols because its thermostable properties allow the reverse transcriptase reaction to be carried out at temperatures as high as 60°C. At such temperatures much RNA secondary structure is disrupted, allowing the reverse transcriptase to continue moving on the

RNA template. The reaction conditions in this protocol begin at 37°C in order to allow the downstream primer to anneal fully and to be extended by reverse transcriptase. The reaction temperature is then ramped up by stages until a final temperature of 55°C is reached. A two-step approach may work well on some templates where the first-strand reaction begins at 37°C for a few a minutes before being raised in one step to the final high temperature for the remainder of the reaction. First-strand truncation due to misincorporation of nucleotides during reverse transcription has not been found by this laboratory to be a large problem for viruses with genomes 6 kb or smaller. Addition of a small amount of a proof-reading DNA polymerase has been used by others to generate full-length first strand up to 15 kb in size. (Hawkins et al., 2003).

Anchor ligation to 3′ end of first-strand cDNA

If the 5′ terminal sequences of the viral genome are not known, LA-PCR must be used at this step. In this case T4 RNA ligase is used to ligate the anchor molecule to the 3′ end of the first-strand ssDNA. RNA degradation is no longer a concern at this step. However, the other parameters addressed in the description of LA-PCR at the viral 3′ end remain considerations here.

PCR amplification

Successful PCR amplification of any DNA molecule requires several variables to work in harmony. Annealing temperature, extension time, primer concentration, primer T_m, magnesium concentration, and polymerase quality all need to be optimal. A number of reviews address general optimization of PCR conditions (Kramer and Coen, 2001). Here, the matters found most critical to a successful reverse transcriptase PCR (RT-PCR) or a ligation-anchored PCR (LA-RT-PCR) are addressed.

DNA polymerase: The polymerase must have a proofreading function in order to avoid introducing base changes that could render the full-length clone noninfectious. The DNA polymerase must also be processive enough to amplify the entire genome. By amplifying the entire genome at once, the time-consuming process of piecing together fragments of the genome from subclones is avoided. This also obviates the possibility of introducing lethal mutations at subclone junctions. A trade-off comes at this point, however. In general, the commercially available polymerases claiming the lowest error rate also tend to have a lower maximum fragment length that they are able to routinely amplify. In order to amplify a full-length genome, the authors recommend testing several commercially available enzymes (see Table 16F.3.1) to see which will amplify the genome of interest to high levels with the lowest possible error rate. The authors chose Platinum *Pfx* DNA polymerase (Invitrogen) because it was robust enough to generate clonable quantities of full-length product on a variety of templates. Additionally, Platinum *Pfx* DNA polymerase comes supplied with Platinum anti-*Pfx* antibodies which inhibit polymerase activity until denaturation temperatures are first reached during PCR. This technology provides an automatic and simultaneous "hot start" of all PCR reactions.

Primer concentration: The primer concentration may need to be adjusted to favor amplification of full-length RT-PCR product. If nonspecific or less-than-full-length products are prominent in the PCR reaction, it may help to reduce the amount of primers added to as low as 10 pmol/reaction. One must be aware, however, that some steps taken to speed the cloning process may require careful titrating of the primer concentration. For example, LA-PCR of the 3′ end of a genome is dramatically faster if, without any further purification, a fraction of the anchor ligation reaction mix is added directly to the first-strand reaction and a portion of the first-strand reaction is added directly to the PCR reaction. However, this will transfer some free anchor molecules into the PCR mixture where these small ssDNAs will compete as primer sites for the downstream PCR primer. This can markedly reduce yield of full-length viral product. The problem is solved by increasing the primer concentration to at least 25 pmol/reaction.

Annealing temperature: This is a sensitive parameter when dealing with difficult templates. If set too low, less-than-full-length, nonspecific products may dominate the PCR reaction. If set too high, the reaction may fail to generate any product. With some templates, the workable temperature window is small. Thus, care must be taken to determine the optimal annealing temperature, particularly when attempting to clone full-length genomes of unknown or poorly characterized viral isolates. At times, this parameter needs to be re-examined even after standard PCR operating procedures have been established for the virus of interest. In this laboratory, closely related but distinct isolates of the same virus have required markedly different annealing

temperatures for amplification of clonable amounts of genome. Fortunately, this otherwise time consuming, trial-and-error optimization process is accomplished very rapidly with the use of the gradient block PCR technology described above.

Other paramters: Several remaining PCR variables require brief comment. The extension time used during PCR will vary with template. As a rule of thumb, allow for 1 min/kb of genome. Some DNA polymerases are fairly sensitive to magnesium concentration. Normally, instruction is provided in the product literature for trying and assessing several magnesium levels. Finally, use of any of several available primer designer programs is necessary in order to check for primer problems such as potential for false priming, primer dimers, low G/C content, or low T_m. However, many PCR reactions have worked in the hands of the authors with primer pairs that were rejected by computer primer designing programs.

Cloning

Traditional cloning of PCR products by restriction enzyme digestion, followed by ligation into a plasmid vector linearized with compatible enzymes is time-consuming and often inefficient. The ends of the PCR product must be prepared by restriction digestion in order to provide a phosphorylated "sticky" end. TA cloning, which is sometimes used to avoid this step, is not useful here, because the proofreading DNA polymerase used during the PCR amplification leaves true blunt ends on the PCR product. Restriction digestion generally must be followed by gel purification to separate the main PCR product from the small end fragments liberated by the restriction enzyme. Similarly, the vector must be prepared by restriction digestion, possible gel purification, and treatment with an alkaline phosphatase. Ratios of vector to insert must be determined followed by ligation and transformation of a bacterial host. Preparation of the vector and insert can take days, with multiple steps where problems can arise that reduce the ultimate number of clones obtained.

In contrast to restriction enzyme-based cloning, the TOPO cloning system (Invitrogen), is fast and efficient. The vector comes precut and ready for use. If PCR yields a single band or a dominant band with minor background, then the PCR product can be added directly to the TOPO reaction without further manipulation. If gel purification of the PCR product is needed, the appropriate band can be purified quickly using commercial purification kits (Qiagen) and then used immediately without further preparation of the ends. Nonproofreading DNA polymerases add a single dATP residue to the 3′ end of each strand of the PCR product. The proofreading DNA polymerase used in this protocol "polishes" the ends by removing the overhanging adenosine residue. Thus, a population of cDNA molecules is created during PCR where the majority have true blunt ends, ideally compatible with the Zero Blunt TOPO vectors. The topoisomerase is very efficient: a 6-kb fragment, representing a full-length viral genome, which was extremely difficult to clone using traditional ligation methods, was readily cloned in this laboratory using the TOPO system on the first try. The PCR amplified genome was inserted into a TOPO vector and transformed into *E. coli* within 3 hr.

Anticipated Results

Starting with ≥250 ng of purified viral RNA (genome size ≤6 kb) this protocol can quickly generate full-length clones of an RNA virus. Properly optimized, the protocol allows amplification and cloning of the entire genome without the assembling of multiple subclones. Though the procedures have not been tested on larger genomes, it is anticipated that full-length clones of genomes >6 kb can be constructed using these methodologies.

Time Considerations

The Basic Protocol, resulting in full-length clones ready for screening, can be executed in 1 to 2 days. First-strand synthesis requires 1 to 2 hr. Amplification takes 2 to 5 hr, depending on the size of the viral genome. TOPO-mediated insertion of the amplified fragment into the vector takes 1 to 4 hr depending on if gel purification is needed. If LA-PCR is required to determine the ends sequences of the genome, the 5′ and 3′ ends should be cloned simultaneously. Clones of the end fragments can be obtained in 3 days.

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Identification of Viroids by Gel Electrophoresis

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ABSTRACT

Two-dimensional PAGE involving an initial fractionation under nondenaturing conditions followed by a second electrophoresis under denaturing conditions provides a powerful means to detect viroids and other small circular RNAs. This unit describes a method known as "R(eturn) PAGE" in which denaturation is achieved by simultaneously raising the temperature and lowering the ionic strength during the second electrophoresis. Under denaturing conditions, circular RNAs migrate more slowly than their corresponding linear forms. Following fractionation, RNAs are visualized by staining with ethidium bromide, SYBR Gold, or silver nitrate. Unlike nucleic acid hybridization or RT-PCR, viroid identification by R-PAGE requires no nucleotide sequence information. *Curr. Protoc. Microbiol.* 10:16G.1.1-16G.1.9. © 2008 by John Wiley & Sons, Inc.

Keywords: viroids • polyacrylamide gel electrophoresis (PAGE) • circular RNAs • (R)eturn PAGE

INTRODUCTION

Viroids are the smallest autonomously replicating pathogens yet described. These small, unencapsidated, circular RNA molecules lack mRNA activity yet are able to induce a wide range of disease symptoms in susceptible plant hosts. Although molecular hybridization or polymerase chain reaction (PCR) techniques have now largely replaced polyacrylamide gel electrophoresis (PAGE) for routine detection of well characterized viroids, electrophoresis remains an essential tool for the detection of viroid-like molecules of unknown sequence. Unlike these other techniques, PAGE requires no nucleotide sequence information.

The migration rate of a macromolecule during PAGE is determined by three factors: charge, size, and shape. Two-dimensional PAGE (initial fractionation under nondenaturing conditions followed by electrophoresis under denaturing conditions) provides a powerful means to detect viroids and other small circular RNAs because they migrate more slowly than linear RNAs under denaturing conditions. This unit describes a method known as "R(eturn) PAGE" in which denaturation is achieved by simultaneously raising the temperature and lowering the ionic strength during the second electrophoresis. Following fractionation, RNAs are visualized by staining with ethidium bromide, SYBR Gold, or silver nitrate.

STRATEGIC PLANNING

Although viroids have been isolated from a wide range of plant species, no animal or human infections have been reported. Thus, Biosafety Level 1 (BSL-1) precautions are adequate for most laboratory manipulations. In the United States, laboratory and greenhouse studies require a permit from the Animal and Plant Health Inspection Service (PPQ Form 526). Experimental protocols involving viroids should also be reviewed by the local Institutional Biosafety Committee (IBC).

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RNA ISOLATION

No single RNA extraction protocol works well with every possible viroid/host combination. As described by Hanold et al. (2003), the challenge is to prepare undegraded viroid RNA in concentrations sufficient to be visible on the gel, yet containing a sufficiently low level of impurities so as not to interfere with R-PAGE. Viroid concentrations are usually highest in young, symptomatic tissue. Small amounts of leaf tissue (0.1 to 1.0 g) can be conveniently powdered in liquid N_2 using a mortar and pestle and extracted with TRIzol reagent as described by the manufacturer (Invitrogen). Total RNA yields usually range between 250 to 1000 μg/g tissue fresh weight. Ribosomal and other large single-stranded RNAs are insoluble in 2 M LiCl; thus, LiCl precipitation is often used to enrich an RNA preparation for viroids prior to R-PAGE.

Materials

White quartz sand (50 to 70 mesh; Sigma, cat. no. 14808-60-7 or equivalent)
Liquid nitrogen
Plant tissue (fresh or frozen)
TRIzol reagent (Invitrogen)
Chloroform (molecular-biology-grade)
Isopropanol (molecular-biology-grade)
70% (v/v) ethanol/0.1 M sodium acetate (see recipe)
RNase-free H_2O
4 M LiCl
Absolute ethanol

Mortar and pestle (autoclaved)
Spectrophotometer

Additional reagents and equipment for measuring A_{260} using a spectrophotometer (Gallagher and Desjardins, 2006)

1. Add an appropriate amount of sand (0.2 g/g tissue) to an autoclaved mortar, carefully fill the mortar with liquid N_2, add the plant tissue (fresh or frozen), and grind until the tissue has been reduced to a fine powder and the liquid N_2 has evaporated.

   Although not essential, addition of sand facilitates the grinding process and helps ensure that the tissue is finely powdered.

2. Add the appropriate amount of TRIzol reagent (1 ml/100 mg tissue), quickly mix the reagent with the powdered tissue to form a thick paste, and allow to thaw.

   CAUTION: *Use gloves and eye protection when working with TRIzol reagent. Avoid contact with skin or clothing and use in a chemical fume hood. Avoid breathing vapor.*

3. Continue the extraction process, following the manufacturer's protocol as described in the data sheet that accompanies each bottle of TRIzol reagent.

   TRIzol reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. A mono-phasic solution of phenol and guanidine isothiocyanate, this reagent is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Kingston et al., 1996). During sample homogenization, TRIzol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase and is recovered by precipitation with isopropyl alcohol. Dispose of the lower organic phase containing chloroform and phenol with other hazardous waste from the laboratory.

4. Wash RNA pellets with 70% (v/v) ethanol/0.1 M sodium acetate, dissolve in RNase-free H_20, and measure A_{260} using a spectrophotometer (see Gallagher and Desjardins, 2006).

5. Assuming that an A_{260} of 1.0 indicates 40 μg/ml single-stranded RNA, adjust RNA to a final concentration of 1 to 2 mg/ml, add an equal volume of 4 M LiCl, and incubate overnight at 0°C to 4°C.

   IMPORTANT NOTE: *Chill RNA on ice before starting LiCl fractionation and add the 4 M LiCl solution dropwise with constant mixing. A flocculent precipitate containing primarily rRNA and large mRNA should appear only SLOWLY (after 1 to 2 hr). Too rapid precipitation may nonspecifically trap viroid RNA.*

6. Centrifuge 5 min at 13,000 × *g*, 4°C, and remove the supernatant, which contains low-molecular-weight RNA (including most but not all viroids) plus small amounts of contaminating DNA.

7. Add 2.5 vol absolute ethanol and precipitate overnight at −20°C. Collect the low-molecular-weight RNA pellet by centrifuging 10 min at 13,000 × *g*, 4°C, rinse pellet with 70% ethanol/0.1 M sodium acetate, and dissolve in RNase-free water.

   IMPORTANT NOTE: *It is very important to avoid introducing RNase into solutions used to isolate, store, or analyze RNAs. Use sterile, nuclease-free plasticware and pipet tips. Where possible, wear disposable latex or nitrile gloves and change them frequently. Practice good microbiological technique to avoid contaminating solutions with bacteria or fungi. RNAs are most stable when stored as ethanol precipitates at −20°C. Store aqueous solutions frozen at −20° or −70°C (preferable).*

R-PAGE ANALYSIS

BASIC PROTOCOL 2

As described by Schumacher et al. (1986) and Singh and Boucher (1987), R-PAGE was originally performed using a precursor to the current Bio-Rad PROTEAN II xi vertical electrophoresis cell and required large volumes of running buffer. The procedure described below has been adapted for use in a "minigel" format and requires only 300 ml of buffer.

Materials

GlassPlus or equivalent glass cleaner
95% ethanol
Gel solution (see Table 16G.1.1)
1× TBE
Isolated RNA (Basic Protocol 1)
6× gel loading buffer (see recipe)
0.5 μg/ml ethidium bromide in water

Compatible plates, spacers, and combs designed for Bio-Rad's Mini PROTEAN 3 apparatus (available from Bio-Rad Laboratories)
JGC-3 Joey Gel Casting System (Owl Separation Systems), optional
P8DS Emperor Penguin Vertical Electrophoresis System (Owl Separation Systems)
Micropipet equipped with a drawn-out plastic tips
Haake Model DC10-P5/U circulating baths (available from Fisher Scientific) or any circulating bath capable of maintaining temperatures between 25° and 100°C is also acceptable

Cast the gel

1. Clean glass plates as necessary with GlassPlus or equivalent glass cleaner followed by 95% ethanol.

2. Assemble glass plates and spacers.

   Although not required, use of the Mini PROTEAN 3 casting frame and stand (Bio-Rad Laboratories) or the JGC-3 Joey Gel Casting System (Owl Separation Systems) eliminates many potential problems with leakage during polymerization.

   For many purposes, a 10-well, 0.75- or 1.0-mm comb, and spacers work well.

Table 16G.1.1 Volumes of Reagents Used to Cast Polyacrylamide Gels for R-PAGE

Component	5%	7.5%	10%
40% (w/v) acrylamide stock (see recipe)	3.1 ml	4.7 ml	6.25 ml
10× TBE buffer (see recipe)	2.5 ml	2.5 ml	2.5 ml
H_2O	19.3 ml	17.7 ml	16.1 ml
TEMED[a]	12.5 µl	12.5 µl	12.5 µl
10% (w/v) ammonium persulfate (APS)[b]	125 µl	125 µl	125 µl
Total	25 ml	25 ml	25 ml

[a] N,N, N′,N′-Tetramethylethylenediamine.
[b] APS should be added last.

3. Calculate the volume of gel solution required and prepare according to the recipes given in Table 16G.1.1, taking care to add the ammonium persulfate last. Quickly mix.

   CAUTION: *Acrylamide is a neurotoxin. See UNIT 1A.3 for guidelines on handling and contact your local safety officer concerning guidelines for disposal. It is not necessary to deaerate the acrylamide solution before adding ammonium persulfate. Deaeration may lead to incomplete polymerization around the comb.*

4. Tilt the glass plate-spacer assembly slightly, fill with gel solution, and insert the comb. Avoid trapping air bubbles under the teeth of the comb or against the glass plates.

5. Allow polymerization to proceed for 30 to 60 min at room temperature.

   When polymerization is complete, a refractive line will appear 1 to 2 mm below the surface of the acrylamide between the teeth of the comb.

6. Carefully remove the comb, first wetting it with a small amount of 1× TBE to avoid disturbing the wells.

   If the gel is not to be used immediately, place a laboratory tissue soaked in 1× TBE over the comb, wrap the entire gel cassette in plastic wrap, and store at 4°C.

Apply the sample and begin electrophoresis

7. Place the gel assembly in the electrophoresis apparatus, tighten the side clamps, and fill the upper and lower reservoirs with 1× TBE.

   Using a Pasteur pipet or disposable syringe and needle, quickly flush the wells to prevent polymerization of trapped acrylamide solution. Remove any bubbles trapped against the bottom edge of the gel in the lower buffer reservoir.

8. Circulate coolant at room temperature (20° to 25°C) through central cooling block.

9. Mix aliquots of RNAs to be analyzed with appropriate volumes of 6× gel loading buffer and load samples into the wells using a micropipet equipped with a drawn-out plastic tip.

   Two factors (sample well dimensions and RNA preparation type) govern the amount of RNA applied to each well. For gels that are 0.75 to 1 mm in thickness and contain 10 wells, apply 2 to 3 µg total or 2 to 5 µg low-molecular-weight (i.e., 2 M LiCl-soluble) RNA in a total volume of 10 to 15 µl.

10. Connect electrodes to a power supply, set the voltage at 100 V, and run until the blue xylene cyanol FF marker dye reaches the bottom of the gel.

    Running time should be ~1 hr 15 min (constant voltage).

11. Turn off power and disconnect leads from power supply.

Denature samples and resume electrophoresis

12. Discard upper and lower reservoir buffers and increase temperature setting in the circulating bath from 20° to 70°C.

13. Refill the upper and lower buffer reservoirs with 0.11× TBE preheated to 80°C.

    Approximately 30 min before the first electrophoresis ends, add 50 ml 1× TBE to 400 ml H_2O in a 1-liter Erlenmeyer flask and place flask on electric hot plate. Monitor temperature with a thermometer and remove flask from hot plate when temperature reaches 80°C.

14. Reconnect electrodes making sure to **REVERSE THE POLARITY** and wait 5 min for RNA denaturation to occur before resuming electrophoresis at 100 V.

    During electrophoresis under denaturing conditions, RNAs migrate from the bottom of the gel toward the top. Thus, it is essential to REVERSE THE LEADS, connecting red (+) to black (−) and black (−) to red (+).

15. Turn off power and disconnect the leads when the xylene cyanol marker dye reaches the top of the gel.

    Running time should be ∼45 min (constant voltage).

16. Discard upper and lower reservoir buffer and remove gel assembly.

VISUALIZE SMALL CIRCULAR RNAs

BASIC PROTOCOL 3

RNAs and DNAs are most commonly visualized by staining with ethidium bromide after electrophoresis (see Brown et al., 2004). For many purposes, the sensitivity of ethidium bromide staining (i.e., ∼100 pg/band) is entirely adequate. Use of SYBR Gold (Invitrogen–Molecular Probes) increases sensitivity ∼10-fold. Staining with either of these dyes is faster and less complicated than silver staining. Silver staining can detect proteins as well as nucleic acids, and many different protocols have been described. The protocol described by Schmitz and Riesner (2006) is particularly simple and easy to use.

Materials

Electrophoresed gel (Basic Protocol 2)
Staining solution (0.5 μg/ml ethidium bromide in water)

Scalpel *or* single-edged razor blade
Plastic or glass dish for staining
Platform shaker
Plastic wrap
UV transilluminator

Additional reagents and equipment for photographing the gel (Voytas, 2000)

1. Lay the gel assembly on the benchtop, remove the spacers (if necessary), and carefully separate the glass plates using a scalpel or single-edged razor blade.

   In most cases, the gel remains attached to the lower plate.

2. Transfer the gel to an appropriate staining solution, using either a plastic (ethidium bromide or SYBR Gold) or glass (silver stain) dish.

   Use only dishes with smooth bottoms to avoid scratching the gel.

3. For ethidium bromide, use sufficient staining solution (0.5 μg/ml ethidium bromide in water) to just float the gel and gently agitate for 15 to 30 min on a platform shaker.

   CAUTION: *Ethidium bromide is a potent mutagen; see UNIT 1A.3 for guidelines on handling and disposal.*

4. Remove the staining solution and destain in water for 10 to 15 min.

5. Place a piece of plastic wrap on the surface of a UV transilluminator and transfer the gel to the plastic wrap, being careful to remove any trapped air bubbles before photographing.

6. Photograph the gel (see Voytas, 2000).

REAGENTS AND SOLUTIONS

Use autoclaved, deionized water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see the* ***SUPPLIERS APPENDIX****.*

Acrylamide, 40% (39% acrylamide, 1% N,N′-methylene-bis-acrylamide)

39 g Acrylamide
1 g N,N′-methylene-bis-acrylamide
Dissolve in H_2O and adjust volume to 100 ml
Filter to remove insoluble particulates
Store up to 6 months at 4°C (protected from light)

Ethanol, 70% (v/v)/0.1 M sodium acetate

75 ml absolute ethanol
27 ml H_2O
2.7 ml of 3 M sodium acetate (see recipe)
Store at −20°C

Gel loading buffer, 6×

0.25% (v/v) bromphenol blue
0.25% (v/v) xylene cyanol FF
0.5× TBE buffer (*APPENDIX 2A*)
60% (w/v) sucrose
Store in small aliquots at −20°C

Sodium acetate, 3 M

3 M sodium acetate
10 mM disodium EDTA
Adjust pH to 5.2 with glacial acetic acid

TBE buffer, 10×

0.89 M Tris base
0.89 M boric acid
25 mM EDTA
Store up to 6 months at 4°C

pH of 1× TBE should be ∼8.3.

COMMENTARY

Background Information

The use of denaturing PAGE to identify viroids exploits the unusual circular nature of these small (246 to 400 nucleotides), highly structured RNA molecules. Under denaturing conditions, circular RNAs migrate much more slowly than the corresponding linear forms. Either high temperature or chemicals such as urea and/or formamide can be used as the denaturant. Sambrook and Russell (2001) provides an excellent introduction to the principles of nucleic acid electrophoresis, especially the analysis of single-stranded RNA and DNA molecules under denaturing conditions. Safety considerations are discussed by Gallagher (2006).

In S(equential) PAGE (Rivera-Bustamante et al., 1986), a strip of polyacrylamide containing viroid-sized RNAs is cut from a nondenaturing gel and transferred to the top of a denaturing gel containing 8 M urea. R-PAGE eliminates the need to transfer the viroid from a urea-free to a urea-containing gel; instead, denaturation is achieved by simultaneously

raising the temperature and lowering the ionic strength of the gel buffer.

Critical Parameters

Running time

It is obviously important that the molecule of interest not run off the gel during the first electrophoresis. Perhaps not quite so obvious is the need to run as many of the small linear cellular RNAs as possible off the bottom of the gel so that they do not later clutter the viroid window. Their strong secondary structure causes viroids to behave anomalously during electrophoresis under nondenaturing conditions; thus, choosing an appropriate set of RNA markers is critical.

If available, low-molecular-weight RNA preparations containing one or more viroids of known size (i.e., 246 to 403 nucleotides) are ideal. Where the existence of circular RNAs <250 nt (i.e., *Avocado sunblotch viroid*) or >600 nt (i.e., the "slow-slow" dimer of *Coconut cadang-cadang viroid*) is suspected, it may be wise to construct a series of self-circularizing RNAs of precisely known size, derived from the less abundant form of tobacco ringspot virus satellite RNA (Feldstein et al., 1997). At the present time, circular RNA markers are not commercially available.

Viroid concentration

Under favorable growing conditions, concentrations of *Potato spindle tuber viroid* (PSTVd) in diseased tissue are high enough that the viroid can be easily detected by ethidium bromide staining after analysis of total cellular RNA. Where R-PAGE of a total RNA preparation fails to reveal an obvious band in the viroid window, the analysis should be repeated using low-molecular-weight RNA. Removing rRNA and other high-molecular-weight RNAs by LiCl precipitation allows one to load the RNA from several-fold more tissue onto a single lane, thereby increasing the sensitivity of the analysis (Fig 16G.1.1C, compare lanes 4 and 6). Staining with SYBR Gold or silver rather than ethidium bromide can yield an additional increase in sensitivity.

Troubleshooting

Optimizing R-PAGE for viroids of different size

R-PAGE analysis using a 5% gel works well for viroids that are similar to PSTVd in size and/or structural properties. Provided that appropriate circular RNA markers are available, changing the gel concentration or run times to improve the separation is straightforward. One potential source of confusion stems from the

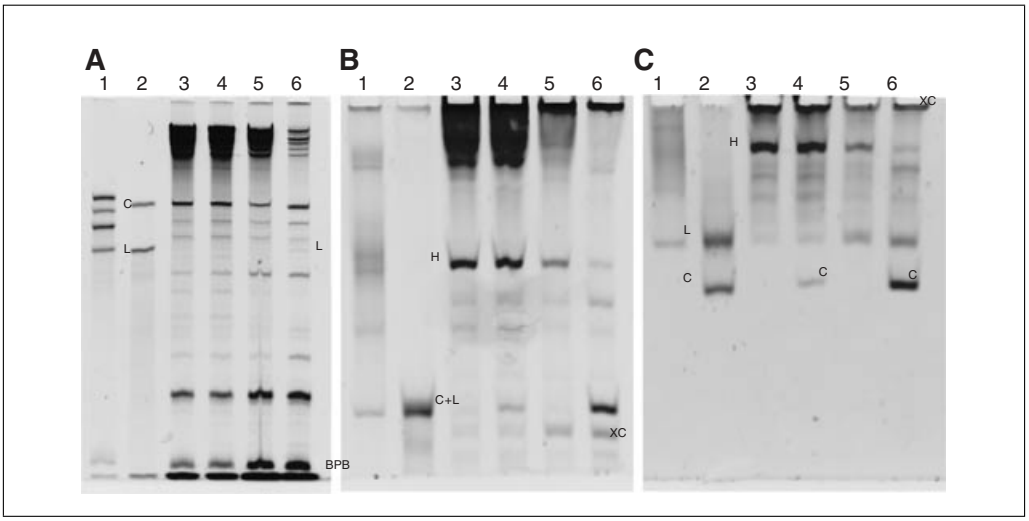

Figure 16G.1.1 Identification of PSTVd by R(eturn)-PAGE. (**A**) Electrophoresis under completely denaturing conditions (1× TBE, 8 M urea, 55°C), (**B**) first (i.e., nondenaturing) R-PAGE electrophoresis, and (**C**) second R-PAGE electrophoresis. RNAs were visualized by ethidium bromide staining after electrophoresis, and the positions of circular PSTVd (C), linear PSTVd (L), and a linear host RNA (H) of unknown size are marked. Lane 1, precisely-full-length (i.e., 359 nt) PSTVd RNA synthesized in vitro; lane 2, purified PSTVd marker containing both circular and linear molecules; lane 3, ~2 μg total RNA from uninfected tomato; lane 4, ~2 μg total RNA from PSTVd-infected tomato; lane 5, ~3 μg low-molecular-weight RNA from uninfected tomato; lane 6, ~3 μg low-molecular-weight RNA from PSTVd-infected tomato. Positions of the bromphenol blue (BPB) and xylene cyanol (XC) tracking dyes are marked.

failure of the particular combination of temperature and low ionic strength used during the second electrophoresis to completely denature an unknown viroid RNA. On the positive side, Singh and Boucher (1987) have exploited this situation to separate closely related sequence variants of PSTVd. Different banding patterns may reflect differences in either nucleotide number or nucleotide sequence. For general PAGE troubleshooting, please see Sambrook and Russell (2001) and Gallagher (2006).

Viroids with branched secondary structures

Viroids with branched structures are insoluble in 2 M LiCl. Three of the 29 viroid species currently known have branched secondary structures, and titers of at least one of these branched viroids (i.e., *Chrysanthemum chlorotic mottle viroid*) in infected tissue are low. Where such a situation is suspected, readers should consult Navarro and Flores (1997) for additional guidance.

Anticipated Results

Figure 16G.1.1 compares the ability of conventional one-dimensional electrophoresis under completely denaturing conditions (panel A) and R-PAGE (panels B and C) to resolve a complex mixture of viroid and host RNAs. Results from both the nondenaturing (Fig. 16G.1.1B) and denaturing (Fig. 16G.1.1C) phases of R-PAGE are shown. Figure 16G.1.1A illustrates two important points. First, examination of lane 2 reveals that the circular and linear forms of PSTVd are well-resolved by electrophoresis under denaturing conditions; second, the presence of comigrating host RNAs may mask the presence of viroid(s) in a preparation of total RNA (compare lanes 2 to 4). R-PAGE effectively eliminates this uncertainty while still allowing the separation of linear and circular forms of the viroid.

As shown in Figure 16G.1.1B, PSTVd (359 nt) migrates slightly more slowly than the xylene cyanol tracking dye during the first electrophoresis in R-PAGE. Note that the PSTVd marker (lane 2) yields only a single band. Under nondenaturing conditions, the linear and circular forms of PSTVd assume an identical rod-like secondary structure and cannot be resolved. Figure 16G.1.1C shows the position of PSTVd after the second electrophoresis, this time under denaturing conditions. Comparison of the lanes containing RNA from uninfected (lanes 3 and 5) and PSTVd-infected tissue (lanes 4 and 6) reveals that all host RNAs are now located in the upper portion of the gel, leaving behind the slowly migrating circular viroid molecules in a "viroid window" in the middle of the gel. The potential advantages of analyzing 2 M LiCl-soluble low-molecular-weight rather than total RNA are also apparent. Lanes 5 and 6 each contain ~3 μg low-molecular-weight RNA derived, while lanes 3 and 4 each contain ~2 μg total RNA. Note the dramatic difference in the intensities of the PSTVd band between lanes 4 and 6. Analysis of low-molecular-weight RNA is much less prone to artifacts related to sample overloading.

Time Considerations

Isolation of total RNA using TRIzol reagent is rapid and convenient. Provided that the number of samples to be processed is limited (i.e., <10), preparations of total RNA can be ready for electrophoresis within 2 to 3 hr. LiCl fractionation involves an overnight incubation at 0° to 4°C; nevertheless, preparations of low-molecular-weight RNA should be ready for electrophoresis before noon on the second day. When carried out in a minigel format as described in Basic Protocol 2, R-PAGE requires only 3 to 3.5 hr from sample application to photography of the ethidium- or SYBR Gold-stained gel.

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R-PAGE and other electrophoretic methods are compared with other detection techniques such as molecular hybridization and RT-PCR.

Internet Resources

http://subviral.med.uottawa.ca/

Subviral RNA Database. Maintained by Martin Pelchat [RNA Group/Groupe ARN, Département de biochimie, Université de Sherbrooke, Sherbrooke (Québec) J1H 5N4, Canada], this website contains the sequences of more than 2400 viroids and related RNAs.

http://www.ictvonline.org

The Universal Database of the International Committee on the Taxonomy of Viruses is a convenient source of current information on viroid (and virus) taxonomy.

http://www.probes.invitrogen.com/handbook/

Invitrogen Detection Technologies: Contains an informative discussion of nucleic acid staining using SYBR Gold, SYBR Green I, and SYBR Green II.

Isolation and Cloning of Small RNAs from Virus-Infected Plants

RNA silencing is an evolutionarily conserved, RNA-mediated gene regulatory system in eukaryotic organisms. RNA silencing is induced by double-stranded (ds)RNA that is processed into short 21- to 26-nucleotide RNAs by an RNase III–like enzyme called Dicer. These small RNAs are known as short interfering RNAs (siRNAs) or micro RNAs (miRNAs). Most siRNAs are derived from long dsRNA duplexes or extended hairpins, while miRNAs are processed from transcripts that can form local hairpin precursor structures. These small RNAs can provide sequence specificity for the effector complexes of the silencing machinery. Both miRNAs and siRNAs can incorporate into the RNA-induced silencing complex (RISC) and guide it to control gene expression by base pairing to specific mRNAs, resulting in either RNA cleavage or arrest of protein translation. In general, siRNAs repress the expression of homologous genes, while miRNAs negatively regulate the expression of genes that are distinct from which they originate. In addition, siRNAs can cause another effector complex, the RNA-induced initiation of transcriptional gene silencing (RITS) complex, to direct the chromatin modification of homologous DNA sequences.

Plant viruses are strong inducers as well as targets of RNA silencing. Virus infection is always associated with the accumulation of virus-specific siRNAs in both locally inoculated and systemically infected tissues, and the elevated small-RNA level correlates with the reduction of virus titer; thus, RNA silencing acts as an antiviral system in plants. Consequently, successful virus infection requires suppression of the induced silencing response (see *UNIT 16H.1*).

The majority of plant viruses have a single-stranded (ss)RNA genome. It is believed that these RNA viruses replicate their genomes via dsRNA intermediates that may activate the RNA silencing machinery, resulting in equal accumulation of positive- and negative-strand-derived siRNAs along the length of the viral genome; however, cloning and sequence analyses of the positive-stranded RNA virus *Cymbidium* ringspot virus (CymRSV) siRNAs revealed that the majority of small RNAs derive from the coding strand of the virus and that the virus-specific siRNAs can be grouped to several clusters that likely represent hotspots for siRNA production (Molnár et al., 2005). Moreover, analyses of two other unrelated positive-stranded RNA viruses displayed the same asymmetry in siRNA accumulation as observed in the case of CymRSV siRNAs. These observations indicate that virus-derived siRNAs are predominantly produced by direct Dicer cleavage of imperfect duplexes originating from highly base-paired structures from the positive-strand viral genomic RNA. Like RNA-silencing suppressor proteins, the abundant, hotspot-derived viral siRNAs may also influence the symptom development associated with virus infection by altering the expression of endogenous genes.

This unit provides a detailed protocol for isolating and cloning small RNAs from virus-infected plant tissues. Small RNAs can be routinely collected from 800 to 1000 μg total nucleic acid extract separated on a 15% denaturing polyacrylamide gel. The isolated small RNAs can be used either for hybridization studies as a probe or for directional cloning to get detailed information about their sizes, origins, and functions.

The small-RNA cloning procedure is based on adapter ligation (see Fig. 16H.2.1). The adapter oligonucleotides are used for priming reverse transcription and for defining the orientation and sequence of the cloned small RNAs. In this unit, two isotope-free

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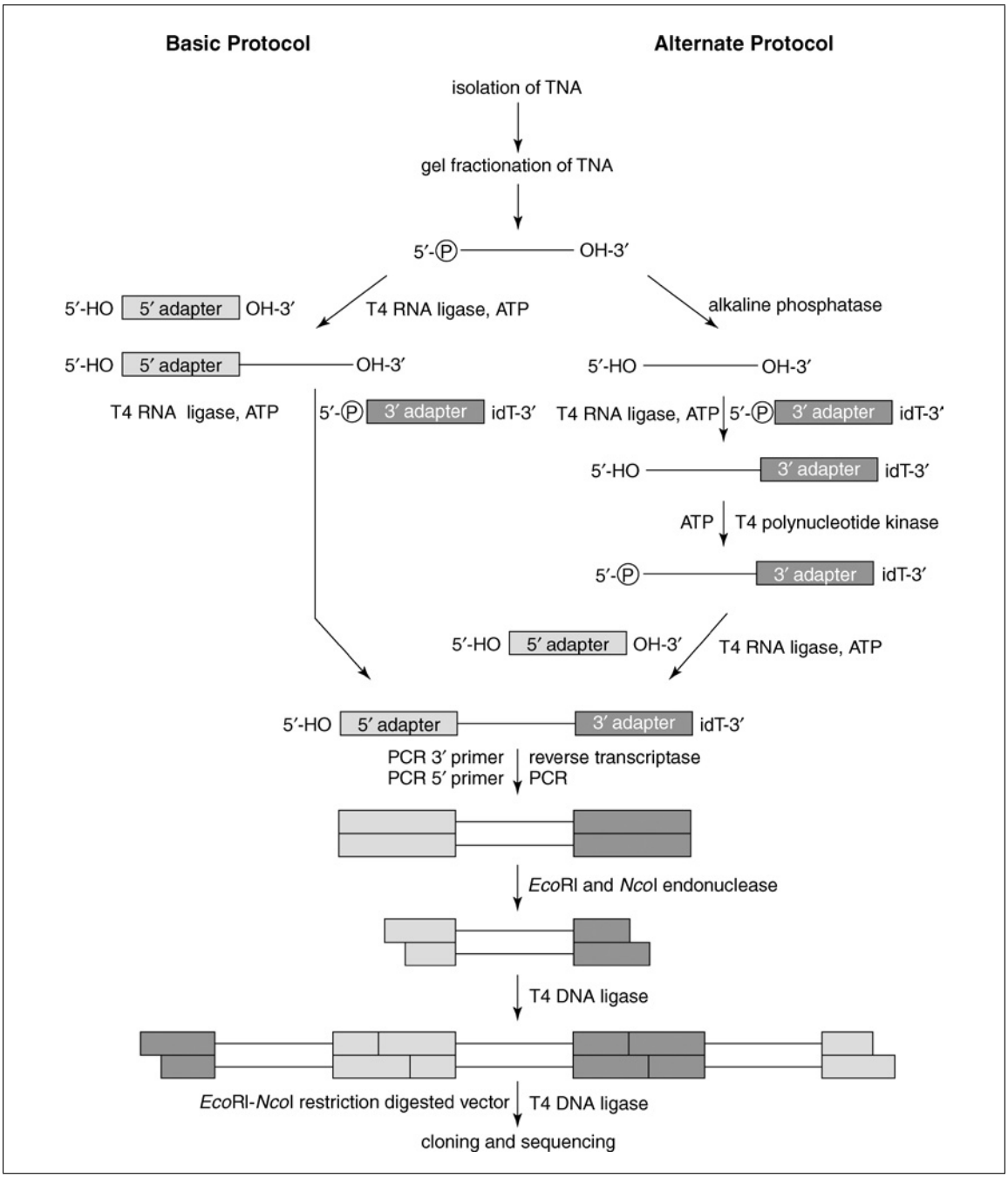

Figure 16H.2.1 Schematic diagram of small-RNA isolation and cloning steps in the Basic Protocol and Alternate Protocol.

protocols for small-RNA cloning are described. The Basic Protocol uses unmodified small RNAs, taking advantage of the fact that small RNAs processed by Dicer have both a 5′-phosphate and 3′-hydroxyl terminus. In the Alternate Protocol, the small RNAs are dephosphorylated prior to ligation of the 3′ adapter. Dephosphorylation can enhance the efficiency of ligation reaction by preventing the circularization of small RNAs. However, this step also increases the chance of cloning RNA degradation products generated by endogenous RNases. The Alternate Protocol may be used to clone small RNAs from high quality RNA extracts of plants infected with low abundance viruses.

ISOLATING AND CLONING UNMODIFIED SMALL RNAs FROM VIRUS-INFECTED PLANT TISSUES

In this protocol, gel-purified small RNAs are ligated directly to a nonphosphorylated 5′-adapter oligonucleotide using T4 RNA ligase. The ligation products are separated from the excess of 5′ adapter on a 15% denaturing polyacrylamide gel and are subsequently ligated to a 5′-phosphorylated 3′-adapter oligonucleotide with a blocked 3′-hydroxyl terminus. The final ligation products are separated from the excess of 3′ adapter and are subjected to reverse transcription and PCR amplification. The gel-purified PCR products are digested with *Eco*RI and *Nco*I restriction enzymes and subsequently concatamerized using T4 DNA ligase. The concatamers are ligated into a cloning vector digested with *Eco*RI and *Nco*I, and then TOP10 cells are transformed with the recombinant plasmids. Individual colonies are screened for the size of concatamer inserts by PCR, and selected PCR fragments are purified and submitted for sequencing. The small-RNA sequences are extracted from the sequence manually or automatically, using software tools (e.g., Staden Package, *http://staden.sourceforge.net*; or software developed in-house).

Materials

Virus-infected leaf tissue
Liquid nitrogen
Extraction buffer (see recipe)
Buffered phenol, pH 8.0 (*APPENDIX 2A*)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*APPENDIX 2A*)
24:1 (v/v) chloroform/isoamyl alcohol
4 M sodium acetate, pH 5.2
Absolute ethanol
80% ethanol
70% ethanol in a wash bottle
RNase-free H_2O (*APPENDIX 2A*) *or* double-distilled autoclaved H_2O
15% denaturing polyacrylamide gels, fifteen well, 20 × 16 × 0.15 cm (see recipe; also see Ellington and Pollard, 2001)
0.5× TBE buffer (*APPENDIX 2A*): sterilize by autoclaving
Gel-loading solution (see recipe)
10-nucleotide DNA oligo ladder (see recipe)
10 mg/ml ethidium bromide (*APPENDIX 2A*)
0.3 M NaCl, RNase-free
20 mg/ml glycogen (Roche)
100 μM 5′ adapter (see Table 16H.2.1)
Dimethyl sulfoxide (DMSO)
10× PAN ligation buffer (see recipe)
31 U/μl RNAguard (Amersham Pharmacia)
40 U/μl T4 RNA ligase (Amersham Pharmacia)
100 μM 3′ adapter (Table 16H.2.1)
100 μM PCR 3′ primer (Table 16H.2.1)
0.1 M dithiothreitol (DTT)
5× first-strand buffer (Invitrogen)
2 mM deoxyribonucleoside triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP, 2 mM each; *APPENDIX 2A*)
200 U/μl reverse transcriptase (Superscript II, RNase H^-, Invitrogen)
150 mM KOH/20mM Tris base
150 mM HCl
1× and 10× TE buffer (see *APPENDIX 2A* for 1×)
10× PCR buffer (Roche)
PCR 5′ primer (Table 16H.2.1)

Table 16H.2.1 Oligonucleotides and Their Corresponding Sequences

Oligonucleotide	Sequence[a]
5′ adapter	5′ TGGGAATTCCTCACTrArArA 3′
3′ adapter	5′ ℗rUrUrUCTATCCATGGACTGTidT 3′
PCR 5′ primer	5′ CATGGGAATTCCTCACTAAA 3′
PCR 3′ primer	5′ TACAGTCCATGGATAGAAA 3′
M13 F primer	5′ TTCCCAGTCACGACGTT 3′
M13 R primer	5′ CAGGAAACAGCTATGAC 3′
20-nt DNA marker	5′ GCGCTCTTGACTCGTTGTGC 3′
30-nt DNA marker	5′ ACGTGTCGACATCACGCTGGAAATGATACA 3′
40-nt DNA marker	5′ GATAATACGACTCACTATAGGGCCAGGGCGCAGATTGAGC 3′
50-nt DNA marker	5′ ACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTA CTTTCTCTTA 3′
61-nt DNA marker	5′ ATGGATCCCTCGAGGTCGACCCTAGGTGGTCTCATCCTCA GTTCGAGAAGTAACCCGGGAT 3′
71-nt DNA marker	5′ ATGACTAGTAGTAGGCTCTCTCTGTCCTTGAGGATGATGC TCAAGTCTAGCTCTAAGCTGCTCAAGCTCTC 3′
80-nt DNA marker	5′ AGTCTTCTCATCCATAGAAGCAGTAGTAGGAATATCGTAA TCAAGAGCACCAGATGAAGAGATCTTCTTGAATCTGTTAG 3′

[a] A, C, G, T, DNA residues; rA, rU, RNA residues; ℗, 5′ phosphate; idT, 3′-inverted deoxythymidine.

5 U/μl *Taq* DNA polymerase (Roche)
6× DNA loading solution (Fermentas)
15% native polyacrylamide gel, fifteen well, 20 × 16 × 0.15 cm (see recipe; also see Chory and Pollard, 1999)
20-bp PCR low DNA ladder (Sigma)
10× buffer H (Roche)
10 U/μl *Eco*RI (Roche)
10 U/μl *Nco*I (Roche)
2% (w/v) standard agarose gel with 0.25 μg/ml ethidium bromide (also see Voytas, 1992)
10× T4 DNA ligase buffer (New England Biolabs)
400 U/μl T4 DNA ligase (New England Biolabs)
QIAquick PCR purification kit (Qiagen)
10 mM Tris·Cl, pH 8.5 (*APPENDIX 2A*)
pGEM-T Easy cloning vector (Promega)
Rapid DNA ligation kit (Roche)
TOP10 cells (Invitrogen)
SOC medium (*APPENDIX 4A*)
LB agar plates (*APPENDIX 4A*) with 100 μg/ml ampicillian
2% (w/v) Xgal
20% (w/v) IPTG
Primer M13 F (Table 16H.2.1)
Primer M13 R (Table 16H.2.1)
1% (w/v) W-1 (Invitrogen)
100-bp DNA ladder (Fermentas)
Pestle and mortar
15-ml polypropylene conical tube, sterile (i.e., Falcon Blue)
Refrigerated laboratory centrifuge with swinging-bucket rotors (e.g., Sigma 4K15C)

90°, 65°, 50°, and 42°C water baths
360-nm UV transilluminator
Plastic wrap (e.g., Saran)
Rotary shaker or rocker
1.7-ml siliconized polypropylene microcentrifuge tubes (Sigma)
Tabletop centrifuge, refrigerated (e.g., Eppendorf 5415 D)
200-µl PCR tubes
16°C incubator
96-well thermal cycler–compatible microtiter plates

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 1992), denaturing polyacrylamide gel electrophoresis (Ellington and Pollard, 1998), nondenaturing (native) polyacrylamide gel electrophoresis (Chory and Pollard, 1999), and the polymerase chain reaction (Kramer and Coen, 2001)

NOTE: Use normal precautions for working with nucleic acids, including sterile solutions and autoclaved utensils. Avoid exposure to ribonucleases. Also see Critical Parameters and Troubleshooting.

Isolate total nucleic acid (TNA)

1. Using a pestle, grind 2 to 2.5 g virus-infected leaf tissue with liquid nitrogen in a mortar. Add 6 ml extraction buffer to the powdered tissue and mix thoroughly until the frozen sample completely thaws.

2. Transfer homogenized material to a sterile 15-ml polypropylene conical tube containing 6 ml buffered phenol. Mix immediately by vortexing for 15 sec. Centrifuge 15 min at 3500 × *g*, room temperature.

   The maximum centrifugal force that can be used for the recommended centrifuge tubes is 3500 × g. This can be increased if glass tubes are used.

3. Transfer the aqueous upper phase to a new tube, avoiding precipitated material from the interface. Add 6 ml of 25:24:1 phenol/chloroform/isoamyl alcohol, vortex 15 sec, and centrifuge 5 min at 3500 × *g*, 4°C.

   CAUTION: *Phenol is extremely toxic and highly corrosive, and can cause severe burns (UNIT 1A.3). Wear appropriate gloves, goggles, and protective clothing. Always use in a chemical fume hood.*

   The interface contains precipitated proteins. If a large precipitate forms after the first phenol/chloroform/isoamyl alcohol extraction, additional extraction may be required to remove all proteins including endogenous RNases.

4. Transfer the aqueous upper phase to a new tube. Add 6 ml of 24:1 chloroform/isoamyl alcohol. Vortex 15 sec and centrifuge 5 min at 3500 × *g*, 4°C.

5. Again, transfer the aqueous upper phase to a new tube and precipitate the total nucleic acid by adding 1/20 vol. of 4 M sodium acetate, pH 5.2, and 3 vol absolute ethanol. Mix by inverting the tube, and incubate 10 to 15 min on ice or store at −20°C overnight.

6. Recover the total nucleic acid (TNA) by centrifuging 30 min at 3500 × *g*, 4°C. Remove the supernatant by aspiration.

7. To remove residual salts from the pellet, slowly add an equal volume of 80% ethanol down the side of the tube, and centrifuge immediately 5 min at 3500 × *g*, 4°C.

   Avoid resuspending the TNA pellet in 80% aqueous ethanol, because short RNAs are soluble to some extent in 80% ethanol in the absence of salt.

8. Remove the ethanol by aspiration without disturbing the pellet, and collect the residual ethanol at the bottom of the tube by an additional 10-sec centrifugation. Remove the residual liquid completely using a small pipet tip and air dry the pellet 5 to 10 min at room temperature.

9. Place the tube on ice and dissolve the pellet in 200 µl RNase-free water. Dilute 2 µl TNA in 1 ml water to estimate the concentration of the total RNA by measuring the absorbance (A_{260}) in a 1-cm quartz cuvette (Gallagher, 2004). Transfer the remaining TNA extract to a siliconized microcentrifuge tube and store up to 1 year at –70°C.

   Regular reaction tubes may absorb the low-molecular-weight nucleic acids to some extent. Use siliconized reaction tubes where possible.

   Quality of RNA can be checked by denaturing 2 to 5 µl sample with 1 vol gel-loading solution at 65°C for 5 min and analyzing on a 1% agarose gel.

   The authors have found that the phenol/chloroform/isoamyl alcohol extraction provides the highest yield, although other RNA isolation methods (e.g., Trizol reagent) may also be used. The relatively small amount of genomic DNA in TNA will not interfere with further processes. Avoid commercial RNA isolation kits with silica-gel-membrane purification because the small-RNA fraction does not bind to the column. LiCl precipitation must also be avoided because small RNAs do not coprecipitate with long RNA transcripts.

Purify small RNA from total nucleic acid

10. Before assembling the electrophoresis apparatus, wash with detergent and rinse with sterile distilled water followed by 70% ethanol.

11. To heat up the system and remove persulfate, prerun the 15%, fifteen-well denaturing polyacrylamide gel 30 min at ~350 V (7 W) using 0.5× TBE buffer. Carefully wash every well with 0.5× TBE buffer using a syringe immediately prior to loading the samples.

    See Ellington and Pollard (1998) for general protocols on denaturing gel electrophoresis.

12. Mix 800 to 1000 µg TNA with an equal volume of gel-loading solution. Denature the nucleic acid solution, as well as 40 µl of 10-nucleotide DNA oligo ladder, by incubating the tube for 5 to 10 min at 65°C.

13. Load the sample in the center wells of the gel. Load 40 µl denatured 10-nucleotide DNA oligo ladder. Leave one well empty between the sample and the size marker to avoid contamination.

    It is important to apply the 10-nucleotide DNA oligo ladder in a similar volume as the TNA sample to ensure similar gel-running behavior.

14. Separate the small RNAs by running the gel ~2.5 hr using at ~350 V (7 W) using 0.5× TBE until the bromphenol blue dye in the gel-loading solution reaches the bottom of the gel.

15. Dismantle the electrophoresis apparatus and stain the gel by soaking in 100 ml of 0.5× TBE buffer containing 0.25 µg/ml ethidium bromide (dilute from 10 mg/ml stock) for 10 min. Visualize the nucleic acids in the gel using a 360-nm UV transilluminator. To protect the gel from RNase contamination, cover the transilluminator with plastic wrap.

    CAUTION: *Ethidium bromide is a powerful mutagen and is toxic (UNIT 1A.3). Wear appropriate gloves when working with solutions that contain ethidium bromide.*

    CAUTION: *UV light is mutagenic and carcinogenic, and can damage the retina of the eyes. Wear UV-protective glasses or face shield, and appropriate gloves.*

A small portion of nucleic acids can diffuse into the solution during staining. To reduce the loss of small RNAs and subsequent adapter ligation products, the DNA size markers may be visualized separately, by slicing off the marker lane, staining it, and returning it to its original position.

16. Excise the gel slice encompassing 18- to 28-nucleotide small RNAs defined by the mobility of the 20- and 30-nucleotide DNA size markers.

    RNA migrates slightly more slowly than DNA of the same sequence and length, necessitating the adjustment of size estimates when excising the small RNAs. Avoid unnecessarily long UV exposure, which will damage the nucleic acids.

17. Cut the gel slice into 1 × 1–mm squares, transfer to a preweighed 15-ml polypropylene conical tube, and weigh the gel slices. Elute the small RNAs from the gel by adding 3 vol RNase-free 0.3 M NaCl and incubate the tube overnight at 4°C under constant agitation (i.e., using rotary shaker or rocker).

18. Transfer the supernatant to a fresh tube (keep at 4°C) and repeat the elution of the gel with 2 vol RNase-free 0.3 M NaCl for 6 to 8 hr at 4°C.

    The more elution buffer, the higher the efficiency of recovery.

19. Combine the supernatants by pipetting and pellet residual polyacrylamide by centrifuging 10 min at 3500 × *g*, 4°C. Transfer the supernatant to a new tube, avoiding the polyacrylamide from the bottom.

    A 3-ml volume of supernatant can be precipitated in a 15-ml conical tube. Large volumes of gel may require greater amounts of extraction buffer for efficient elution, which can either be precipitated in a larger tube or split into multiple 15-ml tubes.

20. Precipitate the RNA from the supernatant by adding glycogen solution to a final concentration of 2 μg/ml and 3 vol absolute ethanol. Incubate samples overnight at −20°C.

Ligate the 5′ adapter to small RNAs

21. Collect the RNA pellet as described in steps 6 to 8. Place the tube on ice and dissolve the pellet in 24 μl RNase-free water by adding the water on top of the pellet and leaving the tube undisturbed for 10 to 15 min. Vortex briefly.

    Immediate, rapid vortexing might result in loss of the small-RNA pellet since it can stick to the side of the tube.

22. Add 4 μl of 100 μM 5′ adapter (see Table 16H.2.1) and 6 μl DMSO.

23. Denature both the isolated small RNA and the 5′ adapter by incubating the tube for 1 min at 90°C. Place the tube immediately on ice for 1 min.

24. Add 4 μl of 10× PAN ligation buffer, 1 μl of 31 U/μl RNAguard, and 1 μl of 40 U/μl T4 RNA ligase. Mix gently and incubate the tube for 1 hr at 37°C.

25. Add an equal volume of gel-loading solution and denature the nucleic acids by incubating the tube 5 min at 65°C. Denature the DNA ladder as in step 12.

26. Prerun the gel and wash the wells as in step 11. Load the sample in two center wells of a fifteen-well, 15% denaturing polyacrylamide gel (Ellington and Pollard, 1998). Load the 10-nucleotide DNA oligo ladder as described in step 13.

27. Run the gel ~2.5 hr at ~350 V (7 W) using 0.5× TBE until the bromophenol blue dye in the gel-loading solution reaches the bottom of the gel.

28. Stain the gel and visualize the nucleic acids as described in step 15.

29. Excise the gel slice encompassing 36- to 46-nucleotide ligated small RNAs defined by the mobility of the 30- and 60-nucleotide DNA size markers.

30. Elute the ligation products from the gel slice with RNase-free 0.3 M NaCl as described in steps 17 and 18.

31. Combine the supernatants in one tube and pellet residual polyacrylamide by centrifuging ≥10 min at ≥3500 × *g*, 4°C. Transfer the supernatant to a fresh tube(s), avoiding the polyacrylamide from the bottom.

32. Precipitate the RNA from the supernatant by adding 20 mg/ml glycogen solution to a final concentration of 2 μg/ml and 3 volumes absolute ethanol, and incubate at −20°C overnight.

Ligate the 3′ adapter to small RNAs

33. Collect the RNA pellet as described in steps 6 to 8. Place the tube on ice and dissolve the pellet in 24 μl RNase-free water as described in step 21. Transfer the RNA to a siliconized microcentrifuge tube and place on ice.

34. Add 4 μl of 100 μM of 3′ adapter (see Table 16H.2.1) and 6 μl DMSO.

35. Denature the RNA by incubating the tube for 1 min at 90°C. Place the tube immediately on ice for 1 min.

36. Add 4 μl of 10× PAN ligation buffer, 1 μl RNAguard, and 1 μl T4 RNA ligase. Mix gently and incubate the tube 1 hr at 37°C.

37. Add an equal volume of gel-loading solution and denature the nucleic acids by incubating the tube 5 min at 65°C. Denature the DNA ladder as described in step 12.

38. Load the sample in two center wells of a 15%, fifteen-well denaturing polyacrylamide gel (also see Elllington and Pollard, 1998). Load the 10-nucleotide DNA oligo ladder as described in step 13.

39. Run the gel ∼2.5 hr at ∼350 V (7 W) using 0.5× TBE until the bromophenol blue dye in the gel-loading solution reaches the bottom of the gel.

40. Stain the gel and visualize the nucleic acids as described in step 15. Excise the gel slice encompassing 54- to 64-nucleotide ligated small RNAs defined by the mobility of the 50- and 80-nucleotide DNA size markers).

41. Elute the ligation products from the gel slice with RNase-free 0.3 M NaCl as described in steps 17 and 18.

42. Combine the supernatants in one tube and pellet residual polyacrylamide by centrifuging ≥10 min at ≥3500 × *g*, 4°C. Transfer the supernatant to fresh tubes, avoiding the polyacrylamide from the bottom.

43. Precipitate the RNA from the supernatant by adding 1 μl of 100 μM PCR 3′ primer (primer for reverse transcription; see Table 16H.2.1), 20 mg/ml glycogen to a final concentration of 2 μg/ml, and 3 vol absolute ethanol. Incubate overnight at −20°C.

Perform reverse transcription on adapter ligated small RNA

44. Collect the RNA pellet as described in steps 6 to 8. Place the tube on ice and dissolve the pellet in 11.1 μl RNase-free water as described in step 21.

45. Transfer the RNA to a siliconized microcentrifuge tube and place on ice. Denature the nucleic acids by incubating the tube for 1 min at 90°C. Place the tube immediately on ice for 1 min.

46. Add 3 μl of 0.1 M DTT, 6 μl of 5× first-strand buffer, and 8.4 μl of 2 mM dNTPs. Incubate the tube for 3 min at 50°C. Add 1.5 μl of 200 U/μl reverse transcriptase and incubate 30 min at 42°C and then 30 min at 50°C in a thermal cycler.

47. Hydrolyze the RNA by adding 80 μl of 150 mM KOH/20 mM Tris-base and incubate for 10 min at 90°C.

48. To neutralize the solution add 80 μl of 150 mM HCl.

49. Add 100 μl of 1× TE buffer and 300 μl of 25:24:1 phenol/chloroform/isoamyl alcohol. Mix by vortexing for 10 sec and centrifuge 5 to 10 min at maximum speed (16,000 × *g*) in a tabletop centrifuge.

50. Transfer the aqueous upper phase to a fresh tube, avoiding precipitated material from the interface. Add 300 μl of 24:1 chloroform/isoamyl alcohol. Vortex 10 sec and centrifuge 3 min at 16,000 × *g*.

51. Again, transfer the aqueous upper phase to a new siliconized tube and precipitate the DNA by adding 1/20 volume of 4 M sodium acetate, pH 5.2, glycogen to a final concentration of 2 μg/ml, and 2.5 to 3 vol absolute ethanol. Mix by inversion and incubate at least 1 hr on ice or store at –20°C overnight.

    The regular reaction tubes may absorb the low-molecular-weight nucleic acids to some extent. Use siliconized reaction tubes where possible.

Amplify the cDNAs by PCR

52. Recover the DNA by centrifuging 15 min at 16,000 × *g*, 4°C in a tabletop centrifuge.

53. Rinse the pellet with 1 ml of 80% ethanol and centrifuge immediately for 5 min at 16,000 × g, 4°C.

54. Remove the ethanol completely by aspiration, dry the tube for 5 min at room temperature, and dissolve the pellet in 50 μl water.

55. Add the following reagents to the dissolved pellet:

    50 μl 10× PCR buffer
    50 μl 2 mM dNTPs
    5 μl 100 μM PCR 5′ primer
    5 μl 100 μM PCR 3′ primer
    330 μl H_2O
    10 μl 5 U/μl *Taq* DNA polymerase.

56. Divide the reaction mixture among five 200-μl PCR tubes and run a polymerase chain reaction (PCR; also see Kramer and Coen, 2001) using the parameters listed below:

1 cycle:	2 min	94°C	(initial denaturation)
25 cycles:	1 min	94°C	(denaturation)
	1 min	50°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	72°C	(final extension).

57. Recombine the PCR reactions in one 1.7-ml microcentrifuge tube, and perform one 500-μl 25:24:1 phenol/chloroform/isoamyl alcohol extraction and one 500-μl 24:1 chloroform/isoamyl alcohol extraction, as described in steps 49 and 50.

58. Transfer the aqueous upper phase to a fresh siliconized tube and precipitate the DNA as described in step 51.

Purify the cDNAs from native polyacrylamide gel

59. Recover the DNA by centrifuging 15 min at 16,000 × *g*, 4°C. Rinse the pellet with 1 ml of 80% ethanol and centrifuge immediately for 5 min at 16,000 × *g*, 4°C.

60. Remove the supernatant completely by aspiration but do not allow the pellet to dry. Dissolve the pellet in 50 μl water.

    If the pellet is completely dried, the short DNA molecules will be denatured. Renaturation may result in imperfect rehybridization, which interferes with subsequent steps.

61. Add 10 μl of 6× DNA loading solution and load the sample in two to four center wells of a 15%, fifteen-well native polyacrylamide gel (also see Chory and Pollard, 1999).

    The volume that can be loaded is determined by the size of the wells. The wells should not be fully loaded, and more wells may be needed to load the total volume.

    A native polyacrylamide gel is used to separate the cDNAs in nondenaturing conditions, addition of formamide to the sample, denaturation by heating and prerunning of the gel are not required.

62. Load 1 μg of 20-bp PCR low DNA ladder in a similar volume as the cDNA sample to ensure similar gel running behavior. Leave one well empty between the sample and the size marker to avoid contamination.

63. Separate the cDNAs by running the gel ~3 hr at ~350 V (8 W) using 0.5× TBE buffer until the bromophenol blue dye of the gel-loading solution reaches the bottom of the gel.

64. Dismantle the gel-running apparatus and stain the gel by soaking in 100 ml of 0.5× TBE buffer containing 0.25 μg/ml ethidium bromide for 10 min. Visualize the nucleic acids in the gel using a 360-nm UV transilluminator.

    A double band, ~40 and ~60 bp, is often visible in the gel. The ~40-bp band corresponds to amplified ligation product of directly joined 5′- and 3′-adapter oligonucleotides without a small-RNA insert. Avoid contamination by this product in gel slices.

65. Excise the gel slice encompassing 59- to 65-bp cDNAs defined by the mobility of the DNA size markers.

66. Elute the DNA from the gel slice with 0.3 M NaCl as described in step 17 and 18.

67. Combine the supernatants and pellet residual polyacrylamide by centrifuging ≥10 min at ≥3500 × *g*, 4°C. Transfer the supernatant to a fresh tube(s), avoiding the polyacrylamide from the bottom.

68. Precipitate the DNA from the supernatant by adding 1/20 vol of 4 M sodium acetate, pH 5.2, 20 mg/ml glycogen solution to a final concentration of 2 μg/ml, and 3 vol absolute ethanol. Incubate at −20°C overnight.

Digest cDNA with EcoRI and NcoI restriction enzymes

69. Collect the DNA pellet as described in steps 6 and 7. Remove the supernatant completely by aspiration but do not allow the pellet to dry.

70. Dissolve the pellet in 90 μl water. Keep 10 μl undigested material for further analyses and for second PCR amplification if necessary.

71. Add 10 μl of 10× buffer H, 5 μl of *Eco*RI, and 5μl of *Nco*I restriction endonuclease and incubate the tube 4 to 5 hr at 37°C.

72. Analyze whether the restriction digestion is complete by separating 5 μl digested sample and 5 μl undigested sample on a 2% agarose gel containing ethidium bromide (also see Voytas, 1992). Use a 20-bp DNA ladder as a size marker.

The undigested PCR product should be ~ 60 bp, the digested PCR product should be ~50 bp.

73. Add 200 µl of 1× TE buffer and perform a 300-µl 25:24:1 phenol/chloroform/isoamyl alcohol extraction and one 300-µl 24:1 chloroform/isoamyl alcohol extraction as described in steps 49 to 50.

74. Precipitate the DNA by adding 1/20 vol of 4 M sodium acetate, pH 5.2, 20 mg/ml glycogen solution to a final concentration of 2 µg/ml, and 2.5 vol absolute ethanol. Mix by inversion and incubate at least 1 hr on ice.

Concatamerize EcoRI- and NcoI-digested DNA (optional)

75. Collect and rinse the DNA by centrifugation as described in step 52 and 53.

76. Remove the ethanol completely by aspiration but do not allow the pellet to dry. Dissolve the pellet in 8 µl water.

77. Add 1 µl of 10× T4 DNA ligase buffer and 1 µl of 400 U/µl T4 DNA ligase. Incubate overnight at 16°C.

78. Stop the reaction by adding 40 µl of 1× TE buffer and purify the DNA with QIAquick PCR purification kit as described by the manufacturer. Elute the DNA with 30 µl of 10 mM Tris·Cl, pH 8.5, and concentrate the sample to one third of the original volume using a Speedvac concentrator.

This step is important to enrich for long concatamers. Only DNA fragments longer than 100 bp can be recovered from QIAquick column.

Ligate concatamers into EcoRI and NcoI restriction-digested cloning vector

79. Digest 3 µg pGEM-T easy cloning vector with 1.5 µl *Eco*RI and 1.5 µl *Nco*I restriction endonuclease in 50 µl of 1× buffer H for 2 hr at 37°C. Verify that the restriction digestion is complete by separating 3 µl digested vector and 180 ng undigested vector on a 2% agarose gel containing ethidium bromide (Voytas, 1992).

80. Purify the *Eco*RI and *Nco*I restriction-digested cloning vector with QIAquick PCR Purification kit according to the manufacturer's instructions. Elute the DNA with 50 µl of 10 mM Tris·Cl, pH 8.5.

81. Ligate 5 µl purified concatamer DNA into *Eco*RI and *Nco*I restriction-digested cloning vector using Rapid DNA ligation kit as described by the manufacturer.

82. Transform TOP10 (or other supercompetent *E. coli*) cells using 5 µl ligation reaction according to the manufacturer. Add 500 µl SOC medium and plate out 50-µl aliquots on LB agar plates with ampicillin, and with 40 µl Xgal solution and 7 µl IPTG solution spread on the surface, for blue-white selection. Grow colonies overnight at 37°C.

Screen colonies for concatamer inserts by PCR

83. Fill the wells of a 96-well thermal cycler–compatible microtiter plate with 20 µl PCR mixture containing the following ingredients:

2 µl 10× PCR buffer
2 µl 2 mM dNTPs
0.2 µl 100 µM primer M13 F (Table 16H.2.1)
0.2 µl 100 µM primer M13 R (Table 16H.2.1)
0.7 µl 1% W-1
14.7 µl H_2O
0.2 µl *Taq* DNA polymerase.

84. Pick individual white colonies from the agar plate using a pipet tip and restreak on a master plate. Transfer the same pipet tips to the wells of the microtiter plate filled with the PCR mixture and resuspend the remaining colony by pipetting up and down.

The cells in the colonies will be lysed during the first PCR cycle. W-1 is a detergent.

85. Run a PCR (also see Kramer and Coen, 2001) using the following parameters:

1 cycle:	2 min	94°C	(initial denaturation)
30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	72°C	(final extension).

86. Separate 5 μl of the PCR products on a 2% standard agarose gel (Voytas, 1992) using a 100-bp DNA ladder as a size marker.

PCR products equivalent or longer than 400 bp contain at least three small-RNA sequences. The expected size of the PCR product from the empty vector is 249 bp.

87. Purify the remaining PCR products with the QIAquick PCR purification kit as described by the manufacturer. Sequence or submit the purified PCR product for automated sequencing using M13 F or M13 R primers.

Instead of using QIAquick column SAP/Exo I reaction may be used to prepare PCR products for sequencing.

ALTERNATE PROTOCOL

DEPHOSPHORYLATING AND CLONING SMALL RNAs FROM VIRUS-INFECTED PLANT TISSUES

In this technique, the gel-purified small RNAs are first dephosphorylated to prevent circularization and then ligated to a 5′-phosphorylated-3′-adapter oligonucleotide with a blocked 3′-hydroxyl terminus. The ligation product is recovered from separation on a 15% denaturing polyacrylamide gel and is subsequently 5′ phosphorylated and subjected to 5′-adapter ligation. The final ligation product is separated from the excess of 5′ adapter and then reverse transcription (RT)-PCR amplified and cloned according to the Basic Protocol.

