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Zhou Songyang *Editor*

# Telomeres and Telomerase

## Methods and Protocols

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Edited by

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# Preface

A gap of 40 years separates the first identification of telomeres and the discovery of the telomerase. Our knowledge of the telomerase and how telomeres are maintained, however, has undergone exponential growth since then. The telomeres are coming into sharper focus as we look deeper and wider at how telomere maintenance is critically linked to cell growth, proliferation, aging, and diseases such as cancer. For example, in the majority of human cancers, normal telomere maintenance is often bypassed. And in many human cell types, telomere erosion is thought to limit the proliferative capacity of transformed cells. This book has come at an exciting time when the 2009 Nobel Prize in Physiology or Medicine was just awarded to Drs. Elizabeth H. Blackburn, Carol W. Greider, and Jack W. Szostak for their pioneer work on telomeres and telomerase.

New and rapid advances in technology have equipped us with a variety of tools and platforms to ask fundamental questions of telomere regulation and have allowed investigators to carry out experiments using diverse model systems. For example, proteomic, genomic, and molecular approaches have afforded us unprecedented insight into the complex protein interaction networks at work on the telomere chromatin and the detailed information regarding telomere dynamics in response to stress or stimuli.

While the first volume of *Telomeres and Telomerase: Methods and Protocols* (MiMB Vol. 191) focused mostly on telomerase assays, this volume expands the scope to encompass many different assays that allow investigators to query the function of telomere proteins and the responses of the telomere DNA. Biochemical, molecular, and proteomic approaches are detailed so that investigators may easily follow these protocols. It is our belief that this work will prove useful and informative.

***Houston, TX*** ***Zhou Songyang***

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# Chapter 1

# Introduction to Telomeres and Telomerase

Zhou Songyang

## Abstract

Telomeres are ends of chromosomes that play an important part in the biology of eukaryotic cells. Through the coordinated action of the telomerase and networks of other proteins and factors, the length and integrity of telomeres are maintained to prevent telomere dysfunction that has been linked to senescence, aging, diseases, and cancer. The tools and assays being used to study telomeres are being broadened, which has allowed us to derive a more detailed, high-resolution picture of the various players and pathways at work at the telomeres.

**Key words:** Telomeres, Telomerase, Telomere interactome, Telomere dysfunction

## 1. Telomere Structure

The seminal work of Müller and McClintock (1, 2) led to the first recognition of the importance of chromosome ends – the telomeres. And work from Blackburn and Greider (3, 4) further demonstrated that it was the telomerase that helps to maintain chromosomal end sequences. In the years that followed, tremendous amount of information has been gained regarding the mechanisms of how telomere maintenance is achieved, and the consequences of disrupting telomere homeostasis.

In most eukaryotes, telomeric DNA consists of tandem repeat sequences with the terminus ending in a single-stranded G-rich 3′ overhang. Both the length and exact sequence of the repeat sequence and overhang may differ from species to species. In mammalian cells, the 5′-TTAGGG-3′ repeat sequences can reach hundreds of kilobases (such as certain mouse strains), with the 3′ overhangs range in length between 75 and 300 bases (5, 6). In between the repetitive telomeric ends and chromosome-specific

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gene sequences are the subtelomeric regions that exhibit great diversity and complexity between organisms and may contain genes (7) and contribute to genetic diversity (8–12).

Telomeres appear to adopt a specialized structure with the help from various telomere-binding proteins, as revealed by EM and biochemical studies (13). In this case, invasion by the 3′ overhang into the double-stranded telomere DNA leads to the formation of the t-loop, while one strand is displaced to form the D-loop (13, 14). Such configurations may protect telomere ends from nuclease attack and prevent chromosome end-to-end fusion (15). The long G overhangs of mammalian telomeres may form the G-quadruplex, which has been shown to inhibit telomerase access (16–18).

## 2. The Telomere Interactome: An Integrated Telomere Signaling Network

One inherent problem during mammalian cell DNA synthesis is the replication of linear chromosomal ends (19, 20). Together with the telomerase (TERT and TERC), the telomeric nucleoprotein complex serves to inhibit loss of genetic information due to incomplete end replication (21–25). Tremendous progress has been made in the last few years in our understanding of the complex protein networks at the telomeres – the telomere interactome (26). The interactome incorporates the various telomere signaling pathways and represents the molecular machinery that regulates mammalian telomeres. Key protein–protein interaction hubs within the interactome include the telomerase, TRF1, and TRF2.

The telomerase contains a highly conserved reverse transcriptase (TERT) and a template RNA (TERC or TR) (22). Using the 3′ telomere overhang as a primer to align with TERC sequences, TERT adds telomeric repeats to chromosome ends. A number of proteins and factors have been shown to interact with the telomerase, including dyskerin and TCAB1 (27, 28). Cells that lack TERT extend their telomeres through the alternative lengthening of telomeres (ALT) pathway (29–31).

TRF1 and TRF2 specifically bind to telomeric DNA through their Myb domains (32–35). It has been shown that TRF1 counts and controls the length of telomere repeats, probably through complexing with TIN2, Tankyrase, PINX1, TPP1, and POT1 (36–47). In comparison, TRF2 has an essential role in end protection, recruiting the BRCT domain containing protein RAP1, the nucleotide excision repair protein ERCC1/XPF, BLM helicase, and DNA repair proteins PARP-1, Ku70/80, MCPH1, and the Mre11/Rad50/NBS1 (MRN) complex (45, 48–56). TRF2 is thought to be critical for maintaining the t-loop that may be important for shielding the ends from being recognized as DNA

breaks (35, 57, 58). Furthermore, TRF2 associates with the RecQ-like helicase WRN that regulates end protection, the D-loop structure and replication of the G-rich telomere strand (55, 59–61). In fact, six telomere-targeted proteins, TRF1, TRF2, TPP1, TIN2, RAP1, and POT1, can complex together and form the core of the telomere interactome (26), coordinating protein–protein interactions and crosstalk between protein complexes. These proteins in turn recruit a multitude of factors to dynamically regulate telomeres.

These protein–protein interaction studies have also offered clues as to the posttranslational modifications that are important to telomere maintenance. For example, kinases (such as ATM and DNA-PK) have been shown to be recruited to the telomeres (62–68), while poly-ADP ribosylation plays an important role in regulating TRF1 and TRF2 (40, 69, 70). Furthermore, ubiquitination may add another level of control for telomere proteins, as is the case for the TRF1-specific E3-ligase FBX4 (71). Further studies should shed light on the signals that activate these modifying enzymes and whether other modifications are also involved.

## 3. Telomere Dysfunction, Genome Stability, and Diseases

Without telomere protection, exposed or critically short chromosomes may result in chromosome end-to-end fusion, formation of dicentric chromosomes and ultimately aneuploidy (72). The unprotected ends can also activate DNA damage response pathways, cell cycle checkpoints, senescence, or apoptosis (67, 68, 73–76). Cancer development and aging is often found to associate with changes in telomeres. Telomere erosion, found in many human cell types, is thought to limit the proliferative capacity of transformed cells. In humans, telomerase expression appears tightly regulated; in >90% of human cancers normal telomere maintenance is often bypassed and TERT expression is upregulated (25). The strongest evidence to date for the importance and function of telomerase and telomere maintenance comes from studies in human diseases and mouse models. Patients with the rare human disease dyskeratosis congenita syndrome (DKC) have abnormally short telomeres and lower telomerase activity (77), with clinical manifestations of premature aging phenotypes and increased incidence of cancer (77–80). X-linked DKC is due to a mutation in dyskerin that can bind to the telomerase RNA template (TERC) (81). And autosomal-dominant DKC is caused by mutations in the *TERC* gene itself (79). Recently, mutations in one of the telomere core proteins TIN2 have also been found in DKC patients (82). In addition, some patients with leukemia have mutations in the *hTERC* and *hTERT* genes (77, 78).

Several DNA repair proteins can localize to telomeres and interact with telomere binding proteins (78), a number of which are mutated in human genome instability syndromes that are characterized by premature aging, increased cancer susceptibility, and critically short telomeres. For example, the gene defective in the autosomal recessive Werner syndrome (WS) is *WRN*, a 3′–5′ helicase and exonuclease that participates in DNA replication, repair, recombination, and transcription (59, 83–90). WS cells display premature senescence, accelerated telomere attrition, and defective telomere repair (91). As in WS, the gene mutated in Bloom syndrome (BS) also encodes a helicase (BLM) with diverse functions (92–94). Both WRN and BLM have been demonstrated to interact with the telomere binding protein TRF2 in ALT (alternative lengthening of telomeres) cells (51, 52). Ataxia telangiectasia (AT) and the Nijmegen breakage syndrome (NBS) share many characteristics, including developmental retardation and predisposition to lymphoid malignancy (95–100). Cells from these patients exhibit pronounced genome instabilities such as chromosome end-to-end fusions. The gene products responsible for NBS (NBS1) and AT (ATM) have been shown to interact with a number of telomere proteins (49, 65, 67, 68, 101–104). Fanconi anemia (FA) is a heterogeneous disorder characterized by bone marrow failure and high incidence of developmental abnormalities and cancer (105–107). In FA patients, there is a strong correlation between telomere dysfunction and hematopoietic defects (108–110).

Several mouse models of the diseases mentioned above have been generated. Mutations in a number of telomere regulators (e.g., Rte1, RAD51D, ATM family kinases, and Ku) have provided important evidence linking telomere dysfunction to the development of diseases such as cancer (111–115). Homozygous knockout for BLM is lethal (116), whereas $TERC^{-/-}$ and $WRN^{-/-}$ mice are initially normal (117). However, successive breeding of $TERC^{-/-}$ mice leads to progressive telomere loss, chromosome end-to-end fusions, and various age-related diseases affecting highly proliferative tissues (118, 119). Furthermore, inactivation of the gene encoding TERC in combination with the *BLM* hypomorphic mutation and/or *WRN* null mutation, result in accelerated pathology compared with $TERC^{-/-}$ (120, 121). Similarly, mice homozygous knockout for TERT also exhibit accelerated telomere shortening and genomic instability (122). Homozygous inactivation of the core telomeric proteins TIN2 or TRF1 is lethal (123–125). In conditional POT1-knockout mice, mouse POT1a and POT1b are shown to function distinctly, and both are required for normal telomere maintenance (126, 127). In adrenocortical dysplasia (*acd*) mice (a spontaneous autosomal recessive mutant), a mutation in the *TPP1* gene results in aberrant splicing of TPP1, leading to adrenal dysplasia, skin abnormalities, and defects in embryologic and germ cells (128).

## 4. Tools for Studying Telomere Biology

Over the years, numerous tools and platforms have been developed for telomere studies, affording us unprecedented ability to probe into the many aspects of telomere biology. At the same time, techniques commonly used in other fields are being incorporated and adapted for telomere studies. We are now able to assay changes in telomeres and telomere proteins in larger scale, at higher resolution (at the single cell or chromosome level), and with better sensitivity. The tools commonly used for telomere studies can be roughly divided into three categories. One set focuses on the study of telomere DNA itself, biochemical approaches such as EM and NMR seek to probe the structure and property of telomere DNA. For example, G-quadruplexes may play an important part in telomere structure. In the current series, we have added methods for detecting the formation and structure of G-quadruplexes. Assays to measure telomere length are the workhorse for telomere biology, since telomere length is a major indicator of telomerase function. A number of methods have been developed to determine the length of telomere repeat DNA (and its overhangs) under different conditions and in a variety of systems. These assays allow us to look at telomere length changes in a population of cells or in single cells. In addition to TRF assays and STELLA, this series has added newly developed approaches to measure the length of the 3′ overhangs, which can provide more accurate information regarding changes in telomere protection.

The second set of tools focuses on the proteins that are important for telomere function. The most important enzyme at the telomeres is the telomerase, and the previous series provided a number of methods to assay telomerase activity. In the last few years, other telomere-associated proteins have also garnered a lot of attention. And biochemical and molecular approaches have been utilized to analyze these telomere factors. Examples include proteomic tools to study the network of proteins at the telomeres and how they interact with each other, and the spatial and temporal information regarding how proteins are targeted to the telomeres. This series has added a fluorescent protein complementation assay among others that facilitates our investigation into the protein–protein interaction network.

The last set of tools seeks to understand the consequences of telomere dysfunction. While telomere length changes may reflect such dysfunction, it usually takes longer for the effect to become apparent in a population of cells. On the other hand, immediate consequences as a result of decapping or deprotection at the telomeres may be assayed by a variety of tools, many of which rely on microscopy to visualize the damage and changes at the telomeres.

For example, one important indication of telomere dysfunction is the telomeric recruitment of proteins involved in DNA damage responses. This response can be examined by the telomere dysfunction-induced foci (TIF) assay. The number of telomere-specific DNA-damage foci can then be quantified and compared. When used with a telomere-specific probe, chromosome orientation fluorescence in situ hybridization (CO-FISH) can determine the absolute 5′ to 3′ pter–qter direction of a DNA sequence. And the technique has proven especially powerful to assay abnormalities associated with telomere dysfunction, including telomere sister chromatid exchange (T-SCE) and telomere fusion. These methods are important additions in the current series.

In conclusion, we have in our possession an ever-expanding arsenal that continues to aid us in dissecting the function of telomerase and telomere-binding proteins, probing the changes in telomeres, and elucidating the consequences of telomere dysfunction.

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# Chapter 2

# Analysis of Average Telomere Length in Cultured Human Cells

**Dan Liu**

## Abstract

Telomeres play an important role in ensuring the integrity of the genome. Telomere shortening can lead to the loss of genetic information and trigger DNA damage responses. Cultured mammalian cells have served as critical model systems for studying the function of telomere binding proteins and telomerase. Tremendous heterogeneity can be observed both between species and within a single cell line population. Here, we describe the assay that analyzes the average length of telomeres in cultured cells (TRF analysis).

**Key words:** Telomere length, TRF, Telomere maintenance

## 1. Introduction

In eukaryotic cells with linear chromosomes, the chromosomal ends – telomeres – are maintained and protected through the coordinated action of telomerase and telomere binding proteins (1, 2). Perturbations in this intricate telomere interacting network can lead to changes in telomere structure and exposed chromosomal ends (3, 4). In telomerase active cells such as cancer cells and during development, such changes in turn impact on the length of telomeres and the status of the cell such as its replicative potential (5, 6).

Different organisms display remarkable variability in the make-up and exact length of the repetitive telomeric elements in their telomere DNA sequences. For example, in yeast, the sequence is $350 \pm 75$ bp of $C_{1-3}A/TG_{1-3}$ (7), whereas mammalian telomeres contain $(TTAGGG)_n$. Among mammalian species, mouse telomeres can be up to 150 kb, while somatic human cells have telomeres of

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5–15 kb in length (8). Even in a relatively homogenous population such as cultured mammalian cell lines, telomeres exhibit great heterogeneity in length. Genomic Southern blotting has been adapted to assess the average length of telomeres in populations of cultured mammalian cells. Here, genomic DNA is digested with frequent cutting restriction enzymes, to which repetitive telomeric sequences are resistant, thereby allowing for the analysis of the length of chromosomal terminal restriction fragments (TRF analysis). The final results reflect the estimation of both the telomeric repeats and subtelomeric regions that do not contain the particular restriction digest sites.

## 2. Materials

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO4$, 1.4 mM $KH_2PO_4$, pH 7.3.
2. Restriction digestion enzymes *Rsa*I and *Hin*fI and DNase-free RNase.
3. 50× TAE buffer: Mix 242 g Tris base, 57.1 mL acetic acid, and 18.6 g EDTA in $ddH_2O$ to final volume of 1 L. Make 1× TAE buffer from this stock solution.
4. DNA molecular weight markers: 1 kb DNA ladder and CHEF DNA size standard-5 kb ladder.
5. Ethidium bromide stock solution at 10 mg/mL.
6. Agarose gel electrophoresis apparatus.
7. Depurination solution: 0.25 M HCl.
8. Denaturation solution: 0.4 M NaOH.
9. Neutralization solution: 1.5 M NaCl, 0.5 M Tris–HCl, pH = 7.5.
10. 20× SSC stock solution: 3 M NaCl, 0.3 M sodium citrate, pH = 7. Dissolve 175.3 g NaCl and 88.2 g trisodium citrate (citric acid) in $ddH_2O$ to make 1 L.
11. Hybond-$N^+$ nylon membrane (GE Science) or equivalent.
12. Prehybridization and hybridization buffer: 0.5 M phosphate buffer (pH 7.2), 7% SDS, 1 mM EDTA (pH 8). 1 M phosphate buffer (pH 7.2) stock solution can be made by mixing 17.1 mL of $Na_2HPO4$ and 7.9 mL of $NaH_2PO4$, store the hybridization solution at –20°C.
13. $(TTAGGG)_3$ telomeric probe. Labeling may be carried out in a 20 μL reaction with 2 μL of T4 polynucleotide kinase (NEB), 1× kinase reaction buffer (NEB), 7 μL $\gamma$-$^{32}P$ ATP (3,000 Ci/mmol), and 10 pmol of the oligonucleotide probe

for 1 h at 37°C. Remove unincorporated labels with QIAquick nucleotide removal kit (QIAGEN).

14. Low-stringency wash buffer: 4× SSC (from 10)/0.1% SDS.
15. High-stringency wash buffer: 2× SSC (from 10)/0.1% SDS.

## 3. Methods

### 3.1. Sample Preparation

1. Harvest at least 300,000 cultured cells and wash with PBS. Collect the cells in a microcentrifuge tube (if possible). Spin at $1{,}000 \times g$ in a microcentrifuge at room temperature, wash in 1× PBS, and collect the pellet (see Note 1). Cell pellets can be assayed immediately, or directly frozen and stored at –80°C.
2. Extract genomic DNA using the QIAGEN DNeasy Tissue kit (QIAGEN). Standard genomic DNA extractions usually require several phenol/chloroform extraction steps, which makes processing multiple samples (routine for TRF analysis) time consuming (see Note 2). Estimate the amount of DNA based on the number of cells used (see Note 2).
3. Mix ~2–5 μg of extracted genomic DNA with 15 U each of *Rsa*I and *Hin*f1, and 1 μg of RNase A. Incubate at 37°C for ≥12 h. The digested DNA mixture may be stored at –20°C until further use (see Note 3).

### 3.2. Electrophoresis and Transfer

1. Prepare a large agarose gel (0.7%, 20–25 cm long) (roughly 300 mL) in 1× TAE buffer containing ethidium bromide (2–3 μL) (see Note 4).
2. Load 1–2 μg of digested genomic DNA per lane. Load DNA molecular weight markers (preferably mixed with $1–3 \times 10^5$ cpm radiolabeled DNA marker) to aid visualization under UV and to facilitate quantification steps (see Note 5). Run the gel at 1.5 V/cm until the 1 kb marker is at the bottom of the gel (see Note 6).
3. Visualize and document the gel under UV. Handle with care as the gel can be fragile and prone to breakage. Use a ruler to note the positions of the DNA ladder relative to the wells.
4. Soak the gel in depurination buffer for 15–20 min with gentle agitation (see Note 7).
5. Discard the solution, briefly rinse the gel in $ddH_2O$, and soak the gel in denaturation buffer for 30 min with gentle agitation.
6. Discard the solution, rinse the gel in $ddH_2O$, and neutralize the gel in neutralization solution for 30 min with gentle agitation.

7. Equilibrate the gel in 2× SSC for 5–10 min, and wet the Hybond $N^+$ nylon membrane in 2× SSC. Mark the gel and membrane for easy orientation during hybridization and analysis. Set up capillary transfer in 2× SSC for >12 h (see Note 8).
8. Disassemble the transfer assembly, and UV crosslink the DNA to the membrane (120 mJ/$cm^2$) with the DNA side facing up. The membrane can be stored in a sealed plastic bag with support at −20°C until ready to use (see Note 9).

### 3.3. Hybridization and Analysis

1. Prehybridize the membrane in hybridization buffer in a sealed bag or roller bottle at 50°C for ≥2 h. Use 10–20 mL of buffer depending on the size of the blot.
2. Prepare purified radiolabeled telomeric probe as described in Subheading 2, item 13. Determine the specific activity of the probe using a liquid scintillation counter (see Note 10).
3. Discard the prehybridization solution, add fresh hybridization solution (10–20 mL) along with the labeled probe (~1–5 × $10^6$ cpm/mL), and incubate at 50°C for at least 12 h.
4. Properly dispose the hybridization solution. Rinse the membrane briefly in low-stringency wash buffer to remove excess probes and hybridization solution. Then wash the membrane in succession with low- and high-stringency wash buffers. A minimum wash should have two low-stringency and two high-stringency buffer washes. Please see Note 11 for a guide to the wash steps as the length and temperature of each wash step should be empirically determined.
5. Blot-dry the membrane to get rid of excess wash buffer, wrap it in plastic wrap. Autoradiograph using KODAK X-OMAT film or equivalent for densitometric analysis, or expose the membrane in a PhosphorImager cassette for visualization and quantification on a PhosphorImager (see Note 12).
6. Use the Telorun spreadsheet to calculate average telomere length. Follow the instructions at the homepage of the Shay and Wright laboratory (http://www4.utsouthwestern.edu/cellbio/shay-wright/research/sw_lab_methods.htm).

## 4. Notes

1. In general, $10^6$ mammalian cells yield roughly 6 µg of genomic DNA. For genomic Southern blotting analysis, at least 2 µg of DNA is needed. Typically 5 µg of DNA is ideal for the analysis. This may serve as a guide for calculating the number of cells needed per assay.

2. The genomic DNA may also be extracted using standard genomic DNA extraction protocols. An example using proteinase K is given below. Please note that while spectrophotometric measurements are usually used to assess the quality and quantity of genomic DNA, many genomic DNA preparations often contain significant amount of RNA, which can skew the results.
   - Harvest cells in a DNase-free clean microcentrifuge tube.
   - Resuspend cells in 100 µL 1× PBS.
   - Add 200 µL of Lysis buffer [0.3 M Tris–HCl (pH 8), 0.15 M EDTA (pH 8), 1.5% SDS] plus 15 µL of freshly added proteinase K (10 mg/mL).
   - Mix and incubate at 55°C for 2–12 h. Heat the sample to 70°C for 30 min to inactive proteinase K.
   - Briefly centrifuge the tube to collect all liquid. Add 200 µL of lysis buffer and mix.
   - Add 500 µL of phenol/chloroform/isopropanol (1:1:1).
   - Mix thoroughly by vortexing and spin in microcentrifuge at top speed for 5 min.
   - Transfer the top aqueous phase to a new tube containing 500 µL chloroform/isopropanol (1:1). And repeat spinning and transfer step.
   - Add 200 µL 7.5 M ammonium acetate and 800 µL of 100% ethanol, mix by inverting the tube multiple times.
   - Spin in microcentrifuge at top speed for 5 min.
   - Wash the pelleted DNA with cold 70% ethanol and repeat spinning step.
   - Resuspend the DNA pellet in 100 µL TE or appropriate buffers.
3. The reaction volume will depend on the amount of DNA and enzymes used. If TE is used as the final elution buffer for DNA extraction, the EDTA concentration will need to be diluted (at least tenfold) to ensure complete digestion. Likewise, the amount of glycerol in the enzymes will dictate that their combined volume not exceed 10% of total reaction volume.
4. Handle the gel with care as it contains ethidium bromide. The gel should not be overly thick or thin. A thin large gel may be too fragile to handle and can break easily during subsequent steps, whereas a thick gel can hinder DNA transfer. Generally, a thickness of 0.5 cm is good. Take care to select combs with the right thickness and width, which should permit sufficient loading of samples.

A small gel (less than half the size) may also be prepared to verify complete digestion of the genomic DNA samples. Since 4 bp cutters are used here, they are expected to cut every $4^4$ bp. As a result, a completely digested sample should show a smear below the 1 kb DNA marker band. For incompletely digested samples, more enzymes may be added for additional incubation at 37°C. Some samples may appear resistant to digestion (they float out the well when being loaded). Repeat purification steps to get rid of salt and other contaminants (such as phenol if using the protocol in Note 3) may help.

Electrophoresis may also be carried out in 0.5–1 × TBE buffer (1× TBE buffer: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). While TBE buffer has better buffering capacity, it is best for resolving smaller-sized DNA fragments.

5. A radiolabeled DNA marker will be visible by autoradiography or phosphorImager exposure, which facilitates the calculations in TRF analysis. Radiolabeled DNA markers may be obtained through random priming labeling reactions using Klenow, or end-labeled using T4 polynucleotide kinase (as described below). The latter can be performed along with the telomere probe as described in Subheading 2, item 14.

   The 1 kb DNA ladder should first be dephosphorylated using calf intestinal phosphatase (CIP, NEB) (1 μg of DNA ladder, 0.5 U CIP, 1× NEB buffer, 60 min at 37°C). The dephosphorylated DNA should be purified through either gel purification, spin column, or phenol extraction. End labeling is then carried out in a 20 μL reaction with 1 μL of T4 polynucleotide kinase (NEB), 2 μL 10× kinase reaction buffer (NEB), 3 μL $\gamma$-$^{32}$P ATP (3,000 Ci/mmol), and 0.5–120 μg of DNA ladder for 30–60 min at 37°C. Unincorporated labels are removed with QIAquick nucleotide removal kit (QIAGEN). Determine the specific activity of the labeled marker using a liquid scintillation counter.

6. Depending on the size of the gel, type of running buffer, power supply, and gel apparatus, the electrophoresis process can take 24 h or longer. TAE buffer generally requires lower voltage and longer running time. In addition, slow low-voltage electrophoresis leads to better resolution.

7. We find it easier to carry out steps 3–6 with the gel still in the casting tray. There is no need to slide the gel on and off during these steps, which can lead to gel breakage.

8. There is no need to flip the gel upside down for the transfer. Carefully slide it off onto the transfer surface. Make sure to place several layers of 2× SSC soaked Whatman paper (cut to the correct size) followed by several dry layers on top of the membrane to ensure even transfer and minimize bubbles.