Additional Materials *(also see Basic Protocol)*

10× phosphatase buffer (New England Biolabs)
20 U/μl calf intestinal alkaline phosphatase (CIP, New England Biolabs)
10× T4 polynucleotide kinase (PNK) buffer (New England Biolabs)
10 U/μl T4 polynucleotide kinase (New England Biolabs)
100 mM ATP, pH 7.0

Dephosphorylate small RNAs

1. Dissolve the small-RNA pellet from the Basic Protocol, step 21, in 44 μl RNase-free water. Add 5 μl of 10× phosphatase buffer and 1 μl of 20 U/μl CIP. Incubate 30 min at 50°C.

2. Add 250 μl of 1× TE buffer, and perform one 300-μl 25:24:1 phenol/chloroform/isoamyl alcohol extraction and one 300-μl 24:1 chloroform/isoamyl alcohol extraction, and precipitate the RNA as described in Basic Protocol steps 49 to 51.

Ligate phosphorylated 3′ adapter to dephosphorylated small RNAs

3. Collect and wash the RNA pellet as described in Basic Protocol, steps 52 to 54, except dissolve the pellet in 24 μl RNase-free water.

4. Ligate phosphorylated 3′ adapter to dephosphorylated small RNAs as described in Basic Protocol, steps 34 to 40. Excise the gel slice encompassing 36- to 46-nucleotide ligated small RNAs defined by the mobility of the 30- and 60-nucleotide DNA size markers. Elute the RNA as described in Basic Protocol, steps 17 to 20.

Perform 5′ phosphorylation of the ligation product

5. Collect the RNA pellet as described in Basic Protocol, step 21. Dissolve the pellet in 31.9 μl RNase-free water.
6. Transfer the RNA to a siliconized microcentrifuge tube placed on ice, and add 4 μl of 10× PNK buffer, 0.8 μl 100 mM ATP, 1.3 μl RNAguard, and 2 μl T4 polynucleotide kinase. Incubate the reaction 30 min at 37°C.
7. Add 260 μl of 1× TE buffer. Perform one 300-μl 25:24:1 phenol/chloroform/isoamyl alcohol extraction and one 300-μl chloroform extraction. Precipitate the RNA-DNA hybrid as described in Basic Protocol steps 49 to 51.

Ligate 5′ adapter to small RNA-3′- adapter hybrid

8. Collect and wash the RNA pellet as described in Basic Protocol, steps 52 to 54, and dissolve the pellet in 24 μl RNase-free water.
9. Ligate 5′ adapter as described in Basic Protocol, steps 22 to 28.
10. Excise the gel slice encompassing 54- to 64- nucleotide ligated small RNAs defined by the mobility of the 50- and 80-nucleotide DNA size markers.
11. Elute the RNA and proceed with Basic Protocol, steps 41 to 87.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Denaturing polyacrylamide gel, 15%

21 g urea
2.5 ml 10× TBE buffer (*APPENDIX 2A*)
18.75 ml 40% (w/v) 19:1 acrylamide:bis-acrylamide

Adjust volume to 50 ml with water, dissolve the urea by mixing with magnetic stirring. Add 350 μl of 10% (w/v) ammonium persulfate (APS) and 17.5 μl of TEMED. Mix thoroughly and pour immediately.

CAUTION: *Acrylamide and bis-acrylamide are potent neurotoxins and are absorbed through the skin. The use of pre-made solutions is recommended. When weighing powdered acrylamide and methylene-bis-acrylamide, wear appropriate gloves and a face mask to avoid inhalation.*

General protocols for denaturing gel electrophoresis are presented in Ellington and Pollard (1998).

DNA oligo ladder, 10-nucleotide

Mix DNA markers (see Table 16H.2.1), 3 μM each, in water
Add 1 vol gel-loading solution (see recipe)
Store up to 1 year at −20°C

EB, 10×

1 M glycine
100 mM EDTA
1 M NaCl
Adjust to pH 9.5, autoclave and store at room temperature, avoiding direct sunlight

Extraction buffer

1× EB (see recipe)
2% SDS
Prepare just before use

Gel-loading solution

10 ml deionized formamide
200 μl 0.5 M EDTA, pH 8.0
1 mg xylene cyanol FF
1 mg bromphenol blue
Store up to 1 year at 4°C

CAUTION: *Formamide is teratogenic. Wear appropriate gloves and safety glasses.*

Native polyacrylamide gel, 15%

2.5 ml 10× TBE (*APPENDIX 2A*)
18.75 ml 40% (w/v) 19:1 acrylamide:bis-acrylamide
28.75 ml water
Add 350 μl of 10% (w/v) ammonium persulfate (APS) and 17.5 μl of TEMED
Mix thoroughly and pour immediately

CAUTION: *Acrylamide and bis-acrylamide are potent neurotoxins and are absorbed through the skin. The use of pre-made solutions is recommended. If you must weigh powdered acrylamide and methylene-bis-acrylamide, wear appropriate gloves and a face mask to avoid inhalation.*

General protocols for native (nondenaturing) gel electrophoresis are presented in Chory and Pollard (1999).

PAN ligation buffer, 10×

0.5 M Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.1 M $MgCl_2$
0.1 M 2-mercaptoethanol
2 mM ATP
1 mg/ml acetylated BSA
Store up to 1 year at −20°C

COMMENTARY

Background information

The first small RNA to be discovered that controls gene regulation (lin-4) was identified by a genetic screen in *Caenorhabditis elegans* (Lee et al., 1993). It was thought unique and specific to nematode development until Hamilton and Baulcombe (1999) revealed that post-transcriptional gene silencing is also associated with the accumulation of siRNAs in plants. Later the Tuschl group demonstrated (Elbashir et al., 2001) that the dsRNA-induced RNA silencing is mediated by 21- and 22-nucleotide double-stranded small RNAs (siRNAs) leading to effective degradation of cognate mRNAs.

The first small-RNA cloning protocol was developed to study the processing of long exogenous dsRNAs into siRNAs using *Drosophila melanogaster* embryo extract that retained RNA silencing activity (Elbashir et al., 2001). In addition to siRNAs generated from the exogenous dsRNAs, high levels of cellular 21-nucleotide small RNAs were also identified (Lagos-Quintana et al., 2001). The endogenous small RNAs were derived from short hairpin RNA precursors and were characterized as miRNAs regulating the expression of various distinct mRNAs. This approach opened the way to deciphering the mechanism and function of gene silencing by analyzing the small-RNA profile of various organisms including worm, human, fly, mouse, fission yeast, plant, frog, and virus-infected tissues (Lau et al., 2001; Lagos-Quintana et al., 2001, 2002; Llave et al., 2002; Reinhart and Bartel, 2002; Pfeffer et al., 2004; Watanabe et al., 2005; Molnár et al., 2005).

This protocol is isotope free, utilizes unmodified small RNAs and is routinely used to characterize miRNAs and siRNAs from various plant tissues. The TNA isolation is adapted from White and Kaper (1989). The small-RNA cloning protocol results from modifications of protocols originally published by the Tuschl, Bartel, and Carrington groups (Elbashir et al., 2001; Lau et al., 2001; Llave et al., 2002; Pfeffer et al., 2003).

Critical Parameters and Troubleshooting

One of the most critical parameters of small-RNA cloning is the quality of total nucleic acid extract. Virus-infected plant tissues displaying severe symptoms such as necrosis and chlorosis contain large amounts of apoptotic cells. The small-RNA library prepared from those tissues would mainly comprise degradation products of abundant endogenous RNAs, including rRNAs and tRNAs. It is, therefore, recommended to harvest plant material at an early stage of infection. Analyses of the quality of TNA on an agarose gel and the detection of viral RNA by Northern hybridization are recommended before starting the cloning protocol. High-quality TNA reveals a clear banding pattern, representing RNAs without any sign of degradation (smearing).

The success of small-RNA cloning also depends on the quality of adapter oligonucleotides. To avoid mis-ligation caused by truncated adapter oligonucleotides, their gel purification is recommended prior to ligation. Alternatively, gel purified adapter oligonucleotides can be purchased from RNA synthesis companies.

If the viral genome contains either *Eco*RI or *Nco*I recognition sites, these regions will not be represented in the small-RNA library. Design adapter oligonucleotides and PCR primers containing alternative restriction endonuclease recognition sites that are not in the genome to ensure that every possible small-RNA sequence will be represented.

Concatamerization can reduce the expense of sequencing small RNAs; however, small RNA libraries enriched in concatamers might be less representative of all small RNAs present in the cells. Therefore, direct cloning of restriction enzyme digested cDNAs may be performed.

Avoid nuclease contamination throughout the procedure. Use sterile solutions and disposable plasticware, keep the tubes containing RNA solution on ice prior to enzymatic reactions.

The low amounts of small RNAs and ligation products can be absorbed by the walls of untreated reaction tubes. To increase recovery rate after precipitation steps, always use siliconized tubes where possible.

If hundreds of small-RNA sequences will be analyzed, the development of software tools for analyzing and archiving them is recommended.

Anticipated Results

Depending on the size of the concatamerized DNA, every recombinant plasmid should yield at least three small-RNA sequences. Sequence analyses of a typical clone are shown in Figure 16H.2.2.

Time Considerations

The small-RNA cloning is a technically demanding multistep procedure. If one step fails, the small-RNA clones will never be obtained. The protocol can be safely interrupted at every ethanol precipitation step. The precipitated nucleic acid can be stored in ethanol at −20°C for several days prior to collecting the pellet by centrifugation.

The cloning procedure can be performed within two weeks. Isolation and quality analyses of TNA extract require 1 day. Gel purification of small RNA with repeated elution from the gel requires 2 days. Ligation of the 5′ adapter to small RNA and purification of the first ligation product with repeated elution from the gel require 2 days. Ligation of the 3′ adapter to the 5′-adapter-small-RNA hybrid and purification of the final ligation product with repeated elution from the gel requires 2 days. Reverse transcription and PCR amplification of the cDNA require 1 day. Gel purification of the cDNA corresponding to the 5′-adapter-small-RNA-3′-adapter ligation product with repeated elution from the gel requires 2 days. *Eco*RI and *Nco*I restriction digestion of the PCR product and concatamerization of *Eco*RI and *Nco*I restriction-digested DNA require 1 day. Cloning of concatamers into *Eco*RI and *Nco*I restriction-digested vector requires 1 day. PCR screening of colonies and sequencing of concatamer inserts require 1 day.

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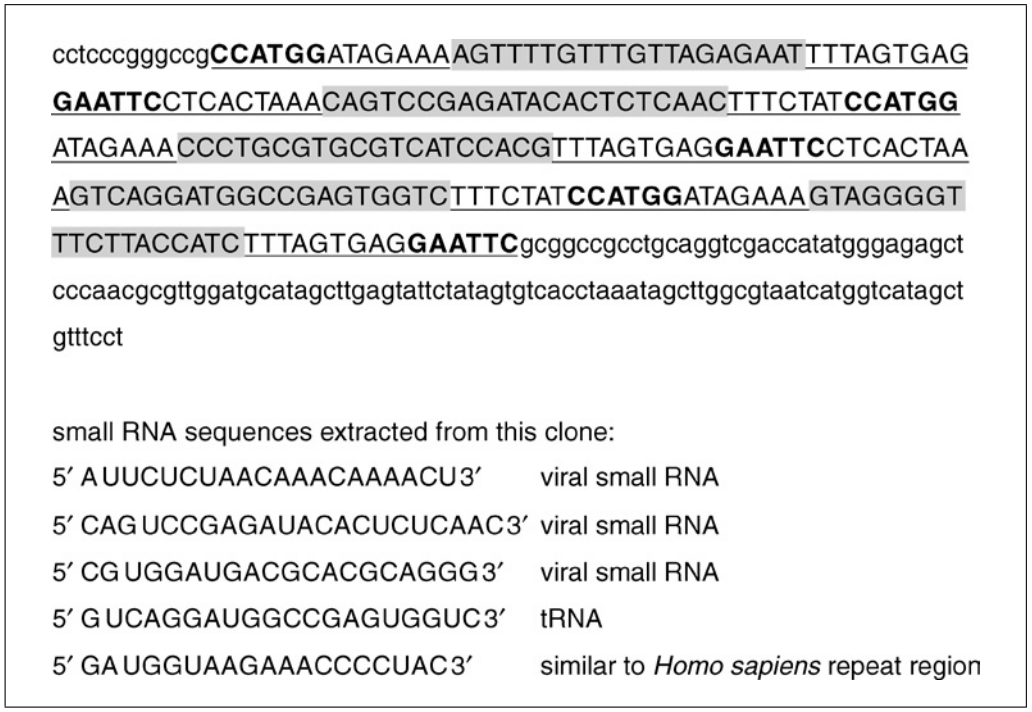

Figure 16H.2.2 Example of a concatamer sequence after cloning small RNAs from viral infected *Nicotiana benthamiana* leaf tissue. The lowercase letters represent the vector sequence while the uppercase letters represent the concatamer sequence. Adapter sequences are underlined and the *Eco*RI and *Nco*I restriction sites are emboldened. Small-RNA sequences are highlighted (dark gray).

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Use of Potato Virus X (PVX)–Based Vectors for Gene Expression and Virus-Induced Gene Silencing (VIGS)

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ABSTRACT

Potato virus X–based vectors are a well established system for rapid in planta studies. The vectors can be used for expression of proteins in plants and to down-regulate genes through virus-induced gene silencing. The development of binary-based vectors for *Agrobacterium* delivery makes this system well suited to high-throughput studies. Protocols are given for establishing infections to achieve expression and VIGS through in vitro transcription and *Agrobacterium* delivery to glasshouse and in vitro–grown plant material. *Curr. Protoc. Microbiol.* 8:16I.1.1-16I.1.13. © 2008 by John Wiley & Sons, Inc.

Keywords: potato virus X • virus vector • gene silencing • *Agrobacterium tumefasciens* • solanaceous plants

INTRODUCTION

Vectors based on potato virus X (PVX) have been the most widely used plant virus–based vectors. This is in part due to the fact that they have been made freely available through The Sainsbury Laboratory (*http://www.ayeaye.tsl.ac.uk/*), where PVX-based vectors were originally constructed. PVX-based vectors have proven valuable experimental tools. They can be used for virus-induced gene silencing (VIGS) through the insertion of partial cDNA sequences, or for the expression of proteins through the insertion of full-length cDNAs from plants, of cDNAs encoding therapeutic proteins, or of cDNAs for other foreign proteins (e.g., derived from other plant pathogens).

Various strategies have been tested for the production of vectors from PVX. The most successful strategy is that originally developed: expression of the foreign gene is achieved by its insertion between a duplication of the subgenomic promoter sequence that, in the wild-type RNA virus, directs the production of a subgenomic mRNA from which the viral coat protein is translated. This unit describes procedures for infecting plants with PVX-based vectors that use this strategy to achieve protein expression or VIGS.

In early PVX-based vectors, such as P2C2S (Figure 16I.1.1), full-length cDNA copies of PVX were cloned into plasmids under the control of a T7 RNA polymerase promoter sequence. The vector P2C2S differs from later binary-based vectors (pGR106 and pGR107 described further) in that it contains a shorter promoter sequence to drive expression of the coat protein gene. Such vectors give higher levels of foreign protein expression (S. Santa Cruz, unpub. observ.), possibly due to reduced promoter competition. In Basic Protocol 1, a method is given in which such a plasmid is linearized, the linear DNA transcribed in vitro, and the run-off transcripts produced used for manual inoculation of plants. Subsequently PVX-based vectors, e.g., pGR106 and pGR107 (Figure 16I.1.1), were produced in which genetically engineered, full-length, cDNA copies of PVX were

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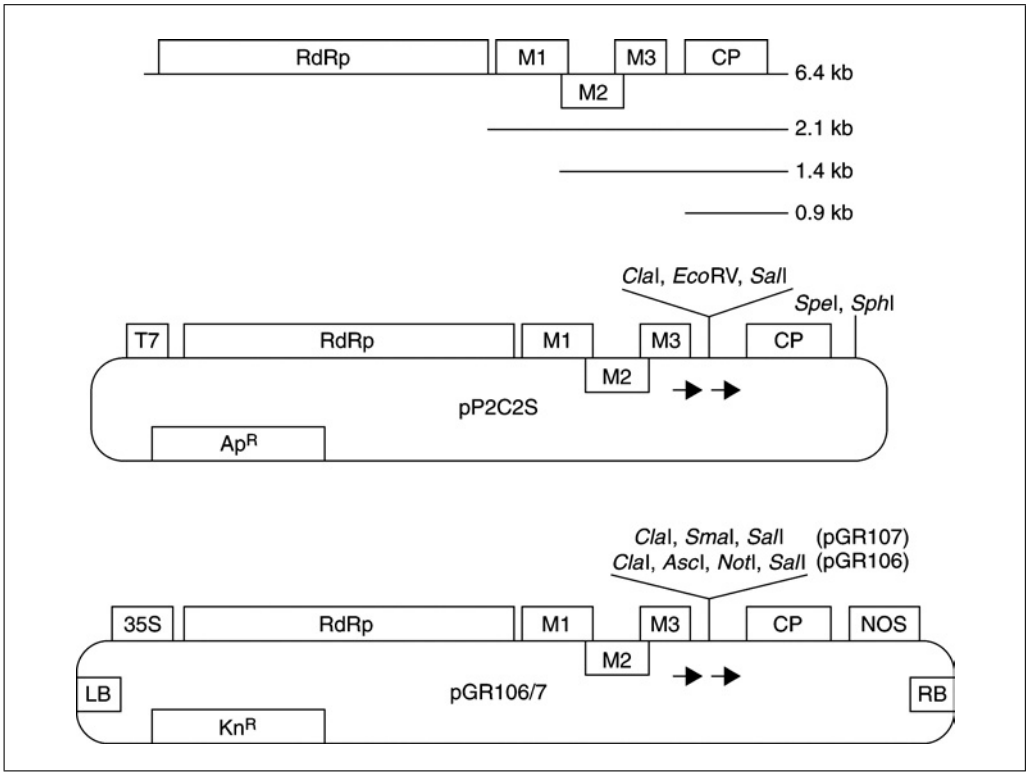

Figure 16I.1.1 Schematic representations of PVX and PVX-based vectors. Genome organization of wild-type PVX (**A**). The five major ORFs in the 6.4-kb genome are indicated by boxes. The subgenomic mRNAs from which the 5′ distal ORFs are expressed are indicated by lines below, with the sizes of the mRNAs. Organizations of pP2C2S (**B**) and pGR106/7 (**C**). The positions of the duplicated, subgenomic promoter sequences are indicated by arrows. The restriction enzyme sites in the multiple cloning sites are listed above. Features shown are: viral RNA-dependent RNA polymerase gene (RdRp); viral movement protein genes (M1, M2, M3); viral coat protein gene (CP); T7 bacteriophage (T7) and cauliflower mosaic virus 35S (35S) promoters; nopaline synthase transcriptional terminator (NOS); T-DNA left and right borders (LB, RB); β-lactamase gene (Ap^R) conferring ampicillin resistance; and neomycin phosphotransferase II gene (Kn^R) conferring kanamycin resistance.

positioned between a cauliflower mosaic virus 35S promoter and nopaline synthase terminator sequence in binary vectors to allow *Agrobacterium tumefasciens*–mediated initiation of infections. This system provides a more robust and cheaper method for accomplishing infections than in vitro transcription. Beginning with the insertion of a foreign gene sequence into the binary pGR106, Basic Protocol 2 describes the transformation of *Agrobacterium*, propagation of transformed bacteria, and infiltration of such bacteria into plant leaves to initiate infection. A similar protocol is found in *UNIT 16B.2*. This method is of particular utility when pervasive infections of inoculated leaves and plants are required.

Tubers produced by potatoes are the world's fourth largest crop. In Basic Protocol 3, a modification of the *Agrobacterium*-mediated infection process, stab inoculation, is used to initiate infections to achieve VIGS in potato microtubers, a model for the crop tissue. Stab inoculation has previously been used in high-throughput studies, involving multiple stab inoculations of a single leaf, to screen clones for a phenotype that is easily assessable at the site of inoculation (Takken et al., 2000) and for facile initiation of systemic infections of large numbers of plants with individual clones for high-throughput VIGS studies (Lu et al., 2003).

PVX-based vectors do not in themselves present a risk to human health, but, as genetically engineered plant pathogens, they present a risk to the environment. So far, PVX-based

vectors have been found to be unfit in comparison to wild-type virus and to revert to wild-type virus through homologous recombination between the duplicated promoter sequences. PVX is found in potato-growing regions worldwide. Experiments involving PVX-based vectors are likely to require BL2-P containment; however researchers should carry out a risk assessment and comply with local and national regulations (see *UNIT 1A.1*).

INFECTION OF PLANTS WITH IN VITRO TRANSCRIPTS OF RECOMBINANT PVX

BASIC PROTOCOL 1

This protocol begins with a plasmid vector (pP2C2S, Figure 16I.1.1) containing the gene of interest inserted in the multiple cloning site between the duplicated coat protein subgenomic promoters. This recombinant plasmid is then linearized and transcribed in vitro with T7 RNA polymerase. After confirmation of the integrity of the in vitro run-off transcripts, they are manually inoculated onto host plant leaves. After incubation to allow the recombinant virus to move locally and/or systemically, the plants are observed for symptom development and analyzed for expression of the gene of interest.

Materials

pP2C2S-derived recombinant plasmid (The Sainsbury Laboratory; *http://www.ayeaye.tsl.ac.uk*; follow links for David Baulcombe, then for "PVX vectors")
*Spe*I restriction endonuclease and 10× buffer
25:24:1 (v/v/v) phen4ol:chloroform:isoamyl alcohol saturated with 10 mM TE buffer, pH 8.0 (*APPENDIX 2A*)
3 M sodium acetate pH 5.2 (*APPENDIX 2A*)
100% and 70% ethanol
10 mM TE buffer, pH 8.0 (*APPENDIX 2A*)
0.8% and 0.7% nondenaturing agarose gels (Voytas, 2000) prepared in 1× TBE buffer (*APPENDIX 2A*)
5× T7 RNA polymerase buffer
0.1 M dithiothreitol (DTT)
10× A/C/U/gTP mix (see recipe)
5 mM cap analog (m7G[5′]ppp[5′]G)
50 U/μl T7 RNA polymerase
20 mM GTP
Plants to be infected (Table 16I.1.1)
Aluminum oxide F400WP (Washington Mills Electro Minerals Company; *http://www.washingtonmills.com/*)

0.5-ml microcentrifuge tubes
Erlenmeyer flask
Gauze
Containment glasshouse or growth chamber

Additional reagents and equipment for agarose gel electrophoresis and ethidium bromide staining of gels (Voytas, 2000)

Linearize plasmid DNA

1. In a 0.5-ml microcentrifuge tube, digest 25 μg of plasmid DNA with 25 U *Spe*I restriction endonuclease using an appropriate buffer in a final volume of 50 μl. Incubate overnight at 37°C.

   The SpeI site is preferred for linearization of pP2C2S as it lies immediately downstream of the 3′ poly(A) tail of the infectious clones. The SphI site is located just downstream of the SpeI site and can be used instead if an SpeI site is present in the inserted cDNA, but addition of extra nucleotides after the poly(A) tail is detrimental to transcript infectivity.

2. Purify the digested DNA as follows:

   a. Add 35 μl of buffer-saturated 25:24:1 phenol:chloroform:isoamyl alcohol.
   b. Vortex thoroughly, centrifuge 5 min at 13,000 × *g* to separate phases, and collect the upper aqueous phase.
   c. Add 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, mix, and place on ice for 15 min.
   d. Microcentrifuge 15 min at 13,000 × *g*, 4°C., and remove supernatant.
   e. Wash pellet with cold 70% ethanol, centrifuge briefly, remove all of the supernatant, evaporate residual ethanol, and dissolve DNA pellet in TE buffer to a concentration of 0.5 μg/μl.

3. Electrophorese a 1 μl aliquot of the dissolved DNA alongside suitable DNA molecular weight standards on a 0.8% agarose/1× TBE gel (Voytas, 2000). Stain with ethidium bromide (also described in Voytas, 2000) to check DNA recovery and the efficiency of linearization.

   Efficient linearization is required because circularized DNA acts as a better template for T7 RNA polymerase, but the 3′ extended transcripts produced from undigested DNA have very poor infectivity.

Transcribe linearized DNA

4. Assemble transcription reaction components in a 0.5-ml microcentrifuge tube at room temperature:

   10 μl 5× T7 RNA polymerase buffer
   2.5 μl 0.1 M DTT
   5 μl 10× A/C/U/gTP mix
   5 μl 5mM cap analog
   12.5 μl H_2O
   10 μl 0.5 μg/ml linearized template DNA (step 2, substep e).

   Mix, incubate at 37°C for 5 min, then add 5 μl of T7 RNA polymerase. Mix by gently pipetting up and down, and continue incubation at 37°C.

   As spermidine in the enzyme buffer has the potential to precipitate DNA, the reaction components should be assembled in the order indicated, at room temperature and not on ice.

   Optionally, 20 U of ribonuclease inhibitor can be added to the reaction.

   The authors have also used the Ambion mMESSAGE mMACHINE T7 kit which provide good yields of transcripts.

5. After incubating at 37°C for 20 min, chase the reaction with GTP by pipetting 5 μl of 20 mM GTP onto the surface of the reaction. Do not mix, and continue incubating for 40 min at 37°C.

   The initial GTP concentration is reduced to promote incorporation of the cap analog, as uncapped transcripts are not infectious.

6. Purify the transcription reaction products by adding 55 μl buffer-saturated 25:24:1 phenol:chloroform:isoamyl alcohol, vortexing thoroughly, and centrifuging 5 min at 13,000 × *g* to separate phases. Collect ~45 μl of the upper aqueous phase, avoiding carrying over any of the phenolic phase. Set aside 2 μl for gel analysis and use the remainder of the transcripts immediately for plant inoculation.

   Optionally, if the transcripts are not to be used immediately for plant inoculation, add 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol, mix, and store at –20°C. Prior to use, microcentrifuge for 15 min at 13,000 × g, 4°C, remove supernatant, wash pellet with cold 70% ethanol, centrifuge briefly, remove all supernatant, evaporate residual ethanol, and dissolve the RNA pellet in 45 μl of RNase-free or DEPC-treated sterile distilled water.

7. Electrophorese the 2-μl aliquot of transcription reaction products on a nondenaturing, 0.7% agarose/1× TBE gel and stain with ethidium bromide (procedures described in Voytas et al., 2000) to quickly check transcript yield and integrity.

   The transcripts should migrate as a single band with slightly higher mobility than the template DNA. The intensity of the transcript band should be greater than that of the template DNA, usually by an order of magnitude. If degradation is apparent, this is indicative of an RNase contamination problem.

Inoculate plants

8. Mark leaves to be inoculated by punching a hole in each of them with a pipet tip. Very lightly dust the leaves with aluminum oxide using an Erlenmeyer flask topped with a double layer of gauze.

   CAUTION: *Due to the fine particulate nature of the aluminum oxide suitable respiratory protection should be worn.*

   The choice of leaves for inoculation is important. Young leaves are generally more susceptible to virus infection, but also more easily damaged by the manual inoculation process. For inoculation of Nicotiana benthamiana, select plants that are about 12 cm across and at the nine-leaf stage (4 to 5 weeks post sowing), and inoculate the seventh leaf.

9. Pipet 5 μl of transcript to an inter-veinal region on the upper side of the leaf near the base. Wearing gloves, support the leaf from below with one hand. Spread the inoculum over the leaf surface by drawing it to the leaf apex with a finger.

   Ten gentle strokes should be adequate for this; more may result in excessive damage to the leaf.

10. Place plants under controlled growth conditions (in a containment glasshouse or growth chamber) and leave until systemic infection results in the development of a mild, chlorotic mosaic on uninoculated leaves.

    IMPORTANT NOTE: *Containment level and standard operating procedures required for propagation of PVX-infected plants must be in accordance with the health and safety regime defined by local and national regulations covering work with genetically modified organisms in contained facilities (as an example for the U.K., http://www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/).*

11. When systemic symptoms are well developed, assess accumulation of foreign protein in inoculated and systemically infected leaves by suitable analytic methods, e.g., through immunoblotting of total protein extracts.

    The stability of the recombinant PVX-based vector can be assessed through reverse transcription of extracted poly(A) RNA and PCR amplification with primers flanking the duplicated promoter sequences, i.e., a sense primer 5′ of position 5580 in the PVX genome and an antisense primer 3′ of position 5680.

INFECTION OF PLANTS BY AGROINFILTRATION OF RECOMBINANT PVX VECTOR

BASIC PROTOCOL 2

This protocol begins with a plasmid vector (pGR106 or pGR107, Figure 16I.1.1) containing the sequence of interest inserted in the multiple cloning site between the duplicated subgenomic promoters. This recombinant plasmid is then introduced into electrocompetent agrobacteria cells previously transformed with the helper plasmid pSoup. The transformed agrobacteria are propagated and induced, and their density adjusted prior to pressure infiltration of leaves. After incubation to allow the recombinant virus to move locally and/or systemically, the plants are observed for symptom development and analyzed for expression or down-regulation of the gene of interest.

Materials

Electrocompetent *A. tumefasciens* strain GV3101 (Hellens et al., 2000) harboring pSoup plasmid (*http://www.pgreen.ac.uk/JIT/JIT_fr.htm*; follow links for "binary vectors," then for "pGreen")

pGR106 or pGR107-derived recombinant plasmid

LB plates (*APPENDIX 4A*) with 50 μg/ml kanamycin

PVX- or insert-specific primers (see information on supply and sequence data at *http://www.ayeaye.tsl.ac.uk/*; follow links for David Baulcombe, then for "PVX vector"; pGR106 and pGR107 GenBank accession numbers are, respectively, AY297843 and AY297842)

LB liquid medium (*APPENDIX 4A*) with 50 μg/ml kanamycin

Agromix (see recipe)

N. benthamiana plants (or other Solanaceae; see Table 16.I.1.1) to be inoculated

28°C incubator

Bench-top centrifuge

Spectrophotometer

2-ml syringes

Containment glasshouse or growth chamber

UV lamp: Blak-Ray model B100AP (UVP) for green fluorescent protein visualization

Additional reagents and equipment for transformation of *Agrobacterium* (*UNIT 16B.2*), the polymerase chain reaction (PCR; Kramer and Coen, 2001), and agarose gel electrophoresis (Voytas, 2000)

Transform of Agrobacterium tumefasciens

Also see Hellens et al. (2000a) and *http://www.pgreen.ac.uk/JIT/JIT_fr.htm* (follow links for "binary vectors," then for "pGreen").

1. Transform electrocompetent *A. tumefasciens* strain GV3101 harboring helper plasmid pSoup with 1 μg of recombinant pGR106/107 plasmid DNA (*UNIT 16B.2*).

   The plasmids pGR106 and pGR107 are derived from the binary vector pGreen0000, which carries a kanamycin selectable marker and replicates to high copy number in Escherichia coli. For replication in Agrobacterium, these plasmids require a helper plasmid pSoup (or pJIC Sa_Rep), which provides a replicative function (RepA) in trans and carries a tetracycline selectable marker (Hellens et al., 2000b; http://www.pgreen.ac.uk/JIT/pSoup.htm). Agrobacteria can be transformed with pSoup and pGR106/107 simultaneously; however, for simplicity, it is recommended that Agrobacteria already harboring pSoup be transformed with the binary-PVX constructs. Instead of GV3101, Agrobacterium strains LBA4404 or AGL1 can be used. A. tumefasciens strain GV3101, LBA4404, or AGL1 can be obtained from the e-mail addresses and Web sites mentioned in Hellens et al. (2000a).

2. Plate aliquots of the transformation on LB plates supplemented with 50 μg/ml kanamycin for selection and incubate at 28°C for 2 to 3 days.

3. *Optional:* Screen colonies of transformed cells by PCR with PVX- or insert-specific primers to confirm their identity; to do this, pick colonies with a pipet tip, restreak to LB plates supplemented with kanamycin, and dip tip into a PCR tube containing PCR mix prior to thermal cycling (Kramer and Coen, 2001) and agarose gel analysis (Voytas, 2000).

   If PCR screening is not performed, simply identify positive clones as the colonies that grow on the kanamycin plate.

Infiltrate leaves

4. Inoculate 20-ml aliquots of LB liquid medium supplemented with 50 μg/ml kanamycin with the positive clones using a sterile pipet tip. Incubate cultures overnight at 28°C with agitation.

   Typically 20 ml of overnight culture is sufficient to inoculate up to 20 plants.

5. Pellet the agrobacteria by centrifugation for 10 min at 2250 × *g*, 16°C.

6. Remove the supernatant, resuspend the bacterial pellet in 1 to 2 ml of Agromix, and let the suspension stand at room temperature for 1 to 2 hr.

7. Adjust the optical density of the resuspension to an OD_{600} of 0.5 with Agromix.

8. Puncture the surface of leaves to be inoculated (at least once per leaf to be infiltrated) with a pipet tip or hypodermic needle to facilitate entry of the bacterial resuspension. Place a 2-ml syringe (without a hypodermic attached) containing the bacterial resuspension over the puncture sites and gently infiltrate the resuspension while applying a counter-pressure on the opposite side of the leaf with a finger.

   For most but not all species, infiltration of the lower leaf surface is easiest. When using vectors for overexpression, choose N. benthamiana at the same developmental stage as described in Basic Protocol 1. For VIGS, younger plants with a diameter of 5 cm and about six leaves are preferred.

9. Place plants under controlled growth conditions (in a containment glasshouse or growth chamber).

   IMPORTANT NOTE: *Containment level and standard operating procedures required for propagation of PVX-infected plants must be in accordance with the health and safety regime defined by local and national regulations covering work with genetically modified organisms in contained facilities (as an example for the U.K., http://www.hse.gov.uk/biosafety/gmo/acgm/acgmcomp/).*

   Symptoms of systemic infection, typically a chlorotic mosaic, should be observable on N. benthamiana by 1 week post inoculation.

   The time that virus infections are allowed to propagate prior to sampling depends on the purpose of the experiment, the host, and the construct, in particular the size of the foreign insert. As an indication, in expression studies, inoculated leaves should be sampled between 1 and 2 weeks after inoculation, and systemically infected leaves up to 3 weeks after inoculation. In VIGS studies, systemic leaf tissue should be sampled at 3 weeks or more post inoculation, while potato tubers should be sampled at ∼2 months after inoculation.

   Similar infections carried out in parallel with a vector designed to express the green fluorescent protein (GFP) or a vector designed to silence phytoene desaturase (PDS) through the inclusion of a partial cDNA sequence in antisense orientation provide useful indicators as to the progression of viral infections and VIGS responses, respectively. Expression of GFP can be visualized by illumination of infected tissue with a hand-held UV lamp (e.g., Blak-Ray from UVP), while VIGS of PDS can be discerned through the consequent photobleaching. Furthermore a vector designed to express GFP can be an appropriate control in functional and VIGS studies for virus-induced changes in the host plant.

INDUCTION OF VIGS IN POTATO MICROTUBERS WITH RECOMBINANT PVX VECTORS

BASIC PROTOCOL 3

This protocol begins from step 3 of Basic Protocol 2, with a pGR106 derivative containing a partial plant cDNA inserted in antisense orientation into the polylinker that has been transformed into *Agrobacterium* with the helper plasmid pSoup. Viral infections of in vitro–grown potato explants are initiated by the transformed *Agrobacterium* at sites of mechanical damage on leaves. The explants are then grown to allow the virus to propagate and a VIGS response to spread. Cuttings from the silenced explants are transferred to

new medium to induce microtuber formation; when these have formed, the effects of silencing are analyzed (Faivre-Rampant et al., 2004).

Materials

pGR106 or pGR107-derived recombinant plasmid in *A. tumefasciens* GV3101 harboring plasmid pSoup (Basic Protocol 2)

In vitro–grown potato explants (Xu et al., 1998; in U.K. available from Scottish Agricultural Science Agency; *http://www.sasa.gov.uk*; *info@sasa.gsi.gov.uk*) propagated in potato explants propagation medium (see recipe) by internode cuttings (Xu et al., 1998; Hendriks et al., 1991)

Microtuberization medium (see recipe)

Plant growth cabinet at 18° to 22°C with 16-hr photoperiod and light intensity 110 μE $m^{-2}sec^{-1}$

Plant growth cabinet at 16°C with 8-hr photoperiod and light intensity 80 μE $m^{-2}sec^{-1}$

Nescofilm (Karlan; *http://www.karlan.com*) or other laboratory film, e.g., Parafilm

Perform stab inoculation in vitro

1. Using sterile toothpicks or pipet tips, pick individual, clonal, transformed agrobacteria colonies.
2. Inoculate all leaves of in vitro–grown potato explants at the two- to four-leaf stage as follows (Takken et al., 2000):
   a. Open a vessel containing a potato explant under sterile conditions in a laminar flow hood.
   b. Wipe gloved hands with 70% ethanol.
   c. Supporting a leaf with a gloved finger, stab leaf in several locations with the pipet tip or toothpick used to pick the agrobacteria colony.
3. Incubate the plants for 4 weeks at 18° to 22°C with a 16-hr photoperiod and light intensity of 110 μE $m^{-2}sec^{-1}$.

   VIGS will occur in the upper, uninoculated leaves within 4 weeks of stab inoculation.

Induce microtuberization

4. Collect plant tissue from above the inoculated leaves under sterile conditions. Cut off leaves and cut stem into pieces containing single nodes. Place nodal cuttings on the surface of a Petri dish containing microtuberization medium and seal plate with Nescofilm. Incubate at 16°C with an 8 hr photoperiod of 80 μE $m^{-2}sec^{-1}$.
5. After 7 days wrap petri dishes in aluminum foil to exclude light and continue incubating at 16°C for a further 28 days.
6. Harvest the microtubers by pulling them from the agar medium and characterize the silencing response by appropriate analytical methods, e.g., semiquantitative or quantitative RT-PCR, northern blotting, and immunoblotting.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

A/C/U/gTP mix, 10×

20 mM ATP
20 mM CTP
20 mM UTP
2 mM GTP
Store in aliquots up to 6 months at −20°C

Agromix

10 mM $MgCl_2$ (add from 1 M filter-sterilized stock; store up to 1 year at 4°C)
150 μM acetosyringone (add from 10 mM stock in ethanol; store up to 1 year at −20°C)
10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; add from 0.5 M filter-sterilized stock, pH adjusted to 5.6 with KOH; store up to 1 year at 4°C)
Prepare fresh

Microtuberization medium

Murashige & Skoog (MS) basal medium (Sigma)
6% (w/v) sucrose
0.8% (w/v) Bacto Agar (BD Difco)
Adjust pH to 5.8
Autoclave
Aseptically add filter-sterilized 1 M chlorocholine chloride (Sigma) to final concentration of 7 mM
Aseptically add filter-sterilized 1 mg/ml benzylaminopurine (Sigma) to final concentration of 8 μM
Store up to 1 month at 18° to 20°C

Potato explants propagation medium

Murashige & Skoog (MS) basal medium, with macro- and micronutrients and vitamins (Sigma)
2% (w/v) sucrose
0.8% (w/v) Bacto Agar (BD Difco)
Adjust pH to 5.8
Autoclave
Store up to 6 months at 4°C

COMMENTARY

Background Information

PVX-based vectors were developed to facilitate rapid, transient expression of foreign genes in plants (Chapman et al., 1992) and to down-regulate genes through antisense approaches. Plant virus–based vectors were produced to circumvent the lengthy procedures involved in generating transgenic plants and to achieve higher levels of foreign protein expression. The rapidity of such vectors is still one of their primary advantages, and the levels of expression achievable with some tobacco mosaic virus (TMV)–based vectors are still greater than those achievable by any other means (Marillonnet et al., 2004). The development of *Agrobacterium* infiltration techniques has made transient expression and gene silencing possible without virus-based vectors (Johansen and Carrington, 2001; Voinnet et al., 2003). However, when virus-based vectors are used in conjunction with *Agrobacterium*, they are still advantageous in terms of levels of expression, the strength of the silencing responses they induce in host plants, and their adaptability to high-throughput studies. However, plant virus–based vectors have a major limitation in terms of the size of insert they can be used to express, as well as host-range limitations. This is not an important factor for silencing, but means that *Agrobacterium* infiltration is a more attractive option when expression of large inserts is desired.

Potato virus X is the type member of the potexvirus group. It is a positive sense RNA plant virus that forms flexuous, filamentous viral particles. The single PVX genomic RNA of 6.4 kb contains five major open reading frames (ORFs). The most 5′ ORF, encoding an RNA-dependent RNA polymerase necessary for viral replication, is translated from the genomic RNA. The other ORFs are expressed from three subgenomic RNAs produced during in planta replication of the virus. The three internal ORFs encode proteins necessary for cell-to-cell movement of the virus, while the most 3′ ORF encodes the viral coat protein, which protects the viral RNA and is also required for cell-to-cell movement of the virus.

Initial vector strategies included replacement of the coat protein gene, which was of

limited utility because of the role of the coat protein in intercellular movement, and insertion of foreign genes between a duplication of the coat protein subgenomic promoter sequence to generate an additional subgenomic RNA from which the foreign gene could be translated. Subsequently, other strategies for vector construction were tested: expression of a foreign protein via an intermediate fusion to the coat protein using the foot-and-mouth-disease virus autoproteolytic peptide to generate two separate proteins (Santa Cruz et al., 1996); inclusion of an internal ribosome entry site to allow expression of two genes from one subgenomic RNA (Toth et al., 2001); and transgenic complementation of vectors with gene replacements. None of these more recent strategies has proven as effective as duplication of the subgenomic promoter. One problem with this strategy is that homologous recombination between the duplicated sequences results in regeneration of wild-type virus that is competitively more fit than the progenitor vector, though this may also be perceived as an advantage with respect to containment.

Infections with early PVX-based vectors were initiated with T7 RNA polymerase–derived in vitro transcripts. This procedure is expensive, and because of RNA lability, not extremely robust. These problems were circumvented with the development of infectious DNA constructs (Baulcombe et al., 1995) in which vector sequences were placed between CaMV 35S promoter and nopaline synthase terminator sequences. This approach was further improved upon by insertion of the DNA sequences into binary vectors for *Agrobacterium*-mediated delivery, rather than manual inoculation or bombardment of plasmid DNAs. This enhancement has facilitated the inoculation of large numbers of plants and obviated the need for viral passage during which foreign genes can be deleted.

PVX-based vectors are dually functional: they can be used for both overexpression of foreign genes and VIGS. The levels of foreign gene expression achievable with PVX-based vectors are lower than those achievable with TMV-based vectors, such as 30B (*UNIT 16I.2*), which replicate to higher levels in infected plants. This may be a disadvantage when gross overproduction is required, e.g., in the expression of therapeutic proteins, but may be an advantage in functional studies when excessive expression might be more perturbing to the host plant. Similarly, vectors based on tobacco rattle virus (*UNIT 16I.6*) may be better for VIGS studies, as they give less symptomatic infections and induce more pervasive and sustained VIGS responses (Ratcliff et al., 2001).

Although PVX is distributed in potato-growing regions worldwide, the disease that it causes is not of major economic importance. In potatoes, PVX causes mild mosaic symptoms or is latent, but can cause much more severe symptoms in mixed infections with other viruses, such as potato virus Y. PVX is readily, mechanically transmissible; this is probably its major route of spread, as it does not have known vectors. Potato cultivars are protected against infection by strain-specific hypersensitivity genes, including *Nx*, and extreme resistance (*Rx*) genes that protect against all but two strains of PVX.

Critical Parameters and Troubleshooting

Insert sequences

Insertion of foreign sequences places a genetic load on the virus that reduces its fitness. This load is dependent on insert size, with larger inserts reducing viral replication and expression levels. Further, vectors with larger inserts are more susceptible to lose the foreign insert through in planta recombination. The largest insert successfully expressed from a PVX-based vector is the 2-kb *uidA* gene encoding β-glucuronidase. As PVX is a cytoplasmically replicating virus, it is necessary that sequences introduced for expression not contain introns; thus, if of eukaryotic origin, these are usually cDNAs. There are a number of additional considerations in cloning inserts into the PVX-based vectors. First, if the transcriptional clone P2C2S is to be used, it should be checked that the insert sequence does not contain an *Spe*I site, as this will be required for linearization of the plasmid prior to transcription. Second, if the *Cla*I sites in the vector polylinkers are to be used, it should be borne in mind that these are in methylation-sensitive contexts. Thus, for efficient cutting, the plasmid should be propagated in a *dam*$^-$ *E. coli* strain. Third, for expression of foreign inserts, the initiating methionine should be the first ATG triplet encountered after the *Cla*I site, and this should preferably be in a good translational context, e.g., A-A-A-A-ATG-G. Fragments as short as 33 nucleotides in length can trigger VIGS (Thomas et al., 2001), but inserts of about 400 nucleotides, in antisense orientation, are recommended for more reliable silencing.

In vitro transcription

As with most procedures involving RNA, it is essential to avoid RNase contamination, and a number of precautions are advised to avoid this. First, gloves should be worn during all procedures to prevent transfer of RNases from hands. Second, nuclease-free solutions and ultrapure RNase-free water and chemicals should be used. These can be purchased from suppliers, or where appropriate, solutions can be treated with diethylpyrocarbonate (DEPC). Third, equipment, such as microcentrifuge tubes, pipet tips, and glassware should be RNase free. The latter can be baked to inactivate RNases. Fourth, standard laboratory equipment that cannot be baked (e.g., that used in gel electrophoresis), should be cleaned thoroughly. Optionally, an RNase decontamination solution, such as RNaseZap (Ambion), can be used to remove RNase from glassware and plastic surfaces, including microcentrifuge tubes.

In Basic Protocol 1, a method for the production of in vitro transcripts is presented; alternatively, commercially available kits, such as T7 mMESSAGE mMACHINE (Ambion), can be used to produce high yields of capped transcript.

Choice of host plant

N. benthamiana and *N. clevelandii* are two highly susceptible hosts that allow replication of PVX-based vectors to high titer and high levels of foreign insert expression; therefore these two hosts are recommended for overexpression studies. Furthermore, the ease with which gene silencing can be induced in *N. benthamiana* makes it the host of first choice for VIGS studies. Host plants that have been used for overexpression and VIGS studies with PVX-based vectors are listed in Table 16I.1.1. When infecting potatoes, it should be borne in mind that all the PVX vectors are based on the UK3 strain of PVX, which is a group 3 strain. Therefore, cultivars carrying *Nx* or *Rx* resistance genes should be avoided, as they will be resistant to the virus.

Plant propagation

PVX is found in temperate regions and does not replicate to high titer at high temperatures. A recommended growth regime for host plant

Table 16I.1.1 Host Plants Used for PVX-Based Systemic Expression and Virus-Induced Gene Silencing

Genus	Species	Vectors	Expression	VIGS
Nicotiana	*benthamiana*	P2C2S pGR106	+	+
	clevelandii		+	Not tested
	paniculata		+	Not tested
	glutinosa		+	Not tested
	rustica		+	Not tested
	tabacum		+	Not tested
Lycopersicon	*esculentum* cultivars MoneyMaker, Marmande, Rossol, Rio Grande	P2C2S pGR106	+	Not tested
	pimpinellifolium		+	Not tested
Solanum	*tuberosum* cultivars Desiree, Bintje, Stirling	P2C2S pGR106	+	+
	bulbocastanum		+	+
	phureja accessions DB337, 71P10, 80CP23, 375/1		Not tested	+
	phureja accessions DB271, DB371		Not tested	–
	chacoense accessions 3886, 5915, 7211, 7234		Not tested	+
Capsicum	annuum	P2C2S	+	Not tested

propagation is a 16-hr light period of 400 to 1000 μE $m^{-2}sec^{-1}$ at 24°C and an 8-hr dark period at 18°C.

Infectivity of PVX constructs

Observation of mosaic symptoms in highly permissive hosts is often the easiest way to determine efficient infection and systemic invasion by PVX-based vectors. However, in less permissive hosts such as *Solanum tuberosum*, obvious systemic symptoms may not be observed. Therefore, it is prudent to confirm the infectivity of constructs by parallel inoculation of a permissive host, such as *N. benthamiana*, or local-lesion hosts such as *Chenopodium quinoa* or *Chenopodium amaranticolor*.

Stability and passage inoculation

As mentioned above, the PVX-based vectors lose inserted foreign sequences through in planta recombination, and this is more of a problem with larger inserts and less permissive hosts. Thus, for overexpression of large inserts, only highly permissive hosts should be used. Further, as wild-type recombinants will predominate with time, it is wise to sample only inoculated leaves and the first few systemically infected leaves displaying early signs of mosaic symptoms (youngest sink leaves by 7 to 10 days post inoculation). When inoculation techniques involving *Agrobacterium* are used, the amount of inoculum available is unlikely to be limited, and there is no need to carry out passage inoculations unless the increased infectivity of viral preparations is required to establish infections on poorly susceptible hosts. However, if transcription clones are used, the high cost of preparing in vitro–transcribed inoculum may necessitate passage inoculation to obtain sufficient numbers of infected plants and amounts of infected material. If this is the case, primary inoculations should be performed on highly susceptible hosts. In addition, new inoculum should be prepared only from inoculated leaves and within 7 days of inoculation. After homogenization of the infected tissue in buffer, the absence of wild-type revertants should be confirmed through RT-PCR. Pooling of inoculated leaves will result in contamination of all the inoculum if reversion has occurred in one leaf; thus, it is wise to prepare separate passage inocula from individual leaves.

Anticipated Results

The levels of overexpression or VIGS achieved with PVX-based vectors are not predictable and will vary from gene to gene. Previously, accumulation of a foreign protein up to 1.5% of total soluble protein has been achieved. However, with other foreign proteins, much lower levels have been found; in part this is probably a reflection of the in planta stability of these proteins. As a production platform, vectors based on PVX are probably not the best choice, but they have proven themselves for high-throughput functional screening. In previous studies involving VIGS of potato *pds* genes, normalized mRNA levels have been lowered by 70% to 85% in the leaves of glasshouse-grown plants, resulting in 5- to 10-fold increases in phytoene levels. Likewise, normalized mRNA levels have been lowered by about 70% in the leaves of in vitro–grown potato plants, resulting in 3- to 8-fold increases in phytoene levels and up to 20-fold increases in phytoene levels in microtubers. As before, the PVX-based vectors have proven themselves valuable for high-throughput VIGS screening.

Time Considerations

Once plasmid DNAs or transformed *Agrobacterium* have been obtained, the inoculation procedures described here take a few hours if only a few different constructs are being tested. Obviously, the time taken will increase if library screening is undertaken. A major component of the time taken is required for in planta virus propagation. This can range from less than 1 week for overexpression studies on inoculated leaves to up to 2 months to achieve VIGS in microtubers. The time required for analysis will depend on the gene of interest and the method of analysis. Immunoblotting of extracted proteins in protein overexpression studies and real-time RT-PCR in VIGS studies can be expected to take 2 to 3 days.

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Internet Resources

http://www.ayeaye.tsl.ac.uk

Provides a source to obtain PVX-based vectors. Also gives sequence information for vectors and methods for their use.

http://www.pgreen.ac.uk/JIT/pSoup.htm

Provide a source to obtain pSoup helper plasmid. Also gives sequence information for pSoup- and pGREEN-derived vectors.

Using Vectors Derived from Tomato Bushy Stunt Virus (TBSV) and TBSV Defective Interfering RNAs (DIs)

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ABSTRACT

This unit describes principles and protocols for expressing a gene of interest in plant cells using gene vectors that are derived from an infectious full-length cDNA plasmid of the tomato bushy stunt virus (TBSV) genomic RNA, and from defective interfering RNAs (DIs). The TBSV gene vector system permits convenient cloning, allows modification and abundant expression of the gene of interest, and facilitates biosecure containment of the gene vectors. These vectors can be employed for functional genomics studies and for analyzing the biochemical properties and subcellular distribution of expressed RNAs and/or their cognate proteins. As with other plant virus gene vectors, recombination and deletion of the gene of interest during virus multiplication limits the application of the TBSV gene vectors to the inoculated cells or leaves. *Curr. Protoc. Microbiol.* 7:16I.4.1-16I.4.16. © 2007 by John Wiley & Sons, Inc.

Keywords: tomato bushy stunt virus • defective interfering RNAs • gene vectors • gene of interest

INTRODUCTION

At the present time, expression of foreign genes in plants is routinely achieved in many laboratories through *Agrobacterium*-mediated transformation or biolistic delivery followed by the regeneration of transgenic plants. This approach results in a stable, genetically inherited trait. As an alternative methodology, transient expression techniques are also being used. For this purpose, *Agrobacterium*-mediated agroinfiltration or biolistic delivery through (hand-held) gene guns (*UNIT 16B.3*) can be applied to achieve high levels of temporary gene expression at local sites. These methods are expedient and do not need to await the time-consuming process of selecting transgenic lines.

As reviewed in detail in the literature (Pogue et al., 2002; Scholthof et al., 2002), in order to conveniently obtain expression of foreign genes in a relatively short time span, plant viruses have also been explored as gene vectors. Advantages of virus-mediated gene delivery include high levels of replication and concurrent expression of foreign genes, and the repeatable application of the same gene vector for inoculation on multiple susceptible plants without the necessity for genetic transformation of each host species. A number of plant viruses have been investigated for their utility as gene vectors (as described in the other units of Section 16I), and each has specific advantages and disadvantages. Some of these can be predicted, but the success often depends on the virus-host combination, and, more importantly, on the type and size of the insert, because these factors substantially influence the stability and expression of foreign genes in the recombinant virus. In this unit, we will describe methods of utilizing gene vectors derived from tomato bushy stunt virus (TBSV) and its defective interfering RNAs (DIs).

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TBSV is the type member of the *Tombusvirus* genus in the Tombusviridae, with a very broad host range. The TBSV genome consists of a single-stranded, plus-sense RNA (4.8 kb) that essentially functions as an mRNA for translation of the two replication-associated proteins, P33 and P92 (Fig. 16I.4.1). The capsid protein (CP) is translated from *p41* on subgenomic messenger RNA1 (sgRNA1), and not only is required for encapsidation, but also plays an active role in virus transport (Desvoyes and Scholthof, 2002; Qu and Morris, 2002). TBSV encodes two additional proteins, P22 and P19, that are expressed via a second subgenomic mRNA (sgRNA2) which encodes two nested open reading frames (ORFs) for translation of the *p22* and *p19* genes (Figs. 16I.4.1 and 16I.4.2). P22 is required for cell-to-cell movement, whereas P19 is involved in host-specific systemic invasion and acts as a suppressor of post-transcriptional gene silencing (Desvoyes et al., 2002; Qiu et al., 2002; Turina et al., 2003). An intriguing and exploitable property of TBSV is that, during infection of laboratory hosts, it can generate high levels of DIs that are coreplicating deletion mutants of ~400 nucleotides (White and Morris, 1999). DIs are composed of four contiguous regions (I, II, III, and IV) derived from the helper TBSV genome. The abundant proliferation of DIs interferes with the accumulation of the genomic and subgenomic RNAs, which generally results in attenuated symptoms on infected plants.

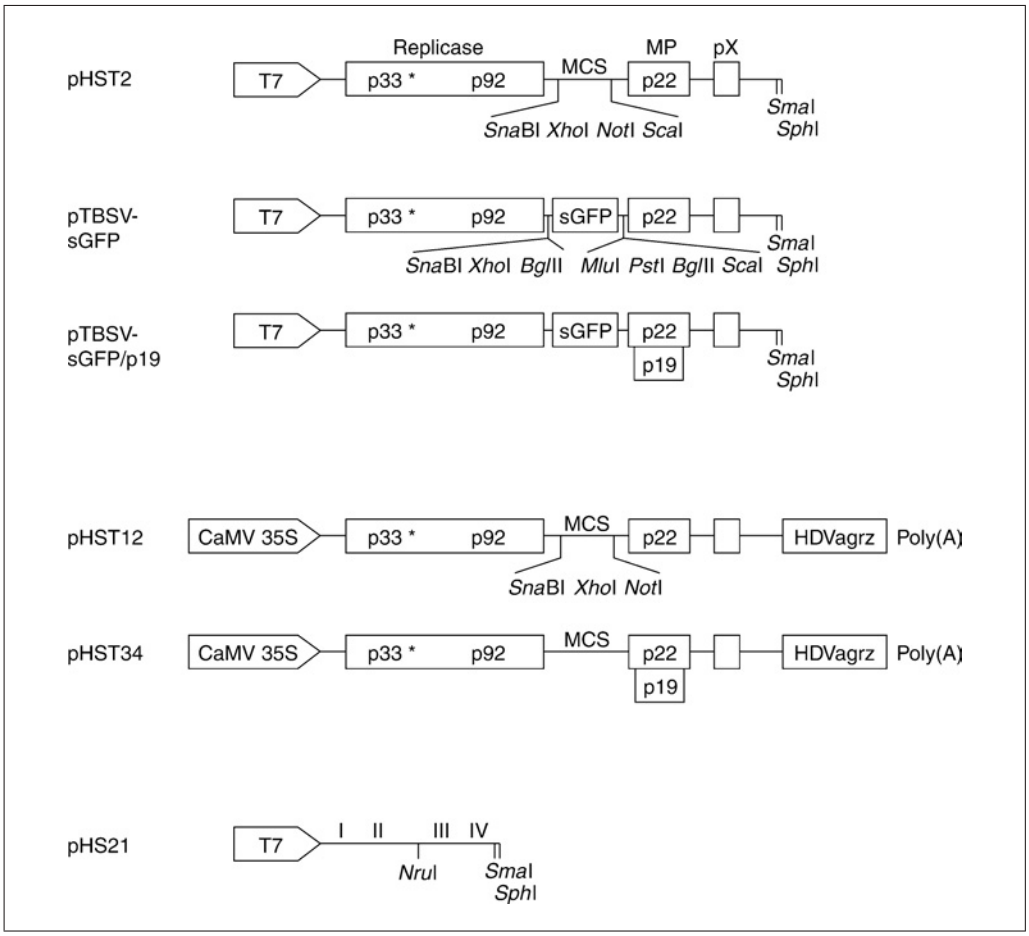

Figure 16I.4.1 TBSV-based gene vectors. Schematic diagram of TBSV RNA- and DNA-based vectors to illustrate the viral genome and the composition of defective interfering RNAs (DIs). The function of each protein (numbers represent molecular mass in kDa) is listed on top of the diagram. Rectangles denote coding regions of the TBSV genome, and lines represent untranslated sequences. The four regions (I, II, III, and IV) of DIs are presented on top of the diagram for the plasmid pHS21. Unique restriction sites for cloning purposes are listed below each diagram. Abbreviations: MSC, multiple cloning sites; MP, movement protein; sGFP, synthetic green fluorescent protein; HDVagrz, hepatitis delta virus antigenomic ribozyme; CaMV 35S, the cauliflower mosaic virus 35S promoter; T7, the DNA-dependent RNA polymerase promoter of the T7 bacteriophage.

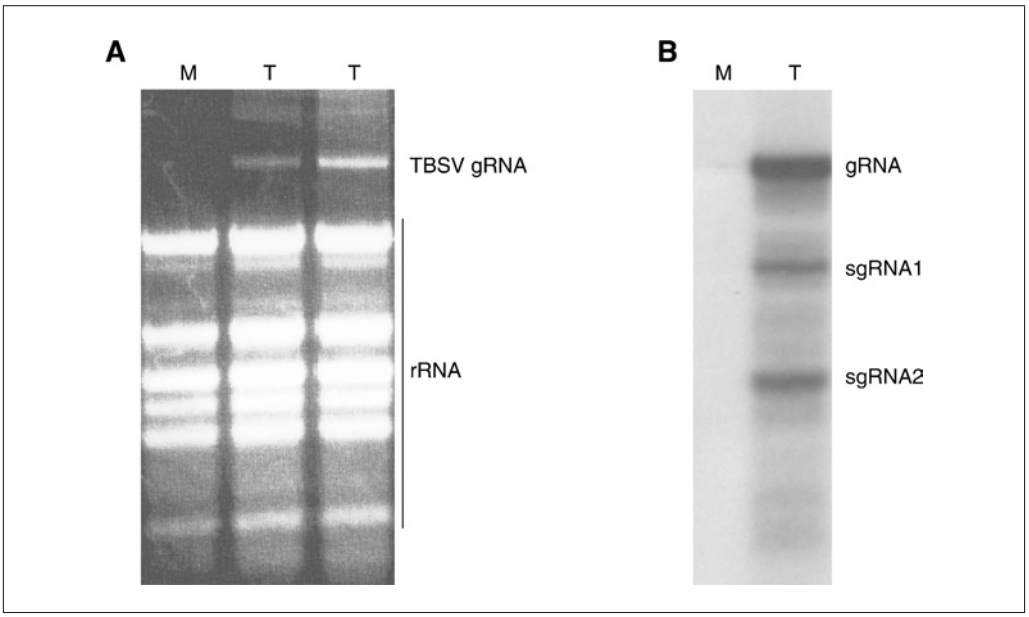

Figure 16I.4.2 Levels of TBSV RNA accumulation. (**A**) An agarose gel image is shown to illustrate that TBSV genomic RNA accumulates abundantly and can be readily visualized upon ethidium bromide staining. (**B**) The identity of the single-stranded genomic (gRNA) and subgenomic RNAs (sgRNA1 and sgRNA2) can be confirmed by hybridization with TBSV-specific probes. These particular results were obtained with wild-type TBSV viral RNAs that accumulate in infected *Nicotiana benthamiana* protoplasts. Abbreviations: M, mock-inoculated; T, TBSV-infected. The double-stranded genomic RNA is visible above the gRNA in the lanes labeled T on the ethidium bromide-stained gel.

The concept of using TBSV as a gene vector for expressing foreign genes was first demonstrated by replacement of a large portion of the TBSV CP gene with reporter genes (Scholthof et al., 1993). This application was based on a discovery that the TBSV CP is not required to establish an infection of certain *Nicotiana* species (Scholthof et al., 1993; Russo et al., 1994); this has since also been found to be the case for other species (Table 16I.4.1). In the prototype version of the TBSV gene vector, the foreign gene was inserted as an in-frame fusion with the 5′ end of the CP gene. This resulted in high levels of expression due to the efficient initiation of translation from the native CP start codon. However, the absence of multiple cloning (restriction enzyme) sites (MCS) precluded convenient cloning of foreign genes of choice. For this purpose, a new version of the TBSV gene vector was engineered in which an MCS was incorporated to substitute the major part of the CP gene, and the native start codon of the CP gene was inactivated (Fig. 16I.4.1). This strategy permits translational initiation from the endogenous start codon of any inserted foreign gene (Scholthof et al., 1996).

The plasmid of the new TBSV gene vector (referred to as pHST2) contains a 4103-bp TBSV-derived cDNA (Fig. 16I.4.1). Infection of plants with this gene vector is initiated by inoculating T7 polymerase–generated RNA that is transcribed from linearized pHST2 DNA templates. Thus, pHST2 represents an RNA-based TBSV gene vector. To provide an alternative T7 polymerase–independent method of infecting plants with TBSV gene vectors, a DNA-based gene vector was developed. In this vector, the TBSV cDNA backbone of pHST2 is positioned downstream of the universally applied cauliflower mosaic virus (CaMV) 35S promoter and upstream of the hepatitis deltavirus antigenomic ribozyme and the nopaline synthase poly(A) signal (Scholthof, 1999). The DNA-based TBSV gene vector is referred to as pHST12 (Fig. 16I.4.1). Direct rub-inoculation of the recombinant pHST12 plasmid DNA results in an infection that also sustains expression of the foreign gene in inoculated leaves (Scholthof, 1999). For expression of small

Table 16I.4.1 Examples of Using TBSV Gene Vectors to Express Foreign Genes[a]

Foreign genes	Cloning strategy	Host plants infected	References
CaMV gene II	Replacement of CP	*N. tabacum* protoplasts	Blanc et al. (1996)
CAT	In-frame fusion to CP N-terminal and remainder of CP replaced	*N. benthamiana* *N. clevelandii*	Scholthof et al. (1993)
GFP	Replacement of CP	*N. benthamiana*	Qiu et al. (2002)
GUS[b]	In-frame fusion to CP N-terminal and remainder of CP replaced.	*Nicotiana benthamiana* *Chenopodium amaranticolor* *N. clevelandii* *Vigna unguiculata* *C. quinoa* *Spinacia oleracea*	Scholthof et al. (1993) Scholthof (1999) Zhang et al. (2000)
HIV-1 gp120 V3 loop	In-frame fusion to the C-terminal of CP	*N. benthamiana*	Joelson et al. (1997)
HIV-1 p24	In-frame fusion to N-terminal of CP	*N. benthamiana*	Zhang et al. (2000)
PMV CP[b]	Replacement of CP	*C. quinoa*	Scholthof (1999)
RCNMV RNA-2	Replacement of CP	*N. benthamiana*	Sit et al. (1998)
sGFP	Replacement of CP	*N. benthamiana*	Sit et al. (1998)
SPMV CP	Replacement of CP	*N. benthamiana*	Qiu and Scholthof (2004)
TEV P1	Replacement of CP	*N. benthamiana*	Hou and Qiu (2003)

[a]Abbreviations: CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; GUS, β-glucuronidase; RCNMV, red clover necrotic mosaic virus; PMV, Panicum mosaic virus; sGFP, synthetic GFP; SPMV, satellite panicum mosaic virus; TEV, tobacco etch virus; HIV, human immunodeficiency virus.
[b]These foreign genes were cloned in both RNA- and DNA-based TBSV gene vectors.

proteins, TBSV has also been used as an epitope-presentation vector whereby peptides of interest decorate the surface of the particles (Joelson et al., 1997; Zhang et al., 2000). The examples in Table 16I.4.1 illustrate various applications of the TBSV-derived gene vectors.

This unit focuses on strategies employing RNA- and DNA-based TBSV gene vectors to express a gene of interest in plants. The protocols below represent the general procedures for expressing a gene of interest from TBSV gene vector pHST2 or pHST12 in *Nicotiana benthamiana*, a model plant species for testing the infectivity of plant viruses and for expression of foreign genes. Figure 16I.4.1 illustrates the main important features of these gene vectors. The Basic Protocol details the application of the RNA-based gene vector system, starting with the purification of pHST2 plasmid DNA and going on to describe strategies for cloning the gene of interest into the pHST2 or its derived plasmids, generating RNA transcripts in vitro, and inoculation of these transcripts onto *N. benthamiana* plants. The Strategic Planning section introduces two assays for detecting the presence of the gene of interest–encoded RNA and/or protein in the infected plants. Alternate Protocol 1 briefly describes the additional unique features associated with the DNA-based gene vector system, and Alternate Protocol 2 introduces a method of cloning a gene-of-interest fragment into a DI gene vector (pHS21) for RNA interference (RNAi) assays.

STRATEGIC PLANNING

Protoplasts as Alternative Expression Systems

In addition to whole plants, RNA transcripts can also be inoculated directly into protoplasts for expression of a foreign gene (Fig. 16I.4.3). For general-application protocols to prepare protoplasts from host plants and for inoculating protoplasts with RNA transcripts, we refer to Section 16D (particularly *UNIT 16D.4*). However, it is worthwhile to include a few salient notes for transfecting *N. benthamiana* protoplasts with TBSV RNA transcripts: (1) use $\sim 4 \times 10^5$ protoplasts for transfection; (2) treat RNA transcripts with RNase-free DNase after the completion of in vitro transcription reaction; (3) use a polyethylene glycol (PEG, mol. wt. 1540)–mediated transfection procedure (40% PEG, pH 5.6, 3 mM $CaCl_2$); (4) harvest protoplasts at 24 hr after inoculation and extract total RNAs using protoplast RNA extraction buffer (see recipe in Reagents and Solutions), followed by standard phenol/chloroform extraction and ethanol precipitation. Users can also refer to *UNIT 16E.2* for extracting viral RNA from protoplasts.

When preparing the PEG solution, no chemicals should remain insoluble (giving a slight cloudy appearance). It is best to autoclave PEG and a stock of $CaCl_2$ separately prior to preparation of the final solution.