9. The membrane may be further incubated in NaOH solution for 5–10 min to denature any remaining DNA, neutralized again, and rinsed in 2× SSC before crosslinking.
10. While both the G and C probes can be used, the G probe generally yields better and stronger signals. The specific activity of the probe should be ~$0.5–1 \times 10^6$ cpm/μL.
11. The membrane is first washed in low-stringency buffer once at room temperature and once at 37°C, and then in high-stringency buffer at least twice at room temperature. The stringency of the wash may be further raised by increasing the number of washes, or the temperature for high-stringency wash (to 37°C or 50°C if needed). The membrane should be checked with a Geiger counter periodically. A good signal ratio between DNA bound vs. unbound portions of the membrane coupled with minimum signals from DNA-free portions of the membrane would indicate readiness. Prolonged and overly stringent washes may result in weak signals that require extended exposure time.
12. For samples with exceptionally long telomeres such as those from inbred laboratory mice, agarose plugs (available from commercial sources) with embedded cells should be prepared to aid the digestion with protease and restriction enzymes.

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# Chapter 3

# Telomere Length Analysis by Quantitative Fluorescent In situ Hybridization (Q-FISH)

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## Abstract

Length is a functional parameter of telomeres, the nucleoprotein structures that protect chromosome ends. The availability of highly specific, high-affinity probes for telomeric repeated sequences allowed the development of quantitative approaches aimed at measuring telomere length directly on chromosomes or in interphase nuclei. Here, we describe a general method for telomere quantitative FISH on metaphase chromosomes and discuss its most common applications in research.

**Key words:** Telomere, FISH, Q-FISH, Length, PNA, LNA

## 1. Introduction

The number of telomeric repeats at chromosome ends is important for telomere function (1–3). These repeats serve as a platform for the assembly of a protective protein complex, called shelterin (4) or telosome (5) that will protect the extremity against degradation and fusion (6). The telomeric protein complex is also required for replication and repair and to prevent recombination (7). Maintaining telomere length is thus crucial to preserve function. However, telomere repeats are lost at every cycle of DNA replication due to both the "end replication problem" and the necessary processing of newly replicated ends to recreate a 3′ overhang (8). In cells expressing telomerase, the dedicated enzyme able to add de novo telomere repeats, an equilibrium is reached between shortening and lengthening kinetics, thus defining a dynamic point of length homeostasis (9). However, most human somatic cells do not express, or else at low levels, telomerase activity and telomeres inexorably shorten with cell division (10). As soon as telomere length is insufficient to ensure protection, a signal is

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sent to command cell arrest and entry in mitotic senescence (11). Telomere length, therefore, directly influences cell proliferation potential.

In vivo, telomere lengths decrease with aging and premature aging syndromes are associated with accelerated telomere shortening (12). Both observations have lent support to the hypothesis that cell senescence triggered by short telomeres is responsible of most, if not all, manifestations connected to aging (12). The discovery, which mutations in components of the telomere enzyme are implicated in aging syndromes and aging-related manifestations, has established a definitive link between short telomeres and organismal aging (13). As a result, telomere length has become an obliged biomarker in all studies related to aging (14).

Classically, telomere length is measured by telomere restriction fragment (TRF) analysis in Southern blots, which, in spite of its technical problems and relative imprecision, remains the gold standard (15, 16). On the other hand, PCR-based quantitative analyses, which require little material, have been developed and used with success by some laboratories (17, 18). Both methods, though, have the inconvenience of providing a rough estimate of either mean telomere length or total telomere repeat content of a cell population, thus underestimating the relative proportion of short, potentially dysfunctional, telomeres which may be sufficient to trigger cell senescence in any single cell (19, 20). In fact, telomere length heterogeneity is the rule in normal somatic cells (21–23) and single telomere lengths are allele-specific, transmitted to the offspring and stable throughout life, thus representing a constitutive characteristic of any given individual (24, 25). It has been also shown that chromosome arms carrying the shortest telomeres are the first to become unstable upon unrestrained cell proliferation (2). Moreover, the distribution of single telomere lengths among chromosome arms determines the way a particular cell accumulates chromosome abnormalities during tumor transformation and has a major impact on the karyotypic characteristics of spontaneously immortalized cells in vitro (2, 26).

Therefore, telomere length analysis on chromosome metaphase provides not only an estimation of telomere length of the cell population but allows the evaluation of other parameters such as heterogeneity of telomere lengths and the presence of critically short telomeres. Telomere length heterogeneity tends to be reduced and almost disappears when cells express high levels of telomerase (tumor cells or cells expressing hTERT transgene) (21). On the other hand, telomere length heterogeneity is characteristically exaggerated in cells using recombination to maintain telomeres (ALT) so that very long telomeres coexist with chromosome extremities on which no telomere signals are detectable (signal-free end, SFE) (27). Interestingly, the reexpression of telomerase activity in these cells leads to a significant reduction of SFEs thus

constituting a readout for telomerase activity, a test which can be used to evaluate the performance of modified telomerase complexes (28, 29). Finally, allele-specific analyses can be carried out to study length differences between homologous chromosomes (21).

The method described, hereafter, is based on the original method described by the group of Lansdorp (30). Very little modifications need to be introduced, if PNA probes are used. However, it is also possible to use telomeric LNA probes, which also have high specificity and high affinity for telomere repeats (31). Contrary to PNA, LNA probes tolerate a few mismatches (32); and therefore, signals may no longer exclusively originate from canonical sequences but may instead comprise telomere repeats variants, whose composition and length presumably vary from an extremity to another and, therefore, between individuals (33, 34). However, the number of juxtatelomeric variants is limited, relative to the number of canonical repeats and, therefore, the contribution to the total fluorescence intensity from probes hybridizing to variant repeats is most likely negligible.

## 2. Materials

### 2.1. Preparation of Metaphase Suspensions

1. 0.1 µg/mL colcemid (KaryoMax, Invitrogen).
2. Trypsin/EDTA solution: Trypsin 0.05%, EDTA 0.53 mM.
3. Hypotonic solutions (see Note 1): 8 g/L Na citrate, 0.075 M KCl.
4. Fixative solution for metaphase preparation: ethanol/acetic acid (3/1, V/V) (see Note 2).

### 2.2. Metaphase Spreads

1. Clean superfrost slides (Menzel-Gläser from D. Dutscher S.A.) with pure ethanol.
2. Coverslip 24×60 (VWR).

### 2.3. PNA Probe

PNA probes can be ordered from Panagene (http://www.panagene.com). The most common telomeric probe is $(CCCTAA)_3$ which can be labeled with red (Cy3) or green (Fam/FITC) fluorochromes (Note 3).

### 2.4. Q-FISH Procedure

1. Pepsin solution: 1 mg/mL, pH 2. For 100 mL: 100 mg pepsine (Sigma – P7000) – 10 mL 0.5 M citric acid pH 2 – water (see Note 4).
2. Fixative solution: 3.7% formaldehyde/PBS 1×.

   For 100 mL: 10 mL PBS 10× – 10 mL formaldehyde 37% (SIAL from Sigma – F1635) – water.

3. Hybridization solution: 70% formamide, 20 mM Tris pH 7.4 with blocking agent (see Note 5)

   For 1 mL: 700 µL formamide (SIAL from Sigma – 47670-1L-F) – 20 µL 1 M Tris pH 7.4 – 20 µL of blocking agent – water.

4. First two washes: formamide 70% Tris 20 mM pH 7.4 (see Note 6).

   For 200 mL: 140 mL formamide – 4 mL 1 M Tris pH 7.4 – water.

5. Following washes: 50 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween 20.

   For 300 mL: 15 mL 1 M Tris pH 7.4 – 9 mL 5 M NaCl – 150 µL Tween 20 – water.

6. Ethanol baths: 70, 80, 90, and 100%.

### 2.5. Image Capture and Analysis

Computer-piloted or manual UV epifluorescence microscope, equipped both with filters of appropriate wavelengths, typically for blue: excitation, 360 nm–emission, 460 nm, red: excitation, 545 nm–emission, 610 nm, and green: excitation, 490 nm–emission, 520 nm and with a cooled CCD video camera (see Note 7).

Image analysis software (see Note 8).

## 3. Methods

### 3.1. Preparation of Metaphase Suspensions

1. Add 0.1 µg/mL colcemid to the in vitro cell culture for at least 1 h (see Note 9).
2. If cells are adherent, incubate with a trypsin/EDTA solution for 10–30 min at 37°C to detach them (Trypsin 0.05% and EDTA 0.53 mM). Recover the cell suspension in PBS.
3. Spin the cells down (300 rcf×5 min).
4. Remove medium leaving about 0.2 mL above the cell pellet.
5. Resuspend the cell pellet in the remaining supernatant.
6. *Hypotonic shock*: Slowly add (drop-wise) the prewarmed hypotonic solution to the resuspended cells while gently vortexing. Incubate at 37°C for 30–40 min (see Note 10).
7. *Pre-Fixation*: Add a few drops of freshly prepared fixative ethanol/acetic acid (3/1, V/V) and invert to mix. Spin the cells down at slow speed (200 rcf, 5 min).
8. Remove most of the supernatant and resuspend the cell pellet in the remaining liquid.
9. *Fixation*: With gentle vortexing, slowly add (drop-wise) 8 mL of fixative ethanol/acetic acid (3/1, V/V). Spin the cells down

(200 rcf, 5 min). Repeat the fixation (8 mL)/centrifugation (200 rcf, 5 min) step three times.

10. Spread metaphases or store cell suspensions at –20°C in at least 5 mL of fixative (see Note 11).

### 3.2. Metaphase Spreads

1. Bring cell suspensions at RT before spinning down (200 rcf, 5 min).
2. Resuspend in fixative (8 mL) and centrifuge (200 rcf, 5 min). Repeat the step two more times.
3. Resuspend the cells in a small volume of fixative (see Note 12).
4. The cell suspension is dropped onto clean slides, at optimal ambient humidity, and slides are let to air dry (see Note 13).
5. Check under the phase contrast microscope for the presence of correctly spread metaphases.
6. Let the slides air dry overnight.
7. Slides can be stored in a slide box at –20°C (see Note 14).

### 3.3. Q-FISH Procedure

1. Rehydrate slides 5 min in PBS 1×.
2. Fix spreads 2 min in 3.7% formaldehyde/PBS 1×.
3. Wash three times 5 min in PBS 1×.
4. Digest 10 min with pepsin (1 mg/mL – pH 2) at 37°C (see Note 15).
5. Quick wash in PBS 1×.
6. Fix cells 2 min in 3.7% formaldehyde/PBS 1× (same solution as previous step).
7. Wash three times 5 min in PBS 1×.
8. Dehydrate slides in successive ethanol baths (70, 80, 90, and 100%), 2 min each.
9. Let the slides air dry in the dark.
10. *PNA hybridization*: If lyophilized, prepare a master tube of PNA probe, 1 μM, in hybridization buffer (70% formamide and 20 mM Tris pH 7.4). Vortex for 2 min and heat at 70°C for 5 min, spin 3–4 min at top speed. Prepare a working solution at 10 nM final in hybridization solution containing blocking agent (see Note 16).
11. Distribute at least 35 μL of the hybridization solution per slide and overlay with a coverslip avoiding bubbles.
12. Denature by heating at 80°C for 3 min.
13. Incubate for 2 h at RT in the dark (see Note 17).
14. Wash two times 15 min in formamide 70%, Tris 20 mM pH 7.4 (with agitation) (see Note 18).

15. Wash three times 5 min in 50 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween 20.
16. Dehydrate slides in successive ethanol baths (70, 80, 90, and 100%), 2 min each.
17. Let the slides air dry in the dark.
18. Mount coverslip with 20 μL of Vectashield (DAPI 0.2–0.5 μg/mL) (see Note 19).
19. Slides can be stored at 4°C.

### 3.4. Visualization of Metaphases and Image Analysis

1. Search for metaphases under the UV microscope using the DAPI channel. Depending on the number of metaphases available, you may choose those in which individual chromosomes are in good shape and well spread so that a karyotype analysis may be eventually carried out. Visualize telomeric signals using the appropriate filter (red for Cy3 and green for FITC/Fam) (Fig. 1). Set the camera gain to a maximum and choose a time of exposure that allows exponential acquisition of most signals while avoiding saturation of the strongest ones. Once you have determined the most adequate time of exposure, apply it to all pictures taken across all samples belonging to the same experiment (see Note 20). 25–30 metaphases should allow a statistical analysis of telomere

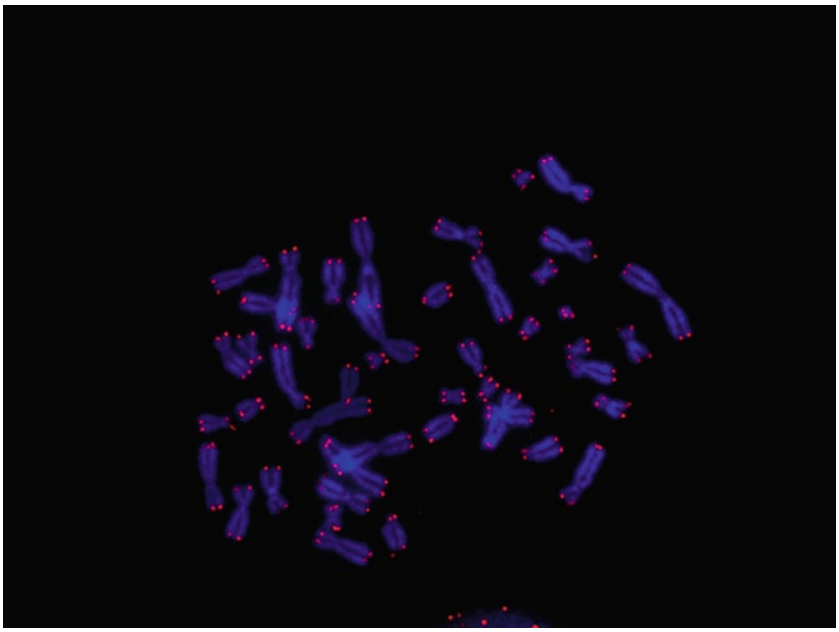

Fig. 1. Metaphase chromosomes processed for Q-FISH using a C-rich PNA telomeric probe $(CCCTAA)_3$ labeled in *red* (Cy3).

intensity. Metaphases are captured using the appropriate channels and the original, unmodified black and white images are saved for later analysis.

2. The quantitative analysis is carried out with the help of image softwares. Any software able to determine the mean intensity of a pixel can be used. However, analysis is facilitated by the use of software that allows both the merging of original images into color pictures, the segmentation of signals based on pixel values and the writing of scripts that allow a semiautomatic quantification. Briefly, single telomeric signals are segmented either manually or automatically and the mean pixel intensity for all telomeres or for single telomeres is calculated through available software functions. Background fluorescence is measured by segmenting the interstitial regions of chromosomes. The data is exported in text format, which can be recovered on an "excel" sheet for statistical treatment. The mean fluorescence intensity of the cell sample is calculated, once the background of the metaphase has been subtracted, using the mean intensity of metaphases or the mean intensity of single telomeres (see Note 21). Distributions of single telomere intensities are also informative (for examples, see ref. 35). Statistical comparisons are best carried out using Wilcoxon rank-sum tests.

## 4. Notes

1. Several formulae have been proposed, including a mixture of (V/V/V) Na citrate 8 g/L/KCl 0.075 M/$H_2O$, 1/1/1. The sensitivity to hypotonic swelling by any of these solutions should be tested for each cell type or cell line.
2. To reduce toxicity, we have replaced methanol, classically used in this preparation, by ethanol. Always use freshly prepared fixative.
3. Telomere-specific LNA probes (obtained from EXIQON, http://www.exiqon.com/) can also be used. For instance, it is possible to use a G-rich LNA probes to reveal the C-rich strand instead of the G-rich strand. The following sequence have been successfully used for quantitative purposes (36): GGGT+TAGGG+T+TAG+GGTTAGGG+T+TAG+GG+T+TAGGG+TTA, where the LNA bases are preceded by a + sign. Fluorochrome modifications [either green (fam) or red (cy3)] are added at both 3′ and 5′ extremities to increase sensitivity.
4. Bringing the solution to pH 2 may be a little laborious and therefore an alternative is to prepare a solution of water with a few drops of concentrated HCl. However, buffering is potentially important to preserve chromosome structure and allow optimal pepsin enzymatic activity.

5. Many types of blocking agents for hybridization reactions are commercially available. Any of those, as well as a BSA solution, can be used to decrease background.
6. Formamide should be deionized before use. However, we only do this routinely for formamide solutions that are used to prepare hybridization reactions. The current quality of commercially available formamide is enough to be directly used in wash steps. After deionization, formamide solutions should be stored at –20°C.
7. Most types of CCD cameras allow the acquisition of digitalized image files adapted to quantification analysis. A good dynamic range can be obtained with 12-bit gray-scale images.
8. The original dedicated computer program TFL-TELO developed by Lansdorp et al. (30) is available from the BC Cancer Agency (Vancouver, Canada). Other freewares such as NIH image or ImageJ can be also used for quantification purposes. Commercial softwares are also available (see Note 21).
9. Depending on the mitotic index, the incubation time with colcemid varies. If cells divide slowly, this time can be increased up to 12 h. Nocodazol can also be used.
10. It is necessary to determine the best combination of hypotonic solution and incubation time for every cell line. For instance, some embryonic stem cells are extremely sensitive, whereas some fibroblast-derived cell lines are much more resistant. The Na citrate solution is gentler than the KCl hypotonic solution. As an example, we incubate HT1080 cells as well as human primary fibroblasts in 10 mL of 8 g/L Na citrate for 40 min at 37°C.
11. Although metaphase suspensions in fixative are very stable, long-term storage is not recommended for quantitative analysis of telomere fluorescence.
12. A slightly cloudy suspension is most of the time appropriate. However, if the mitotic index is high, much more diluted suspensions can be used.
13. Conditions for optimal metaphase spreading are not obvious to master. Dropping the cell suspension from a high height is not an absolute requirement since dropping from a few centimeters high is enough to get good spreading. Humidity seems more crucial and placing the perfectly clean, dried glass slide on top of a wet napkin often helps one to obtain acceptable spreading from certain difficult-to-work-with suspensions or when the ambient air is too dry.
14. Long-term storage is not recommended for quantitative analysis of telomere fluorescence.
15. Pepsin should be prepared freshly. Digestion time can be prolonged, if cytoplasmic material is visible under phase contrast

microscope. Eliminating this cytoplasmic material greatly reduces background problems.

16. Master solutions of PNA probes can be kept for 2–3 months. They, however, have a tendency to precipitate. Heating increases solubility. The final concentration can be increased up to 50 nM without substantially increasing the background. The hybridization solution can be prepared in advanced, aliquoted and stored at –20°C. We usually prepare the hybridization buffer with deionized formamide and the blocking reagent is added just to working dilution of the probe. LNA probes are more soluble than PNA probes, but master solutions are probably less stable for long-term storage. The final concentration for LNA probes is 50 nM in 50% formamide, 2× SSC, and blocking reagent.
17. The kinetics of hybridization of PNA probes is very fast and reactions are probably completed after 30 min.
18. When LNA probes are used, these washing steps are made in 50% formamide, 2× SSC. 2 h is also sufficient for LNA probes.
19. A strong DAPI staining will appreciably bleed into the green channel thus interfering with signal quantification, if a green fluorophore is used to label the probe.
20. Because of both day-to-day strength fluctuations and wearing off of the UV lamp, comparisons between images captured on different sessions (days) are difficult, even if the same time of exposure has been used, although intensity fluctuations appear to be less marked with hybrid mercury/xenon lamps. To correct for these variations, independent controls (such as fluorescent beads or hybridization samples with known telomere repeat content – i.e., plasmids) are to be included in every acquisition session to normalize fluorescence intensity values.
21. The TFL-TELO package offers the possibility of intersession calibration using plasmids with sized telomere inserts (30). Other packages use fluorescent signals obtained with centromeric probes as a reference signal (Isis – Telomere Analysis Software). Results may be expressed as fluorescence arbitrary units or, if using the TFL-TELO package and, as telomere fluorescence units (1 U = 1 kb).

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# Chapter 4

# Telomere Strand-Specific Length Analysis by Fluorescent In Situ Hybridization (Q-CO-FISH)

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## Abstract

The implementation of quantitative approaches in telomere chromosome-oriented FISH (telomeric CO-FISH) allows the assessment of the relative efficiency of lagging versus leading strand telomere replication and thus provides information on the implicated mechanisms. Here, we describe a simple method for telomere strand-specific analyses and discuss its potential applications.

**Key words:** Telomere, FISH, CO-FISH, Q-CO-FISH, Replication, Leading, Lagging, PNA, LNA

## 1. Introduction

Telomere length is an important parameter for telomere function (1–3). Telomeres shorten at every cell division and the rate of shortening directly influences cell proliferation potential (4, 5). The kinetics of telomere shortening differs from one type of cell to another and between individuals (6, 7). It is the direct consequence of both the efficiency of replication, the rate of telomeric repeat addition by telomerase (if present) and the processing reactions that remodel telomeres after replication (8). Of all three mechanisms, though, replication efficiency is probably the most fundamental one since any obstruction to the progression of the replication fork will cause an abrupt telomere shortening that can compromise telomere stability and thus jeopardize cell proliferation capacity (9, 10). Understanding the mechanisms that allow fork progression and replication completion at telomeres is, therefore, crucial to understand telomere metabolism and homeostasis.

Replication at telomeres is unique in the sense that replication forks are typically unidirectional, thus making impossible the

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rescuing of replication fork stalling by convergent replication forks (10). It has been proposed that particular mechanisms are at play during telomere replication to alleviate potential obstacles to fork progression. Remarkably, it has been recently shown that TRF1 (a telomere-specific protein which directly binds the telomere tract (11)) is required for normal fork progression at telomeres (12), thus demonstrating that components of the telomere-specific nucleoprotein complex, instead of constituting an obstacle, facilitate replication. On the other hand, ancillary factors, such as the WRN helicase or the exonuclease Apollo, have also been shown to be required for normal telomere replication (13–15).

The CO-FISH technique, which was introduced into the telomere field by Bailey, Cornforth, and collaborators (16, 17) and allow the specific visualization of G-rich and C-rich strands on metaphase chromosomes, has become instrumental in the characterization of recombination phenomena at telomeres (18, 19), and also in the demonstration of fundamental differences in the metabolism of telomeres that have been replicated either by lagging (parental G-rich strand) or leading (parental C-rich strand) mechanisms (16, 20, 21). For instance, absence of particular telomere factors induces telomere instability through recombination reactions that preferentially affect one or the other strand (16, 20).

More recently, a quantitative approach was used for telomeric CO-FISH to more precisely define the role of WRN helicase during telomere replication (21). While qualitative CO-FISH analyses had previously suggested that WRN was required in a stochastic way for the replication of G-rich strands (15), a quantitative analysis actually revealed that WRN is absolutely required for the complete replication of virtually every telomeric G-rich strand (21). Interestingly, in the absence of WRN, single G-rich strands accumulate while DNA polymerization continues on C-rich strands. This uncoupling of DNA polymerization, which presumably occurs exclusively at telomeric replication forks, requires POT1, the G-rich single-strand binding protein (21).

The CO-FISH technique is based on the incorporation of base substitutes into the DNA only during one round of replication (22). Metaphase spreads are prepared after this single S-phase and chromosomes are treated to eliminate the newly synthesized strand and to obtain single-stranded sister chromatids. The use of differentially labeled, high affinity, single-strand telomeric probes that recognize G-rich and C-rich strands allows to measure the fluorescence intensity associated with each sister chromatid and estimate the relative length of the parental strand. It is assumed that, if replication proceeds normally and processing reactions to remodel telomeres are under control, the fluorescence intensities of these parental strands should be about the same than at the start of the S-phase. On the other hand, a lower telomere fluorescence intensity will indicate that replication was incomplete, that the level of degradation is increased, or both.

To obtain reproducible results, the quantitative CO-FISH (Q-CO-FISH) technique requires that cells carry relatively long telomeres (23). This is because the degradation procedure involved in CO-FISH decreases the intensity of telomere signals that can be obtained and, therefore, the procedure is less sensitive than the classic Q-FISH. Also, although C-rich PNA probes can still be used to reveal the G-rich strand, G-rich PNA probes that reveal the C-rich strand tend to be less soluble and yield high background. This is why we recommend here the use of LNA telomeric probes, which have high affinity and specificity allowing their use for quantitative purposes, even if they are somewhat less efficient than PNA and therefore less sensitive. Consequently, it is best to carry out the Q-CO-FISH technique in telomerase positive cells. It should be noted that any elongation due to telomerase during the examined S-phase will lead to the incorporation of base substitutes, and therefore the elongated strand and its complementary will be degraded by the CO-FISH procedure thereby restricting the detection to the original parental strand only.

Finally, many of the telomerase positive cell lines currently available carry relatively short telomeres, which may prevent a correct quantification of signals by the Q-CO-FISH approach. In such cases, an alternative consists in carrying out a classic Q-FISH analysis first, followed by a CO-FISH experiment to specifically reveal the mode of replication of sister telomeres. In this way, telomeres that replicated using either mechanism will be identified and specifically quantified. This combined approach (Q-FISH/CO-FISH) requires that cells had been in contact with base substitutes for one cell cycle and that the captured metaphases after Q-FISH be easily retrieved after the CO-FISH procedure. Contrary to Q-CO-FISH, any elongation due to telomerase activity will be taken into account in the Q-FISH/CO-FISH analysis. However, it is likely that the number of repeats added by the enzyme represents a limited proportion of the total signal, thus allowing to detect major defects on lagging versus leading telomere replication mechanisms.

## 2. Materials

### 2.1. Preparation of Metaphase Suspensions

1. Trypsin/EDTA solution: Trypsin 0.05% and EDTA 0.53 mM.
2. 0.1 µg/mL colcemid (KaryoMax, Invitrogen).
3. Hypotonic solutions (see Note 1): 8 g/L Na citrate, 0.075 M KCl.
4. Fixative solution for metaphase preparation: ethanol/acetic acid (3/1, V/V) (see Note 2).

### 2.2. Metaphase Spreads

1. Clean superfrost slides (Menzel-Gläser from D. Dutscher S.A. – 100204B) with pure ethanol.
2. Coverslip 24 × 60 (VWR – 631-0666).

### 2.3. Single-Stranded Telomere Probes

C-rich PNA telomeric probe: $(CCCTAA)_3$ labeled with red (Cy3) or green (Fam/FITC) fluorochromes (http://www.panagene.com).

G-rich LNA telomeric probe: GGGT+TAGGG+T+TAG+GG TTAGGG+T+TAG+GG+T+TAGGG+TTA, where the LNA bases are preceded by a + sign. Fluorochrome modifications [green (Fam/FITC) or red (Cy3)] are added in both 3 and 5 extremities to increase sensitivity (see Note 3).

### 2.4. CO-FISH Procedure

1. Pepsin solution: 1 mg/mL Pepsin pH 2. For 100 mL: 100 mg pepsine (Sigma – P7000)–10 mL 0.5 M citric acid pH 2–water (see Note 4).
2. Fixative solution: 3.7% formaldehyde/PBS 1×. For 100 mL: 10 mL PBS10×–10 mL formaldehyde 37% (SIAL from Sigma–F1635)–water.
3. Hybridization solution for PNA probe: 70% formamide, 20 mM Tris pH 7.4 with blocking agent (see Note 5). For 1 mL: 700 µL formamide (SIAL from Sigma – 47670-1L-F)–20 µL 1 M Tris pH 7.4–20 µL of blocking agent–water.
4. First two washes after PNA hybridization: formamide 70%, Tris 20 mM pH 7.4 (see Note 6). For 200 mL: 140 mL formamide–4 mL 1 M Tris pH 7.4–water.
5. Following washes after PNA hybridization: 50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween 20. For 300 mL: 15 mL 1 M Tris pH 7.4–9 mL 5 M NaCl–150 µL Tween 20–water.
6. Hybridization solution for LNA probe: 50% formamide, 2× SSC with blocking agent. For 1 mL: 500 µL formamide–100 µL SSC 20×–20 µL of blocking agent–water.
7. First two washes after LNA hybridization: formamide 2× SSC. For 200 mL: 100 mL formamide–20 mL SSC 20×–water.
8. Following washes after LNA hybridization: 50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween 20. For 300 mL: 15 mL 1 M Tris pH 7.4–9 mL 5 M NaCl–150 µL Tween 20–water.

### 2.5. Image Capture and Analysis

1. Computer piloted or manual UV epifluorescence microscope equipped both with filters of appropriate wavelengths, typically for blue: excitation, 360 nm–emission, 460 nm, red: excitation, 545 nm–emission, 610 nm, and green: excitation, 490 nm–emission, 520 nm and with a cooled CCD video camera (see Note 7).
2. Image analysis software (see Note 8).

## 3. Methods

### 3.1. Preparation of Metaphase Suspensions

1. Add 30 μM BrdU/10 μM BrdC to the cell culture and let it go for another 24 h (see Note 9).
2. Add 0.1 μg/mL colcemid for at least 1 h (see Note 10).
3. If cells are adherent, incubate with a trypsin/EDTA solution for 10–30 min at 37°C to detach them. Recover the cell suspension in PBS.
4. Spin the cells down (300 rcf×5 min) and remove medium leaving about 0.2 mL above the cell pellet.
5. Resuspend the cell pellet in the remaining supernatant.
6. Hypotonic shock: Slowly add (drop-wise) the pre-warmed hypotonic solution to the resuspended cells while gently vortexing. Incubate at 37°C for 30–40 min (see Note 11).
7. Prefixation: Add a few drops of freshly prepared fixative ethanol/acetic acid (3/1, V/V) and invert to mix. Spin the cells down at slow speed (200 rcf, 5 min).
8. Remove most of the supernatant and resuspend the cell pellet in the remaining liquid.
9. Fixation: With gentle vortexing, slowly add (drop-wise) 8 mL of fixative ethanol/acetic acid (3/1, V/V). Spin the cells down (200 rcf, 5 min). Repeat the fixation (8 mL)/centrifugation (200 rcf×5 min) step three times.
10. Spread metaphases or store cell suspensions at –20°C in at least 5 mL of fixative (see Note 12).

### 3.2. Metaphase Spreads

1. Bring cell suspensions at RT before spinning down (200 rcf, 5 min).
2. Resuspend in fixative (8 mL) and centrifuge (200 rcf, 5 min). Repeat the step two more times.
3. Resuspend the cells in a small volume of fixative (see Note 13).
4. The cell suspension is dropped onto clean slides at optimal ambient humidity and slides are let to air dry (see Note 14).
5. Check under the phase contrast microscope for the presence of correctly spread metaphases.
6. Let the slides air dry overnight.
7. Slides can be stored in a slide box at –20°C (see Note 15).

### 3.3. CO-FISH Procedure

1. Rehydrate slides 5 min in PBS 1×.
2. Fix spreads 2 min in 3.7% formaldehyde/PBS 1×.
3. Wash three times 5 min in PBS 1×.
4. Digest 10 min with pepsin (1 mg/mL – pH 2) at 37°C (see Note 16).

5. Quick wash in PBS 1×.
6. Fix cells 2 min in 3.7% formaldehyde/PBS 1× (same solution as previous step).
7. Wash three times 5 min in PBS 1×.
8. Immerse slides in a solution of Hoechst 33258 (0.5 μg/mL in PBS) for 15 min.
9. Quick wash in PBS 1×.
10. Expose slides to a 306 nm UV source for 30 min. Typically, the slides are placed on a heating plate (55°C) covered with excess PBS and a coverslip (to avoid desiccation), and placed under a flipped-over UV box. The slides should be 1 cm away from the UV source.
11. After UV exposure, immerse slides in PBS 1× to help the coverslip to come off and the slides to cool down.
12. After draining all the PBS, treat slides with 3 U/μL of ExoIII for 5 min at room temperature (use at least 40 μL/slide and put a coverslip on to ensure even distribution) (see Note 17).
13. Wash two times 2 min in PBS 1×.
14. Dehydrate slides in successive ethanol baths (70, 80, 90, and 100%), 2 min each.
15. Let the slides air dry in the dark.
16. *PNA hybridization*: If lyophilized, prepare a master tube of PNA probe, 1 μM, in hybridization buffer (70% formamide, 20 mM Tris pH 7.4). Vortex for 2 min and heat at 70°C for 5 min, spin 3–4 min at top speed. Prepare a working solution at 10 nM final in hybridization solution containing blocking agent (see Note 18).
17. Cover the slides with at least 30 μL of the hybridization solution and overlay with a coverslip avoiding bubbles.
18. Denature by heating at 80°C for 3 min (see Note 19).
19. Incubate for 2 h at RT in the dark (see Note 20).
20. Wash two times 15 min in wash buffer (70% formamide, 20 mM Tris pH 7.4) (with agitation).
21. Wash three times 5 min in 50 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween 20.
22. Dehydrate slides in successive ethanol baths (70, 80, 90, and 100%), 2 min each.
23. Let the slides air dry in the dark.
24. *LNA hybridization*: If lyophilized, prepare a master tube of PNA probe, 1 μM, in hybridization buffer (70% formamide, 20 mM Tris pH 7.4). Vortex for 2 min and heat at 70°C for 5 min, spin 3–4 min at top speed. Prepare a working solution at 50 nM final in 50% deionized formamide, 2× SSC and blocking agent.

25. Distribute at least 35 µL of the hybridization solution per slide and overlay with a coverslip avoiding bubbles.
26. Incubate for 2 h at RT in the dark (see Note 21).
27. Wash two times 15 min in formamide 50% SSC 2× (with agitation).
28. Wash three times 5 min in 50 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween 20.
29. Dehydrate slides in successive ethanol baths (70, 80, 90, and 100%), 2 min each.
30. Let the slides air dry in the dark.
31. Mount coverslip with 20 µL of Vectashield (DAPI 0.2–0.5 µg/mL) (see Note 22).
32. Slides can be stored at 4°C.

### 3.4. Visualization of Metaphases and Image Analysis

1. Search for metaphases under the UV microscope using the DAPI channel. Depending on the number of metaphases available, you may choose those in which individual chromosomes are in good shape and well spread so that a karyotype analysis may be eventually carried out. Visualize telomeric signals using the appropriate filter (red for Cy3 and green for FITC/Fam) (Fig. 1). Set the camera gain to a maximum and choose a time of exposure, specific for each color, that allows exponential

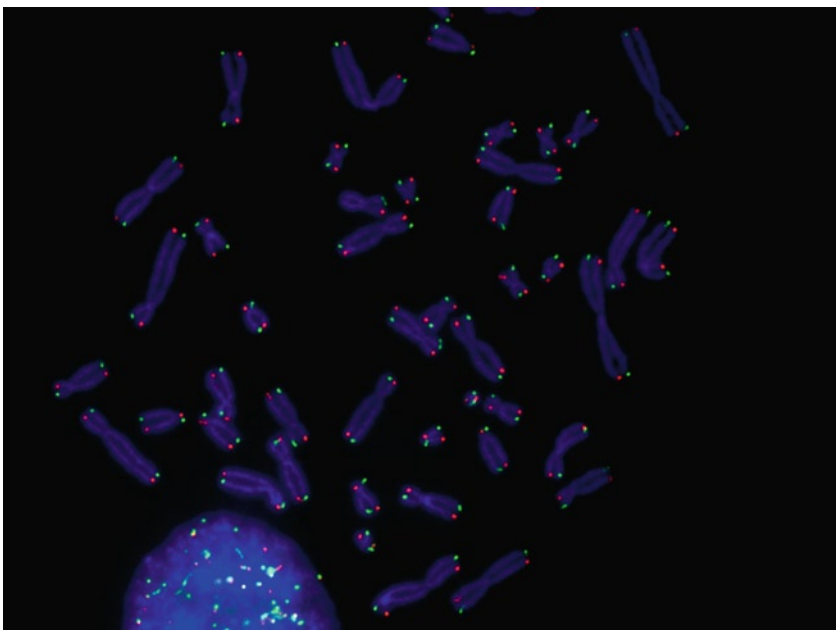

Fig. 1. Metaphase chromosomes after the CO-FISH procedure, hybridized with both a C-rich PNA telomeric probe $(CCCTAA)_3$ and a G-rich LNA telomeric probe.

acquisition of most signals while avoiding saturation of the strongest ones. Once you have determined the most adequate time of exposure for green and red signals, apply it to all pictures taken across all samples belonging to the same experiment (see Note 23). 25–30 metaphases should allow a statistical analysis of telomere intensity. Metaphases are captured using the appropriate channels and the original, unmodified black and white images are saved for later analysis.

2. The quantitative analysis is carried out with the help of dedicated software. Any software able to determine the mean intensity of a pixel can be used. However, analysis is facilitated by the use of software that allows both the merging of original images into color pictures, the segmentation of signals based on pixel value and the writing of scripts that allow a semiautomatic quantification. Briefly, single telomeric signals are segmented either manually or automatically, assigning different segment colors to G-rich and C-rich strands, and the mean pixel intensity for all telomeres replicated by lagging or by leading mechanisms is calculated through available software functions. Background fluorescence is measured by segmenting the interstitial regions of chromosomes. The data is exported in text format, which can be recovered on an "excel" sheet for analysis. The mean fluorescence intensity for G-rich or C-rich signals is calculated, once the background of the metaphase has been subtracted (see Note 24). Distributions of single telomere intensities are also informative (e.g., see ref. (21). Statistical comparisons are best carried out using Wilcoxon rank-sum tests.

### 3.5. Q-FISH Followed by CO-FISH

In cases where telomere length is limiting, thus preventing the recording of reliable fluorescence intensities, it is possible to apply a telomeric CO-FISH procedure (using G-rich and C-rich probes) after a first hybridization for telomeric Q-FISH (that used a single telomeric probe, usually a C-rich PNA probe) (Fig. 2). This allows the identification of replication mechanisms used by sister telomeres (by CO-FISH) and the comparison of fluorescence intensities

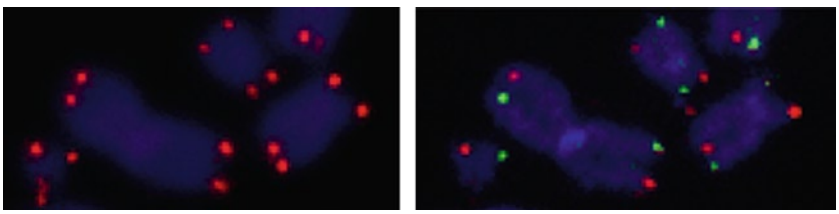

Fig. 2. Example of chromosomes with telomeres revealed after a Q-FISH procedure (*left*) and later after a CO-FISH procedure (*right*).

(recorded in the Q-FISH procedure) associated with chromatids replicated by lagging and leading. In this case, a direct comparison between sister telomeres is possible since the Q-FISH has revealed on each of them the same strand (G-rich or C-rich) with the same probe.

The procedure is as follows:

1. A Q-FISH protocol is applied (as described in the dedicated chapter) to cells that have been exposed to base substitutes BrdU/BrdC (first step of the CO-FISH protocol). When capturing metaphases, record the position coordinates. Microscopes with a motorized stage piloted by a module in phase with the imaging software constitute the best option. However, manual annotations of XY-coordinates are also possible through the Vernier scales typically present in any manual stage. Before the second procedure, clean as thoroughly as possible, the immersion oil from the coverslip.
2. Immerse slides in 2× SSC until the coverslip come off gently. Change the bath once to also wash the mounting medium off.
3. Dehydrate slides in successive ethanol baths (70, 80, 90, and 100%), 2 min each.
4. Let the slides air dry in the dark.
5. In a hot (78°C) 70% formamide/10 mM Tris pH 7.4 bath, denature slides for 5 min (do not immerse too many slides at the same time so that the bath remains at the same temperature).
6. Quick wash in 50 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween 20.
7. Fix cells 2 min in 3.7% formaldehyde in PBS.
8. Wash 3 × 5 min in PBS.
9. You can resume the CO-FISH protocol at the Hoechst treatment step.
10. After the procedure is finished, retrieve the metaphases using the recorded coordinates. Using color-merged CO-FISH images showing blue/green/red signals, identify the strands replicated by either leading or lagging mechanisms. On the color-merged Q-FISH images, manually segment telomere signals with different colors dependent on their mode of replication. Also include a few segments on interstitial parts of chromosomes to measure background. Quantify the mean intensity associated with specific sister telomeres.

## 4. Notes

1. Several formulae have been proposed, including a mixture of (V/V/V) Na citrate 8 g/L/KCl 0.075 M/$H_2O$, 1/1/1. The sensitivity to hypotonic swelling by any of these solutions should be tested for each cell type or cell line.
2. To reduce toxicity, we have replaced methanol, classically used in this preparation, by ethanol. Always use freshly prepared fixative.
3. Both C-rich- and G-rich-specific probes are required to reveal the telomeric strands that had replicated by lagging and leading mechanisms, respectively. G-rich PNA probes, however, are rather insoluble, and therefore we prefer to use telomere-specific LNA probes to reveal the strand replicated by leading. It is also possible to use LNA probes to reveal both G-rich and C-rich strands. A C-rich LNA probe used with success is: CCCTA+AC+CCT+A+AC+CCTAAC+CCT+A+ACCCT+A +ACCCT+AA, where the LNA bases are preceded by a + sign. The label, on both 3 and 5 , should be different from that on the G-rich probe.
4. Bringing the solution to pH 2 may be a little laborious, and therefore an alternative is to prepare a solution of water with a few drops of concentrated HCl. However, buffering is potentially important to preserve chromosome structure and allow optimal pepsin enzymatic activity.
5. Many types of blocking agents for hybridization reactions are commercially available. Any of those, as well as a BSA solution, can be used to decrease background.
6. Formamide should be deionized before use. However, we only do this routinely for formamide solutions that are used to prepare hybridization reactions. The current quality of commercially available formamide is enough to be directly used in wash steps. After deionization, formamide solutions should be stored at –20°C.
7. Most types of CCD cameras allow the acquisition of digitalized image files adapted to quantification analysis. A good dynamic range can be obtained with 12-bit gray-scale images. Since retrieval of images may become a crucial issue, it is best to have a module able to control (at least on XY-axes) a motorized stage and record slide positions.
8. The original dedicated computer program TFL-TELO developed by Lansdorp et al. (24) is available from the BC Cancer Agency (Vancouver, Canada). Other freewares such as NIH image or Image J can also be used for quantification purposes.
9. The incubation time with base substitutes should cover the entire S-phase. Therefore, depending on the doubling time,

incubations can be adjusted to 18–30 h. However, it is best to synchronize cells with a double thymidine (or thymidine/aphidicolin) block and to release them in S-phase in the present of the base substitutes, as described (23). This requires, though, a rather precise characterization of the cell cycle by FACS so that cells are collected as they accumulate in M upon colcemid treatment.

10. Depending on the mitotic index, the incubation time with colcemid varies. However, if cells divide slowly, avoid long incubation times with colcemid since this may result in metaphases preparations from cells that have not been in contact with base substitutes for the entire S-phase. It is better to synchronize cells, in which case 1 h should be sufficient. Nocodazol can also be used.
11. It is necessary to determine the best combination of hypotonic solution and incubation time for every cell line. For instance, some embryonic stem cells are extremely sensitive, whereas some fibroblast-derived cell lines are much more resistant. The Na citrate solution is gentler than the KCl hypotonic solution. As an example, we incubate HT1080 cells as well as human primary fibroblasts in 10 mL of Na citrate (8 g/L) for 40 min at 37°C.
12. Although metaphase suspensions in fixative are very stable, long-term storage is not recommended for quantitative analysis of telomere fluorescence.
13. A slightly cloudy suspension is most of the time appropriate. However, if the mitotic index is high, much more diluted suspensions can be used.
14. Conditions for optimal metaphase spreading are not obvious to master. Dropping the cell suspension from a high height is not an absolute requirement since dropping from a few centimeters high is enough to get good spreading. Humidity seems more crucial and placing the perfectly clean, dried glass slide on top of a wet napkin often helps one to obtain acceptable spreading from certain difficult-to-work-with suspensions or when the ambient air is too dry.
15. Long-term storage is not recommended for quantitative analysis of telomere fluorescence.
16. Pepsin should be prepared freshly. Digestion time can be prolonged if cytoplasmic material is visible under phase contrast microscope. Eliminating this cytoplasmic material greatly reduces background problems.
17. Incubation time is crucial. Most of the time, 5 min is enough to achieve a complete degradation of substituted strands, even in cells with long telomeres. However, the time of incubation can be adjusted if incomplete degradations are observed. On the other hand, the ExoIII enzyme is quite

labile, and therefore it should be replaced if doubts persist about the completeness of the reaction.

18. When combining PNA and LNA probes for CO-FISH, the PNA probe should be used first, since hybridization buffer and washing conditions are more astringent (higher formamide concentration and lower ionic strength). Master solutions of PNA probes can be kept for 2–3 months. They, however, have a tendency to precipitate. Heating increases solubility. The final concentration can be increased up to 50 nM without substantially increasing the background. The hybridization solution can be prepared in advanced, aliquoted and stored at –20°C. We usually prepare the hybridization buffer with deionized formamide and the blocking reagent is added just to working dilution of the probe. LNA probes are more soluble than PNA probes, but master solutions are probably less stable for long-term storage. The final concentration for LNA probes is 50 nM in 50% formamide, 2× SSC, and blocking reagent.
19. Although a denaturation step is theoretically no longer required since sister chromatids have been converted into single strands through degradation of the newly synthesized strand by ExoIII, denaturing clearly improves hybridization efficiency.
20. The kinetics of hybridization of PNA probes is very fast and reactions are probably completed after 30 min. 2 h is also sufficient for LNA probes.
21. Do not denature! 2 h incubation should be enough for LNA hybridization.
22. A strong DAPI staining will appreciably bleed into the green channel thus interfering with signal quantification, if a green fluorochrome-labeled probe is used.
23. Because of both day-to-day strength fluctuations and wearing off of the UV lamp, comparisons between images captured on different sessions (days) are difficult, even if the same time of exposure has been used, although intensity fluctuations appear to be less marked with hybrid mercury/xenon lamps. To correct for these variations, independent controls (such as fluorescent beads or hybridization samples with known telomere repeat content – i.e., plasmids) are to be included in every acquisition session to normalize fluorescence intensity values. Because of the many differences between G-rich and C-rich hybridizations (base composition, affinity, and fluorochromes), it is not possible to make direct comparisons between fluorescence intensities associated with lagging and leading strands. Comparisons should be done either between leading strands (control versus treated) or between lagging

strands (control versus treated). For direct comparisons between these strands, the Q-FISH/CO-FISH approach, described hereafter, is more suitable.

24. We have used the mathematical functions of the iVision software (BioVision technologies). It is possible that freely available softwares such as TLF-TELO (24) can also be used.

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# Chapter 5

# Telomere G-Overhang Length Measurement Method 1: The DSN Method

**Yong Zhao, Jerry W. Shay, and Woodring E. Wright**

## Abstract

Telomeres terminate in 3′ single-stranded G-overhangs that function in telomere end protection and telomerase action. An accurate measurement of overhang length is challenging due to the presence of many kilobases of double-stranded telomere DNA. Here, a simple method is described that utilizes duplex-specific nuclease (DSN) to digest all genomic DNA including telomeres, leaving the single-stranded overhangs intact. The telomere single-strand G-rich overhang length can then be determined by Southern blot-based assays.

**Key words:** Telomere, G-overhang, Duplex-specific nuclease, Genomic DNA, Southern blot

## 1. Introduction

Mammalian telomeric DNA consists of repetitive hexamers of TTAGGG, ending in a single-stranded G-overhang at the 3′ ends of the chromosome (1). Telomeric overhangs are thought to be involved in the formation of T-loops, in which the single-stranded overhang invades the double-stranded telomere, forming a so-called D-loop (2). This structure is thought to hide the chromosome ends from being recognized as DNA double-stranded breaks. Telomeric overhangs play an essential role in the protection of telomeres from end-to-end fusions, abnormal recombination, degradation (3), and serve as the substrate of telomerase in vivo.

As G-overhangs perform many critical functions, it is important to have an assay that can accurately determine the length of telomeric overhangs. Current overhang assays fall into two groups: relative analysis and absolute length measurements. With relative analysis such as the non-denaturing hybridization (4) and HPA methods (5), the relative strength of overhang signals with respect

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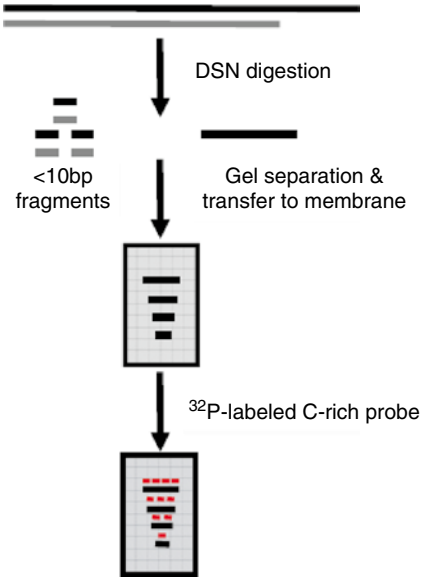


Fig. 1. Strategy of DSN assay to determine the length of telomeric overhangs.

to total telomeric DNA is obtained. In comparison, the absolute length measurements, such as primer extension–nick translation (PENT) (6), telomeric-oligonucleotide ligation assay (T-OLA) (7), and electron microscopy methods (1), provide an actual overhang length but fail to detect very short overhangs. In this chapter, we present details of a new method for the direct measurement of overhang length that utilizes Kamchatka crab duplex-specific nuclease (DSN). DSN is a newly characterized endonuclease that is highly specific for double-stranded (ds) DNA and is practically inactive on single-stranded (ss) DNA (8). DSN is able to digest double-stranded DNA into <10 bp fragments while leaving the single-stranded telomeric overhangs intact (9). These single-stranded overhangs can then be used for size determination on Southern blots upon hybridization with a telomere-specific probe (Fig. 1). This procedure has been successfully applied to analyze overhang lengths as short as 12 nt in human cells (9).

## 2. Materials

### *2.1. Cell Harvest and DNA Isolation*

1. 1× PBS, pH 7.4, made from 10× PBS (Gibco).
2. DNeasy blood and tissue kit (Qiagen).
3. RNase A (100 mg/mL, Qiagen).
4. 100% Ethyl alcohol.
5. Millipore water with 5 mM Tris–HCl, pH 8.0.

### 2.2. DSN Digestion

1. Exonuclease I, 20 U/μL (Epicentre Biotechnologies).
2. Duplex-specific nuclease DSN (EVROGEN, Moscow, Russia) prepared as 0.2 U/μL following instructions provided by manufacture and stored at −20°C.
3. 10× DSN buffer provided by the manufacture containing 500 mM Tris–HCl pH 8.0, 50 mM $MgCl_2$, and 10 mM DTT.
4. 0.5 M EDTA, pH 8.0.
5. 37°C Water bath or PCR machine.

### 2.3. Electrophoresis on Alkaline Agarose Gel and Transfer to Membrane

1. 10 M NaOH and 0.5 M EDTA.
2. 2× Loading dye: 100 mM NaOH, 2 mM EDTA, 5% ficoll (type 400), and 0.05% bromophenol blue (Bio-Rad).
3. Alkali electrophoresis buffer: 50 mM NaOH and 1 mM EDTA.
4. UltraPure™ Agarose (Invitrogen).
5. Telomeric DNA markers: synthesized 36, 54, and 96-mer oligonucleotides of repetitive telomere sequence were used as low MW DNA markers; high MW telomeric markers were made as described by Chai et al. (10) (see Note 1).
6. Amersham hybond™-XL (GE Healthcare).
7. 10× SSC (transfer buffer), made from 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
8. 3MM Whatman paper.

### 2.4. Making a High-Specific Activity Telomere C-Rich Probe

1. 10 pmol/μL $GTU_4$ oligonucleotide dissolved in TE buffer: 5′-GGGUUAGGGUUAGGGUUAGGGAAA-3′.
2. 100 pmol/μL $T_3C_3$+9 oligonucleotide ($T_3C_3$ is complementary to the 3′ end of $GTU_4$ above, and +9 refers to 9 nt of telomeric repeats) dissolved in TE buffer: 5′-TTTCCCTAA CCCTAA-3′.
3. 1 M NaCl.
4. 10× buffer M: 100 mM Tris–Cl (pH 7.5), 100 mM $MgCl_2$, 500 mM NaCl, and 10 mM dithioerythritol.
5. 2 M Tris–HCl, pH 7.4–7.6.
6. 10 mg/mL BSA (Ambion, Inc.).
7. 1.25 mM dAdT: 1.25 mM each of dATP and dTTP.
8. [α-$^{32}$P]dCTP (3,000 Ci/mmol) (PerkinElmer).
9. 5 U/μL Klenow large fragment of *Escherichia coli* DNA polymerase I (NEB).
10. 1 U/μL Uracil deglycosylase (UDG) (Invitrogen).
11. PCR machine.