Alternative Hosts

This unit mostly focuses on the use of *N. benthamiana* plants, but an apparent advantage of the system relates to the broad host range of TBSV. The virus will establish infections upon rub-inoculation of transcripts onto a variety of plants (see Table 16I.4.1 for examples). Best results are obtained when using young plants with three or four fully

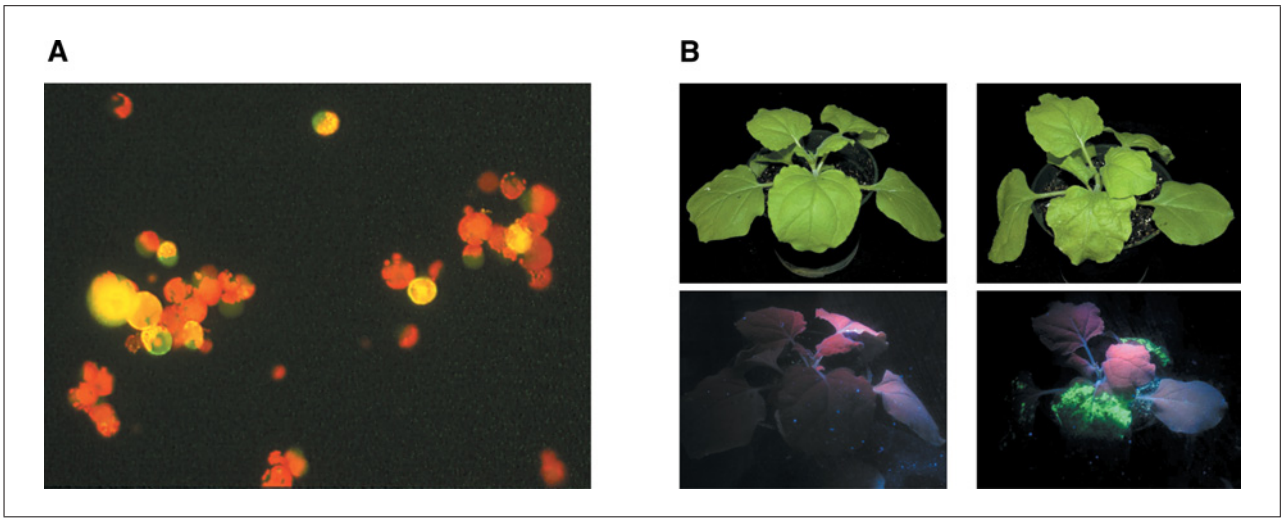

Figure 16I.4.3 TBSV-mediated expression of green fluorescent protein (GFP) from a TBSV vector reminiscent of pHST2 (construct kindly provided by Teresa Rubio and Andy Jackson). (**A**) Protoplasts prepared from leaf tissues of *Nicotiana benthamiana* transfected with a TBSV vector expressing GFP. The protoplasts shown in the image were illuminated with blue light. The red-colored cells (appearing as dark in the black and white reproduction) represent uninfected protoplasts containing autofluorescent chloroplasts. The green and yellow color (green superimposed on red; gray in the black and white version) that is due to GFP expression can be observed in some of the protoplasts. The image serves as an example to illustrate that protoplasts can be infected with RNA transcripts of TBSV gene vectors for transient expression of a foreign gene and for inspecting infectivity of a recombinant TBSV virus. (**B**) *N. benthamiana* leaves 5 days post-inoculation with TBSV expressing GFP (right), compared to mock-inoculated leaves (left). The bright-field images (top) show the slight chlorotic symptoms typical upon inoculation of this host with TBSV vectors; the UV-illuminated panels (bottom) show the GFP fluorescence. Images courtesy of Yi-Cheng Hsieh. For the color version of this figure go to *http://www.currentprotocols.com*.

expanded leaves after growing under conditions of 14 hr light at 24°C/10 hr dark at 20°C, light intensity: 120 μmol $m^{-2}sec^{-1}$, and often it is advisable to use plants that have been placed in the dark for 1 to 2 days prior to inoculation. Two leaves are inoculated with transcripts for each plant. However, whether a vector containing a specific gene of interest will be infectious and yield high levels of expression in a given plant species cannot be predicted, and will have to be empirically determined on a case-by-case basis.

Monitor the Expression of the Gene of Interest

Appearance of symptoms

Depending on the host, chlorotic or necrotic lesions or areas are expected to appear on the inoculated leaves at 4 to 7 days post inoculation with RNA transcripts. These symptoms are an indication that the recombinant TBSV has successfully established an infection (Fig. 16I.4.3B).

RNA analyses

Extract total RNA from infected leaf tissues and perform RNA gel blot assays essentially as described in *UNIT 16E.1* and *UNIT 16E.3*. However, some important points deserve attention. Total nucleic acid can be isolated and used for standard 1% agarose gel electrophoresis in TBE buffer. There is no need to use denaturing gels if the intention is to detect the presence of single-stranded TBSV viral genomic and subgenomic RNAs. The quantity of viral RNAs is generally comparable to that of ribosomal RNAs, and the double-stranded and single-stranded TBSV RNAs can often be directly observed upon ethidium bromide staining (Fig. 16I.4.3).

For RNA hybridization, TBSV-specific probes can be used to detect the presence of TBSV genomic RNA and subgenomic RNAs. For this purpose, it is recommended that a probe be selected that is derived from a 3′ region present in equimolar amounts on genomic and subgenomic RNAs. A suitable region is the sequence from *Nco*I to *Sal*I (nt 3886-4500) and the sequence for primers can be obtained from the TBSV accession (GenBank: NC001554). Similarly, the gene of interest–specific probes can be derived from the original gene of interest plasmid or from PCR products containing the gene of interest. Quantitative real-time reverse transcription PCR can also be used to monitor the RNA level of the gene of interest.

Detection and Purification of Gene of Interest-Encoded Protein

Expression of reporter genes (CAT, GUS, GFP) can be monitored following standard procedures (Scholthof et al., 1993; Qiu et al., 2002). If antibodies to the gene of interest protein are available, immunoblot assays can be employed to monitor the expression level of the gene-of-interest protein. For this purpose, extract total proteins and analyze them by employing standard techniques for SDS-PAGE (Gallagher, 2006) and immunodetection (i.e., western blot analysis; Gallagher et al., 2004). Fusion of the gene of interest to a FLAG epitope tag (see Kimple and Sondek, 2004) will allow the use of corresponding commercially available antibodies for indirect detection of the gene of interest protein.

To enable the application of standard purification protocols, it is advisable to fuse a His-tag to the gene of interest protein. Protein affinity chromatography purification protocols can then be applied to isolate the protein (see Kimple and Sondek, 2004). For example, the authors have used this methodology to purify TBSV-expressed His-tagged GFP from infected plants (W. Qiu and H.B. Scholthof, unpub. observ.). Extraction buffers and other reagents are the same as those prescribed by the suppliers of chromatography materials (e.g., GE Healthcare), with the only variation being that the plant material is pulverized in a mortar and pestle or in a blender, which is followed by a low speed-centrifugation to remove insoluble debris.

CLONING INTO THE EXPRESSION PLASMIDS

BASIC PROTOCOL

This section describes a basic protocol routinely used in the laboratory of the authors for TBSV-derived gene vectors. The protocol includes conventional cloning of the gene of interest into the TBSV gene vector, generating DNA templates for in vitro transcription, synthesizing transcripts, inoculation of transcripts into plants, and detection of virus infection and gene of interest-derived RNA or protein.

Materials

TBSV gene vector (pHST2) DNA (can be obtained from the authors at *http://scholthoflab.tamu.edu*)
E. coli DH5α competent cells
LB medium containing 50 to 100 μg/ml ampicillin (*APPENDIX 4A*)
Column-based plasmid purification kit (e.g., QIAprep Spin Miniprep kit; Qiagen)
Gene-of-interest DNA fragment
Restriction endonucleases *Xho*I, *Sac*I, and *Sna*BI (and other restriction enzymes as needed) and corresponding buffers
Forward and reverse PCR primers for gene of interest (see Kramer and Coen, 2001)
*Pfu*UltraII Fusion HS DNA polymerase (Stratagene, cat. no. 600670; optional)
Klenow fragment of DNA polymerase I
Plasmid harboring Kan^r gene (e.g., pKAN-2 or Gateway pENTR)
1% agarose gel prepared in TBE buffer (Voytas, 2000)
TE buffer (*APPENDIX 2A*)
50 U/μl T7 RNA polymerase (Invitrogen, cat. no. 18033-19) and 5× T7 buffer
100 mM dithiothreitol (DTT)
10 mM NTP mix (Invitrogen, cat. no. 18109-017)
RNAsin ribonuclease inhibitor (Promega)
RNA inoculation buffer (see recipe)
Nicotiana benthamiana plants
Climate-controlled growth chambers (Conviron; *http://www.conviron.com*)

Additional reagents and equipment for transformation of *E. coli* (Seidman et al., 1997), plasmid DNA isolation (Engebrecht et al., 1991), polymerase chain reaction (PCR; Kramer and Coen, 2001), insertion of DNA fragments into plasmids (Struhl, 1991), use of Klenow fragment of DNA polymerase I to generate blunt ends (Tabor et al., 1997), phenol/chloroform extraction and ethanol precipitation of DNA (Moore and Dowhan, 2002), agarose gel electrophoresis (Voytas, 2000), spectrophotometric quantitation of nucleic acids (Gallagher and Desjardins, 2006), and inoculation of RNA transcripts into whole plants (*UNIT 16B.1* in this manual)

Amplify and purify the plasmid

1. Amplify pHST2 DNA as a regular plasmid in *E. coli* DH5α competent cells cultured in LB medium containing 50 to 100 μg/ml ampicillin overnight.

   Heat shock (Seidman et al., 1997) is the preferred method of transformation.

2. Purify plasmid DNA by a conventional plasmid preparation method (Engebrecht et al., 1991) or using a column-based plasmid purification kit (such as QIAprep Spin Miniprep kit).

   The TBSV gene vector plasmids described in this unit contain the Amp^r gene as the selection marker.

Clone gene of interest into plasmid

Steps 4a-c to 7a-c, below, describe three methods of cloning the gene of interest into pHST2. As an alternative to pHST2, plasmids pTBSV-sGFP and pTBSV-sGFP/p19, which were developed by Sit et al. (1998) and W. Qiu (unpub. observ.), respectively, can also be used for cloning purposes. pTBSV-sGFP is derived from pHST2 and contains

a modified version (sGFP) of the green fluorescent protein (GFP) gene in place of the CP gene. The pTBSV-sGFP/p19 construct is similar, but with the added capability of expressing P19. Both plasmids have additional *Bgl*II, *Mlu*I, and *Pst*I sites to increase cloning flexibility (Fig. 16I.4.1). The sGFP gene can simply be substituted with the gene of interest, and the resultant recombinant plasmid is essentially the same as the one made by inserting the gene of interest fragment into the pHST2 plasmid.

3. Design a strategy for cloning the gene of interest into the pHST2 plasmid based on the restriction sites flanking the gene of interest (either derived from another plasmid or as a PCR product) and the available MCS of pHST2 (Fig. 16I.4.1; also see Ausubel et al., 2007).

   To permit detection of protein expression (Palmer and Wingfield, 2004) it might be practical to fuse a tag sequence to the open reading frame of the gene of interest prior to insertion into the TBSV vectors.

   For restriction enzyme digestion of plasmids, gel purification of DNA fragments containing the gene of interest, ligation of the gene of interest insert into pHST2, transformation of E. coli, screening of recombinant plasmids, and other routine cloning methods, see relevant units in Current Protocols in Molecular Biology (Ausubel et al., 2007).

   IMPORTANT NOTE: *A condition for using the pHST2 gene vector is that the gene-of-interest DNA fragment not contain either an SmaI or a SphI site. Those sites are located at the 3′ end of the TBSV cDNA (Fig. 16I.4.1), and one of those will be used to linearize the plasmid to prepare a DNA template for the in vitro transcription reaction (step 13).*

For directional cloning

4a. Provided that the gene of interest does not contain internal *Xho*I and *Sac*I sites, prepare a gene-of-interest DNA fragment with flanking *Xho*I and *Sac*I sites from a separate plasmid containing the gene of interest by releasing the gene-of-interest fragment with the digestion of restriction enzymes *Xho*I and *Sac*I.

5a. Alternatively, add *Xho*I and *Sac*I restriction sites to the gene of interest–specific forward and reverse primers, respectively. Perform PCR (Kramer and Coen, 2001).

6a. Digest the gene-of-interest PCR products with the restriction enzymes *Xho*I and *Sac*I.

7a. Insert the resulting DNA fragment directionally between the *Xho*I and *Sac*I sites of pHST2 (Struhl, 1991).

For blunt-end cloning

Alternatively, a gene of interest–derived PCR product or restriction enzyme fragment can be treated with Klenow fragment of DNA polymerase I to generate blunt ends that can then directly be cloned into the *Sna*BI site of the pHST2 plasmid.

4b. Obtain blunt-end gene-of-interest DNA fragments using one of the following strategies.

   i. PCR amplification (Kramer and Coen, 2001) with a polymerase (e.g., the high-fidelity *Pfu*Ultra II Fusion HS DNA polymerase) that does not generate single nucleotide overhangs.
   ii. Treatment of cohesive ends or overhangs at the termini of the gene-of-interest DNA fragments with DNA polymerase Klenow fragment (utilizing either the 3′-5′ exonuclease or 5′-3′ polymerase activity; Tabor et al., 1997).
   iii. By designing blunt-end restriction enzyme sites on both ends of the gene-of-interest DNA fragment.

5b. Digest pHST2 with *Sna*BI to obtain a linearized plasmid with blunt ends.

6b. Insert the blunt-end gene-of-interest DNA fragment into the plasmid (Struhl, 1991).

7b. Check the orientation of the gene of interest fragment after cloning by using an internal unique restriction enzyme site and the unique *Sma*I or *Sph*I site located 3′ distal to the location of the insertion (Fig. 16I.4.1).

Alternative cloning method to avoid purification of DNA fragments from agarose gels

4c. First, clone the gene of interest into an intermediate plasmid harboring the Kan^r gene (Struhl, 1991)—e.g., pUC-derived Kan^r vectors such as pKAN-2 that was used in a previous study (Qiu and Scholthof, 2004) or the Gateway pENTR vectors that allow directional cloning of PCR products into a Kan$^\gamma$ background.

5c. Subsequently, digest the intermediate pKAN-gene of interest and pHST2 using the same restriction enzymes (such as *Xho*I and *Sac*I).

6c. Perform phenol/chloroform extraction and ethanol precipitation of the products (Moore and Dowhan, 2002).

7c. Mix the DNA fragments and perform a ligation reaction (Struhl, 1991).

8. Transform *E. coli*, then select transformed bacterial colonies on ampicillin-containing medium (Seidman et al., 1997).

Prepare templates for in vitro transcription

9. Purify plasmid DNA by a conventional plasmid preparation method (Engebrecht et al., 1991) or using a column-based plasmid purification kit (such as QIAprep Spin Miniprep kit).

Since plasmid DNA isolated in this step will be used as templates for synthesis of RNA transcripts, avoid adding RNase in any step of the purification procedure. If RNase is a component of any solution in a commercial plasmid purification kit, it is advisable to prepare a substitute solution, without addition of RNase, for the purification. Alternatively, the purified plasmids can be subjected to standard phenol/chloroform extraction followed by ethanol precipitation to remove RNase. To avoid any of this, a conventional method involving cesium chloride equilibrium density gradient centrifugation is a time-consuming, but in fact preferred, method to purify DNA to be used as a template for the in vitro transcription reaction. Nevertheless, it remains advisable to routinely extract purified plasmid DNA with phenol/chloroform. This extraction is also commonly included in this step as an enhanced security measure to preclude any RNase contamination.

Linearize, purify, and quantify plasmid

10. Digest DNA (20 μg is regularly used in laboratories of the authors) with either *Sma*I or *Sph*I and verify the digestion by electrophoresis of a small aliquot on a 1% agarose-TBE gel (Voytas, 2000).

When using SphI, it is advisable to treat the reaction with Klenow fragment of DNA polymerase (Tabor et al., 1997) to flush the 3′ overhang after the restriction enzyme digestion is complete.

11. Perform two phenol/chloroform extractions, then ethanol precipitate the linearized plasmids. Collect precipitated DNA by centrifugation, vacuum dry the preparation, and dissolve the DNA template in 20 μl of TE buffer.

The above procedures are described in Moore and Dowhan (2002).

12. Measure the concentration of DNA with a UV spectrophotometer (Gallagher and Desjardins, 2006) and adjust the DNA concentration to ~0.5 μg/μl with TE buffer.

To generate infectious RNA transcripts, plasmids must be linearized at the 3′end of the full-length TBSV cDNA. Complete linearization of the plasmids ensures the synthesis of RNA transcripts of full-length TBSV units. Furthermore, the highest RNA yields and best infections are obtained when the 3′ end of the linearized template is blunt ended. The transcripts do not need to be provided with a 5′ cap analog because they are infectious without capping.

Synthesize infectious RNAs in in vitro transcription reaction

13. Prepare the reaction mixture according to the following recipe:

    1 µl (0.5 µg) DNA template (from step 12)
    5 µl 5× T7 buffer
    1 µl 100 mM DTT
    1.5 µl 10 mM NTP mix
    0.5 µl RNasin
    0.5 µl 50 U/µl T7 RNA polymerase
    15.5 µl distilled H_2O
    Total volume, 25 µl.

    Incubate reaction 1 hr at 37°C.

14. Analyze RNA transcripts by loading 2 µl of the incubated reaction mixture from step 13 on a standard 1% agarose-TBE gel, performing electrophoresis, and evaluating the quantity and integrity of RNA transcripts by ethidium bromide staining (procedures described in Voytas, 2000).

    A single high-intensity fluorescent band of the expected size (~4 kb of empty gene vector plus size of insert) indicates high quality of RNA transcripts. A fainter band of higher size represents the input template DNA.

    For routine infections of protoplasts or plants with RNA transcripts, it is not necessary to remove the DNA templates or to quantify the RNA concentration. However, if a precise measure of RNA concentration is required, remove DNA templates by adding RNase-free DNase to the whole reaction and incubate at room temperature for 20 min. RNA transcripts can then be purified using the phenol/chloroform procedure or with spin columns, and the RNA concentration can be determined by UV spectrophotometry. When using the RNA transcripts to infect protoplasts, this is often followed by monitoring infections with RNA hybridization. In that case, DNA input templates can cause background hybridization signals that can be avoided by removing the templates with RNase-free DNase just prior to inoculation; no phenol/chloroform extraction is required for this application.

Inoculate RNA transcripts onto whole plants (also see UNIT 16B.1)

15. Mix RNA transcripts 1:5 with RNA inoculation buffer, and gently rub-inoculate the mixture onto *N. benthamiana* plants as described in UNIT 16B.1. Allow plants to recover 16 hr in the dark at room temperature with 100% humidity.

    The rub-inoculation with the mixture containing Celite will introduce microscopic wounds that serve as the entry points for the RNA transcripts present in the inoculum.

16. After the 16-hr recovery period, maintain the inoculated plants in a climate-controlled growth chamber with 14 hr daytime at 24°C, and 20°C night temperature.

    The 100% humidity during recovery improves infectivity of RNA transcripts, and this can simply be achieved by covering the RNA-inoculated plants with moist paper sheets. It is advisable to inoculate plants with transcripts from "empty" pHST2 plasmids to provide a negative control sample for subsequent analysis of the gene of interest RNA and/or protein expression. At the same time, inoculation of plants with these "empty" transcripts will serve as a positive control to verify that the transcription reaction yields infectious RNA transcripts. If RNA transcripts are not infectious on plants, it is recommended to test their ability to infect protoplasts (see Strategic Planning) to determine if the gene of interest insert has detrimental effects on replication and/or viral transcription.

ALTERNATE PROTOCOL 1

Using Vectors Derived from TBSV and TBSV DIs

DNA-BASED TBSV GENE VECTORS

To avoid the linearization of recombinant TBSV gene vector plasmids and to alleviate the necessity of generating RNA transcripts by the in vitro transcription reaction, a method has been adapted for directly delivering plasmid DNA of TBSV gene vectors into plants (Scholthof, 1999; Fig. 16I.4.1). In this DNA-based TBSV gene vector system, the gene of

interest is cloned into pHST12 or pHST34 as in the Basic Protocol. The pHST12 contains the same TBSV cDNA as pHST2, while pHST34 is a modified version of pHST12 with the added feature of expressing P19. Figure 16I.4.1 illustrates the main features of pHST12 and pHST34. After confirming that the gene of interest is correctly inserted into the vector, purify plasmid DNAs and directly rub-inoculate these onto leaves of host plants as in the Basic Protocol for transcript inoculation. Normally, 10 μg of plasmid DNA is sufficient for rub-inoculation of a Celite-dusted leaf. The subsequent phases of recovery, monitoring infectivity, and evaluating expression of the gene of interest, are the same as in Basic Protocol 1.

How the rub-inoculated DNA is transferred to the nucleus for in vivo transcription is not known, but it most likely involves a nonspecific translocation event in the damaged cell. Upon transfer into the nucleus, transcription occurs and the viral RNA is properly processed and transported out of the nucleus for translation in the cytoplasm. This is then followed by viral transcription and replication to establish an infection. The DNA-based gene vector can also be used in combination with agroinfiltration (see *UNIT 16B.2*), whereby *Agrobacterium* is used to transfer and integrate the viral DNA into the plant nucleus. Subsequent transcription is then followed by nuclear exit of the RNA into the cytoplasm to initiate an infection.

DI AS A GENE VECTOR FOR RNAi

ALTERNATE PROTOCOL 2

To use DI as a gene vector for RNA interference (RNAi), insert a small DNA fragment (normally <200 bp long) of an endogenous host gene into the *Nru*I site of the plasmid pHS21 (Fig. 16I.4.1). Linearize plasmid DNA of the chimeric DI construct with *Sma*I or *Sph*I as in the Basic Protocol. Mix RNA transcripts that are synthesized from the helper TBSV template and from the DI template at a 2:1 ratio of TBSV to DI and inoculate these into *N. benthamiana* plants or other hosts as in the Basic Protocol. Refer to Hou and Qiu (2003) and Qiu et al. (2002) for detailed technical instructions. Subsequently, measure the accumulation of chimeric DIs by RNA hybridization assays. Case-dependent experiments are needed to investigate the biological consequences and symptomatology resulting from DI-induced RNAi.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Protoplast RNA extraction buffer

Dissolve 1.92 g ammonium carbonate in 100 ml distilled water, adjust the solution to pH 9.0 using ammonium or potassium hydroxide, add 2 g SDS, 400 μl of 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*), and 0.02 g Bentonite (Fisher). Store up to 1 year at 4°C.

RNA inoculation buffer

0.05 M K_2HPO_4
0.05 M glycine
1% (w/v) Bentonite (Fisher)
1% Celite (J.T. Baker)
Adjust pH to 9.0 using potassium hydroxide
Divide into aliquots in 1.5-ml tubes
Store up to 1 year at −20°C

COMMENTARY

Background Information

TBSV is a model system for virology, and examples of its utility in plant virus research over the years are plentiful (Hull and Matthews, 2001). For example, TBSV was one of the first viruses for which the X-ray crystallographic structure was resolved to reveal the details of the quasi-equivalent T = 3 isometric particles. It is also one of the primary systems for studying replication and transcription of viral RNA, as well as a model system to study the biology of subviral DIs. In addition, TBSV provides an ideal tool to analyze the intercellular movement of viruses and the molecular mechanisms of virus-host interactions.

Because the genomic RNA of TBSV essentially functions as an mRNA for the two replicase proteins (P33 and P90), infectious RNAs can be obtained from a full-length cDNA clone of TBSV upon in vitro or in vivo transcription (see *UNIT 16F.3* for principles of constructing infectious full-length cDNA clones of RNA viruses). Furthermore, in vitro–generated RNA of DIs is also "infectious," provided that the helper virus is present (Knorr et al., 1991), and DNA-based constructs also have been shown to launch DI accumulation in the presence of helper virus (Rubio et al., 1999).

The original full-length TBSV cDNA clone was constructed from a cherry strain of TBSV (Hearne et al., 1990), and the backbone of the TBSV gene vector described in this unit is derived from that original material. The strategies for exploiting TBSV gene vectors discussed in this unit were developed based on the following three observations. First, T7 polymerase–generated RNA transcribed from the full-length TBSV cDNA clone was infectious (Hearne et al., 1990). Secondly, the CaMV 35S promoter-driven full-length constructs were also shown to be infectious upon rub-inoculation of the plasmid DNA (Scholthof, 1999). Thirdly, the CP gene of the full-length TBSV construct was shown to be dispensable for (at least local) infection of several plants (Scholthof et al., 1993). Thus, as described in this unit, we currently have TBSV-derived CP replacement gene vectors available that can be rub-inoculated as RNA transcripts, or directly as DNA, onto plants, resulting in expression of foreign genes.

RNA- and DNA-based TBSV gene vector systems have provided convenient molecular tools for transient expression of foreign genes in plants. Examples and references are listed in Table 16I.4.1. To briefly summarize a few, TBSV gene vector-mediated expression of reporter genes has been very useful as a sensitive method to track virus movement during infection. TBSV-mediated expression of the CaMV gene II protein was used to determine the colocalization of this protein with cytoskeletal elements. The TBSV gene vector was also effective in verifying that specific RNA elements on RNA2 of a dianthovirus had *trans*-activation properties for transcription from RNA1. TBSV has also been used in displaying antigenic peptides on the surface of the TBSV particle by fusing the encoding region of a gene with N- or C-terminal portions of the CP (Table 16I.4.1).

In addition, the TBSV gene vector system also includes the utilization of the DIs as a bonus tool for delivering small RNA fragments into a plant cell. For example, insertion of small fragments between regions II and III of an infectious prototype TBSV DI construct pHS21 resulted in chimeric DI molecules that were amplified by the helper TBSV (Qiu et al., 2001). More recent experiments showed that DI-based gene vectors serve as a powerful tool to induce post-transcriptional gene silencing (Qiu et al., 2002; Hou and Qiu, 2003), providing evidence that a subviral derivative of a plant virus is capable of efficiently activating RNAi.

Considering the demonstrated utility of RNA- or DNA-based TBSV gene vectors, a number of applications can be envisioned. For example, cellular distribution of a host or pathogen protein that is overexpressed using TBSV can be investigated by EM or by fluorescence, light, or confocal microscopy using immunolabeling or by fusing the genes to GFP. Moreover, the TBSV gene vector system and DIs form a novel and potent system for RNAi assays and for investigating the suppressive activity of a protein on gene silencing (Hou and Qiu, 2003). Another potential utility of the TBSV gene vector system is that an entire cDNA or expressed sequence tag (EST) library of a specific plant tissue can be transferred to the TBSV (or DI) gene vector system. Subsequently, the putative function of each cDNA can be annotated or inferred by the appearance of deviated phenotypes as a result of gene silencing–mediated disturbance of normal physiological processes.

The TBSV gene vector system provides the following advantageous features: (1) it permits rapid cloning and analyses of the expressed product in plants; (2) it provides a system for

genetically modifying a foreign gene through site-directed mutagenesis to study the structure and function of the foreign protein; (3) it has a broad host range, allowing investigation of host-dependent biological phenomena (e.g., see Table 16I.4.1 for a subset of these); and (4) it does not have a biological vector that is known to transmit TBSV, readily facilitatating biosecure containment of the TBSV gene vectors.

These advantages have to be considered within the context of the disadvantage that TBSV displays a notorious propensity for recombination. Although this does not have to preclude high levels of foreign gene expression in inoculated leaves (see Critical Parameters, discussion of local versus systemic infection), most systemic infections are characterized by the presence of TBSV RNA from which large portions of the foreign RNA may have been removed, thus diminishing the expression of foreign genes in systemically infected tissue.

Critical Parameters

Cloning strategy

There are several options for cloning a gene of interest into the MCS of TBSV gene-vector constructs (Fig. 16I.4.1). Transferring the gene of interest from the recombinant plasmid directly into the TBSV gene vector is preferred because this method ensures the true-to-origin sequences of the gene of interest. If restriction sites are incompatible or unavailable, cloning gene-of-interest PCR products amplified by a high-fidelity DNA polymerase into the TBSV gene vector is an option. To further improve general utility of the TBSV gene vector system, TBSV gene vector can be converted into a Gateway-compatible destination vector with the Gateway Vector Conversion System (Invitrogen, cat. no, 11828-029). In this novel cloning system, the gene of interest can first be cloned into pENTR/TOPO plasmid, and then transferred to the TBSV gene vector by the convenient recombination reaction. Refer to *http://www.invitrogen.com* for details about the Gateway technology

Another factor to be considered is the size of the gene of interest. Smaller-sized inserts of <1 kb are generally preferred, but secondary-structure features of the inserted RNA are also influential, yet thus far mostly unpredictable. These structural properties are important within the context of gene-vector stability in plants.

RNase-free laboratory materials and reagents

The RNA-based TBSV gene vectors require generation of RNA transcripts for inoculation. Therefore, avoiding contamination with RNase in each step is critical for successfully establishing a TBSV infection in plants. In addition, RNA gel-blot assays also dictate the use of RNase-free solutions and a clean laboratory environment.

Selection of host plants

TBSV is able to infect a broad spectrum of plant species but expression of foreign genes from engineered TBSV gene vectors has thus far been mostly investigated with *Nicotiana* species. However, promising incidental results have also been obtained for instance with spinach, *Chenopodium* sp., pepper, and soybean. Some plants, such as cowpea, are very suitable for achieving high levels of local infections (i.e., local lesions). Cowpea represents a particularly sensitive species that is very useful for verifying infectivity of TBSV gene-vector constructs. The bottom line is that the purpose of the study dictates the types of host plants that should be chosen for inoculation.

Local versus systemic infection

Instability and potential mutation of foreign inserts is an innate "virus-defense" property that appears to be associated with the majority of plant virus gene vectors (Scholthof et al., 1996). Likewise, the TBSV gene vector pHST2 expresses high levels of foreign proteins in the inoculated leaves, but fails to maintain sustainable levels of expression of those inserts in the upper, noninoculated leaves (Scholthof et al., 1996). Thus, TBSV gene vectors are best employed as a delivery system for transient expression of a foreign gene in inoculated leaves. If the objective entails high levels of systemic infection, systems other than TBSV-derived gene vectors may be preferred.

Beneficial role of the suppressor

TBSV-mediated foreign gene expression can be achieved in absence of P19 (Scholthof, 1999). However, due to the role of P19 in assisting virus spread, expanding host range (Chu et al., 2000; Turina et al., 2003), and delaying the onset of virus-induced gene silencing (Qiu et al., 2002), it may often be beneficial to use vectors such as pTBSV-sGFP/p19 or pHST34 that express P19 (Fig. 16I.4.1). On

the other hand, users should be cautioned that P19 is an elicitor of severe symptoms, and the presence of P19 can complicate the interpretation of the functional analysis of a protein. Therefore, it is advisable to perform the analyses both in presence and absence of P19. However, when the TBSV system is used to induce RNAi, one may want to avoid expression of P19, which is a suppressor of RNAi.

Troubleshooting

If various cloning attempts fail to yield TBSV constructs with inserts, it is likely that the insert has negative effects on proper multiplication of plasmids in bacteria (the authors have experienced unexplainable recalcitrance of certain inserts with respect to cloning into the pHST2 plasmid). In that case, cloning of subfragments may be an option, but only if the purpose is to examine RNA-mediated effects.

To troubleshoot a failure in obtaining little or no in vitro–generated RNA transcripts, users may refer to *UNIT 16F.3*. Transcripts of pHST2 containing the gene of interest may fail to infect plants while the transcripts of the control pHST2 RNA are infectious. A most likely cause is that the insert disturbs or alters the TBSV genomic RNA structure to render the recombinant TBSV virus unable to establish an infection. Whether this is an effect on replication or movement can be tested by examining the RNA accumulation in protoplasts.

Not all plants are equally susceptible to inoculation with RNA or DNA. As mentioned earlier, young plants are generally more susceptible, and incubation of plants in the dark for 48 hr at room temperature conditions the leaf tissue to be more susceptible. To alleviate recurring problems with rub-inoculations, in preliminary tests the authors have used a self-assembled laboratory air-driven handheld gene-gun device for biolistic delivery of coated RNA or DNA coated onto tungsten particles (*UNIT 16B.2*). It also has to be mentioned that it is not known if the CP is dispensable in all hosts. Therefore, as indicated earlier, control experiments need to be conducted with the empty gene vectors to determine if a given host is susceptible to infection with the TBSV vector.

What if the RNA or protein of the gene of interest cannot be detected with northern or western blot assays, respectively, whereas TBSV-specific probes detect the viral RNA? In this case, it is highly possible that the inserted gene of interest was rapidly deleted from the TBSV genome. Alternatively, the expression level is just below the detection limits, or the expressed protein may be unstable. If the protein is detectable by immunoblot assay, but no protein can be purified with affinity, ion-exchange, or gel-filtration chromatography protocols, it is most likely that the protein is insoluble. In that case, it is possible that expression of truncated versions may alleviate the problem, but if that is not an option, then alternative expression systems should be pursued.

Under certain circumstances, P19 is able to enhance the accumulation level of TBSV (Qiu et al., 2002). Therefore, as indicated earlier, if pHST2- or pHST12-mediated expression is suboptimal, it is worthwhile to employ pTBSV-sGFP/p19 or pHST34 gene vectors that express P19, unless the aim is to induce RNAi.

Anticipated Results

Nucleic acid biochemistry

If the TBSV gene-vector infection successfully establishes in inoculated plants, then it is expected that relatively high and readily detectable levels of viral RNAs will accumulate. When the gene-of-interest insert is maintained, then the size of the genomic RNA and sgRNA1 that hybridize with gene of interest-specific probes should be of the same size as those that hybridize with TBSV-specific probes in parallel tests.

Protein biochemistry

If the objective involves expression of protein, then the high levels of TBSV accumulation will ensure detection of the foreign protein by the gene of interest–specific antiserum in immunoblot assays, and the proteins (if soluble) can be purified.

RNAi

Even when the gene-of-interest inserts are deleted during infection, studies with *N. benthamiana* showed that the initial rounds of replication can be sufficient to induce RNAi (Qiu et al., 2002; Hou and Qiu, 2003).

Time Considerations

In anticipation of cloning success, the first action should involve planting seed. For hosts such as *N. benthamiana*, it takes 6 to 8 weeks before plants have grown to a suitable size with three or four fully expanded leaves for inoculation. As little as 2 days can be sufficient to clone the gene of interest into the TBSV gene vector plasmid and confirm the insertion of the

gene of interest in the recombinant plasmid. Purification of plasmid DNAs and preparation of linearized template for in vitro transcription requires 1 additional day. Synthesis and inoculation of RNA transcripts onto plants or protoplasts can be performed within 3 to 4 hr.

Following inoculation, local symptoms are expected to appear on inoculated leaves 5 to 10 days after inoculation depending on the host plants. It takes another 3 days to verify the expression of the gene of interest by RNA hybridization and/or immunoblot assays.

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Presents an example of using TBSV gene vectors to induce silencing, allowing for functional studies of coexpressed suppressor protein.

Hou and Qiu, 2003. See above.

A case study of applying both TBSV and defective interfering RNA (DI) as gene vectors in gene-silencing research.

Using Satellite Tobacco Mosaic Virus Vectors for Gene Silencing

The unit describes the application of satellite RNA virus vectors for induction of transient gene silencing in the tobacco species *Nicotiana tabacum*. It demonstrates the power of using RNA virus vectors for highly efficient gene function discovery or validation studies in plants.

Satellite tobacco mosaic virus (STMV) is a plus single-stranded RNA virus of 1059 nucleotides. It encodes its own coat protein but depends on helper virus proteins for replication and movement. The combination of native autonomous helper viruses, such as TMV-U2 (which is used in this protocol), with a tailor-made infectious vector of a naturally occurring satellite virus, such as STMV, permits the uncoupling of the components needed for virus replication from those components that are essential for durable silencing induction. Such a two-component silencing system enhances stable and highly efficient performance. The authors refer to this TMV and STMV silencing system as a satellite virus–induced silencing system (SVISS).

The selected virus combination used in these protocols works efficiently in *Nicotiana tabacum SR1* plants, producing gene silencing phenotypes in 10 to 12 days after inoculation (see Basic Protocol 3). In this tobacco species, the helper virus (TMV-U2; see Support Protocol 2) causes no or only minor symptoms (see Critical Parameters and Troubleshooting). It is crucial for the successful use of the system that the tobacco plants are cultivated under good standard growth conditions (see Support Protocol 1) before and after virus infection. Physiologically weak or stressed plants are less prone to allowing the accumulation of high levels of viruses and therefore will not induce gene silencing to a desired extent. It is also crucial that the target gene insert size does not exceed 250 bp. Insertion of fragments >250 bp results in significantly lower levels of satellite virus RNA and thus less-efficient silencing induction. In vitro transcription (see Basic Protocol 1) and RNA isolation and analysis (see Basic Protocol 4) follow standard protocols. Because satellite virus RNA is neither 5′ capped nor 3′ polyadenylated in vivo, satellite virus RNA that is synthesized in vitro does not require end protection. This is highly advantageous with respect to time and resource constraints.

In this unit, the authors describe only the TMV-STMV-based system (see Basic Protocol 2). They expect that other naturally occurring helper virus–satellite virus combinations can, however, be converted into transient silencing systems and can be implemented for plant species other than tobacco. They also include a short description of combining helper virus inoculation and satellite virus agroinfiltration (see Alternate Protocol 1). This combination permits the delivery of the satellite virus RNA, including the target gene insert, in a form that is protected by bacterial structures and becomes transiently expressed in the inoculated leaves via the strong CaMV 35S promoter. The authors have observed that this approach results in less variability of the obtained silencing phenotype among individual plants. Therefore, the combination of helper virus inoculation with satellite virus agroinfiltration can be a preferred method when highly uniform silencing phenotypes of a larger number of plants are required. A method for infecting plants with sap or total RNA from leaves that show a silencing phenotype is also included (see Alternate Protocol 2).

CAUTION: Although TMV and STMV are not pathogenic to humans and animals, both viruses can easily be spread by direct contact from humans to plants, or from infected

Contributed by Véronique Gosselé and Michael Metzlaff
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to noninfected plants (see Critical Parameters and Troubleshooting). Caution has to be taken not to contaminate equipment, laboratories, growth rooms, or plants. This can be achieved by wearing protective clothes (lab coats, gloves, shoes) while handling virus-infected plants. All infected material and equipment, including soil and rinsing water, must be collected in specific containers for decontamination or destruction.

NOTE: Performing gene silencing experiments with TMV-U2 wild-type and chimeric STMV constructs requires legal permission to work with plant viruses and recombinant DNA in accordance with the laws of the country in which the experiments are carried out (see *APPENDIX 1B*).

NOTE: When working with RNA, all reagents should be high-quality molecular biology grade and should be RNase-free. Likewise, all plasticware should be RNase-free, and great care should be taken to minimize contamination by RNases. Also see *APPENDIX 2A*.

BASIC PROTOCOL 1

PREPARATION OF SATELLITE TOBACCO MOSAIC VIRUS CONSTRUCTS AND HELPER VIRUS FOR RNA SILENCING

The authors can provide the satellite tobacco mosaic virus (STMV) vectors pVE349 and pVE350 in *E. coli* strain MC1061 for academic research purposes via a Material Transfer Agreement. The strains are grown overnight in LB medium (*APPENDIX 4A*) with 100 μg/ml ampicillin for plasmid DNA preparation. Vector maps are shown in Figures 16I.5.1 and 16I.5.2. The vector pVE349 carries the full-length STMV cDNA sequence between the T7 and SP6 promoter sequences. An *Sbf*I-*Not*I multiple cloning site is inserted in the *Age*I site, which is located in the STMV coat protein coding sequence. Vector pVE350 has the same polylinker inserted at the *Age*I site but lacks 275 bp of the STMV coat protein sequence downstream from this *Age*I site.

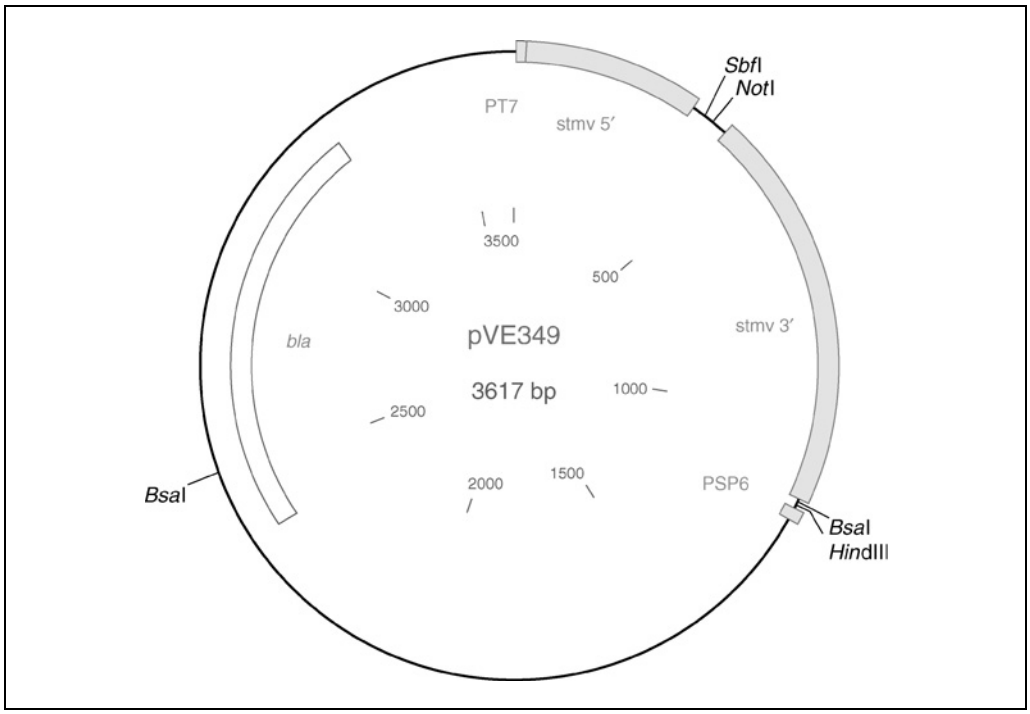

Figure 16I.5.1 Physical map of satellite tobacco mosaic virus (STMV) vector pVE349. The authors have obtained the best results with pVE349 and recommend using this vector first when setting up SVISS. Abbreviations: *bla*, β-lactamase; PSP6, SP6 promoter sequence; PT7, T7 promoter sequence; stmv 5′ and 3′, full-length STMV cDNA sequence, with a polylinker inserted.

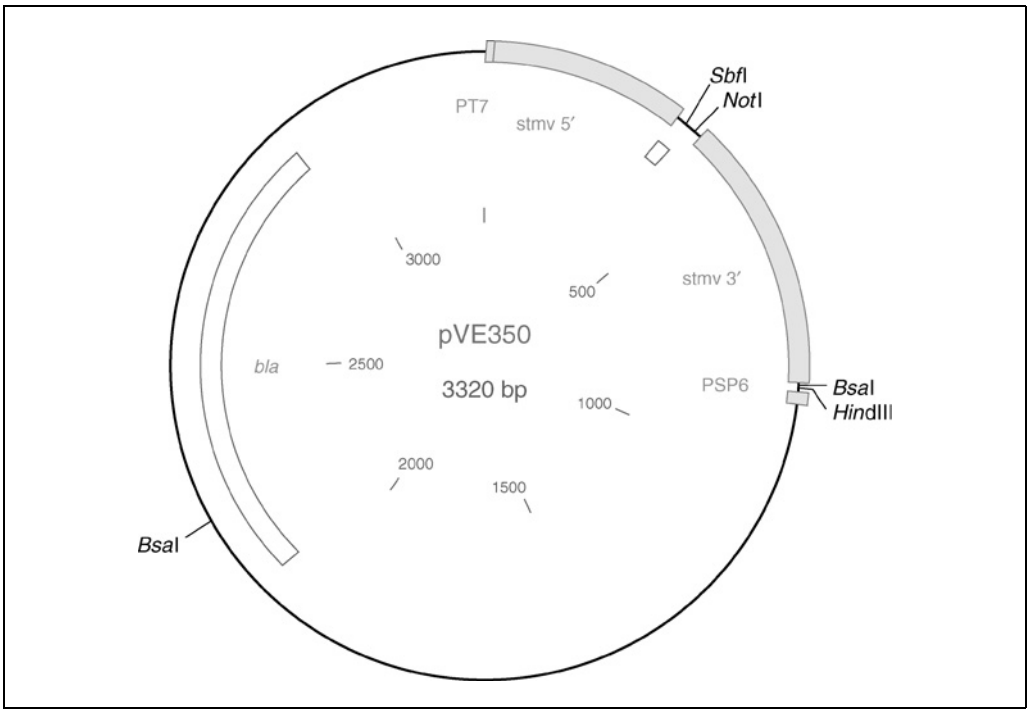

Figure 16I.5.2 Physical map of STMV vector pVE350. Abbreviations: *bla*, β-lactamase; PSP6, SP6 promoter sequence; PT7, T7 promoter sequence; stmv 5′ and 3′, STMV cDNA sequence, without 275 bp of the STMV coat protein, with a polylinker inserted.

Fragments of plant genes targeted for gene silencing can be inserted into the STMV vectors using the *Sbf*I and *Not*I sites. The most straightforward way is to amplify the target gene fragment using primers that contain the *Sbf*I and *Not*I recognition sites at the 5′ and 3′ ends, respectively, and to insert the fragment into the *Sbf*I-*Not*I double-digested vector.

Materials

*Sbf*I and *Not*I and *Bsa*I or *Hin*dIII restriction enzymes and appropriate buffers
Satellite tobacco mosaic virus (STMV) vectors pVE349 or pVE350 (Figs. 16I.5.1 and 16I.5.2)
Buffered phenol (*APPENDIX 2A*)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*APPENDIX 2A*)
24:1 (v/v) chloroform/isoamyl alcohol
5 M sodium perchlorate ($NaClO_4$)
Isopropanol
RNase-free H_2O
MEGAscript high-yield T7 transcription kit (Ambion)
Additional reagents and equipment for PCR, subcloning DNA fragments, DNA sequencing (optional), agarose gel electrophoresis, measuring DNA and RNA concentration by UV spectrophotometry, denaturing formaldehyde agarose gel electrophoresis (*UNIT 16E.3*)

1. Choose a target gene fragment of 50 to 250 bp for which an optimal primer combination can be designed. Add an *Sbf*I recognition sequence to the 5′ primer and a *Not*I recognition sequence to the 3′ primer.

   The recognition sequence of SbfI is 5′-CCTGCAGG-3′, and that of NotI is 5′-GCGGCCGC-3′.

Fragments >250 bp should be avoided because replication efficiency of the resulting STMV RNA will be low. Fragments in the size range of 50 to 250 bp work equally well. Although the authors generally use the sense orientation for the insert, they have not seen a significant influence of either sense or antisense orientation on silencing efficiency.

2. Amplify target gene sequence using standard PCR techniques.

   During PCR amplification, point mutations may occur in the target gene insert. They can be tolerated because stretches of 21-mers with up to two mismatches are still sufficient for silencing induction.

3. Digest PCR fragment with *Sbf*I and *Not*I and digest vector pVE349 or pVE350 with the same enzymes. Gel purify the PCR fragment.

   The digested vector can be used without gel purification.

4. Ligate vector and insert fragments following standard protocols. Check the correct insertion of the fragment in the resulting clones by restriction enzyme and/or sequence analysis.

5. Digest 10 μg STMV vector plasmid DNA with *Bsa*I or *Hin*dIII. Check on a gel whether digest is complete.

   For efficient synthesis of chimeric STMV in vitro transcripts, a fully linear plasmid DNA template should be used. The STMV vectors pVE349 and pVE350 contain a BsaI and HindIII recognition site downstream of the STMV coding sequence and upstream of the SP6 promoter sequence (Figs. 16I.5.1 and 16I.5.2). Depending on the sequence of the inserted target, either BsaI or HindIII is chosen for linearization of the template. The chosen enzyme recognition site should not be present in the target sequence.

   The HindIII digest creates a single band of a fully linear plasmid. The BsaI digest generates two bands (~1300 and ~2300 bp) because of the presence of a second BsaI site further downstream in the vector (Figs. 16I.5.1 and 16I.5.2). The smaller band is needed, but the presence of the larger band does not affect the following steps. No fragment purification is needed.

6. Clean linearized STMV vector by phenol treatment as follows:

   a. Extract once with 1 vol buffered phenol.
   b. Extract once with 1 vol 25:24:1 phenol/chloroform/isoamyl alcohol.
   c. Extract once with 1 vol 24:1 chloroform/isoamyl alcohol.
   d. Precipitate with 1/10 vol of 5 M sodium perchlorate and 1 vol isopropanol. Centrifuge immediately for 10 min at ~18,000 × *g*, room temperature.

   Resuspend obtained pellet in 17 μl RNase-free water. Check 1 μl on an agarose gel or measure the concentration with a UV spectrophotometer. Store template indefinitely at −20°C until needed.

   Purified template with a concentration of ~0.5 μg/μl should be obtained.

7. Carry out an in vitro transcription reaction using a MEGAscript high-yield T7 transcription kit. Recover the in vitro transcript RNA using lithium chloride precipitation solution as described by the manufacturer. Resuspend RNA in RNAse-free water and measure the concentration.

   The authors use the Ambion kit because it produces the highest yields in their hands. They have also obtained good yields with in vitro transcription kits from other suppliers. Alternatively, transcripts can be prepared using T7 polymerase without a kit (e.g., see UNIT 16D.5).

8. Check the quality of the RNA on a denaturing formaldehyde agarose gel. Load 0.5 to 1 μg transcript.

   A single ~1300-nucleotide band should be visible for the full-length in vitro RNA. If partial degradation has occurred during in vitro synthesis, the band would be accompanied by a smear. Partially degraded in vitro RNA results in poor performance of satellite virus–induced silencing system (SVISS), and the transcripts should be remade.

9. Store intact in vitro transcripts indefinitely at −20°C until they are needed for inoculation.

CO-INOCULATING SATELLITE TOBACCO MOSAIC VIRUS IN VITRO TRANSCRIPTS AND TMV-U2-INFECTED TOBACCO LEAF SAP INTO TOBACCO LEAVES

BASIC PROTOCOL 2

The authors normally co-inoculate five to ten plants per construct with in vitro satellite tobacco mosaic virus (STMV) RNA and TMV-U2 helper virus leaf sap. As controls, one or two plants are infected with helper virus only and one or two plants are mock inoculated with phosphate buffer only. To avoid degradation of in vitro transcripts, the time taken to manipulate the in vitro transcript should be kept as short as possible.

NOTE: Before thawing STMV and TMV-U2 stock solutions (see Basic Protocol 1 and Support Protocol 2), check and choose the plants to be inoculated carefully. Use only well-developed, nondamaged plants for infection. Label the pots and decide in advance for each plant which leaf should be inoculated. Choose the youngest fully developed leaf for inoculation. Labeling the pots at the side of the inoculated leaf will help to identify the inoculated leaf later during phenotype screening.

NOTE: Plants should be cultivated before and after virus infection under the most optimal growth conditions to obtain physiologically fit and unstressed plants. Do not use plants that show signs of physiological weakness, such as chlorosis, atonic leaves, or extended internodes. This is the major prerequisite for stable and optimal performance of the satellite virus–induced silencing system (SVISS).

Materials

STMV in vitro transcript of interest (see Basic Protocol 1)
0.2 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*), autoclaved
Carborundum powder (e.g., silicon carbide, 400 grinding compound; Alfa Aesar cat. no. 39800)
Nicotiana tabacum SR1 plants (see Support Protocol 1), each with leaf chosen for inoculation
TMV-U2-containing sap (see Support Protocol 2), store on ice
Plastic squirt bottle containing water
~0.5-cm (diameter) brush, does not need to be sterilized

1. Dilute STMV in vitro transcript of interest in 0.2 M sodium phosphate buffer to a concentration of 50 μg/ml and a final volume of 50 μl per each plant to be inoculated. Keep diluted in vitro transcript on ice for ≤1 hr while preparing plants for inoculation.

2. Using a 0.5-cm brush, apply carborundum powder on the upper side of a *Nicotiana tabacum SR1* leaf or leaves (for multiple plants) that will be inoculated in one series. Carefully brush over the complete leaf surface.

   Always inoculate five plants per construct in one go. If more than five plants need to be infected at a time, they should be inoculated in series of five.

3. Immediately pipet ~50 µl in vitro transcript on each brushed leaf. Spread RNA over the complete surface of the leaves with your gloved finger. Remove glove and replace with a clean glove.

   A clean glove needs to be used when applying the helper virus in the next step.

   The virus-contaminated gloves, together with all used pipet tips and rinsing water, should be collected for safe disposal.

4. Pipet ~50 µl TMV-U2-containing sap on the same leaves. Again, spread the sap over the complete surface. Remove glove and replace with a clean glove.

   Because the sap can not be easily pipetted with small (yellow) pipet tips, the larger (blue) pipet tips should be used.

5. Wait for a few minutes and then rinse inoculated leaves thoroughly with water using a plastic squirt bottle. Rinse leaves before they are completely dry.

   The water in the squirt bottle does not need to be sterile.

6. Apply carborundum powder as described (step 2) to leaves from one or two plants each to inoculate with TMV-U2 alone and to mock inoculate.

7. Pipet ~50 µl TMV-U2-containing sap on a carborundum-brushed leaf or leaves for the TMV-U2 control. Spread the virus with your gloved finger, wait a few minutes, and rinse thoroughly. Remove glove and replace with a clean glove.

8. Mock inoculate one or two plants by pipetting 50 µl of 0.2 M sodium phosphate buffer on a carborundum-brushed leaf. Spread the buffer with your gloved finger, wait a few minutes, and rinse.

SUPPORT PROTOCOL 1

CULTIVATION OF TOBACCO PLANTS

Nicotiana tabacum SR1 seeds are sown on agar plates and cultivated at 24°C. After ~2 to 3 weeks, the agar-grown seedlings are transferred into soil. Germinating plants that are first grown on agar plates result in plants of a more similar size. For high-throughput experiments in which high numbers of plants are required, seeds can also be sown immediately into soil. This is less time consuming, but plants may be more variable in size. Although the authors have not tested this, the satellite virus–induced silencing system (SVISS) should work in all *Nicotiana* species that are susceptible to tobacco mosaic virus (TMV).

Materials

Nicotiana tabacum SR1 seeds (available from many seed suppliers)
5% (v/v) bleach solution
Tween 20
H_2O, sterile
14-cm sterile polystyrene plates (e.g., Falcon 3025) containing 100 ml *N. tabacum* germination medium (see recipe)
12-cm flowerpots containing soil
Plant growth room (*UNIT 16A.3*), 24°C, equipped with timer-controlled grow lights and controlled relative humidity

1. Sterilize 32 *Nicotiana tabacum SR1* seeds in a 1.5-ml microcentrifuge tube by adding 1 ml of 5% bleach solution and 1 drop of Tween 20. Keep seeds in bleach solution for 20 min. Remove solution with an automatic pipet and wash seeds three times with ~1 ml sterile water.

2. Sow 32 sterile seeds on a 14-cm plate containing 100 ml *N. tabacum* germination medium.
3. Grow the plants for 2 to 3 weeks on the plate in a growth room at 24°C, with 18 hr light and 6 hr dark.
4. Transfer small plants into 12-cm flowerpots containing soil.

   Flowerpots and soil do not need to be sterilized, but they should never be reused after virus infection.
5. Grow plants in a growth room at 24° to 25°C, with the same light/dark cycle and relative air humidity of 60% to 80% (*UNIT 16A.4*). Water regularly but avoid continuously wet soil, which leads to water stress and root damage.

   Depending on the growth conditions, the plants reach the 5- to 6-leaf stage in 2 to 3 weeks. This is the best stage for virus inoculation.

   Young plants should be used for inoculation. Using older plants will result in lower efficiency of inoculation and gene silencing induction. Because of this, silencing phenotypes that are less pronounced may result.

   Suboptimal conditions can result in slower plant growth. As long as plants remain healthy, this should not affect the infection and silencing efficiency.

ISOLATING HELPER VIRUS LEAF SAP FROM TMV-U2-INFECTED TOBACCO PLANTS

SUPPORT PROTOCOL 2

The leaf sap of TMV-U2-infected plants contains high concentrations of virus particles and can therefore be used for infection of new tobacco plants (as described in Section 16B). The inoculated leaf and all upper leaves can be harvested 1 to 2 weeks after inoculation of plants with leaf sap from infected plants. The harvested leaves must be frozen immediately in liquid nitrogen. They can be stored for up to 10 years or longer at −70°C. For longer storage and shipment of material, the leaf material should be freeze-dried. The authors can supply interested users of SVISS with freeze-dried tobacco leaves infected with TMV-U2 to start with.

Materials

Leaf material from TMV-U2-infected plants, frozen at −70°C or freeze-dried
0.2 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2A*), autoclaved
Mortar and pestle, prechilled

Additional reagents and equipment for preparing total RNA from leaves (*UNIT 16E.1*), measuring RNA concentration by UV spectrophotometry, and denaturing agarose gel electrophoresis

1. Transfer an ~4-cm^2 portion of frozen or freeze-dried leaf material from TMV-U2-infected plants into a prechilled mortar.

   An additional amount of leaf material will be needed to measure the amount of TMV RNA that is present (step 3).
2. Add ~0.5 ml of 0.2 M sodium phosphate buffer and grind leaf material with a prechilled pestle. Add ~2 ml more sodium phosphate buffer so fluid can easily be pipetted with an automatic pipet. Transfer sap into 1.5- or 2.2-ml microcentrifuge tubes and keep on ice.

   This sap can be used for inoculation of new tobacco plants (see Basic Protocol 2) or can be stored indefinitely as a stock at −70°C. For storage, the sap should be divided into small aliquots; repeated freeze-thawing should be avoided.

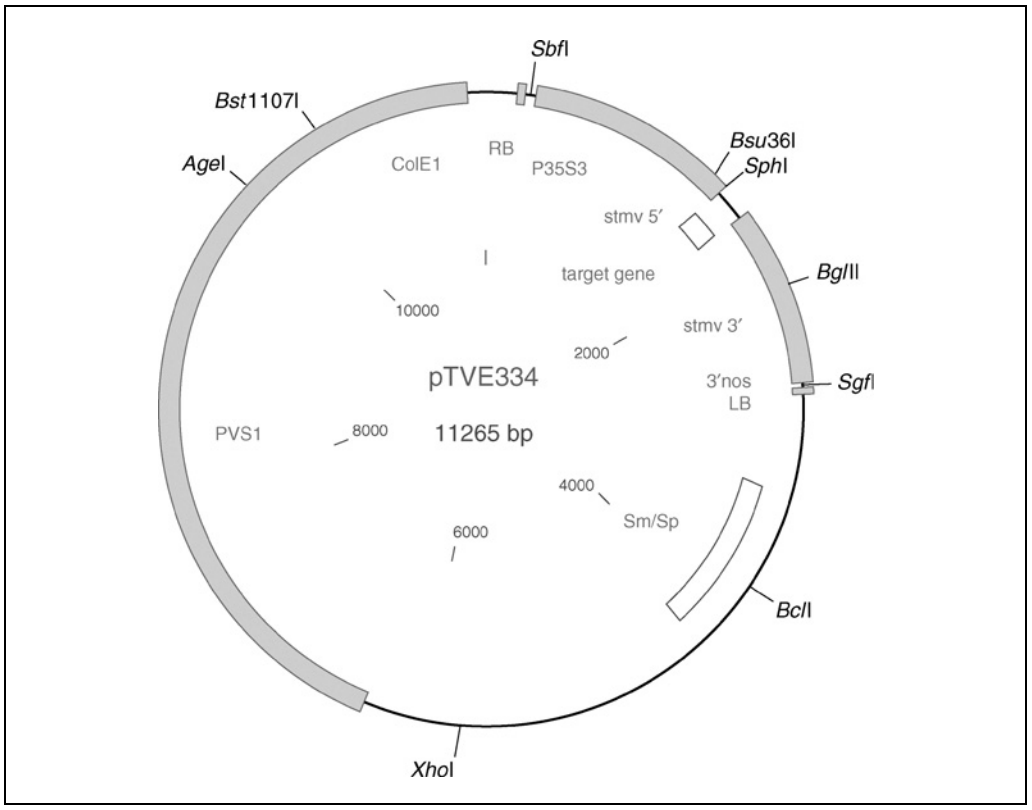

Figure 16I.5.3 Physical map of the agroinfiltration vector pTVE334. Abbreviations: ColE1, origin of replication of the naturally occurring *E. coli* plasmid ColE1; LB, left border of T-DNA; P35S3, cauliflower mosaic virus 35S promoter; Rb, right border of T-DNA; Sm/Sp, streptomycin/spectomycin resistance gene; stmv, full-length satellite tobacco mosaic virus cDNA sequence; 3′nos, 3′ untranslated region of the nopaline synthase gene.

3. To check whether the sap contains sufficient levels of TMV, prepare total RNA from an additional sample of the leaf material (*UNIT 16E.1*).
4. Run 5 to 10 μg RNA, as determined by UV spectrophotometry, on a denaturing (formaldehyde) agarose gel.

   The TMV-U2 RNA should be visible after ethidium bromide staining as a separate band that runs slower than all ribosomal bands (the length of TMV RNA is 6355 nucleotides).

ALTERNATE PROTOCOL 1

SUPPLYING SATELLITE TOBACCO MOSAIC VIRUS CONSTRUCTS TO TOBACCO VIA AGROINFILTRATION

Satellite tobacco mosaic virus (STMV) can also be supplied by agroinfiltration. The authors have observed that this results in highly uniform phenotypes of all infected plants. Agroinfiltration of STMV constructs is the delivery method of choice when large numbers of plants have to be repeatedly inoculated with the same construct. For agroinfiltration, a tobacco-infecting *Agrobacterium tumefaciens* strain is needed, such as $C_{58}C_1$Rif (Van Larebeke et al., 1974) containing the Ti plasmid pGV3000 and a T-DNA vector plasmid in which the STMV sequences can be expressed via a plant promoter. The authors have constructed a T-DNA vector in which STMV is transcribed via the *cauliflower mosaic virus* 35S promoter (Fig. 16I.5.3). Because agroinfiltration of cloned plant viruses has been described in detail in *UNIT 16B.2*, this protocol provides only a short overview of the method.

Additional Materials *(also see Basic Protocol 2)*

T-DNA vector (e.g., pTVE334; available from the authors: *Michael.Metzlaff@bayercropscience.com*)
Chimeric STMV of interest (see Basic Protocol 1, steps 1 to 4)
Tobacco-infecting *Agrobacterium tumefaciens* strain
LB medium (*APPENDIX 4A*)
Agroinfiltration medium (see recipe)

Incubator, 28°C, with shaking
Spectrophotometer, 600 nm
Razor blades
1- or 2-ml syringes

Additional reagents and equipment for subcloning DNA fragments and transforming *Agrobacterium* (*UNIT 16B.2*),

1. Make a T-DNA construct that contains a chimeric STMV of interest under control of a constitutive plant promoter.
2. Transform a tobacco-infecting *Agrobacterium tumefaciens* strain with the T-DNA construct.
3. Grow the strain overnight at 28°C with shaking in LB medium, centrifuge the culture, and resuspend it in agroinfiltration medium to an OD_{600} of 1 to 1.5. Keep the culture at room temperature for 3 hr.

   The 3-hr incubation significantly increases the infectivity of Agrobacterium.
4. After the 3-hr incubation, brush the tobacco leaves with carborundum and inoculate with TMV-U2-containing sap as described (see Basic Protocol 2, step 2 and step 4).

   TMV-only and mock-inoculated controls should be set up as described (see Basic Protocol 2, steps 6 to 8).
5. Immediately after TMV inoculation, make small, shallow cuts (≤5 mm in length) on the lower side of the inoculated leaf using a razor blade. Make six to eight cuts parallel to the leaf veins. Inject 100 µl *Agrobacterium* culture into the cuts with a 1- or 2-ml syringe without needle.

   Cutting is essential for achieving efficient agroinfiltration.

   The razor blade can be used repeatedly.
6. After a few minutes, rinse off carborundum and excess TMV-U2-containing sap with water using a plastic squirt bottle.

SUPPLYING CHIMERIC SATELLITE TOBACCO MOSAIC VIRUS AS SAP OR AS TOTAL RNA FROM PREVIOUSLY INFECTED PLANTS

ALTERNATE PROTOCOL 2

Plants can also be efficiently infected with sap or with total RNA from leaves that show a silencing phenotype. This is very easy to perform and results in highly uniform phenotypes of all inoculated plants. Prepare sap of the silenced leaf tissue (see Support Protocol 2) and inoculate the plants as described for TMV-U2 control inoculations (see Basic Protocol 2). Because the sap contains TMV particles, there is no separate TMV inoculation needed. Alternatively, isolate total RNA from infected leaves (e.g., *UNIT 16E.1*) and directly apply the total RNA preparations to tobacco plants as described earlier for in vitro transcripts (see Basic Protocol 2). Because the total RNA preparations contain both STMV and TMV-U2 RNAs, no separate TMV infection is needed. The authors have obtained good results with a volume of 50 to 100 µl and a concentration of 100 µg total RNA/ml 0.2 M sodium phosphate buffer, pH 7.0.

BASIC PROTOCOL 3

SCREENING FOR SILENCING PHENOTYPES

Using the conditions in Basic Protocol 2 for *Nicotiana tabacum SR1* plants, the gene silencing phenotypes for leaf-expressed plant target genes normally occur 10 to 12 days postinoculation in the third emerging leaf above the inoculated leaf. All plants inoculated with the satellite virus–induced silencing system (SVISS) should be screened visually for phenotypic changes as compared with tobacco mosaic virus (TMV)–only and mock-inoculated control plants on a daily basis from day 10 postinoculation forward. The phenotype becomes visible first in tissues surrounding the major leaf veins but quickly spreads during the following 2 days into most areas of the leaf. This leaf normally does not show a uniform phenotype, however, but rather remains variegated. The next three emerging leaves show the strongest phenotype by being almost uniformly silenced. Later in plant development (2 to 3 weeks after inoculation), plants recover from virus infection and start to lose the silencing phenotype. At this stage, newly emerging leaves again show a variegated phenotype and finally do not show the silencing phenotype any more. Phenotypic changes should be documented by a series of photographs. A sample from the inoculated leaf of each plant, as well as samples of upper leaves with and without the silencing phenotype, should be taken and frozen immediately in liquid nitrogen. The frozen leaves should be stored indefinitely at −70°C until needed for northern blot (see Basic Protocol 4) and biochemical analyses.

In many cases, silencing by SVISS does not result in a complete knockout of the targeted plant gene, and the plants survive, recover, and flower at later stages of development. These plants do, however, produce sufficient tissue for carrying out molecular studies of the effect of silencing on gene expression. If needed, more silenced tissue can be produced by cutting off the apex of the main branch. By doing this, secondary shoots develop within a few days out of almost all axillary buds of tobacco. The emerging side branches normally show a drastic silencing phenotype. In cases where the targeted gene is involved in plant development, a partial and transient silencing by SVISS can lead to drastic disturbances in growth and development. These plants may stop growing and show leaf distortions; they do not flower, and eventually they die. These plants also produce enough tissue to perform detailed molecular analyses. The authors have also targeted genes predominantly expressed in flower tissue (e.g., chalcone synthase A). In those cases, there was no obvious phenotype detected in leaves. Only when the flowers emerged (∼6 weeks postinoculation) did loss of flower pigmentation indicate target gene–specific gene silencing. TMV and satellite TMV (STMV) also move to roots. Targeting of root-specific genes by SVISS is therefore feasible.

BASIC PROTOCOL 4

ANALYZING VIRUS RNA AND TARGET GENE MESSENGER RNA LEVELS IN SILENCED VERSUS NONSILENCED TISSUE

Inoculated leaves usually contain high levels of tobacco mosaic virus (TMV) and satellite TMV (STMV) RNA, often up to 10 ng/μg total RNA. The upper leaves normally have lower amounts. Plants that will display a strong silencing phenotype contain high STMV and TMV RNA levels 3 to 10 days postinoculation (i.e., before the phenotype is visible). In silenced tissue, the mRNA of the targeted gene is lower than in wild-type tissue. Viral RNA levels and target gene mRNA levels can be determined by northern blot analysis as described in detail in *UNIT 16E.3*.

CAUTION: When working with ^{32}P, investigators should use appropriate shielding and frequently check themselves and the work area for radioactivity using a hand-held monitor. All proper guidelines for radioisotopes should be followed. Radioactive waste should be placed in appropriately designated areas for disposal. Follow the guidelines provided by your local radiation safety adviser. See *APPENDIX 1C* for more information.

Materials

Frozen leaf samples, harvested at appropriate time points (see Basic Protocol 3), including noninoculated leaf samples for transcript quantification only

STMV in vitro transcript (see Basic Protocol 1), for transcript quantification only

Hybond N+ filter (e.g., Amersham Biosciences)

STMV or target gene probe, either subcloned in an appropriate vector or as PCR fragment

Additional reagents and equipment for preparing RNA from leaf samples (*UNIT 16E.1*) and northern analysis of plant RNA (*UNIT 16E.3*)

1. Prepare total RNA from frozen leaf samples (including TMV-only and mock-inoculated samples) using a standard protocol (*UNIT 16E.1*). Prepare RNA from non-inoculated leaves if quantification of transcripts is desired (step 3).

   Usually, the third upper leaf is the first leaf that shows a clear silencing phenotype and still has high STMV RNA levels. The next three emerging upper leaves show a stronger phenotype but lower STMV RNA levels. This indicates that gene silencing is at its height in these upper leaves, degrading most of the target gene mRNA and chimeric STMV RNA.

2a. *For detection of STMV RNA:* Load 1 μg total RNA on a denaturing agarose gel.

2b. *For detection of target gene mRNA:* Load 5 to 10 μg total RNA on a denaturing agarose gel.

3. *Optional:* Load a dilution series of transcripts in the same gel for quantification. Use STMV in vitro transcript diluted 0.5 pg to 5 ng and each mixed with 1 μg total RNA isolated from noninoculated tobacco leaves. For mRNA detection, adjust dilution series to lower quantities, depending on the abundance of the mRNA in leaves.

   It is especially important to carry out target gene mRNA detection when no silencing phenotype is observed. This will determine whether the absence of a phenotype is due to loss of STMV replication.

4. Run the gel and blot onto a Hybond N+ filter using standard procedures.

   TMV RNA can be seen after electrophoresis in ethidium bromide–stained gels as an extra slow-moving band that is located above all rRNA bands. STMV RNA runs within the region of rRNA and can be seen only after hybridization with an STMV-specific probe.

5. Hybridize the filter with an STMV probe or a probe for detecting the target gene mRNA.

   For STMV hybridization, the authors use an STMV 5′ leader sequence riboprobe. This probe contains the first 200 nucleotides of the STMV cDNA. The authors cloned this fragment into a pGEM vector for general use as a probe, but PCR fragments can also be used directly.

   For detecting the target gene mRNA, an appropriate target gene probe should be designed and prepared. A 3′ end fragment of the STMV sequence is not recommended for use as a probe because it is partially homologous with a specific region of the TMV-U2 sequence. An STMV 3′ end probe will therefore also hybridize with TMV-U2 RNA.

6. Expose the hybridized filter after washing to an X-ray film. The levels of STMV RNA are usually very high and therefore short exposures (for a few hours) are sufficient. For mRNA detection, longer exposures will be needed.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agroinfiltration medium

4.3 g/liter Murashige and Skoog salts (Duchefa Biochemie)
20 g/liter sucrose
500 μM 2-(*N*-morpholino)ethanesulfonic acid (MES)
Adjust pH to 5.6 with 3 M KOH
Autoclave for 15 min
Add 10 mM acetosyringone (filter sterilized with 0.45-μm filter) before use
Store up to 6 months at room temperature

This medium can be used for most plant species.

N. tabacum germination medium

50% (w/w) Murashige and Skoog medium including vitamins (2.2 g/liter; Duchefa Biochemie)
15 g/liter sucrose
6 g/liter agar
Adjust pH to 7 with 1 M KOH
Autoclave for 15 min

The medium can be stored at room temperature for up to 6 months and melted in a microwave when needed.

COMMENTARY

Background Information

RNA interference (RNAi) has recently been identified as a natural mechanism for regulation of gene expression in all higher organisms from plants to humans (for review, see Baulcombe, 2004). Technologies have emerged for making use of RNAi for discovering or validating gene functions and for deregulation of biochemical pathways. Because RNAi acts in a highly sequence-specific fashion, the selective modulation of biochemical pathways underlying phenotypes has become feasible.

The common goal of all RNAi technologies in plants is to produce sufficient amounts of double-stranded RNA (dsRNA) that has homology with endogenous mRNAs and can trigger the initiation of the silencing mechanism. The most efficient delivery methods for dsRNA in plants today are (1) the inoculation of plants with engineered plant viruses that produce dsRNA intermediates during their life cycles (Muangsan and Robertson, 2004) and (2) the transformation of plants with transgene constructs from which the RNA transcripts are folded into dsRNA structures (Waterhouse et al., 1998). In both approaches, accumulating dsRNA activates the plant dsRNA-specific RNase, DICER, which cleaves the dsRNA into small RNA fragments with a size of ~21 nucleotides (Bernstein et al., 2001). These small RNAs, which are called small interfering RNAs (siRNAs), become integrated into an enzyme complex called the RNA-induced silencing complex (RISC) and then guide the complex to all homologous plant RNAs for degradation. The degree of degradation of the targeted plant RNAs can vary from partial to complete degradation and depends on exogenous factors (e.g., temperature) as well as endogenous factors (e.g., the physiological status of the plant). This enables studies of the effects of downregulating the expression of a gene at various levels. Also, the downregulation of genes for which a complete knockout would be lethal becomes feasible.

Currently, dsRNA vectors based on plant RNA viruses are the most efficient tools for induction of RNAi in plants. A number of RNA viruses have been identified that are not toxic to plants and cause no or only mild symptoms in infected plants. Some of them have been converted into vectors in which fragments of plant genes can be integrated to form chimeric viruses. RNA from these chimeric viruses can be produced in vitro by performing in vitro RNA transcription. These in vitro transcripts can also be used for infecting plants by

inoculating viral in vitro RNA into young leaves, which results in local virus infection. The viral RNA replicates in vivo to high levels, and within a few days the virus spreads throughout the plant. As a result of this amplification and spreading, the plant becomes systemically infected. The systemic infection induces a range of plant defense mechanisms, one of which is the gene silencing or RNAi mechanism. Because the chimeric viruses also carry small fragments of a plant gene, the viruses and the corresponding plant gene are both targeted for silencing. Within ~10 to 12 days, the expression of the targeted gene becomes downregulated to a level that causes physiological changes that are manifested as a visible change in phenotype. In an RNAi screening procedure, the infected plants are continuously screened for phenotypic changes. Plants with phenotypic changes are selected and channeled into biochemical assays for further studies.

To date, only a few plant viruses have been successfully converted into functional RNAi vectors because of their sensitivity to changes in their genome and resulting in vivo stability problems. The authors therefore tested the idea of uncoupling the function of virus replication and induction of gene silencing in a two-component system that could overcome these stability problems. A well-studied natural viral two-component system is the helper virus–satellite RNA virus association of tobacco mosaic virus (TMV) and satellite TMV (STMV). STMV can be converted into an infectious vector, and in vitro transcripts synthesized using the vector as template are highly infectious (Routh et al., 1995). The authors converted STMV into a gene-silencing vector for tobacco, which efficiently replicates and spreads in the presence of TMV-U2 helper viruses. The authors call this two-component system the satellite virus–induced silencing system (SVISS; Gosselé et al., 2002). For induction of gene silencing of endogenous plant genes, chimeric vector plasmids are produced by integrating small fragments of plant genes into a multicloning site located within the coat protein region of the satellite virus. The chimeric viral in vitro RNAs are co-inoculated together with TMV-U2 into young tobacco plants. Within 10 to 12 days, changes are observed in plant phenotypes according to the function of the targeted gene. An analysis of the level of viral RNAs present in the phenotypic plants shows high levels of both the helper virus RNA and the satellite virus RNA. Correspondingly, the observed phenotypes are intense and persistent. In proof-of-concept experiments, numerous genes involved in leaf and flower pigmentation, cell wall synthesis, and flower development have been efficiently downregulated with SVISS constructs (Gosselé et al., 2002).

Critical Parameters and Troubleshooting

Target gene fragment insertions

Today there is only limited sequence information for tobacco available in public databases (see Internet Resources). To obtain fragments for silencing of specific tobacco genes, sequence data may be obtained by sequencing short stretches of tobacco expressed sequence tags (ESTs). Remember, only fragments as small as 50 to 250 bp are needed for performing SVISS experiments. Alternatively, fragments from related *Solanaceae* species (e.g., tomato, potato) may be sufficiently homologous for inducing silencing of tobacco genes. For example, the authors have successfully silenced the phytoene desaturase gene of tobacco using a fragment of the corresponding tomato gene (Gosselé et al., 2002). Although any part of the coding region can be targeted for silencing, highly conserved regions (e.g., the catalytic center) should be chosen when heterologous fragments from other species are used. It has to be kept in mind that these conserved regions may cross-silence related genes with similar conserved regions. In cases where the tobacco gene sequence is fully or partially known, conserved regions should be avoided.

Primer selection for PCR amplification of the target gene fragment follows the standard rules. Standard software programs for creating optimal primer pairs do exist and can be purchased from several suppliers. As described in Basic Protocol 1, *Sbf*I and *Not*I recognition sites should be added to the ends of the amplified fragments. All insert components should not exceed a total insert length of 250 bp. During PCR amplification, point mutations may occur. This is not crucial because the complementarity of the amplified fragment will still be high enough for silencing induction.

The authors have inserted fragments in the sense and antisense orientation. In most cases, they did not observe an advantage for one or the other orientation with regard to vector stability or silencing frequency. In only a few cases did the sense or antisense orientation show an advantageous effect on gene silencing induction. This is most likely due to viral

RNA secondary structures that are formed differentially depending on the orientation of the insert. The authors therefore suggest producing constructs in the sense and antisense orientation for each target gene and inoculating separate plants with each construct. For subsequent experiments, the construct with the highest frequency of gene silencing induction can be used.

The authors have also produced chimeric vectors for simultaneous silencing of two target genes by inserting small fragments for both target genes in tandem orientation. Again, the total insert size should not exceed 250 bp. This approach resulted in simultaneous downregulation of the expression of both genes, although the frequency and extent of gene silencing were lower than for vectors with a single target gene insert.

In vitro transcription of infectious STMV clones

For in vitro transcription, the authors use standard kits and have obtained the best results with the Ambion MEGAscript T7 kit. The template plasmid DNA should be fully linear. Contamination with circular plasmid significantly reduces the final concentration of in vitro transcripts. Therefore, all restriction enzyme digests of template DNA should be checked on agarose gels.

STMV RNA forms stable stem-loop structures at both ends and is not 5′ capped or 3′ polyadenylated. Therefore, STMV in vitro transcripts do not need in vitro capping and polyadenylation. It is important, however, that only sterilized equipment and RNase-free solutions are used for synthesis and subsequent handling of STMV in vitro RNA. Always check the quality of the STMV in vitro transcripts on denaturing formaldehyde agarose gels. If STMV in vitro transcripts are partially degraded, a smear occurs. Use only fully intact in vitro transcripts for inoculation to obtain best results for SVISS. In vitro transcripts can be stored at −20°C for up to 12 weeks. For long-term storage (up to several years), the transcripts should be stored at −70°C.

Propagation and storage of TMV helper virus stocks

The strain TMV-U2 is highly infectious for tobacco and also other plant species, and is naturally spread by physical contact. It can easily be spread by direct contact between infected and noninfected plants or by touching noninfected plants with contaminated gloves or equipment used for inoculation. To avoid contamination, it is therefore essential to avoid any physical contact with TMV-infected material. In most host plants, infection causes only mild symptoms, which are often not visible. This is advantageous for phenotype screening of SVISS-silenced plants but carries the risk that contamination with TMV-U2 will remain undetected. In young tobacco plants that are preinfected with TMV-U2, the systemic defense system against biotic stresses is already active before the plants are inoculated with SVISS helper and satellite viruses. This results in low levels of these viruses and causes poor performance of SVISS. Noninoculated tobacco plants should therefore be grown in a separate growth room and should be transferred to a virus growth room for inoculation. All contaminated material including pots in which the tobacco plants were grown during SVISS experiments should be collected for decontamination. Only personnel trained in handling plant viruses should have access to virus growth rooms and should carry out SVISS experiments. Change lab coats when moving between infected and noninfected areas. The authors also advise decontaminating growth rooms at regular times.

To obtain leaf sap stocks with high levels of TMV-U2, it is best to inoculate young plants (5- to 6-leaf stage) and to harvest leaves of infected plants 1 to 2 weeks postinoculation. The optimal time point may depend on growth conditions. It can be determined by isolating total RNA from infected leaves at different time points within that period and determining the TMV RNA concentration in formaldehyde agarose gels. If the TMV levels are high, a TMV RNA band can be observed above the ribosomal RNA bands after ethidium bromide staining. Infected leaf material can be stored at −70°C for long periods (up to 10 years or longer). Leaf sap stored at −70°C also contains high virus levels even after long periods (e.g., 10 years or longer). Freeze-drying of infected leaf material is an alternative that is recommended when infected leaf material has to be shipped.

Host plant cultivation

As discussed earlier, optimal growth conditions of the host plants are crucial for optimal SVISS performance. The authors cultivate *Nicotiana tabacum SR1* plants with good results with 18-hr days of cool white light (60 to 80 μmol $m^{-2}sec^{-1}$ light reaching the surface of the leaves) and a high relative air humidity of up to 80%. Low air humidity in particular reduces the systemic spread of viruses in

tobacco and, thereby, the induction of gene silencing in upper leaves.

Tobacco seeds can be sown directly into soil. To obtain more equally sized plants, the authors recommend germinating seeds first on agar plates for 2 to 3 weeks and then transferring seedlings into soil. The roots of tobacco seedlings are very fragile. Care has to be taken during transfer from agar plates to soil. After transfer into soil, excess water harms young roots. Water young tobacco plants sparingly.

TMV-STMV co-inoculation

To avoid STMV in vitro transcript degradation and cross-contamination during the inoculation procedure, it is crucial to execute all steps of the protocol without delay:

1. Prelabel all selected plants.
2. Keep TMV leaf sap and diluted STMV in vitro transcripts on ice.
3. Carry out inoculations fast, starting with the STMV in vitro transcript first, followed by TMV-infected leaf sap.
4. Do not inoculate more than five plants at once.
5. Do not damage leaves with your gloved fingers when rubbing in the inocula.
6. Remove carborundum thoroughly by extensive rinsing with distilled water.
7. Carefully collect all virus-contaminated solid and liquid waste for decontamination.
8. Arrange plants in an order that prevents contact between plants after inoculation (keep in mind that plants will grow quickly).

Agroinfiltration of STMV

For critical parameters and troubleshooting of agroinfiltration, see *UNIT 16B.2*. In addition, be sure to make the cuts on the lower side of the leaves as small as possible ($\leq$5 mm). Larger cuts or cuts that go through all layers of the leaf cause irreversible leaf damage and thereby often lead to early loss of the inoculated leaves.

Phenotype screening

For genes expressed in leaves, the silencing phenotype starts to develop 10 to 12 days postinoculation (Fig. 16I.5.4). The first phenotypic changes normally occur around the major veins of the third emerging leaf above the inoculated leaf. They develop quickly in the next 2 to 3 days. The authors normally take a series of photos with a digital camera and carefully document the time and type of observed changes. The subsequent emerging upper leaves normally show a strong silencing phenotype. They are the best source of leaf material to be harvested for molecular and biochemical assays. It is crucial to compare SVISS-inoculated plants with TMV-only and mock-inoculated control plants to judge whether the virus infection or the inoculation procedure has an influence on the observed phenotype. This is especially important when the phenotypic changes caused by silencing of the target gene are minor. Depending on the target gene, the other parts of the plant (e.g., stems, roots, flowers) should be screened carefully for phenotypic changes to obtain a comprehensive picture of the biological consequences of silencing a specific target gene.

Virus RNA and target gene mRNA analysis

The RNA analyses performed on SVISS-silenced tobacco plants follow standard protocols. The general precautions for working with RNA (e.g., use of sterilized equipment and RNase-free water) have to be taken. The levels of TMV and STMV are normally very high in infected plants. Thus, the gels should not be overloaded with total RNA to avoid nonspecific hybridization with radioactive probes used for quantification of virus RNA levels.

Anticipated Results

SVISS is an easy-to-perform, transient gene-silencing tool. As discussed earlier, the performance of the system is very much dependent on the efficiency of infection with the helper and satellite viruses, which in turn depends on the physiological conditions of the plants used for infection. Once these conditions are established and all steps of the protocol are standardized, the output of gene silencing data can be very high, considering that most of the experimental steps described in the protocols can be performed in parallel for numerous target genes. It must be remembered that SVISS is a transient, virus-based system. Thus, the silencing phenotype will in most cases disappear over time because of recovery of the plant from virus infection, and the silencing phenotype will not occur in all tissues (e.g., in the case of leaf-expressed genes, most leaves will show a variegation phenotype). When used to determine gene function, SVISS should be regarded as a screening tool for identifying candidate genes, which can then be channeled into detailed gene function validation studies.

Time Considerations

Depending on the containment space that is available for growing virus-infected plants, hundreds of transient gene silencing experiments can be performed in a few months. PCR amplification of target gene

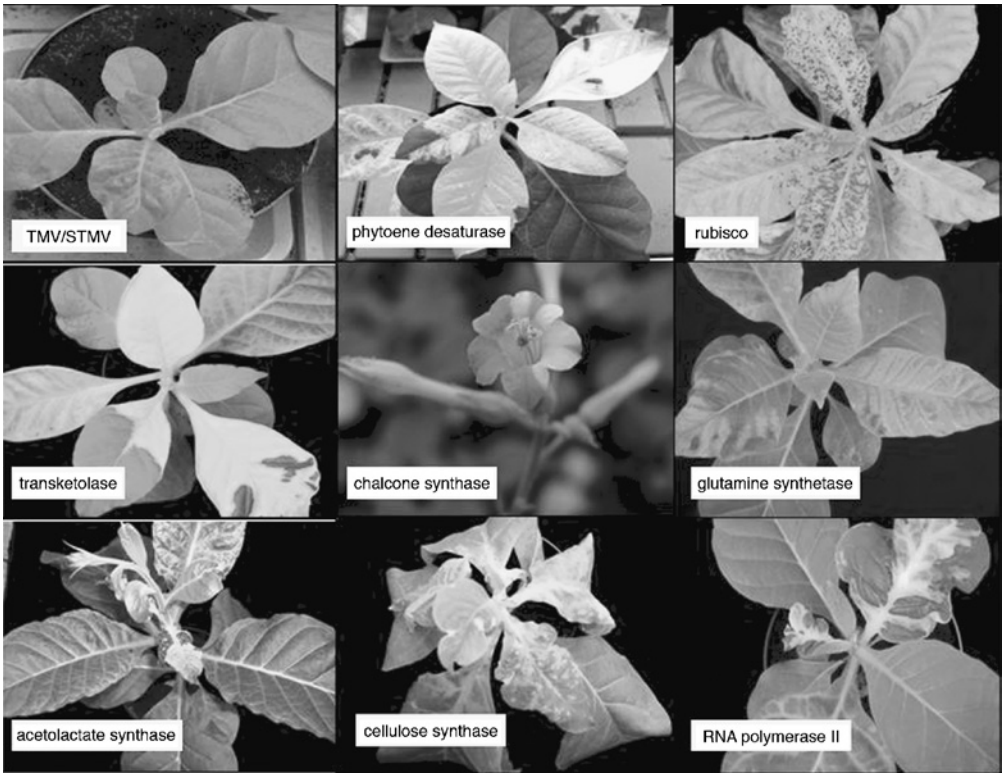

Figure 16I.5.4 Satellite virus–induced silencing system (SVISS) tobacco knockout plants, 4 weeks after inoculation with chimeric satellite tobacco mosaic virus (STMV) in vitro transcripts and TMV helper virus leaf extract. The wild-type TMV- and STMV-inoculated plant shows almost no viral symptoms, whereas plants co-inoculated with wild-type TMV leaf extract and target gene–specific chimeric STMV in vitro transcripts show numerous changes in pigmentation, leaf structure, and development. The targeted genes are indicated for each photograph. (This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to *http://www.interscience.wiley.com/c_p/colorfigures.htm*.)

Table 16I.5.1 Timeline for Satellite Virus–Induced Silencing System

Procedure	Time required
PCR amplification and purification of the target gene fragment from total RNA or cDNA or plasmid DNA (see Basic Protocol 1)	1 day
Chimeric SVISS plasmid isolation and in vitro transcription (see Basic Protocol 1)	2 days
Cultivation of young *Nicotiana tabacum SR1* plants (see Support Protocol 1)	4-6 weeks
Isolation of TMV-U2 helper virus (see Support Protocol 2)	$^1/_2$ day
Co-inoculation of in vitro transcripts with TMV-U2 helper virus (see Basic Protocol 2 and see Alternate Protocols 1 and 2)	$^1/_2$ day
Occurrence of gene silencing phenotype and subsequent screening (see Basic Protocol 3)	10-12 days postinoculation
Analyzing virus RNA and target gene mRNA (see Basic Protocol 4)	4 days

sequences and insertion of the resulting PCR fragments into an SVISS vector can be performed in parallel for numerous targets. Once a chimeric vector plasmid is ready, it can be stored for long periods until being used for inoculation in parallel with many other constructs. For a single transient silencing experiment, the time estimation provided in Table 16I.5.1 can be used as a general guideline. In summary, within ~14 days after

identification of a target gene, phenotypic and molecular studies of the silencing event can be started.

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Routh, G., Dodds, J.A., Fitzmaurice, L., and Mirkov, T.E. 1995. Characterization of deletion and frameshift mutants of satellite tobacco mosaic virus. *Virology* 212:121-127.

Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R.A., and Schell, J. 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. *Nature* 252:169-170.

Waterhouse, P.M., Graham, M.W., and Wang, M.B. 1998. Virus resistance and gene silencing can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci. U.S.A.* 95:13959-13964.

Key References

Baulcombe, D.C. 1999. Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* 2:109-113.

Describes how the technology of virus-induced gene silencing is being refined and adapted as a high-throughput procedure for functional genomics in plants.

Routh et al., 1995. See above.

Describes the first conversion of a satellite RNA virus into an infectious vector.

Internet Resources

http://www.ambion.com/catalog

Provides much information on RNA, RNAi, and enzymes for in vitro RNA procedures.

http://www.esi-topics.com/genesil

Summarizes publication and citation data from ISI Essential Science Indicators for the analysis of research trends and performance in gene silencing.

http://tgi.ncsu.edu

Provides information on the progress of the Tobacco Genome Initiative, launched in 2003, with the aim to sequence the whole genome of Nicotiana tabacum.

Contributed by Véronique Gosselé and Michael Metzlaff
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Gent, Belgium

Using Viral Vectors to Silence Endogenous Genes

Over the past decade, researchers have developed various methods for exploiting viruses to study gene function. This unit will explain how viral vectors are used for virus-induced gene silencing (VIGS) of an endogenous gene. Many different VIGS vectors have been developed over the past decade, including vectors based on tobacco mosaic virus (TMV), potato virus X (PVX), tomato golden mosaic virus (TGMV), and tobacco rattle virus (TRV). TRV-based silencing provides the most robust, persistent, and consistent silencing results without causing severe viral symptoms; therefore, protocols optimized for TRV-based silencing in plant species belonging to the family *Solanaceae*, of which tobacco and the tomato are representatives, are provided in this unit. TRV is a bipartite virus and requires two vectors containing either RNA1 or RNA2. RNA1 encodes two replicases, a movement protein, and a small (16-kDa) protein of unknown function (Fig. 16I.6.1). RNA2 encodes the coat protein and two nonessential proteins. The nonessential proteins in RNA2 were removed and replaced with a multiple cloning site that can be used to insert the gene of interest (Fig. 16I.6.2). The protocols below rely on transforming *Agrobacterium tumefaciens* with a modified TRV vector containing a segment of a gene targeted for VIGS. *Agrobacterium* is used as delivery system for the TRV-based binary vectors to avoid laborious in vitro transcription of viral RNA and inoculation

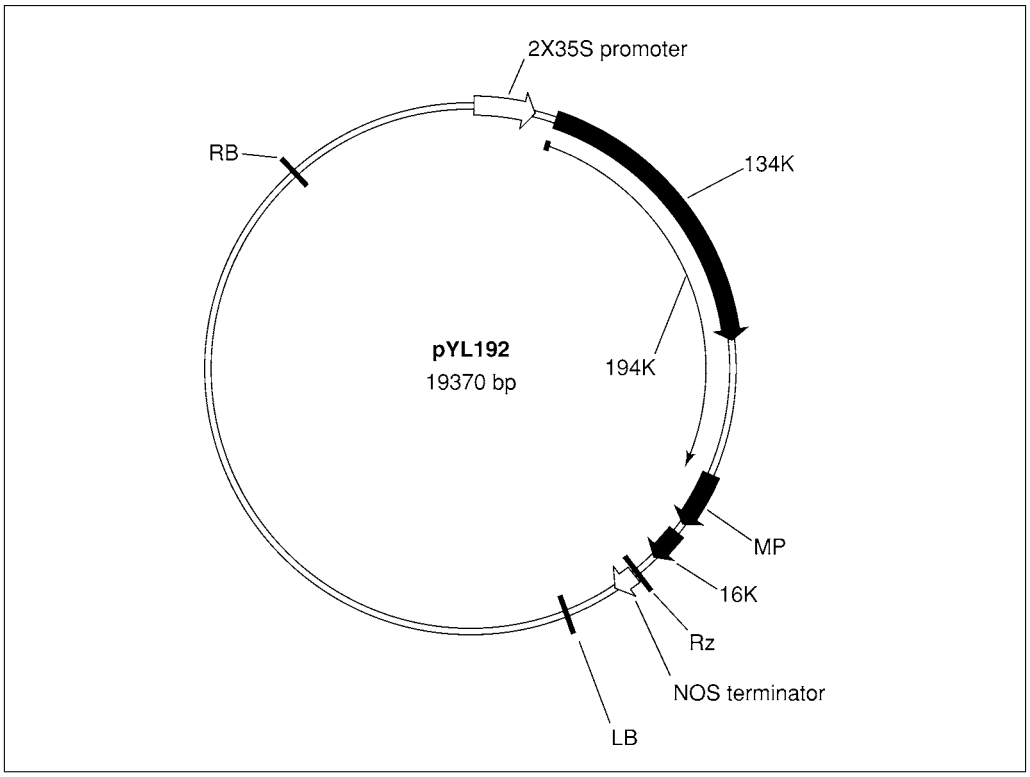

Figure 16I.6.1 Physical map of the tobacco rattle virus RNA1 vector pYL192. The RNA1 vector in *Agrobacterium* must be coinfiltrated with an RNA2 vector. Abbreviations: RB, right border; LB, left border; 2X35S promoter, two copies of the cauliflower mosaic virus (CaMV) 35S promoter; 134K, 134-kDa replicase; 194K, 194-kDa replicase translated as a read-through of the 134K stop codon; MP, movement protein; 16K, 16-kDa protein; Rz, ribozyme; NOS terminator, nopaline synthase terminator.

Contributed by Jeffrey Caplan and S.P. Dinesh-Kumar
Current Protocols in Microbiology (2006) 16I.6.1-16I.6.13

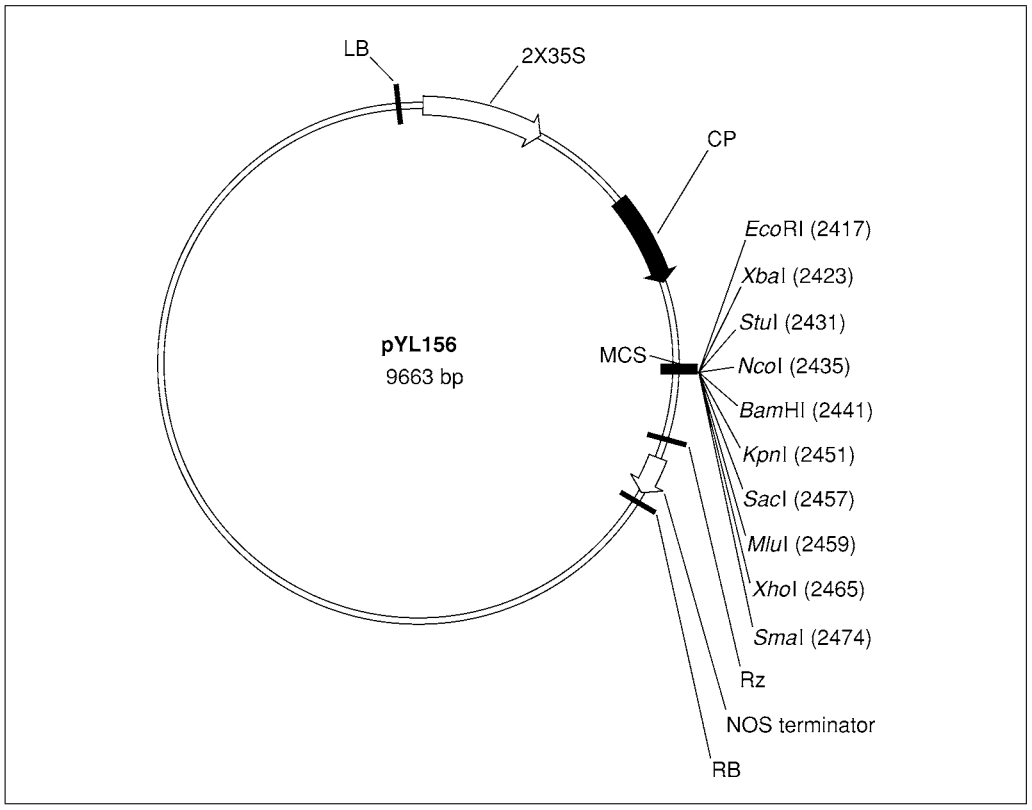

Figure 16I.6.2 Physical map of the tobacco rattle virus RNA2 vector pYL156. A fragment of the gene of interest will be inserted into the multiple cloning site. Abbreviations: RB, right border; LB, left border; 2X35S promoter, two copies of the cauliflower mosaic virus (CaMV) 35S promoter; CP, coat protein; MCS, multiple cloning site; Rz, ribozyme; NOS terminator, nopaline synthase terminator.

with unstable RNA. The four protocols described below differ in the method used for inoculation of *Agrobacterium* into the plant. Basic Protocol 1 introduces the *Agrobacterium* via syringe infiltration (agroinfiltration), and is the most robust method for VIGS in *Nicotiana benthamiana*. Basic Protocol 2 uses an *Agrobacterium* drench (agrodrench) method that has been tested in seven different *Solanaceae* species. This unit also describes two alternative methods which result in higher success rates when silencing in *Lycopersicon esculentum* (tomato). Alternate Protocol 1 inoculates *Agrobacterium* by a spray-inoculation method, and Alternate Protocol 2 inoculates *Agrobacterium* via a vacuum-infiltration method. These protocols are complemented by support protocols for culturing *Agrobacterium* for VIGS (Support Protocol 1), construction of a TRV vector containing a segment of target gene for silencing (Support Protocol 2), and growing the two plants most commonly used for VIGS, *N. benthamiana* (Support Protocol 3) and *L. esculentum* (Support Protocol 4).

CAUTION: Agrobacterium is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans, and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

VIRUS-INDUCED GENE SILENCING BY SYRINGE INFILTRATION

BASIC PROTOCOL 1

The syringe-infiltration method results in a silencing success rate near 100% in *N. benthamiana* (Liu et al., 2002a). The protocol uses the *Agrobacterium* strain GV2260; however, either GV2260 or GV3101 may be used for VIGS in *N. benthamiana*. In tomato, the silencing success rate of this protocol is between 20% and 30% using GV3101, and very low (<10%) using GV2260.

Materials

Agrobacterium GV2260 containing pTRV1 vector (Support Protocol 1)
Agrobacterium GV2260 containing pTRV2 with target gene (pTRV2-TARGET; Support Protocol 1)
VIGS agroinfiltration medium (see recipe)
N. benthamiana or tomato plants (Support Protocol 2 or 3)
1-ml syringe without needle

1. Separately resuspend pelleted *Agrobacterium* GV2260 (see last step of Support Protocol 1) containing the TRV1 or TRV2-TARGET vector in 5 ml VIGS agroinfiltration medium.

   For discussion of controls, see Critical Parameters.

2. Using VIGS agroinfiltration medium, adjust the OD_{600} to 1.0 for *N. benthamiana* or 1.5 to 2.0 for tomato.

3. Incubate cultures 4 to 6 hr at room temperature.

4. Mix the cultures of *Agrobacterium* containing pTRV1 and pTRV2 vectors in a 1:1 ratio.

5. Load a 1-ml syringe (without needle) with the mixture of *Agrobacterium*.

6. Using the corner of a new razor blade, make a 0.1-mm hole in the two lower leaves of *N. benthamiana* or tomato plants.

7. Place the opening of the 1-ml syringe (without needle) containing the *Agrobacterium* mixture over the hole.

8. Using a finger from the other hand, apply light pressure on the opposite face of the leaf to form a seal between the tip of the syringe and the leaf. Squeeze the syringe to infiltrate the *Agrobacterium* mixture into the leaf.

   The penetration of the Agrobacterium into the plant cells can be clearly seen on the underside of the leaf.

9. Repeat steps 5 to 8 until the entire two lower leaves are infiltrated with the *Agrobacterium* mixture.

10. Maintain the growth temperature at 25°C for *N. benthamiana* and between 18° to 21°C for tomato.

11. Quantify or visualize VIGS of the target gene (see Critical Parameters) at 6 to 10 days post infiltration of *Agrobacterium*.

VIRUS-INDUCED GENE SILENCING BY AGRODRENCH INOCULATION

BASIC PROTOCOL 2

The agrodrench method was developed by Ryu et al. (2004) and is a simple, efficient, and fast inoculation method for *Agrobacterium* in VIGS experiments. *Agrobacterium* is grown to an OD_{600} of 1.0, then 3 to 5 ml of the TRV-containing *Agrobacterium* is applied to the crown of the plant. The *Agrobacterium* drenches the soil around the roots and eventually infects the plant and integrates the TRV vectors. The technique has been

systematically tested in many different members of the Solanaceae family. The silencing success rate is near 100% in *N. benthamiana* and between 60% and 70% in tomato, *N. tabacum* (tobacco), *Petunia hydrida*, *Solanum tuberosum* (potato), *Capsicum annuum* (pepper), and eggplant (Ryu et al., 2004). The agrodrench method does not require fully expanded leaves; consequently, VIGS can be conducted on plants as young as 1 week old. Furthermore, because the agrodrench method is faster and requires fewer materials than the other protocols described in this unit, it may be the best method for high-throughput VIGS-based forward genetic screens.

Materials

Agrobacterium GV2260 containing pTRV1 vector (Support Protocol 1)
Agrobacterium GV2260 containing pTRV2 with target gene (pTRV2-TARGET; Support Protocol 1)
VIGS agroinfiltration medium (see recipe)
Plant of *Solanaceae* family: *N. benthamiana* (Support Protocol 3), tomato (Support Protocol 4), pepper, potato, petunia, or eggplant
10-ml pipet

1. Separately resuspend pelleted *Agrobacterium* GV2260 (see last step of Support Protocol 1) containing the TRV1 or TRV2-TARGET vector in 5 ml VIGS agroinfiltration medium.

   For discussion of controls, see Critical Parameters.

2. Adjust the OD_{600} of each suspension to 1.0 with VIGS agroinfiltration medium.
3. Incubate cultures 4 to 6 hr at room temperature.
4. Mix the cultures of *Agrobacterium* containing pTRV1 and pTRV2 vectors in a 1:1 ratio.
5. Fill a 10-ml pipet with the *Agrobacterium* mixture.
6. Apply 3 to 5 ml of the *Agrobacterium* mixture to the crown of the plant.
7. Maintain temperature between 18°C to 21°C for tomato and between 24° to 26°C for all other *Solanaceae* species.
8. Quantify or visualize VIGS of the target gene (see Critical Parameters) 10 to 14 days post drenching of *Agrobacterium*.

ALTERNATE PROTOCOL 1

VIRUS-INDUCED GENE SILENCING BY SPRAY INOCULATION

Although VIGS in *N. benthamiana* provides the most robust and efficient silencing, cloning the genes to silence from *N. benthamiana* is often challenging. In contrast, VIGS in tomato (*L. esculentum*) is less efficient and more difficult, but the large repository of tomato expressed sequence tags (ESTs) makes it easier to clone genes for silencing. Furthermore, tomato may be a better model organism for studying certain biological questions.

This protocol details a method for introducing *Agrobacterium* into *L. esculentum*. Although the protocol requires additional equipment, the success rate of silencing is greater than 90% in tomato (Liu et al., 2002b) and *S. tuberosum* (Brigneti et al., 2004).

Materials

Agrobacterium GV3101 containing pTRV1 vector (Support Protocol 4)
Agrobacterium GV3101 containing pTRV2-TARGET vector (Support Protocol 4)
VIGS agroinfiltration medium (see recipe)
Carborundum

Tomato plants (see Support Protocol 4)

Artist's airbrush (Model V180; Paasche; *http://www.paascheairbrush.com*)
Portable air compressor (Campbell Hausfeld; *http://www.chpower.com/*)

CAUTION: Carborundum may cause eye, skin, respiratory-tract, or digestive-tract irritation, as well as lung damage. It may cause cancer, based on the results of animal studies. Read the Material Safety Data Sheet (MSDS) before use.

1. Separately resuspend pelleted *Agrobacterium* GV3101 (see last step of Support Protocol 1) containing the TRV1 or TRV2-TARGET vector in 5 ml VIGS agroinfiltration medium.

   For discussion of controls, see Critical Parameters.

2. Adjust the OD_{600} of each suspension to 2.0 with VIGS agroinfiltration medium.
3. Incubate the cultures for 4 to 6 hr at room temperature.
4. Mix the cultures of *Agrobacterium* containing pTRV1 and pTRV2 vectors in a 1:1 ratio.
5. Add a pinch (~75 to 100 mg) of carborundum to the mixed culture.
6. Attach an artist's airbrush to a pressure compressor set to 80 psi. Load the *Agrobacterium* mixture into the reservoir of the airbrush.
7. Spray the underside of the two lower leaves of each tomato plant for ~1 to 5 sec from a distance of ~8 in. (~20 cm). Shake the suspension in the airbrush reservoir periodically to prevent settling of the carborundum.
8. Maintain growth temperature between 18° to 21°C.
9. Visualize or quantify VIGS of the target gene (see Critical Parameters) at 8 to 10 days post infiltration of *Agrobacterium*.

VIRUS-INDUCED GENE SILENCING BY VACUUM INFILTRATION

ALTERNATE PROTOCOL 2

A second effective method for *Agrobacterium* inoculation into tomato is via vacuum infiltration (Ekengren et al., 2003). The silencing success rate of vacuum infiltration is 90% to 100%, and provides the most uniform and robust silencing in tomato.

Materials

Agrobacterium GV3101 containing pTRV1 vector (Support Protocol 1)
Agrobacterium GV3101 containing pTRV2-TARGET vector (Support Protocol 1)
VIGS agroinfiltration medium (see recipe)
Tomato plants (see Support Protocol 4)
Silwet L-77

500-ml plastic beaker
Desiccator (240 mm diameter; Bel-Art Co.)
Vacuum pump (Model FB DVP0352; Fisher)

1. Separately resuspend pelleted *Agrobacterium* GV3101 (see last step of Support Protocol 1) containing the TRV1 or TRV2-TARGET vector in 5 ml VIGS agroinfiltration medium.

   For discussion of controls, see Critical Parameters.

2. Adjust the OD_{600} of the pTRV1 culture to 0.4 and that of the pTRV2 culture to 0.2 with VIGS agroinfiltration medium.
3. Incubate the cultures 4 to 6 hr at room temperature.

4. Mix the cultures containing pTRV1 and pTRV2 vectors in a 1:1 ratio. Transfer the culture mixture to a 500-ml plastic beaker. Add Silwet L-77 to a final concentration of 0.04% (v/v).

   It is not necessary to completely fill the beaker. Mixed Agrobacterium from two 50-ml cultures is sufficient.

5. Completely submerge the tomato plant by placing it upside down in the plastic beaker and immediately continue to the next step.

6. Place the beaker containing the submerged plant into a vacuum desiccator. Attach the desiccator to the vacuum pump and pull with a maximum vacuum of 29.5 in. Hg (749.3 mmHg) for 90 sec.

7. Maintain growth temperature between 18° to 21°C.

8. Visualize or quantify VIGS of the target gene (see Critical Parameters) at 6 to 10 days post infiltration of *Agrobacterium*.

SUPPORT PROTOCOL 1

GROWTH OF *AGROBACTERIUM* CONTAINING EITHER TRV1 OR TRV2 VECTOR FOR VIRUS-INDUCED GENE SILENCING

Follow this support protocol for *Agrobacterium* growth to prevent the loss of the helper vector containing the virulence genes (*vir*) or the TRV vector.

Materials

TRV1 or TRV2 vector (available by request from Dr. S. Dinesh-Kumar; *savithramma.dinesh-kumar@yale.edu*)
Kit for BP Gateway or LR Gateway reactions (Invitrogen)
Agrobacterium strain GV2260 or GV3101
LB plates (*APPENDIX 4A*) containing 50 mg/liter kanamycin, 25 mg/liter rifampicin, 50 mg/liter streptomycin, and 50 mg/liter carbenicillin (for strain GV2260) *or* 50 mg/liter kanamycin and 15 mg/liter gentamicin (for strain GV3101)
LB medium (*APPENDIX 4A*) containing 50 mg/liter kanamycin, 25 mg/liter rifampicin, 50 mg/liter streptomycin, and 50 mg/liter carbenicillin (for strain GV2260) *or* 50 mg/liter kanamycin and 15 mg/liter gentamicin (for strain GV3101)
200 mM acetosyringone (see recipe)
1 M MES (see recipe)
Additional reagents and equipment for transformation of *Agrobacterium* (*UNIT 16B.2*)

1. Transform the TRV1 or TRV2 vector into either *Agrobacterium* strain GV2260 or GV3101.

2a. *For TRV vectors in Agrobacterium GV2260:* Streak *Agrobacterium* containing either the TRV1 or TRV2 vector on LB plates containing 50 mg/liter kanamycin, 25 mg/liter rifampicin, 50 mg/liter streptomycin, and 50 mg/liter carbenicillin.

2b. *For TRV vectors in Agrobacterium GV3101:* Streak *Agrobacterium* containing either the TRV1 or TRV2 vector on LB plates containing 50 mg/liter kanamycin and 15 mg/liter gentamycin.

3. Grow plates for 1 to 2 days at 28°C.

4a. *For TRV vectors in Agrobacterium GV2260:* Inoculate a colony from the plate into 5 ml LB medium containing kanamycin (50 mg/liter), rifampicin (25 mg/liter), streptomycin (50 mg/liter), and carbenicillin (50 mg/liter).

4b. *For TRV vectors in Agrobacterium GV3101:* Inoculate a colony from the plate into 5 ml LB medium containing kanamycin (50 mg/liter) and gentamycin (15 mg/liter).

5. Grow cultures overnight at 28°C.
6. Transfer the 5 ml culture into 50 ml fresh liquid LB medium containing the same antibiotics used in step 4a or 4b. Supplement the culture with 500 μl of 1 M MES (10 mM final) and 5 μl of 200 mM acetosyringone (20 μM final).
7. Grow cultures overnight at 28°C.
8. The following day, centrifuge 10 min at 3000 × *g*, room temperature, to pellet the *Agrobacterium*.

CONSTRUCTION OF TRV VECTOR CONTAINING TARGET GENE FOR VIRUS-INDUCED GENE SILENCING

SUPPORT PROTOCOL 2

A gene of interest may be silenced by inserting a piece of the target gene sequence into a TRV2 vector such as pYL156 or pYL279 (Liu et al., 2002a,b). The following protocol provides brief instructions for creating a TRV2-TARGET vector and transforming it into *Agrobacterium*.

The pYL156 vector is an improved TRV2 cloning vector. It contains a double 35S promoter and a ribozyme at the 3′ end, which increase silencing efficiency and infectivity, respectively. pYL279 is designed to work with the Gateway cloning system (Invitrogen) and is optimal for high-throughput cloning of genes for VIGS.

Materials

Target gene of interest
TRV2 vector pYL156 or pYL279 (available by request from Dr. S. Dinesh-Kumar; *savithramma.dinesh-kumar@yale.edu*)
pDONR221 (Invitrogen)
Competent *Agrobacterium* strain GV2260 or GV3101 (available by request from Dr. S. Dinesh-Kumar; *savithramma.dinesh-kumar@yale.edu*)
LB plates (*APPENDIX 4A*) containing 50 mg/liter kanamycin, 25 mg/liter rifampicin, 50 mg/liter streptomycin, and 50 mg/liter carbenicillin (for strain GV2260) *or* 50 mg/liter kanamycin and 15 mg/liter gentamicin (for strain GV3101)
Additional reagents and equipment for PCR (Kramer and Coen, 2001), restriction digestion (Bloch and Grossman, 1995) and ligation (Tabor, 1987), and transformation of *Agrobacterium* (*UNIT 16B.2*)

1. Design primers to amplify a 500 to 700 bp segment of the target gene to be silenced. Amplify the gene (Kramer and Coen, 2001).

   Cloning into pYL156 or pTV00 will require the addition of restriction sites to the 5′ ends of the primers. Use unique restriction sites located in the multiple cloning site (Fig. 16I.5.2).

   Cloning into pYL279 will require the addition of att-B1 (GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TNN) to the forward primer and att-B2 (GGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTN) to the reverse primer.

2. Clone the fragment into the TRV2 vector pYL156 or pYL279. If cloning into TRV2 vector pYL156, cut the fragment and the vector with appropriate restriction enzymes (Bloch and Grossman, 1995) and ligate (Tabor, 1987). If cloning into pYL279, transfer the fragment into pDONR221 entry clone using a BP Gateway reaction. Next, transfer the fragment from pDONR221 to pYL279 using an LR Gateway reaction.

   Refer to http://www.invitrogen.com/content/sfs/manuals/pdonr_man.pdf for more information about using the pDONR221 plasmid in Gateway reactions.

3. Transform the TRV2 construct into competent *Agrobacterium* strain GV2260 or GV3101 by electroporation (*UNIT 16B.2*).

4a. *For strain GV2260:* Select transformants on LB plates containing 50 mg/liter kanamycin, 25 mg/liter rifampicin, 50 mg/liter streptomycin, and 50 mg/liter carbenicillin.

4b. *For strain GV3101:* Select transformants on LB plates containing 50 mg/liter kanamycin and 15 mg/liter gentamycin.

5. Grow cells at 28°C.

   Colonies will take ~2 days to form.

6. Check transformants by colony polymerase chain reaction (cPCR).

   Protocols for cPCR can be found at http://www.protocol-online.org.

SUPPORT PROTOCOL 3

GROWTH OF *N. BENTHAMIANA* PLANTS FOR VIRUS-INDUCED GENE SILENCING

The four-leaf stage is the optimal stage for VIGS in *N. benthamiana*.

Materials

N. benthamiana seeds
Super Fine Germinating Mix (Conrad Fafard, Inc.; *http://www.fafard.com*)
Professional Growth Medium No. 2 (Conrad Fafard, Inc.; *http://www.fafard.com*)
1-pint (~500-ml) pots, 4 in. (~10 cm) square
Pot trays
Clear plastic domes
Light source (40-W Gro-Lux fluorescent light bulb, Sylvania)

1. Place germination growth medium into a 1-pint pot. Spread approximately 40 to 50 *N. benthamiana* seeds onto the surface of the germination growth medium. Place pot on a tray and cover with a clear plastic dome.
2. Remove plastic dome immediately after seeds germinate.
3. Grow seedlings under continuous light at 24° to 26°C for 10 to 12 days. Water plants every 2 to 3 days by adding 0.5 to 1 in. (1.25 to 2.5 cm) of water to the pot trays.
4. Transplant seedlings into individual 1-pint pots containing professional growth medium and cover with clear plastic dome for 2 to 3 days.
5. Remove plastic dome and grow plants until they reach the four-leaf stage (~3 weeks).

SUPPORT PROTOCOL 4

GROWTH OF TOMATO PLANTS FOR VIRUS-INDUCED GENE SILENCING

The two-leaf stage is the optimal stage for VIGS in tomato.

Materials

VF36 or MicroTom tomato seeds (Tomato Genetics Resource Center, TGRC; *http://tgrc.ucdavis.edu)*
Professional Growth Medium No. 2 (Conrad Fafard, Inc.; *http://www.fafard.com*)
Vermiculite
1-pint (~500-ml) pots, 4 in. (~10 cm) square
Pot trays
Clear plastic domes
Light source (40-W Gro-Lux fluorescent light bulb, Sylvania)

1. Plant 1 to 2 VF36 or MicroTom tomato seeds per 1-pint pot containing professional growth media. Cover with 0.5 in. (1.27 cm) of vermiculite. Place pots on a tray and cover with a clear plastic dome.
2. Remove plastic dome immediately after seeds germinate.
3. Germinate seeds under a 16-hr light/8-hr dark cycle at 24° to 26°C for 12 to 14 days.
4. Keep one seedling per 1-pint pot.
5. Grow plants to the two-leaf stage (~4 weeks).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetosyringone, 200 mM

Dissolve 0.1962 g of 3′,5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone) in 5 ml dimethylformamide (DMF).

It is advisable to make this stock fresh; however, it may be stored for up to one month at −20°C.

MES, 1 M

Add 19.62 g of 2-[*N*-morpholino]ethanesulfonic acid (MES) to 100 ml water. Store up to several months at room temperature.

VIGS agroinfiltration medium

10 ml 1 M $MgCl_2$ (10 mM final)
10 ml 1 M MES (see recipe; 10 mM final)
0.75 ml 200 mM acetosyringone (see recipe; 150 μM final)
Prepare fresh (do not store)

A 1 M solution of $MgCl_2$ can be prepared by adding 20.33 g $MgCl_2 \cdot 6H_2O$ to 100 ml of water. Store this stock up to several months at room temperature.

COMMENTARY

Background Information

Viruses are some of the most insidious and devastating plant pathogens. They harbor extraordinary diversity, ranging from DNA to RNA genomes and from small monopartite to large tripartite capsulation (Hull, 2002). Millions of years of plant evolution have resulted in a sophisticated arsenal of antiviral defense mechanisms. One of the most effective antiviral defenses is post-transcriptional gene silencing (PTGS), which is similar to quelling and RNA interference (RNAi; Waterhouse and Helliwell, 2003). The PTGS defense system is activated by dsRNA originating from transgenes or viruses. The dsRNA is cleaved into short, 23-nucleotide segments called short interfering RNA (siRNA) that associate with an RNAi silencing complex (RISC). The RISC complex is guided by the siRNA to degrade RNA containing 100% homology to the siRNA (Meister et al., 2004).

This endogenous PTGS system has been exploited by scientists to study gene function. Kumagai et al. (1995) discovered that recombinant TMV carrying a segment of *phytoene desaturase* (*PDS*) sequence could cause VIGS of the endogenous *PDS* gene in *N. benthamiana.* However, TMV infection causes severe disease symptoms and has varying levels of silencing efficiency. Consequently, VIGS vectors were quickly developed from four RNA viruses, one RNA satellite virus, and two DNA viruses (Burch-Smith et al., 2004).

VIGS was pioneered in *N. benthamiana* using vectors based on TMV, PVX, and TGMV. However, most VIGS in *N. benthamiana* is now done with TRV-based vectors. More persistent and efficient silencing can be obtained with TRV-based VIGS because TRV spreads quickly through the plant and causes minor viral symptoms (Burch-Smith et al., 2004). TRV has a large host range and has been used for

VIGS in seven different *Solanaceae* species (Ryu et al., 2004). VIGS in *Arabidopsis* may be conducted using a geminivirus, i.e., the cabbage leaf curl virus (CbLCV; Turnage et al., 2002). In tobacco, a more efficient VIGS has been observed using a bipartite system consisting of a satellite virus and a TMV helper virus (Gossele et al., 2002). For silencing in monocots, the only vector available is based on the Barley stripe mosaic virus (BSMV; Holzberg et al., 2002).

VIGS has many advantages and disadvantages. The most obvious disadvantage of VIGS is that it only down-regulates a gene and does not completely knock it out; therefore, negative results using VIGS are often inconclusive. The lack of an observable phenotype may be caused by incomplete silencing. Additionally, it may be difficult to determine if VIGS is down-regulating one gene or multiple highly homologous genes. Furthermore, VIGS is a transient assay and does not result in a stable loss-of-function line.

The advantages of VIGS often outweigh the disadvantages. VIGS is an extremely powerful technique that can overcome many of the limitations of traditional mutagenesis. For example, VIGS may be used to study highly homologous genes with redundant function that are often missed by traditional mutagenesis screens. VIGS does not require expensive mapping and sequencing. This not only makes VIGS cheaper and faster, but makes it possible to study plant species in which it is extremely difficult to map mutations because they are tetraploid, aneuploid, or lack a sequenced genome. Moreover, the gene function between divergent plant ecotypes, species, or even families can be quickly compared. The speed of VIGS is probably its strongest feature. It does not require the time-consuming generation of transgenic lines or mapping. Therefore, VIGS can be used for forward genetic screens and functional genomics (Burch-Smith et al., 2004).

Critical Parameters and Troubleshooting

VIGS in plant species outside of the Solanaceae family

The protocols in this unit are optimized for VIGS in the *Solanaceae* plant family. TRV has a broad host range, but it may not spread rapidly and uniformly in all species. Thus, a VIGS vector system should be chosen and optimized for each plant species. A VIGS vector that does not include any strong suppressors of silencing should be chosen.

Growth conditions

Growth conditions can affect silencing efficiency and reproducibility. Light and temperature are the two most important variables and should be kept constant between experimental sets. Conduct seed germination, plant growth, and gene silencing in growth chambers that have controlled light and temperature. VIGS in *Nicotiana* sp. is favored by continuous light, while VIGS in other *Solanaceae* species requires a 16 hr light/8 hr dark cycle.

The optimal temperature for *Agrobacterium* T-DNA insertion and VIGS may differ. Lower temperatures between 16° to 21°C may result in greater efficiency of T-DNA insertion, and, therefore, a higher success rate of VIGS. VIGS in tomato is more sensitive to high temperature because the silencing success rate drops dramatically at temperatures above 21°C. Conversely, higher temperatures, above 24°C, have been shown to increase the abundance of siRNAs that are associated with VIGS (Szittya et al., 2003). Thus, the protocols provided here may be optimized by inoculating the *Agrobacterium* at 16° to 21°C and then shifting the plants to a higher temperature 24° to 26°C after 1 to 2 days. The authors of this unit have not tested this protocol, but it should be considered if optimizing VIGS for a new plant species.

Age or developmental stage

The age of the plant inoculated with *Agrobacterium* can affect silencing efficiency. The four-leaf and two-leaf stages are the optimal ages for *N. benthamiana* and tomato respectively. Syringe infiltration of younger leaves in *N. benthamiana* may result in severe necrosis of the inoculated leaf. If the VIGS phenotype needs to be observed at a younger stage in development, then use the agrodrench method (Basic Protocol 2).

Choosing a gene segment for silencing

In order for VIGS to efficiently silence an endogenous gene, a fragment of the gene must be chosen. In theory, 23 nucleotides of complete identity should be sufficient for silencing. In practice, a fragment between 300 and 1500 bp should be cloned into the TRV2 vector. Below 300 bp in length, the silencing efficiency is greatly reduced. Above 1500 bp in length, the insert may be lost from the virus and viral movement or replication may be impaired. The optimal size of the insert is 500 to 700 bp. At least 30 bp of the mRNA target sequence should be excluded from the construct, so RT-PCR may be used to check the silencing efficiency. Design two primers to check

the silencing efficiency by RT-PCR. The first primer must be outside of the gene segment chosen for VIGS. The second primer for RT-PCR may be outside or within the segment chosen for VIGS.

If silencing a gene results in lethality before the desired phenotype can be observed, a shorter segment may decrease the silencing efficiency and severity of the phenotype. Alternatively, the gene region used for VIGS may be cloned from a plant species within the same family. For example, using a tomato gene to silence an *N. benthamiana* gene often results in a lower silencing efficiency (Liu et al., 2004). Alternatively, a less efficient VIGS vector system, such as PVX, will result in lower silencing efficiency.

VIGS may be used to suppress several highly homologous genes or a single gene. To silence a family of homologous genes that may have functional redundancy, clone a highly conserved region of the gene into TRV2. To silence a single gene that has highly conserved homologs, clone a segment of the untranslated region (UTR) or a segment of the gene that does not contain stretches of exact identity greater than 22 nucleotides.

Agrobacterium strains, growth, and inoculation

The optimal *Agrobacterium* strain for VIGS varies with different plant species. Usually, the best strain for creating transgenic lines is the best strain for introducing the VIGS vectors into the plant. However, it is advisable to test multiple *Agrobacterium* strains if applying VIGS to a new plant species. GV2260 is the best for VIGS in *N. benthamiana* and GV3101 is the best for VIGS in tomato.

Always grow *Agrobacterium* on an LB plate containing the required antibiotics before inoculating a liquid culture. Inoculation of a liquid culture from a glycerol stock can cause inconsistent results. Alternatively, the *Agrobacterium* may be spread evenly on an LB plate containing antibiotics and harvested by scraping and then resuspending in infiltration media. This has been tested for the syringe-infiltration protocol and the agrodrench protocol (Ryu et al., 2004). *Agrobacterium* should always be grown at temperatures at or below 28°C for no longer than 2 days. High temperatures and an excess of antibiotics may result in the loss of the helper plasmid containing the virulence (*vir)* genes or the TRV vector.

It is essential to determine the concentration of *Agrobacterium* that results in sufficient silencing but does not cause necrosis of the inoculated leaves. Higher concentrations of *Agrobacterium* generally results in a higher success rate of silencing (Ryu et al., 2004). *Agrobacterium* at an OD_{600} greater than 1.0 or 2.0 may cause severe necrosis in *N. benthamiana* or tomato respectively.

The optimal *Agrobacterium* inoculation method varies with different plant species. The syringe infiltration is the best inoculation method for *N. benthamiana*. Conversely, the spray-inoculation or vacuum-infiltration methods provide better silencing results in tomato. It is also possible to transcribe viral RNA in vitro and inoculate plants by rubbing the viral RNA onto the leaves (Holzberg et al., 2002).

Controls

It is essential to include controls to test the efficiency and reproducibility of VIGS. TRV2 without a gene insert should be used as negative control. TRV2 containing a segment of the *PDS* gene is a good positive control. Silencing of *PDS* causes white bleaching that is easily visible with the naked eye, and, therefore, the efficiency and location of VIGS can be qualitatively observed (Kumagai et al., 1995). Silencing the *SU* gene necessary for chlorophyll production also produces easily discernable visible phenotypes (Peele et al., 2001). The efficiency of VIGS should be quantitatively determined by RT-PCR. Levels of EF1α and tubulin mRNA should not be altered by VIGS, and, therefore, they are good negative controls for VIGS. The silencing efficiency may be gauged by RT-PCR of a positive control, such as *PDS*. If the VIGS of *PDS* is low, then the plant growth conditions, *Agrobacterium* inoculation protocol, and the VIGS vector system should be optimized.

If the RT-PCR analysis suggests the VIGS of *PDS* was efficient, but the VIGS of the target gene was inefficient, then the construction of TRV-TARGET vector should be analyzed. A larger fragment of the target gene may lead to more efficient silencing.

Anticipated Results

It is nearly impossible to obtain complete knockout of a gene using VIGS. A gene's transcript level is often reduced by 75% to 95% with VIGS. This level of down-regulation is often sufficient to disrupt a gene's function. The phenotype may be a loss of function or a reduction of function. The results may vary depending on the plant species, age, or the gene targeted by VIGS.

VIGS has the highest success rate and silencing efficiency in *N. benthamiana*. If the syringe-infiltrate method for VIGS in *N. benthamiana* is used, then a success rate near 100% and a silencing efficiency between 85% and 95% can be expected. VIGS of *NbCTR1* (constitutive triple response 1) and *NbEDS1* (enhanced disease susceptibility 1) resulted in a >95% reduction of their endogenous mRNA (Liu et al., 2002a,b). However, the silencing efficiency varies with different genes and constructs. For example, a reduction of only 78% was obtained when silencing with TRV2-*NPR1/NIM1* (Liu et al., 2002a).

If the spray-infiltration method is used for VIGS in tomato, a success rate >90% and a silencing efficiency between 75% and 85% can be obtained. Endogenous *CTR1* was reduced by 85% and the highly transcribed ribulose bisphosphate carboxylase (RbcS) was reduced by 76% using TRV-based VIGS (Liu et al., 2002b). Similar results were obtained in *Solanum* species and other members of the *Solanaceae* family (Brigneti et al., 2004; Ryu et al., 2004).

Figure 16I.5.3 shows the effects of VIGS of endogenous genes in *N. benthamiana* plants.

Time Considerations

The amount of time it takes to complete a VIGS experiment is dependent on the plant's generation time and the developmental stage at which the silencing phenotype can be observed. Silencing in *N. benthamiana* or tomato can be completed in ~4 to 5 weeks.

It is important to consider the amount of time it takes to transform the TRV2 vector into *Agrobacterium*. Transformations should be done ~1 week before the plants are ready for silencing. A time commitment of 1 day will be required for the transformation, 2 days for the transformed *Agrobacterium* to grow,

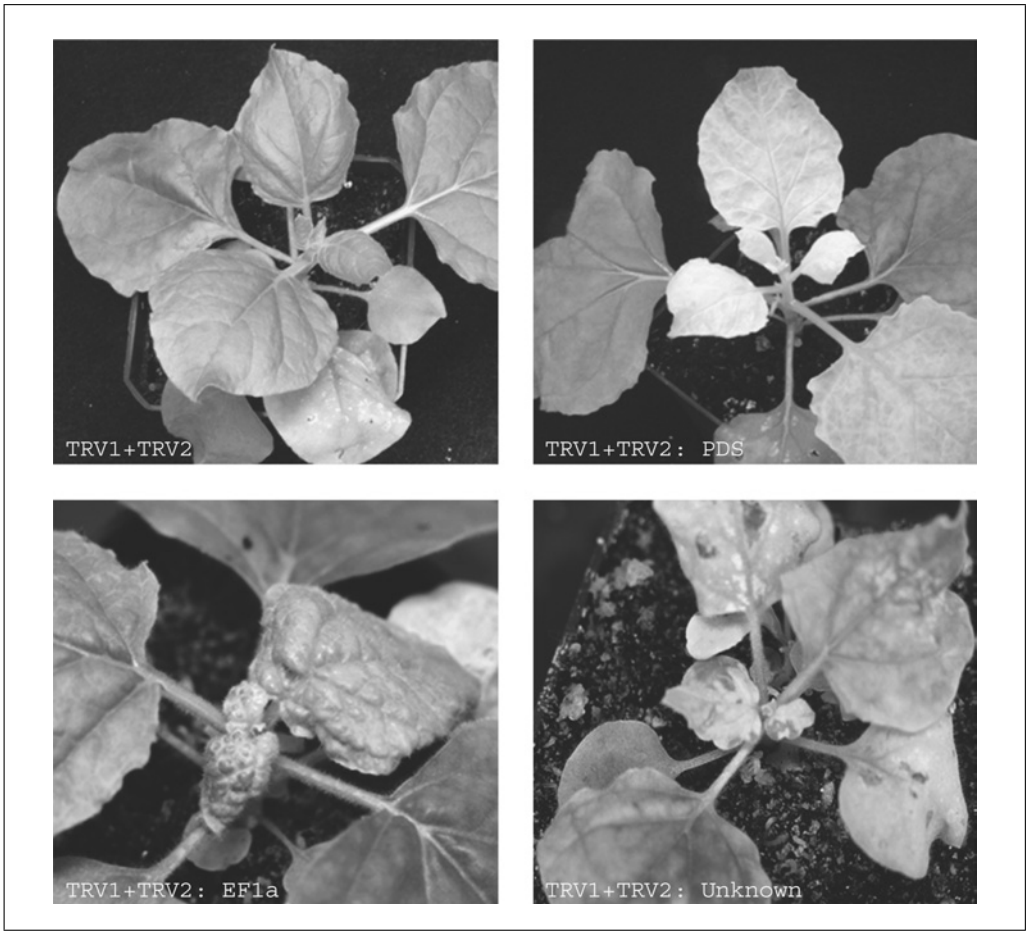

Figure 16I.6.3 VIGS of endogenous genes in *N. benthamiana*. Pictures were taken 9 days post syringe infiltration of TRV1- and TRV2-vector-containing *Agrobacterium*. All of the TRV2 constructs were coinfiltrated with TRV1. Empty TRV2 vector was used as a control. TRV2 containing phytoene desaturase (PDS) resulted in bleaching of the leaves. TRV2 containing elongation factor 1a (EF1a) or an uncharacterized gene caused severe stunting and deformation of the leaf structure. For the color version of this figure go to *http://www.currentprotocols.com*.

1 day for cPCR to check the transformation, and another 2 days for growth in liquid culture.

The best stopping point in the protocols provided in this unit is the 4- to 6-hr induction period. A 4- to 6-hr induction at room temperature is optimal; however, this time may be extended. In *N. benthamiana*, the silencing success rate was still 100% after a 12-hr overnight induction at room temperature using the syringe-infiltration method of agroinoculation (J. Caplan, unpub. observ). If the cells are induced for longer than 6 hr, the *Agrobacterium* should be pelleted and resuspended in fresh VIGS agroinfiltration medium before inoculation.

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In Vitro Translation of Plant Viral RNA

Many plant viruses have positive-strand RNA as their genetic material. These viral RNAs serve as messenger RNAs upon infection and are the templates for synthesis of proteins necessary for replication of the virus. Analysis of the translation of plant viral RNAs using in vitro protein synthesis systems are very useful for biochemical analysis of the efficiency of the RNAs and the effects of 5′ UTRs, 3′ UTRs, m^7GpppG cap groups, and other elements. The protocols described here are for the large-scale preparation of a wheat germ extract (see Basic Protocol 1) and in vitro transcription of plant viral RNAs from phage promoters (see Basic Protocol 2). The wheat germ extract is a preparation of all the soluble components of the translational apparatus and includes ribosomes, initiation factors, elongation factors, and aminoacyl tRNA synthetases. Wheat germ, the embryo of the wheat kernel, is a dormant system and as such has a very low level of endogenous messenger RNA. The use of in vitro transcription to prepare RNAs is an excellent way to prepare large amounts of RNA to translate in the wheat germ system. Variations of RNA, capped or uncapped, changes in UTRs, or mutations may be easily prepared by in vitro transcription and compared in the wheat germ extract. The translation assay described (see Basic Protocol 3) may be used to optimize the amount of extract, amount of RNA, and concentrations of Mg^{2+} and K^+ for efficient translation in vitro.

NOTE: All solutions that have been stored should be mixed well by inversion before use. Use a graduated cylinder of appropriate size for measurement and preparation. Unless otherwise indicated, store all solutions at −20°C.

PREPARATION OF WHEAT GERM EXTRACT (S30)

BASIC PROTOCOL 1

The preparation of a high-activity wheat germ extract is dependent upon several factors: the wheat germ, the extraction and purification processes, and the purity of the reagents (also see Critical Parameters and Troubleshooting). This preparation evolved from several previously described procedures for wheat germ extracts (Roberts and Paterson, 1973; Marcu and Dudock, 1974; Marcus et al., 1974; Walthall et al., 1979; Lax et al., 1986).

Materials

Wheat germ column elution buffer (see recipe)
Wheat germ extraction buffer (see recipe)
14.3 M 2-mercaptoethanol (2-ME; Sigma)
2- liter Sephadex G-25 column (see Support Protocol)
Wheat germ (e.g., Randolph & James Flax Mills), chilled: store up to 6 months at −20°C
Soybean trypsin inhibitor (STI; Sigma)
50 mM PMSF (see recipe)
Powdered dry ice *or* dry ice/isopropanol bath or liquid nitrogen

Blender, chilled
Slender kitchen spatula (optional)
50-ml centrifuge tubes, chilled
High-speed centrifuge (capacity up to ∼250 ml)
Cheese cloth

1. One day before beginning the preparation, prepare wheat germ column elution and wheat germ extraction buffers. Chill overnight at 4°C.

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2. On the morning of extraction, add 0.2 ml of 14.3 M 2-ME (7 mM final) to 4 liters column elution buffer. Start washing 2-liter G25 column with the buffer. Allow at least 2.0 liters to pass through the column (~45 to 75 min, then turn off until step 9.).
3. Weigh 200 g chilled wheat germ and place in a chilled blender.
4. Add 0.175 ml of 2-ME, 35 mg STI, and 3.5 ml of 50 mM PMSF to the wheat germ extraction buffer immediately prior to use.
5. Grind the wheat germ to a fine powder using four to five 15-sec bursts at high speed. Stir the wheat germ powder with a plastic stirring rod or spatula between bursts.
6. Slowly add 340 ml extraction buffer to ground wheat germ and blend 15 to 30 sec at medium speed.

   A slender kitchen spatula works well to mix the edges during addition of the extraction buffer.

   The mixture will be a thick paste.
7. Spoon the mixture into chilled 50-ml centrifuge tubes (~14 tubes) and centrifuge 20 min at 25,700 × *g*, 4°C.

   The extract should be kept at 4°C or on ice at all times.
8. Gently push aside the fatty layer on the top of the supernatant with a slender spatula and pour the liquid through a layer of cheese cloth to catch any residual fat.

   Approximately 200 ml should be recovered in a chilled beaker or graduated cylinder.
9. Layer the supernatant on top of the G25 column (step 2), taking care not to disturb the top of the resin bed. Once all of the supernatant has entered the G25 matrix, gently layer the column elution buffer on top. Reconnect buffer reservoir containing column elution buffer.