### 2.5. Hybridization and Washing Buffer

1. Hybridization buffer: 6× SSC, 5× denhardt's solution, and 0.5% (w/v) SDS.
2. Washing buffer 1: 2× SSC and 0.1% SDS.
3. Washing buffer 2: 0.5× SSC and 0.1% SDS.
4. UVC 500 Crosslinker (GE Healthcare, Inc.).
5. Hybridization oven (Hybridiser HB-1D, Techne, Inc.).

### 2.6. Image Capture and Data Analysis

1. Typhoon™ (GE Healthcare).
2. Phosphor screen (Molecular Dynamics).
3. Software: ImageQuant 5.2 and IQTools 3.0.

## 3. Methods

### 3.1. Cell Harvest and DNA Purification (see Note 2)

1. Harvest cells by trypsinizing and spinning at 500 × *g* for 5 min, wash the cell pellet once with 1× PBS and resuspend the cells in 1× PBS ($5 \times 10^6$ cells/200 μL).
2. Isolate genomic DNA following the DNeasy kit instructions with a slight modification: instead of adding RNase A before lysing the cells, add it afterwards and incubate at RT for at least 10 min.
3. Reprecipitate the DNA by adding two volumes of 100% ethanol and spinning down.
4. Wash DNA pellet twice with 70% ethanol.
5. Let DNA pellet dry (not too dry) at room temperature. It will take 10 min to 1 h depending on the size of the pellet.
6. Resuspend the DNA pellet in deionized water (Millipore) containing 10 mM Tris–HCl, pH 8.0.
7. Incubate DNA in a 37°C water bath overnight to allow genomic DNA to completely dissolve in solution and to disrupt any potential overhang structures.
8. Measure DNA concentration. (The ratio of 260/280 must be between 1.8 and 1.9.)

### 3.2. DSN Digestion of Genomic DNA

1. Prepare samples for DSN digestion:

| | |
|---|---|
| Genomic DNA(5 μg) | χ μL (dependent on DNA concentration) |
| 10× DSN buffer | 2 μL |
| $H_2O$ | 17–χ μL |
| DSN (0.2 U/μL) | 1 μL |
| Total | 20 μL |

2. As a control, add 10 U (0.5 μL) ExoI to genomic DNA instead of DSN in the above reaction solution and incubate at 37°C for 1 h to digest 3′ overhangs. Then add DSN.
3. Perform DSN digestion at 37°C for 2 h (see Note 3).
4. Stop reaction by adding 0.5 μL 0.5 M EDTA (pH 8.0).

### 3.3. Electrophoresis of Telomeric Overhangs on Alkaline Agarose Gels and DNA Transfer to Membranes (see Note 4)

1. Add the correct amount of agarose to a measured quantity of boiling Millipore water to make a 1.2% gel.
2. Cool the solution to ~50°C, add 10 M NaOH and 0.5 M EDTA to a final concentration of 50 and 1 mM, respectively.
3. Make the gel. After it sets, add sufficient alkaline electrophoresis buffer to cover the gel.
4. Add the same volume (20 μL) of 2× loading dye to each sample.
5. Load the samples; telomeric DNA markers are loaded on both sides of the gel, perform electrophoresis in a cold room (4°C) at low voltage (1–2 V/cm) until the dye has migrated approximately 6–8 cm.
6. Set up a blotting tray for the capillary transfer as shown in Fig. 2.
7. Carefully place the gel on 10× SSC presoaked Whatman paper with both of its sides draped in transfer buffer and remove all bubbles in between gel and paper.
8. Cut a piece of Hybond™-XL membrane and two pieces of 3MM Whatman paper slightly larger than gel, wet the membrane in $dH_2O$ and then soak it in transfer buffer until ready, wet Whatman filter paper in transfer buffer.
9. Cut four strips of SaranWrap™ to cover around the edge of the gel.
10. Carefully overlay membrane on top of the gel, roll away all bubble between gel and membrane.
11. Lay wet Whatman filter paper, one at a time, on the top of the membrane, making sure no bubbles are introduced.

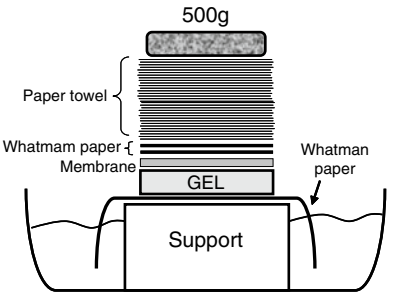


Fig. 2. Illustration of upward capillary transfer.

12. Place a stack of paper towels (5–10 cm high, cut to appreciate size) over the Whatman filter paper. Put glass plate on top of paper towels and apply a weight (such as a 2 in. book) on top.
13. Allow capillary transfer to occur overnight.
14. Remove the membrane from the gel and let it dry in air for 1 h.
15. Fix DNA on the membrane by UV cross-linking at 70 mJ/cm$^2$ in a commercial UV crosslinker.

### *3.4. Making a High-Specific Activity Telomere C-Rich Probe (see Note 5)*

1. Make 1.7 pmoL/μL annealed oligonucleotide template by mixing the following:

| | |
|---|---|
| 10 pmol/μL GTU4 oligonucleotide | 3.4 μL |
| 100 pmol/μL T3C3+9 oligonucleotide | 15.6 μL |
| 1 μL 1 M NaCl | 1 μL |
| Total | 20 μL |

2. Incubate for 1 min at 99°C, 15 min at 37°C, and 15 min at room temperature, the annealed template is stored at –20°C.
3. Make 8× modified buffer M, by mixing the following:

| | |
|---|---|
| 10× buffer M | 500 μL |
| 2 M Tris–HCl, pH 7.4–7.6 | 100 μL |
| 10 mg/mL BSA | 25 μL |
| Total | 625 μL |

4. Prepare the reaction mixture:

| | |
|---|---|
| 8× buffer M | 3.125 μL |
| Annealed template oligo | 1 μL |
| 1.25 mM dAdT (final 50 μM) | 1 μL |
| $dH_2O$ | 13.875 μL |
| [α-$^{32}$P)dCTP | 5 μL |
| Klenow large fragment | 1 μL |
| Total | 25 μL |

5. Incubate the reaction mixture at room temperature for 30 min and then 95°C for 5 min.
6. Add 0.5 μL of 1 U/μL UDG and incubate for 10 min at 37°C and then 10 min at 95°C, the probe is ready to use.

### 3.5. Hybridizing, Washing, Exposing on PhosphorImager Screens and Imaging

1. Prehybridize the membrane at 42°C for at least 30 min using hybridization buffer.
2. Add 5–10 μL high-specific activity telomere C-rich probe to hybridization buffer to a concentration of 1 μL/mL.
3. Hybridize at 42°C overnight.
4. After the hybridization, wash the membrane by incubating twice, 15 min each, in washing buffer 1, followed by washing buffer 2 for 15 min at 42°C.
5. Remove the membrane from the last wash, drain and wrap in SaranWrap, expose to PhosphorImager screens for an appropriate time (unusually 2–3 h is enough to get a good signal, but overnight exposure is recommended to obtain better images).
6. After exposure, scan PhosphorImager screen on Typhoon.

### 3.6. Analysis of Data to Obtain Mean Length of the Telomeric Overhang

1. Divide each lane into 100–300 intervals from the highest MW to the lowest using ImageQuant 5.2.
2. Determine the MW for each interval by fitting the molecular weight ($Y$) and migration distance ($X$) of each marker to one phase exponential decay to generate a standard curve using the following function:

$$Y = (Y_0 - \text{Plateau}) \times \exp(-KX) + \text{Plateau},$$

where $K$, $Y_0$, and Plateau are the constants for each fitting.

3. Use the ExoI treated sample as a background and subtract its signal from that of the untreated sample at each measured size interval.
4. Calculate the average overhang length as the weighted mean using the following formula:

$$\text{Mean overhang length} = \frac{\Sigma(\text{OD}_i)}{\Sigma(\text{OD}_i / L_i)},$$

where $\text{OD}_i$ is the signal intensity and $L_i$ is the size of the overhang at position $i$.

## 4. Notes

1. Telomeric DNA markers can be replaced by commercial DNA markers with 5′-ends labeled by $^{32}$P.
2. DSN is highly sensitive to ionic strength, and the presence of salt results in a dramatic decrease in catalytic activity. Therefore, it is important to avoid any unnecessary salt input. To get rid of any potential contaminations from the purification process,

we reprecipitate the genomic DNA, wash with 70% ethanol twice and dissolve in low-salt solution. If DNA is from other sources, the same protocol is highly recommended.

3. The maximum activity of DSN is 65°C, but this will melt small DNA fragments well before the limit size of <10 bp is reached.
4. Overhangs vary in size and give a smear. This smear is more compact and easier to see using 1.2% agarose gels compared with 8% polyacrylamide gels, but either can be used.
5. This primer extension protocol is designed for synthesis of super-sensitive probes that have six $^{32}$P nucleotides incorporation (for details, see ref. 11). Using regular $^{32}$P end-labeled C-rich probes may not be sensitive enough to detect telomeric overhangs from 5 μg genomic DNA. The use of dU and uracil deglycosylase to remove the G-strand template maximizes the hybridization of the single-stranded C-rich probe to the G-rich overhangs.

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# Chapter 6

# Telomere G-Overhang Length Measurement Method 2: G-Tail Telomere HPA

Hidetoshi Tahara

## Abstract

Both telomere length and telomere G-tail length are altered in human diseases such as cancer and age-related disease. While most methods for the measurement of G-tail and telomere length require electrophoresis, centrifugation, radioisotope labeling, and autoradiography, G-tail telomere HPA provides a convenient and useful tool for the examination of G-tail length with a high-throughput platform using genomic DNA or cell lysate. G-tail telomere HPA may be applicable for clinical diagnostics as well as drug target screening.

**Key words:** G-tail, Hybridization protection assay, High-throughput, Risk assessment, Cancer, Aging

## 1. Introduction

Mammalian telomeric DNA consists of 5′-$(TTAGGG)_n$-3′ double-stranded repeats, followed by up to several hundred bases of G-rich single-stranded 3′-overhang, the so-called telomere G-tail (1, 2). Telomeres are gradually shortened with cell division due to problems with end replication (3, 4). Telomere repeat sequences are synthesized by the telomerase, a cellular ribonucleoprotein reverse transcriptase (5). Expression of the catalytic subunit of the human telomerase gene, hTERT, correlates with the presence of telomerase activity in human cells (6), and introduction of the hTERT gene alone into normal cells is sufficient to induce telomerase activity, followed by telomere elongation and cell immortalization (7, 8). The single-stranded telomere G-tail is a key structure that protects telomere DNA from DNA damage and chromosome instability. The G-tail invades into double-stranded telomere DNA, leading to the formation of the so-called

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t-loop. G-tail length is essential for chromosome capping (9). In addition, shelterin proteins TRF1, TRF2, POT1, TPP1, TIN2, and Rap1 also required for t-loop formation (9). Knockdown of these genes can affect G-tail length alterations and chromosome stability (10, 11). For example, when either TRF2 or POT1 is inhibited by dominant negative forms of the proteins, the overall amount of G-tail is diminished (12, 13). In the case of TRF2 inhibition, ERCC1/XPF-deficient cells retained the G-tail after TRF2 inhibition, suggesting that the loss of G-tail might involve ERCC1/XPF NER endonuclease which can remove 3′-overhang DNA (14). Taken together, G-tail alterations may be a good indicator for stability of the t-loop structure that protects chromosome ends.

Hybridization protection assay (HPA) utilizes probes that are labeled with a nonradioactive chemiluminescence compound – acridinium ester (AE) detector molecule that emits a chemiluminescent signal, and has been used for the detection of total telomere length and telomerase activity (15, 16). HPA reaction does not require physical separation of unhybridized vs. hybridized probe, therefore, HPA-based assays including G-tail telomere HPA can be applicable for high-throughput screening.

## 2. Materials

1. Hybridization buffer: 0.1 M succinic acid, 0.23 M lithium hydroxide monohydrate, 2% lithium lauryl sulfate, 1.2 M lithium chloride, 20 mM EDTA·2Na, 20 mM EGTA, 15 mM 2,20-dithiodipyridine, adjusted to pH 4.7 with HCl.
2. Hydrolysis buffer: 0.6 M boric acid, 182 mM NaOH, 1% Triton X-100, adjusted to pH 8.5 with NaOH.
3. Synthesized human telomere oligonucleotide: an 84mer of 5′-$(TTAGGG)_{14}$-3′ or a 35mer of 5′-$(TTAGGG)_6$-TTAGG-3′.
4. Acridinium ester (AE)-labeled telomere probe: 5′-CCCTAACCCTAACC*CTAACCCTAACCCTA-3′ [*AE position, $8 \times 10^7$ rlu/pmol probe DNA made by Fujirebio, Inc., (Tokyo, Japan)].
5. GEN-PROBE detection reagent kit: Catalog No. 1791, Kit contains Detection Reagent I (0.1% hydrogen peroxide in 0.001 N nitric acid) and Detection Reagent II (1 N sodium hydroxide).
6. Spectrophotometer such as NanoDrop (Thermo scientific).
7. Luminometer (Leader I, Gen-Probe, Inc., San Diego, CA).

## 3. Methods

### 3.1. Preparation of Genomic DNA for G-Tail Telomere HPA

1. Purify genomic DNA from cultured cells or clinical samples including tissues and blood using a phenol–chloroform-based method (see Note 1).
2. Store genomic DNA at 4°C or –20°C until use, or proceed directly to step 3.
3. Shear genomic DNA using a 24 G syringe at least ten times. Be sure to confirm that DNA solution is homogenous. This step does not have an effect on G-tail length or total telomere length.
4. Centrifuge at 8,000 × *g* for 5 min.
5. Collect the supernatant and transfer to new tube.
6. Measure DNA concentration by NanoDrop.
7. Store at –20°C until ready to use.

### 3.2. Preparation of Diluted Telomere Oligonucleotide and Diluted Genomic DNA Samples for G-tail Telomere HPA

1. Make a series of dilutions of the synthesized human telomere oligonucleotide for making a standard curve of the assay: 1.0, 0.5, 0.1, 0.05, 0.01, and 0.005 nM (see Note 2).
2. Transfer 10 μL of each dilution of the oligonucleotide into a 5 mL polypropylene tube and add 90 μL of sterilized water. Make a duplicate for each dilution, and a pair of blanks without oligonucleotide. Proceed to Subheading 3.3, step 1, after preparation of diluted genomic DNA.
3. For the detection of telomere G-tails in genomic DNA, 5 μg of non-denatured human genomic DNA is typically used for each assay. Dilute purified genomic DNA with TE buffer to 5 μg/100 μL. Prepare 400 μL of diluted genomic DNA, if both G-tail and total telomere length are measured (see Note 3).

### 3.3. G-Tail Measurement by G-Tail Telomere HPA (Fig. 1)

1. Transfer 100 μL of each diluted genomic DNA sample (Preheat at 50°C is recommended) to a Falcon BD 352053 tube.
2. Transfer 100 μL (containing 5 μg) of the non-denatured genomic DNA to 5 mL polypropylene tubes. Make a triplicate for each sample.
3. Denature 80 μL of remaining genomic DNA at 99°C for 10 min, and then immediately transfer to iced water and cool for 2 min.
4. For the detection of double-stranded telomere regions with G-tails (total telomere) in genomic DNA, 1 μg denatured human genomic DNA is typically used. Transfer 20 μL (containing 1 μg) of the genomic DNA to a 5 mL polypropylene tube, and add 80 μL of sterilized water. Make a triplicate for each sample.

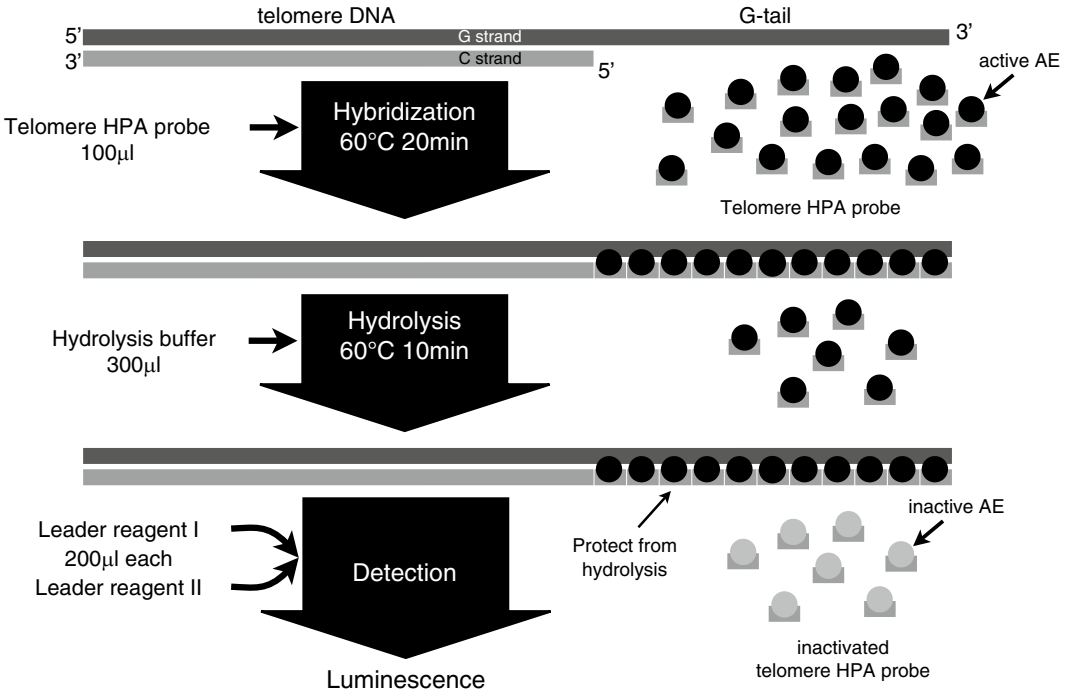


Fig. 1. The principle of G-tail telomere HPA. G-tail telomere HPA consists of three steps: "hybridization," "Hydrolysis," and "Detection" for the measurement of relative G-tail length from purified genomic DNA as well as nonpurified cell lysate using single tube. First step, "hybridization step": incubation of non-denatured genomic DNA with acridinium ester-labeled telomere HPA probe at 60°C for 20 min. Second step, "Hydrolysis step": incubation of reaction solution at 60°C for 10 min after adding hydrolysis buffer to inactivate unhybridized telomere HPA probe. Hybridized telomere HPA probe can be protected the inactivation of acridinium ester from hydrolysis. Last step, "Detection step": detection of luminescence from hybridized probe after adding Detection buffer I and Detection buffer II using autoinjector-equipped luminometer.

5. Dilute AE-labeled telomere HPA probe to $3 \times 10^5$ rlu (relative light units)/mL.
6. Add 100 μL of diluted telomere HPA probe to the bottom of each tube for detecting standard telomere oligo, G-tails, and total telomere (final $3 \times 10^5$ rlu/assay). Vortex at maximum speed for 5 s.
7. Incubate all the tubes simultaneously in a 60°C water bath for 20 min without agitation. Cover top of tubes with aluminum foil and protect from light (see Note 4).
8. Remove all tubes to room temperature from the water bath and let them sit for 10 min.
9. Add 100 μL of hydrolysis buffer. Vortex at maximum speed for 5 s (see Note 5).
10. Incubate all the tubes simultaneously in the 60°C water bath for 10 min.
11. Transfer all tubes to ice cold water and leave for over 1 min.

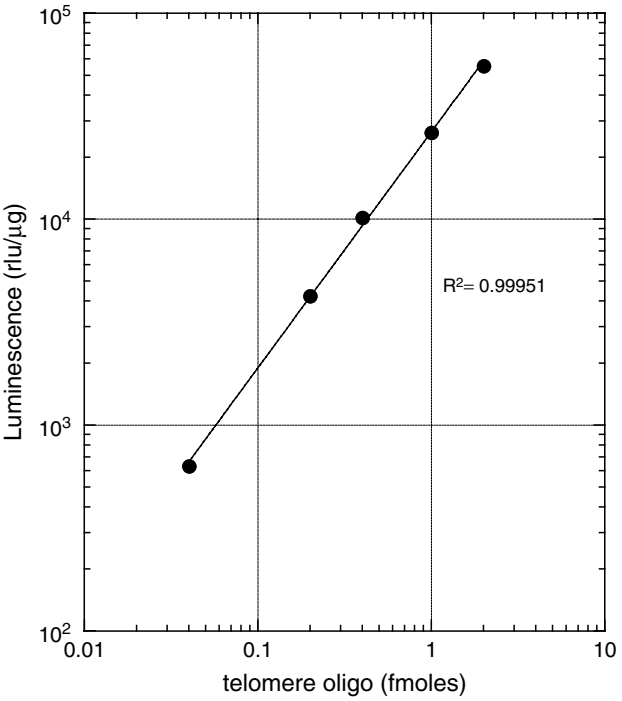


Fig. 2. The typical data of standard curve of G-tail telomere HPA using telomere oligonucleotide.

12. Measure chemiluminescence after injection of Detection Reagent I and Detection Reagent II using the autoinjector for 2 s per tube with a luminometer (see Note 6).
13. Luminescence value, RLU (relative luminescence unit), should be normalized to actual genomic DNA concentration as measured in Subheading 3.1, step 6.
14. Typical results of standard curves using telomere oligonucleotides are shown in Fig. 2. R2 is typically nearly 0.9999.
15. Typical results of the G-tail assay using genomic DNA are shown in Fig. 3. The luminescence signal (RLU) was normalized with the amount of genomic DNA in the tubes.

## 4. Notes

1. Do not use any genomic purification kits that utilize filters or beads.
2. Diluted oligonucleotide cannot be reused even if stored at –20°C or –80°C. Make fresh every time.

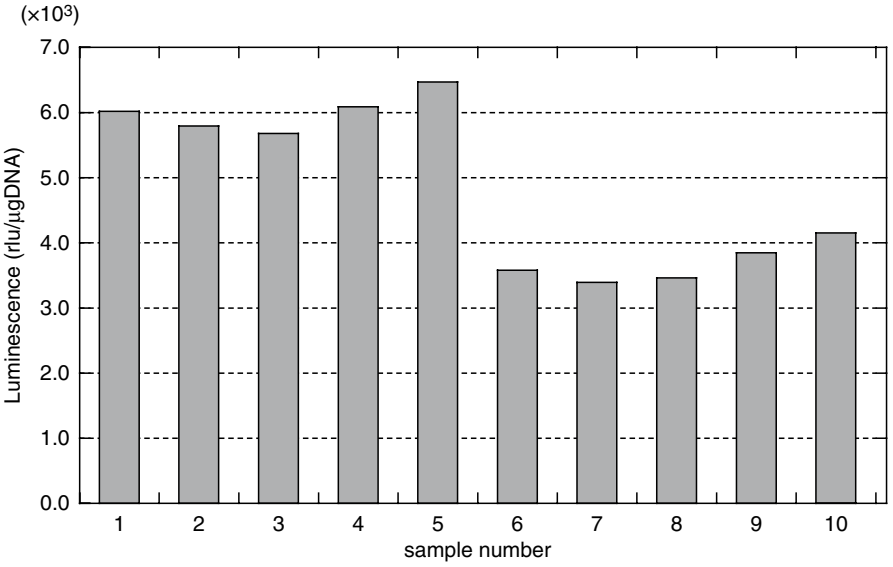


Fig. 3. The typical data of relative G-tail signals of human genomic DNA samples isolated from different persons using G-tail telomere HPA.

3. To normalize by the amount of genomic DNA, take 2 μL of DNA solution before starting following step. This step eliminate to measure DNA exact amount in each tube.
4. Do not use a heat block without water for this incubation step.
5. Make sure all reaction solution should be mixed with hydrolysis buffer, residual solution may cause nonspecific signals due to insufficient inactivation of unhybridized probe.
6. An autoinjector is essential for this assay. Luminescence should be counted from 0 to 2 s. Follow the instruction manual of the equipment.

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# Chapter 7

# Telomere Terminal G/C Strand Synthesis: Measuring Telomerase Action and C-Rich Fill-In

**Yong Zhao, Jerry W. Shay, and Woodring E. Wright**

## Abstract

Telomerase is present in most human cancers, and proliferative stem cells including germline cells. Telomerase plays an essential role in tumorigenesis by maintaining/elongating telomeric DNA, and thus preventing the telomere shortening that results in replicative senescence. Understanding telomerase action in vivo has important implication for both cancer and aging, but there are not robust methods for monitoring telomerase action. By combining a series of cell biological and biochemical approaches, and taking advantage of the enzyme DSN that specifically cuts double-stranded DNA and releases the telomeric overhangs, we have developed a method to monitor telomerase action during one cell cycle. Here, we describe this method using HeLa carcinoma cells as an example.

**Key words:** Telomere, Telomerase, Overhang, C-rich fill-in, Cell synchronization, BrdU, CsCl gradients, Duplex-specific nuclease

## 1. Introduction

Human telomeres are composed of several kilobases of double-stranded TTAGGG/AATCCC DNA terminating in a 12–300 nt single-stranded G-rich sequence (the G-rich overhang) (1, 2). In normal somatic cells, telomeres shorten at the rate of 50–200 bp/PD (population doubling) due to the end replication problem of linear chromosome, processing and stochastic events. Eventually, critically short telomeres trigger replicative senescence or apoptosis (3). To counteract this continuous telomere loss, most cancer and proliferative stem cells express telomerase, a ribonucleoprotein complex that exhibits reverse transcriptase activity that can extend telomeres by adding hexameric repeats of GGTTAG to telomeric overhangs (4, 5). This extension is finished

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by a C-rich strand fill-in that makes some of the extended G-overhang double-stranded.

Most cancer cells maintain a constant telomere length, where telomerase is only adding enough to compensate for the rate of shortening. Techniques to measure telomere length, such as telomere restriction fragment (TRF) assay (6), telomere FISH (7), and single telomere length analysis (STELA) (8), cannot measure telomerase action, if the length is not changing. Telomerase action will extend the G-rich overhangs until C-rich fill-in makes the extended overhangs shorter. By monitoring the change of G-overhang length during the cell cycle, one should be able to tell when and how telomerase extension and C-rich fill-in occur. Using duplex-specific nuclease (DSN), which specifically digests double-stranded DNA to <10 bp fragments while keeping single-stranded G-overhang intact, we have developed a new method for directly studying the dynamics of the G-overhang during cell cycle.