   The column separates a brown fraction containing the macromolecular components for protein synthesis from a bright yellow fraction of small molecules.
10. Collect ~20-ml column fractions when the brown fraction begins to elute from the bottom of the column and eluate appears cloudy (~0.5 hr).

    After the column run is completed, do not forget to wash the column as described (see Support Protocol). A fraction collector may be used if desired.
11. Measure the absorbance at 260 nm on a 1:100 dilution of every other fraction. Pool fractions containing >90 A_{260}.

    An alternative method is to take six to eight 20-ml fractions until the A_{260} is >90, collect ~200 ml in batch form in a beaker or graduated cylinder, and then start collecting 20-ml fractions again. Read the A_{260} of the last fractions and only keep those >90.
12. Centrifuge the pooled eluate for 20 min at 30,000 × *g*, 4°C to generate the S30 (supernatant of 30,000 × *g*) fraction.
13. Prepare S30 aliquots of appropriate size for storage. Fast freeze by placing container into powdered dry ice if using small aliquots (<10-ml) or into a dry ice/isopropanol bath or liquid nitrogen if using large aliquots (>10-ml). Store at −70°C.

    Powdered dry ice should be deep enough to cover the sample. The dry ice/isopropanol bath should be prepared and allowed to cool for ~15 min. Care should be taken to prevent the isopropanol from seeping into the tubes by keeping the tube top well above the isopropanol level. Liquid nitrogen should also not be allowed to seep into the tubes.

    For long-term storage (i.e., several months to years before use), 50-ml aliquots are used. These are then thawed and subaliquoted to 10 ml, 1 ml, or smaller aliquots as desired.

PREPARATION OF A 2-LITER G-25 COLUMN

SUPPORT PROTOCOL

The following protocol details preparation of a 2-liter G-25 column. This column may be reused many times, but should be washed with at least 6 liters of column wash buffer containing 0.5% sodium azide after each use to remove all remaining contaminants and to prevent microbial growth. Every three to four uses, or prior to long-term storage, the column should be disassembled and the G25 washed with water to remove detritus. The cleaned resin should then be transferred in water to a 4-liter beaker and autoclaved to kill any fungal or bacterial contaminants. After the G25 has cooled, add sodium azide to 0.5% and store at 4°C until use.

If after the column has been poured, it runs dry within reach of a sturdy glass rod, stir the bed and allow to resettle. If the crack is 8 to 12 inches from the top, add water or buffer to the column, seal the ends of the adaptors, remove the column from the clamps and gently tilt it back and forth until the crack in the column disappears. Allow the column to resettle. If the column runs completely dry or >50% down the column, as soon as possible, add buffer or water to the top, remove the bottom adaptor, and allow the liquid to displace the resin into a 4-liter beaker placed under the column; repour the column.

CAUTION: Dispose of sodium azide as described in *UNIT 1A.3*.

NOTE: The column should be prepared at least one day prior to use.

Materials

Sephadex G-25 slurry (Sigma, cat. no. G-25-150)
Wheat germ column wash buffer (see recipe)

2.35-liter (50 × 1200–mm) ACE glass column (e.g., Ace Glass, cat. no. 5820-58; *http://www.aceglass.com*)
Top and bottom Luer adaptors (Ace Glass, cat. no. 5837-21) with appropriate tubing and stopcock
Buffer reservoir (e.g., 6-liter flat-style carboy) with appropriate tubing

1. Weigh out ~425 g of Sephadex G-25 and allow to swell in 2.5 liters deionized water for at least 3 hr at room temperature or overnight at 4°C. If at any time during the swelling process there is not an excess of water, add more.
2. Set up a clean 2.35-liter ACE glass column with top and bottom Leur adaptors in a cold room.

   The fully loaded column is heavy so adequate clamping to a column rack is necessary.

3. Close the bottom adaptor and pour ~200 ml water into the column. Check for leaks.

   The column must not leak or the column will run dry and have to be repoured.

4. Gently swirl the G25 resin in a large beaker to resuspend, minimizing the amount of liquid, but having enough to allow the resin to move freely. Immediately pour about half of the G-25 slurry into the column. Allow 10 to 15 min for the resin to begin settling. Open the bottom adaptor to allow a slow flow rate (~1.0 ml/min).
5. Continue to add the slurry of G-25 until all is poured into the column.

   Make every effort to prevent the bed of G-25 from settling completely until all the slurry is added. If the bed does settle, use a very sturdy glass rod to stir the top of the bed before adding more G-25.

6. After all the G-25 is hand-poured, allow the bed to completely settle. Turn off the column until needed (up to 24 hr) or proceed immediately to step 7.

   Do not leave column for more than 24 hr without adding 0.5% sodium azide to prevent microbial growth.

7. Attach a reservoir of buffer ~12 to 18 in. above the column using appropriate tubing to attach it to the top Luer adaptor.
8. Wash the column with 6 to 8 liters of wheat germ column wash buffer (~24 hr).
9. Close the bottom adaptor and store (no more than 24 hr) until use at 4°C.

BASIC PROTOCOL 2

IN VITRO TRANSCRIPTION OF PLANT VIRAL RNAs

In vitro transcription systems to prepare large amounts of a specific mRNA are a very easy way to prepare plant viral RNAs and mutants for analysis by in vitro protein synthesis. This method describes the use of a phage polymerase system to prepare large amounts of a capped or uncapped mRNA. A transcription kit is recommended for novice users; more experienced users may wish to prepare their own reagents as described in Struhl (2003) or Pokrovskaya and Gurevich (1994).

Materials

Plasmid DNA
Restriction enzyme and buffer appropriate for linearizing plasmid DNA
Sterile water
Ambion MegaScript (uncapped RNA) or MessageMachine (capped RNA) T7 or SP6 transcription kit or equivalent
Sephadex G100 slurry (see recipe)
RNA column elution buffer (see recipe)
ACE-saturated phenol (see recipe)
Chloroform
80% and 100% ethanol, −20°C
20-ml plastic columns and frits (BioRad)
Stopcocks (Baxter), sterile
1.5-ml microcentrifuge tubes, sterile
Tabletop centrifuge
13-ml sterile centrifuge tubes
Additional reagents and equipment for purifying DNA (Moore and Dowhan, 2002) and separating RNA on a urea gel (Ellington and Pollard, 1998)

NOTE: All reagents must be prepared with ultrapure, sterile water to prevent nuclease contamination. Autoclave all buffers, resin, columns, tubes, and other reagents and equipment to prevent nuclease contamination. Gloves should be worn and all efforts made to prevent nuclease contamination from personnel or air sources.

1. Linearize 100 to 200 μg plasmid DNA with an appropriate restriction enzyme (5′ overhang or blunt end) for run-off transcription.
2. Phenol-chloroform extract the reaction, ethanol precipitate the DNA in the presence of ammonium acetate or other salt, and collect by centrifugation (Moore and Dowhan, 2002). Resuspend in 100 to 200 μl sterile water.

   The author has not found it necessary to use diethylpyrocarbonate (DEPC)-treated water, as autoclaved, ultrapure water appears to be sufficient. However, if nuclease is a problem, DEPC-treated water may help (see APPENDIX 2A). Plasmids can be prepared by several different methods for use as template; however, methods that avoid RNase in the procedure are preferred. PCR-derived template also works well.

3. Following the manufacturer's instructions for the transcription kit, assemble components for a 300- to 1000-μl transcription reaction (1/2 of a 25-reaction kit or up to a full 40-reaction kit). Add enzyme mixture to start the transcription reaction. Incubate 2 hr at 37°C.

Alternatively, in vitro transcription can be performed as described in Struhl (2003) or Pokrovskaya and Gurevich (1994).

The purification of larger amounts of RNA is more efficient and the purified RNA is stable when stored for up to 2 years at −70°C. Long-term storage of RNA should be in aliquots of 100 μl or larger to avoid desiccation of the sample.

4. While the transcription reaction is incubating, pour 20 ml Sephadex G100 resin into a sterile 20-ml plastic column fitted with a sterile stopcock.

5. After the bed has settled, push a sterile frit down on top of the resin. Wash the column with 30 to 40 ml of sterile RNA column elution buffer (~0.5 hr) and turn off the column.

   This procedure is carried out at room temperature. Make every effort to ensure that nothing falls into the column or buffer during the procedure. The frit on top of the column protects the top from being disturbed when washing, loading, and eluting.

6. Following completion of the transcription reaction (step 3), degrade the template by adding DNase (included in the kit) for 15 min at 37°C.

7. Adjust volume to 0.5 to 1.0 ml with sterile water if necessary.

8. Load the entire reaction mixture onto the G100 column (step 5) and elute with ~10 to 15 ml of RNA column elution buffer at a flow rate of ~1 ml/min.

   The G100 column removes unincorporated ribonucleotides, deoxynucleotides from degradation of the template, and salts. The column elution buffer contains potassium chloride as the salt to precipitate the RNA. Potassium chloride is compatible with protein synthesis assays.

9. Collect twelve ~1-ml fractions by hand in sterile 1.5-ml microcentrifuge tubes.

   Fractions are collected by hand to prevent contamination from tubing or other sources using a fraction collector.

10. Prepare a 1:300 dilution (2 μl to 598 μl water) of each fraction and measure the A_{260}. Discard the dilutions and pool the fractions (generally three) with the highest A_{260} in a 13-ml centrifuge tube.

    The RNA is usually in fractions six to eight and the yield should be 1 to 2 mg for an uncapped mRNA and 0.5 to 1 mg for a capped mRNA. The columns are only used once for RNA work; however, the resin and column may be reused for non-RNA purposes.

11. Extract pooled fractions with an equal volume of ACE-saturated phenol. Centrifuge the extraction mixture in a tabletop centrifuge 10 min at 3500 × *g*, room temperature. Remove the supernatant, avoiding the interface.

12. Extract the supernatant two times with an equal volume of chloroform. Precipitate the RNA in the supernatant by adding 2.5 vol of 100% ethanol at –20°C. Store 1 to 2 hr at –20°C.

    The phenol extraction removes T7 or SP6 polymerase, deoxyribonuclease, and any ribonuclease contamination that may have been introduced during the handling procedures. No additional salt is necessary for precipitation of the RNA. The 150 mM KCl in the column elution buffer is sufficient. Potassium is the preferred salt, as it is compatible with in vitro translation conditions. Sodium or ammonium ion may be inhibitory under certain conditions. The RNA should be visible as a snowy, fluffy, white precipitate in the ethanol. Overnight precipitation is also acceptable, but additional salt or other contaminants may also precipitate.

13. Collect the RNA by centrifuging 20 min at 8000 × *g*, 4°C. Rinse the precipitate gently with 80% ethanol at –20°C. Remove as much liquid as possible and dry the pellet for 10 to 15 min at 37°C.

14. Reconstitute in an appropriate amount of sterile water to give a final concentration of 0.5 to 2 mg/ml based on the A_{260} from the column.

The RNA pellet should be a translucent, white pellet and may streak up the side of the tube. Be sure to resuspend any material on the side of the tube. The RNA pellet often spins around on the surface of the water as it dissolves. If there is insoluble material or the RNA appears granular, then some of the protein layer was accidentally taken during the phenol extraction. If this occurs, repeat the phenol extraction.

15. Make a 1:300 dilution (2 µl to 598 µl water) and read the A_{260} to confirm the concentration. Check the integrity of 1 to 2 µg RNA by electrophoresis on an appropriate percentage (e.g., 4% for large mRNAs, >1000 nucleotides) urea gel (Ellington and Pollard, 1998).

The ultimate assay is to use the mRNA in in vitro protein synthesis assays (Basic Protocol 3).

The "activity," or how much protein is synthesized from the mRNA of the mRNA preparation, will vary depending on several factors, including the level of contaminants from phenol extraction (phenol, chloroform, ethanol, excess salt) and, more importantly, the integrity of the RNA. At least three preparations should be compared to get an idea of the range of an mRNA's activity in in vitro protein synthesis. A low yield of RNA may lead to poor protein synthesis as the contaminants present become inhibitory at higher concentrations of RNA.

BASIC PROTOCOL 3

PROTEIN SYNTHESIS ASSAY

The protein synthesis assay requires the addition of small molecules including ATP, GTP, amino acids, buffer, DTT, and spermine. To simplify the addition of all these components, a supermix is prepared and added as one assay component.

Materials

Supermix (–Leu or –Met; see recipe)
1 M potassium acetate (see recipe)
0.1 M magnesium acetate (see recipe)
0.6 mM [^{14}C]leucine (~170 µCi/µmol, ~200 cpm/pmol) *or* 0.6 mM [^{35}S]methionine (~1300 µCi/µmol, ~ 2000 cpm/pmol)
mRNA
Wheat germ column elution buffer (see recipe)
S30 (Basic Protocol 1)
5% and 1% TCA (see recipe; for filter binding assays) in acid-resistant squeeze bottles
Scintillation fluid (e.g., Econo-Safe, Research Products International; for filter binding analysis)
SDS sample buffer (*APPENDIX 2A*; for SDS-PAGE analysis)
12 × 75–mm glass test tubes (for filter binding assay) or 1.5-ml microcentrifuge tubes (for SDS-PAGE analysis)
27°C water bath
90°C heating block (for filter binding analysis)
Glass fiber filters (Schleicher and Schuell; for filter binding analysis): wet with 5% TCA prior to use
Vacuum filtration manifold (for filter binding analysis)
100°C drying oven (for filter binding analysis)
Scintillation vials (for filter binding analysis)
Additional reagents and equipment for SDS-PAGE (Gallagher, 1999)

Table 16K.1.1 Reaction Mixture for S30 Activity Assay

Component	µl/tube[a]	Contribution to concentration	
		K^+	Mg^{2+}
Water	19		
Supermix (–Leu)[b]	7.5		
1 M potassium acetate[c]	4.7	94 mM[c]	
0.1 M magnesium acetate	0.25		0.5 mM
[^{14}C]leucine	3		
mRNA (0.5–10 pmol)	0.5		
Contribution from 15 µl S30 column buffer[d]		36 mM	1.5 mM
Total	35	130 mM	2 mM

[a]Always prepare one more tube than is actually needed so as not to run out of reaction mixture. For example, the assay in Table 16K.1.2 would require a 7× reaction mixture. Note that the mixture specified in this recipe is for a single reaction.
[b]The supermix may also be made with 19 amino acids but without methionine if [^{35}S]methionine labeling is desired.
[c]Be sure to check that the amount of K^+ and Mg^{2+} being added is compensated correctly for a final concentration of 130 mM and 2 mM, respectively, in 50 µl. Avoid adding more than 30 mM chloride from any source, as chloride ions become inhibitory above 30 mM.
[d]The amount of S30 to be added depends upon the quality of the extract: a range of 7.5, 10, 12, and 15 µl or higher should be assayed to determine the optimum amount.

To measure activity of S30 extract

1a. Prepare sufficient reaction mixture as outlined in Table 16K.1.1. Add reactants in the order indicated (top to bottom) and mix well.

Keep all components of the reaction mixture on ice during preparation and distribution to 12 × 75–mm glass test (for filter binding) or 1.5-ml microcentrifuge (for SDS-PAGE) assay tubes.

The activity of the S30 extract should be measured at several levels to optimize the amount of extract needed. This may vary from preparation to preparation. This is done with an mRNA template preferably known to be "active." Ideally, the S30 extract is compared to another extract known to function in vitro translation (i.e., is "active"). Using a commercial kit for initial comparisons would provide both an mRNA control template of known activity and an extract for comparison purposes.

2a. Add the reaction components shown in Table 16K.1.2 in order from left to right. Gently flick the tube to mix components well.

See Table 16K.1.2 for an example of an assay for activity of S30.

To measure activity of mRNA

Once the amount of a particular extract has been optimized, then an mRNA may be assayed at different levels to determine the optimum amount of mRNA to be used.

1b. Prepare sufficient reaction mixture as outlined in Table 16K.1.3. Add reactants in the order indicated and mix well.

Keep all components of the reaction mixture on ice during preparation and distribution to 12 × 75–mm glass test (for filter binding) or 1.5-ml microcentrifuge (for SDS-PAGE) assay tubes.

2b. Add the reaction components shown in Table 16K.1.4 in order from left to right. Gently flick tube to mix components well. Proceed to step 3.

See Table 16K.1.4 for an example of an assay for RNA activity.

Table 16K.1.2 Sample Assay for S30 Activity[a]

Tube no.	Wheat germ column elution buffer (μl)	Reaction mixture[b] (μl)	S30 (μl)
1	15[c]	35	0
2	7.5	35	7.5
3	5	35	10
4	2.5	35	12.5
5	0	35	15
6	15[c]	35	0

[a]The standard S30 reaction contains in a final volume of 50 μl: 24 to 32 mM HEPES/KOH, pH 7.6, 2.4 mM DTT, 0.1 mM spermine, 2 mM magnesium acetate, 130 mM potassium acetate, 1 mM ATP, 0.2 mM GTP, 34 μM [^{14}C]leucine, 50 μM 19 amino acids (–Leu), 7.8 mM creatine phosphate, 1.5 μg creatine kinase, 0.38 A_{260} units of bakers yeast tRNA.
[b]See Table 16K.1.1.
[c]Two blanks are included for averaging.

Table 16K.1.3 Reaction Mixture for mRNA Activity Assay

		Contribution to concentration	
	μl/tube[a]	K^+	Mg^{2+}
Water	12.1		
Supermix (–Leu)[b]	7.5		
1 M potassium acetate[c]	5	101.2 mM[c]	
0.1 M magnesium acetate	0.4		0.8 mM
[^{14}C]leucine	3.0		
S30[d]	12	28.8 mM	1.2 mM
Total	40.0	130 mM	2 mM

[a]Always prepare one additional tube than is actually needed so as not to run out of reaction mixture. For example, the assay in Table 16K.1.4 would require a 6× reaction mixture. Note that the mixture specified in this recipe is for a single reaction.
[b]The supermix may also be made with 19 amino acids but without methionine if [^{35}S]methionine labeling is desired.
[c]Be sure to check the amount of K^+ and Mg^{2+} being added is compensated correctly if the amount of S30 changes.
[d]The amount of S30 (in column elution buffer) to be added depends upon the quality of the extract: a range should be assayed to determine the optimum amount (see Table 16K.1.2).

3. Remove the reaction tubes from ice and incubate 30 min in a 27°C water bath or the desired time(s) for a time-course experiment.

For analysis of activity by filter binding

Filter binding is a quick quantitative method for measuring the amount of radioactive label incorporated into protein. It is a good way to determine quickly how "active" an extract or mRNA preparation is. However, it does not provide any information about the size or integrity of the protein product(s) being synthesized.

4a. Terminate the reaction by adding ~2 ml of 5% TCA and heating 10 min at 90°C to deacylate the tRNAs. Vortex the tubes gently to resuspend the precipitate.

Table 16K.1.4 Sample RNA Assay[a]

	Water (μl)	Reaction mixture (μl)	mRNA (μl)[b]
1	10	40	0
2	7.5	40	2.5
3	5	40	5
4	0	40	10
5	10	40	0

[a]The standard S30 reaction contains in a final volume of 50 μl: 24 to 32 mM HEPES/KOH, pH 7.6, 2.4 mM DTT, 0.1 mM spermine, 2 mM magnesium acetate, 130 mM potassium acetate, 1 mM ATP, 0.2 mM GTP, 34 μM [^{14}C]leucine, 50 μM 19 amino acids (–Leu or −Met), 7.8 mM creatine phosphate, 1.5 μg creatine kinase, and 0.38 A_{260} units of bakers yeast tRNA.
[b]0.5 to 20 pmol or as appropriate for mRNA being investigated.

5a. Pass the mixture through a glass fiber filter on a vacuum filtration manifold. Rinse the tubes two times with ∼3 ml of 1% TCA, each time pouring the TCA through the filter.

The 5% and 1% TCA are placed in acid-resistant squeeze bottles for convenience and the 3 ml may be estimated. The vacuum manifold should be set up while the tubes are heating and the filters wetted with 5% TCA prior to the pouring of the terminated reaction mixtures.

6a. Rinse the filters again with ∼3 ml of 1% TCA and allow to slightly dry in the manifold (∼2 to 3 min).

7a. Remove the filters with forceps, being sure to only handle the edges of filters. Dry 10 min in a small 100°C drying oven. Place in 10 ml scintillation fluid and count in a scintillation counter.

The filters must be completely dry. Wet spots may cause anomalies in the scintillation counting.

For analysis of activity by SDS-PAGE

SDS-PAGE may be used when the size and integrity of the protein product(s) is important.

4b. Alternatively, terminate the reaction (or a portion of the reaction) by mixing with SDS sample buffer and analyzing by SDS PAGE (Gallagher, 1999).

5b. Visualize the in vitro–synthesized protein by autoradiography (overnight for ^{35}S or several days for ^{14}C) or by immunoblotting with an antibody to the synthesized protein.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

ACE buffer

1.67 ml 3 M sodium acetate, pH 5.1 (final 10 mM)
1.46 g NaCl (final 50 mM)
3 ml 0.5 M EDTA (final 3 mM)
Adjust the volume to 500 ml with H_2O
Autoclave and store up to 1 year at room temperature

ACE-saturated phenol

Combine 6 ml ACE buffer (see recipe) and 20 ml molecular-biology-grade phenol (Fisher). Store up to 1 year at –20°C in the dark.

Amino acid mix minus leucine (AA–Leu), 5 mM each

Combine 200 µl of a 0.1 M solution of each of the amino acids (store each up to 1 year at −20°C) except leucine and tryptophan. Add 400 µl of a 0.05 M stock of tryptophan. Divide into 0.25-ml aliquots and store up to 1 year at –20°C. Use once and discard.

Amino acid mix minus methionine (AA–Met), 5 mM each

Combine 200 µl of a 0.1 M solution of each of the amino acids (store each up to 1 year at −20°C) except methionine and tryptophan. Add 400 µl of a 0.05 M stock of tryptophan. Divide into 0.25-ml aliquots and store up to 1 year at –20°C. Use once and discard.

HEPES (1 M)/KOH, pH 7.6

Place 1600 ml water in a 2-liter beaker. Add a magnetic stir bar and place on a magnetic stirrer. Slowly add 476.6 g of HEPES (USB) and stir until dissolved. Slowly add ∼65 g KOH pellets and stir until dissolved. Make a 1:10 dilution and measure the pH, which should be 7.6. If necessary, add more KOH and check the pH of a 1:10 dilution (pH of a 1 M solution will be ∼8.1 when the pH of a 1:10 dilution is 7.6). Adjust volume to 2 liters with water and let sit overnight at 4°C. Recheck pH of 1:10 dilution at room temperature; adjust pH if necessary. Store 1 liter for short term (1 to 3 weeks) at 4°C and 1 liter at −20°C. If used for RNA work, autoclave the buffer for 20 min. Unopened autoclaved solutions may be stored 1 to 2 months at room temperature in the dark.

Magnesium acetate, 1 or 0.1 M

Prepare a 1 M solution by dissolving 107.2 g magnesium acetate (Sigma) in 500 ml water. Prepare a 0.1 M solution by dissolving 21.5 mg magnesium acetate per milliliter water. Store up to 1 year at −20°C. Mix thoroughly after thawing.

The solid is very hydroscopic and is stored in a metal can with desiccant.

Potassium acetate, 1.0 M

Dissolve 98.15 mg potassium acetate (Fisher) per milliliter water. Store up to 1 year at –20°C. Mix thoroughly after thawing.

Phenylmethylsulfonyl fluoride (PMSF), 50 mM

Dissolve 52.3 mg phenylmethylsulfonyl fluoride (Sigma) in 6 ml isopropanol; prepare immediately before use.

PMSF has a half-life of minutes in aqueous solution.

RNA column elution buffer

10 ml 1 M HEPES/KOH, pH 7.6, sterile (see recipe; final 20 mM)
0.5 ml 0.1 M EDTA, pH 8.0, sterile (Sigma; final 0.1 mM)
5.6 g KCl (Fisher; final 150 mM)
Adjust volume to 500 ml with H_2O
Autoclave and store up to 1 year unopened at room temperature
Once bottle is opened, use within 1 week or discard

Sephadex G100 slurry

Suspend 4 g nucleic-acid-grade Sephadex G100 (Sigma) in 100 ml water. Autoclave and store unopened up to 1 year at room temperature. Once bottle is opened, use within 1 week or discard.

Sodium acetate, pH 5.1 (3 M)

In a 100-ml beaker, add ~10 ml water and ~40 ml glacial acetic acid. While stirring, add either 40.82 g of trihydrate (136.08 g/mol) or 24.61 g of anhydrous sodium acetate. While monitoring on a pH meter, add glacial acetic acid until the pH is ~5.3. Measure the volume and adjust the volume to ~95 ml with water. Mix and check the pH. Adjust to a final value of 5.1 if necessary. Add water to a total volume of 100 ml and autoclave. Store at room temperature if sterile, or at 4°C after bottle is opened.

Most of the volume of this solution is acetic acid; adding water close to the final pH may change the pH drastically.

Supermix (–Leu or –Met)

Add reagents in order given and mix after each addition:
7.5 ml sterile H_2O
2.45 ml 1 M HEPES, pH 7.6 (USB; final 0.163 M)
1.0 ml 5 mM amino acid mix–Leu (or –Met) (see recipe; final 0.33 mM)
37 mg DTT (final 16 mM)
3.5 mg spermine (Sigma; final 0.67 mM)
55.1 mg ATP (Amersham Biosciences; final 6.67 mM)
10.5 mg GTP (Sigma; final 1.33 mM)
255.3 mg creatine phosphate (Roche Diagnostics; final 52 mM)
3 mg creatine kinase (Roche Diagnostics; final 0.2 mg/ml)
37.3 mg bakers yeast tRNA (Sigma; final 20.1 A_{260}/mg; 50 A_{260}/ml)
dH_2O to 15 ml
Divide into 0.5-ml aliquots. Store at −70°C until ready to use. Store up to 1 year at −70°C.

Supermix may be quick frozen and thawed up to three times before discarding

TCA, 20%, 5%, or 1% (w/v)

Prepare a 20% (w/v) solution by dissolving 1 bottle (454 g) trichloroacetic acid (TCA) into 227 ml water. Prepare a 5% (v/v) solution by diluting 250 ml of 20% TCA to 1 liter water. Prepare a 1% (v/v) solution by diluting 50 ml of 20% TCA to 1 liter water. Store all TCA solutions up to 1 year at room temperature.

Wheat germ column elution buffer

400 ml glycerol (Fisher; final 10%)
80 ml 1 M HEPES/KOH, pH 7.6 (see recipe; final 20 mM)
20 ml 1 M magnesium acetate (see recipe; final 5 mM)
47.2 g potassium acetate (Fisher; final 120 mM)
Adjust volume to 4 liters with H_2O
Store up to 24 hr at 4°C
Just prior to use, add 2.0 ml of 14.3 M 2-ME (Sigma, final 7 mM)

Wheat germ column wash buffer

360 ml 1 M Tris·Cl, pH 7.6 (Sigma; final 20 mM)
90 ml 1 M magnesium acetate (see recipe; final 5 mM)
161.1 g KCl (Fisher; final 0.12 M)
Adjust volume to 18 liters with dH_2O
Store up to 24 hr at 4°C

Wheat germ extraction buffer

17.5 ml 1 M HEPES/KOH, pH 7.6 (see recipe; final 50 mM)
3.13 g KCl (Fisher; final 0.12 M)
0.7 ml 1 M magnesium acetate (see recipe; final 2 mM)
0.7 ml 1 M $CaCl_2$ (Sigma; final 2 mM)
Adjust volume to 350 ml with H_2O
Store up to 24 hr at 4°C
Just prior to use, add:
0.175 ml 14.3 M 2-ME (Sigma; 7 mM final)
35 mg STI (Sigma)
3.5 ml 50 mM PMSF (see recipe; final 0.5 mM)

COMMENTARY

Background Information

In vitro protein synthesis systems were crucial for unraveling the genetic code in the 1960s. A translation system is a relatively crude extract of some type of cell. The extract is generally referred to as an "S30" extract, which refers to the supernatant of the 30,000 × *g*–centrifugation. This centrifugation step is sufficient to remove particulate matter, membranes, and unbroken organelles, but not to remove polysomes or ribosomes. The extracts are then dialyzed or passed over a gel-filtration column to remove small molecules, that may be toxic, and amino acids, so that radiolabel is more readily incorporated into the synthesized protein.

In vitro protein synthesis systems from *E. coli*, wheat germ, rabbit reticulocytes, HeLa cells, and yeast have been used to biochemically characterize the components of the translational apparatus and to study translational efficiency of various RNAs. The advent of cloning and harnessing of phage RNA polymerase and promoters (T7, SP6, T3) made it possible to prepare large amounts of messenger RNAs in vitro. Protein synthesis assays of plant viral RNAs are very useful for determining the requirements for a cap, the specific part of a 5′ or 3′ UTR that affects translational efficiency, and to determine the size of the protein product(s).

Critical Parameters and Troubleshooting

The most difficult part of the wheat germ preparation is finding good, fresh wheat germ. The source of the raw wheat germ may be a local health food store. Try several sources and compare activities of the wheat germ extracts with a test RNA to determine the best sources. High variability of the quality of the wheat germ may be due to many factors, such as field conditions during growth, harvesting, or extraction of the germ during processing. It has also been reported that the quality of the extract may be affected by the amount of ribosome inactivating protein present in the endosperm contamination (Madin et al., 2000). It may also be useful to purchase a commercial wheat germ translation kit to compare activity of the extract if in vitro translation assays have never been carried out before. This kit would be a good source of control RNA for comparison purposes. The protocol described here is for large-scale preparation; however, the extraction procedure and column may be scaled down proportionately for the desired amount of S30 extract.

Once a reproducible and active wheat germ extract is obtained, then various RNAs prepared by in vitro transcription may be translated. Several RNA preparations should be compared to determine what range of activity is to be expected for a specific mRNA. Some preparations of RNA may have low activity due to excessive phenol, chloroform, ethanol, or salt contamination, especially if the yield of RNA is low.

The translation of some mRNAs may have to be optimized for Mg^{2+} and K^+ concentrations (although the conditions of 130 mM KCl and 2 mM magnesium acetate are generally favorable for a variety of mRNAs; Table 6K.1.5). This may be easily accomplished by omitting either Mg^{2+} or K^+ from the reaction mixture. The magnesium acetate or potassium acetate becomes the variable instead of mRNA.

To simplify certain types of assays, the mRNA may be added to the reaction mixture or added individually as desired. The volume of reaction mix added to a reaction may be varied as needed by adjusting the amount of water, as long as the final volume of the reaction is 50 μl.

Anticipated Results

The amount of protein synthesis will be dependent on the efficiency of the mRNA template. Some mRNAs, especially viral RNAs, are very efficient in vitro and several rounds of initiation will occur; however, other mRNAs are not as efficient and may only be translated one time. The amount of protein product (pmol) may be estimated from the radioactivity incorporated based on the number of [^{14}C]leucine or [^{35}S]methionine residues in the protein product. The efficiency of the RNA is calculated as picomoles protein synthesized per picomoles RNA. For example, if a viral RNA template (10 pmol) encodes a protein containing 18 leucine residues, and the amount of radioactive [^{14}C]leucine obtained by filter binding is 45,000 cpm, then the calculation would be as follows:

45,000 cpm/200 cpm/pmol [^{14}C]leucine = 225 pmol [^{14}C]leucine

225 pmol [^{14}C]leucine/18 leucine per pmol protein = 12.5 pmol protein synthesized

12.5 pmol protein synthesized/10 pmol mRNA = 1.25 pmol protein synthesized per pmol of mRNA

This may indicate that 100% of the mRNA was translation competent and initiated only once, or that only 10% of the mRNA was translation competent and re-initiated about ten times.

Time Considerations

The preparation of the wheat germ extract requires 2 days (solution preparation on day 1 and extraction/purification on day 2). The preparation of the RNA requires ~4 to 5 hr if the solutions are prepared/autoclaved ahead of time. The assays generally take ~1.5 to 2 hr (depending on incubation time) if all the reagents are prepared.

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In Vivo Translation Studies of Plant Viral RNAs Using Reporter Genes

Viral messenger RNAs must compete with cellular RNAs to be translated on ribosomes and to support viral gene expression. Translational expression of an RNA is typically controlled by its 5′- and 3′- untranslated regions (UTRs). Replacing all or most of the viral coding region with a reporter gene is the most convenient way to study the role(s) of UTRs in directing translation. Among available reporter systems, firefly luciferase (LUC) has strong advantages, most notably an easy assay procedure and superior sensitivity (also see Brasier and Fortin, 1996). Two protocols for introduction of in vitro transcribed RNAs into protoplasts are described in this section. The Basic Protocol illustrates electroporation of cowpea mesophyll protoplasts, and the Alternate Protocol describes polyethylene glycol (PEG)–mediated transfection. In addition, LUC activity measurement and normalization by protein concentration, as well as experimental design considerations and data interpretation, are described.

EXPRESSION OF LUCIFERASE FROM REPORTER RNA TRANSCRIPTS INTRODUCED INTO PROTOPLASTS BY ELECTROPORATION

BASIC PROTOCOL

In this protocol, luciferase (LUC) reporter RNA transcripts are introduced into cowpea protoplasts by electroporation. The RNA constructs are mixed with protoplasts in cold electroporation buffer and are then subjected to an electrical pulse with high capacitance. After electroporation, the protoplasts are transferred to growth medium and incubated to allow LUC expression. LUC activity and total protein are assayed in extracts made from harvested protoplasts. The procedure describes assays based on 96-well plates for accommodating large numbers of determinations. Detailed discussion of protoplast preparation and electroporation are found in Section D of Chapter 16 (e.g., *UNIT 16D.4*).

Materials

8- to 10-day-old cowpea (*Vigna unguiculata*) seedlings, e.g., California Blackeye 46
30% ethanol
Protoplast digestive enzyme solution (see recipe)
MMC buffer (see recipe)
Protoplast electroporation buffer (see recipe)
5 mg/ml fluorescein diacetate in acetone (store indefinitely at –20°C)
In vitro–transcribed RNA (2 pmol/10 μl; see Critical Parameters and Troubleshooting) containing luciferase reporter gene, suspended in H_2O: chill on ice
Protoplast growth medium (see recipe)
Passive Lysis Buffer (Promega cat. no. E1941)
Luciferase assay reagent (see recipe)
Bradford dye-binding protein assay reagent
BSA protein standards: dilute BSA in 1× Passive Lysis Buffer to produce stock concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/ml
Whatman 3MM filter paper
Fine-point tweezers
Petri dish, 15 cm diameter
Platform rotator
30°C incubator
Miracloth (Calbiochem)
50-ml polypropylene conical tubes

Contributed by Daiki Matsuda and Theo W. Dreher
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Clinical centrifuge
Hemacytometer
UV/visible fluorescence microscope (*UNIT 2A.1*)
Electroporation cuvettes (2-mm gap), chilled on ice
Electroporation system with capacitance extender (CE) module (Bio-Rad GenePulser Xcell)
Pasteur pipets, autoclaved (one per electroporation)
Refrigerated centrifuge
Standard 96-well plate (optional)
Clear-bottom black 96-well plates (Costar #3631)
Microplate luminometer: e.g., Wallac Microbeta 1450 or Turner BioSystems Veritas (*http//www.turnerdesigns.com*)
Microplate spectrophotometer (e.g., Molecular Devices SpectraMax 250)
Additional reagents and equipment for electroporation of protoplasts (*UNIT 16D.4*) and protein assay (*APPENDIX 3A*)

NOTE: Avoid exposure to ribonucleases in all procedures. Use gloved hands at all times, including when handling chemical ingredients of solutions, and make all solutions with MilliQ-grade (Millipore) water. Alternatively, treat solutions with DEPC as described in Appendix 2A.

Prepare and electroporate the protoplasts

1. Harvest the first leaves of young cowpea seedlings (preferably 8- to 10-days-old). Dip the leaves in 30% ethanol to sterilize the surface, shaking off the excess ethanol.

   The cowpea protoplast system is very robust; leaves are easy to peel and this results in a better yield of intact protoplasts than when leaves are diced. The choice of cowpea is also based on the ability of these protoplasts to replicate TYMV. Suspension cultures have been used and are suitable for translation studies.

2. On Whatman 3MM filter paper, cut each leaf into halves and remove the midrib. Hold a pair of sharp fine-point tweezers near-horizontal to the leaf. Lift a minor vein and peel parallel to it to remove the epidermis.

   The lower epidermis is peeled from the leaves to permit access of the protoplast digestive enzyme solution to mesophyll cells. This is a more gentle procedure than dicing, which is necessary for the leaves of some types of plants. Cowpea leaves are easy to peel and the protoplasts are particularly reliable to prepare; however, the authors have used essentially the same procedures described here to study LUC expression in protoplasts made from other plants and from suspension cell culture.

3. Put eight to ten half-leaves in a large petri dish containing 25 ml protoplast digestive enzyme solution.

   About 10^6 protoplasts can be obtained from two half-leaves.

4. Incubate 4 to 5 hr at 30°C in the dark with gentle agitation at 40 to 50 rpm on a platform rotator.

   The leaves will not be completely digested; partially digested leaves (somewhat patchy) should be floating on the surface of the enzyme solution at the end of the incubation period.

5. Swirl the petri dish gently three to four times to release the digested protoplasts into the medium.

6. Strain the protoplast suspension through one layer of Miracloth (prewetted with MMC buffer) into a 50-ml polypropylene conical tube.

   Tilt the conical tube so that the protoplast suspension moves down the wall of the tube. Gentle treatment of protoplasts will prevent rupture.

7. Centrifuge 5 min at 50 × *g*, room temperature, in a clinical centrifuge. Remove the supernatant with a pipet and resuspend the protoplasts with 25 ml MMC buffer.

   After removal of each supernatant, the protoplast pellet should be initially resuspended in the residual solution with gentle shaking before making up the full volume.

8. Centrifuge 5 min at 50 × *g*, room temperature, in a clinical centrifuge. Remove the supernatant with a pipet and resuspend the protoplasts in 40 ml protoplast electroporation buffer.

9. Centrifuge again, remove the supernatant, and resuspend the pellet in 5 ml protoplast electroporation buffer.

10. Dilute 10 µl protoplast suspension with 90 µl protoplast electroporation buffer and add 2 µl of 5 mg/ml fluorescein diacetate in acetone. Load onto a hemacytometer and use a UV/visible fluorescence microscope to determine the number of viable protoplasts.

    Healthy, live cells exhibit evenly distributed fluorescence, while dead protoplasts do not emit green fluorescence. The viability of the protoplasts is often 85% and should be >70%; batches with lower viability should be discarded. If a fluorescence microscope is not available, cell viability can be assessed by the ability of live cells to exclude the dye Evans blue (0.1% w/v).

11. Adjust the protoplast concentration to 1×10^6 cells/ml with protoplast electroporation buffer. Aliquot 300 µl (i.e., 0.3×10^6 cells) into a 1.7-ml microcentrifuge tube for each electroporation to be performed.

    This amount is sufficient for up to eight harvest time points (see step 16).

12. At 35- or 45-sec intervals, place each microcentrifuge tube on ice. Incubate 15 min before electroporation.

    This step establishes equal timing of samples on ice (i.e., 15 min 45 sec if working alone), which is especially important when handling large numbers of samples. Steps 13 and 14 take ~45 sec per sample when working alone or 35 sec if another person is available to help. If early harvest time points (e.g., 15 to 20 min) are taken, they may overlap with the electroporation points of later samples. This should be taken into consideration when planning the work schedule. Also see Time Considerations.

13. Transfer the 300-µl protoplast suspension to a microcentrifuge tube containing 2 pmol RNA in a 10-µl volume. Mix with one stroke of a 1000-µl pipettor.

14. Transfer immediately to a prechilled electroporation cuvette. Remove water/ice from the surface of the cuvette with a tissue and apply a pulse with Bio-Rad GenePulser Xcell with the following settings: exponential decay pulse with 90 V, 50 msec time constant (which typically gives 900 to 950 µF; note that CE module is required).

    Different electroporation settings may be needed for protoplasts from other plant sources. The three variables for optimization are KCl concentration in electroporation buffer, voltage (range of 100 to 1400 V/cm), and time constant (30 to 100 msec). Optimize voltage first at two KCl concentrations (40 and 80 mM), and then optimize the time constant. Monitor LUC activity and cell viability.

15. Use a fresh, sterile, disposable glass Pasteur pipet to transfer protoplasts from the cuvette to a microcentrifuge tube containing 1.1 ml protoplast growth medium.

    To recycle cuvettes, submerge the cuvettes in water overnight, brush the inside with a test tube brush, rinse six times with distilled, deionized water, and leave them in 70% alcohol until use. Three hours before use, remove the ethanol and dry completely.

16. Incubate under fluorescent light at room temperature. Aliquot the protoplasts at this time into separate microcentrifuge tubes according to the desired number of harvest time points (e.g., eight aliquots of 170 µl for eight harvest times).

    Fluorescent lights should be used because photosynthesis is thought to be necessary for the reaction.

17. Harvest the protoplasts by centrifuging 3 min at 3000 × *g*, room temperature.

18. Aspirate the supernatant, centrifuge again as in step 17, and carefully remove remaining supernatant.

    At this point, the protoplast pellets can be stored at −80°C (stable at least 1 week) for later LUC assay.

19. Add at least 20 µl of 1× Passive Lysis Buffer per 4×10^4 protoplasts. Vortex briefly and shake vigorously for 5 min at room temperature.

    The minimal volume of protoplast lysis buffer to lyse all the 4×10^4 cowpea protoplasts was found to be 20 µl. A greater volume of protoplast lysis buffer is sometimes used in order to perform enough replicates of luciferase readings, protein concentration determinations, etc. However, using a larger volume results in dilution of expressed luciferase, which correspondingly gives lower signals. This becomes particularly crucial if the sample is from an early time point or if the RNA has low ability to support translation.

20. Centrifuge 1.5 min at 12,000 × *g*, 4°C.

21. Transfer supernatant to another microcentrifuge tube or a 96-well plate, and store at –80°C for subsequent LUC assay.

    The extracts are stable for at least 1 month.

Assay luciferase and normalize to total protein concentration

The following LUC and total protein assays are described for 96-well plate analyses, permitting the handling of the large numbers of samples that result when time courses of several reporter RNAs are studied in replicate. The authors typically analyze twelve reporter RNAs in duplicate with time courses of six to eight time points each. Protoplast extracts are stored in 96-well plates to facilitate the use of multichannel pipettors.

22. Thaw the luciferase assay reagent 30 min at room temperature.

23. Using a multichannel pipettor, place 10 µl protoplast extract (step 21) in each well of a black, clear-bottom, 96-well plate.

24. Add 50 µl luciferase assay reagent to each well at 3-sec intervals.

25. Measure luciferase activity in each sample for 1 sec using a microplate luminometer.

    The luminometer that the authors use (Wallac Microbeta 1450) reads a new sample every 3 sec when set to read for 1 sec; other instruments may advance to the next sample with a different timing that should be reflected in the above interval. At most, 24 wells can be read without losing LUC signal in an instrument that lacks a reagent injector. The signal declines by <10% over ~2 min.

26. Using a multichannel pipettor, place 195 µl Bradford protein assay reagent in 96-well plates. Fill enough wells to perform at least duplicate determinations for each standard (i.e., ten wells; see step 28) and sample, as well as eight wells to allow determination of the appropriate volume of sample to use (see step 29)

    See APPENDIX 3A for more information regarding the Bradford assay.

27. Let stand ~5 min to allow bubbles to settle down. Read the background at 595 nm using a microplate spectrophotometer.

28. Add 5 µl BSA standard to each well set aside for this purpose to produce a standard curve from 2.5 to 0.156 µg BSA.

29. Add 2, 3, 4, and 5 µl of two of the experimental samples to separate wells.

    These determinations will be used to find the appropriate volume of sample to use, i.e., the volume which will produce measurements falling within the linear range of the standard curve.

30. Read the plate and plot the standard curve. Determine the linear range by linear regression, obtaining the slope, *y*-axis intercept, and regression coefficient (R^2).

31. Referring to the standard curve, decide the appropriate sample volume to assay based on the resulting measurements from the wells prepared in step 29.

32. Using a multichannel pipettor, in duplicate add that amount of sample into the appropriate wells containing Bradford reagent (see step 26). Determine the protein concentration by reading in a microplate spectrophotometer.

33. Using the equation obtained from the standard curve, determine the protein concentration for each sample.

34. Divide LUC readings in relative light units (RLU) obtained for each sample by the total protein (mg) present in the same volume of sample (typically 10 µl). LUC activity is thus reported as RLU/mg protein.

PEG-MEDIATED PROTOPLAST TRANSFECTION OF REPORTER RNA TRANSCRIPTS

ALTERNATE PROTOCOL

The use of polyethylene glycol (PEG) is an alternative way to introduce RNA into protoplasts when an electroporator is not available. Although this method is economical, it requires more time and is thus not recommended if the early time course of reporter gene expression is to be studied.

Additional Materials *(also see Basic Protocol)*

PEG solution (see recipe)
15-ml round-bottom glass or polypropylene tubes
Wide-mouth 1000-µl pipet tips

1. Prepare protoplasts as described in the Basic Protocol, steps 1 to 7.

2. Centrifuge 5 min at 50 × *g*, room temperature. Remove the supernatant with a pipet, and resuspend the protoplast pellet in a minimal volume of MMC buffer (~5 ml, depending on the density of protoplasts).

    The dual resuspension removes residual digestive enzyme.

3. Dilute 10 µl protoplast suspension with 90 µl MMC buffer and add 2 µl of 5 mg/ml fluorescein diacetate in acetone. Load onto a hemacytometer and use a UV/visible fluorescence microscope to determine the number of viable protoplasts.

    See step 10 of the Basic Protocol for a discussion of how to determine protoplast viability.

4. Aliquot $0.5-2 \times 10^6$ protoplasts for each transfection into separate 15-ml round-bottom glass or polypropylene tubes. Centrifuge 5 min at $50 \times g$, room temperature. Remove sufficient supernatant to leave 170 µl total volume in the tube. Place on ice until use.

5. Using a wide-mouth pipet tip, take 500 µl PEG solution and leave the pipet on the bench.

   The aliquot of PEG solution must be taken in advance because of the viscosity of the solution, which makes pipetting a slow process. The PEG should be added immediately after the RNA is mixed with protoplasts in step 6.

6. Add 30 µl RNA sample (1 to 10 pmol depending on the experiments; usually 2 pmol) to protoplasts, and mix gently. Immediately add PEG solution, mix the contents throughout with rocking or rotating motion, and incubate the tube 20 to 30 sec at room temperature.

   Alternatively, a repeating pipettor can be used to transfer the PEG solution.

7. Add 5 ml MMC buffer at once (allow to run down the wall of the tube), and mix the contents gently by inverting the tube five times. Let the tubes sit at room temperature for 15 min.

8. Pellet protoplasts by centrifuging 5 min at $50 \times g$, room temperature. Remove the supernatant and resuspend the pellet in 3 ml protoplast growth medium.

9. Pellet the protoplasts by centrifuging 5 min at $50 \times g$, room temperature. Remove 2 ml supernatant (leaving about 1 ml).

10. Incubate under fluorescent light at room temperature (see step 16 of the Basic Protocol).

11. Harvest protoplasts (see Basic Protocol, steps 17 to 21) and perform LUC assay (see Basic Protocol, steps 22 to 25) and protein concentration assay (see Basic Protocol, steps 26 to 32).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Luciferase assay reagent

20 mM Tricine, pH 7.8
5 mM $MgCl_2$
0.1 mM EDTA (*APPENDIX 2A*)
3.3 mM DTT
270 µM coenzyme A
500 µM luciferin (Promega)
500 µM ATP
Store up to 1 year in aliquots at −80°C

This reagent can also be obtained at http://www.promega.com/pnotes/44/luerhsen/luerhsen.html.

MMC buffer

0.6 M D-mannitol
0.1% (w/v) MES
5 mM $CaCl_2$
Adjust to pH 5.8 with KOH
Autoclave for 15 min and store at room temperature up to 6 months

PEG solution

40% (w/v) PEG 8000 (RNase-free)
100 mM $Ca(NO_3)_2$
0.5 M D-mannitol
Heat to 50°C to dissolve, but do not overheat
Filter-sterilize; do not autoclave
Store at room temperature in the dark up to 6 months

Protoplast digestive enzyme solution

1% (w/v) cellulase (Onozuka)
0.05% (w/v) macerase-pectinase (Calbiochem)
Dissolve in MMC buffer (see recipe) by stirring in the dark 30 min
Filter-sterilize and store at −20°C for up to 1 month in aliquots for single use

Protoplast electroporation buffer

0.6 M D-mannitol
0.1% (w/v) MES
5 mM $CaCl_2$
40 mM KCl
Adjust to pH 5.8 with KOH and autoclave for 20 min
Store up to 6 months at room temperature

This buffer (400 μl) in a 2-mm electroporation cuvette should have a resistance of ~37 Ω at room temperature.

Protoplast growth medium

1 pack of MS salts (Invitrogen) per liter
0.44 M mannitol
3% (w/v) sucrose
0.01% (w/v) inositol
1 mg/liter thiamine
5 μM 2,4-dichlorophenoxyacetic acid (Sigma)
0.1 μM kinetin (Sigma)
Adjust to pH 5.8 with NaOH
Autoclave for 15 min and store at room temperature up to 1 month

COMMENTARY

Background Information

Investigation of the properties of the untranslated regions (UTRs) of an mRNA is of interest because of their contribution to translational regulation, such as enhancement of gene expression, and to the regulation of mRNA stability (Dreher, 1999). In order for the genomic RNAs of positive-strand RNA viruses to be successfully translated, and to initiate the replication cycle, the 5′- and/or 3′-UTRs of the viral mRNA typically provide strong translation signals. These allow competitive translation in the presence of host cellular mRNAs containing a 5′-cap and 3′-poly(A) tail, which are known to strongly stimulate translation. The use of a reporter system has been a common method to study the translational control of an mRNA (Gallie and Kobayashi, 1994; Guo et al., 2000; Gallie, 2002; Matsuda et al., 2004), and the reporter protein coding region is introduced in place of the viral coding region, between the 5′- and/or 3′-UTRs. The reporter mRNA construct can be expressed in vitro (see *UNIT 16K.1*) and in vivo (this unit) to investigate the translational properties of the UTRs. Although the coding regions usually do not influence translational expression, this possibility will need to be borne in mind when drawing conclusions from studies with reporter constructs (see below).

Several reporter genes have been used to study translational regulation, including β-glucuronidase, β-galactosidase, chloramphenicol acetyltransferase, and firefly (*Photinus*) and sea pansy (*Renilla*) luciferases.

Among these, firefly luciferase (LUC) stands out as a result of its superior sensitivity (10^{-20} mol detection limit), wide linear range of detection (eight orders of magnitude), and low background. Detection assays have been formulated to provide compatibility with a wide range of detectors, allowing automated or manual assay in a luminometer or even in a liquid scintillation counter. The most comprehensive support for various applications of the LUC reporter system is provided by Promega, whose reporter plasmids are also a convenient source of the *Photinus pyralis* LUC coding region (GenBank M15077).

Firefly LUC is a 61-kDa protein which emits light at 560 nm in the presence of ATP, O_2, and the substrate luciferin. The luciferase assay reagent includes coenzyme A, which increases the enzyme turnover rate and stabilizes the light emission. This makes the assay amenable to various detection formats, including the use of luminometers that do not have an autoinjector for timed substrate delivery.

Reporter constructs can be delivered to protoplasts in the form of DNA or RNA. RNA-dependent expression simplifies interpretation and is directly applicable to studies of positive-strand-RNA-viral-gene expression, as conducted by the authors. DNA-based expression requires a promoter that directs transcription in the nucleus. Interpretation of gene expression is complicated by potential variation in splicing and the efficiency of mRNA export from the nucleus (Kozak, 2001). Kinetic studies are also far more straightforward with RNA transfection because RNA is made available inside the cell in a single, short pulse.

The authors' laboratory has used two methods to introduce RNAs to protoplasts from various plant sources: electroporation and PEG-mediated delivery. More consistent results have been obtained with the electroporation method (see Basic Protocol), probably due to the less complicated steps of this method. Additionally, the delivery of RNAs to protoplasts via electroporation is quicker than via PEG (see Alternate Protocol), as the latter method requires extra washing steps to remove residual PEG (see Alternate Protocol, steps 8 to 9). Electroporation allows quicker handling of samples, an important consideration when conducting experiments with many samples (various reporter RNAs and replicates). The rapidity of electroporation also makes this method suitable for monitoring early expression of LUC in time courses. On the other hand, electroporation requires a costly electroporator and the continuous expense of disposable cuvettes, although they may be reused to some extent. PEG-mediated transfection is a viable alternative when an electroporator is not available.

Critical Parameters and Troubleshooting

RNA quality and quantity

The quality of the inoculum RNA can be very important for optimal results. The authors usually synthesize RNA transcripts in duplicate and monitor the RNA quality by agarose gel electrophoresis. RNAs are best delivered in the form they exist naturally. Capped transcripts are prepared with a 6:1 concentration ratio of ^{m7}GpppG cap structure to GTP. The concentrations of the other nucleotides are lowered to a similar level as GTP in order to minimize misincorporation of nucleotides by T7 RNA polymerase. The template plasmid DNA and protein are removed by treatment with deoxyribonuclease I and phenol/chloroform, followed by precipitation with ethanol and ammonium acetate. Unincorporated nucleotides are removed with a desalting spin column. In order to minimize freeze-thaw cycles, prepared RNA should be aliquotted into several tubes and stored at −80°C.

In the authors' system, there is a linear response between LUC activity and input RNA up to 30 pmol RNA for both electroporation and PEG-mediated transformation. Working RNA amounts should fall in the middle of the linear range. The RNA (1 to 30 pmol) is in 10 μl of water. The volume (and number) of protoplasts is always constant at 300 μl for electroporation. Uncapped forms of normally capped RNAs, which are sometimes studied for mechanistic reasons, typically show poor mRNA activity and thus may require input levels in the upper part of the linear range in order to obtain signals comfortably higher than background. It is important that LUC activity be fully responsive to differing inputs, verifying that the system is not saturated and that it is capable of supporting increased LUC activity, e.g., generated from a highly efficient mRNA. Delivering large quantities of RNA may also be required when many harvest time points are taken (i.e., protoplasts are aliquotted into many tubes). If more harvesting time points are taken, the number of protoplasts (and LUC signal) at each time point becomes less. This consideration becomes important at early harvesting time points when the luciferase signal is low and relatively close to background.

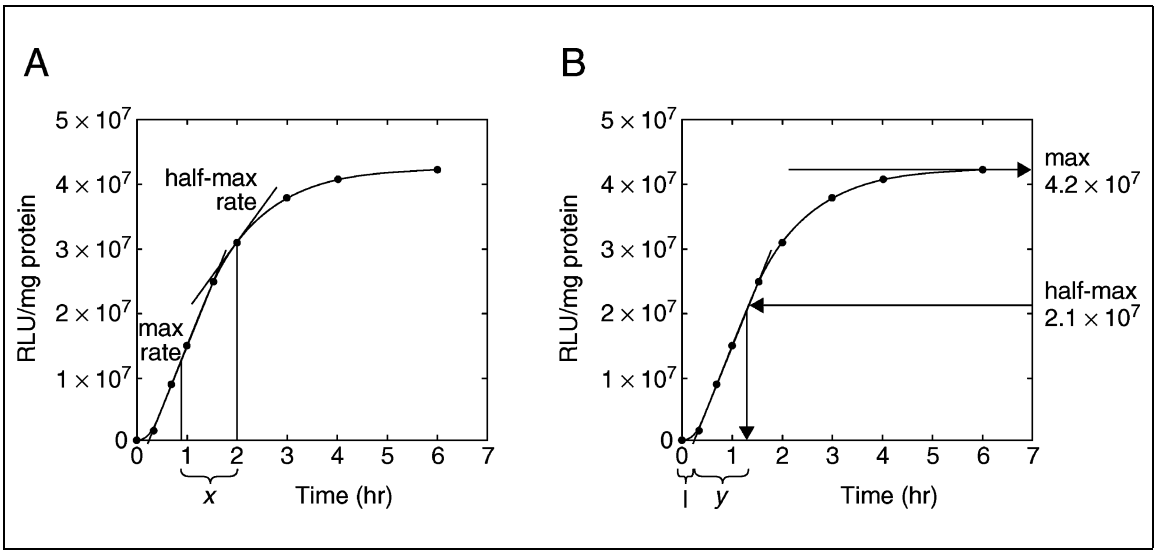

Figure 16K.2.1 A typical LUC expression profile in protoplasts and determination of mRNA functional half-life. Early in the time course, LUC expression enters a near-linear stage of accumulation (usually by 40 min post electroporation) and reaches plateau at around 4 hr. The LUC expression half-life can be determined as (**A**) the time for the maximal rate to fall to half-maximal (interval *x*) or (**B**) the time to reach the half-maximum accumulation of LUC less the lag time, represented by *l* (interval *y*). When studying reporter RNAs making stable forms of LUC, the LUC expression half-life represents the mRNA functional half-life. However, when comparisons involve the expression of modified forms of LUC with shorter half-lives, the mRNA functional half-life is under-estimated.

Protoplasts

The quality of protoplasts can be critical for optimal results. The viability of the protoplasts is typically 70% to 80% after overnight digestion of the leaves at room temperature and >85% after 4 to 5 hr digestion at 30°C. Although overnight digestion is convenient, the LUC yield per cell is usually lower than from protoplasts prepared with the short digestion period.

LUC fusion proteins

In some instances, part of the coding region in mRNAs may be involved in translational control (Kozak, 1990; Frolov and Schlesinger, 1996; Fang et al., 2004). When investigating such cases, a portion of the coding region can be fused in-frame to the LUC coding region to make N-terminal fusion proteins. This translational fusion can potentially result in altered specific activity of the LUC enzyme, which can be determined upon in vitro translation by comparing its light emission activity to incorporation of radiolabeled amino acids into the LUC band on an SDS-PAGE gel (Matsuda et al., 2004). Very little variation in specific activity for a range of N-terminal LUC fusion proteins has been seen, but new variants are routinely tested. LUC fusion proteins may also possess an altered stability or turnover rate, which may result in underestimated expression unless a time course of LUC expression is performed (see below). The authors have observed different stabilities resulting from added N-terminal sequences. Note that the amino acid following the N-terminal methionine residue is an important determinant of protein stability (Varshavsky, 1996; Bradshaw et al., 1998).

LUC expression

LUC expression can be determined on the basis of single time point measurements or more extensive time courses. Since the half-life of the firefly LUC protein has been reported as ~20 hr in tobacco protoplasts at room temperature (Gallie, 1991), some researchers have conveniently harvested the electroporated protoplasts the following morning. However, if there is significant variation in the half-lives of either RNA or LUC protein, misleading results can be obtained from single time-point assays. Aside from exploratory experiments, the authors routinely conduct time courses up to 6 to 9 hr post electroporation. These have the benefit of simultaneously providing information on both translational efficiency and LUC expression half-life, as illustrated in Fig. 16K.2.1. Translational efficiency is represented by the greatest rate of increase of LUC activity, which typically occurs 1 to 2 hr after RNA delivery. This rate is estimated from the line of best fit plotted through values obtained from a time course performed

in duplicate. The half-life of LUC expression is represented by the time taken for the rate of LUC accumulation to fall to half, determined by the time taken for the maximal rate mentioned above to fall to half-maximal (Fig. 16K.2.1A, interval *x*).

For comparisons in which the same forms of LUC are being produced, differences in half-life represent differences in RNA stability. Because the estimate derives from the capacity of the RNA to support protein synthesis, it has been termed the "functional" stability (Gallie and Kobayashi, 1994; Gallie, 2002). RNA functional half-lives are typically estimated to be 1 to 3 hr, considerably shorter than the half-life of LUC protein in plant cells. Functional half-lives determined from rate estimates require several time points and high-quality data to be satisfactorily determined. In many cases, similar values for functional half-lives can be determined as the time taken to reach the half-maximum accumulation of LUC less the lag period (Fig. 16K.2.1B, interval *y*). The maximum accumulation represents the plateau that is reached as input RNA has become largely degraded. With N-terminal LUC fusions (see above), short-lived plateaus are frequently observed, with decreases in relative light unit (RLU) levels occurring in the later part of a time course as the LUC fusion protein turns over with an apparent half-life considerably shorter than 20 hr.

The measured LUC activity, expressed as RLU, can be normalized by the amount of protein present in the extract. (Note that actual RLU values differ depending on the luminometer used.) In this case, the final result is usually expressed as RLU/mg total protein. Some LUC extraction buffers are not compatible with a particular protein assay reagent; for example, the level of Triton X-100 in CCLR (*http://www.promega.com/pnotes/44/luerhsen/luerhsen.html*) makes this buffer incompatible with the standard Bradford assay. The authors currently use a combination of Passive Lysis Buffer (Promega) and Bradford protein assay kit (Bio-Rad). Although Promega does not recommend the use of Passive Lysis Buffer for studies with plant cells, the authors find that it produces excellent results.

Assessing stability of input RNA

As an alternative to estimating the RNA functional half-life, one can assess the physical stability of input RNA. This can be monitored by northern blot, quantitative RT-PCR, or by inspection on denaturing agarose gels if the input RNAs are radiolabeled. Note that this method may be assessing the status of RNAs that are cell-associated following electroporation but that are not accessible to the translational apparatus.

The dual LUC reporter system

Another way to normalize the firefly LUC reading is with the use of an internal control reporter RNA encoding *Renilla* LUC that is co-introduced into the protoplasts. The dual LUC reporter system can also be used to study bicistronic RNA constructs useful in identifying sequences with internal ribosome entry site activity (Qin and Sarnow, 2004).

Anticipated Results

LUC activity can be detected as early as 15 min post electroporation, in the authors' experience. More RNA and protoplasts can be used in order to observe the signal earlier if desirable. In most cases, the LUC expression profile enters a steady stage of near-linear increase at ~40 min post transfection and reaches plateau or maximum at ~4 to 6 hr (Fig. 16K.2.1). The time taken to reach the maximal rate of increase in LUC activity (lag time) can differ from one RNA construct to another; a short lag time for a well translated RNA suggests that the RNA may have a superior ability to actively recruit translation initiation factors and ribosomes (Gallie, 2002).

The functional half-life of input RNAs varies from 0.7 (uncapped RNAs) to 2.5 hr in the authors' system. Translation can be said to be "enhanced" if the difference in LUC expression from two mRNAs is significantly greater than the difference in their functional half-lives.

Time Considerations

The preparation and analysis of input RNA may take 5 to 6 hr total. The leaf-peeling process takes 0.5 to 1.5 hr, depending on the number of leaves and the experimenter's experience. Digestion to produce protoplasts takes 4.5 hr or overnight (see Critical Parameters and Troubleshooting for discussion of the two choices). Another 1.5 hr are expected after digestion in preparing the cells for electroporation. With a 35-sec interval, it takes ~20 min to electroporate 24 samples into protoplasts and dispense samples for multiple harvest time points. Harvesting and lysis of protoplasts takes ~20 min.

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Internet Resources

http://www.promega.com

The Promega Inc. Web site is a searchable resource with information on various aspects of the use of LUC as a reporter.