We have found that telomerase extension is coupled to telomere replication, which occurs throughout S phase in human cells, whereas C-rich fill-in is delayed until S/G2 (9, 10). The time-lag between these two events allowed us to investigate telomerase action during S phase and C-rich fill-in at S/G2. Cells synchronized at G1/S are released into S phase in the presence of BrdU. Telomere replication incorporates BrdU into both leading and lagging daughters. The leading daughter's G-strand is newly synthesized, and its overhangs will be fully BrdU substituted regardless of whether telomerase has acted (Fig. 1). However, the

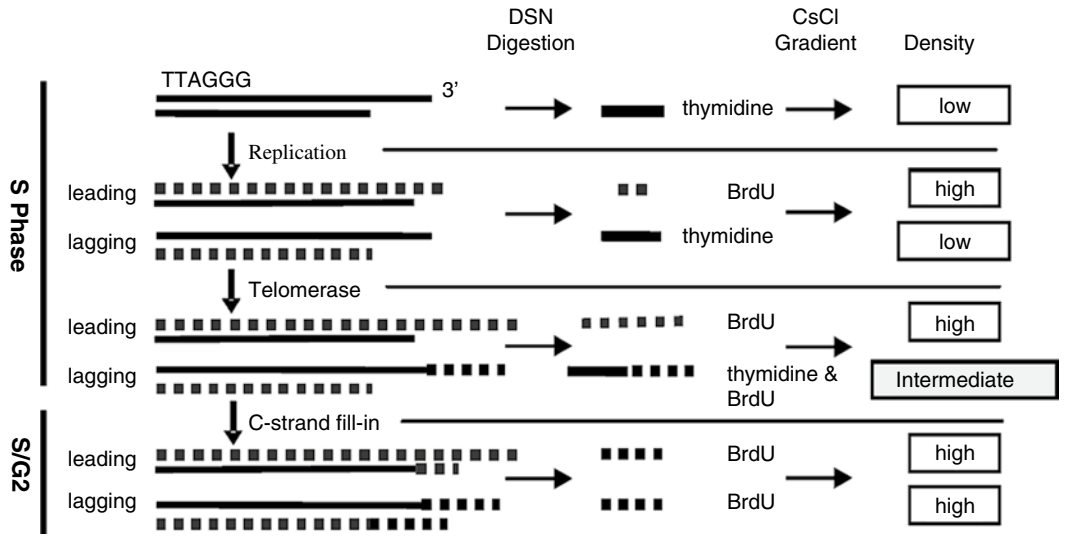


Fig. 1. Strategy for detecting telomerase extension and C-rich fill-in on lagging daughter telomere.

parental G-strand provides the template for lagging strand synthesis, and lagging daughters initially have overhangs that contain only thymidine. If telomerase acts, it will incorporate BrdU into the newly synthesized GGTTAG repeats, thus the overhangs of lagging daughters will contain a mixture of thymidine and BrdU containing repeats and should have an intermediate density on CsCl gradients (Fig. 1). At S/G2, C-rich fill-in makes the thymidine-containing portion of the overhang double-stranded, leaving a fully BrdU-substituted overhang that has the highest density. During S phase, by monitoring what fraction of lagging overhangs are of intermediate density versus low density, one can determine the percentage of telomeres extended by telomerase. In addition, the intermediate density per se indicates the ratio of thymidine to BrdU in an overhang that can be used to calculate the average size of telomerase extension (the BrdU part of the overhang) once the total length of the overhang has been determined.

Figure 2 shows an outline of the different steps needed for this analysis. A brief explanation is provided for each step prior to a detailed description of the protocols. HeLa cells are used as an example.

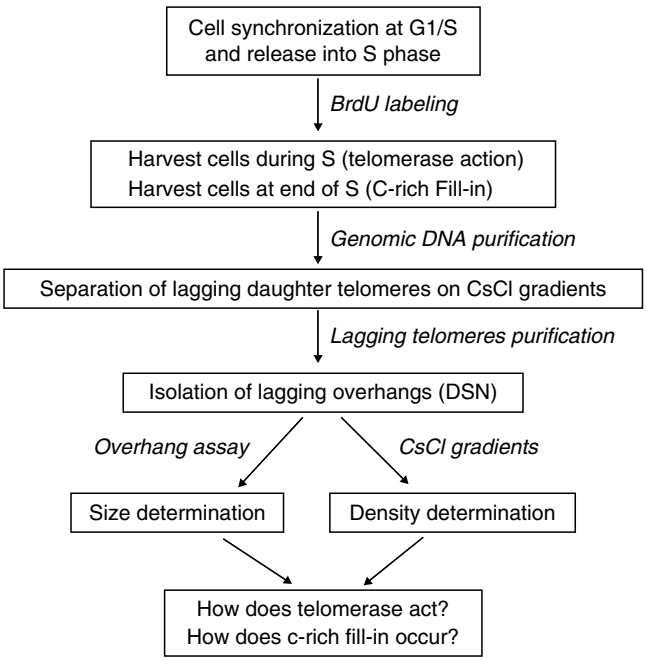


Fig. 2. Outline of steps for the study of telomerase action and C-rich fill-in.

## 2. Materials

### 2.1. Cell Culture and Synchronization

1. DMEM with high glucose (Hyclone) (see Note 1).
2. Cosmic calf serum (Hyclone).
3. Thymidine (Sigma-Aldrich), 100 mM stock in 1× PBS, filter-sterilized.
4. Solution A: HEPES 30 mM (pH 7.4), glucose 4 mM, KCl 3 mM, NaCl 122 mM, $Na_2HPO_4$ 1 mM, phenol red 0.5 mM (see Note 2).
5. Bromodeoxyuridine (BrdU), 100 mM stock in DMSO.

### 2.2. Genomic DNA Purification

1. Blood and cell culture DNA midi kit (Qiagen) (see Note 3).
2. Ethyl alcohol (70%).
3. Millipore water with 5 mM Tris–HCl, pH 8.0.
4. Water bath, 37°C.

### 2.3. Restriction Enzyme Digestion and CsCl Gradients

1. 10× NEB buffer 2 (NEB, Inc.).
2. Restriction enzymes: *Hin*fI, *Rsa*I, *Msp*I, *Hae*III, *Alu*I, and *Hha*I (NEB).
3. CsCl (USB, Inc.).
4. 1 M Tris–HCl (pH 8.0).
5. 0.5 M EDTA (pH 8.0).
6. Ultracentrifuge L8-M with VTi80 vertical rotor (Beckman, Inc.) (see Note 4).
7. Quick-seal centrifuge tubes (13×51 mm) (Beckman).
8. Light mineral oil (Sigma-Aldrich).
9. 27 guage 1/2″ and 21 guage 1″ needles, 5-mL syringe and 0.5-mL tube.
10. Refractometer (Milto Roy).

### 2.4. Slot Blot and Hybridization with C-Rich Telomeric Probe

1. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
2. 1 M NaOH.
3. Protran BA 85 nitrocellulose transfer membrane (Watman).
4. Minifold® II Slot blot (Schleicher & Schuell, Inc.) (see Note 5).
5. 80°C oven.
6. Hybridization buffer: 6× SSC, 5× Denhardt's solution, and 0.5% (w/v) SDS.
7. C-rich telomeric probe made as described in Subheading 3.4 of Chapter 5.
8. Washing buffer 1: 2× SSC and 0.1% SDS.

9. Washing buffer 2: 0.5× SSC and 0.1% SDS.
10. Hybridization oven (Hybridiser HB-1D, Techne, Inc.).
11. Phosphor screen (Molecular Dynamics).
12. Typhoon™ (GE Healthcare).

### 2.5. Purification of Lagging Daughter Telomeres from CsCl Gradients

1. Ultrapure™ agarose (Invitrogen).
2. Ethanol (100 and 70%).
3. NaCl (5 M).
4. Centrifuge with temperature control.
5. DSN 1× buffer: 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT.

### 2.6. Second CsCl Gradients, Slot Blot and Hybridization

Materials required are the same as listed in Subheadings 2.3 and 2.4.

### 2.7. Data Analysis

Software requirement: ImageQuant 5.2 and IQTools 3.0 for the quantification of slot blot and software that can perform Gaussian distribution analysis, e.g., Graphpad prism 5.

## 3. Methods

All experiments need to be performed in the absence of fluorescent light, which damages BrdU.

### 3.1. Cell Synchronization at G1/S and BrdU Labeling (see Note 6)

Telomeres replicate and telomerase acts throughout S phase, whereas C-rich fill-in only happens at the end of S and G2. Cells are synchronized at G1/S and released into S phase in the presence of BrdU to label DNA. In HeLa cells, S phase lasts 6–8 h, so cells are harvested 4 h after release (during S phase) to study telomerase action or 8 h after release (S/G2) to study C-rich fill-in.

1. Grow cells to 30–40% confluence in DMEM medium with 10% calf serum.
2. Add thymidine to 2 mM final and incubate for 19 h.
3. Wash cells three times with prewarmed solution A and refeed with fresh serum-rich DMEM medium.
4. Incubate for 9 h before adding 2 mM thymidine.
5. Incubate for 16 h and wash three times with prewarmed solution A, refeed with fresh medium, and add BrdU to a final concentration of 100 μM.
6. Harvest cells after 4 or 8 h.

### 3.2. Genomic DNA Purification

1. Purify genomic DNA following the manufacture's instruction.
2. Wash the DNA pellets twice with 70% ethanol.
3. Let DNA dry in air.
4. Dissolve genomic DNA in Millipore water with 5 mM Tris–HCl (pH 8.0), overnight incubation at 37°C ensure that all DNA is completely solubilized and any 3′ end structures are resolved.

### 3.3. Separation of Lagging Daughter Telomeres on CsCl Gradients

Due to the absence of restriction enzyme recognition sites within the TTAGGG tandem repeat sequence, telomeric DNA can be released by restriction enzyme digestion which cleaves genomic DNA into <300 bp fragments while leaving telomere DNA uncut. In the presence of the thymidine analog BrdU, DNA replication incorporates BrdU into the daughter strands. Lagging synthesis of CCCTAA only incorporate one BrdU per six nucleotides, whereas leading synthesis of TTAGGG incorporates two BrdU per six nucleotides. Leading telomeres thus incorporate more of the heavier BrdU nucleotide and thus have a greater density compared with lagging telomeres, permitting separation on CsCl density gradients (11).

1. Digest genomic DNA with six restriction enzymes (Subheading 2.3). Start with at least 400 μg DNA. DNA is incubated with six enzymes in 1× NEB buffer 2 at 37°C overnight to get complete digestion (or at least 8 h) (see Note 7).
2. Make CsCl solution (~8 M, density ~1.788) containing 5 mM Tris–HCl (pH 8.0) and 1 mM EDTA.
3. Mix-digested genomic DNA with CsCl solution and adjust the final density to be ~1.766 using CsCl powder or water. Inject mixed solution into centrifugation tube and seal the tube.
4. Place tube into rotor and spin at 55 K RPM (120,443 × *g*) for 20 h at 25°C, let rotor slow down without braking (usually it takes 45 min to completely stop).
5. Make a small hole on the bottom of tube using 27 G 1/2″needle, push the sample out by injecting mineral oil into tube from top of tube (see Fig. 3).
6. Collect the fractions at 100 μL per fraction (approximately 50 fractions total).
7. Measure the density of each fraction. Read the refractive index from refractometer following the instruction provided by the manufacture, convert the refractive index of each fraction to density using the following formula:

$$Density = 10.8601 \times RI - 13.4974$$

where RI is the refrective index.

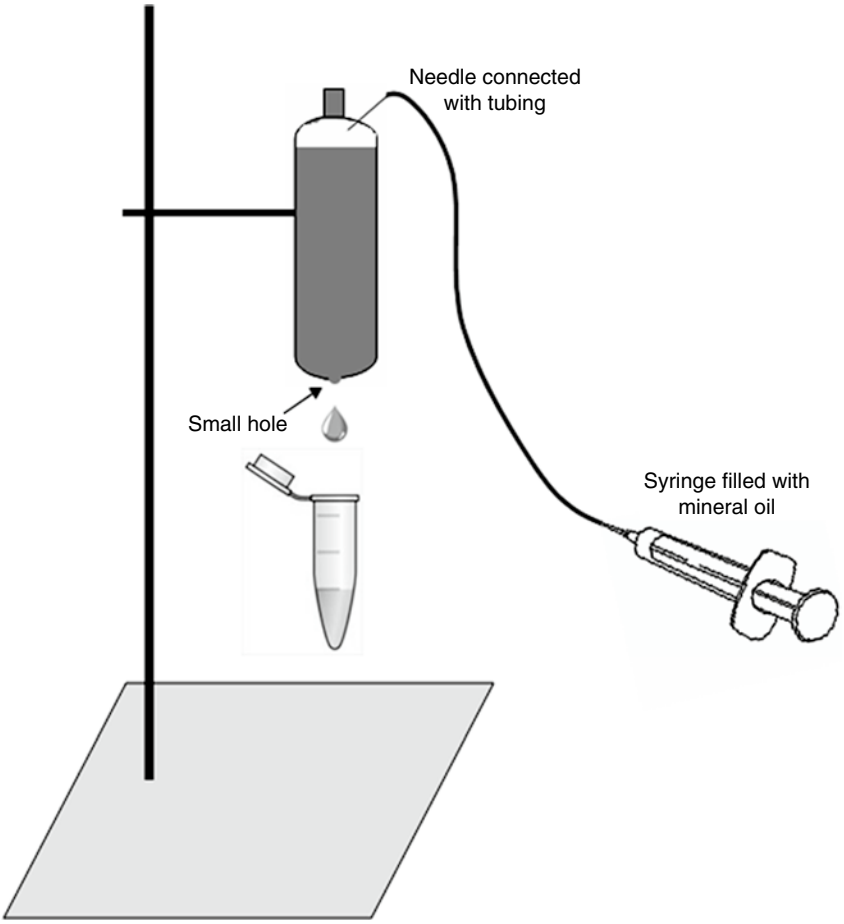


Fig. 3. Illustration of how to collect the fractions from CsCl gradients.

### *3.4. Slot Blot to Determine Fractions Containing Lagging Strand Telomeres*

After spinning, DNA with different densities will shift to the corresponding densities of CsCl. To determine which fractions contain lagging telomeres, the aliquots of DNA are denatured by NaOH, neutralized with SSC and loaded on a slot blot. After hybridizing to a telomeric-specific C-rich probe, the fractions that have telomere DNA will be located.

1. Dilute aliquots of DNA (5 μL) from each fraction with water to 50 μL, add the same volume (50 μL) of 0.2 M NaOH and incubate the mixture at 37°C for 10 min.
2. Neutralize the sample by adding 100 μL 12× SSC.
3. Assemble slot blot apparatus according to manufacturer's instructions. The membrane needs to be prewetted in 20× SSC.

4. Apply vacuum, wash the wells with 400 μL 20× SSC, and load-treated samples to wells in the sequence with which they were collected.
5. Wash each well with 400 μL 20× SSC twice, let vacuum run for additional 10 min.
6. Disassemble the slot blot apparatus and carefully remove the membrane.
7. Bake the membrane at 80°C for 2 h to immobilize DNA on membrane. Do not use UV to crosslink the DNA to the membrane since BrdU is much more reactive to UV than thymidine.
8. Prehybridize membranes at 42°C for at least 30 min using hybridization buffer.
9. Add 5–10 μL high-specific activity telomere C-rich probe to hybridization buffer to a concentration of 1 μL/mL.
10. Hybridize at 42°C overnight.
11. After thybridization, wash the blots at 42°C for 15 min in washing buffer 1, then 15 min in washing buffer 2.
12. Remove the blot from the last wash, drain and wrap in plastic wrap such as SaranWrap®, expose to PhosphoImager screens for appropriate time (30 min to 2 h).
13. Scan screens on the Typhoon PhosphoImager®.
14. Quantify the signal intensity, draw graph by using the density of each fraction (Subheading 3.3, step 7) as the *X*-axis and the intensity as the *Y*-axis.

### *3.5. Purification of Lagging Strand Telomere DNA from CsCl Fraction*

Leading daughter telomeres are located at a density of ~1.788, lagging daughter DNA is at a density of 1.766, while unreplicated telomeres are at a density of ~1.744 (see Note 8). To purify lagging telomere DNA, the fractions containing lagging DNA are pooled together, surface dialyzed to reduce salt concentration and then ethanol precipitated (see Note 9).

1. Pool 4–5 fractions of lagging peak together (see Note 10).
2. Melt 25 mL 2% agarose in water containing 5 mM Tris–HCl (pH 8.0) and pour gel into 50-mL tube.
3. Load-pooled DNA fractions on the top of gel, rock the tube for 1 h at room temperature on a rocking platform as used for western blots. The tube is maintained in a "vertical" position so the DNA solution is maximally exposed to the agarose surface.
4. Remove dialyzed DNA to a 2-mL tube, mix with 1/25 volume of 5 M NaCl and two volumes of ice-cold ethanol, store at –20°C for at least 1 h.
5. Centrifuge at 13,000 RPM (18,000 × *g*) for 30 min at 4°C.

6. Discard the supernatant, wash the DNA pellet with 70% ice-cold ethanol twice.
7. Dry pellet in air, usually it takes 10–15 min at room temperature.
8. Dissolve DNA in 50 μL 1× DSN buffer. Incubation at 37°C for 10 min helps DNA dissolve.
9. Measure DNA concentration, usually 10–20 μg DNA containing the lagging telomere daughters can be recovered from 400 μg genomic starting DNA.

### 3.6. Determine the Length of Lagging Daughter Overhang

The overhang length of the lagging daughter can be measured by following the method described in Chapter 5, step 2.

### 3.7. Determine Density of Lagging Overhangs Extended by Telomerase

Lagging overhangs containing only thymidine have a density of ~1.744, whereas the density of overhangs with thymidine fully substituted by BrdU is ~1.831. For lagging daughters, the initial overhang contains only thymidine, but telomerase incorporates BrdU into each new synthesized repeat, making the overhang heavier. Therefore, depending on the initial overhang length and the number of repeats telomerase added, the overhang's density should be between ~1.744 and ~1.830. Assuming that the density shift is proportional to the percentage of incorporated BrdU in the overhang, one can calculate the ratio of thymidine to BrdU by measuring its density.

1. Add 1–2 U DSN (0.2 U/5 μg) to purified lagging DNA, and incubate at 37°C for 2 h.
2. Make CsCl solution (density ~1.783) containing 1 mM EDTA (pH 8.0) (see Note 11).
3. Mix DSN-digested DNA with CsCl solution, inject them into tube and seal tube.
4. Spin at 60 K RPM (143,337 × *g*) for 20 h at 25°C, let rotor slow down without braking.
5. Make a small hole on the bottom of tube using 27 G 1/2″ needle, push the sample out by injecting mineral oil into tube from top of tube.
6. Collect the fractions at 100 μL per fraction (approximately 50 fraction in total).
7. Measure density of each fraction as described above.
8. Assemble slot blot, prewash with 20× SSC, load each fraction into slots, and wash with 20× SSC twice. It is not necessary to denature the samples since only single-stranded DNA survives DSN digestion.
9. Let membrane dry in air for 2–3 h.

10. UV-crosslink DNA on membrane by setting energy to be 70 mJ/cm$^2$ in a commercial UV crosslinker. (The crossliking efficiency following baking is not as good as with UV, possibly because the DNA is single-stranded).
11. Prehybridize membrane at 42°C for at least 30 min using hybridization buffer.
12. Add 5–10 μL high-specific activity telomere C-rich probe to hybridization buffer to a concentration of 1 μL/mL (see Note 12).
13. Hybridize at 42°C overnight.
14. After the hybridization, wash the blots with washing buffer 1 twice, 15 min each, followed by washing buffer 2 for 15 min at 42°C.
15. Remove the blot from the last wash, drain and wrap in SaranWrap®, expose to PhosphoImager screens for several hours to overnight.
16. Scan screen on Typhoon PhosphoImager®.

### 3.8. Data Analysis

The central questions regarding telomerase action are the following: first, how many telomeres in cells have been extended by telomerase during one cell cycle? Second, on average, how many repeats of GGTTAG did telomerase add to each telomere? To answer these questions, three parameters need to be extracted from the experimental data: the relative amount of unextended and extended overhangs, expressed in these experiments as the relative signal intensity of the thymidine overhang peak versus the intermediate peak; the density of intermediate peak – this is extremely useful for determining the percentage of BrdU in lagging overhangs; and the lagging overhangs length as measured in Subheading 3.6. In this section, methods to extract these parameters from the raw data are described.

1. Quantify the signal intensity for each fraction on slot blot.
2. Using density as the $X$-axis and intensity as the $Y$-axis, draw the graph.
3. Fit the data to the function of "sum of two Gaussian distributions" using the built-in equation in software GraphPad Prism:

$$Y = \left(\frac{\text{AREA1}}{\text{SD1}\times(2\times\pi)^{0.5}}\right)\times\exp\left(-0.5\times\left(\frac{X-\text{Mean1}}{\text{SD1}}\right)^2\right) + \left(\frac{\text{AREA2}}{\text{SD2}\times(2\times\pi)^{0.5}}\right)\times\exp\left(-0.5\times\left(\frac{X-\text{Mean2}}{\text{SD2}}\right)^2\right),$$

where AREA1 and 2 are peak areas, Mean1 and 2 represent the densities at peaks, SD1 and 2 are standard deviations, and π is a constant.

4. From fitting, the density of the intermediate peak will be given (Mean2), as well as the relative amounts of unextended and extended overhangs that were expressed as AREA1 and AREA2.
5. Draw a linear regression line between the highest density (1.831, 100% BrdU) and the lowest density (1.744, 0% BrdU) using density and percentage of BrdU as $X$- and $Y$-axis, respectively, then the following linear regression equation can be obtained:

$$BrdU\% = 11.49D - 20.04$$

where BrdU% is the percentage of BrdU in the overhang and $D$ is density of intermediate peak.

6. Input intermediate density (Mean2) to linear regression equation and export the percentage of BrdU (BrdU%).
7. Calculate the average length of extension (ALE) using the following expressions:

$$ALE = OL \times BrdU\%,$$

where OL is overhang length determined in Subheading 3.6.

8. Calculate the percentage of extended telomeres (PEE) using the following expressions:

$$PEE = \frac{\text{AREA2} \times (1 - BrdU\%)}{\text{AREA2} \times (1 - BrdU\%) + \text{AREA1}}.$$

## 4. Notes

1. DMEM medium with 10% calf serum works well for HeLa cell synchronization and BrdU incorporation. It is also suitable for other cancer cell lines such as H1299 and A549. However, different medium may be used if DMEM does not work well on your cells.
2. 1× PBS as wash buffer can also be used.
3. Any genomic DNA purification kit should be good for this purpose.
4. The vertical rotor (VTi80, 90) significantly shortens spinning time. One can also use a swinging bucket rotor, but it will take several days to form gradients.

5. Other slot blot equipment may work, but the one described in this method gives a low background and a high reproducibility.
6. The method described here is optimized for the synchronization of normal HeLa cells, which produced synchronization of >90% cells at G1/S. If double thymidine blocking strategy does not work well for your cells, different approaches such as aphidicolin should be tried. If a high percentage of synchronization cannot be obtained, a minimum of 50% cells synchronized should be good enough as long as there are no cells leaking from S phase to G2 before the end of S.
7. The concentration of each enzyme used is 0.5 U/μg. Use of just two enzymes (*Hin*fI and *Rsa*I), works almost as well as six enzyme digestion.
8. The density of each peak may change from time to time, but the variation should not be beyond ±0.0055. It is also worth noticing that the distance (in density) between leading and lagging peaks should be equal to the distance between lagging and unreplicated peaks.
9. This method will give a recovery of ~40% in purifying DNA from CsCl.
10. With more fractions pooled, there is an increased probability of contamination by leading telomeres and unreplicated DNA.
11. Overhangs may locate on any density ranging from ~1.744 (pure thymidine overhang) to 1.831 (fully BrdU-substituted overhang) depending on how many repeats were added by telomerase. The CsCl gradients generated for overhang analysis should thus cover all possible densities. To this end, increase the initial density of CsCl (~1.783) and the spinning speed (60,000).
12. Using fresh isotope to make C-rich telomeric probe is recommended. Isotope exceeding one half-life should not be used.

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# Chapter 8

# G-Quadruplex Structures and G-Quadruplex-Interactive Compounds

**Raveendra I. Mathad and Danzhou Yang**

## Abstract

G-quadruplexes are noncanonical secondary structures formed in DNA sequences containing consecutive runs of guanines. DNA G-quadruplexes have recently emerged as attractive cancer therapeutic targets. It has been shown that the 3′ G-rich single-stranded overhangs of human telomeres can form G-quadruplex structures. G-quadruplex-interactive compounds have been shown to inhibit telomerase access as well as telomere capping. Nuclear magnetic resonance (NMR) spectroscopy has been shown to be a powerful method in determining the G-quadruplex structures under physiologically relevant conditions. We present the NMR methodology used in our research group for structure determination of G-quadruplexes in solution and their interactions with small molecule compounds. An example of a G-quadruplex structure formed in the human telomere sequence recently solved in our laboratory is used as an example.

**Key words:** Human telomeres, G-quadruplex structures, Structure polymorphism, G-quadruplex-interactive compounds, Telomerase inhibitors, Anticancer drug targets

## 1. Introduction

### *1.1. G-Quadruplex Structures*

G-quadruplexes are noncanonical secondary structures formed in DNA sequences containing consecutive runs of guanines and have recently emerged as attractive cancer therapeutic targets (1). A G-quadruplex is a four-stranded DNA secondary structure that contains stacked G-tetrad planes of four guanines connected by a network of Hoogsteen hydrogen bonding (Fig. 1a). G-quadruplexes can be formed with one, two, or four G-rich strands (Fig. 1b). G-strands in a G-quadruplex structure can be parallel or antiparallel, and G-tetrad guanines can adopt *anti* or *syn* conformations around the glycosidic bonds depending on the orientation of DNA strands. G-tetrad guanines from parallel G-strands adopt the same glycosidic conformation, while those

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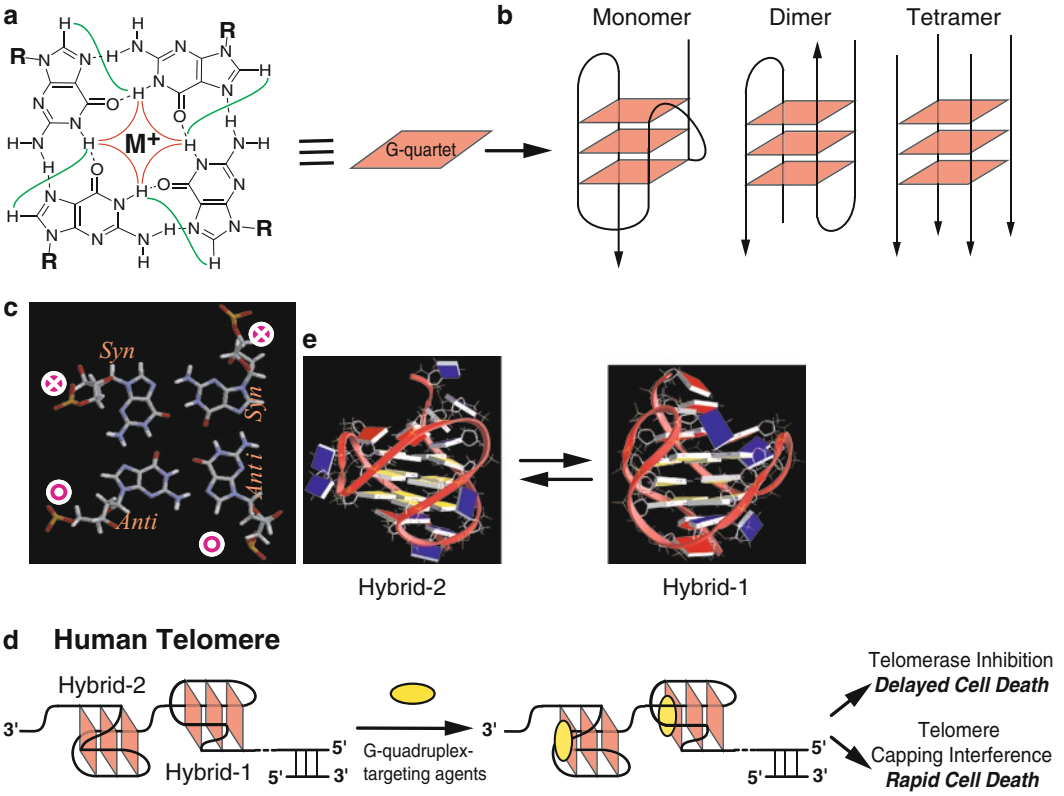



Fig. 1. (**a**) Schematic diagram of a G-tetrad containing a square planar alignment of four guanines connected by cyclic Hoogsteen hydrogen bonding between the N1, N2 and O6, N7 of guanine bases. Curved lines show the H1–H1 and H1–H8 connectivity pattern detectable in NOESY experiments. (**b**) Schematic diagrams of monomeric (intramolecular), dimeric, and tetrameric G-quadruplexes. (**c**) The guanines in a G-tetrad can adopt either syn or anti glycosidic conformation depending on the orientation of DNA strands. (**d**) A schematic model of human telomeres composed of compact-stacking multimers of DNA G-quadruplex secondary structure, and the proposed mechanism of drug-mediated inhibition of telomerase and interference of telomere capping by G-quadruplex-stabilizing compounds. (**e**) Two hybrid-type intramolecular G-quadruplex structures coexist in human telomeres in $K^+$ solution. The molecular structures are determined by NMR in pH 7.0, 95 mM $K^+$ solution.

from antiparallel G-strands adopt the opposite glycosidic conformation (Fig. 1c). G-quadruplex formation requires monovalent cations, in particular $K^+$ and $Na^+$, to coordinate with the eight electronegative O6 atoms of the adjacent stacked G-tetrads (2) (Fig. 1a). In general, $K^+$ is more preferred than $Na^+$ by G-quadruplex as $K^+$ has a better coordination with eight guanine O6s and a lower dehydration energy (3). The $K^+$ form is considered to be more biologically relevant due to the higher intracellular concentration of $K^+$ (~140 mM) than that of $Na^+$ (5–15 mM).

Intramolecular G-quadruplexes are of intensive current research interest due to their potential formation in telomeres and oncogene promoter regions (4–6). Intramolecular G-quadruplex structures form quickly in solution and are found

to be DNA sequence specific, exhibiting great conformational diversity, such as folding topologies, loop conformations, and capping structures (1). High-field NMR spectroscopy has been shown to be a powerful method in determining the G-quadruplex structures under physiologically relevant conditions.

### 1.2. G-Quadruplexes Formed in Human Telomeres

The first biologically relevant G-quadruplex formation was observed in telomeric DNA (7). Human telomeres consist of tandem repeats of the hexanucleotide $d(TTAGGG)_n$ 5–25 kb in length, which terminate in a single-stranded 3′-overhang of 35–600 bases (8, 9). Telomeric DNA is extensively associated with various telosome proteins, and the structure and stability of telomeres are closely related with cancer (4), aging (10) and genetic stability (11). Vertebrate telomeric DNA repeats are highly conserved, which has been suggested to be related to their ability to form the DNA G-quadruplex (4). The most direct evidence of the *in vivo* existence of G-quadruplexes was established by using specific antibodies against parallel and antiparallel G-quadruplexes formed in telomeric DNA of the ciliate *Stylonychia lemnae* (12). Using the same antibody in ciliates, it was shown *in vivo* that the telomere end-binding proteins TEBPα and TEBPβ are required to control G-quadruplex formation and that TEBPβ phosphorylation is needed to resolve G-quadruplex structures during replication (13). A number of other proteins have been found to specifically interact with telomeric G-quadruplex structures (14). In addition, G-quadruplex formation was detected *in vivo* at human chromosomal ends by using the radiolabeled G-quadruplex ligand 360A (15) and the fluorescent G-quadruplex ligand BMVC (16).

In normal somatic cells, each cell replication results in a 50- to 200-base loss of the telomere. After a critical shortening of the telomeric DNA is reached, the cell undergoes apoptosis (17). However, telomeres of cancer cells do not shorten on replication, due to the activation of a reverse transcriptase, telomerase, that extends the telomeric sequence at the chromosome ends (18). Telomerase is activated in 80–85% of human cancer cells (19) and has been suggested to play a key role in maintaining the malignant phenotype (20). Formation of intramolecular G-quadruplex in human telomeric DNA induced by $K^+$ ion has been shown to inhibit the telomerase activity (21).

### 1.3. G-Quadruplex Structures in Human Telomeres

Intramolecular G-quadruplexes formed by single-stranded DNA in human telomeres are considered to be attractive anticancer drug targets due to their association with telomerase function and telomere end-capping (see Refs. 4, 6 for reviews) (Fig. 1d). Understanding of the human telomeric G-quadruplex structure under physiologically relevant $K^+$ solution has been the subject of intense investigation. A 22-nt human telomeric DNA 5′-$AGGG(TTAGGG)_3$ (wtTel22) (Fig. 2a) has been

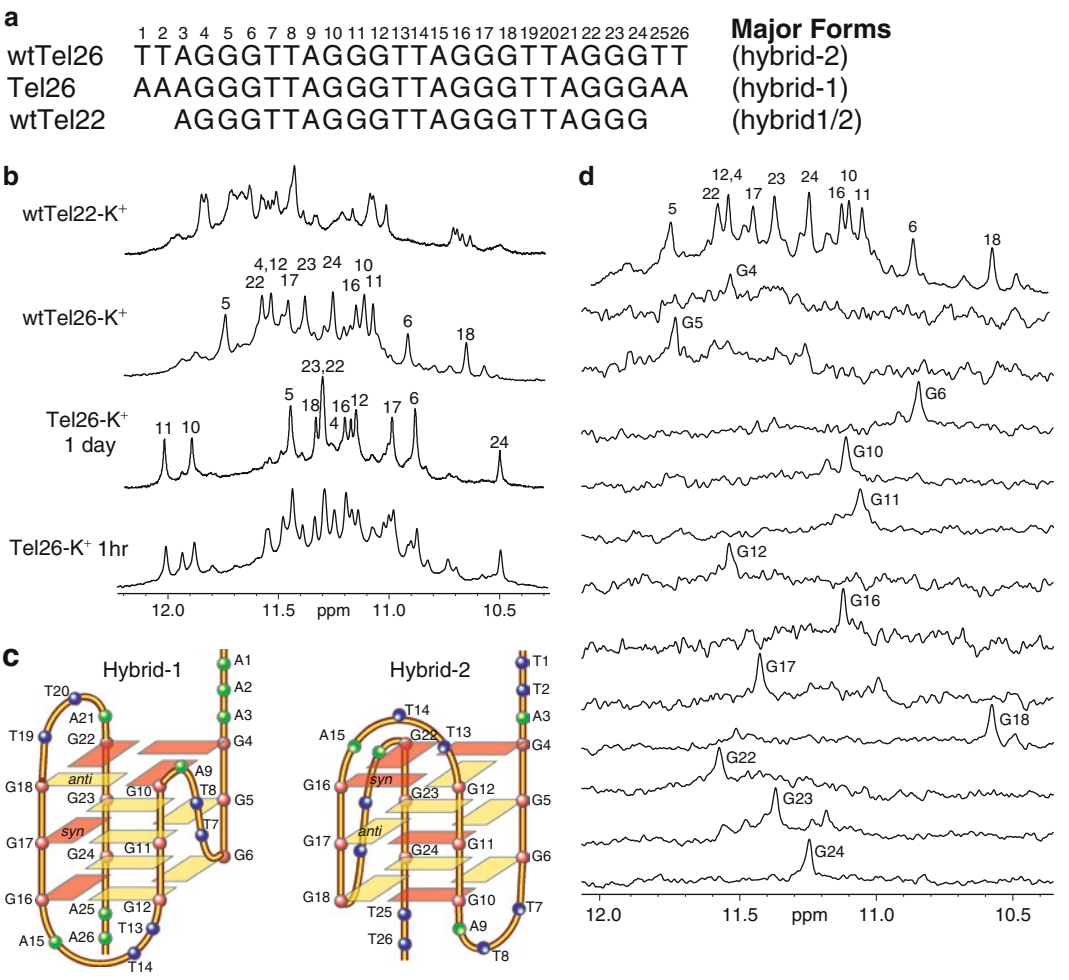



Fig. 2. (**a**) Human telomeric DNA sequences containing four G-tracts that were used for structure determination. The sequence numbering system is shown above wtTel26. The major conformations formed in each sequence are specified. (**b**) The imino regions of 1D $^{1}$H-NMR spectra of wtTel22, wtTel26, and Tel26 (1 week and 1 h samples) in $K^+$ solution. The assignment of imino protons is shown for wtTel26 and Tel26. (**c**) Schematic drawing of the folding topologies of the hybrid-1 and hybrid-2 intramolecular telomeric G-quadruplexes in $K^+$ solution. Dark box=(syn) guanine, light box=(anti) guanine. (**d**) The imino proton region with the assignment of the 1D $^{1}$H NMR spectrum of wtTel26 in $K^+$ solution (top), and imino proton assignments of wtTel26 using 1D $^{15}$N-filtered experiments on site-specific labeled oligonucleotides. Conditions: 25 mM phosphate, 95 mM potassium, pH 7.0, 25°C, 0.5–0.6 mM DNA.

shown by NMR to form a basket-type three-tetrad intramolecular G-quadruplex in $Na^+$ solution (22); the same 22-nt telomeric sequence has been shown to form a parallel-stranded three-tetrad G-quadruplex in the crystalline state in the presence of $K^+$ (23). However, wtTel22 does not appear to form a single G-quadruplex structure in $K^+$ solution (Fig. 2b). More recent work from our lab (24–26) and others' (27–30) has shown that the major G-quadruplexes formed in human telomeres in $K^+$ solution are the hybrid-type intramolecular structures, distinct from the $Na^+$

solution structure or the $K^+$ crystal structure (see ref. 31 for a review). Two hybrid-type structures appear to coexist in $K^+$ solution, with the hybrid-2 form being the major one for the extended four-G-tract human telomeric DNA (25) (Fig. 1e). The kinetics of the interconversion between the two forms appears to be rather slow, as shown by NMR experiments (25). Both hybrid-type structures contain three parallel G-strands and one antiparallel G-strand, five *syn* guanines, and asymmetrical G-arrangements (Fig. 2c). They differ in loop arrangements, strand orientations, G-tetrad arrangements, and capping structures which may provide specific ligand binding sites (Fig. 1e) (25, 26). The hybrid-form structures provide an efficient scaffold for a compact-stacking structure of multimers in the 3′ single-stranded overhang of the human telomeric DNA (Fig. 1d). While short loop sizes of 1 and 2 nt are in usually favored for the double-chain-reversal loop conformation (32, 33), the presence of a 3-nt double-chain-reversal TTA loop in the hybrid-type telomeric G-quadruplexes allows the 5′ and 3′ ends to point in opposite directions (Figs. 1d and 2c). However, this may contribute to the structural flexibility and polymorphism of telomeric G-quadruplexes. Nature may have chosen the human telomeric sequence with the low energy difference between various forms (see our recent reviews for more information (6, 31)).

### 1.4. G-Quadruplex-Interactive Ligands

Recognition of the biological significance of DNA G-quadruplexes has intensified research and development of G-quadruplex-interactive compounds. Targeting of DNA G-quadruplex secondary structures represents an entirely new approach for cancer therapeutics, with the first report of targeting G-quadruplexes for inhibiting telomerase activity in 1997 (34). Formation of diverse G-quadruplex structures offers an opportunity to design small molecules/ligands that can bind different G-quadruplexes selectively. Structure-based drug design has been playing an important role in the development of G-quadruplex-interactive compounds. In fact, G-quadruplex inhibitors themselves have contributed immensely to understanding G-quadruplexes as a therapeutic target. Most G-quadruplex compounds are the stacking ligands, which consist of aromatic systems that can stack onto the terminal G-tetrads. Additionally, stacking ligands may contain positively charged side-chain substituents that interact with phosphate groups in G-quadruplex grooves. More recently, unfused aromatic ring systems that can adopt flexible conformations have been shown to bind into G-quadruplex grooves (35, 36). A number of small molecules that can specifically bind and stabilize telomeric G-quadruplexes have been developed, and some have become prospective anticancer agents that display relatively low cytotoxicity. For example, BRACO19 is a trisubstituted acridine

derivative, rationally designed by considering the unique structural features of DNA G-quadruplexes (37). Telomestatin, one of the most potent G-quadruplex stabilizing compounds, has been shown to be a highly potent inhibitor of telomerase (38). TMPyP4, another G-quadruplex-interactive compound by structure-based rational drug design (39), has been shown to inhibit human telomerase in HeLa cell extracts. A number of other G-quadruplex-interactive compounds, including BMVC, RHPS4, 360A, and 12459 have been shown to inhibit telomerase activity with various levels of potency. It is noteworthy that Quarfloxacin, a G-quadruplex-targeting compound originated from rational drug design and developed by Cylene Pharmaceuticals (San Diego, CA), shows excellent *in vivo* activity in various solid tumors and is currently in Phase II clinical trials (see recent review (1, 6) for more information).

In this chapter, we focus on practical steps involved in the NMR structural determination of G-quadruplexes formed in human telomeric DNA in $K^+$ solution. In addition, NMR analysis of drug interactions is also presented.

## 2. Materials

### 2.1. Reagents

1. Phosphoramidites, resins, and other reagents (Glen Research, Sterling, VA) (see Note 1).
2. $^{15}N$ Guanine phosphoramidites (Cambridge Isotopes Laboratories, Andover, MA).
3. $D_2O$ and DMSO-$d_6$ solvents (Sigma-Aldrich, St. Louis, MO).
4. Phosphate buffer: 25 mM phosphate, 95 mM potassium, pH 7.0 (see Note 2).

### 2.2. NMR Sample Preparation

1. Lyophilized DNA samples are dissolved in aqueous solution containing 25 mM phosphate, 95 mM potassium, pH 7.0 (see Note 3).
2. For $H_2O$ samples, DNA powder is dissolved in 0.6 ml of 90/10% $H_2O/D_2O$. For $D_2O$ samples, DNA is dissolved in 0.6 ml of 99% $D_2O$.
3. The final NMR samples contain 0.1–3 mM DNA with a final $K^+$ concentration of 95 mM (see Note 4).
4. DNA oligonucleotides are annealed by heating to 95°C for 15 min, then slowly cooling to room temperature.
5. Drug stock solutions (10–40 mM) can be prepared in $H_2O$ or DMSO, depending on their solubility (see Note 5).

## 3. Methods

NMR spectroscopy is widely employed for structural studies of G-quadruplexes under physiologically relevant conditions. Significantly, unlike long thread-like double-helical DNA structures, e.g., B-DNA, that lack global interaction information, global interaction information is well observed in G-quadruplex structures because of extensive DNA folding. Thus, a G-quadruplex structure can be derived from extended single-stranded DNA conformation by NMR-based structure calculation, analogous to a globular protein with global folding that can be well defined by the NMR-restrained structure calculation.

### 3.1. Synthesis and Purification of DNA with and Without Labeling

1. The DNA oligonucleotides are synthesized at the 1 μmol scale on an Expedite™ 8909 Nucleic Acid System from Applied Biosystems (Foster City, CA) in DMT-on mode using solid-phase β-cyanoethylphosphoramidite chemistry (40) (see Note 6).
2. For labeled DNA oligonucleotide synthesis, $^{15}$N-guanine phosphoramidite is used for site-specific $^{15}$N-labeling of individual bases in DNA sequences at a 6% low-level enrichment. This low enrichment is sufficient to perform special NMR experiments such as JR-HMQC (see Note 7).
3. The oligonucleotides are cleaved from the solid support by treatment with concentrated ammonia for 15 h at 55°C (see Note 8).
4. DMT-on oligonucleotides are purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a $C_{18}$ column (250 × 10.0 mm VARIAN Dynamax Microsorb 300-10) with a linear gradient of 5–45% acetonitrile over 45 min in 1% triethylammonium acetate (TEAA), pH 7.0, with a flow rate of 3 ml/min. DNA is monitored with UV at 254 nm. The DMT-on DNA is collected at 25 min and is then lyophilized.
5. DMT protection groups are removed from DNA by treating with 2 ml of 80% acetic acid for 2 h. The sample is then diluted with 1 ml of water and extracted with 10 ml of ether to remove DMT (see Note 9). The aqueous phase containing DNA is collected and concentrated on a Speedvac.
6. The concentrated DMT-off DNA samples are purified by reverse-phase HPLC on the $C_{18}$ column with a linear gradient of 5–30% acetonitrile over 30 min in 1% TEAA, pH 7.0, with a flow rate of 3 ml/min. DMT-off DNA is collected at 10 min.
7. After HPLC purification, the DMT-off DNA samples are further processed by sequential dialysis in glass beakers

with water, low salt (10 mM NaCl), high salt (150 mM NaCl), and water (see Notes 10–12). A weak base (10 mM NaOH) can be used to remove persistent secondary structures. The purified and dialyzed samples are lyophilized to a homogeneous powder (see Note 13).

### 3.2. Determination of the G-quadruplex Formation by 1D $^1$H-NMR

NMR spectroscopy has a unique strength in studying DNA G-quadruplex secondary structures. The imino peaks associated with guanines in G-tetrad formation give rise to characteristic chemical shifts around 10.5–12 ppm. This chemical shift region is completely separated from imino chemical shifts from any other DNA conformations, such as duplex DNA, single-stranded DNA, or other secondary DNA structures. Therefore, the imino regions of G-quadruplex guanines provide a direct and clear monitoring system for not only the formation of a G-quadruplex structure but also its drug binding interactions.

1. As a first step in the NMR structure determination process, it is important to determine the G-quadruplex formation, sample purity and line widths by $^1$H-NMR spectra in solution. Formation of G-quadruplexes can be identified by chemical shifts of imino H1 protons in $^1$H-NMR in $H_2O$ solution. The appearance of imino peaks in the downfield region 10.5–12 ppm indicates their involvement in a Hoogsteen hydrogen-bonded network and is characteristic for the formation of G-quadruplex. The number of imino resonances corresponds to the number of G-tetrad-associated guanines present in the system (see Note 14).
2. For samples in aqueous solution, the $^1$H-NMR experiments are performed with water suppression techniques such as Watergate or Jump-and-Return, and the relaxation delay is set to 3 s.

### 3.3. Sequence Design and Selection

It is important to note that the intramolecular G-quadruplex structure formed in a G-rich sequence can be complex. Not only do different sequences adopt different structures, but also a given sequence can fold into a variety of different conformations, as in the case of the human telomeric DNA sequence (see below). A DNA sequence that forms a major stable structure with good NMR spectral properties in solution, i.e., sharp line widths, is necessary for successful structure determination by NMR. NMR and sequence analysis are unique in being able to identify the major stable G-quadruplex structures under physiologically relevant ion conditions (pH 7, 100 mM KCl).

1. The 22-nt human telomeric DNA 5′-AGGG(TTAGGG)$_3$ (wtTel22, Fig. 2a), which has been shown to form a basket-type G-quadruplex in $Na^+$ solution (22) or a parallel-stranded intramolecular G-quadruplex in the crystalline state in the presence of $K^+$ (23), was examined by 1D $^1$H NMR (Fig. 2b top).

The 1D $^{1}$H NMR shows that wtTel22 does not form a single G-quadruplex structure in $K^+$ solution.

2. We have screened a large number of variant four G-tract sequences containing the core wtTel22 with different flanking segments by $^{1}$H NMR (24). A few sequences, including the 26-nt sequence Tel26 (Fig. 2a), were found to form a major hybrid-1 type intramolecular G-quadruplex in $K^+$ solution. The 1D $^{1}$H NMR spectrum of the Tel26 sequence in $K^+$ solution (Fig. 2b) shows a major intramolecular G-quadruplex structure with 12 resolved imino proton resonances between 10.5 and 12 ppm (see Note 15). The folding topology and molecular structure of the hybrid-1 structure was determined by NMR (PDB ID 2HY9) (24, 26) (Figs. 2c and 1e).
3. The wtTel26 sequence was found to form a major hybrid-2 type intramolecular G-quadruplex in $K^+$ solution. The folding topology and molecular structure of this hybrid-2 structure was determined by NMR (PDB ID 2JPZ) (25) (Figs. 2c and 1e).
4. CD (Circular Dichroism) can be used as a complementary method to determine the G-quadruplex formation. Antiparallel-stranded G-quadruplexes have a CD spectrum characterized by a positive ellipticity maximum at 295 nm and a negative minimum at 265 nm, while the parallel-stranded ones have a positive maximum at 264 nm and a negative minimum at 240 nm (31).

### 3.4. Assignment of Guanine Base Resonances Using Labeled DNA

Only sequences showing well-resolved NMR spectra are subjected to further structural investigation. Herein, we use the telomeric DNA sequence wtTel26 as an example for structure determination by NMR spectroscopy. NMR structure determination is composed of NMR resonance assignment and NMR-restrained structure calculation. NMR resonance assignment of G-quadruplexes includes guanine base proton assignment using site-specific $^{15}$N-labeling and complete spectral assignment using 2D NMR methods. After the complete spectral assignment, the folding topology of a G-quadruplex is first determined, followed by three-dimensional structure determination using NMR-derived distance restraints in combination with MD calculations.

1. The 1D $^{1}$H NMR spectrum of the wtTel26 sequence in $K^+$ solution (Fig. 2b second top) shows a major intramolecular G-quadruplex structure with 12 resolved imino proton resonances between 10.5 and 12 ppm, indicating the formation of G-quadruplex with three G-tetrads (see Note 16).
2. The imino H1 and base aromatic H8 protons of 12 guanine residues can be unambiguously assigned by site-specific low enrichment (6%) using $^{15}$N-labeled guanine nucleoside at each guanine position in the wtTel26 sequence. The guanine

imino H1 and aromatic H8 proton resonances have one-bond coupling to N1 and two-bond coupling to N7, respectively (Fig. 1a). Both H1 and H8 protons of the site-specific labeled guanine are readily detected by 1D $^{15}$N-filtered JR-HMQC experiments (Fig. 2d) (25).

3. The assignment of guanine H8 protons can be further confirmed by long-range through-bond correlations with the already assigned imino H1 via $^{13}$C5, using 2D $^{1}$H-$^{13}$C HMBC experiment at natural abundance.