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USEFUL INFORMATION AND DATA

Abbreviations Used in this Manual

A adenine or adenosine; one-letter code for alanine
A_{260} absorbance at 260 nm
7-AAD 7-aminoactinomycin D
Ab antibody
ABSA American Biological Safety Association
ABSL Animal Biosafety Level
ADP adenosine 5′-diphosphate
Ads adenoviruses
AEBSF 4-(2-aminoethyl)benzenesulfonyl fluoride
Ag antigen
AI-2 autoinducer-2
AIDS acquired immune deficiency syndrome
ALI air-liquid interface
AMP adenosine 5′-monophosphate
AMSMIC Association of Medical School Microbiology and Immunology Chairs
ANOVA analysis of variance
AOTF acousto-optical tunable filter
APMSF *p*-amidinophenylmethanesulfonyl fluoride
Apr ampicillin resistant
APS ammonium persulfate
ARDRA amplified rDNA restriction analysis
ATCC American Type Culture Collection
ATP adenosine 5′-triphosphate
BA *Brucella abortus*
BALO *Bdellovibrio* and like organisms
BBS BES-buffered solution; borate-buffered saline
BCIP 5-bromo-4-chloro-3-indolyl phosphate
BDB benzidine
BES *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
β-gal β-galactosidase
Bisacrylamide *N*,*N*′-methylene-bisacrylamide
Bis-Tris 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
BLAST Basic Local Alignment Research Tool
BMBL Biosafety in Microbiological and Biomedical Laboratories
BMP bitmap (file format)
bp base pair
Bq Becquerel
BrdU 5-bromodeoxyuridine
BSA bovine serum albumin
BSC biological safety cabinet
BSL Biosafety Level
C cytosine or cytidine; one-letter code for cysteine
CA3 chromomycin A3
cAMP adenosine 3′,5′-cyclic-monophosphate
CAPS cyclohexylaminopropane sulfonic acid
CAT chloramphenicol acetyltransferase
CDC Centers for Disease Control and Prevention
cDNA complementary deoxyribonucleic acid
CDP cytidine 5′-diphosphate
CFA complete Freunds adjuvant
cfu colony forming units
CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
CHES 2-(*N*-cyclohexylamino)ethanesulfonic acid
CHO Chinese hamster ovary
Ci curie
CLSM confocal laser scanning microscopy
cM centimorgans
CMFDA chloromethylfluoroscein diacetate
CMP cytidine 5′-monophosphate
CMV cytomegalovirus
CNBr cyanogen bromide
CPC cetylpyridinium chloride
CPE cytopathic effect
cpm counts per minute
CSF colony-stimulating factor
CTAB cetyltrimethylammonium bromide
CTD C-terminal domain
CTP cytidine 5′-triphosphate
CFP cyan fluorescent protein
D dextrorotatory
Da Dalton
dA deoxyadenosine
DAB diaminobenzidine
DABCO 1,4-diazabicyclo[2,2,2]-octane
dAMP deoxyadenosine monophosphate
DAPI 4′,6-diamidino-2-phenylindole
dATP deoxyadenosine triphosphate
dC deoxycytosine

DCA dichloroacetic acid
dCF 2′-deoxycoformycin
dCMP deoxycytidine monophosphate
dCTP deoxycytidine triphosphate
ddATP dideoxyadenosine triphosphate
ddCTP dideoxycytidine triphosphate
ddGTP dideoxyguanosine triphosphate
ddNTP dideoxynucleoside triphosphate
ddTTP dideoxythymidine triphosphate
DEA diethylamine
DEAE diethylaminoethyl
DEPC diethylpyrocarbonate
DFP diisopropyl fluorophosphate
dG deoxyguanosine
dGTP deoxyguanosine triphosphate
dH_2O deionized water
DMC dialysis membrane chamber
DMEM Dulbecco's minimum essential medium
DMF dimethylformamide
DMS dimethyl sulfate
DMSO diemthyl sulfoxide
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DNB diluted nutrient broth
dNTP deoxynucleoside triphosphate
DPA diphenylamine
dpm disintegrations per minute
ds double stranded
dT deoxythymidine
DTT dithiothreitol
dTTP deoxythymidine triphosphate
dUMP deoxyuridine monophosphate
DUS DNA uptake sequence
dUTP deoxyuridine triphosphate
EB elementary body
EBSS Earl's balanced salt solution
EBV Epstein-Barr virus
EDTA ethylenediaminetetraacetic acid
EGTA ethylene glycol-bis(-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid
EHEC enterohemorrhagic *Escherichia coli*
EIA enzyme immunoassay plate
ELISA enzyme-linked immunosorbent assay
EMBL European Molecular Biology Laboratory
EMEM Eagle minimal essential medium
EMS ethyl methanesulfonate
EtBr ethidium bromide
F Farad
FAQ frequently asked questions
FBS fetal bovine serum
FCS fetal calf serum
FISH fluorescence in situ hybridization
FITC fluorescein isothiocyanate
FLIP fluorescence loss in photobleach
FOA fluoroorotic acid
FPLC fast protein, peptide, and polynucleotide liquid chromatography
FRAP fluorescence recovery after photobleach
FRET fluorescent resonant energy transfer
FTIR Fourier transform infrared (spectroscopy)
FTP File Transfer Protocol
Fuc L-fucose
FUdR 5-fluoro-2′-deoxyuridine
G gauge; guanine or guanosine; one-letter code for glycine
g gravity (unit of centrifugal force)
Gal D-galactose
Gb gigabyte
GCB gonococcal base medium
GDP guanosine 5′-diphosphate
GFP green fluorescent protein
GIF Graphics Interchange Format
Glc D-glucose
GlcA D-glucuronic acid
GMP guanosine monophosphate
GSSA Glaxo selective supplement A
GST glutathione S-transferase
GTP guanosine 5′-triphosphate
Gy Gray (radioactivity unit)
hBD human β-defensins
HBSS Hank's balanced salt solution
HeBS HEPES-buffered saline
HeNe helium-neon (laser)
HEPA vacuuming high-efficiency particulate air vacuuming
HEPA high-efficiency particulate air (filter)
HBV hepatitis B virus
HEPES *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]
HGA human granulocytic anaplasmosis
HI host independent (organisms)
HIV human immunodeficiency virus
HPLC high-pressure liquid chromatography
HTML hypertext markup language
Hz hertz
i.d. inner diameter
i.m. intramuscular
IAA 3-β-indoleacrylic acid
IACUC Institutional Animal Care and Use Committee
IBC Institutional Biosafety Committee
IFU infectious forming units
Ig immunoglobulin
IMDM Iscove's modified Dulbecco's medium
IP Internet Protocol
IPTG isopropyl-1-thio-β-D-galactoside
IR infrared
IRES internal ribosomal entry site

ISDN integrated services digital network
ISH in situ hybridization
ISP Internet service provider
JPEG Joint Photographic Experts Group
kb kilobase
K_d dissociation constant
kDa kilodalton
KHz kilohertz
L levorotatory
LAN local area network
LB Luria-Bertani (medium)
LC liquid chromatography
LCM laser capture microdissection
LCSM laser scanning confocal microscope; laser scanning confocal microscopy
LGV lymphogranuloma venereum
ln natural logarithm
M relative molecular weight
mA milliampere
MAb monoclonal antibody
MALDI matrix-assisted laser desorption/ionization (mass spectrometry)
MALDI-TOF matrix-assisted laser desorption/ionization time-of-flight (mass spectroscopy)
MALT mucosa-associated lymphatic tissue
Man D-mannose
Mb megabase, megabyte
Mbp megabase pair
MEM minimal essential media
2-ME 2-mercaptoethanol
MES 2-(*N*-morpholino)ethanesulfonic acid
MHz megahertz
μF microfarad
miRNA microRNA
MMS methyl methanesulfonate
mmu millimass unit or one thousandth of a Dalton
MNNG *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
MOI multiplicity of infection
MOMP major outer membrane protein
MOPS 3-(*N*-morpholino)propane sulfonic acid
mp melting point
MPA mycophenolic acid
MRI magnetic resonance imaging
mRNA messenger RNA
MS/MS tandem mass spectrometry
MS mass spectroscopy
MSDS material safety data sheet
MSX methionine sulfoximine
MWCO molecular weight cutoff
NA not applicable
NBT nitroblue tetrazolium
NCBI National Center for Biotechnology Information
NCS newborn calf serum
ND not determined
neo neomycin gene (selectable marker)
NEPHGE nonequilibrium pH gradient electrophoresis
NIH National Institutes of Health
NK natural killer (cells)
NLM National Library of Medicine
NMR nuclear magnetic resonance
NP-40 Nonidet P-40 (detergent)
NRC Nuclear Regulatory Commission
nt nucleotide
NTP nucleoside triphosphate
o.d. outer diameter
OD optical density (subscript specifies wavelength, e.g., optical density at 600 nm is abbreviated as OD_{600})
oligo(dT) oligodeoxythymidylic acid
oligo oligonucleotide, a short, single-stranded DNA or RNA
ONPG *o*-nitrophenyl-β-D-galactosidase
Opps oligopeptide permease proteins
ORC origin recognition complex
ORF open reading frame
ori origin of replication
OspA outer surface protein A
OspC outer surface protein C
PACT photodynamic antimicrobioal chemotherapy
PAGE polyacrylamide gel electrophoresis
PaGFP photosensitive green fluorescent protein
PBS phosphate-buffered saline
PCR polymerase chain reaction
PCR polymerase chain reaction
PDB Protein Data Bank
PE phycoerythrins
PEG polyethylene glycol
PEI polyethylenimine
PEL permissible exposure limit
PFA paraformaldehyde
pfu plaque forming unit
PI propidium iodide
pI isoelectric point
PIPES piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)
PMA phorbol 12-myristate 13-acetate
PMSF phenylmethylsulfonyl fluoride
PMT photomultiplier tube
poly(A) polyadenylic acid or polyadenylate
Pp polypeptone (medium)
PPD *p*-phenylenediamine
ppm parts per million
PPO 2,5-diphenyloxazole
PSF point-spread functions
PSGL-1 P-selectin glycoprotein ligand-1
PSTVd potato spindle tuber viroid

Pu purine
Py pyrimidine
Q-RT-PCR quantitative reverse transcription PCR
RB reticulate body
RBS ribosome-binding site
RCF relative centrifugal force
RFP red fluorescent protein
RI refractive index
RNA ribonucleic acid
RNAi RNA interference
RNase ribonuclease
RP reversed phase (HPLC)
rRNA ribosomal ribonucleic acid
RT reverse transcriptase
RTF reduced transport fluid
RT-PCR reverse transcription PCR
SD standard deviation
SDS sodium dodecyl sulfate or sodium lauryl sulfate
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SED standard enzyme diluent
siRNA short interfering RNA
SLS sodium lauryl sulfate
SRBC sheep red blood cells
ss single stranded
SSC sodium chloride/sodium citrate (buffer)
STI soybean trypsin inhibitor
T thymine or thymidine; one-letter code for threonine
TAE Tris/acetate (buffer)
Taq *Thermus aquaticus* DNA (polymerase)
TB terrific broth
TBE Tris/borate (buffer)
TBS Tris-buffered saline
TBST Tris-buffered saline containing Tween-20
TCA trichloroacetic acid
TCP Transmission Control Protocol
TE Tris/EDTA (buffer)
TEA triethanolamine acetate
TEAE triethylaminoethyl
TEM transmission electron microscopy
TEMED *N*,*N*,*N*′,*N*′-tetramethylethylenediamine
TEN sodium chloride in TE buffer
TFA trifluoroacetic acid
TIFF Tagged-Image File Format
TK thymidine kinase
TLC thin-layer chromatography
TLCK *N*α-*p*-tosyl-L-lysine chloromethyl ketone
T_m melting (or midpoint) temperature; thermal denaturation
TMAC tetramethylammonium chloride
TMP trimethyl phosphate
TPCK *N*α-*p*-tosyl-L-phenylalanine chloromethyl ketone
Tris tris(hydroxymethyl)aminomethane
Tris·Cl Tris hydrochloride
tRNA transfer ribonucleic acid
TSB trypticase soy broth
TTP thymidine 5′-triphosphate
U unit (enzyme); uracil or uridine
UDP uridine 5′-diphosphate
UMP uridine 5′-monophosphate
USDA United States Department of Agriculture
UTP uridine 5′-triphosphate
UTR untranslated leader region
UV ultraviolet
V_0 void volume
Vent *Thermococcus litoralis* DNA (polymerase)
vol/vol, v/v volume/volume
WHO World Health Organization
wt/vol; w/v weight/volume
WT wild-type
WWW World Wide Web
Xgal 5-bromo-4-chloro-3-indolyl-β-D-galactoside
YFP yellow fluorescent protein

Resources for International Biosafety Guidelines and Regulations

Determining the appropriate safety guidelines and regulations relevant to a given experiment is the first step in any research study. To facilitate this process when working with microorganisms, the reader is refered to *http://www.absa.org/resguides.html*, the Biosafety Guidelines website maintained by the American Biological Safety Association (ABSA). This comprehensive site not only provides direct links to several high-impact references, but also to the compendium maintained by the International Biosafety Working Group (IBWG), which researchers can use to search through an extensive list of international biosafety resources, including other databases appropriate to the investigation. Note that the IBWG compendium is currently hosted by the European Biosafety Association (EBSA; *http://www.ebsa.be*), which is itself a valuable resource.

The reader is also strongly advised to explore the ABSA home page (*http://www.absa.org/index.shtml*) and Resources and Tools page (*http://www.absa.org/restool.html*), both of which provide a wealth of biosafety information.

Table A.1B.1 provides a list of the resources available on the ABSA website. For general safety information, the reader is referred to the first section of Chapter 1, including *UNIT 1A.1*, which provides a discussion of general biosafety.

IMPORTANT NOTE: While an effort has been made to provide a starting point for the reader to begin their investigation into the appropriate safety precautions for their experiment, the resources presented here and at the websites mentioned should not be considered comprehensive. Ultimate responsibility for safety lies with the individual researcher. Please consult all appropriate safety resources prior to beginning any experiment.

Table A.1B.1 Resources Available from the American Biological Safety Association Biosafety Guidelines Website[a]

Resource	Responsible Agency
Laboratory Biosafety Manual, Third Edition	World Health Organization
NIH Guidelines on Recombinant DNA Molecules	U.S. Department of Health and Human Services, National Institutes of Health
Primary Containment for Biohazards: Selection, Installation and Use of Biological Safety Cabinets	U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, *and* National Institutes of Health
Biosafety in Microbiological and Biomedical Laboratories, Fourth Edition	U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, *and* National Institutes of Health
Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens	World Health Organization, Division of Emerging and Other Communicable Diseases Surveillance and Control
The Laboratory Biosafety Guidelines, Third Edition	Public Health Agency of Canada, Centre for Emergency Preparedness and Response, Office of Laboratory Security
NIH Design Criteria for Laboratory Construction website	U.S. Office of Research Facilities
International Biosafety Working Group website[b]	International Biosafety Working Group

[a] Available at *http://www.absa.org/resguides.html*.

[b] This site is currently hosted by the European Biosafety Association (EBSA; *http://www.ebsa.be*) and is accessed by selecting International Biosafety from the EBSA homepage.

COMMONLY USED REAGENTS AND EQUIPMENT

Commonly Used Reagents

This section describes the preparation of buffers and reagents commonly used in this manual. For a list of where to find formulations of media, please refer to *APPENDIX 2C*. When preparing solutions, use deionized, distilled water and reagents of the highest available grade. Sterilization—by filtration through a 0.22-μm filter or by autoclaving—is recommended for most solutions stored at room temperature and is essential for cell culture applications. Where storage conditions are not specified, store up to 6 months at room temperature. Discard any reagent that shows evidence of contamination, precipitation, or discoloration.

CAUTION: Handle strong acids and bases with care. See *UNIT 1A.3* for more information concerning the use of hazardous chemicals.

Acid, concentrated stock solutions

See Table A.2A.1.

Acid precipitation solution

1 M HCl (Table A.2A.1)
0.1 M sodium pyrophosphate

Nucleic acids can also be precipitated with a 10% (w/v) solution of trichloroacetic acid (TCA; see recipe); however, this recipe is cheaper, easier to prepare, and just as efficacious.

Table A.2A.1 Molarities and Specific Gravities of Concentrated Acids and Bases[a]

Acid/base	Mol. wt.	% by weight	Molarity (approx.)	Specific gravity	1 M solution (ml/liter)
Acetic acid (glacial)[b]	60.05	99.6	17.4	1.05	57.5
Ammonium hydroxide	35.0	28	14.8	0.90	67.6
Formic acid[b]	46.03	90	23.6	1.205	42.4
		98	25.9	1.22	38.5
Hydrochloric acid	36.46	36	11.6	1.18	85.9
Nitric acid	63.01	70	15.7	1.42	63.7
Perchloric acid	100.46	60	9.2	1.54	108.8
		72	12.2	1.70	82.1
Phosphoric acid[b]	98.00	85	14.7	1.70	67.8
Sulfuric acid	98.07	98	18.3	1.835	54.5

[a] *CAUTION:* Handle strong acids and bases carefully (see *UNIT 1A.3*).
[b] Also see Table A.2A.3.

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Alsever's solution

20.5 g dextrose (114 mM)
7.9 g sodium citrate·$2H_2O$ (27 mM)
4.2 g NaCl (71 mM)
H_2O to 1 liter
Adjust to pH 6.1 with 1 M citric acid (Table A.2A.3) and filter sterilize
Store indefinitely at 4°C

Ammonium hydroxide, concentrated stock solution

See Table A.2A.1

Ammonium acetate, 10 M

Dissolve 385.4 g ammonium acetate in 150 ml H_2O
Add H_2O to 500 ml

Ammonium sulfate, saturated

76 g ammonium sulfate
100 ml H_2O
Heat with stirring to just below boiling point
Let stand overnight at room temperature

ATP, 100 mM

1 g ATP (adenosine triphosphate)
12 ml H_2O
Adjust pH to 7.0 with 4 M NaOH
Adjust volume to 16.7 ml with H_2O
Store in aliquots indefinitely at −20°C

Base, concentrated stock solutions

See Table A.2A.1.

BBS (BES-buffered solution), 2×

50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; Calbiochem)
280 mM NaCl
1.5 mM sodium phosphate buffer, pH 6.95 (see recipe)
800 ml H_2O
Adjust pH to 6.95 with room temperature 1 N NaOH
H_2O to 1 liter
Filter sterilize through a 0.45-μm nitrocellulose filter (Nalgene)
Store in aliquots at −20°C (can be frozen and thawed repeatedly)

The pH of this solution is critical (pH 6.95 to 6.98). When a new batch of 2× BES buffer is prepared, its pH should be checked against a reference stock prepared (and tested) earlier.

BCIP, 5% (w/v)

Dissolve 0.5 g 5-bromo-4-chloro-3-indolyl phosphate disodium salt (stored at −20°C) in 10 ml of 100% dimethylformamide (DMF). Store wrapped in aluminum foil up to 6 months at 4°C.

The BCIP may not dissolve completely. Vortex the solution immediately before use and pipet with a wide-mouth pipet tip.

Discard solution if it turns pinkish.

BSA (bovine serum albumin), 10% (w/v)

Dissolve 10 g BSA (e.g., Sigma) in 100 ml H_2O. Filter sterilize using a low-protein-binding 0.22-μm filter. Store indefinitely at 4°C.

Lower-concentration stock solutions (e.g., 1%), which are useful for various applications, can be made by diluting 10% stock appropriately with sterile water.

BSA is available in various forms that differ in fraction of origin, preparation, purity, pH, and cost; the most commonly used is fraction V. Use the form that is appropriate for the application; this may need to be optimized empirically.

$CaCl_2$, 1 M

147 g $CaCl_2 \cdot 2H_2O$
H_2O to 1 liter

Carbonate buffer

1.6 g Na_2CO_3 (15 mM final)
2.9 g $NaHCO_3$ (35 mM final)
0.2 g NaN_3 (3.1 mM final)
H_2O to 1 liter
Adjust to pH 9.5

CAUTION: Sodium azide is poisonous; follow appropriate precautions for handling, storage, and disposal.

Denhardt solution, 100×

10 g Ficoll 400
10 g polyvinylpyrrolidone
10 g bovine serum albumin (Pentax Fraction V; Miles Laboratories)
H_2O to 500 ml
Filter sterilize and store at −20°C in 25-ml aliquots

DEPC (diethylpyrocarbonate)-treated solutions

Add 0.2 ml DEPC to 100 ml of the solution to be treated. Shake vigorously to dissolve the DEPC. Autoclave the solution to inactivate the remaining DEPC.

CAUTION: Wear gloves and use a fume hood when using DEPC, as it is a suspected carcinogen.

Many investigators keep the solutions they use for RNA work separate to ensure that "dirty" pipets do not go into them.

Do not treat solutions containing Tris with DEPC, as Tris inactivates the DEPC.

DNase I, RNase-free (1 mg/ml)

Prepare a solution of 0.1 M iodoacetic acid plus 0.15 M sodium acetate and adjust pH to 5.3. Filter sterilize. Add sterile solution to lyophilized RNase-free DNase I (e.g., Worthington) to give a final concentration of 1 mg/ml. Heat 40 min at 55°C and then cool. Add 1 M $CaCl_2$ to a final concentration of 5 mM. Store at −80°C in small aliquots.

dNTPs: dATP, dTTP, dCTP, and dGTP

Concentrated stocks: Purchase deoxyribonucleoside triphosphates (dNTPs) from a commercial supplier either as ready-made 100 mM solutions (the preferred form for shipping and storage) or in lyophilized form. If purchased lyophilized, dissolve dNTPs in deionized water to an expected concentration of 30 mM, then adjust to

continued

Table A.2A.2 Molar Extinction Coefficients of DNA Bases

Base	Molar extinction coefficient (ε)[a]
Adenine	15,200
Cytosine	7050
Guanosine	12,010
Thymine	8400

[a] 1 M solution measured at 260 nm; see Wallace and Miyada (1987).

pH 7.0 with 1 M NaOH (to prevent acid-catalyzed hydrolysis). Determine the actual concentration of each dNTP by UV spectrophotometry at 260 nm, referring to the molar extinction coefficients given in Table A.2A.2.

Working solutions: Prepare working solutions of desired concentration (commonly 2 mM) for each dNTP by diluting concentrated stocks appropriately. Remember that the molarity of the 3dNTP and 4dNTP mixes refers to the concentration of *each* precursor present in the solution.

4dNTP mixes: Prepare mixed dNTP solutions containing equimolar amounts of all four DNA precursors; e.g.:

2 mM 4dNTP mix: 2 mM *each* dATP, dTTP, dCTP, and dGTP
1.25 mM 4dNTP mix: 1.25 mM *each* dATP, dTTP, dCTP, and dGTP.

3dNTP mixes: Prepare stocks lacking one particular dNTP but containing equimolar amounts of the remaining three precursors; e.g.:

2 mM 3dNTP mix (minus dATP): 2 mM *each* dTTP, dCTP, and dGTP.

Store dNTPs and dNTP mixtures as aliquots at −20°C (stable for ≤1 year).

DPBS (Dulbecco's phosphate-buffered saline)

8.00 g NaCl (0.137 M)
0.20 g KCl (2.7 mM)
0.20 g KH_2PO_4 (1.1 mM)
0.10 g $MgCl_2 \cdot 6H_2O$ (0.5 mM)
2.16 g $Na_2HPO_4 \cdot 7H_2O$ (8.1 mM)
0.10 g anhydrous $CaCl_2$ (0.9 mM)
H_2O to 1 liter

DPBS may be made or purchased without Ca^{2+} and Mg^{2+} (CMF-DPBS). These components are optional and usually have no effect on an experiment; in a few cases, however, their presence may be detrimental. Consult individual protocols to see if the presence or absence of these components is recommended.

Also see recipes for phosphate-buffered saline (PBS) and phosphate-buffered saline containing potassium (KPBS).

DTT (dithiothreitol), 1 M

Dissolve 1.55 g DTT in 10 ml water
Filter sterilize
Store in aliquots at −20°C

EDTA (ethylenediaminetetraacetic acid), 0.5 M (pH 8.0)

Dissolve 186.1 g disodium EDTA dihydrate in 700 ml water. Adjust pH to 8.0 with 10 M NaOH (~50 ml; add slowly). Add water to 1 liter and filter sterilize.

Begin titrating before the sample is completely dissolved. EDTA, even in the disodium salt form, is difficult to dissolve at this concentration unless the pH is increased to between 7 and 8.

Ethidium bromide staining solution

Concentrated stock (10 mg/ml): Dissolve 0.2 g ethidium bromide in 20 ml water. Mix well and store at 4°C in the dark or in a foil-wrapped bottle. Do not sterilize.

Working solution: Dilute stock to 0.5 μg/ml or other desired concentration in electrophoresis buffer (e.g., 1× TBE or TAE; see recipes) or in water.

Ethidium bromide working solution is used to stain agarose gels to permit visualization of nucleic acids under UV light. Gels should be placed in a glass dish containing sufficient working solution to cover them and shaken gently or allowed to stand for 10 to 30 min. If necessary, gels can be destained by shaking in electrophoresis buffer or water for an equal length of time to reduce background fluorescence and facilitate visualization of small quantities of DNA.

Alternatively, a gel can be run directly in ethidium bromide by using working solution (made with electrophoresis buffer) as the solvent and running buffer for the gel.

CAUTION: Ethidium bromide is a mutagen and must be handled carefully. See UNIT 1A.3 for more information.

FBS (fetal bovine serum)

Thaw purchased fetal bovine serum (shipped on dry ice and kept frozen until needed). Store 3 to 4 weeks at 4°C. If FBS is not to be used within this time, aseptically divide into smaller aliquots and refreeze until used. Store ≤1 year at −20°C.

FBS is shipped on dry ice and kept frozen until needed. Repeated thawing and refreezing should be avoided, as it may cause denaturation of the serum.

FBS, heat inactivated

Heat FBS (see recipe) 30 min to 1 hr in a 56°C water bath with periodic gentle swirling during the first 10 to 15 min to ensure uniform heating.

Treating FBS in this manner inactivates complement protein and thus prevents an immunological reaction against cultured cells, making it useful for a variety of purposes.

This reagent can also be purchased commercially.

Formamide loading buffer, 2×

Prepare in deionized formamide
0.05% (w/v) bromphenol blue
0.05% (w/v) xylene cyanol FF
20 mM EDTA
Do not sterilize
Store at −20°C

HBSS (Hanks' balanced salt solution)

0.40 g KCl (5.4 mM final)
0.09 g $Na_2HPO_4 \cdot 7H_2O$ (0.3 mM final)
0.06 g KH_2PO_4 (0.4 mM final)
0.35 g $NaHCO_3$ (4.2 mM final)

continued

0.14 g $CaCl_2$ (1.3 mM final)
0.10 g $MgCl_2 \cdot 6H_2O$ (0.5 mM final)
0.10 g $MgSO_4 \cdot 7H_2O$ (0.6 mM final)
8.0 g NaCl (137 mM final)
1.0 g D-glucose (5.6 mM final)
0.2 g phenol red (0.02%; optional)
Add H_2O to l liter and adjust pH to 7.4 with 1 M HCl (Table A.2A.1) or 1 M NaOH
Filter sterilize and store up to 1 month at 4°C

HBSS may be made or purchased without Ca^{2+} and Mg^{2+} (CMF-HBSS). These components are optional and usually have no effect on an experiment; in a few cases, however, their presence may be detrimental. Consult individual protocols to see if the presence or absence of these components is recommended.

Bottles should be kept tightly closed to prevent CO_2 loss and subsequent alkalinization.

HCl, 1 M

Mix the following in order:
913.8 ml H_2O
86.2 ml concentrated HCl (Table A.2A.1)

HeBS (HEPES-buffered saline) solution, 2×

16.4 g NaCl
11.9 g HEPES acid (Table A.2A.3)
0.21 g Na_2HPO_4
800 ml H_2O
Titrate to pH 7.05 with 5 M NaOH
Add H_2O to 1 liter
Filter sterilize through a 0.45-μm nitrocellulose filter
Store in 50-ml aliquots at −20°C

If the solution is to be used for transfection, the pH should be between 7.05 and 7.12, and should be tested for transfection efficiency.

KCl, 1 M

74.6 g KCl
H_2O to 1 liter

2-ME (2-mercaptoethanol), 50 mM

Prepare 1 M stock:
0.5 ml 14.3 M 2-ME
6.6 ml H_2O
Prepare 50 mM stock:
5 ml 1 M 2-ME
95 ml H_2O
Store at 4°C

$MgCl_2$, 1 M

20.3 g $MgCl_2 \cdot 6H_2O$
H_2O to 100 ml

$MgSO_4$, 1 M

24.6 g $MgSO_4 \cdot 7H_2O$
H_2O to 100 ml

MOPS buffer

0.2 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 7.0 (Table A.2A.3)
0.5 M sodium acetate
0.01 M EDTA
Store in the dark and discard if it turns yellow

NaCl, 5 M

292 g NaCl
H_2O to 1 liter

NaOH, 10 M

Dissolve 400 g NaOH in 450 ml H_2O
Add H_2O to 1 liter

PCR amplification buffer, 10×

500 mM KCl
100 mM Tris·Cl, pH 8.3 (see recipe)
x mM $MgCl_2$
0.1% (w/v) gelatin
Store in aliquots at −20°C

This solution can be sterilized by autoclaving. Alternatively, it can be made from sterile water and stock solutions, and the sterilization omitted.

15 mM $MgCl_2$ is the concentration (x) used for most PCR reactions. However, the optimal concentration depends on the sequence and primer of interest and may have to be determined experimentally.

Phenol, buffered

Add 0.5 g of 8-hydroxyquinoline to a 2-liter glass beaker containing a stir bar. Gently pour in 500 ml liquefied phenol or melted crystals of redistilled phenol (melt in a water bath at 65°C). The phenol will turn yellow due to the 8-hydroxyquinoline, which is added as an antioxidant. Add 500 ml of 50 mM Tris base. Cover the beaker with aluminum foil and stir 10 min at room temperature using a magnetic stirrer on low speed. Let phases separate at room temperature. Gently decant the top (aqueous) phase into a suitable waste receptacle (*UNIT 1A.3*). Remove what cannot be decanted with a 25-ml glass pipet and a suction bulb. Add 500 ml of 50 mM Tris·Cl, pH 8.0 (see recipe). Repeat equilibration with 500 ml of 50 mM Tris·Cl, pH 8.0, twice. Check the pH of the phenol phase with indicator paper to determine if it is 8.0. If it is not, repeat equilibration until this pH is obtained. Add 250 ml of 50 mM Tris·Cl, pH 8.0, or TE buffer, pH 8.0 (see recipe), and store at 4°C in brown glass bottles or clear glass bottles wrapped in aluminum foil.

CAUTION: Phenol can cause severe burns to skin and damage clothing. Gloves, safety glasses, and a laboratory coat should be worn whenever working with phenol, and all manipulations should be carried out in a fume hood. A glass receptacle should be available exclusively for disposing of used phenol and chloroform (see UNIT 1A.3).

Phenol prepared with 8-hydroxyquinoline as an antioxidant can be stored ≤2 months at 4°C.

Phenol must be redistilled before use, because oxidation products of phenol can damage and introduce breaks into nucleic acid chains. Redistilled phenol is commercially available. Regardless of the source, phenol must be buffered before use.

Table A.2A.3 pK_a Values and Molecular Weights for Some Common Biological Buffers[a]

Name	Chemical formula or IUPAC name	pK_a	Useful pH range	Mol. wt. (g/mol)
Phosphoric acid[b]	H_3PO_4	2.12 (pK_{a1})	—	98.00
Citric acid[c]	$C_6H_8O_7$ (H_3Cit)	3.06 (pK_{a1})	—	192.1
Formic acid[b]	HCOOH	3.75	—	46.03
Succinic acid	$C_4H_6O_4$	4.19 (pKa_1)	—	118.1
Citric acid[c]	$C_6H_7O_7^-$ (H_2Cit^-)	4.74 (pKa_2)	—	
Acetic acid[b]	CH_3COOH	4.75	—	60.05
Citric acid[c]	$C_6H_6O_7^-$ ($HCit_2^-$)	5.40 (pK_{a3})	—	
Succinic acid	$C_4H_5O_4^-$	5.57 (pK_{a2})	—	
MES	2-(*N*-Morpholino]ethanesulfonic acid	6.15	5.5-6.7	195.2
Bis-Tris	bis(2-Hydroxyethyl)iminotris (hydroxymethyl)methane	6.50	5.8-7.2	209.2
ADA	*N*-(2-Acetamido)-2-iminodiacetic acid	6.60	6.0-7.2	190.2
PIPES	Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)	6.80	6.1-7.5	302.4
ACES	*N*-(Carbamoylmethyl)-2-amino-ethanesulfonic acid	6.80	6.1-7.5	182.2
Imidazole	1,3-Diaza-2,4-cyclopentadiene	7.00	—	68.08
Diethylmalonic acid	$C_7H_{12}O_4$	7.20	—	160.2
MOPS	3-(*N*-Morpholino)propanesulfonic acid	7.20	6.5-7.9	209.3
Sodium phosphate, monobasic	NaH_2PO_4	7.21 (pK_{a2})	—	120.0
Potassium phosphate, monobasic	KH_2PO_4	7.21 (pK_{a2})	—	136.1
TES	*N*-tris(Hydroxymethyl)methyl-2-aminoethanesulfonic acid	7.40	6.8-8.2	229.3
HEPES	*N*-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)	7.55	6.8-8.2	238.3
HEPPSO	*N*-(2-Hydroxyethyl)piperazine-*N*′-(2-hydroxypropanesulfonic acid)	7.80	7.1-8.5	268.3
Glycinamide·HCl	$C_2H_6N_2O$·HCl	8.10	7.4-8.8	110.6
Tricine	*N*-tris(Hydroxymethyl)methylglycine	8.15	7.4-8.8	179.2
Glycylglycine	$C_4H_8N_2O_3$	8.20	7.5-8.9	132.1
Tris	Tris(hydroxymethyl)aminomethane	8.30	7.0-9.0	121.1
Bicine	*N*,*N*-bis(2-Hydroxyethyl)glycine	8.35	7.6-9.0	163.2
Boric acid	H_3BO_3	9.24	—	61.83
CHES	2-(*N*-Cyclohexylamino)ethane-sulfonic acid	9.50	8.6-10.0	207.3
CAPS	3-(Cyclohexylamino)-1-propane-sulfonic acid	10.40	9.7-11.1	221.3

continued

Table A.2A.3 pK_a Values and Molecular Weights for Some Common Biological Buffers[a] , *continued*

Name	Chemical formula or IUPAC name	pK_a	Useful pH range	Mol. wt. (g/mol)
Sodium phosphate, dibasic	Na_2HPO_4	12.32 (pK_{a3})	—	142.0
Potassium phosphate, dibasic	K_2HPO_4	12.32 (pK_{a3})	—	174.2

[a]Some data reproduced from Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems (Mohan, 1997) with permission of Calbiochem.

[b]See Table A.2A.1 for more information.

[c]Available as a variety of salts, e.g., ammonium, lithium, sodium.

Phenol/chloroform/isoamyl alcohol, 25:24:1 (v/v/v)

25 vol buffered phenol (bottom yellow phase of stored solution; see recipe)
24 vol chloroform
1 vol isoamyl alcohol
Store up to 2 months at 4°C

Phosphate-buffered saline (PBS)

0.23 g NaH_2PO_4 (anhydrous; 1.9 mM)
1.15 g Na_2HPO_4 (anhydrous; 8.1 mM)
9.00 g NaCl (154 mM)
Add H_2O to 900 ml
Adjust to desired pH (7.2 to 7.4) using 1 M NaOH or 1 M HCl (see recipe and Table A.2A.1)
Add H_2O to 1 liter

Also see recipes for phosphate-buffered saline containing potassium (KPBS) and Dulbecco's phosphate-buffered saline (DPBS).

Phosphate-buffered saline containing potassium (KPBS)

8.00 g NaCl (0.137 M)
0.20 g KCl (2.7 mM)
0.24 g KH_2PO_4 (1.4 mM)
1.44 g Na_2HPO_4 (0.01 M)
H_2O to 1 liter

Also see recipes for phosphate-buffered saline (PBS) and Dulbecco's phosphate-buffered saline (DPBS).

PMSF (phenylmethylsulfonyl fluoride), 100 mM

Dissolve 0.174 g PMSF in 10 ml of 100% ethanol, isopropanol, or methanol. Store in aliquots up to 2 years at −20°C.

CAUTION: Phenylmethylsulfonyl fluoride is toxic.

Make fresh dilutions from the alcohol stock for each use, because the half-life of PMSF in aqueous solution is <30 min at room temperature and a few hours on ice.

If PMSF is being added to a solution without detergent, the solution should be stirred vigorously during addition because PMSF has a tendency to form an insoluble precipitate in aqueous solution.

Potassium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid (Tables A.2A.1 and A.2A.3) per liter (0.2 M) in water.

continued

Table A.2A.4 Preparation of 0.1 M Sodium and Potassium Acetate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

[a]Adapted with permission from CRC (1975).

Solution B: 19.6 g potassium acetate ($KC_2H_3O_2$) per liter (0.2 M) in water.

Referring to Table A.2A.4 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check the pH by diluting an aliquot of concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.4, prepare the closest higher pH, then titrate with solution A.

Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH_2PO_4 (Table A.2A.3) per liter (0.2 M final) in water

Solution B: 34.8 g K_2HPO_4 (Table A.2A.3) per liter (0.2 M final) in water

Referring to Table A.2A.5 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This buffer may be made as a 5- or 10-fold concentrate simply by scaling up the amount of potassium phosphate in the same final volume. Phosphate buffers show concentration-dependent changes in pH, so check the pH of the concentrate by diluting an aliquot to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.5, prepare closest higher pH, then titrate with solution A.

RNase A stock solution, DNase-free, 2 mg/ml

Dissolve RNase A (e.g., Sigma) in DEPC-treated H_2O (see recipe) to 2 mg/ml. Boil 10 min in a 100°C water bath. Store up to 1 year at 4°C.

The activity of the enzyme varies from lot to lot; therefore, prepare several 10-ml aliquots of each dilution to facilitate standardization.

Table A.2A.5 Preparation of 0.1 M Sodium and Potassium Phosphate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5
5.8	92.0	8.0
5.9	90.0	10.0
6.0	87.7	12.3
6.1	85.0	15.0
6.2	81.5	18.5
6.3	77.5	22.5
6.4	73.5	26.5
6.5	68.5	31.5
6.6	62.5	37.5
6.7	56.5	43.5
6.8	51.0	49.0
6.9	45.0	55.0
7.0	39.0	61.0
7.1	33.0	67.0
7.2	28.0	72.0
7.3	23.0	77.0
7.4	19.0	81.0
7.5	16.0	84.0
7.6	13.0	87.0
7.7	10.5	90.5
7.8	8.5	91.5
7.9	7.0	93.0
8.0	5.3	94.7

[a]Adapted by permission from CRC (1975).

Saline, 0.9%

9 g NaCl (154 mM final; 0.9% w/v)
H_2O to 1 liter

Saponin, 10% (w/v)

Dissolve 1 g saponin in 10 ml PBS (see recipe)
Store in 500-µl aliquots at −20°C

Once thawed, the 10% solution is stable for several months when stored at 4°C.

SDS, 20% (w/v)

Dissolve 20 g sodium dodecyl sulfate (SDS; also known as sodium lauryl sulfate, SLS) in H_2O in a total volume of 100 ml with stirring. Filter sterilize using a 0.45-µm filter.

It may be necessary to heat the solution slightly to fully dissolve the powder.

Table A.2A.6 Preparation of SDS Sample Buffer

Ingredient	2×	4×	Final conc. in 1× buffer
0.5 M Tris·Cl, pH 6.8[a]	2.5 ml	5.0 ml	62.5 mM
SDS	0.4 g	0.8 g	2% (w/v)
Glycerol	2.0 ml	4.0 ml	10% (v/v)
Bromphenol blue	20 mg	40 mg	0.1% (w/v)
2-Mercaptoethanol[a,b,c]	400 µl	800 µl	~300 mM
H_2O	to 10 ml	to 10 ml	—

[a] See recipe.
[b] Alternatively, dithiothreitol (DTT; see recipe), at a final concentration of 100 mM, can be substituted for 2-mercaptoethanol.
[c] Add just before use.

SDS sample buffer

See Table A.2A.6

SED (standard enzyme diluent)

20 mM Tris·Cl, pH 7.5 (see recipe)
500 µg/ml bovine serum albumin (Pentax Fraction V)
10 mM α-mercaptoethanol
Store at 4°C for up to 1 month

Sodium acetate, 3 M

Dissolve 408 g sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$) in 800 ml H_2O
Adjust pH to 4.8, 5.0, or 5.2 (as desired) with 3 M acetic acid
Add H_2O to 1 liter
Filter sterilize

Sodium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid (Tables A.2A.1 and A.2A.3) per liter (0.2 M) in water.

Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) per liter (0.2 M) in water.

Referring to Table A.2A.4 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 100 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check the pH by diluting an aliquot of concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.4, prepare closest higher pH, then titrate with solution A.

Sodium phosphate buffer, 0.1 M

Solution A: 27.6 g $NaH_2PO_4 \cdot H_2O$ (Table A.2A.3) per liter (0.2 M final) in water

Solution B: 53.65 g $Na_2HPO_4 \cdot 7H_2O$ (Table A.2A.3) per liter (0.2 M) in water

continued

Referring to Table A.2A.5 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml. Filter sterilize if necessary. Store up to 3 months at room temperature.

This buffer may be made as a 5- or 10-fold concentrate by scaling up the amount of sodium phosphate in the same final volume. Phosphate buffers show concentration-dependent changes in pH, so check the pH by diluting an aliquot of the concentrate to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2A.5, prepare the closest higher pH, then titrate with solution A.

Silanized glassware

For smaller items: In a well-vented fume hood, place glassware or plasticware (e.g., tubes, tips) in a dedicated vacuum desiccator with an evaporating dish containing 1 ml dichlorodimethylsilane. Apply vacuum with an aspirator and allow ~50% of the liquid to evaporate (several minutes). Turn off aspirator and allow items to remain under vacuum for 30 min. Remove the lid and allow fumes to vent into the hood for ~30 min. If desired, autoclave silanized items.

Do not leave the desiccator attached to the vacuum pump. This will suck away the silane, minimizing deposition and damaging the pump.

For larger items: Silanize items that do not fit in a desiccator by briefly rinsing with or soaking in a solution of ~5% dichlorodimethylsilane in a volatile organic solvent (e.g., chloroform, heptane). Remove organic solvent by evaporation, allowing deposition of dichlorodimethylsilane.

This approach is particularly useful for treating glass plates for denaturing polyacrylamide sequencing gels.

Treatment of glassware, plasticware, or equipment with dichlorodimethylsilane introduces a short polymer of dimethylsiloxane onto its surface. Polydimethylsiloxane is silicone oil. Autoclaving or rinsing with water removes the reactive chlorosilane end of the dimethylsiloxane polymer generated by dichlorodimethylsilane.

CAUTION: Dichlorodimethylsilane vapors are toxic and highly flammable. Always perform in a fume hood.

SSC (sodium chloride/sodium citrate), 20×

Dissolve the following in 900 ml H_2O:
175 g NaCl (3 M final)
88 g trisodium citrate dihydrate (0.3 M final)
Adjust pH to 7.0 with 1 M HCl (see recipe and Table A.2A.1)
Adjust volume to 1 liter
Filter sterilize
Store up to 6 months at room temperature

SSPE (sodium chloride/sodium phosphate/EDTA), 20×

175.2 g NaCl
27.6 g $NaH_2PO_4 \cdot H_2O$
7.4 g disodium EDTA
800 ml H_2O
Adjust pH to 7.4 with 6 M NaOH, then bring volume to 1 liter with H_2O
Filter sterilize
Store up to 6 months at room temperature

The final sodium concentration of 20× SSPE is 3.2 M.

T4 DNA ligase buffer, 10×

500 mM Tris·Cl, pH 7.6 (see recipe)
100 mM $MgCl_2$
10 mM DTT
10 mM ATP
250 μg/ml BSA
Store in aliquots at −20°C

TAE buffer, 50×

242 g Tris base
57.1 ml glacial acetic acid (Tables A.2A.1 and A.2A.3)
37.2 g $Na_2EDTA{\cdot}2H_2O$ (2 mM)
H_2O to 1 liter

This solution does not normally need to be sterilized. The Tris base and acetic acid correspond to 40 mM Tris·acetate.

TBE (Tris/borate/EDTA) buffer, 10×

108 g Tris base (890 mM)
55 g boric acid (890 mM; Table A.2A.3)
960 ml H_2O
40 ml 0.5 M EDTA, pH 8.0 (20 mM final; see recipe)

TBS (Tris-buffered saline)

100 mM Tris·Cl, pH 7.5 (see recipe)
0.9% (w/v) NaCl
Store up to several months at 4°C

TCA (trichloroacetic acid), 100% (w/v)

500 g TCA
227 ml H_2O

TE (Tris/EDTA) buffer

10 mM Tris·Cl, pH 7.4, 7.5, or 8.0 (or other pH; see recipe)
1 mM EDTA, pH 8.0 (see recipe)

TEA (triethanolamine) solution

50 mM triethanolamine, pH ∼11.5
0.1% (v/v) Triton X-100
0.15 M NaCl
Add Triton X-100 from a 10% stock (see recipe).

Tris·Cl, 1 M

Dissolve 121 g Tris base in 800 ml H_2O
Adjust to desired pH with concentrated HCl (Table A.2A.1)
Adjust volume to 1 liter with H_2O
Filter sterilize if necessary
Store up to 6 months at 4°C or room temperature

Approximately 70 ml HCl is needed to achieve a pH 7.4 solution and ∼42 ml for a solution that is pH 8.0.

IMPORTANT NOTE*: The pH of Tris buffers changes significantly with temperature, decreasing approximately 0.028 pH units per 1°C. Tris-buffered solutions should be adjusted to the desired pH at the temperature at which they will be used. Because of the pK_a, Tris should not be used as a buffer below pH ∼7.2 or above pH ∼9.0 (see Table A.2A.3).*

Triton X-100, 10% (w/v)

1 g Triton X-100
H_2O to 10 ml
Stir to dissolve
Filter sterilize through a 0.45-μm filter
Store protected from light up to 6 months at room temperature

TTBS (Tween 20/TBS)

Dissolve 0.1% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20) in TBS (see recipe). Store up to several months at 4°C.

Urea loading buffer, 2×

5 mg bromphenol blue (0.05% w/v)
5 mg (w/v) xylene cyanol FF (0.05% w/v)
4.8 g urea (8 M)
186 mg EDTA (50 mM)
H_2O to 10 ml
Do not sterilize
Store up to 6 months at room temperature

Literature Cited

Chemical Rubber Company (CRC). 1975. CRC Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data, 3rd ed., Vol. 1. CRC Press, Boca Raton, Fla.

Mohan, C. (ed.). 1997. Buffers: A Guide for the Preparation and Use of Buffers in Biological Systems. Calbiochem, San Diego, Calif.

Wallace, R.B. and Miyada, C.G. 1987. Oligonucleotide probes for the screening of recombinant DNA libraries. *In* Methods of Enzymology, Vol. 152: Guide to Molecular Cloning Techniques (S.L. Berger and A.R. Kimmel, eds.) pp. 432-442. Academic Press, San Diego.

Standard Laboratory Equipment

Listed below are pieces of equipment that are standard in the modern microbiology laboratory—i.e., items used extensively in this manual and thus not usually included in the individual materials lists. No attempt has been made to list all items required for each procedure in the Materials list of each protocol; rather, those lists present items that might not be readily available in the laboratory or that require special preparation. See *SUPPLIERS APPENDIX* for contact information for commercial vendors of laboratory equipment.

Aluminum foil
Applicators, cotton-tipped and wooden
Autoclave
Bag sealer
Balances, analytical and preparative
Beakers
Bench protectors, plastic-backed (including "blue" pads)
Biohazard disposal containers and bags
Biological safety cabinet (BSC), must be appropriate for the organism under study
Bottles, glass, plastic, and squirt
Bunsen burners
Cell culture dishes, 60 and 100 mm
Cell culture flasks, T75
Cell spreader
Centrifuges, low-speed (to 20,000 rpm) refrigerated, ultracentrifuge (20,000 to 80,000 rpm), large-capacity low-speed, tabletop, with appropriate rotors and adapters
Centrifuge tubes and bottles, plastic and glass, various sizes
Clamps
Conical centrifuge tubes, plastic and glass
Containers, assortment of glass and plastic, for gel and membrane washes
Coplin jars, glass, for 25 × 75–mm slides
Cryovials, sterile (e.g., Nunc)
Cuvettes
Desiccator and desiccant
Dry ice
Electrophoresis equipment, agarose and acrylamide, full-size and mini, with power supplies
Film developing system and darkroom
Filtration apparatus
Flasks
Forceps
Fraction collector
Freezers, −20°C, −70°C, and liquid nitrogen
Fume hood
Geiger counter
Gel dryer
Gloves, disposable plastic and heat resistant
Graduated cylinders
Heating blocks, thermostatically controlled for test tubes and microcentrifuge tubes
Hemacytometer and/or electronic cell counter
Homogenizer
Humidified CO_2 incubator
Ice bucket
Ice maker
Immersion oil for microscopy
Incubator, 37°C
Inoculating loop
Kimwipes
Lab coats
Laboratory glassware
Light box
Light microscope with 10×, 40×, and 100× oil immersion objectives
Liquid nitrogen
Lyophilizer
Magnetic stirrer, with and without heater, and stir bars
Markers, including indelible markers, china-marking pens, and luminescent markers
Microcentrifuge, Eppendorf-type with 12,000 to 14,000 rpm maximum speed
Microcentrifuge tubes, 0.2-, 0.5-, 1.5-, 2-ml
Microscope slides, glass, 25 × 75–mm, and coverslips
Microtiter plate reader

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Microwave oven
Mortar and pestle
Ovens, drying and microwave
Paper cutter, large
Paper towels
Parafilm
Pasteur pipets and bulbs
PCR thermal cycler and tubes
pH meter
pH paper
Pipets, graduated
Pipettors, adjustable delivery, 0.5- to 10-µl, 10- to 200-µl, and 200- to 1000-µl
Polaroid camera or video documentation system
Power supplies, 300-V for polyacrylamide gels, 2000- to 3000-V for other applications
Racks, for test tube and microcentrifuge tubes
Radiation shield, Lucite or Plexiglas
Radioactive waste containers for liquid and solid wastes
Refrigerator, 4°C
Ring stand and rings
Rubber policemen or plastic scrapers
Rubber stoppers
Safety glasses
Scalpels and blades
β-Scintillation counter
Scissors
Shakers, orbital and platform, room temperature or 37°C
Sharps disposal container
Spectrophotometer, visible and UV range
Speedvac evaporator
Syringes and needles
Tape, masking, electrician's black, autoclave, and Time tape
Test tubes, glass and plastic, various sizes, with and without caps
Timer
Toolbox with common tools
Trays, plastic and glass, various sizes
Tubing, rubber and Tygon
UV light sources, cross-linking and long- and short-wavelength
UV transilluminator
UV transparent plastic wrap (e.g., Saran Wrap)
Vacuum desiccator
Vacuum oven
Vacuum supply
Vortex mixers
Waring blendor
Water bath with adjustable temperature
Water purification system
X-ray film cassettes and intensifying screens

Where to Find Recipes for Media

Table A.2C.1 presents a list of media used throughout this manual and the unit or appendix in which the formulation can be found. It is important to note that while the names of two media may be identical, the formulations may differ, sometimes greatly.

This appendix will be revised regularly; however, due to the constantly updating nature of *Current Protocols in Microbiology*, at any given time there may be media formulations which exist in the book but are not listed here. Likewise, listings may be provided for recipes in units which are in preparation and expected to be published soon. Readers are urged to refer to the Main Index and Supplement Index for the most current information.

CAUTION: Prior to beginning any experiment, the reader is strongly urged to read through Section 1A of this manual, in particular *UNIT 1A.1* which presents information regarding biosafety and *UNIT 1A.3* which details safe handling of commonly encountered chemicals.

NOTE: For formulations of commonly used reagents, refer to *APPENDIX 2A*. For common culture techniques, refer to *APPENDIX 4*.

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology*

Medium	Location
199V medium	*UNIT 14E.1*
7H9 liquid culture medium (for *M. marinum*; also see Middlebrook media)	*UNITS 10B.1 & 10B.2*
7H10 agar plates (for *M. marinum*; also see Middlebrook media)	*UNITS 10B.1 & 10B.2*
7H11 agar plates (for *M. tuberculosis*)	*UNIT 10A.5*
AB medium	*UNIT 1C.1*
Agroinfiltration medium	*UNIT 16I.5*
Agroinfiltration medium, VIGS	*UNIT 16I.6*
Alkaline peptone water (APW)	*UNIT 6A.5*
Anacker and Ordal liquid or solid medium	*UNIT 13B.1*
Apple juice agar plates (for *Drosophila*)	*UNIT 3A.4*
Artificial gingival crevicular fluid (GCF)	*UNIT 1B.5*
Artificial saliva	*UNIT 1B.5*
AT medium	*UNIT 1C.2*
Bacto heart infusion (BHI) blood agar plates (for *C. trachomatis*)	*UNIT 11A.1*
Blood agar plates (for *Helicobacter* species)	*UNIT 8B.1*
Blood agar plates (for *P. gingivalis*)	*UNIT 13C.2*
Brain heart infusion (BHI) broth (2×)/2% (v/v) Supplement B	*UNIT 9D.5*
Brain heart infusion (BHI) medium	*UNIT 9B.4*
BSK-II medium, complete	*UNIT 12C.1*
BSK-II medium, incomplete (basal), 1× and 2×	*UNIT 12C.1*
Caco-2 medium	*UNIT 9B.4*

continued

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Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
Callus maintenance medium 1% agar plates (CM plates)	*UNIT 16D.1*
CDFF culture suspension medium	*UNIT 1B.5*
Chemically defined medium (CDM), liquid (for *N. gonorrhoeae*)	*UNIT 4A.1*
Chemically defined medium (CDM), solid (for *N. gonorrhoeae*)	*UNIT 4A.1*
Chlamydial transport buffer	*UNIT 11A.1*
Columbia blood agar plates	*UNIT 13A.1*
Columbia broth	*UNIT 13A.1*
Columbia broth base	*UNIT 13A.1*
Complete DMEM medium containing 10% FBS	*UNIT 14E.2*
Complete MEM with Hanks' salts, 2×	*UNIT 15B.1*
Complete RPMI medium containing 10% FBS	*UNIT 14E.2*
Culture medium for mesophyll protoplasts	*UNIT 16D.2*
CVE plates	*UNIT 13A.1*
Diluted nutrient broth (DNB)	*UNIT 7B.1*
DMEM/5% FBS or NCBS	*UNIT 14E.1*
DMEM with supplements	*UNIT 15F.2*
DMEM-5	*UNIT 14E.1*
DMEM-10, complete	*UNIT 15J.1*
DMEM, complete/7.5% BSA	*UNIT 15G.1*
DMEM/HEPES	*UNIT 5A.1*
EBV-A9 selection medium A and B	*UNIT 14E.2*
EBV-infected-cell freezing medium	*UNIT 14E.2*
EMEM, complete, 1× and 2×	*UNIT 15B.1*
EMEM, incomplete, serum-free, 1× and 2×	*UNIT 15B.1*
EMJH agar plates (also see Leptospira medium, solid)	*UNIT 12E.4*
EMJH basal salt solution	*UNITS 12E.1 & 12E.2*
EMJH, base	*UNIT 12E.4*
EMJH medium (liquid)	*UNITS 12E.1, 12E.2, & 12E.4*
FEA plates	*UNIT 13A.1*
Freezing medium, M & M and EMEM	*UNIT 15B.1*
GC medium base	*UNIT 4A.2*
GCB plates	*UNIT 4A.2*
GCBL medium	*UNIT 4A.2*
GCMB solid medium	*UNIT 4A.1*
GCP solid medium	*UNIT 4A.1*
Gelatin agar	*UNIT 6A.5*
HeLa cell growth medium	*UNIT 5A.1*
HeLa cell infection medium	*UNIT 5A.1*
HM buffer	*UNIT 7B.1*

continued

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
HM plates	*UNIT 7B.1*
HM top agar	*UNIT 7B.1*
Hsu-Shotts liquid or solid medium	*UNIT 13B.1*
HUVEC culture medium, complete, containing 20% FBS	*APPENDIX 4B*
Influenza virus growth medium	*UNIT 15G.1*
Influenza virus plaque assay medium, 2×	*UNIT 15G.1*
Influenza virus plaque assay wash medium	*UNIT 15G.1*
J774 and L2 medium	*UNIT 9B.4*
Kliger iron agar (KIA)	*UNIT 6A.5*
L. monocytogenes plaquing medium, 2×	*UNIT 9B.4*
LB (Luria-Bertani) medium and plates	*APPENDIX 4A*
LB medium and plates (for agroinfiltration)	*UNIT 16B.2*
Leptospira medium, semisolid	*UNIT 12E.1*
Leptospira medium with 5-fluorouracil, semisolid	*UNIT 12E.2*
Leptospira medium, solid	*UNIT 12E.1*
Leptospira storage medium	*UNIT 12E.1*
MDCK growth medium	*UNIT 15G.1*
M. marinum freezing medium	*UNIT 10B.1*
M9/glucose/bicarbonate medium	*UNIT 5A.1*
M9 minimal salts, 5×	*UNIT 5A.1 & APPENDIX 4A*
MAC-T growth medium	*UNIT 9C.4*
MAT liquid or solid medium	*UNIT 13B.1*
MEM with Hanks' salts, serum-free, 2×	*UNIT 15B.1*
Methyl Red/Voges-Proskauer (MR-VP) medium	*UNIT 6A.5*
Microtuberization medium	*UNIT 16I.1*
Middlebrook 7H9 liquid medium (for *M. tuberculosis*; also see 7H9 medium)	*UNIT 10A.1*
Middlebrook 7H10 solid medium (for *M. tuberculosis*; also see 7H10 medium)	*UNIT 10A.1*
Middlebrook 7H11 solid medium (for *M. tuberculosis*)	*UNIT 10A.1*
Middlebrook top agar	*UNIT 10A.2*
Minimal medium agar supplemented with 50 μg/ml X-gal (for quorum quenching)	*UNIT 1C.3*
Mitsuhashi and Maramorosch (M & M) medium, 1×, serum-free and complete	*UNIT 15B.1*
Modified nutrient agar	*UNIT 6A.5*
Modified Stuart medium	*UNIT 12E.1*
Moeller decarboxylase base	*UNIT 6A.5*
MTYGVS plate medium	*UNIT 12B.1*
Murashige and Skoog medium with 0.4 M mannitol	*UNIT 16D.3*

continued

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
Murashige and Skoog medium with phytagel	*UNIT 16D.3*
M. tuberculosis strain storage medium	*UNIT 10A.1*
N. tabacum germination medium	*UNIT 161.5*
NDV overlay medium	*UNIT 15F.2*
NDV overlay medium with trypsin	*UNIT 15F.2*
Noble agar solution, 12%	*UNIT 12E.4*
NOS 1.5% LMP agarose semisolid medium	*UNIT 12B.1*
NOS basal medium, 1× and 2×	*UNIT 12B.1*
NOS-GN 1% agar semisolid medium	*UNIT 12B.1*
OADC plates	*UNIT 10A.1*
OMIZ-P4 basal medium	*UNIT 12B.1*
Peptone saline maintenance medium	*UNIT 3B.1*
Polypeptone 20 (Pp) medium	*UNIT 7B.1*
Polypeptone 20 (Pp) plates	*UNIT 7B.1*
Polypeptone 20 (Pp) top agar	*UNIT 7B.1*
Potato explants propagation medium	*UNIT 16I.1*
Potato infusion agar (PIA)	*UNIT 3B.1*
Protoplast culture medium (PCM)	*UNIT 16D.1*
Protoplast culturing solid medium	*UNIT 16D.4*
Protoplast culturing solution	*UNIT 16D.4*
Protoplast growth medium	*UNIT 16K.2*
Protoplast inoculation medium (PIM)	*UNIT 16D.1*
Purple broth base	*UNIT 6A.5*
PYE medium	*UNIT 7B.1*
PYE plates	*UNIT 7B.1*
Reduced transport fluid (RTF)	*UNITS 12B.1 & 13A.1*
RPMI-10 or -20, complete	*UNIT 15J.1*
Sauton liquid medium	*UNITS 10A.1 & 10B.1*
Shieh liquid or solid medium	*UNIT 13B.1*
SOB medium	*UNIT 16B.2*
SOC medium	*APPENDIX 4A*
SOC medium (for agroinfiltration)	*UNIT 16B.2*
Spinner medium, complete	*UNIT 14C.1*
Stabilizing medium	*UNIT 3B.1*
SWYE plates	*UNIT 7B.1*
Tellurite taurocholate gelatin agar (TTGA) plates	*UNIT 6A.5*
Thiosulfate citrate bile-salts sucrose (TCBS) agar	*UNIT 6A.5*
Treponema denticola preservation medium	*UNIT 12B.1*
T80/40/LH medium	*UNIT 12E.1*
TB (Terrific broth)	*APPENDIX 4A*

continued

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
THY medium and plates, with and without 1000 μg/ml streptomycin	*UNIT 9D.5*
Transport medium for *Helicobacter*	*UNIT 8B.1*
Tryptic soy agar (TSA)	*UNITS 3B.1 & 9C.4*
Tryptic soy agar (TSA) blood plates	*UNIT 3B.1*
Tryptic soy broth (TSB)	*UNITS 3B.1 & 9C.4*
Tryptic soy broth (TSB)/50% (v/v) glycerol	*UNIT 3B.1*
Tryptic soy broth (TSB), supplemented, to grow *P. gingivalis*	*UNIT 13C.2*
Tryptose phosphate broth (TPB)	*UNITS 15B.1 & 16G.1*
TYES solid or liquid medium	*UNIT 13B.1*
TYGVS basal medium	*UNIT 12B.1*
YEB medium with and without 50 μg/ml kanamycin and 10 μg/ml tetracycline	*UNIT 1C.3*
YEP plates	*UNIT 16B.2*
Zebrafish embryo medium	*UNIT 10B.2*

Where to Find Recipes for Media

Table A.2C.1 presents a list of media used throughout this manual and the unit or appendix in which the formulation can be found. It is important to note that while the names of two media may be identical, the formulations may differ, sometimes greatly.

This appendix will be revised regularly, however, due to the constantly updating nature of *Current Protocols in Microbiology*, at any given time there may be media formulations which exist in the book but are not listed here. Likewise, listings may be provided for recipes in units which are in preparation and expected to be published soon. Readers are urged to refer to the Main Index and Supplement Index for the most current information.

CAUTION: Prior to beginning any experiment, the reader is strongly urged to read through Section 1A of this manual, in particular *UNIT 1A.1* which presents information regarding biosafety and *UNIT 1A.3* which details safe handling of commonly encountered chemicals.

NOTE: For formulations of commonly used reagents, refer to *APPENDIX 2A*. For common culture techniques, refer to *APPENDIX 4*.

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology*

Medium	Location
199V medium	*UNIT 14E.1*
7H10 plates	*UNITS 10B.1 & 10B.2*
7H9 liquid culture medium	*UNITS 10B.1 & 10B.2*
AB medium	*UNIT 1C.1*
Agroinfiltration medium	*UNIT 16I.5*
Agroinfiltration medium, VIGS	*UNIT 16I.6*
Alkaline peptone water (APW)	*UNIT 6A.5*
Apple juice agar plates (for *Drosophila*)	*UNIT 3A.4*
AT medium	*UNIT 1C.2*
Bacto heart infusion (BHI) blood agar plates (for *C. trachomatis*)	*UNIT 11A.1*
Blood agar plates (for *Helicobacter* species)	*UNIT 8B.1*
Brain heart infusion (BHI) broth (2×)/2% (v/v) Supplement B	*UNIT 9D.5*
Brain heart infusion (BHI) medium	*UNIT 9B.4*
BSK-II medium, complete	*UNIT 12C.1*
BSK-II medium, incomplete (basal), 1× and 2×	*UNIT 12C.1*
Caco-2 medium	*UNIT 9B.4*
Callus maintenance medium 1% agar plates (CM plates)	*UNIT 16D.1*
Chlamydial transport buffer	*UNIT 11A.1*
Columbia blood agar plates	*UNIT 13A.1*
Columbia broth	*UNIT 13A.1*
Columbia broth base	*UNIT 13A.1*
Complete MEM with Hanks' salts, 2×	*UNIT 15B.1*

continued

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
Culture medium for mesophyll protoplasts	*UNIT 16D.2*
CVE plates	*UNIT 13A.1*
Diluted nutrient broth (DNB)	*UNIT 7B.1*
DMEM/5% FBS or NCBS	*UNIT 14E.1*
DMEM with supplements	*UNIT 15F.2*
DMEM-5	*UNIT 14E.1*
DMEM-10, complete	*UNIT 15J.1*
DMEM, complete/7.5% BSA	*UNIT 15G.1*
EMEM, complete, 1× and 2×	*UNIT 15B.1*
EMEM, incomplete, serum-free, 1× and 2×	*UNIT 15B.1*
EMJH basal salt solution	*UNITS 12E.1 & 12E.2*
EMJH medium (liquid)	*UNITS 12E.1 & 12E.2*
FEA plates	*UNIT 13A.1*
Freezing medium, M & M and EMEM	*UNIT 15B.1*
GC medium base	*UNIT 4A.2*
GCB plates	*UNIT 4A.2*
GCBL medium	*UNIT 4A.2*
Gelatin agar	*UNIT 6A.5*
HM buffer	*UNIT 7B.1*
HM plates	*UNIT 7B.1*
HM top agar	*UNIT 7B.1*
HUVEC culture medium, complete, containing 20% FBS	*APPENDIX 4B*
Influenza virus growth medium	*UNIT 15G.1*
Influenza virus plaque assay medium, 2×	*UNIT 15G.1*
Influenza virus plaque assay wash medium	*UNIT 15G.1*
J774 and L2 medium	*UNIT 9B.4*
Kliger iron agar (KIA)	*UNIT 6A.5*
L. monocytogenes plaquing medium, 2×	*UNIT 9B.4*
LB (Luria-Bertani) medium and plates	*APPENDIX 4A*
LB medium and plates (for agroinfiltration)	*UNIT 16B.2*
Leptospira medium, semisolid	*UNIT 12E.1*
Leptospira medium with 5-fluorouracil, semisolid	*UNIT 12E.2*
Leptospira medium, solid	*UNIT 12E.1*
Leptospira storage medium	*UNIT 12E.1*
MDCK growth medium	*UNIT 15G.1*
M. marinum freezing medium	*UNIT 10B.1*
M9 minimal salts, 5×	*APPENDIX 4A*
MAC-T growth medium	*UNIT 9C.4*
MEM with Hanks' salts, serum-free, 2×	*UNIT 15B.1*

continued

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
Methyl Red/Voges-Proskauer (MR-VP) medium	*UNIT 6A.5*
Minimal medium agar supplemented with 50 μg/ml X-gal (for quorum quenching)	*UNIT 1C.3*
Mitsuhashi and Maramorosch (M & M) medium, 1×, serum-free and complete	*UNIT 15B.1*
Modified nutrient agar	*UNIT 6A.5*
Modified Stuart medium	*UNIT 12E.1*
Moeller decarboxylase base	*UNIT 6A.5*
MTYGVS plate medium	*UNIT 12B.1*
Murashige & Skoog (MS) medium	*UNIT 16D.4*
N. tabacum germination medium	*UNIT 161.5*
NDV overlay medium	*UNIT 15F.2*
NDV overlay medium with trypsin	*UNIT 15F.2*
NOS 1.5% LMP agarose semisolid medium	*UNIT 12B.1*
NOS basal medium, 1× and 2×	*UNIT 12B.1*
NOS-GN 1% agar semisolid medium	*UNIT 12B.1*
OMIZ-P4 basal medium	*UNIT 12B.1*
Peptone saline maintenance medium	*UNIT 3B.1*
Polypeptone 20 (Pp) medium	*UNIT 7B.1*
Polypeptone 20 (Pp) plates	*UNIT 7B.1*
Polypeptone 20 (Pp) top agar	*UNIT 7B.1*
Potato infusion agar (PIA)	*UNIT 3B.1*
Protoplast culture medium (PCM)	*UNIT 16D.1*
Protoplast culturing solid medium	*UNIT 16D.4*
Protoplast culturing solution	*UNIT 16D.4*
Protoplast growth medium	*UNIT 16K.2*
Protoplast inoculation medium (PIM)	*UNIT 16D.1*
Purple broth base	*UNIT 6A.5*
PYE medium	*UNIT 7B.1*
PYE plates	*UNIT 7B.1*
Reduced transport fluid (RTF)	*UNITS 12B.1 & 13A.1*
RPMI-10 or -20, complete	*UNIT 15J.1*
SOB medium	*UNIT 16B.2*
SOC medium	*APPENDIX 4A*
SOC medium (for agroinfiltration)	*UNIT 16B.2*
Spinner medium, complete	*UNIT 14C.1*
Stabilizing medium	*UNIT 3B.1*
SWYE plates	*UNIT 7B.1*
Tellurite taurocholate gelatin agar (TTGA) plates	*UNIT 6A.5*
Thiosulfate citrate bile-salts sucrose (TCBS) agar	*UNIT 6A.5*
Treponema denticola preservation medium	*UNIT 12B.1*

continued

Table A.2C.1 Media Formulations Detailed in *Current Protocols in Microbiology, continued*

Medium	Location
T80/40/LH medium	*UNIT 12E.1*
TB (Terrific broth)	*APPENDIX 4A*
THY medium and plates, with and without 1000 μg/ml streptomycin	*UNIT 9D.5*
Transport medium for *Helicobacter*	*UNIT 8B.1*
Tryptic soy agar (TSA)	*UNITS 3B.1 & 9C.4*
Tryptic soy agar (TSA) blood plates	*UNIT 3B.1*
Tryptic soy broth (TSB)	*UNITS 3B.1 & 9C.4*
Tryptic soy broth (TSB)/50% (v/v) glycerol	*UNIT 3B.1*
Tryptose phosphate broth (TPB)	*UNITS 15B.1 & 16G.1*
TYGVS basal medium	*UNIT 12B.1*
YEB medium with and without 50 μg/ml kanamycin and 10 μg/ml tetracycline	*UNIT 1C.3*
YEP plates	*UNIT 16B.2*
Zebrafish embryo medium	*UNIT 10B.2*

COMMONLY USED TECHNIQUES

Assays for Total Protein

Total protein assays are used to analyze hundreds of industrial, agricultural, and biotechnology products. They are also basic for research purposes, especially for determining the specific activity (i.e., total activity/total protein) of enzymes, antibodies, and lectins. Clearly, accuracy and precision of specific activity measurements depend as much on accurate measurement of total protein as on determination of total activity.

In performing total protein assays, there are five issues of concern: (1) sensitivity and technique of the method, (2) clear definition of units, (3) interfering compounds, (4) removal of interfering substances before assaying samples, and (5) correlation of information from various techniques. These concerns are discussed at length in the Commentary section.

This appendix describes two copper-based assays to quantitate total protein: a variation of the Lowry method (Hartree-Lowry method; see Basic Protocol 1) and the bicinchoninic acid (BCA) assay (see Basic Protocol 2). Ultraviolet spectrophotometry is used to measure total protein (see Basic Protocol 3) and evaluate samples for the presence of contaminants. The Coomassie-dye binding, or Bradford, assay (see Basic Protocol 4) is quite simple and frequently quite sensitive, although it sometimes gives a variable response depending on how well or how poorly the protein binds the dye in acid pH. TCA precipitation to precipitate and concentrate proteins, and to remove low-molecular-weight contaminants is also described (see Basic Protocol 5).

Table A.3A.1 presents a brief summary of the assays described in this unit, including detection range and required sample size.

HARTREE-LOWRY ASSAY FOR QUANTITATION OF TOTAL PROTEIN

BASIC PROTOCOL 1

The original Lowry method for total protein analysis was first described in one of the most cited papers in biochemistry (Lowry et al., 1951). The technique is a colorimetric assay based on cupric ions and Folin-Ciocalteau reagent for phenolic groups. The method has been reinvestigated many times and sometimes improved. Most of these studies were designed to discern how interfering compounds distort the assay and how detergents solubilize otherwise insoluble proteins. The literature related to this assay was comprehensively reviewed by Peterson (1983). This protocol describes Hartree's version of the Lowry assay (Hartree, 1972), which is somewhat less laborious than the original assay but maintains its sensitivity. This version uses three reagents instead of five, produces more intense color (increased sensitivity) with some proteins, maintains a linear response over a larger concentration range (30% to 40% greater), is less easy to overload, and

Table A.3A.1 Summary of Assays for Quantitating Total Protein

Assay	Sample size	Detection range (μg/ml)
Hartree-Lowry	1 ml	100-600
BCA	100 μl	200-1000
UV absorption	1 ml	30-300
Coomassie dye binding	100 to 200 μl	60-300

Contributed by Rex Lovrien and Daumantas Matulis
Commonly Used Techniques (2005) A.3A.1-A.3A.14

overcomes the reagent salt coprecipitation problem encountered with Lowry reagents. Finally, the reagent formulation offers some advantages in storage stability.

Materials

Calibration standard: 300 μg/ml crystalline BSA in water
Buffer or solvent used to prepare the protein-containing sample
Sample containing protein at 100 to 600 μg/ml
Hartree-Lowry reagents A, B, and C (see recipes)
50°C water bath
Spectrophotometer and 1-cm cuvettes

1. Prepare a dilution series of calibration standard in the same buffer or solvent used to prepare the sample to give concentrations of 30 to 150 μg/ml. Also include reference standard (blank) containing no protein.

   Concentrations of albumin in the final assay volume (f.a.v.) of 5 ml may range from ~30 to 300 μg protein/5 ml f.a.v. to produce A_{650} readings from ~0.20 to 0.80.

2. Add 1.0 ml of protein-containing sample, calibration standard, or reference standard to 0.90 ml of Hartree-Lowry reagent A in separate test tubes. Incubate 10 min in a 50°C water bath.

   It is recommended to make triplicate protein samples to obtain reliable values.

3. Cool the tubes to room temperature.

4. Add 0.1 ml Hartree-Lowry reagent B to each tube and mix. Incubate 10 min at room temperature.

5. Rapidly add 3 ml Hartree-Lowry reagent C to each tube and mix thoroughly. Incubate 10 min in a 50°C water bath, then cool to room temperature.