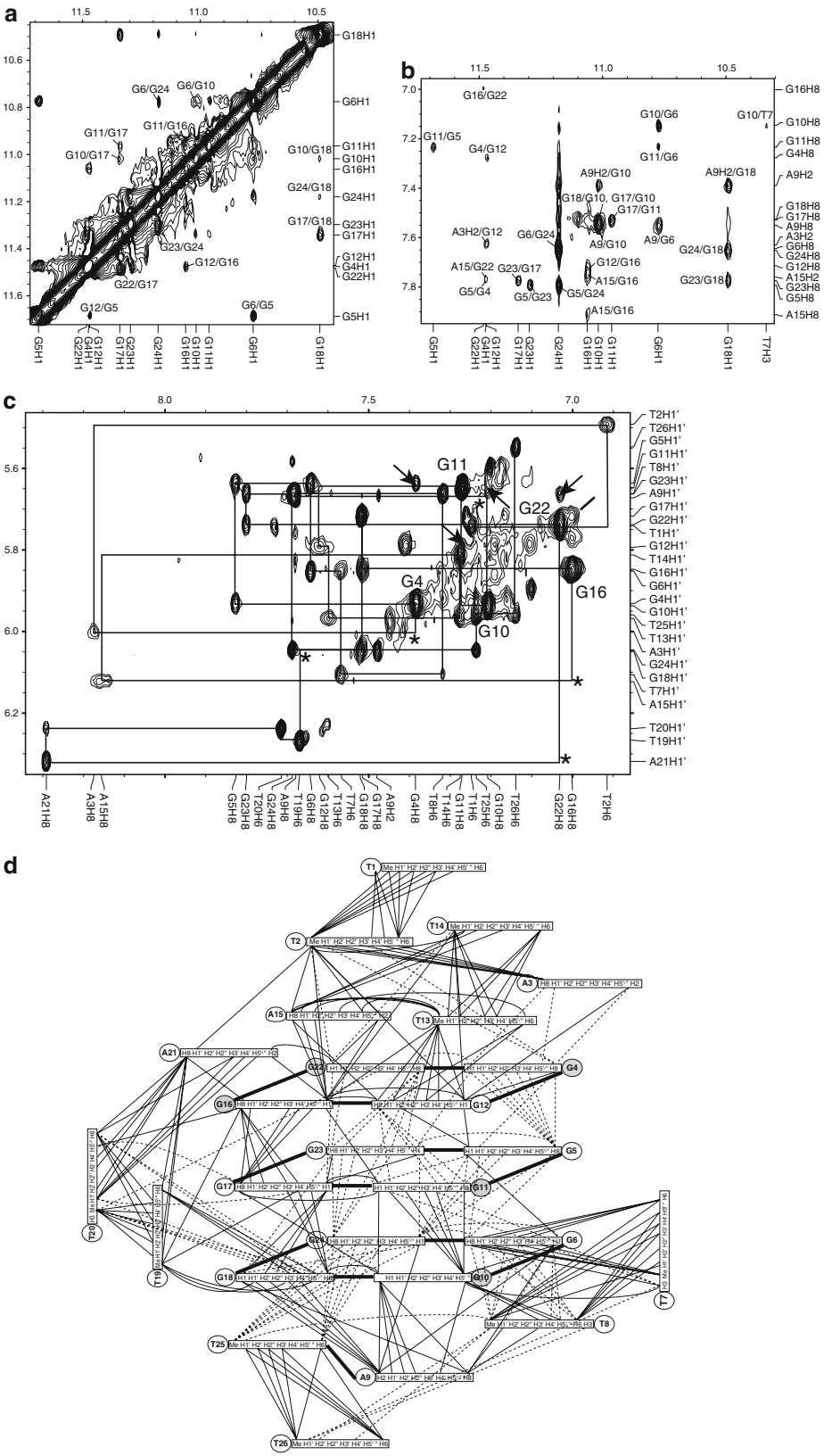
### 3.5. Assignment of Thymine/Cytosine Base Resonances

1. Thymine residues can be identified by a strong cross-peak between H6 and Me in COSY/TOCSY and NOESY spectra.
2. Cytosine residues can be identified by a strong cross-peak between H5 and H6 in COSY/TOCSY and NOESY spectra.
3. Base H6 protons of thymines or cytosines in a sequence can be unambiguously assigned by site-specific substitution for each thymine with deoxyuridine (dU) or for each cytosine with dT or dU one at a time (see Note 17).

### 3.6. Complete Spectral Assignment

1. After the site-specific assignment of base protons, the complete assignment of the base and sugar protons of a DNA sequence can be accomplished with 2D-NMR experiments (Fig. 3). Standard homonuclear 2D-NMR experiments are used to assign the nonexchangeable proton chemical shifts of the DNA, including DQF-COSY, TOCSY, and NOESY. The mixing times are set at 50, 100, 150, and 200 ms for NOESY and at 40 and 80 ms for TOCSY at various temperatures. The exchangeable proton chemical shifts are assigned for $H_2O$ samples using 2D-NOESY experiments with WATERGATE or jump-return (NOE11) water suppression techniques. The relaxation delay is set to 2 s. The acquisition data points are set to 4,096×512. The 60° shifted sine bell functions are applied to both dimensions of NOESY and TOCSY spectra. The fifth-order polynomial functions are employed for the baseline corrections.
2. Assignment of base protons H8/H6 (see Subheadings 3.4 and 3.5) gives rise to the assignment of H1–H1 (Fig. 3a) and H1–H8 crosspeaks (Fig. 3b), which can be used for the determination of the folding topology of a G-quadruplex (see Subheading 3.8).

Fig. 3. The expanded H1–H1 (**a**) and H1–H8/H2/H6 (**b**) regions of the exchangeable proton 2D-NOESY spectrum of wtTel26 in $K^+$ solution. (**c**) The expanded H8/H6–H1′ region of the nonexchangeable 2D-NOESY spectrum of wtTel26 in $K^+$ solution. The sequential assignment pathway is shown. The H8–H1′ NOEs of the guanines with *syn* conformation are labeled with residue names. Missing connectivities are labeled with *asterisks*. The characteristic G(*i*)H8/G(*i*+1)H1′ NOEs for the *syn* G(*i*)s are labeled by *arrows*. Conditions: 15°C, 25 mM phosphate, 95 mM potassium, pH 7.0, 2.5 mM DNA. (**d**) Schematic diagram of interresidue NOE connectivities of wtTel26 G-quadruplex formed in $K^+$ solution. The guanines in *syn* conformation are represented using *gray circles*.


a
b
c
d
G11
G22
G4
G16
G10

3. The assignment of guanine H1 and H8 protons can give rise to the assignment of the base proton H2/H8 of adenine residues that stack with the G-tetrads (Fig. 3b).
4. A standard DNA sequential assignment procedure is used for the assignment of nonexchangeable protons (Fig. 3c). The assignment of the aromatic H8 can lead to the direct assignment of H1′ and H2′/H2″ and then can be extended to H3′, H4′, and H5′/H5″.
5. Sequential NOEs can be assigned for the neighboring residues in a DNA sequence. For example, G*n*H8-G$n_{-1}$ H1′/H2′/H2″ NOEs allow the assignment of sequential guanines in the DNA (Fig. 3c).
6. The glycosidic torsion angles (*syn*/*anti*) of G-tetrad guanines can be determined by the intensity of the intraresidue H8-H1′ NOEs in NOESY spectra with different mixing times. Observation of a weak intraresidue H8-H1′ NOE at a short mixing time indicates an *anti* conformation, while a strong H8-H1′ NOE indicates a *syn* conformation (Fig. 3c). In addition, a characteristic downfield shift is observed for H2′/H2″ of guanines that are in *syn* conformation. In wtTel26, there are five *syn* tetrad guanines out of a total of 12 tetrad guanines (Fig. 2c), clearly shown in the NOESY spectrum (Fig. 3c).
7. Based on the complete spectral assignment, critical interresidue NOE interactions can be schematically drawn as in Fig. 3d. This schematic diagram of NOE interactions clearly defines the overall shape of the hybrid-2 G-quadruplex formed in wtTel26 in $K^+$.

### 3.7. $^{31}$P Heteronuclear NMR

1. $^{31}$P heteronuclear NMR experiments can be used to refine NMR structure calculation, particularly for loop residues with unusual conformations. $^{31}$P–$^{1}$H correlation experiments provide useful information for torsion angles: $^{31}$P–H5′/H5″ coupling constants define β, while $^{31}$P–$^{1}$H couplings give information about ε torsions (33). All $^{31}$P NMR spectra are collected with an external standard of 85% $H_3PO_4$. The experiments include the 1D $^{1}$H-decoupled $^{31}$P spectrum, 2D-heteronuclear $^{31}$P–$^{1}$H Correlation Spectroscopy (COSY), and Heteronuclear Single Quantum Correlation Spectroscopy (HSQC).

### 3.8. Determination of G-Quadruplex Folding Topology

The characteristic imino H1 and base H8 connectivity pattern lead to the direct determination of the folding topology of a G-quadruplex.

1. The G-tetrad alignments and folding topology of a sequence are determined by the interresidual H1 and H8 NOEs in exchangeable NOESY spectra (Fig. 3a and b). In a G-tetrad

plane, the imino proton H1 of each guanine is in close spatial vicinity to the H1s of the two adjacent guanines, and to the base H8 of one of the adjacent guanines due to the Hoogsteen H-bonded network (Fig. 1a). The formation of three G-tetrads, namely (G4-G12-G16-G22, top), (G5-G11-G17-G23, middle), and (G6-G10-G18-G24, bottom), can be determined for wtTel26.

2. The overall G-quadruplex alignment can be further defined based on the inter-tetrad NOE connections from residues that are positioned far apart in the DNA sequence. The strong NOE interactions of G5H1/G12H1, G11H1/G16H1, and G17H1/G22H1 (Fig. 3a) connect the top two G-tetrads and define their reversed G-arrangements. The sequential inter-tetrad NOE interactions between H1 (Fig. 3a) and H8 proton indicate the same G-conformations of the bottom two G-tetrads. From these data, the folding topology of the G-quadruplex formed in wtTel26 can be unambiguously determined (Fig. 2c).

### 3.9. NMR-Restrained Structure Determination

1. For peak assignments and NOE volume integration (peak fitting function), the software Sparky (UCSF) is used (41). Distances between nonexchangeable protons are estimated based on the NOE cross-peak volumes at 50–300 ms mixing times, with the upper and lower boundaries assigned to ±20% of the estimated distances. Distances between exchangeable protons are assigned with looser boundaries (±1–1.5 Å). The thymine base proton Me–H6 distance (2.99 Å) is used as a reference for wtTel26 (see Note 18). The distances involving the unresolved protons, e.g. methyl protons, are assigned using pseudo-atom notation to make use of the pseudo-atom correction automatically computed by X-PLOR (42). Only relatively isolated peaks are used for NOE-restrained structure calculation.
2. Metric matrix distance geometry (MMDG) calculations are carried out using an X-PLOR protocol to produce and optimize 100 initial structures. An arbitrary global conformation of G-quadruplex is used as a starting model, as a G-quadruplex contains enough global fold to derive a final NMR structure. Substructure embedding is performed to produce a family of 100 DG structures. The embedded DG structures are then subjected to simulated annealing.
3. All of the 100 conformations generated from the DGSA calculations are subjected to NOE-restrained Simulated Annealing refinement in X-PLOR with a distance-dependent dielectric constant. Hydrogen bonds in the G-tetrad planes are restrained with distances corresponding to ideal hydrogen bond geometry. Each individual hydrogen bond is restrained using two distance restraints (heavy atom–heavy atom and

heavy atom–proton). The force constants are scaled at 30 and 100 kcal/mol/A$^2$ for distance restraints and hydrogen bond constraints respectively. All possible intra- and interresidue NOE-distance restraints, together with backbone dihedral torsions and hydrogen-bond restraints for the G-tetrads, are incorporated into the structure calculation.

4. NOE-restrained simulated annealing refinement calculations are initiated at 300 K. The temperature is gradually increased to 1,000 K in 4 ps, equilibrated at 1,000 K for 20 ps, and then slowly cooled to 300 K in 10 ps. The 20 best conformations are selected based both on their minimal energy terms and number of NOE violations and are further subjected to NOE-restrained MD calculations. The resulting averaged structure is subjected to minimization. The ten best molecules are selected, based both on their minimal energy terms and on number of NOE violations, as representative solution structure of G-quadruplex (25).

### 3.10. G-Quadruplex-Interactive Compounds

NMR spectroscopy is an indispensable technique to obtain high-resolution structural information about biologically important molecules and their interaction with ligands in solution. The appropriate sizes and the globular folding of DNA G-quadruplexes offer an excellent molecular system for NMR structural study. In addition, the well-separated characteristic imino regions of G-quadruplex guanines provide a direct and unambiguous detection system for drug binding interactions. Furthermore, NMR allows a direct analysis of drug interactions with G-quadruplexes such as binding stoichiometry and binding kinetics, with direct monitoring of solution conditions such as temperatures and salt concentrations.

1. To study drug interactions of one particular G-quadruplex, the DNA sequence that forms a single major structure should be used (see Note 19). This sequence should have good NMR spectral properties, thus its drug interactions can be unambiguously characterized. For example, to study drug binding of the hybrid-1 telomeric G-quadruplex, the Tel26 sequence can be used; to study drug binding of the hybrid-2 telomeric G-quadruplex, the wtTel26 sequence can be used (Fig. 2a). The molecular structure of the G-quadruplex formed in the free DNA needs to be first determined and serves as the structural basis for studying its drug interactions.
2. 1D $^1$H NMR titration experiments are used to monitor drug binding interactions. 1D NMR spectra of free drug and free DNA will be collected and used as references. Here we use wtTel26 as an example.
3. The purity of the drug is checked using $^1$H-NMR or using analytical-HPLC. Impurities can cause additional peaks in

$^1$H-NMR spectra, which may overlap with the desired peaks or may even bind to wtTel26. The purity of the drug should be >95%.

4. For drug binding studies, concentrated drug stock solutions (20–40 mM) are prepared in $H_2O$ or DMSO-$d_6$ (see Note 20).
5. The DNA (0.1–0.5 mM) solution containing 25 mM phosphate, 95 mM potassium, pH 7.0 is checked for G-quadruplex formation by $^1$H-NMR.
6. The DNA–drug complex is prepared by step-by-step additions of a small volume of concentrated drug stock solution into DNA sample to achieve DNA–drug ratios of 1:0.5, 1:1, 1:2, and 1:3 (see Note 21). The complex sample is annealed if needed.
7. The change in chemical shifts of DNA–drug complex at various drug equivalences is monitored by 1D $^1$H-NMR spectra (Fig. 4). The imino proton regions of tetrad-guanines are well separated from nonexchangeable protons and thus can be used in monitoring drug binding interactions and line width changes in titration profiles.
8. In general, the effects of a drug on a G-quadruplex are readily interpreted by NMR titration methods. A drug that binds specifically to a particular DNA sequence with sufficient affinity will produce an NMR spectrum with well-resolved peaks.

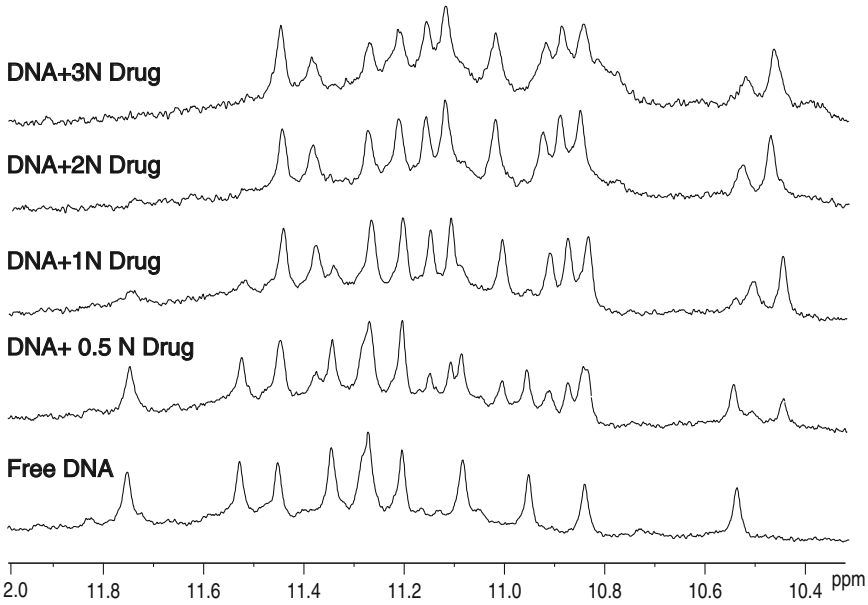



Fig. 4. The 1D $^1$H NMR titration profiles of wtTel26 with a small molecule compound at different ratios. Conditions: 25°C, 0.2 mM DNA, 25 mM phosphate, 95 mM potassium, pH 7.0.

In the case of a slow exchange binding regime, two sets of peaks of the free DNA and the bound DNA can both be observed at a lower drug equivalence, e.g., 0.5; and signals from the free DNA disappear once all the binding sites are occupied. In the case of a medium-to-fast exchange binding regime, only one set of peaks are observed for DNA and drug. In such cases, although the complex structure cannot be determined, some information on binding sites can be obtained. Compounds that do not bind tightly/specifically are readily discerned, as they do not lead to any shifts or cause spectral line broadening.

9. If a drug–DNA complex gives a well-resolved $^{1}$H-NMR spectrum, the binding site(s) or mode(s) can be determined. For example, a stacking compound (with either end of the G-quadruplex) will affect the chemical shifts of the guanine imino protons from the end G-tetrad with which the drug stacks, while an intercalating drug will shift the guanine imino resonances of the two G-tetrads between which the drug intercalates. For the groove-binding drugs, the protons located in the groove region, such as sugar protons and G's H8, rather than the imino protons, will be shifted upon drug binding. The shifting of base protons' chemical shifts will also be different for different binding modes.
10. Once the complex system is optimized, the detailed structure determination can be carried out by following the steps in Subheading 3.

## 4. Notes

1. Phosphoramidites are stored at room temperature until dissolution in acetonitrile.
2. The buffer for NMR sample preparation is stored at room temperature.
3. We used potassium salt as it is considered to be the more physiologically relevant ion. $Na^{+}$ can also be used if needed.
4. A low-concentration DNA sample is usually used for 1D experiments, while a high-concentration sample is needed for 2D NMR, particularly NOESY experiments. It needs to be confirmed that the same conformation of DNA is formed at all concentrations.
5. For DMSO stock solution, deuterated DMSO-$d_6$ is used to minimize the solvent peak in $^{1}$H-NMR spectra.
6. DNA can also be synthesized at a larger scale, e.g., at 15 μmol; however, the yield is anticipated to be lower for the larger scale synthesis.

7. The level of enrichment can vary depending on resonances. An enrichment lower than 6% can be used for detecting resonances with high intensity, while a higher level of enrichment is needed for detecting weaker resonances.
8. Cleavage of blocking groups from synthesized oligonucleotides with ammonium hydroxide can also be done at 62°C for 10 h.
9. Careful extraction of acetic acid-treated oligonucleotides with fresh ether is necessary to obtain the high-quality final material. Presence of cloudiness in the extraction process is an indication of unsuccessful extraction. Addition of 1 ml of high-purity water increases the aqueous phase volume, leading to better phase separation and better recovery of DNA products.
10. Dialysis in glass beakers is necessary to prevent the leaching of chemicals while stirring in plastic beakers, which produces contaminant peaks in NMR spectra.
11. Dialysis should be performed against at least 1,000 volumes of exchange solvent to obtain efficient change of solution conditions.
12. Oligonucleotides greater than 20-mer in lengths can be dialyzed in tubing with 3,000–3,500 MWCO; shorter oligonucleotides should be dialyzed in tubing with a MWCO of 1,000.
13. Lyophilization is a critical step for DNA product quality. All materials should be hard-frozen prior to lyophilization and dried completely before removal from the lyophilizer at all stages. Lyophilized oligonucleotides are stored under desiccation at –20°C until dissolution.
14. It is also important to determine the presence of multiple conformations. From the number of imino peaks arising from a particular sequence, it is possible to determine whether there are multiconformational species in solution and their relative populations.
15. Tel26 was found to form two well-defined G-quadruplex conformations when freshly dissolved in $K^+$ solution, as indicated by two separate sets of relatively sharp guanine imino peaks (Fig. 2b bottom). One conformation (~40%) slowly converts to the other (~60%) overnight, and the complete conversion takes about a day (Fig. 2b). This observation led to the careful examination of the native 26-nt human telomeric sequence wtTel26, $(TTAGGG)_4TT$ (Fig. 2a).
16. However, this major conformation only accounts for ~70% of the total population, with about 30% population of minor conformations shown as weak and broader resonances. Thus,

it is a more challenging process to get a complete resonance assignment for wtTel26 and a large number of different conditions are needed (25).

17. Site-specific substitution for adenines with inosines (dI) can also be used for the assignment of adenine base protons.
18. When applicable, the cytosine base proton H5–H6 distance (2.45 Å) is normally used as a reference.
19. In some cases, the wild-type DNA sequence that forms multiple conformations is preferred for examining specific drug bindings. A drug that binds to one specific conformation may be able to shift the equilibrium of the multiple forms.
20. Highly concentrated drug stock solutions allow the use of small volume additions of drug solutions in the DNA–drug complex, so that the solvent effect is negligible.
21. The DNA–drug ratio of 1:0.5 is important for drug binding studies: for a slow exchange binding regime, two sets of peaks of the free DNA and the bound DNA will be observed, while for a medium-to-fast exchange binding regime, only one set of peaks will be observed.

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# Chapter 9

# Analysis of Yeast Telomerase by Primer Extension Assays

**Min Hsu and Neal F. Lue**

## Abstract

Telomeres are specialized nucleoprotein structures located at eukaryotic chromosomal termini, which are required for chromosome stability and are maintained by a reverse transcriptase named telomerase. Budding yeast has served as an extremely useful model system for analyzing telomere maintenance because the organism offers a wide range of genetic and biochemical tools. Several milestones in telomerase research were reached through investigation of the yeast system. For example, the consequence of telomerase loss was first characterized in the budding yeast *Saccharomyces cerevisiae* (Lundblad and Szostak, Cell 57:633–643, 1989). The catalytic component of telomerase (telomerase reverse transcriptase; TERT) was likewise initially cloned from this organism (Lendvay et al., Genetics 144:1399–1412, 1996). Moreover, much of the current understanding of the structure and function of the telomerase complex was derived from yeast studies (Autexier and Lue, Annu Rev Biochem 75:493–517, 2006). In this chapter, we discuss one of the most useful tools for investigating yeast telomerase mechanisms and regulation: the primer extension assay. This assay can be used to examine the overall activity as well as the processivity of telomerase, which represents a unique aspect of telomerase enzymology (Lue et al., Mol Cell Biol 23:8440–8449, 2003; Bosoy and Lue, Nucleic Acids Res 32:93–101, 2004). It can also be employed to analyze the mechanisms of telomerase regulatory proteins (Zappulla et al., Nucleic Acids Res 37:354–367, 2009; DeZwaan and Freeman, Proc Natl Acad Sci USA 106, 17337–17342, 2009).

**Key words:** Telomerase, DEAE chromatography, IgG-Sepharose pull down, Primer extension assay, TRAP assay

## 1. Introduction

Telomerase is a ribonucleoprotein required for the maintenance of chromosomal terminal repeats in most organisms (1, 2). The reverse transcriptase protein subunit of telomerase (telomerase reverse transcriptase, TERT) catalyzes the addition of nucleotides onto the ends of telomeres in cooperation with an RNA subunit that provides the template (telomerase RNA, TER). The activity of telomerase was first characterized for the enzyme from *Tetrahymena thermophila* using a primer extension assay (3, 4). The assay was based on the

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ability of telomerase to extend telomere-like oligodeoxynucleotides in the presence of dTTP and dGTP (the nucleotides present in the *Tetrahymena* telomere repeat). This assay was subsequently adapted to studies of telomerase in many organisms ranging from yeast (5) to humans (6). However, because of the low levels of telomerase in most cells, activity often cannot be detected in whole cell extracts using this assay. To overcome this difficulty, the TRAP (telomeric repeat amplification protocol) assay, which relies on PCR to amplify the initial telomerase-mediated products, was developed (7). Nevertheless, the TRAP assay cannot be used for detecting telomerases in yeast because these enzymes are generally nonprocessive (8), making it difficult to design appropriate PCR primers for amplifying the initial extension products. In addition, the telomere repeats in some yeast are degenerate. Telomerases from these organisms are thus expected to yield heterogeneous products, again making it difficult to design suitable primers for amplification (9). For these reasons, the study of yeast telomerase has continued to rely on the primer extension assay.

Because of the low levels of telomerase in yeast (10), a purification step following the preparation of whole cell extracts is necessary before the activity can be detected. DEAE anion-exchange chromatography was the first method developed for enriching yeast telomerase and for removing inhibitors (5, 11). Subsequently, following the identification of telomerase protein genes, it became possible to fuse affinity tags to these proteins and employ affinity resins to purify telomerase from cell extracts (12, 13). The DEAE and affinity purification methods, as well as the primer extension assay, have been applied successfully to the telomerase from *Saccharomyces cerevisiae* (5, 12), *Kluyveromyces lactis* (14), *Candida albicans* (15, 16), and *Schizosaccharomyces pombe* (11, 17). In this chapter, we describe in detail our standard method for purifying yeast telomerase and analyzing its primer extension activity. We also outline our method for quantifying telomerase processivity, which allows us to determine the propensity of the enzyme to add the next nucleotide after each extension step. With suitable modifications, these methods can probably be adapted to the investigation of hitherto uncharacterized telomerases from other yeast.

## 2. Materials

### 2.1. Preparation of Yeast Extracts

1. YPD medium: 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (v/v) glucose (see Note 1).
2. TMG buffer: 10 mM Tris–HCl (pH 8.0), 1.2 mM magnesium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 10% (v/v) glycerol, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 1 mM PMSF, and 2 mM benzamidine (see Note 2).

### 2.2. Isolation of Telomerase from Cell Extracts

1. TMG(*n*): TMG buffer with *n* millimolar concentration of sodium acetate.
2. DEAE-agarose (BioRad).
3. Immunoglobulin G (IgG)-Sepharose beads (GE healthcare).
4. Glycine buffer: 0.1 M glycine (pH 2.5).

### 2.3. Primer Extension Assay

1. Reaction buffer (3×): 150 mM Tris–HCl (pH 8.0), 3 mM magnesium chloride, 3 mM spermidine, 3 mM DTT. Store at –20°C.
2. RNase solution: 10 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 0.1 mg/mL RNase A.
3. Proteinase solution: 10 mM Tris–HCl (pH 8.0), 0.5% (v/v) SDS, 0.3 mg/mL proteinase K.
4. Denaturing polyacrylamide gel: 15% acrylamide/bisacrylamide solution (20:1), 7 M urea, 1× TBE (10.8 g/L Tris base, 5.5 g/L boric acid, 1 mM EDTA).
5. Gel loading solution: 80% (w/v) formamide, 0.1× TBE, 0.03% (w/v) xylene cyanol FF, 0.03% (w/v) bromophenol blue.

### 2.4. Quantitation of Telomerase RNA and Protein Levels

1. RNA preparation buffer: TMG (300), 0.5% (v/v) SDS, 200 μg/mL proteinase K, 30 μg/mL tRNA.
2. PCI: Phenol/chloroform/isoamyl alcohol (25:24:1).
3. RT-PCR 2× master mix (USB). Store at –20°C.