   The final assay volume is 5.0 ml.

6. Measure the net absorbance of the sample(s), calibration standards, and reference standard at 650 nm (A_{650}) in 1-cm cuvettes.

7. If the spectrophotometer used does not automatically give net absorbance readings, subtract the value for the reference solution from those obtained for the sample and calibration standards.

8. Prepare a calibration plot by graphing the net A_{650} values for the standards versus protein concentration (micrograms protein/5 ml f.a.v.).

9. Determine the protein concentration of the sample by interpolation from the plot, accounting for any dilutions made in sample preparation.

   Use only the linear portion of the plot. The absorbance plateaus at high protein concentrations, resulting in erroneous calculations.

BASIC PROTOCOL 2

BICINCHONINIC ACID (BCA) ASSAY FOR QUANTITATION OF TOTAL PROTEIN

The bicinchoninic acid (BCA) assay for total protein is a spectrophotometric assay based on the alkaline reduction of the cupric to the cuprous ion by the protein, followed by chelation and color development by the BCA reagent. Either a micro or a semimicro procedure, the latter generating a final assay volume of 2 or 3 ml, may be used; the semimicro procedure is most frequently used. Several companies (e.g., Pierce) sell kits containing all the necessary reagents.

The BCA assay for total protein is somewhat variable: it has differing sensitivities in response to incubation time, incubation temperature, standard protein used for calibration, and other factors (Smith et al., 1985). Certain classes of compounds, such as reducing sugars and ammonium ions, interfere with the assay, sometimes severely. However, if interfering compounds are eliminated (e.g., by dialysis), the BCA assay has a good combination of sensitivity and simplicity, and it has some advantages over the Lowry technique (see Background Information).

Materials

Calibration standard: 1 mg/ml BSA
Buffer or solvent used to prepare the protein-containing sample
Sample containing protein
BCA reagent A/reagent B mix (see recipe)
Spectrophotometer and cuvettes

1. Prepare a dilution series of calibration standard in the buffer or solvent used to prepare the sample to cover the range 0.2 to 1.0 mg/ml. Also include a reference standard (blank) containing no protein.

   It is recommended to make triplicate calibration standards to obtain reliable values.

2a. *For a 2.1-ml final assay volume:* Mix 100 µl protein-containing sample, calibration standard, or reference standard with 2 ml BCA reagent A/reagent B mix in separate tubes.

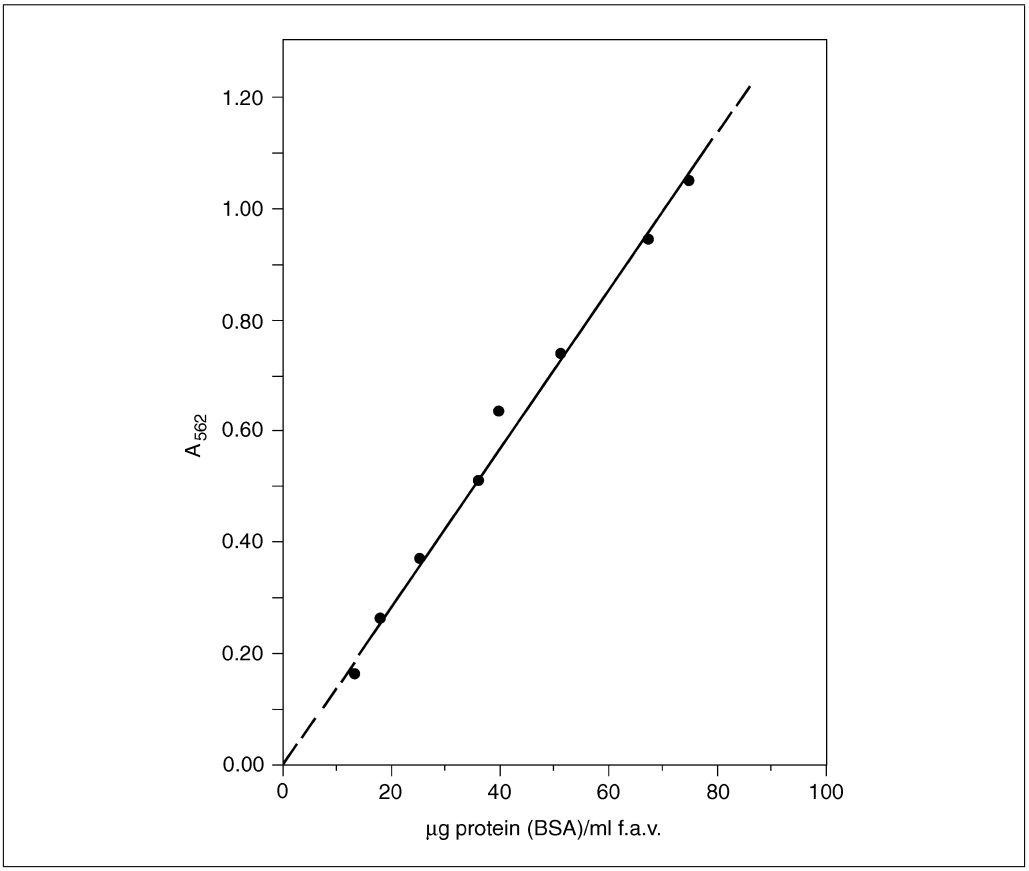

Figure A.3A.1 Bicinchoninic acid assay calibration plot using bovine serum albumin. Slope = 1.5×10^{-2} A_{562} (µg protein/ml f.a.v.)$^{-1}$ cm^{-1}.

2b. *For a 4.2-ml final assay volume:* Mix 200 µl protein-containing sample, calibration standard, or buffer used to prepare the sample (reference standard) with 4 ml BCA reagent A/reagent B mix in separate test tubes.

The final assay volume depends on the size of the available cuvettes.

3. Incubate the samples and standards 30 min at 37°C, then cool to room temperature.
4. Measure the absorbance of the sample, calibration standards, and reference standard at 562 nm (A_{562}).
5. If the spectrophotometer does not automatically give net absorbance readings, subtract the values for the reference standard from those obtained for the sample and calibration standards.
6. Prepare a calibration plot by graphing the net A_{650} values for the standards versus protein concentration (micrograms protein/milliliter f.a.v; see Fig. A.3A.1).
7. Determine the protein concentration of the sample by interpolation from the plot.

Use only the linear portion of the plot. The absorbance plateaus at high protein concentrations, resulting in erroneous calculations.

BASIC PROTOCOL 3

ULTRAVIOLET ABSORPTION TO MEASURE TOTAL PROTEIN

Proteins contain tyrosine and tryptophan side chains that are fairly strong absorbers of light in the 275- to 280-nm (ultraviolet) region. Consequently, after suitable dilution to produce on-scale absorbance readings, total protein can be estimated from UV absorbance spectra using quartz or fused silica cuvettes. Phenylalanine, which has an aromatic side chain, is only weakly absorbing and is usually neglected for most purposes. Weight absorption coefficients, $E^{1\%}$ values (concentration of protein in dry grams per 100 ml volume or weight/volume percent), range between ~3 and 30 A_{280} units $(\text{grams}/100\,\text{ml})^{-1}$ cm^{-1} for most proteins, i.e., if the protein concentration is 10 mg/ml (1 g/100 ml) and the cuvette pathlength is 1 cm, then A_{280} will be between 3 and 30. A useful survey of $E^{1\%}$ values for individual proteins at 275 to 280 nm is available in Sober (1970). If the molecular weight of proteins is known, the molar absorption coefficient, ε_λ in moles/liter is related to the $E^{1\%}$ values at the same wavelength by Equation A.3A.1.

$$10\varepsilon_\lambda = E_\lambda^{1\%} \times \text{molecular weight}$$

Equation A.3A.1

Molar absorption coefficients can be calculated from amino acid composition based on tyrosine and tryptophan contributions to the total molecule. The $\varepsilon_{275\text{-}280}$ values for tyrosine (in neutral or acidic solution) and for tryptophan are close to 1470 and 5700 absorbance units M^{-1} cm^{-1}, respectively. There are minor variations in these values depending on the peptide and the solvent (Bencze and Schmid, 1957).

For example, a 0.04% to 0.12% solution of protein having four to twelve tyrosine groups, two to six tryptophans, and an ε_{280} value of ~8, should have an A_{280} of ~0.2 to 1 with a 1-cm path length cuvette. Hence UV absorption around the 280-nm band provides a fairly sensitive, convenient means for detecting and quantitating pure proteins or mixtures of pure proteins having concentrations in the range of 0.1 to 1 mg/ml.

Materials

Protein sample, pH <8
Spectrophotometer and fused-silica or quartz cuvettes

1. Turn on the spectrophotometer, set the wavelength to 280 nm, and allow it to warm up 30 min.
2. If necessary, dilute the protein sample to give an A_{280} between 0.2 and 1.

   Some spectrophotometers may give precise measurements of A_{280} up to 2 absorption units, but, in general, conditions and concentrations should be adjusted so the instrument is not forced to read absorbances >1.0.
3. Read the absorbance of sample and reference standard (buffer or solvent) at 280 nm in fused-silica or quartz cuvettes.

   Glass and plastic cuvettes are opaque below the near UV range, ~350 mm.

   A complete UV absorption spectrum, ~230 or 240 nm to >310 nm, may be very helpful for detecting interfering compounds. For a solution of one or more pure proteins at acid pH, there should be a deep trough at 250 nm, a peak around 275 to 280 nm, and very little absorption above 310 nm, unless the proteins contain cofactors or prosthetic groups.
4. If necessary, dialyze or precipitate the sample to remove contaminants and interfering compounds and reread the A_{280}.

COOMASSIE DYE–BINDING ASSAY (BRADFORD ASSAY) TO MEASURE TOTAL PROTEIN

BASIC PROTOCOL 4

Coomassie dye (brilliant blue G250) binds to protein molecules in acid pH by two means: the triphenylmethane group binds to nonpolar structures in proteins and the anion sulfonate groups interact with protein cationic side chains (e.g., arginine, lysine) in acid pH (Lovrien et al., 1995). The color change produced when the dye binds to proteins provides a measure of total protein, which is quite sensitive in the case of albumin and certain globular proteins (Bradford, 1976; Sedmak and Grossberg, 1977). The Coomassie dye-binding assay, or Bradford assay, also responds to some interfering substances which are generally unknown unless the experimenter specifically tests for them (Van Kley and Hale, 1977; Kirazov et al., 1993). Nevertheless, because of its apparent simplicity and sensitivity towards many proteins, the Bradford assay is popular and widely used. Using bovine serum albumin (ideally well behaved) to calibrate the Bradford assay produces a calibration plot with a slope of 4.5 to 5.5 $\times 10^{-2}$ (μg protein/ml f.a.v.)$^{-1}$ cm^{-1}. Hence, it is somewhat more sensitive, by a factor of roughly two or three, than the values generally quoted for Lowry, Hartree-Lowry, or BCA assays (see Table A.3A.2).

Materials

Calibration standards: 1.5 mg/ml BSA and 1.5 mg/ml lysozyme
Buffer or solvent used to prepare the protein-containing sample
Sample containing protein
Coomassie dye reagent (see recipe) *or* commercial Coomassie reagent (Bio-Rad, Pierce)

1. Prepare a dilution series of calibration standards in the buffer or solvent used to prepare the sample to cover the range 150 to 750 μg protein/ml. Also include a reference standard (blank) containing no protein.

   Depending on the kind of protein being measured, it may be useful to calibrate with various proteins including ones related to (or even a purified preparation of) the protein being analyzed. For example, if collagen is the analyte, various collagens, high-glycine-hydroxyproline polymers, should be used as calibrating standards. Bovine serum albumin

Table A.3A.2 Slopes of Calibration Plots for Spectrophotometric Assays[a]

Assay	Calibrating compound	Measured slope
Dinitrosalicylate (DNS)	Glucose	5.50 A_{575} (mg sugar/ml f.a.v.)$^{-1}$ cm^{-1}
Nelson-Somogyi reducing sugar	Glucose	6.3×10^{-3} A_{520} (nmol glucose/ml f.a.v.)$^{-1}$ cm^{-1}
Phenol–sulfuric acid neutral sugar	Mannose	8.6×10^{-2} A_{485} (μg sugar/ml f.a.v.)$^{-1}$ cm^{-1}
Biuret protein	BSA	2.3×10^{-4} A_{550} (μg protein/ml f.a.v.)$^{-1}$ cm^{-1}
Hartree-Lowry protein	BSA	1.7×10^{-2} A_{650} (μg protein/ml f.a.v.)$^{-1}$ cm^{-1}
Bicinchoninic acid protein	BSA	1.5×10^{-2} A_{562} (μg protein/ml f.a.v.)$^{-1}$ cm^{-1}
Colorimetric microkjeldahl nitrogen	Ammonium sulfate	1.3 A_{660} (μg nitrogen/ml f.a.v.)$^{-1}$ cm^{-1}

[a]Abbreviations: BSA, bovine serum albumin; f.a.v., final assay volume.

(BSA) is often used as a calibration standard, but it has greater general dye-binding capacity than most proteins.

2. Add 100 μl protein-containing sample, calibration standard, or reference standard to 5 ml Coomassie dye reagent. Mix and incubate 10 min at room temperature.

   It is recommended to make triplicate protein samples to obtain reliable values.

   The ratio of sample to reagent may range from 1:20 to 1:50 (v/v). A microassay can be performed using 5 to 10 μl sample in 250 to 500 μl Coomassie dye reagent.

   If a commercially prepared Coomassie reagent is used, follow the manufacturer's instructions.

3. Measure the absorbance of the sample, calibration standards, and reference standard at 595 nm (A_{595}).

4. If the spectrophotometer does not automatically give net absorbance readings, subtract the values for the reference standard from those obtained for the sample and calibration standards.

5. Prepare a calibration plot by plotting the net A_{595} values for the standards versus protein concentration (microgram protein/milliliter f.a.v.).

6. Determine the protein concentration of the sample by interpolation from the plot.

   At maximal sensitivity (with BSA as a calibration standard) the Coomassie dye-binding assay produces a calibration plot with a slope (sensitivity) of ~4×10^{-2} A_{595} (μg/ml f.a.v.)$^{-1}$ cm^{-1} and falls to a third or half of that value with less responsive proteins.

BASIC PROTOCOL 5

TRICHLOROACETIC ACID PRECIPITATION OF PROTEIN SAMPLES

Trichloroacetic acid (TCA) precipitation can be used to precipitate proteins away from TCA-soluble, low-molecular-weight compounds that may interfere with assays for total protein. The procedure may also be used to concentrate protein from a dilute aqueous solution.

Add 10% (w/v) TCA to a protein sample to give a final concentration of 3% to 4% (v/v). Let stand 2 to 5 min at room temperature. Remove the supernatant and resuspend the precipitate in neutral buffer or alkali, depending on the method for further analysis.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

BCA reagent A

1 g 4,4′-dicarboxy-2,2′-biquinoline, disodium salt (Na_2BCA; Pierce or Sigma; 26 mM final)
2 g $Na_2CO_3 \cdot H_2O$ (0.16 M final)
160 mg disodium sodium tartrate (7 mM final)
0.4 g NaOH (0.1 M final)
0.95 g $NaHCO_3$ (0.11 M final)
100 ml H_2O

After mixing and dissolving the components, adjust the pH to 11.3 ± 0.2, using solid NaOH to increase pH or $NaHCO_3$ to decrease pH. Store this alkaline reagent in a plastic container 1 to 3 weeks at room temperature or longer at 4°C.

Only the disodium salt of Na_2BCA is soluble at neutral pH; the free acid is not readily soluble, even in alkali.

BCA reagent B

4 g $CuSO_4 \cdot 5H_2O$ (16 mM final)
100 ml H_2O
Store up to a few months at room temperature

BCA reagent A/reagent B mix

Mix 50 vol BCA reagent A with 1 vol BCA reagent B (see recipes). Prepare only sufficient mix for a few hours' work because the mixture is not stable.

BCA reagent A/reagent B mix is light apple-green in color.

Coomassie dye reagent

100 mg Coomassie brilliant blue G250 (0.01% w/v)
50 ml 95% ethanol (5% final)
100 ml 85% phosphoric acid (8.5% final)
H_2O to 1 liter
Filter through Whatman no. 2 filter paper
Store up to 1 month at room temperature in a glass container

Coomassie brilliant blue G250 is available from Sigma (as brilliant blue G1), Bio-Rad, Pierce, and others.

Hartree-Lowry reagent A

2 g sodium potassium tartrate·4 H_2O (Rochelle salt; 7 mM final)
100 g Na_2CO_3 (0.81 M final)
500 ml 1 N NaOH (0.5 N final)
H_2O to 1 liter
Store 2 to 3 months at room temperature in a plastic container

Hartree-Lowry reagent B

2 g sodium potassium tartrate·4 H_2O (0.07 M final)
1 g $CuSO_4 \cdot 5H_2O$ (0.04 M final)
90 ml H_2O
10 ml 1 N NaOH
Store 2 to 3 months at room temperature in a plastic container

Hartree-Lowry reagent C

Dilute 1 vol Folin-Ciocalteau reagent (Sigma) with 15 vol water.

Prepare this solution daily in 16-ml quantities or multiples thereof. Do not adjust the pH.

COMMENTARY

Background Information

Many assays for quantitating total protein exist. Several are reliable and straightforward. How to choose the most suitable or optimal method is a recurring problem; the solution frequently requires the use of more than one method or protocol. A good strategy is to compare the results of two methods, such as A_{280} measurements and one of the copper-based chromogenic methods—assays that rely on different chemical properties. Very large differences in total protein estimates from two or more methods occur with crude preparations from bacterial cells, cell cultures, tissues, and food extracts, all of which may be laden with interfering substances. The principle question is often not how sensitive a particular protein assay happens to be, but rather how the assay is affected by interfering substances. The primary concern is how to maneuver around the interfering compounds or how to eliminate them altogether, using analytical protocols to follow the progress. Methods for dealing with some of the more common interfering substances are discussed below.

Individual total protein analytical methods commonly disagree with one another by as much as 5% to 20%, even in the case of a well-behaved protein not laden with interfering compounds. Disagreement in the case of crude samples may be much greater. There are two general ways to deal with such discrepancies. The first is to remove interfering compounds that are likely to upset one, or perhaps both, analyses, or to resort to a third method if there is enough sample to resolve the differences. The second and ultimate resort is to conduct total nitrogen analysis, e.g., by the Kjeldahl method or modern versions of it. It is a generally reliable practice to assume that all proteins and polypeptides contain very close to 16.5% nitrogen by weight, so multiplication of the weight of nitrogen obtained from Kjeldahl analysis by a factor of 6.0 should provide a valid benchmark measure of the weight of protein in an ammonium salt–free sample.

The protocols and related commentaries in this unit describe specific, frequently used methods for quantitating total protein. However, it is important to bear in mind that this field is still evolving.

Recording and reporting data for total protein analysis

Calibration curves for spectrophotometric (colorimetric) assays for total protein are almost always plotted with the vertical axis in units of absorbance at the optimum wavelength (A_λ), which usually ranges between 0 and 1.00, or 0 and 2.0, depending on the spectrophotometer. The horizontal axis is usually labeled in units of protein such as micrograms or milligrams, often without specifying the related volume—the volume of sample introduced into the assay, or the concentration per milliliter of sample or per milliliter of total assay volume. If a 0.50-ml sample is analyzed in a colorimetric assay that adds 3 to 6 ml reagents, there is a >10-fold concentration difference involved. For this reason, it is very important to be clear and specific in defining the appropriate volume units for the values on the horizontal axis. Clear specification of units for calibration curves is also important because the numerical value of the slope is a direct measure of the sensitivity of the method and a reflection of the molecular composition of the calibrating protein(s). Molar absorption coefficients at specified wavelengths (ε_λ) are defined in Equation A.3A.2, where l is cuvette pathlength in centimeters, c is the molar concentration of the chromogen (moles liter^{-1}), and A_λ is the absorbance as read from the spectrophotometer. In most cases, path length l = 1 cm.

$$\varepsilon_\lambda = \frac{A_\lambda}{c \times l}$$

Equation A.3A.2

Because spectrophotometry-based total protein analyses produce calibration plots of A_λ versus concentration (c), the slopes of the plots are either directly equal to molar absorption coefficients (if c is in units of molarity) or directly proportional to molar absorption coefficients (if other units are used for the horizontal axis). A well-characterized pure protein (e.g., bovine serum albumin, BSA) used

for calibration should produce a calibration curve with a slope (absorption coefficient) that is reasonable based on the composition of the protein for the chromogen or prochromogen. BSA has close to eighteen phenolic (tyrosine) groups per protein molecule.

It is not necessary for the horizontal axis to be plotted in units of molarity. However, it is desirable both for planning and for cross-checking results that units include a clear statement of volume so they can be readily converted to other units as needed for reporting and planning. One appropriate unit is micrograms or milligrams per milliliter of final assay volume (per milliliter f.a.v.), which specifies the concentration of the analyzed protein in the operational volume of a solution for spectrophotometric reading after the sample, reagents, and diluents have all been combined.

Table A.3A.2 lists the slopes of calibration curves, equivalent to absorption coefficients and assay sensitivities, for proteins and sugars in some commonly used assays. For some total protein assays, it is a good strategy to monitor sugars in parallel to evaluate removal of interfering compounds. Details for total sugar and reducing sugar assays are outlined in Lovrien et al. (1987) and references therein.

Copper ion–dependent assays for total protein

The Lowry and bicinchoninic acid (BCA) assays are dependent on copper ions, cupric and cuprous. Prochromogenic reagents used in the BCA assay depend on the extent of reduction of cupric ion (Cu^{2+}) to cuprous ion (Cu^{+}) to develop their color (Smith et al., 1985). The brilliant color that BCA reagent develops is the result of formation of a complex with Cu^{+} but not Cu^{2+}. In turn, the amount of Cu^{+} produced depends on the amount of protein present, so color development is a measure of the amount of protein (see Fig. A.3A.2).

Compounds that affect either cupric or cuprous ion chemistry will interfere with the assays. In the BCA assay, the reagent chelates the cuprous ion. Aliphatic amines and ammonia or ammonium ion are strong ligands for copper, at least for Cu^{2+}. Reducing agents such as glucose can certainly be expected to interfere with these assays. Proteins rich in disulfide and sulfhydryl groups such as keratin interfere because reduced sulfur compounds powerfully bind to and reduce Cu^{2+}. Cupric ions in general are avid coordinating metals for foreign compounds, precipitating with them or becoming reduced by them. Large concentrations of ammonium sulfate and some of the phosphates used in purification schemes interfere with cupric ion–based chemistries. A list of interfering compounds, many of them used as buffers or found as metabolites in protein technology, is presented in Smith et al. (1985) and briefly summarized in Table A.3A.3.

The bicinchoninic acid assay

The bicinchoninic acid (BCA) assay (see Basic Protocol 2 and Fig. A.3A.2) developed by P.K. Smith et al. (1985) uses cupric ion in a biuret reaction with proteins in a strong base. The biuret reaction produces cuprous ion from cupric ion, and the cuprous ion is chelated with the BCA reagent. The Cu^{+}-BCA chelate is brilliantly colored with an absorption peak, λ_{max}, at 562 nm. Therefore, A_{562} is directly dependent on protein concentration, when the reaction conditions outlined by Smith et al. (1985) are maintained.

The BCA method compares favorably with the older Lowry or Hartree-Lowry methods in sensitivity and convenience. The sensitivity of the BCA assay can be increased somewhat by using a higher concentration of the BCA reagent (i.e., of bicinchoninic acid disodium salt). However, the BCA reagent is rather expensive, and the fractional increase in sensitivity may not be worth the increase in cost.

The BCA assay is less susceptible than other assays to interference by a number of detergents. For this reason the BCA assay is sometimes favored with detergent-loaded samples, e.g., membrane and cellular proteins extracted by detergent solubilization. On the other hand, the BCA assay is susceptible to interference by reducing sugars, and even by

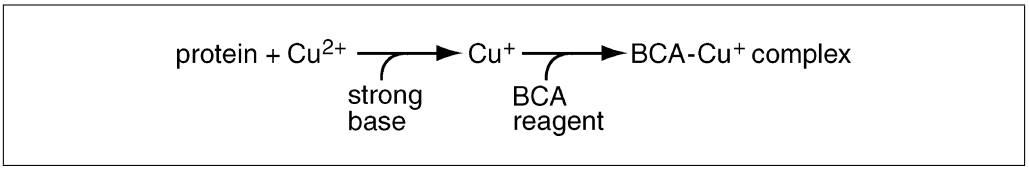

Figure A.3A.2 Reactions in the BCA assay.

Table A.3A.3 Compounds Interfering with Total Protein Quantification Assays[a]

Assay	Interfering compounds
Hartree-Lowry	EDTA Guanidine·HCl Triton X-100 SDS Brij 35 >0.1 M Tris Ammonium sulfate 1 M sodium acetate 1 M sodium phosphate
Bicinchoninic acid	EDTA >10 mM sucrose or glucose 1.0 M glycine >5% ammonium sulfate 2 M sodium acetate 1 M sodium phosphate
UV absorption	Pigments Phenolic compounds Organic cofactors
Bradford	>0.5% Triton X-100 >0.1% SDS Sodium deoxycholate

[a]See Smith et al. (1985) for a more complete discussion.

ostensibly nonreducing sugars such as sucrose (because sucrose releases reducing sugars on partial hydrolysis; Spies, 1957). Some experimental systems use 0.1 to 1 mM sulfhydryl reagents to protect proteins. These reagents can contain significant quantitites of reduced sulfhydryl reagents such as mercaptoethanol that react with cupric ion. Dialysis or other methods for removing organic −SH compounds from the sample may be required.

In order to avoid interfering substances in general, and also to increase protein concentrations, powerful precipitating agents such as TCA (trichloroacetic acid) may be useful (Annand and Romeo, 1976; Beyer, 1983; also see Basic Protocol 5). Most proteins in even dilute solution are quantitatively precipitated by TCA (at a concentration of a few percent), which also concentrates them. The supernatant is discarded, which rids the sample of most low-molecular-weight impurities. The TCA precipitate should be redissolved in base for subsequent BCA assay.

UV spectrum analysis

The ultraviolet (UV) absorption spectrum for a sample protein can be used to quantitate the protein and to evaluate the purity of a sample. Figure A.3A.3 shows the UV absorption spectra for glycyl-L-tyrosine. The molar absorption coefficient ε as a function of wavelength λ is dependent on pH. The glycyl moiety has negligible effect on the spectra, so they are close in general shape to those of tyrosine side chains in proteins. Tryptophan side chains (indole groups) have similar spectra at acid pH. However, tryptophan absorption is considerably more intense at 275 to 280 nm, by a factor of nearly four. Thus, although different proteins have very variable tyrosine-tryptophan compositions, the general shape of their UV absorption spectra in acid are fairly close to those of tyrosine at neutral and acid pH.

Proteins in neutral and acid solutions exhibit a deep trough in absorption at 245 to 250 nm. Most proteins, except heme proteins and proteins with cofactors such as NADH, do not absorb from either tyrosine or tryptophan above 300 to 310 nm. Increased absorption at ≤250 nm and/or ≥300 nm is a strong indication of the presence of compounds that interfere with A_{280} measurements and their interpretation. The effectiveness of techniques such as dialysis and size-exclusion chromatography for removing interfering UV-absorbing compounds can be monitored by analyzing the sample for absorption at 250 nm (trough), 280 nm (peak), and 300 to 310 nm (residual). Ratios of these absorbances are often used to determine whether the UV spectrum is that of

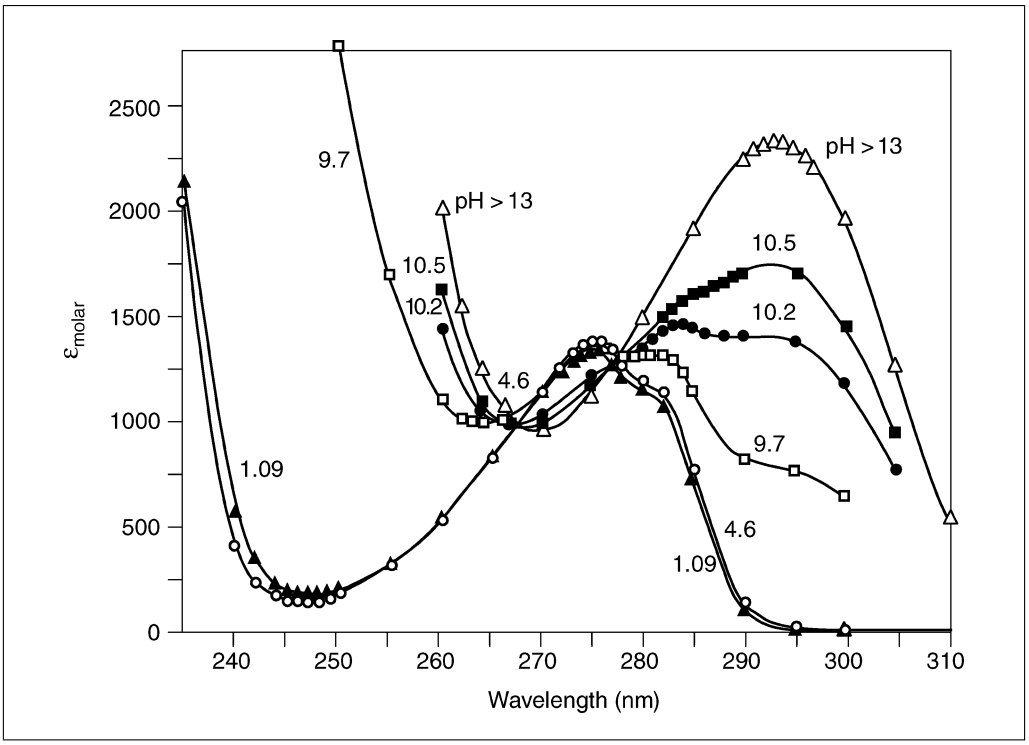

Figure A.3A.3 Absorption spectra of glycyl-L-tyrosine as a function of pH (Craig, 1967).

pure or contaminated protein. For example, the peak/trough ratio (A_{280}/A_{250}) for proteins in neutral and acid solutions is almost always ≥ 2.

When a protein solution is shifted from acidic to alkaline pH, the absorption spectrum for tyrosine changes radically due to alkaline ionization of the tyrosine hydroxyl group. The maximum change in absorption occurs at 295 nm, with a $\Delta\varepsilon_{295}$ of 2470 M^{-1} cm^{-1}. Proteins absorb rather little at 295 nm, except for some contribution by tryptophan groups. However, titration of tryptophan side chains does not produce a shift in A_{295}, so observation of changes in A_{295} with changing pH can be ascribed to tyrosine in the absence of interfering compounds that behave similarly to tyrosine. Analysis of UV absorption spectra, especially at 280 and 295 nm, for a purified protein under acid and alkaline conditions can be used to characterize the tyrosine and tryptophan content of the protein (Bencze and Schmid, 1957). With care, both tyrosine and tryptophan can be quantitated by the Bencze-Schmid method to within 3% to 4% of values obtained from complete analysis of amino acid content by hydrolysis and chromatography (Stein and Moore, 1954; Chang, 1992).

A number of sophisticated UV spectrum–based methods for protein quantitation and characterization use absorption at 205 nm and 224 to 236 nm (Bencze and Schmid, 1957), and second-derivative spectra of enzymatic digests of proteins (Bewley, 1982). It is necessary to remove oxygen from the spectrophotometer by nitrogen purging for measurements at very low wavelengths ("hard" UV, <220 nm).

All proteins absorb strongly at 205 nm, so at first glance this seems an attractive means for quantitating protein. However, most published methods describing hard UV absorbance are based on the behavior of pure, mostly single-subunit proteins. Buffers with carbonyl groups and many other impurities also become strongly absorbing in the hard UV range, and the problems of conventional UV absorbance are magnified.

Coomassie dye binding assay (Bradford assay)

Coomassie blue dye (see Basic Protocol 4) produces a rather large absorption spectrum shift in the visible-light range when it binds to many globular proteins. The strong binding forces are partly, but not entirely, due to electrostatic attraction between dye molecule sulfonate groups (two on the G250 dye molecule) and cationic groups of proteins when they are made quite acidic. The pH of the reaction mixture is low enough to thoroughly convert proteins to cations, but not so low that any sulfonate groups can become neutralized. The low pH of ~0.8 M phosphoric acid also causes most proteins to expand so that they become exposed to the triphenylmethane,

organic, and nonpolar sectors of the dye to enhance binding (Craig, 1967). Proteins that do not bind anionic dye, even in very low pH, may not bind Coomassie dyes. Early research on Coomassie dye–based protein assays used globular proteins, which are known to avidly bind dye and other similar anions such as detergent anions (Edsall and Wyman, 1958). Albumin, hemoglobin, chymotrypsinogen, and cytochrome *c*, which Bradford used to calibrate the method (Bradford, 1976), are particularly able proteins in this regard. However, other classes of proteins are quite unlike these globular, organic anion–binding proteins and these differences may be at the root of some of the failures reported for the Bradford method (Pierce and Suelter, 1977). At any rate, the Bradford method is especially simple and has good sensitivity when it is calibrated with proteins to which it is sensitive (e.g., albumin).

Jernejc et al. (1986) compared four different methods for determining total protein—Kjeldahl nitrogen, biuret, Lowry, and Coomassie dye binding—for each of eight stages in the isolation of proteins from mycelia. They found that the Coomassie dye binding method often grossly underestimated protein content, by factors >2-fold, relative to the other methods tested. Although Coomassie dye binding is an easy and convenient assay, its reliability must be verified for each experimental system.

A similar method is the Udy dye binding method, which uses acid orange 12, a sulfonated azobenzene dye. The technique was originally developed for food proteins, and it apparently compares well with Kjeldahl nitrogen analytic criteria (Udy, 1956).

Critical Parameters

There are two endemic problems in total protein assays. First, there is the issue of interfering compounds and how to remove them or minimize their effects. Second is the intense barrage of advertisements for reagents for measurement of total protein; these promise simplicity, sensitivity, and by implication suggest there is little need to consider what interfering compounds may do, how they operate, or in what concentrations they operate. In crude proteins and at many stages in protein purification, interfering compounds often are present in concentrations one, two, or even three orders of magnitude larger than protein concentrations. Some of the well-advertised means for measuring total proteins are based on calibration with pure proteins such as crystalline bovine serum albumin, which can be expected to behave well. However, good performance in calibration with single, fairly pure proteins frequently does not translate into reliable performance with real samples—tangled mixtures of carbohydrates, lipids, and nucleic acids with proteins and glycoproteins. In some cases, when samples are crude, approximately half the effort needs to be devoted to controls and to simple steps that can be taken to either eliminate interference or at least help understand it.

It should be noted that calibrating a total protein assay using a pure protein, usually bovine serum albumin, as a reference standard is common practice. However, that does not ensure that data from crude samples, taken through the same procedures, accurately quantitates total protein in crude samples.

Variability in BCA analysis

There are a number of factors that contribute to variability in the BCA assay: the temperature and duration of the incubation step just before spectrophotometry, the use of too much protein, the choice of calibration protein, and the presence of interfering compounds in samples of unknown character. It may be helpful to experiment with the temperature and duration of incubation. Sometimes incubating the sample 30 min at 60°C can accelerate color development without loss of color stability (Smith et al., 1985; Goldschmidt and Kimelberg, 1989). However, incubated samples must be cooled adequately before making spectrophotometric measurements because residual temperature gradients in the cuvettes can cause refractive index gradations or striations, which distort the optical path and produce anomalies and errors. These errors can be neutralized by remixing samples until their temperatures are close to the temperature of the cuvette and they appear uniform by simple visual inspection.

BCA calibration plots (see Fig. A.3A.1) show definite curvature downward for most proteins in excess of ~90 to 100 μg protein/ml final assay volume. Such nonlinearity can occur when the protein or polypeptide are present in excess with respect to the amount of reagents (Cu^{2+} and BCA) available to develop color.

There is considerable variation in the slope of calibration plots and therefore in apparent sensitivities, depending on the protein used as a calibration standard. For conventional proteins—e.g., BSA, chymotrypsin, and ribonuclease—slopes can vary by as much

as a factor of two (Smith et al., 1985). If gelatin is used for calibration with an incubation of 30 min at 37°C, the variation can be up to 3-fold. Some preparations of proteins, even "crystalline" and lyophilized proteins, still contain appreciable amounts of water and/or salts used in isolation or crystallization. Some freeze-dried preparations are actually only 95% protein or even less. Accordingly, water and other contaminants in supposedly dried proteins may contribute to lowered slopes, sensitivities, and apparent absorption coefficients.

UV absorption

UV absorption spectra are useful for quantitating and characterizing pure proteins. However, UV absorption is probably the most profoundly and easily distorted analytic method of quantitating proteins because concentrations of UV-absorbing contaminants as low as 10 to 100 μM can affect the spectra. Thus, caution is necessary when analyzing protein samples that are not very pure. Pigments, oxidation products, tannin-like substances, Maillard and other condensation products—all manner of biochemical compounds and natural products—all absorb at ultraviolet wavelengths, sometimes strongly, potentially causing very large errors (at least one order of magnitude). If a protein sample absorbs UV above ~320 nm, it is likely that appreciable and perhaps unacceptably large amounts of interfering compounds are present. Elaborate means have been proposed by a number of authors for UV analysis of total protein (reviewed by Peterson, 1983); nearly all are built on the supposition that no UV-absorbing interfering compounds are present in the protein sample. In practice, this supposition is very shaky, particularly at early and intermediate stages of protein purification. It may be valid for rather well-purified proteins. In general, UV absorption should be used in conjunction with other methods for analysis of total protein to correlate results. Additionally, UV absorption spectra can be used in a reverse sense, in conjunction with other analyses, to estimate the amounts of absorbing impurities (Beyer, 1983).

TCA precipitation

Trichloroacetic acid precipitation may also be affected by reagents used to prepare the sample. Chang (1992) describes a procedure for TCA precipitation of a solution containing large amounts of the strong anionic detergent sodium dodecyl sulfate (SDS). Sometimes it is useful to precipitate the interfering compounds leaving the proteins in solution. Polyethyleneimines (PEI; Sigma) can be used to precipitate nucleic acids, which have a UV absorption peak at 260 nm (Jendrisak, 1987). PEI does not absorb in the ultraviolet range. However, neither TCA nor PEI is free of cross-precipitation, so it may be necessary to experiment with the precipitant concentration, pH, and temperature to optimize a particular precipitation step.

Anticipated Results

Table A.3A.1 presents a summary of the detection ranges to expect for the assays described here.

Time Considerations

Basic Protocols 1 and 2 (Hartree-Lowry and BCA assays) require 1 hr for measuring sample, mixing, incubation, and reading the absorbance. Basic Protocol 3 (UV absorption) is the simplest of the methods described in this unit, taking only about 30 min to make dilutions, warm up the spectrophotometer, and measure the absorbance curve. Basic Protocol 4 (Coomassie dye binding) is also fast, requiring 20 to 30 min.

Acknowledgement

This unit is dedicated to Rex Lovrien, one of its original authors, who passed away prior to its publication in this manual. His insight into the use and advancement of this technology should benefit scientists for many years to come.

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Diagnosis and Treatment of Mycoplasma-Contaminated Cell Cultures

Mycoplasma contamination is a serious and frequent problem in the cell culture laboratory. Although one may suspect mycoplasma contamination when cells fail to thrive, formal diagnosis rests on the polymerase chain reaction (PCR; see Basic Protocol 1) or on the detection of adenosine phosphorylase secretion by infected cell lines (see Alternate Protocol 1). Basic Protocol 2 and Alternate Protocol 2 in this appendix present methods for antibiotic treatment of infected cultures.

NOTE: It is recommended that any positive test for mycoplasma be validated using a second assay.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

TESTING FOR MYCOPLASMA INFECTION BY PCR

BASIC PROTOCOL 1

PCR provides a sensitive and specific method for detecting mycoplasma. The mycoplasma detection kit from Roche can detect at least 10×10^3 colony forming units (cfu) of all species of mycoplasma that have been examined so far, including the five that are most commonly found in contaminated tissue culture: *M. fermentans*, *M. hyorhinis*, *M. orale*, *M. arginini*, and *A. laidlawii* (Wirth et al., 1995; also see package insert/product instructions for Roche Mycoplasma PCR ELISA kit, cat. no. 1663925). The kit utilizes digoxigenin (DIG) labeling of the PCR amplicons immobilized on a 96-well plate. The plate is incubated with anti-DIG antibody conjugated to horseradish peroxidase which is subsequently detected using peroxidase substrate and a microplate absorbance reader. For routine testing of tissue culture supernatants and other cellular samples, the PCR amplification alone is sufficient to detect mycoplasma. In the protocol provided here, reagents from the Roche Mycoplasma PCR ELISA kit are used to amplify mycoplasma using PCR, and the PCR products are then visualized using standard agarose gel electrophoresis. It should be kept in mind that PCR only detects mycoplasma DNA, which could persist in filtered sera that no longer contain viable organisms. Therefore, a positive test result warrants testing of culture medium additives and may also warrant validation via a second method.

Materials

Cell cultures to be tested for contamination
Phosphate-buffered saline (PBS; *APPENDIX 2A*) or culture medium for cells
H_2O, sterile
Roche Mycoplasma PCR ELISA kit (cat. no. 1663925) including:
  Lysis reagent
  Mycoplasma positive control DNA
  Neutralization reagent
  PCR ready-to-go mix
Taq DNA polymerase
Tabletop centrifuge
Thin-walled PCR tubes
Thermal cycler
Additional reagents and equipment for agarose gel electrophoresis (Sambrook and Russell, 2001; Voytas, 2000)

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Prepare culture supernatants

1. Allow cell cultures to overgrow before testing the supernatant.

   Fresh or frozen samples containing cell culture supernatant or other sample(s) of interest can also be used.

2. Centrifuge 1 ml of each culture sample 10 min at 500 × *g*, room temperature. For samples that appear too concentrated, use 50 µl sample and dilute to 1 ml with PBS. Include 1 ml cell culture medium or PBS (depending on the type of sample assayed) as a medium-blank control.

3. Transfer the supernatant to a sterile microcentrifuge tube and microcentrifuge 10 min at maximum speed, 4°C.

4. Carefully remove the supernatant, making sure that the pellet remains at the bottom of the tube.

5. Add 10 µl sterile water and 10 µl lysis reagent to each tube. Include a positive control for the PCR reaction consisting of 10 µl positive control DNA and 10 µl lysis reagent. Also include a negative control for the PCR reaction consisting of 10 µl sterile water and 10 µl lysis reagent.

6. Incubate all samples 1 hr at 37°C.

7. Add 30 µl neutralization reagent to each sample.

Perform PCR

8. For each sample and control to be tested, transfer 25 µl PCR ready-to-go mix and 15 µl sterile water into a thin-walled PCR tube. Add 10 µl control or sample to the corresponding tubes. Add 0.5 µl *Taq* DNA polymerase to each tube.

   Taq DNA polymerase is included in the PCR ready-to-go mix, but after repeated freeze-thawing the enzyme may lose its activity.

9. Transfer each tube to the thermal cycler and perform PCR using the following cycling conditions:

1 cycle:	5 min	95°C	(initial denaturation)
40 cycles:	30 sec	94°C	(denaturation)
	30 sec	62°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension).

10. Following amplification, resolve the PCR products on a 1% agarose gel (see, e.g., Sambrook and Russell, 2001; Voytas, 2000).

    Positive bands are ~500 to 600 kb. A prominent band will be present in the positive control if the PCR reaction was successful, and will also be present in any samples contaminated with mycoplasma.

ALTERNATE PROTOCOL 1

TESTING FOR MYCOPLASMA INFECTION BY DETECTION OF ADENOSINE PHOSPHORYLASE SECRETION

A convenient and accurate method for testing cultured cells for infection with mycoplasma is based on the observation of McGarrity and Carson (1982) that these organisms secrete abundant adenosine phosphorylase, an enzyme capable of converting the nontoxic adenosine analog 6-methylpurine deoxyriboside (6-MPDR) into the potent antimetabolites 6-methylpurine (6-MP) and 6-methylpurine riboside (6-MPR), which are toxic to mammalian cells.

This method can also be used as an alternative to PCR (see Basic Protocol 1) to evaluate conditioned medium for the possible presence of mycoplasma. In the instructions accompanying the MycoTect kit obtained from Life Technologies, 3T6 cells are suggested for use as the indicator cell. However, for many immunology laboratories the SP2/0-Ag14 hybridoma cell line—a widely used fusion partner for generating antibody-secreting hybridomas (Shulman et al., 1978)—is more convenient to use.

In this protocol, a potential problem of differential susceptibility of cells to the toxic effects of 6-MP is avoided by culturing a highly susceptible cell line overnight with culture supernatants from the cells to be tested. Contamination is detected after addition of 6-MPDR—if growth is not observed, mycoplasma is present.

Materials

SP2/0-Ag14 hybridoma cells (ATCC #CRL 1581)
Cells to be tested in appropriate medium without antibiotics
MycoTect (Life Technologies) containing 6-methylpurine deoxyriboside (6-MPDR)
6-methylpurine (6-MP; Sigma)

96-well flat-bottom microtiter plates (Costar, Falcon, or equivalent)

1. Prepare 50-μl triplicate cultures containing 2×10^3 SP2/0-Ag14 cells (4×10^4 cells/ml) in 96-well flat-bottom microtiter plates.
2. Culture cells to be tested in medium without antibiotics in a humidified 37°C, 5% CO_2 incubator for ≥24 hr. Allow cells to overgrow, remove supernatant, and add 50 μl culture supernatant to each well of the microtiter plate (from step 1).

   Routinely, each cell line is tested in triplicate—i.e., cells are cultured in nine microtiter wells—although this may be unnecessary. Each cell line should be tested every 2 months to be certain that contamination has not been inadvertently introduced.

3. Add 100 μl medium to first well (negative control), 100 μl 6-MPDR (40 μM final) to second well, and 100 μl 6-MP (6 μM final) to third well (positive control). Culture cells 2 to 3 days in a humidified 37°C, 5% CO_2 incubator.

   The MycoTect kit also includes adenosine phosphorylase to be used as an additional positive control; use according to the instructions provided.

4. Screen cultures visually using an inverted microscope for growth of indicator SP2/0-Ag14 cells. If mycoplasma is not present, cell growth is observed in the presence of medium and 6-MPDR but not in the presence of 6-MP. If mycoplasma is present, cell growth is observed in the presence of medium but not in the presence of 6-MPDR or 6-MP (see Basic Protocol 2 and Alternate Protocol 2 for treatment).

   Staining cells with crystal violet is recommended in the instructions included in the MycoTect kit, but is usually unnecessary.

TREATMENT OF MYCOPLASMA-CONTAMINATED CELL CULTURES WITH CIPROFLOXACIN

BASIC PROTOCOL 2

In the past, the only effective treatment for mycoplasma contamination was to discard the culture. Alternatively, cell lines could be passaged in vivo, e.g., by inoculation and rescue from a nude mouse. Direct treatment of mycoplasma-contaminated cell cultures has become possible with the development of the antibiotics ciprofloxacin and BM-cyclin (Schmidt et al., 1988). A modified protocol for the use of ciprofloxacin is given in the following method and one for the use of BM-cyclin is given in Alternate Protocol 2. The BM-cyclin regimen is considerably more complicated and BM-cyclin is potentially

more toxic to cells. Nevertheless, there is significant experience indicating the efficacy of BM-cyclin, so use of Alternate Protocol 2 should not be ruled out.

Materials

10 mg/ml ciprofloxacin·HCl (see recipe), sterile
Contaminated cells (see Basic Protocol 1 and Alternate Protocol 1)

1. Add 10 mg/ml ciprofloxacin·HCl to contaminated cells to 10 μg/ml final (a 1:1000 dilution). Keep ciprofloxacin·HCl in the cultures for 12 days in a humidified, 37°C 5% CO2 incubator.
2. After completion of the treatment, freeze aliquots of the mycoplasma-free cells.
3. Test cell line for presence of mycoplasma infection (see Basic Protocol 1 and Alternate Protocol 1).
4. Culture cells in absence of ciprofloxacin·HCl and watch for recurrence of mycoplasma contamination (see Basic Protocol 1 and Alternate Protocol 1).

ALTERNATE PROTOCOL 2

TREATMENT OF MYCOPLASMA-CONTAMINATED CELL CULTURES WITH BM-CYCLIN

Whereas ciprofloxacin (see Basic Protocol 2) blocks bacterial DNA replication by inhibiting DNA gyrase, BM-cyclin is an inhibitor of bacterial protein synthesis.

The BM-cyclin regimen utilizes two solutions. First, the maximum concentration of each solution tolerated by the cells (i.e., which allows cell growth and does not kill cells) is determined. Next, the cells are cultured in BM-cyclin solution 1 for 3 days, then solution 2 for 4 days. Finally, this cycle is repeated ≥2 times, usually resulting in mycoplasma eradication.

Materials

BM-cyclin solutions 1 and 2 (see recipe), sterile
Contaminated cells (see Basic Protocol 1 and Alternate Protocol 1)

1. Determine the maximum amount of BM-cyclin solutions 1 and 2 that can be tolerated by the contaminated cells by setting up a titration curve for each solution in the following manner.
   a. Prepare several different concentrations of each BM-cyclin solution beginning at 20 μl of each BM-cyclin solution per 10 ml cell culture and then halving the concentration for several dilutions (e.g., use 10 μl/10 ml, 5 μl/10 ml, and 2.5 μl/10 ml).
   b. Culture cells at density appropriate for that cell line for 3 days in BM-cyclin solution 1 and 4 days in BM-cyclin solution 2 in a humidified 37°C, 5% CO_2 incubator.
2. Screen cultures visually using an inverted microscope to identify the BM-cyclin solution dose that does not kill cells and allows cell growth.

   Alternatively, an aliquot of cells can be counted using the trypan blue exclusion assay (Phelan, 1996).

3. Culture cells 3 days in the maximum tolerable dose of BM-cyclin solution 1, then 4 days in BM-cyclin solution 2 at the same dose in a humidified 37°C, 5% CO_2 incubator.

   This represents one cycle of treatment. If the cell density has plateaued, split into fresh growth medium plus the appropriate BM-cyclin solution at the maximal tolerated dose.

4. Freeze an aliquot of the treated cells.

   Freeze aliquots of treated cells after each cycle and after the treatment has been completed. This will prevent loss of the cell line to another contaminant.

5. Repeat steps 3 and 4 twice. Screen cultures visually using an inverted microscope after each cycle for signs of improved cell viability and growth. Split cells as their growth rate and density dictate.

   Alternatively, count cells using the trypan blue exclusion assay. In most cases, three cycles are sufficient to eradicate the mycoplasma. Occasionally, additional cycles are necessary. If so, treat cells with one cycle of antibiotics beyond eradication. Chronic administration (>4 weeks) of the antibiotics is not recommended since resistant organisms may develop.

   Treated cells should grow more vigorously and lose symptoms of mycoplasma contamination (see Troubleshooting).

6. After completion of the treatment, freeze aliquots of the mycoplasma-free cells.

7. Test cell line for presence of mycoplasma infection (see Basic Protocol 1 and Alternate Protocol 1).

8. Culture cells in absence of BM-cyclin and watch for recurrence of mycoplasma contamination (see Basic Protocol 1 and Alternate Protocol 1).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

BM-cyclin solutions 1 and 2

Prepare and filter sterilize BM-cyclin (Roche Molecular Biochemicals) solutions 1 and 2 using a 0.45-μm filter. Freeze aliquots indefinitely at −20°C and thaw as needed.

Frequent thawing and freezing of the BM-cyclin solutions does not appear to affect the activity of these antibiotics.

Ciprofloxacin·HCl, 10 mg/ml

Dissolve ciprofloxacin·HCl (Sigma) in water to 10 mg/ml. Filter sterilize through a 0.45-μm filter, aliquot, and store in sterile tubes at −20°C.

COMMENTARY

Critical Parameters

When treating cultures with BM-cyclin, the major critical parameter is that the correct dose of the drug be used (see Basic Protocol 2 and Alternate Protocol 2). In general, toxicity of ciprofloxacin has not been a problem.

Another consideration is whether the desired phenotype of the cell will change during the BM-cyclin or ciprofloxacin treatment. It is possible that a monoclonal antibody–producing hybridoma will lose its secretory capacity during the relatively prolonged treatment and may need to be recloned by limiting dilution (Yokoyama, 1991) during treatment.

Troubleshooting

Sources of Contamination

The most common sources of mycoplasma contamination are previously contaminated cell lines, bovine sources (e.g., FBS), and other animal sources. The best method of eradication is prevention. FBS should be heat-inactivated before use in tissue culture (see *APPENDIX 2A*). Any animals that will be used to generate cell lines or bioactive products should be purchased from specific-pathogen-free (SPF) suppliers and housed in pathogen-free facilities. In general, newly derived cell lines and cell lines from other laboratories

should be quarantined until the investigator is satisfied that the cultures and/or products are mycoplasma free. Suspicious cell lines should not be manipulated at the same time as "clean" cultures, as aerosol inoculation appears to be a frequent mode of cross-contamination with mycoplasma. Finally, a separate hood and tissue culture incubator should be set aside for any certified mycoplasma-free cultures.

Indications of Contamination

Mycoplasma contamination is a frequently overlooked but potentially serious problem. The following symptoms may indicate mycoplasma contamination:

1. A once vigorous cell line grows poorly.
2. An adherent cell line is less adherent.
3. Cell line cannot be grown to high density ($>1 \times 10^6$ cells/ml).
4. B or T hybridomas fail to be generated from cell fusion techniques.
5. Cell lines cannot be cloned by limiting dilution.
6. Large amount of debris is evident in the culture supernatant.

Mycoplasma contamination can interfere in bioassays. The following effects may indicate mycoplasma contamination:

1. Any inhibitory effect of monoclonal antibody or culture supernatant on any proliferation assay.
2. Any costimulatory effect of culture supernatants on B-cell proliferation.
3. Cell lines with higher than expected [^{3}H]thymidine incorporation.
4. Failure of T cell clones or hybridomas to give usual proliferation or lymphokine responses.

If a functional effect of a culture supernatant is eliminated by treatment of the supernatant as indicated below, its source may be mycoplasma contamination. The following treatments are effective in eliminating mycoplasma but obviously may have an effect on the cell viability:

1. Heat-inactivation 30 min at 56°C.
2. Filtration through a 0.1-μm filter.
3. Ultracentrifugation 24 hr at 100,000 × *g*.

Anticipated Results

Most cultures will be relatively free of mycoplasma contamination after the antibiotic treatment. Whether complete eradication has been achieved should be viewed with caution as long as the cell line is used.

Time Considerations

BM-cyclin treatment requires one week for each cycle. At least three cycles are required but sometimes more are necessary.

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Contributed by Helena Harlin and Thomas F. Gajewski
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Gram Staining

The gram stain ranks as one of the most important stains for bacteria. Named after Hans Christian Gram who developed the method in 1884, the Gram stain allows one to distinguish between Gram-positive and Gram-negative bacteria on the basis of differential staining with a crystal violet–iodine complex and a safranin counterstain. The cell walls of Gram-positive organisms retain this complex after treatment with alcohol and appear purple, whereas gram-negative organisms decolorize following such treatment and appear pink. The method described is useful for assessing bacterial contamination of tissue culture samples or for examining the Gram stain status and morphological features of bacteria isolated from mixed or isolated bacterial cultures.

Materials

Sample to be stained
Crystal violet solution (see recipe)
Gram's iodine solution (see recipe)
Decolorizing solution (see recipe)
Counterstain (see recipe)
Bibulous paper (optional)
Microscope with oil immersion objective (*UNIT 2A.1*)

NOTE: A Gram stain kit (e.g., Fisher) may be used in lieu of the staining reagents described above.

Prepare smear

1. Hold an inoculating loop in a flame until red-hot and then allow to cool ~30 sec. Using this sterilized loop, prepare a thin film of the sample to be stained on a glass slide.

   If checking a tissue culture sample for possible bacterial contamination, or a suspension of bacteria growing in culture medium, remove a loopful of sample and apply this directly to the slide. If examining bacteria growing as colonies on plates, pick one colony and resuspend bacteria in a drop of sterile water previously applied to the slide.

2. Air dry the smear then heat-fix by passing the slide over a flame two or three times.

   Do not leave the slide over the flame, as this will overheat the sample.

Stain smear

3. Cover the heat-fixed smear with crystal violet solution. Let stand 30 sec.
4. Wash the stain off gently with flowing water for ~5 sec. Shake off excess water.

   Gently dripping tap water can be used.

5. Cover the smear with Gram's iodine solution for 1 min. Wash with water as in step 4 and shake off excess water.

Decolorize and counterstain smear

6. Decolorize by tilting the slide slightly and slowly dropping decolorizing solution above the smear on the slide, allowing it to run down across the smear. Do this until the purple color just ceases to flow away from the smear.

   It is most important that the smear be neither over- nor underdecolorized. Excessive decolorization may remove enough dye to give false Gram-negatives; insufficient decolorization may give false Gram-positives.

Contributed by Richard Coico

Current Protocols in Microbiology (2005) A.3C.1-A.3C.2

7. Wash with water as in step 4 for ~5 sec. Shake off excess water.
8. Cover the slide with counterstain for 30 sec.
9. Wash with water as in step 4 then shake off excess water. Allow to air dry, or carefully blot with bibulous paper.

   A well-prepared smear should be barely visible to the unaided eye.

Examine slide

10. Examine the bacteria under oil immersion (900× to 1000×) to distinguish between Gram-positive and Gram-negative bacteria.

    Do not use a cover slip. Simply add 1 drop of oil to the center of the slide and observe bacteria. If necessary, slides can be stored in a slide box for many months and reexamined under oil immersion.

    Gram-positive bacteria appear purple as compared with Gram-negatives which appear pink.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Counterstain

Mix 2.5 g safranin O with 100 ml of 95% ethanol. Store up to 1 year at room temperature.

Crystal violet solution

Crystal violet stock (solution A)

Dissolve 20 g crystal violet (85% dye) in 100 ml of 95% ethanol. Store up to 1 year at room temperature.

Oxalate stock (solution B)

Dissolve 1 g ammonium oxalate in 100 ml water. Store up to 1 year at room temperature.

Working solution

Dilute solution A 1:10 with distilled water and mix with 4 vol solution B. Store up to 6 months in a glass bottle at room temperature.

Decolorizing solution

Mix equal volumes of 95% ethanol and acetone. Store up to 1 year in a glass bottle at room temperature.

Gram's iodine solution

Dissolve 1 g iodine crystals (Sigma) and 2 g potassium iodide (Sigma) in 5 ml water, then add 240 ml water and 60 ml of 5% (w/v) sodium bicarbonate solution. Mix well and store up to 6 months in an amber glass or foil-covered bottle at room temperature.

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Direct PCR of Intact Bacteria (Colony PCR)

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ABSTRACT

This protocol describes an efficient method for screening intact bacteria for the presence of desired DNA sequences using polymerase chain reaction (PCR). This method is commonly referred to as colony PCR. *Curr. Protoc. Microbiol.* 9:A.3D.1-A.3D.6. © 2008 by John Wiley & Sons, Inc.

Keywords: colony PCR • bacteria • screen

PCR AMPLIFICATION OF DNA FROM A BACTERIAL COLONY (COLONY PCR)

BASIC PROTOCOL

This unit describes a method for screening individual bacterial colonies for specific DNA sequences using the polymerase chain reaction (PCR; see Kramer and Coen, 2001). This procedure, commonly referred to as colony PCR, is advantageous because it is a very quick and easy way to screen a large number of bacteria to determine which bacteria contain a particular DNA sequence, without first having to purify DNA from all of them. This is especially useful when screening colonies after transformation with recombinant plasmids or after targeted mutagenesis. Colony PCR can be effectively used not only to identify clones with an insertion or deletion, but also to determine the orientation of a DNA insertion, which may be important for proper transcription and translation of the insert. Furthermore, the method can be used to amplify a desired DNA fragment for subsequent sequencing or cloning. While colony PCR is most commonly used in the laboratory to screen transformed *E. coli*, DNA sequences from virtually any bacterial species can be detected and/or isolated regardless of location on the chromosome or a plasmid.

Materials

10× PCR buffer: supplied with DNA polymerase *or* see *APPENDIX 2A*
2.5 mM (each) dNTP mix: supplied with DNA polymerase *or* see *APPENDIX 2A*
30 μM oligonucleotide primer 1 stock (see Critical Parameters)
30 μM oligonucleotide primer 2 stock (see Critical Parameters)
5 U/μl *Taq* DNA polymerase, heat-stable (e.g., Takara *Taq*, Fisher)
Distilled H_2O, sterile
$MgCl_2$ (if required)
Gel electrophoresis loading buffer (see recipe)

200-μl thin-walled PCR tubes (or other appropriate for thermal cycler)
Toothpicks, sterile
Thermal cycler

Additional reagents and equipment for performing agarose (Voytas, 2000) or polyacrylamide (Chory and Pollard, 1999) gel electrophoresis

Procedure

1. Prepare the master mix for PCR amplification (e.g., 100 μl for ten tubes of 10 μl each):

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10 µl of 10× PCR buffer containing 15 mM $MgCl_2$
8 µl of 2.5 mM (each) dNTP mix
5 µl of 30 µM primer 1
5 µl of 30 µM primer 2
1 µl of 5 U/µl heat stable *Taq* DNA polymerase
71 µl sterile distilled water.

The volume of master mix prepared will depend upon the number of reactions and volume of each reaction. For example, if one is performing 20 reactions of 10 µl each, 200 µl master mix will be required. It is best to make a little extra master mix, since pipettors are often slightly inaccurate.

The $MgCl_2$ is often already included in the PCR buffer supplied with the DNA polymerase. If not, the appropriate amount must be added. Optimum $MgCl_2$ concentrations for each PCR reaction may vary and can range from 1.5 mM to 4.5 mM (see Kramer and Coen, 2001).

A high-fidelity Taq polymerase (e.g., Takara Ex Taq, Fisher) should be used if the PCR product is intended for use beyond simple screening, e.g., for DNA sequencing or later cloning into a vector for recombinant protein purification.

2. Dispense the master mix into the PCR tubes.

3. Using a sterile toothpick for each colony, remove a small amount of a bacterial colony directly from the plate to be tested and place in a PCR tube with master mix.

   Be certain to touch just one colony and avoid colonies very close to each other, otherwise this could contaminate the results.

   The amount of bacteria should be just barely visible to the unaided eye. PCR amplification will not be efficient if too few bacteria are transferred into the PCR reaction, while too many bacteria may cause amplification inhibition from other bacterial components. Experience will allow the researcher to know the proper amount of bacteria to allow for efficient PCR amplification. Since some reactions may fail, it is a good idea to simultaneously screen 10 to 20 colonies.

   Colonies may be needed for further study, so spotting each tested colony onto a plate divided into a numbered grid (Riley et al., 2008; also see APPENDIX 4A) is also recommended.

4. Carry out PCR using conditions appropriate for the DNA being tested, with the following amplification cycles as a guideline:

Initial step:	5 min	94°C	(bacterial lysis/ denaturation)
25 cycles:	30 sec	94°C	(denaturation)
	30 sec	50°C-55°C	(annealing)
	1 min	68°C-72°C	(extension)
Final extension:	5 min	72°C.	

   A thermal cycler with a heated lid is recommended for convenience. Otherwise, overlaying the reaction mixture with mineral oil will be required (see thermal cycler instruction manual and Kramer and Coen, 2001).

   Twenty five cycles should be sufficient for production of a detectable amplicon.

   It is important to note that the ideal annealing temperature will vary depending on the GC content of the primers being used. Greater primer GC content requires higher annealing temperatures for specificity.

   Additionally, a general rule of thumb for PCR is to allow 1 min of extension time for every 1 kb of amplified DNA product. For a detailed description of the stages included in the PCR reaction see Kramer and Coen (2001).

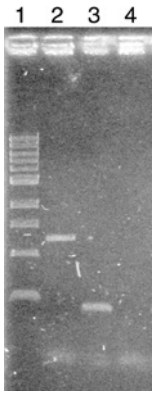

Figure A.3D.1 Representative results of colony PCR. Lane 1: 1-kb ladder (New England BioLabs); Lane 2: ~1.3-kb insert; Lane 3: ~400-bp insert; Lane 4: No insert. The faint bands at the bottom of the gel are unused primers from the PCR reaction.

5. Mix 5 to 10 µl of each reaction mixture with 5 µl gel electrophoresis loading buffer.

   For efficiency, loading buffer may be added directly to the PCR reaction tube. However, if you intend to clone or directly sequence the PCR product, then an aliquot of each reaction should be removed from the reaction tube and mixed with loading buffer.

6. Electrophorese the reaction mixture on an agarose gel (Voytas, 2000) or a nondenaturing, continuous polyacrylamide gel (Chory and Pollard, 1999), depending on the size of the PCR product.

   The optimal percentage of agarose used in gels can vary depending on the size of the DNA product. A 0.8% agarose gel can be used for most PCR products, but if the product is large, a lower percentage gel can be used, and vice versa.

   For PCR products under 500 bp, nondenaturing polyacrylamide gels provide better resolution than agarose gels.

7. Stain the gel with ethidium bromide and use ultraviolet (UV) light to visualize the PCR products (see Voytas, 2000).

   CAUTION: *Gloves should be worn when handling ethidium bromide (a carcinogen) and acrylamide (a neurotoxin).*

   CAUTION: *UV-protectant eyewear should be worn when viewing the gel with UV light.*

   Representative results are shown in Figure A.3D.1.

8. Determine the presence and/or orientation of the insert.

   See Anticipated Results in the Commentary section.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Gel electrophoresis loading buffer

To make 10 ml, combine:
5 ml of 50% glycerol
5 ml of 0.5 M EDTA (*APPENDIX 2A*)
0.0276 g bromphenol blue
0.0214 g xylene cyanol FF
Mix well
Store in 1-ml aliquots up to 12 months at 4°C

CAUTION: *Xylene cyanol is an irritant. Wear gloves when preparing this reagent.*

COMMENTARY

Background Information

The detection or isolation of a specific DNA sequence directly from a colony of *E. coli* or most other bacterial species of interest can be performed using this simple PCR-based method. Most commonly, colony PCR is used to screen *E. coli* transformants for a specific DNA sequence of interest. Screening is important because not all colonies that arise following transformation may contain a plasmid with the desired DNA insert. Additionally, the DNA insert may be in the wrong orientation for proper transcription and translation. Colony PCR is an efficient method for easily identifying the bacteria with the desired DNA, as well as the desired orientation of the insert.

A major advantage of colony PCR is that this procedure does not require DNA purification. Simply, a small aliquot of whole bacteria (e.g., a colony) is added to a PCR mix (specific primers, heat-stable DNA polymerase, dNTPs, and buffer) and then subjected to PCR. The initial PCR step, heating to 94°C, is sufficient to lyse bacteria and liberate their DNA contents.

This procedure is not exclusively for use with bacterial colonies; a small aliquot of bacteria from an actively growing liquid culture, centrifuged and washed with phosphate-buffered saline (PBS; *APPENDIX 2A*), can be used just as easily. A small sample of bacteria from a frozen stock may also be used.

Although, colony PCR is most commonly used to screen *E. coli* transformants, the detection and isolation of a specific DNA sequence from most bacterial species can be performed using this same procedure. The DNA sequence of interest does not have to be located on a recombinant plasmid, but can be located on a chromosome or a native plasmid in the bacterial genome.

Critical Parameters

Researchers commonly use previously constructed plasmid vectors to insert the desired DNA fragment into a specific site on the plasmid, often a multiple cloning site (MCS). Plasmid vectors can be purchased commercially (e.g., the pUC series) or are readily available from other researchers. Cloning of PCR products is generally simplified by use of TA vectors (e.g., pCR2.1; Invitrogen). The MCS or TA-cloning sites of widely used cloning vectors are very often bordered on each side with conserved DNA sequences. Commonly used flanking DNA includes sequences based on the bacteriophages M13 and/or T7.

One can take advantage of conserved vector sequences to minimize the expense of PCR screening by using the same primers for amplification of an insert in any plasmid that contains these common flanking DNA sequences. To determine the oligonucleotide primers that can be used for the plasmid, product information sheets are provided with commercially obtained cloning vector plasmids and are also available at no cost on the manufacturers' Web sites. These Web sites are freely accessible to researchers even if the plasmid was not purchased from the commercial vendor, but obtained from a fellow scientist.

If the DNA insert is being introduced into an expression vector, the proper orientation is required for production of the protein. The orientation can be determined using the same approach as described above, but with a different primer set, as shown in Figure A.3D.2. Two primer sets can be designed, each with one primer complementary to a flanking DNA sequence and the other to a DNA sequence in the inserted fragment (primer sets should be designed to amplify in opposite directions).

Colony PCR using the described methods will allow you to determine if a DNA insert or deletion is in the proper location. However, sequencing the DNA is strongly recommended to ensure no errors were introduced during the cloning processes.

Troubleshooting

Common problems arising during execution of this technique, as well as their potential causes and solutions, are listed in Table A.3D.1.

Anticipated Results

Colony PCR using primers specific for DNA sequences flanking the insertion site will allow you to determine if you have an insertion in the appropriate location and if it is the expected size. PCR amplification with primers specific for DNA on each side of the insert (see Critical Parameters and Fig. A.3D.2) will yield a product the size of the insert plus the distance between the two primer locations and the insertion. Vectors without an insert will yield a small product representing only the distance between the two primers. In general, inserts that are not the desired DNA fragment will yield amplicons of different sizes than the insert.

After performing PCR reactions with either primers 1 + 3 or primers 2 + 4 as diagrammed in Figure A.3D.2, you will obtain a product only if the DNA insert is in the proper

orientation. If the insert is in the wrong orientation, the primers will be aligned in the same direction, yielding no product. If primers 1 + 2 or primers 3 + 4 are used, a PCR product will be produced only if the insert is in the opposite orientation.

Time Considerations

A PCR reaction mix can be prepared in under 10 minutes, once reagents are thawed. For the amplification of a ≤1-kb DNA fragment, 2 hr should be allotted for the reaction to run in the thermal cycler. If the amplicon is larger than 1 kb, the PCR will require additional time, accordingly.

An agarose gel can be prepared in approximately 20 min, with a few minutes required to measure and melt the agarose in the microwave and the rest of the time to allow for the gel to cool and solidify.

Electrophoresis of the PCR products on an agarose gel requires 1 hr, as well as 20 to 30 min to stain the gel in ethidium bromide.

Overnight incubation of bacteria such as *E. coli* is typically required for formation of colonies on plates, but this may vary depending on the specific bacterial species and strain being examined, as well as the growth medium and conditions.

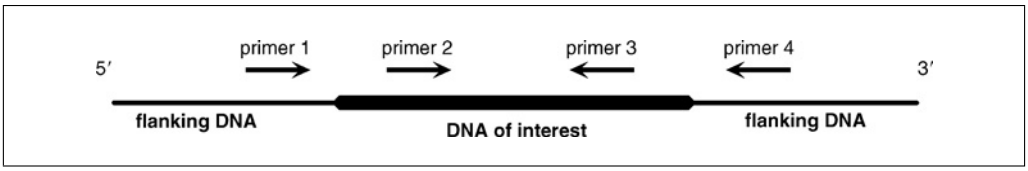

Figure A.3D.2 Diagram of oligonucleotide primer locations for colony PCR. Primers 1 + 4 determine if a DNA insert size is correct. Primers 1 + 3 or primers 2 + 4 can be used to determine if there is a DNA insertion as well as its orientation. For additional confirmation of the orientation of the insert, PCR reactions can be performed with primers 1 + 2 or primers 3 + 4 as well (see Anticipated Results).

Table A.3D.1 Troubleshooting Guide for Colony PCR

Problem	Possible cause	Solution
No PCR product	Too little or too much bacteria.	Adjust amount of bacteria added to the PCR reaction.
	Annealing temperature too high	Decrease temperature 3°C-5°C below primer T_m
	$MgCl_2$ concentration not be optimal for given primer set	Adjust concentration to between 1.5 mM and 4.5 mM
	Primers may not amplify efficiently	Design new, more efficient primers; use no-cost programs from commercial vendors (e.g., *http://www.idtdna.com*)
Multiple PCR products	Nonspecific binding of primers to DNA	Increase annealing temperature 3°C-5°C below primers T_m
	Binding of primers to similar sequences in the genome	Design new primers (see above)
PCR product of undesired size	Insert not the desired DNA fragment	Repeat cloning and transformation
	Mispriming of primer(s) on DNA	Redesign primers (see above)
Colony PCR product of appropriate size but not the desired DNA sequence	Two colonies touching during colony PCR or the DNA extraction for sequencing	Restreak for individual colonies and repeat PCR screen before sequencing
	Another DNA insert of the same size as the desired DNA fragment	Repeat cloning and transformation

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Voytas, D. 2000. Agarose gel electrophoresis. *Curr. Protoc. Mol. Biol.* 51:2.5A.1-2.5A.9.

COMMONLY USED METHODS FOR CELL CULTURE

Common Bacterial Culture Techniques and Media

Bacterial species differ from each other in their metabolic capabilities, requiring researchers to use specific media and culture conditions. As those requirements often vary widely between species, readers should refer to the units in this manual focusing on their particular organism for details on cultivation and media. However, a number of common techniques are used in the study of a wide variety of bacteria, and these are presented in this appendix. In addition, since many strains of *Escherichia coli* serve as important tools for almost all modern biologists, commonly used techniques and media for that species are also included.

ASEPTIC TECHNIQUE

This phrase refers to all of the measures that are taken to prevent the introduction of sepsis (contamination) into cultures. Aseptic technique is sometimes mistakenly called "sterile technique," but this phrase is inappropriate since the goal of bacterial culture is to grow a microorganism rather than maintain a sterile environment devoid of bacteria. It is imperative that the microbiologist practice aseptic technique to ensure that cultures remain uncontaminated; otherwise, resulting studies will be meaningless. Examples of aseptic technique include cleaning and disinfecting laboratory surfaces prior to use, limiting the amount of time during which cultures or media are left uncapped and thus exposed to the air, keeping petri dishes closed whenever possible, effectively sterilizing inoculating loops and other equipment that come into contact with cultures or media, and avoiding breathing on cultures or sterile instruments.

COMMONLY USED TOOLS

Inoculating Loop

Many varieties of loops, made of nickel-chromium or platinum wire, are available from many suppliers. The wire may be permanently attached to a handle or replaceable, and may be obtained as either a straight wire or a twisted loop. Each type has its own advantages and disadvantages, and choice is usually made on the basis of availability or personal preference. The loop shape allows efficient transfer of liquids, which form a film over the loop. Standardized volumetric loops are available for reproducible transfers of specific volumes of liquid.

A loop can be easily made on the end of a straight wire by bending it around the tip of a sharp pencil. The loop itself should be 2 to 3 mm in diameter and must be complete, with the tip of the wire just touching the opposite side of the loop to allow formation of a liquid film for transfer.

The inoculating loop must be sterilized immediately before and immediately after use. To sterilize, first place the end of the wire closest to the handle in the blue (hottest) part of a burner flame, until the wire glows red. Next, slowly draw the wire through the flame, ensuring that the entire length of the wire glows red. The loop can be cooled quickly by

Contributed by Brian Stevenson
Current Protocols in Microbiology (2006) A.4A.1-A.4A.8

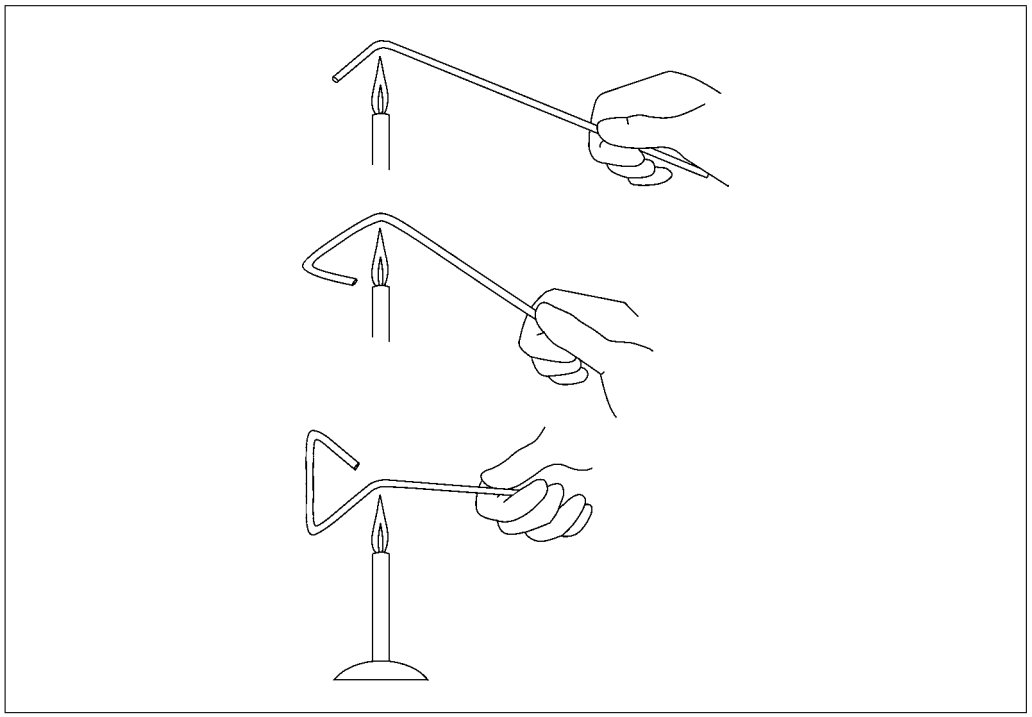

Figure A.4A.1 Making a spreader.

gently touching it to an unused, sterile part of an agar plate or by dipping it in sterile liquid medium. Do *not* blow on a hot loop to cool it off.

Disposable, plastic loops are also available, although they are expensive over the long term and create a large amount of waste. However, use of disposable loops is advisable with certain organisms in order to avoid generation of hazardous aerosols during flaming, or when working in oxygen-deficient environments that preclude use of a burner. See appropriate units of this manual to determine which type of loop is best for each organism being studied.

Culture Spreader

A spreader is used to evenly distribute bacterial cells over the surface of a plate. This will yield either a lawn on a nonselective medium or isolated colonies on a selective medium. A spreader can be easily made by heating and bending a piece of 4- to 5-mm diameter glass rod, as illustrated in Figure A.4A.1. In a pinch, a much less durable spreader can be quickly made by substituting a Pasteur pipet for the glass rod. For safety in handling, melt away sharp ends of the rod as a first step, i.e., prior to bending. A pair of needle-nose pliers or similar tool is useful for forming bends in glass.

Before each use, sterilize the spreader by immersing the triangular end in a beaker of 95% ethanol, passing the spreader through a flame, then allowing the flame to burn out. Be careful not to let the burning ethanol drip into the beaker or onto flammable objects. Cool the spreader by touching to an unused portion of the agar surface. Sterilize again after use by immersing in ethanol.

Presterilized, disposable plastic culture spreaders are also available from a variety of sources. As with plastic inoculating loops (see above), disposable spreaders are expensive over the long run and generate considerable waste, but may be preferable in certain situations.

CULTURE TECHNIQUES

Oxygen Environment

Bacteria differ in their ability to grow in the presence of oxygen. Not all species can utilize oxygen, and many are unable to survive exposure to it. Be sure to follow guidelines described in this book for each species.

Growth of *E. coli* is generally enhanced by the presence of oxygen, with the Krebs cycle and oxidative phosphorylation facilitating complete catabolism of complex carbon sources to carbon dioxide. For this reason, *E. coli* cultures on solid media should be incubated in standard (aerobic, not CO_2) incubators. Liquid cultures should be oxygenated by shaking during incubation. Special Erlenmeyer flasks with baffles on the bottom are ideal, as the baffles perturb liquid flow and help dissolve air into the medium. Caps should be loosely attached, or vented caps or other enclosures used. To maximize exposure of liquid to air, do not fill culture containers more than one-third full.

Monitoring Growth in Liquid Medium

A simple method for quantifying growth is to measure the turbidity of the culture using a spectrophotometer. To perform this technique, measure the absorbance at an appropriate wavelength, e.g., 600 nm is often used for *E. coli*. Zero the spectrophotometer with a blank containing unused culture medium. Klett meters are inexpensive, simple spectrophotometers designed for this purpose. Correlation between absorbance ("optical density" or "OD") and bacterial density varies between species and sometimes between strains due to differences in bacterial sizes and shapes, and so must be determined empirically for each organism.

A hemacytometer, Petroff-Hausser counting chamber, or similar cell counter can be used to determine culture density under a microscope. Such counters consist of a glass slide with a fine grid etched in the glass and a second slide that is suspended above the first; the two hold a specific volume of liquid between them by capillary action. An example of a hemacytometer is given in Figure A.4A.2. Volumes and mathematical ratios vary between units, so be sure to thoroughly read the manufacturer's instructions for the chamber prior to use.

Measurement of colony-forming units (cfu) is probably the most accurate method to determine the number of live bacteria in a culture. To perform this procedure, 10-fold serial dilutions of the culture are individually spread on agar plates (see below) and incubated, and numbers of colonies arising per plate are counted. For accuracy, two to three plates per dilution should be inoculated. This technique is similar to the enumeration of plaque-forming units (pfu) used for quantification of bacteriophage or other virus suspensions.

Positioning Plates within the Incubator

For most bacteria, petri dishes are incubated inverted, with the agar side at the top. The reason for this is that condensation often forms in dishes during incubation. If the agar side is at the bottom, water may drip from the lid onto the agar surface, spreading bacteria around the plate, thus preventing isolation of individual colonies. However, for some bacteria it is actually necessary that plates be incubated with the agar side down, as the extra moisture is beneficial. Be sure to consult the appropriate units of this manual for specific guidelines.

Spreading a Culture on a Plate

Many species of bacteria can be efficiently spread across the surface of an agar plate using a culture spreader (see Commonly Used Tools, above). To do this, place a small

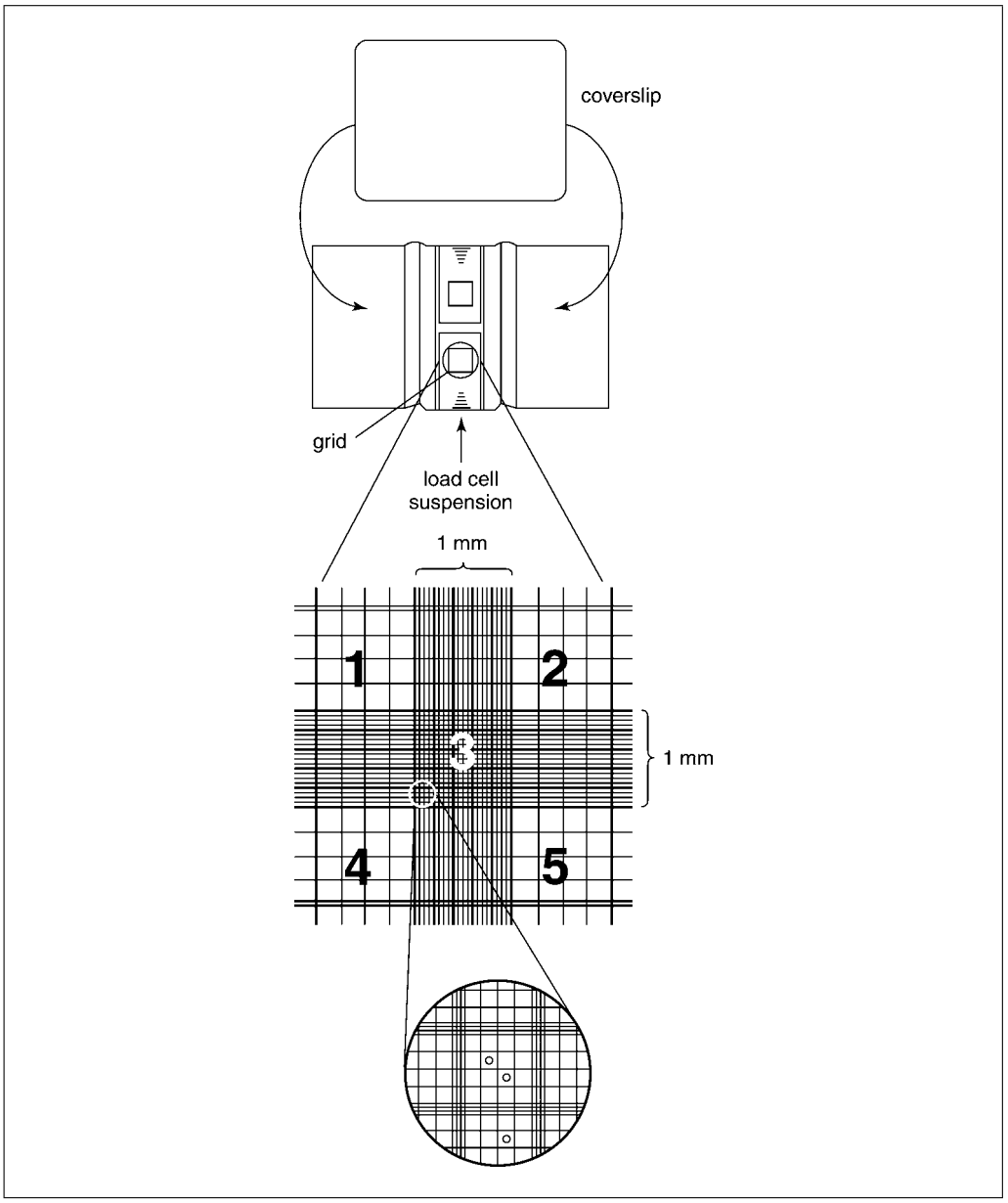

Figure A.4A.2 Example illustrating a gridded counting chamber, a hemacytometer slide (Improved Neubauer), and a coverslip. The coverslip is applied to the slide and the cell suspension is added to the counting chamber using a mechanical pipettor or a Pasteur pipet. Each counting chamber has a 3 × 3–mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added).

volume of culture (50 to 500 μl) in the middle of the agar surface. Sterilize the spreader by immersing the hooked end into 95% ethanol in a small beaker, then burn off the ethanol by passing the spreader through a Bunsen burner flame. Cool the spreader by touching it lightly to a sterile agar surface, then spread the bacterial culture uniformly around the plate. Return the spreader to the beaker with the ethanol and incubate the plate at the appropriate temperature.

Rotating "lazy Susan" devices, which spin the plate, are readily available from many sources, and can aid uniform spreading of cultures. To use such a device, place the petri dish, agar side down, in the center of the rotating device. Flame and cool the spreader, lift the lid off the dish, spin the device with a free hand, and touch the spreader to the agar surface. After a dozen or so rotations, stop the spinning by hand and replace the lid.

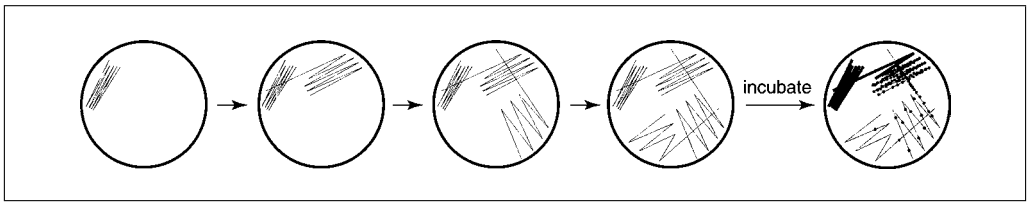

Figure A.4A.3 Proper technique for streaking bacteria on solid medium.

Streaking a Culture on a Plate

For many species of bacteria, clonal populations can be derived by streak plating. Each successive streak dilutes the previous streak, such that isolated colonies (clones) can be obtained (Fig. A.4A.3). To perform this technique, first dip the loop into liquid medium containing the organism of interest or touch the edge to a single colony growing on solid medium. Streak the culture across a small (~2- to 3-cm-long) area near one side of an agar plate. Flame the loop to sterilize. Touch the loop to an unoccupied area of the plate to cool. Lightly drag the loop *once* through the first streak, then continue to drag in a zigzag manner over a section of the agar surface. Be careful not to cross previous lines. Flame the loop again and cool by touching the agar surface. Again, drag the loop *once* through the previous streak, then continue to make a second streak. Repeat for a total of four to five streaks. Incubate plate at appropriate temperature.

Note that some bacteria, such as *Proteus* species, are very motile and will not form isolated colonies on solid medium, but will instead swarm across the entire surface. Other bacterial species will not grow on the surface of solid medium and thus cannot be cultured by this procedure. Consult the appropriate unit of this manual for information specific to the bacterium under investigation.

COMMONLY USED BACTERIAL MEDIA

Several common media used to culture *E. coli*, which are also suitable for cultivation of certain other species, are described below and in Table A.4A.1. Media are generally sterilized by autoclaving; however, certain media or supplements cannot be autoclaved and must instead be sterilized by passage through 0.22-μm filters. Refer to the appropriate units in this manual for details regarding culture medium preparation specific to each organism. *APPENDIX 2C* provides a list of media used in this manual and the corresponding units in which their formulation can be found.

For certain media, components must be sterilized independently, then combined later. Certain mixtures of salts and other compounds can yield toxic byproducts when subjected to autoclave conditions. Other components, such as sugars, are generally filter sterilized to prevent burning (caramelization) that could occur during autoclaving.

For most bacteria, deionized or distilled water is used to make culture media. For less fastidious bacterial species such as *E. coli*, tap water is satisfactory or even preferable for making culture media, as the trace minerals in such water can be beneficial.

CAUTION: Loosen caps of bottles before autoclaving, to prevent explosions.

Liquid Media

LB (Luria-Bertani) medium

10 g tryptone
5 g yeast extract
5 g NaCl
Adjust volume to 1 liter with H_2O

Table A.4A.1 Media Commonly Used for Culture of *E. coli*

Medium	Comments
LB medium	Sometimes also referred to as Luria broth or L broth; a good general-purpose medium for culturing *E. coli*
M9 minimal salts	A simple defined medium useful for selective culture of auxotrophs
SOC medium	A rich medium with high osmolarity; ideal for the recovery step following transformation of *E. coli* and similar bacteria
Terrific broth (TB)	*E. coli* grows rapidly in this rich medium: a very small inoculum in 100 ml will grow to stationary phase overnight, making it a good choice for growing bacteria for plasmid preparations

Sterilize by autoclaving
Store indefinitely at room temperature

Some researchers adjust the pH to ~7 by titrating with 1N NaOH, but this is not necessary.

M9 minimal salts, 5×

30 g Na_2HPO_4
15 g KH_2PO_4
5 g NH_4Cl
2.5 g NaCl
15 mg $CaCl_2$ (optional)
Adjust volume to 1 liter with H_2O
Add ~50 ml chloroform to 5× stock solution as a preservative
Store 5× concentrate up to many months at 4°C

Just before use, dilute 1:5 with water, and sterilize by autoclaving. Cool to <50°C and add the following:

1 ml 1 M $MgSO_4$: filter sterilize; store indefinitely at room temperature
10 ml 20% carbon source (e.g., glucose, lactose, glycerol): filter sterilize; store indefinitely at room temperature
Store indefinitely at room temperature

The chloroform added to the concentrated medium separates into an organic layer at the bottom of the bottle. Be careful not to transfer any of the chloroform when diluting 5× concentrated stock.

SOC medium

20 g Bacto tryptone
5 g Bacto yeast extract
10 ml 1 M NaCl
2.5 ml 1 M KCl
Adjust volume to 980 ml with H_2O
Sterilize by autoclaving
Cool to <50°C and add the following:
10 ml 2 M $MgCl_2$
20 ml 20% (w/v) glucose
Store indefinitely at room temperature

TB (Terrific broth)

12 g Bacto tryptone
24 g Bacto yeast extract
4 ml glycerol

Adjust volume to 900 ml with H_2O
Dispense 90-ml aliquots into screw-cap bottles and autoclave
Store indefinitely at room temperature
Just prior use, add 10 ml TB-potassium salts (see recipe) to each bottle containing a 90-ml aliquot

TB potassium salts

125.5 g K_2HPO_4
23 g KH_2PO_4
Dissolve in 800 ml H_2O, then adjust to a final volume to 1 liter with H_2O
Dispense 100-ml aliquots into screw-cap bottles and autoclave
Store indefinitely at room temperature

Preparing Plates

Media can generally be solidified by addition of 1.5% (w/v) agar or agarose prior to autoclaving. It is easiest to dissolve all other ingredients as described in the appropriate recipes (see, e.g., Liquid Media, above) and then add the agar or agarose immediately before autoclaving, as the autoclaving process will melt the solidifying agent. After autoclaving, swirl gently to evenly disperse ingredients. If solid medium is to be used in plates, cool the medium to ~50°C in a water bath before pouring as this will prevent melting of plastic petri dishes, permit easier handling of the container flask, and reduce condensation in the solidified plates. Dry off any residual water on the outside of the flask before pouring to avoid contamination; briefly passing the lip of the flask through a Bunsen burner flame between plate pours can also help prevent introduction of contaminants. Pour 20 to 25 ml medium per 100-mm diameter petri dish. Swirl plates, if necessary, to cover the entire dish. Bubbles on the medium surface can be removed by quickly passing a burner flame over the surface prior to solidification. Solidified plates should be left at room temperature for 1 to 2 days to allow evaporation of excess moisture; this will reduce condensation during subsequent culture incubations. Alternatively, plates can be dried by incubating at 37°C with the agar side on the bottom for ~30 min with the lids ajar. A perfect plate for growth of *E. coli* has a slightly rippled surface (also see Positioning Plates within the Incubator). Plates can be stored almost indefinitely at 4°C if wrapped in plastic to prevent desiccation.

Table A.4A.2 Antibiotic Stock Solutions for Use with *E. coli* Cultures

Antibiotic	Stock concentration	Working concentration for *E. coli*
Ampicillin[a] (Na salt)	10 mg/ml in H_2O	50 μg/ml
Carbenicillin[a]	10 mg/ml in H_2O	50 μg/ml
Chloramphenicol	6 mg/ml in 100% ethanol	30 μg/ml
Gentamicin	3 mg/ml in H_2O	15 μg/ml
Kanamycin	10 mg/ml in H_2O	50 μg/ml
Rifampicin	20 mg/ml in 100% methanol	100 μg/ml
Spectinomycin	20 mg/ml in H_2O	100 μg/ml
Streptomycin	10 mg/ml in H_2O	50 μg/ml
Tetracycline·HCl	2.5 mg/ml in 50% ethanol	12.5 μg/ml

[a]Carbenicillin is more stable than ampicillin.

If not to be used immediately for pouring plates, solid media can be stored indefinitely at room temperature in tightly capped bottles. To use, loosen cap, melt by autoclaving or by heating in a microwave oven at a high setting (microwave oven times vary according to oven and volume of medium, and must be determined empirically), then proceed as described above.

Antibiotic Supplements for Media

To prevent destruction by excess heat, antibiotics should be added only after the medium has cooled to <50°C. If solid medium is being prepared, cool in a 50°C water bath, add antibiotic(s), then pour plates.

Aqueous antibiotics should be sterilized by passage through 0.22-μm filters. Antibiotics dissolved in ethanol solutions do not require additional sterilization.

For simplicity of use, concentrated stock solutions of each antibiotic should be prepared (Table A.4A.2). Concentrated solutions should be kept no more than 1 month at 4°C or 6 months at −20°C. Media containing antibiotics should be kept for no more than 1 month. Protect media containing light-sensitive antibiotics (e.g., tetracycline) from light by wrapping in aluminum foil or storing in a dark place.

Note that other bacteria/species are often more or less sensitive than *E. coli* to some antibiotics. Refer to appropriate units in this manual for antibiotic requirements when working with other bacteria.

Contributed by Brian Stevenson
University of Kentucky College of Medicine
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Isolation and Culture of Human Umbilical Vein Endothelial Cells (HUVEC)

Cultured human endothelial cells have provided great insights into the biology of coagulation and endothelial activation in various disease states including inflammatory disorders, atherosclerosis, and cancers (Nachman and Jaffe, 2004). However, recent studies suggest that the endothelial cells are also the targets for microbial adhesion and invasion. In some cases, the adhesion is direct (Prasadarao et al., 2003), while in others it may be mediated via bridging molecules such as fibronectin (Peacock et al., 1999; Massey et al., 2001) or fibrinogen (Cheung et al., 1991; Bayer et al., 1995). Activation of endothelial cells would ensue as a result of bacterial adhesion (Grimminger et al., 1997). In some cases, invasion of endothelial cells may ensue after adhesion (Drevets et al., 1995; Prasadarao et al., 1996; Menzies and Kourteva, 1998). Cultured HUVEC have also been used to study viral and fungal infections (Sahni, 2006). Many of these studies on microbe-endothelial cell interaction are conducted with endothelial cells isolated and cultured from source tissue that yields a relatively pure population of cells. The authenticity of these cells can be verified by the presence of a immunohistochemical and functional marker such as von Willebrand factor (Jaffe et al., 1974). Among host tissues, human umbilical vein is by far the most common source of cultured human endothelial cells (Jaffe et al., 1973a; Folkman et al., 1979; Kubota et al., 1988; Fukuda et al., 1989). Due to the success of this procedure, studies on the interaction of microbes with human endothelial cells have received increasing attention. These studies are now the subject of intensive investigation in the field of bacterial pathogenesis and cell biology.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

NOTE: Institutional Review Board (IRB) approval is often required for permission to use human umbilical vein as a source for harvest of HUVEC.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 5% CO_2 humidified incubator.

HARVEST AND CULTURE OF HUVEC

BASIC PROTOCOL

The following protocol is a general HUVEC isolation and propagation procedure. The resulting HUVEC may be used in a variety of assays including those for *S. aureus* (*UNIT 9C.4*). After obtaining appropriate IRB approval, the umbilical cords that are normally discarded after normal vaginal deliveries of newborns provide the source for the HUVEC.

Materials

Human umbilical cord (cords held for more than 3 hr should be discarded)
HEPES-buffered saline (HBS; see recipe), 37°C
1× (0.1% w/v) collagenase, type I (lyophilized; 150 to 200 U/mg; Invitrogen); store in 10-ml aliquots at –20°C
M199 medium (Bio-Whittaker) supplemented with 20% (v/v) FBS
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Complete HUVEC culture medium/20% FBS (see recipe)
0.02% (w/v) EDTA/0.5% (w/v) bovine serum albumin

Contributed by Ambrose L. Cheung
Current Protocols in Microbiology (2007) A.4B.1-A.4B.8

400-ml beakers
Gauze
Nylon tie, 2–0 size, 6-in. (15-cm) length
Cannulas: blunt, hubless 10-G needles
Hemostat
1-, 30- and 50-ml syringes
10-G needles, blunt and hubless
No. 10 surgical blade
Surgical gloves
Polyethylene tubing, 1/8 in. (0.3 cm) outer diameter
50-ml conical polypropylene centrifuge tubes (e.g., Falcon 2070)
Tabletop centrifuge
25-cm^2 gelatin-coated tissue culture flasks (see recipe) and other gelatin-coated tissue culture vessels as needed for passaging

Additional reagents and equipment for phase-contrast microscopy (*UNIT 2A.1*) and cryopreservation of HUVEC (Support Protocol)

NOTE: Utilize aseptic technique in all manipulations of human umbilical cords.

Prepare umbilical cord and assemble materials

1. Place the severed umbilical cord in a sterile 400-ml beaker containing ~200 ml HBS, at 37°C.

2. Place the following sterile items in a sterile tray:

   Gauze
   6-in., 2–0 nylon tie
   Two cannulas (blunt, hubless 10-G needles)
   One hemostat
   Two 50-ml syringes
   One 30-ml syringe
   One no. 10 surgical blade.

3. Pour 150 ml HBS into a beaker. Put on sterile surgical gloves and fill two 50-ml syringes and one 30-ml syringe with HBS from the beaker.

4. Using a hemostat, remove the cord from the container. Wipe clean with sterile gauze. Cut away any clamp marks on the cord.

Obtain HUVEC from umbilical cord

5. Cannulate the umbilical vein (identifiable by the darker venous blood in the blood vessel) with a blunt 10-G needle, secure with a nylon tie, attach the 10-G needle to one of the 50-ml syringes prepared in step 3, and perfuse with 50 ml of HBS twice to wash out the blood.

6. Remove the cannula and repeat this step with the other end of the cord. Remove the second cannula.

7. Cannulate one end of the umbilical vein with a blunt, hubless 10-G needle and attach to a 4-cm length of polyethylene tubing (1/8 in. o.d.).

8. Infuse 10 ml of 0.1% collagenase in HBS (thawed at 37°C) into the umbilical vein, clamp the other end of the umbilical cord shut with a hemostat, place in a 400-ml beaker with ~200 ml PBS at 37°C, and incubate 10 min in a 37°C water bath.

9. Briefly massage the clamped umbilical cord, using a gloved hand, to loosen up the cells. Release the distal hemostat. Flush the collagenase solution from the

Table A.4B.1 Splitting of HUVEC

Starting vessel with confluent HUVEC	Split into
One 25-cm^2 flask	Three 25-cm^2 flasks *or* one 75-cm^2 flask
One 75-cm^2 flask	Three 75-cm^2 flasks
One 35-mm dish	Three 35-mm dishes *or* one 25-cm^2 flask
Three 75-cm^2 flasks	One 850-cm^2 roller bottle

cord with the 30 ml HBS in a syringe (prepared at step 3), and collect the liquid that is flushed out in a 50-ml conical polypropylene tube containing 10 ml M199 medium/20% FBS.

10. Sediment the cells by centrifuging 10 min at 250 × *g*, room temperature. Decant supernatant and resuspend pellet in 4 ml complete HUVEC culture medium/20% FBS.

Culture HUVEC

11. Plate the 4 ml of medium with the cells (from step 10) in a 25-cm^2 flask coated with gelatin.

12. Incubate the flasks, feeding the cells twice a week, each time with a complete change of culture medium, until they reach confluence (which usually takes 4 to 5 days).

Passage HUVEC in culture

13. Upon confluence, wash adherent cell layer gently with 4 ml HBS, and remove buffer.

14. Prepare a 1:1 (v/v) mixture of 0.1% collagenase and 0.02% EDTA/0.5% BSA. Add 2 ml of this solution to the cells and incubate 5 min at 37°C.

15. Add 2 ml complete culture medium/20% FBS, rinse cells off side of flask several times, and transfer cells to a sterile 50-ml conical centrifuge tube.

16. Centrifuge 5 to 10 min at 250 × *g*, room temperature. Remove supernatant, resuspend cells in culture medium, and distribute into gelatin-coated tissue culture vessels as directed in Table A.4B.1 and Table A.4B.2.

Table A.4B.2 Cell Feeding[a]

Vessel	Required volume medium
25-cm^2 flask	4 ml
75-cm^2 flask	10 ml
96–well microtiter plate	100 µl[b]
16-mm dish	800 µl
35-mm dish	2 ml
60-mm dish	8 ml
850-cm^2 roller bottle	30 ml

[a]Feed cells twice per week.
[b]Per well.

Table A.4B.3 Anticipated Yield of HUVEC Cells

Vessel	Cells plated	Cell yield
96-well plate[a]	$0.5–2 \times 10^4$	$2–5 \times 10^4$
24-well plate[a]	$4–6 \times 10^4$	$1–3 \times 10^5$
6-well plate[a]	$0.7–2 \times 10^5$	$3–6 \times 10^5$
25-cm^2 flask	$4–8 \times 10^5$	$1–2 \times 10^6$

[a]Cell numbers listed are on a per-well basis.

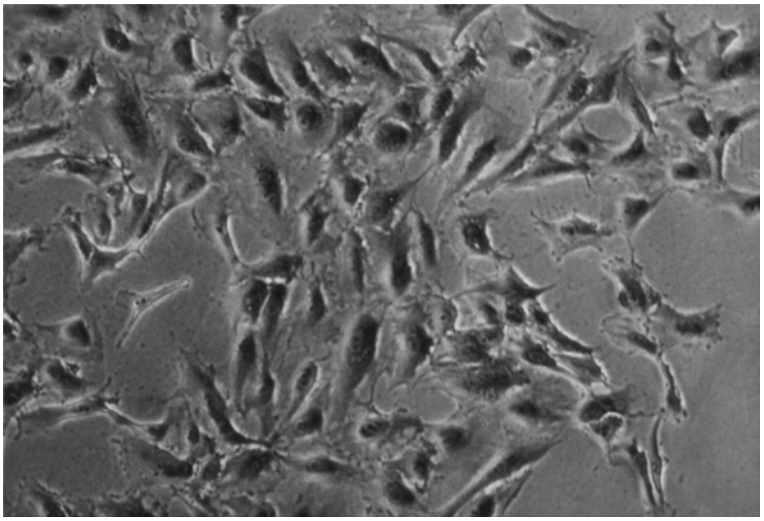

Figure A.4B.1 Culture of human umbilical vein endothelial cells 2 days after passaging. Passage-3 cells at ~70% confluence were fixed with Diff-Quik Fix and stained with Diff-Quik Solution II (Baxter). Cells were viewed using a Zeiss inverted microscope. Originally published in Kleinman and Cid (1998).

17. Perform experiments, generally, with second to fourth-passage cells that have reached confluence as determined by phase-contrast microscopy (*UNIT 2A.1*). If required, cryopreserve HUVEC as in the Support Protocol.

Refer to Tables A.4B.2 and A.4B.3 for more information.

Figure A.4B.1 demonstrates passage-3 cells. Results obtained with HUVEC beyond the fifth passage can be less consistent

SUPPORT PROTOCOL

CRYOPRESERVATION AND THAWING OF HUVEC

Harvested HUVEC can be stored in liquid nitrogen for future propagation, if necessary. In many cases, HUVEC stored in this manner can be retrieved stably even after 7 years of storage.

Additional Materials *(also see Basic Protocol)*

Confluent primary HUVEC growing in 25-cm^2 tissue culture flasks (Basic Protocol)
M199 medium (Bio-Whittaker) supplemented with 20% (v/v) FBS
92.5% (v/v) complete culture medium (containing 20% FBS; see recipe)/7.5% (v/v) DMSO
100% ethanol

50-ml conical polypropylene centrifuge tubes
1.22-ml sterile cryogenic vials (Nalgene)
Cryogenic workstation (Corning)
Liquid nitrogen cryogenic storage system (e.g., Fisher)
15-ml conical polypropylene centrifuge tubes
75-cm^2 tissue culture flasks (Corning), gelatin-coated (see recipe)

1. Prepare a 1:1 (v/v) mixture of 0.1% collagenase and 0.02% EDTA/0.5% BSA. Remove the medium from a 25-cm^2 flask of confluent primary HUVEC and replace with 1 ml of this mixture. Incubate at 37°C for 5 min.
2. Add 2 ml M199 medium/20% FBS to the flask with confluent HUVEC. Rinse cells off side of flask several times and transfer the cell suspension to a sterile 50-ml conical centrifuge tube.
3. Centrifuge 5 to 10 min at 250 × *g*, room temperature. Remove supernatant, resuspend cells in 1 ml 92.5% complete M199 culture/20% FBS7.5% DMSO, and transfer to a 1.2-ml cryogenic vial.
4. Place the cryogenic vial in a cryogenic workstation and transfer the workstation to a −80°C freezer for overnight storage.
5. Transfer the cryogenic vial from the −80°C freezer to a liquid nitrogen cryogenic storage system for prolonged storage.
6. To retrieve the HUVEC from storage, thaw the cryogenic vial in a 37°C water bath for 1 min, then sterilize by submerging the vial briefly in 100% ethanol.
7. Open the vial in a tissue culture hood, transfer the contents to a sterile 15-ml conical centrifuge tube, and add 2 ml complete culture medium/20% FBS.
8. Centrifuge 5 min at 250 × *g*, room temperature. Discard the supernatant and add 10 ml complete culture medium/20% FBS. Transfer the cell suspension to a gelatin-coated 75-cm^2 flask for propagation as described in the Basic Protocol.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Gelatin-coated tissue culture vessels

Add warmed filter-sterilized 2% (w/v) gelatin (J.T. Baker) to the tissue culture vessels in the following quantities:

7 ml for 75-cm^2 flask
3 ml for 25-cm^2 flask
1 ml for 35-mm dish

Let stand overnight at 4°C, then remove the gelatin solution immediately before use.

HEPES-buffered saline (HBS)

11× stock solution:
1.5 M NaCl
44 mM KCl
110 mM HEPES
122 mM glucose
Store at 4°C (stable over 1 year)
1× working solution:

Dilute 11× stock by adding 50 ml of the stock solution to 500 ml water. Store diluted working solution up to 1 year at 4°C.

Final concentration of 1× working solution: 137 mM NaCl, 4 mM KCl, 10 mM HEPES, and 11.1 mM glucose.

HUVEC culture medium, complete, containing 20% FBS

M199 medium (Bio-Whittaker) supplemented with:

20% (v/v) FBS
50 μg/ml endothelial cell growth factor (Sigma) or endothelial mitogen (Biomedical Technologies; *http://www.btiinc.com*); add from 100× stock solution (stored up to 1 month at −20°C)
50 μg/ml heparin (sodium salt, grade 1-A from porcine intestinal mucosa; Sigma); add from 100× stock (stored up to 3 months at 4°C)
2 mM L-glutamine (Mediatech); add from 200 mM stock (stored up to ≥1 year at −20°C)
100 U/ml penicillin
100 μg/ml streptomycin
2 μg/ml amphotericin
Store up to 2 months at 4°C

The antibiotics are added as a 100× combined solution of penicillin, streptomycin, and amphotericin (Bio-Whittaker).

COMMENTARY

Background Information

Under normal conditions, the vascular endothelium maintains an anticoagulant, anti-inflammatory, and antithrombotic barrier between blood and tissue. Because of their strategic location at the interface of blood and tissue, endothelial cells are capable of responding to a variety of pathogens circulating in the bloodstream. Endothelial cells are usually activated during sepsis, presumably due to direct interactions with bacteria or bacterial components such as lipopolysaccharide (LPS) and toxins, or indirect interactions via host mediators such as cytokines and chemokines (Sahni, 2006). Interaction of the vascular endothelium with bacterial pathogens, therefore, is a common phenomenon that likely plays an important role in defining the host response, resultant pathological sequelae, and disease pathogenesis.

Included among pathogenic bacteria that are known to target endothelial cells are obligate intracellular organisms belonging to *Rickettsia* and *Chlamydia* species and other bacterial species such as *Bartonella*, *Listeria, Nisseria*, Group A, Group B and oral *viridans Streptococci* and *Staphylococci* (Sahni, 2006). Clinical and experimental evidence from well characterized animal models of infections clearly implicates vascular endothelia as a primary target of infection for many bacteria. An important step in the pathogenesis is the translocation of circulating bacteria across the endothelium to tissues. This interaction has attracted significant interest as determinants of host defense and pathogenic mechanisms.

The signaling pathways and homeostatic mechanism affected by infection of vascular endothelial cells have been studied with cultured vascular endothelial cells. Jaffe et al. first isolated endothelial cells derived from human umbilical veins in 1973 (Jaffe et al., 1973a). The criterion that has been used to identify the correct cell type is the expression of von Willebrand factor antigen (Jaffe et al., 1973a,b). Human umbilical vein is an ideal source because it is easy to obtain, grows well in pure culture, and is available in abundant quantities. A disadvantage to this procedure is the cost of the medium (e.g., FBS, endothelial cell growth factor) needed to propagate cells. Nonetheless, the ease with which pure cultures of endothelial cells can be obtained with this method has ushered in the rapid growth of studies in microbe-endothelium interactions.

Critical Parameters and Troubleshooting

Most of the studies with HUVEC have been conducted with cells from passage 2 to 4. In general, cells should not be used beyond

passage 5, due to changes in the phenotype. Besides umbilical cords, other blood vessel and tissue sources have also yielded endothelial cells for culture (Folkman et al., 1979; Kubota et al., 1988; Fukuda et al., 1989); however, it should be stressed that there is significant endothelial cell diversity among different sites in the vascular tree (Chi et al., 2003). In particular, large differences in the genotype of large-vessel and microvascular endothelium have been found to exist (Chi et al., 2003). It is also clear that the tissue microenvironment surrounding blood vessels affects endothelial cell phenotype. Recent proteomic studies revealing unique endothelial cell proteins in specific tissues have also lent support to this notion (Oh et al., 2004). Accordingly, the choice of HUVEC to study vascular endothelium biology in different tissues must be interpreted with appropriate caveats.

Anticipated Results

The harvest of endothelial cells from human umbilical vein has become a standard and well accepted technique for acquisition of HUVEC. Provided that sterile techniques are strictly observed and the umbilical cord is fresh (less than 3 hr old), success in acquisition and propagation of HUVEC is expected. The yield of HUVEC as described in Table A.4B.3 is highly achievable.

Time Considerations

It generally takes 4 to 5 days for the HUVEC to propagate from the harvest of umbilical cord endothelial cells to confluence in a T25 flask. The cells can then be passaged every 3 to 4 days upon confluence. Once confluence from the 2nd to 4th passage is reached, the experiment can be performed. In some cases, the experiments can also be performed with the 5th-passage cells. Cells beyond the 6th passage should be discarded.

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Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)

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ABSTRACT

In this appendix, several basic methods are described for preparation of primary B lymphocyte. *Curr. Protoc. Microbiol.* 6:A.4C.1-A.4C.9. © 2007 by John Wiley & Sons, Inc.

Keywords: B lymphocyte • sheep blood cell • T-cell rosetting • CD19

INTRODUCTION

In the protocol for isolating total white blood cells, red blood cells are usually present in the white blood cells. These red blood cells need to be removed as they can otherwise interfere with further experiments in that they can lyse, resulting in binding and sequestering the viral particles nonspecifically hindering infection. This can be achieved by incubating for 5 min with 1 ml NH_4Cl/Tris (0.16 M NH_4Cl, 0.17 M Tris·Cl, pH 7.65) at 37°C. The buffer has been developed for the preferential lysis of red blood cells from human whole blood. This process can be repeated two or three times until the red cells are completely removed.

CAUTION: Follow all appropriate guidelines and regulations for the use and handling of human-derived materials. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

ISOLATION OF MONONUCLEAR CELLS BY FICOLL-HYPAQUE GRADIENT CENTRIFUGATION

BASIC PROTOCOL 1

Peripheral blood is the primary source of lymphoid cells for investigations of the human immune system. This protocol is a simple and rapid method of purifying peripheral blood mononuclear cells (PBMC) that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have a lower density; in contrast, red blood cells (RBC) and granulocytes have a higher density than Ficoll-Hypaque and collect at the bottom of the Ficoll-Hypaque layer. Platelets are separated from the mononuclear cells by subsequent washing or by centrifugation through a fetal bovine serum (FBS) cushion gradient that allows penetration of mononuclear cells but not platelets.

Materials

1000 U/ml heparin
Ficoll or Lymphoprep solution (Amersham Biosciences)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
RPMI-1640 supplemented with 2 mM glutamine and 5% FBS
Alcohol swabs
60-ml syringe
Tourniquet
18-G needle

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Vacutainer blood collection system
50-ml centrifuge tube
10-ml sterile pipet

IMPORTANT NOTE: Blood should be obtained from the patient by an experienced phlebotomist or a licensed physician. IRB approval is necessary to obtain blood from a healthy volunteer with an approved consent form.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique must be used accordingly.

NOTE: Figure A.4C.1 demonstrates some of the needed materials for this procedure.

1. Sterilize the skin area where the needle is to be inserted with alcohol swabs. Place a tourniquet around the upper arm and draw peripheral blood into a 60-ml syringe through an 18-G needle containing 3 ml heparin.
2. Add 20 ml Ficoll separating solution to a 50-ml centrifuge tube.
3. Mix the blood sample with PBS at a ratio of 1:1.
4. Carefully layer above the Ficoll separating solution with the same volume of diluted blood sample avoiding any mixture.
5. Centrifuge 30 min at 1200 × *g*, room temperature.

   Start with the slowest acceleration ramp and switch off the brake!
6. Transfer the lymphocyte-containing band into a new centrifuge tube using a 10-ml sterile pipet (Fig. A.4C.2).

   The washing steps described above usually remove most of the platelets from mononuclear cell suspension. There are certain disease states associated with increased platelet concentrations (mononuclear cell to platelet cell ratio >10:1) in the peripheral blood, and additional steps are needed to remove the extra platelets in these cases. Add 3 ml FBS to a centrifuge tube for each milliliter of mononuclear cells. Layer the cell suspension (1 to 2 × 10^7 cells/ml) over the FBS (alternatively, carefully layer the FBS under the cell suspension, which will rise as FBS is added). Centrifuge 15 min at 800 rpm (200 × g), 18° to 20°C. Discard the supernatant containing the platelets. Resuspend cell pellet in complete RPMI-1640 containing 5% FBS and proceed.

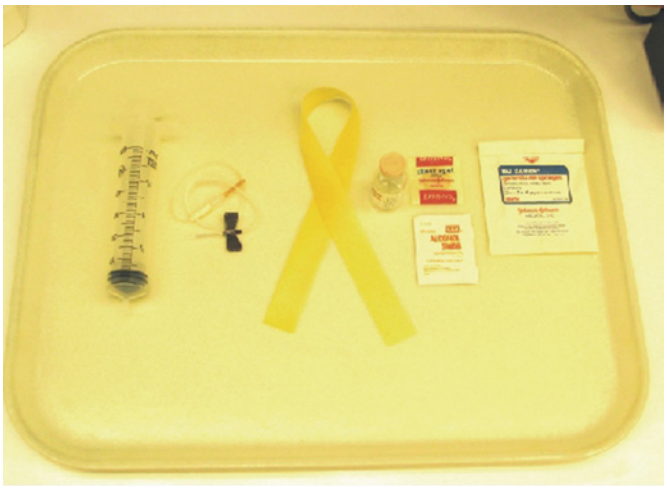

Figure A.4C.1 Components including syringe, vacutainer, tourniquet, heparin, alcohol swab, band aid, and gauze for bloodletting.

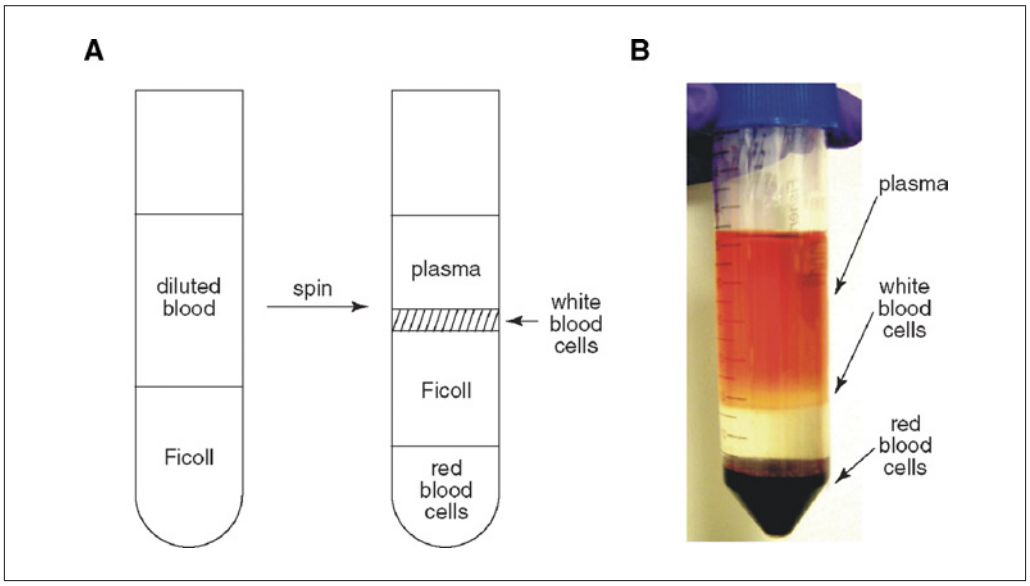

Figure A.4C.2 (**A**) Scheme showing blood separation by Ficoll gradient. (**B**) An actual separation of blood after Ficoll gradient centrifuge.

7. Wash the total lymphocyte population with RPMI 1640 containing 5% FBS three times. Between washes, to pellet down the cells, centrifuge 10 min at 1200 × *g*, room temperature.
8. Dilute the lymphocyte pellet in RPMI 1640 to 10^7 cells/ml and briefly spin the cells 5 min at 200 × *g*, room temperature.

POSITIVE SELECTION OF B LYMPHOCYTES FROM PBMCs USING CD19$^+$ MICROBEADS

BASIC PROTOCOL 2

B lymphocytes can be separated from cells of other hematopoietic lineages either by positive selection or by negative selection (Strelkauskas et al., 1975; Fong et al., 1981). CD19 is a good marker to use in this process because it is differentially expressed at very high levels on B cells compared to other hematopoietic lineages. Positive selection, as the name suggests, separates cells expressing a particular marker from other cells that do not express that marker. Accordingly, an antibody specific for that particular marker is used in this protocol. In negative selection, cells that do not express an array of different markers are separated from cells that express one or more of these markers. In this setup, a mixture of antibodies against all these markers is used. The advantage of positive selection is its simplicity and relatively lower cost in antibodies compared to negative selection. Compared to negative selection, which results in enrichment of the target cells, positive selection yields a highly pure population that can be verified by fluorescence-activated cell sorting (FACS) and/or immunocytochemistry. Alternatively, for an extremely pure population, cells could be enriched using negative selection followed by purification using positive selection. Magnetic microbead-based positive selection protocols are popular, because they do not require the use of expensive laboratory equipment and a large number of cells can be purified in a relatively short time (Fig. A.4C.3).

Materials

Staining buffer (see recipe), ice-cold
Anti-CD19 antibody: Purified mouse anti–human CD19 antibody (BD Pharmingen)
Human Fcγ-receptor blocking antibody
Paramagnetic microbeads conjugated to appropriate secondary antibody
30-μm nylon mesh

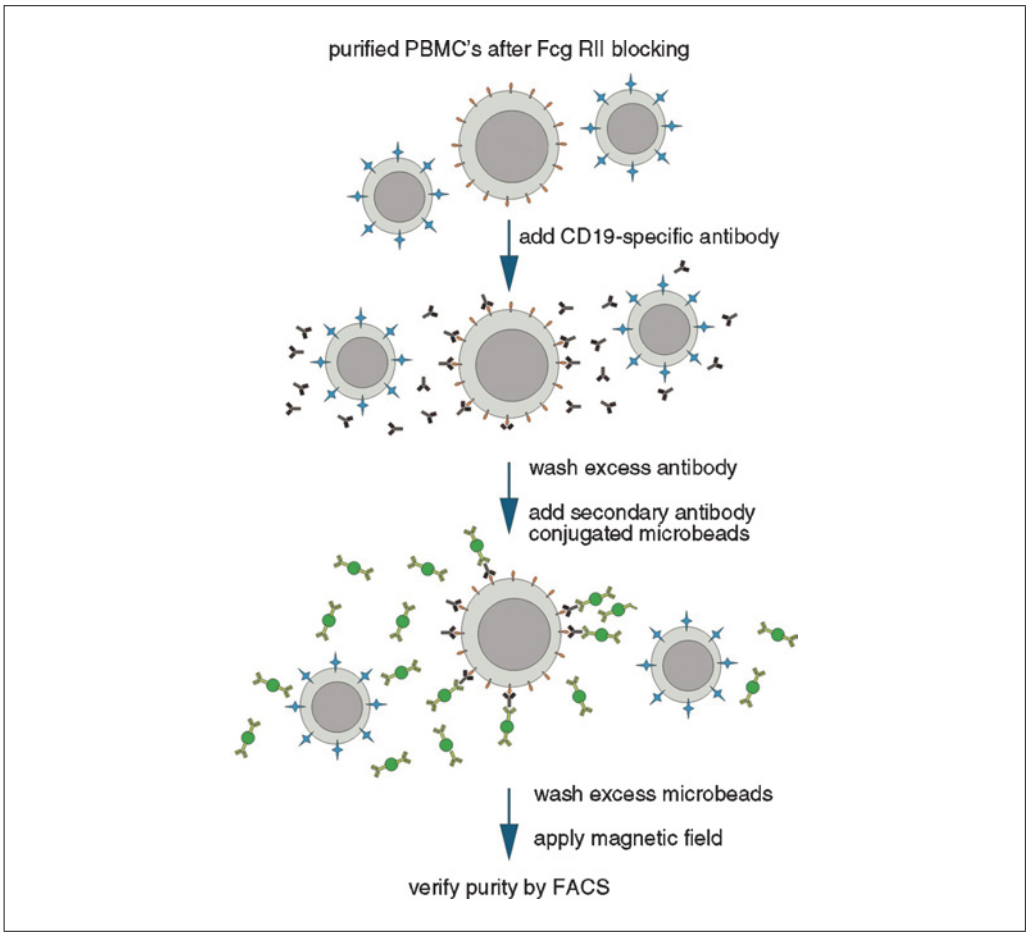

Figure A.4C.3 Schematic showing various steps in positive selection of $CD19^+$ B lymphocytes.

Separation tube
Powerful magnet
Fluorescent-activated cell sorter (FACS)

Additional reagents and equipment for isolating lymphocytes from whole blood (see Basic Protocol 1) and counting cells using a hemacytometer (*APPENDIX 4A*).

Prepare lymphocytes

1. Isolate lymphocytes from whole blood (see Basic Protocol 1).
2. Wash cells once in ice-cold staining buffer.
3. Centrifuge 10 min at 300 × *g*, 4°C to pellet.
4. Count cells in a hemacytometer (*APPENDIX 4A*) and allocate 10^7 cells into a microtube. Centrifuge 10 min at 300 × *g*, 4°C to pellet cells and resuspend cells in 100 µl staining buffer.
5. Filter cells through a 30-µm nylon mesh to get rid of clumps that could result in an impure population.

Add blocking antibody

6. Add Human Fcγ-receptor blocking antibody.

   This step is important to prevent the CD19 antibody from sticking to cells of other hematopoietic lineages via the Fcγ-receptor.

7. Mix well and incubate for 10 min on ice.

8. Wash once with staining buffer and resuspend in 100 μl staining buffer containing anti-CD19 antibody.
9. Mix well and incubate for 10 min on ice for.
10. Wash cells 2 to 3 times in ice-cold staining buffer (use at least 10× the labeling volume).
11. Resuspend cells in 100 μl staining buffer.

Add covalently conjugated microbeads

12. Add paramagnetic microbeads covalently conjugated to appropriate secondary antibody (20 to 80 beads per cell results in an ideal recovery).
13. Mix well and incubate 15 min on ice. Swirl gently every 5 min.

    It is critical to incubate on ice to prevent endocytosis of beads.

14. Wash cells once in ice-cold staining buffer (use at least 10× the labeling volume).
15. Centrifuge 10 min at 300 × *g*, 4°C to pellet.
16. Resuspend cells in 1 ml staining buffer.
17. Place tube horizontally on a magnet (tube is placed horizontally to prevent gravity contamination of the purified population).

    Be sure to keep the separation tube attached to the magnet until step 21.

18. Allow cells attached to paramagnetic microbeads to separate out for 10 min.
19. Discard supernatant (or pellet and save for negative selection).
20. Carefully wash microbead/cell pellet with appropriate medium.
21. Remove magnet and resuspend in appropriate medium.

    Purified cells can be used straight for culturing and/or other downstream application as the magnetic particles are nontoxic and biodegradable. Alternatively, the cells can be trypsinized to remove the attached microbeads.

22. Check for purity using FACS.

RAPID ONE-STEP IMMUNOMAGNETIC SEPARATION OF B LYMPHOCYTES FROM PBMC

BASIC PROTOCOL 3

This protocol is simpler, and can be used if CD19 is the only antigen for positive selection. Basic Protocol 2 is slightly more laborious, but it is more versatile. The same reagents, with the exception of the primary antibody, can be used for positive selection of cells expressing an array of different antigens. If working primarily with one antigen, however, this protocol has the advantage of less noise due to one less antibody incubation.

Materials

Staining buffer (see recipe)
30-μm nylon mesh
Microbeads mix (Miltenyi Biotech GmbH)
Magnetic separator (Miltenyi Biotech GmbH)
Magnetic separation column (Miltenyi Biotech GmbH)
Plunger (Miltenyi Biotech GmbH)
Fluorescence-activated cell sorter (FACS)

Additional reagents and equipment for isolating lymphocytes from whole blood (see Basic Protocol 1)

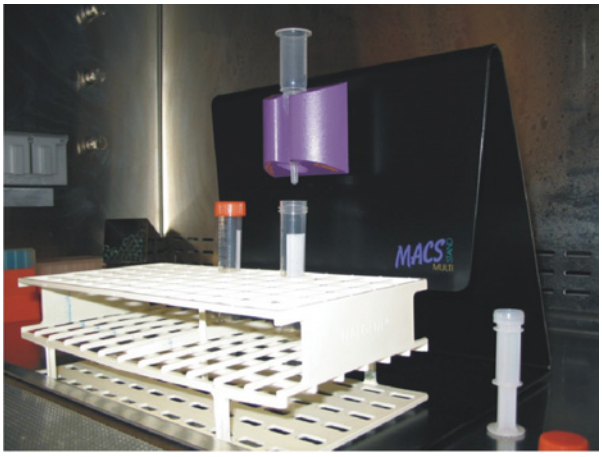

Figure A.4C.4 Picture showing the assembled Miltenyi MACS positive-selection apparatus. The column is vertically mounted in a strong magnet.

NOTE: Figure A.4C.4 shows the assembled MACS positive-selection apparatus.

Prepare lymphocytes

1. Isolate lymphocytes from whole blood (Basic Protocol 1).
2. Wash cells once in ice-cold staining buffer.
3. Centrifuge 10 min at 300 × *g*, 4°C to pellet.
4. Resuspend 10^7-10^8 cells in 800 µl staining buffer.
5. Filter cells through a 30-µm nylon mesh to get rid of clumps that could result in an impure population.

Label cells with microbeads mix

6. Label cells by adding 200 µl microbeads mix (microbeads are directly conjugated to anti-CD19 antibody).
7. Mix well and incubate for 15 min on ice.
8. Wash cells in ice-cold staining buffer (use at least 10× the labeling volume).
9. Resuspend in 1 ml of staining buffer.
10. Place a magnetic separation column in the magnetic separator.
11. Rinse column with 3 ml staining buffer.

Attach labeled cells to the column

12. Add cell suspension to column to attach the labeled cells to the column.
13. Wash three times with 3 ml of staining buffer.
14. Remove column from magnet, place a fresh collection tube below the column and remove attached cells with the plunger.

    If required, cells can be separated from micro-beads, by incubation with 60 µl release reagent (10 min, on ice; Miltenyi Biotec GmbH) and then passing through another magnetic separation column in the magnetic separator. This time the target cells are collected in the effluent, and the unassociated magnetic beads could stick to the column walls.

15. Check for purity using FACS.

ENRICHMENT OF B CELLS FROM PBMC BY REMOVAL OF T CELLS USING 2-AMINOETHYLISOTHIOURONIUM BROMIDE (AET)–TREATED SHEEP RED BLOOD CELL ROSETTING

Following preparation of PBMC using Ficoll-Hypaque density gradient separation of heparinized human blood (see Basic Protocol 1), B lymphocytes need to be enriched for EBV infection. Here the authors describe a simple and efficient method that depletes the T cell population using a rosetting technique to remove T cells. The method exploits the unique ability of cells to bind to and form rosettes with sheep red blood cells (SRBC). Treatment of SRBC with AET prior to missing them with PBMC enhances their ability to form rosettes with T cells (Madsen and Johnsen, 1979).

Materials

Sheep red blood cells (SRBC; usually purchased from Biowhittaker)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Peripheral blood mononuclear cells (PBMC) in complete RPMI-10 medium (see Basic Protocol 1)
RPMI-1640 supplemented with 2 mM glutamine and 5% FBS
0.14 M 2-aminoethylisothirouroniumuronium hydrobromide solution (AET; see recipe)
Tris-buffered NH_4Cl, pH 7.5
Fetal bovine serum (FBS; *APPENDIX 2A*)

Centrifuge with a swinging-bucket rotor
37°C water bath
5-ml pipet, sterile

Additional reagents and solutions for counting cells (*APPENDIX 4A*)

NOTE: Prepare AET treated SRBC in a sterile manner.

NOTE: To prepare a large amount of AET treated SRBC, increase the number of tubes in step one.

Prepare AET-treated SRBC

1. In a 50-ml centrifuge tube wash 15 to 20 ml SRBC with 15 ml PBS three times. After each wash centrifuge 10 min at 300 × *g*, 22°C.
2. Transfer SRBC to a 15-ml centrifuge tube and wash a fourth time with 15 ml PBS and spin 10 min at 300 × *g* (1800 rpm in a swinging-bucket rotor), 22°C.
3. Remove the top layer above the packed SRBC after fourth spin.
4. Add 2 ml packed SRBC to 8 ml AET solution in a 50-ml tube.
5. Mix gently and incubate 15 min in a 37°C water bath.
6. Wash four times with cold PBS and once with RPMI 1640 containing 5% FBS.
7. Resuspend SRBC in RPMI 1640 with 5% FBS and count cells (*APPENDIX 4A*). Store up to 1 week on ice or in a refrigerator.

Rosette T cells with AET-treated SRBC

8. Mix 1 ml of AET-treated SRBC with 10 ml FBS.
9. Mix an equal volume of primary blood mononuclear cells (PBMCs), isolated as in Basic Protocol 1, with a 1% (v/v) mixture of AET-SRBC-FBS in a 50-ml tube (usually 1×10^8 buffy coat PBMCs plus 1.5×10^{10} AET-treated SRBCs).

Buffy coat cells include all white blood cells and platelets. This is essentially all components of human blood other than plasma and red blood cells (RBCs).

10. Incubate 10 min in a 37°C water bath.
11. Centrifuge 10 min at 200 × *g*, 22°C. Make sure that the cells have pelleted. If not, recentrifuge for 5 additional min.
12. Place the tube upright on ice for 60 min or up to overnight on ice.
13. Resuspend cells by gentle pipetting using a sterile 5-ml pipet and layer 15 ml cells on 15 ml of Ficoll.
14. Centrifuge tubes for 40 min at 1200 × *g*, 20°C.
15. Collect B cell fraction at the interface of media and Ficoll.
16. Transfer to fresh 50-ml tubes.
17. Bring volume up to 45 ml with PBS or RPMI 1640 with 5% FBS.
18. Wash three times in 45 ml PBS or RPMI 1640 by centrifuging 5 min at 300× *g*, 22°C.

    At this step, the SRBC-T cell pellet can be discarded or follow the next steps to collect T cells for further experiments.

19. Suspend the SRBC-T cell pellet. Centrifuge 10 min at 1000 × *g*, room temperature.
20. Aspirate all of the supernatant. Break up the cell pellet by gently shaking.

Lyse the SRBC

21. Add 9 ml of distilled water with shaking for 4 sec.
22. Add 1 ml of 10× PBS with shaking.
23. Immediately fill the tube with 1× PBS.
24. Centrifuge 10 min at 300 × *g*, 22°C, and wash twice with PBS.
25. If red cells still remain, incubate the T cell pellet at room temperature in 5 ml Tris-buffered NH_4Cl.
26. Wash three times in 45 ml RPMI 1640 with 5% FBS.
27. Resuspend cells in 10% FBS for use.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

AET

Dissolve 1.967 g 2-aminoethylisothirouroniumuronium hydrobromide (AET) in 35 ml sterile water. Adjust to pH 8.0 with 1.0 N NaOH. Bring volume to 50 ml with sterile water. Filter through a 0.2-μm filter system. Store up to 1 year at 2° to 8°C. Check pH every 2 weeks.

Staining buffer

1× PBS (***APPENDIX 2A***) containing:
2 mM EDTA
0.5% bovine serum albumin (BSA)
Degas for 10 min under vacuum
Sterilize using a 0.2-μm filter
Make fresh every time

COMMENTARY

Background Information

Several methods for isolation of B lymphocytes from whole blood cells have been developed. The T-cell rosetting is a classic method which can separate T cells from B cells and has been widely used. In addition, a B cell magnetic isolation kit has also been developed based on the B cell specific marker CD19.

Critical Parameters and Troubleshooting

Here we discuss the T-cell rosetting method only. If the B cell isolation kit is used, please refer to the manufacturer's information.

For T-cell rosetting, the treatment of SRBC using AET is very critical. With AET treatment, E-rosette formation becomes less dependent on time, temperature, and the presence of serum. The mechanical stability of the rosettes is enhanced, and the number of SRBC attached to each rosette-forming lymphocyte (RFL) is markedly increased, leading to a sharper distinction between RFL and non-RFL. Ultimately, significantly more E-receptor carrying lymphocytes become detectable.

The ratio between AET treated SRBC and PBMCs should be more than 100:1. In this way, the T cell is saturated.

Anticipated Results

In general, 200 million B cells can be obtained from 500 ml of whole blood based on the T-cell rosetting method. However, the yield can vary using B cell isolation kits which depends on the manufacturer.

Time Considerations

Purification of B cells is a consuming process. Firstly, isolation of total white blood cells needs 3 to 4 hr since there is low-speed and long-time spinning and a number of washes. To further isolate B cells, first the SRBC needs to be treated with AET followed by a number of washes. This will take another 2 or 3 hr. The authors suggest that isolation of total WBC and preparation of AET-treated SRBC be initiated at the same time. In this way, the total PBMCs and SRBC can be ready for mixture almost at the same time. In addition, the time for T-cell rosetting is flexible and takes 1 hr to 12 hr based on the schedule of the researcher. Ideally, it takes 24 hr to isolate B lymphocytes from whole blood cell starting from blood letting.

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