#### 2.4.1. RT-PCR Analysis of Telomerase RNA

#### 2.4.2. Western Analysis of Tagged Telomerase Protein Components

1. 10% SDS–PAGE gel.
2. Loading solution (4×): 0.2 M Tris-HCl (pH 6.8), 7% (v/v) SDS, 1.426 M β-mercaptoethanol.
3. Running buffer: 25 mM Tris, 192 mM glycine, 0.1% (v/v) SDS.
4. Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.0075% (v/v) SDS.
5. Tris buffered saline (TBS): 20 mM Tris-HCl (pH 7.5), 200 mM sodium chloride.
6. TBST: TBS with 0.05% (v/v) Tween-20.
7. Blocking buffer: 2% (w/v) fraction V bovine serum albumen (BSA) in TBST.
8. Primary antibody solution: anti-protein A antibodies (Sigma Cat. No. P3775) (1:50,000-fold dilution) in TBST with 0.5% (w/v) BSA.
9. Secondary antibody: anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Cat. No. A9919).
10. BCIP/NBT color development substrate (Promega): 10 mL alkaline phosphatase buffer, 66 μL NBT, and 33 μL BCIP.

## 3. Methods

The primer extension assay is based on the ability of telomerase to add nucleotides to DNA substrates. Using a specific primer whose 3′ end is complementary to telomerase RNA template, the telomerase reverse transcriptase can incorporate radiolabeled nucleotide tripohsphates to the 3′ end of the primer, which can then be detected by denaturing gel electrophoresis and autoradiography/PhosphorImager. However, due to the low levels of telomerase in yeast and the presence other activities and inhibitors, telomerase activity generally cannot be detected in whole cell extracts. Therefore, enrichment of telomerase from whole cell extracts is a crucial first step in the analysis of enzyme activity.

Two methods have been developed for the enrichment of yeast telomerase. The first method is DEAD anion-exchange chromatography. Because almost all yeast telomerases carry a large RNA subunit (generally >1,000 nt) (18), the RNPs typically bind tightly to the DEAE resin and are released only with high salt. Active telomerase can thus be substantially purified by passage through a DEAE-agarose column. The second method is the isolation of tagged telomerase through the high-affinity interaction between protein A and IgG. The protein A tag [either alone or as part of the Tandem Affinity Purification (TAP) tag] can be fused to a telomerase component and the resulting RNP isolated from cell extracts using IgG-Sepharose. Notably, even though other protocols for yeast telomerase purification have been developed, they are all based on the principle of anion-exchange chromatography (e.g. mono-Q) or affinity isolation [e.g., streptavidin binding protein (SBP) tag and calmodulin binding protein (CBP) tag] (Hsu and Lue, unpublished, (19)).

The processivity of telomerase has been quantified using a variety of methods. For telomerases that are capable of adding multiple repeat units, relative processivity is often assessed by plotting the levels of complete repeat addition products against the repeat number on a log scale (20). This procedure is not useful for most yeast telomerases, which are generally incapable of multiple repeat addition. Instead, we developed a protocol to calculate the propensity of telomerase to add the next nucleotide after each extension step (21). This protocol was based on principles that had been used in the analysis of HIV-1 RT processivity (22).

### 3.1. Preparation of Yeast Extracts

1. Typically, 3 L of *S. cerevisiae* or *C. albicans* cell cultures are grown in YPD or YPD + uri to an OD of 1.5–2.0.
2. Cells are harvested, washed with ice-cold water once, and resuspended with equal volume of TMG(0) buffer.

3. The cell suspension is transferred to a 50-mL round bottom centrifuge tube containing glass beads filled to about 1/5 of the total height of the tube, and lysed by vigorous vortexing. 120 s of vortexing at maximum speed is alternated with 180 s of cooling in ice-water bath for a total of 100 min (see Note 3).
4. Cell lysates are transferred to 30-mL PC Oak Ridge centrifuge tubes and spun in a T-865 rotor (Sorvall) at 100,000 × *g* for 1 h.
5. The clear supernatant from each tube is collected, flash frozen in liquid nitrogen and stored at –80°C.

### 3.2. Isolation of Telomerase by DEAE Chromatography or IgG-Sepharose Pull Down

#### 3.2.1. DEAE Chromatography

1. Three milliliters of DEAE-agarose beads are equilibrated in TMG(0) and packed into a 1 × 10 cm Econo-column (BioRad).
2. The cell extract (15 mL) is loaded onto the column at a flow rate of 10 mL/h. The column is then washed successively with 15 mL of TMG(200) and 15 mL of TMG(400).
3. Subsequently, the bulk of telomerase activity is eluted with 15 mL of TMG(900). Typically 1-mL fractions are collected.
4. Peak protein fractions as determined by Bradford assays are pooled, flash frozen in liquid nitrogen and stored at –80°C.

#### 3.2.2. IgG-Sepharose Pull Down

1. Extracts prepared from *S. cerevisiae* or *C. albicans* strains containing protein A or TAP-tagged telomerase subunits are needed for this isolation procedure (13, 16). 45 µL of IgG-Sepharose beads are first equilibrated in glycine buffer and washed with TMG(0) until the pH is greater than 7.0. The beads are treated with 100 µg tRNA at 4°C for 30 min to block nonspecific binding.
2. Whole cell extracts (9.6 mg) are mixed with the IgG-Sepharose beads in 1.2 mL TMG(500) plus 0.05% Tween-20 and subjected to gentle rotation at 4°C for 2 h.
3. The beads are then washed five times in TMG(800) and twice in TMG(0) (see Note 4).
4. The beads are further divided into three equal aliquots. One aliquot is subjected to primer extension assay. The other aliquots are used for RT-PCR and Western blot analysis to quantify the levels of telomerase RNA and protein, respectively.

### 3.3. Primer Extension Assays

The primer extension assay is preformed using a short oligonucleotide as the primer and a radiolabeled nucleotide triphosphate that can be incorporated by the telomerase RNP based on the RNA template sequence and the alignment between the primer and template (see Note 5).

1. The reaction mixture is prepared by adding sequentially the following components to a microcentrifuge tube: 10 μL 3× reaction buffer, 1 μg of primer, 15 μL of column fractions, and 30 μCi [α-$^{32}$P]dNTP (NEN, 3,000 Ci/mmol) (The specific label used depends on the primer).
2. The reaction is allowed to proceed at room temperature for 30 min and stopped by adding 80 μL of the RNase solution. Following incubation at room temperature for 10 min, the mixtures are further digested with 100 μL of the proteinase solution at 37°C for 20 min.
3. The nucleic acids in the reactions are precipitated with the addition of 100 μL 7.5 M ammonium acetate, 10 μg tRNA, and 750 μL absolute ethanol (see Note 6).
4. The pellets are resuspended with 10 μL of loading solution and analyzed on a 15% denaturing polyacrylamide gel. The $^{32}$P-labeled products are visualized by using a PhosphorImager system and quantified using ImageQuant software.

### 3.4. Quantitation of Telomerase RNA and Protein Levels

#### 3.4.1. RT-PCR Analysis of Telomerase RNA

1. Following incubation with extracts containing tagged telomerase, 15 μL of the IgG-Sepharose beads are treated with 330 μL of the RNA preparation buffer at room temperature for 30 min and at 37°C for another 30 min.
2. The mixture is extracted with PCI. The RNA in the mixture is then precipitated by ethanol, and resuspended with 20 μL of nuclease-free water (see Note 7).
3. The level of telomerase RNA is then assayed by semiquantitative RT-PCR. A 20 μL reaction is carried out with 2 μL isolated RNA, 10 μL 2× RT-PCR master mix, and 1 μM primers designed to amplify a specific telomerase RNA fragment (see Note 8).

#### 3.4.2. Western Analysis of the Tagged Telomerase Protein Component

1. Electrophoresis and the transfer of protein to nitrocellulose membrane are performed according to standard procedures.
2. For the detection of a protein A or TAP-tagged telomerase subunit, the membrane is first incubated with α-protein A antibody (Sigma Cat. No. P3775) in primary antibody solution (1:50,000-fold dilution) at room temperature for overnight, and then washed three times in TBST buffer.
3. The secondary antibody (α-rabbit IgG adsorbed with human IgG; Sigma Cat No. A9919) (see Note 9) is added to the membrane at 1:5,000 dilution in TBST and the incubation continued for 40 min at room temperature. The membrane is then washed three times in TBST and twice in TBS buffer.
4. The BCIP/NBT color development substrate solution (Promega) is freshly prepared and added to the membrane until sufficient signals developed on the membrane.

### 3.5. Analysis of Telomerase Processivity

1. The reaction products from a telomerase primer extension assay are identified from a PhosphorImager scan. The relative signals of individual products are quantified using the ImageQuant software.
2. The signal for each band is normalized to the amount of transcript by dividing against the number of labeled residues. For example, if the sequence TGTGGTG was added to the primer and the labeled nucleotide in the reaction was dTTP (Fig. 1), then the +2 to +5 product signals should be divided by 2, and the +6 and +7 product signals divided by 3.
3. Processivity at each position is calculated using the following formula.

$$\text{Processivity } P_i = \frac{\sum_{j=i+1}^{N}(T_j)}{\sum_{i=i}^{N}(T_j)},$$

where *Ti* denotes the amount of transcripts calculated for the primer+*i* position and *N* is the highest number such that a visible signal can be discerned in the PhosphorImager file for the primer+*N* product.

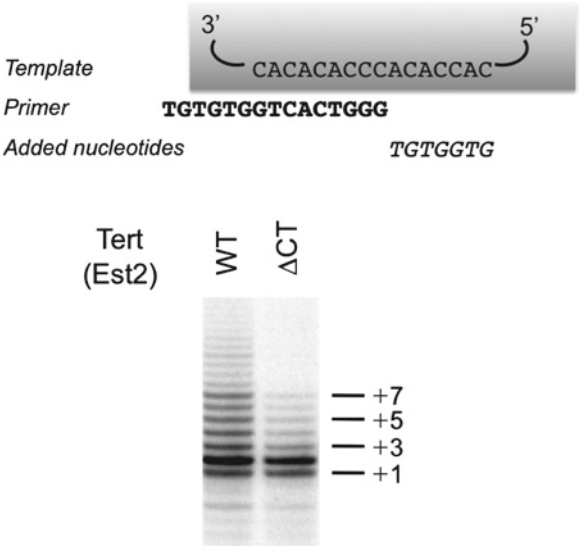


Fig. 1. Multiple nucleotide addition using *S. cerevisiae* telomerase. (*Top*) The RNA template for *S. cerevisiae* telomerase, a primer substrate, and the predicted sequence addition to the primer are illustrated. (*Bottom*) Primer extension assays were performed using wild-type telomerase (WT) and a C-terminal truncation mutant (ΔCT) with reduced processivity. Included in the reactions were 0.2 μM [α-32P]dTTP and 50 μM dGTP. The positions of the various extension products are indicated by horizontal bars to the right of the panel. Applying the method described in Subheading 3.5, we found that the processivity of the wild-type and mutant telomerase at the primer+2 position are ~0.5 and 0.2, respectively.

## 4. Notes

1. The YPD medium can be used for the growth of almost all *S. cerevisiae* strains. Some *C. albicans* strains are auxotrophic for uridine and need to be grown in YPD + uri (YPD supplemented 80 μg/mL uridine).
2. The protease inhibitors Pepstatin A, leupeptin, PMSF, and benzamidine are used to prevent degradation of proteins during extract preparation and telomerase purification. The stock solutions for the protease inhibitors are as follows: 1 mg/mL pepstatin A (in methanol), 1 mg/mL leupeptin (in water), and 100 mM PMSF/200 mM benzamidine (in ethanol). All preparations are stored at −20°C.
3. The glass beads need to be fully stirred up during vortexing to enhance the efficiency of lysis. The extent of lysis can be monitored by measuring the protein concentrations of the lysates. A concentration of ~10 mg/mL or greater is usually reached after 100 min of total vortexing.
4. For binding, the salt concentration of TMG buffer is adjusted by adding 3 M sodium acetate. Because the volume of the beads is quite small, the beads should be carefully collected to the bottom of the tube by performing two spins. After the initial spin, the tube is turned 180° and subjected to a second spin. All the centrifugation steps are done at 4°C and 3,000 rpm for 1 s. For washing, simply pipetting in the buffer vigorously is sufficient to resuspend the beads. Repeated titration may result in the adherence of beads to pipette tips and sample loss. In our experience, a large number of washes (> or = five times) with the high salt TMG buffer are needed to minimize nonspecific binding.
5. The primers are designed based on the telomerase RNA template sequence. Appropriate nucleotide(s) can be included to enable telomerase to add just one or multiple nucleotides to the starting primer. The "single nucleotide addition" assay is often more sensitive because all of the radioactive signals are concentrated in a single band in the gel (Fig. 2). On the other hand, the multiple-nucleotides-addition assay is needed to analyze the elongation ability of telomerase.
6. To monitor sample recovery, a labeled oligonucleotide can be added to each sample before ethanol precipitation to serve as a loading control (Fig. 2).
7. PCI is used for removing proteins from the samples. To minimize protein contamination, the aqueous (upper) phase should be collected very carefully and any insoluble materials at the interphase (beads and precipitates) should be avoided.

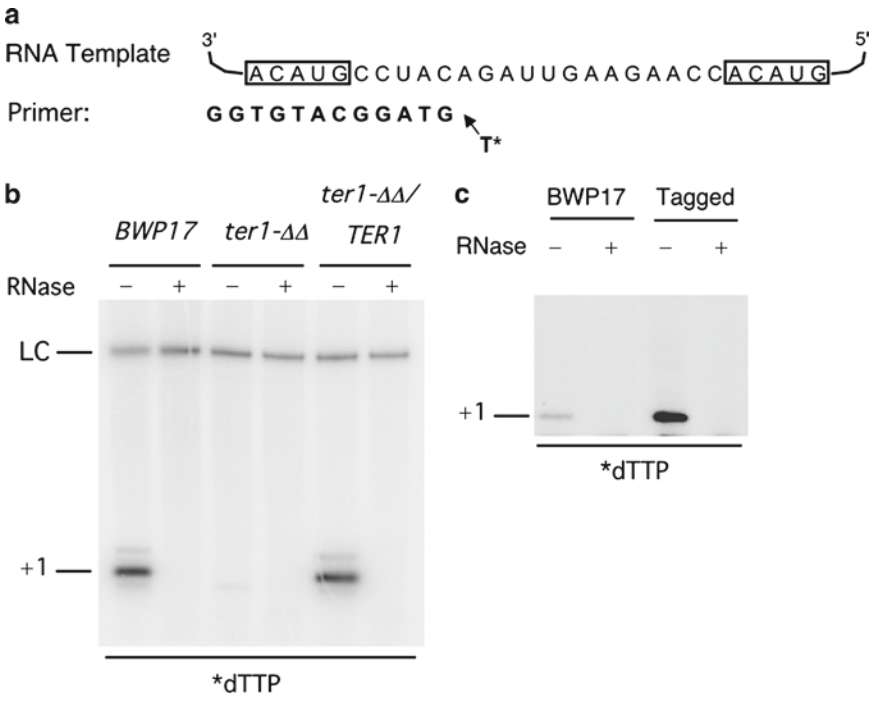


Fig. 2. Single nucleotide addition using *C. albicans* telomerase. (**a**) *C. albicans* telomerase is expected to add one nucleotide to the indicated primer in the presence of dTTP, based on the alignment between the primer and telomerase RNA template. (**b**) *Candia* extracts and DEAE fractions were prepared from the wild-type (*BWP17*), a telomerase RNA knockout mutant (*ter1*-ΔΔ), and a reconstituted strain (*ter1*-ΔΔ/*TER1*), and subjected to primer extension assays in the presence of the indicated primer and $^{32}$P-labeled dTTP. Deletion of the telomerase RNA resulted in complete loss of telomerase activity. Fractions were also pretreated with RNase A before the assays to demonstrate the dependence of the activity on RNA. A 46-mer-labeled oligonucleotide was added to each sample before ethanol precipitation as a control for sample recovery (LC). (**c**) DEAE fractions prepared from *BWP17* and a strain with TAP-tagged telomerase were subjected to IgG-Sepharose pull down. The pull down beads were analyzed directly by primer extension assays to measure telomerase activity. As predicted, higher levels of RNase-sensitive primer extension products were detected in the beads pretreated with the extract containing TAP-tagged telomerase.

8. The linearity of the assay can be confirmed by using different cycle numbers and by titrating the samples.
9. The particular secondary antibody preparation form Sigma (Cat. No. A9919) has been pre-adsorbed with human IgG to reduce the level of cross-reacting antibodies. The use of this secondary antibody should diminish nonspecific signals that are created by the human IgG antibody in the pull down samples.

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# Chapter 10

# Telomeric Repeat Amplification Protocol: Measuring the Activity of the Telomerase

**Huawei Xin**

## Abstract

Levels of telomerase activity can be an indicator of the proliferative potential of somatic cells and may serve as a diagnostic biomarker of malignancy. Telomeric repeat amplification protocol (TRAP) is a fast and sensitive PCR-based assay for detection and measurement of telomerase activity. Since its introduction, the TRAP assay has been widely used in cancer and aging studies. It provides a powerful alternative to other in vitro telomerase detection methods, and has been combined with other molecular techniques to screen telomerase inhibitors, and to study human malignancy and other diseases associated with telomere or telomerase dysfunction.

**Key words:** Telomerase, PCR, Malignancy, Cell proliferation, Senescence

## 1. Introduction

It has been estimated that >90% of human tumors exhibit telomerase activation. In other words, the activity of the telomerase may serve as a critical marker for cell transformation and cancerous growth. The development of the telomeric repeat amplification protocol (TRAP) assay has dramatically improved the ease and accuracy with which we measure telomerase activity (1–3). The TRAP assay can facilitate the drug screening process for telomerase inhibitors. It has also been used to monitor telomerase activity of stem cells and immune cells, in which telomerase activity is closely linked to their senescence and proliferative potential (4).

TRAP is quick and sensitive. Typically, only a small amount of extract from cells, tissue, or other specimen is needed for a simple two-step procedure: telomerase-mediated primer extension and

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amplification of the extended product. Modifications of the standard TRAP assays utilize different techniques to visualize and quantify the extended products, including real-time quantitative (RTQ) TRAP, TRAP–ELISA, and in situ TRAP.

## 2. Materials

### 2.1. Cell Extract Preparation

1. Cells, tissue, or specimen of interest. The telomerase-positive HeLa cells cultured in modified DMEM medium containing 10% FBS as a positive control (see Note 1).
2. DEPC water (see Note 2).
3. $Ca^{2+}$- and $Mg^{2+}$-free PBS: 137 mM sodium chloride, 1.5 mM potassium phosphate, 7.2 mM sodium phosphate, 2.7 mM potassium chloride, pH 7.4.
4. Washing buffer: 10 mM HEPES–KOH, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM DTT in DEPC water. DTT should be added just before use.
5. Lysis buffer: 10 mM Tris–HCl, pH 7.5, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.5–1% CHAPS or NP-40, 10% glycerol, 5 mM beta-mercaptoethanol (β-ME), and 0.1 mM PMSF in DEPC water. β-ME and PMSF should be added fresh.

### 2.2. Assay Reaction and Detection

1. TRAP reaction buffer: 20 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, and 1 mM EGTA in DEPC water (see Note 3).
2. Oligonucleotide primers:

   TS primer (telomerase substrate for extension): 5′-AATCCGT CGAGCAGAGTT-3′

   CX primer (reverse primer for amplification): 5′-CCCTTAC CCTTACCCTTACCCTAA-3′.
3. dNTP mix: 2.5 mM each of dATP, dCTP, dGTP, and dTTP.
4. Hot-start Taq polymerase.
5. Thermal cycler.
6. TBE: 10.8 g/L Tris base, 5.5 g/L boric acid, 1 mM EDTA.
7. 10% Polyacrylamide gel in TBE buffer.
8. Loading buffer: 50% glycerol, 50 mM EDTA, and 0.25% bromophenol blue.
9. SYBR solution.

## 3. Methods

### 3.1. Cell Extract Preparation

1. Harvest the cells (including the positive control HeLa cells) or other specimen such as tissue, wash once with ice-cold PBS, and once with ice-cold Washing buffer. Collect the final cell pellets in microcentrifuge tubes for subsequent steps. At this point, samples may be flash frozen in liquid nitrogen and stored at −80°C (see Note 4).
2. Add 4–5× volume of Lysis buffer relative to pellet size, or 4–5 μL Lysis buffer per milligram of tissue. Resuspend the cells by pipetting up and down repeatedly. Homogenization of tissue sample is performed with pestles.
3. Incubate the sample on ice for 30 min with occasional agitation. Alternatively, shake the sample for 10–30 min at 4°C.
4. Centrifuge the sample in a microcentrifuge tube at 18,000 × *g* for 10 min at 4°C.
5. Transfer the supernatant to a fresh tube, and measure the protein concentration (see Note 5).
6. Divide the cleared lysate into 10 μL aliquots (see Subheading 3.2). Unused aliquots can be quick frozen in liquid nitrogen and stored at −80°C (see Note 6).

### 3.2. TRAP Reaction

1. Each sample is analyzed in pairs, one of which is heat inactivated (85°C for 10 min) before primer extension and PCR. Label this sample as "heat inactivated."
2. Serially dilute (e.g., by twofold) each lysate sample including the heat-inactivated duplicate with TRAP assay buffer. The protein amount of the cell extract should be in the range of 0.001–1 μg.
3. Aliquot 2 μL each into PCR tubes. Include the serially diluted positive controls and a buffer only negative control.
4. Prepare a master reaction mixture. Each 20 μL reaction contains TRAP buffer, 0.125 mM dNTP, 0.25 μM TS primer, 0.25 μM CX primer, and 1 U hot-start Taq polymerase (see Note 7).
5. Add 18 μL of the master reaction solution to each PCR tube that contains the 2 μL of diluted lysate.
6. Put the samples in a thermal cycler. Program it for 30 min of incubation at 30°C, followed by 30–35 cycles of PCR reaction: 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s. The amplified product can be stored at 4 or −20°C before further analysis.

### 3.3. Detection and Analysis

1. Prepare a 10% polyacrylamide gel in TBE buffer.
2. Mix the PCR product with 2 μL of loading buffer. Load 10 μL of this sample mixture per lane. Include positive and negative controls, as well as the heat-inactivated samples (see Note 8).
3. Carry out the electrophoresis at 180–200 V for ~1 h, or until the dye front reaches ¾ length of the gel.
4. Stain the gel with SYBR green and visualize under UV illumination (see Note 9).

## 4. Notes

1. TRAP is designed to detect products of enzymatic activities. As a result, only fresh cells and tissue, or flash frozen samples (stored at −80°C) should be used for the assay.
2. Because the telomerase is a reverse transcriptase with an RNA component, special precaution must be taken to ensure RNase-free manipulation. For example, reagents should be made in DEPC-treated water, and working surfaces and equipment including pipettes and labware should be decontaminated with a solution such as RNase Away.
3. Due to the sensitivity of the PCR-based TRAP assay, contamination should be diligently avoided. Examples include working in a designated area and using barrier tips.
4. Quick frozen samples may be stored for over 12 months. While frozen lysate may be used, it should not be compared directly with freshly prepared lysate for telomerase activity.
5. Bradford assays may be performed to calculate protein concentration.
6. Undiluted lysate can be stored at −80°C for up to 12 months.
7. Modified CX primer may be used for the reduction of primer–dimer formation (5′-GCGCGG$(CTTACC)_3$CTAACC-3′).
8. Special caution should be taken to avoid overloading the lanes, which will result in carryover of the sample into adjacent lanes. This can complicate analysis and interpretation of the data.
9. TS primer can be radio- or fluorescence-labeled for detection by autoradiography or a fluorescence detector. SYBR green may be added to the PCR mixture for real-time detection of formation of amplified double-stranded product.

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# Chapter 11

# CO-FISH, COD-FISH, ReD-FISH, SKY-FISH

**Eli S. Williams, Michael N. Cornforth, Edwin H. Goodwin, and Susan M. Bailey**

## Abstract

Fluorescence in situ hybridization (FISH) has become a powerful tool for exploring genomes at the level of chromosomes. The procedure can be used to identify individual chromosomes, rearrangements between chromosomes, and the location within a chromosome of specific DNA sequences such as centromeres, telomeres, and even individual genes. Chromosome orientation FISH (CO-FISH) extends the information obtainable from standard FISH to include the relative orientation of two or more DNA sequences within a chromosome (Goodwin and Meyne, Cytogenet Cell Genet 63:126–127, 1993). In combination with a suitable reference probe, CO-FISH can also determine the absolute 5′–3′ direction of a DNA sequence relative to the short arm (pter) to long arm (qter) axis of the chromosome. This variation of CO-FISH was originally termed "COD-FISH" (Chromosome orientation and direction FISH) to reflect this fact (Meyne and Goodwin, Chromosome Research 3:375–378, 1995). Telomeric DNA serves as a convenient and absolute reference probe for this purpose, since all G-rich 5′-$(TTAGGG)_n$-3′ telomeric sequences are terminally located and oriented away from the centromere.

In the beginning, CO-FISH was used to detect obligate chromosomal inversions associated with isochromosome formation (Bailey et al., Mutagenesis 11:139–144, 1996), various pericentric inversions (Bailey et al., Cytogenetics and Cell Genetics 75:248–253, 1996), and to confirm the origin of centromeric lateral asymmetry (Goodwin et al., Chromosoma 104:345–347, 1996). More recent and sophisticated applications of CO-FISH include distinction between telomeres produced via leading- vs. lagging-strand DNA synthesis (Bailey et al., Science 293:2462–2465, 2001), identification of interstitial blocks of telomere sequence that result from inappropriate fusion to double-strand breaks (telomere–DSB fusion) (Bailey et al., DNA Repair (Amst) 3:349–357, 2004), discovery of elevated rates of mitotic recombination at chromosomal termini (Cornforth and Eberle, Mutagenesis, 16:85–89, 2001) and sister chromatid exchange within telomeric DNA (T-SCE) (Bailey et al., Nucleic Acids Res 32:3743–3751, 2004), establishing replication timing of mammalian telomeres throughout S-phase (ReD-FISH) (Cornforth et al., In: Cold Spring Harbor Symposium: Telomeres and Telomerase, Cold Spring Harbor, NY, 2003; Zou et al., Proc Natl Acad Sci USA 101:12928–12933, 2004) and in combination with spectral karyotyping (SKY-CO-FISH) (Williams et al., Cancer Res 69:2100–2107, 2009). For more information, the reader is referred to several reviews (Bailey et al., Cytogenet Genome Res 107, 14–17, 2004; Bailey and Cornforth, Cell Mol Life Sci 64:2956–2964, 2007; Bailey, Telomeres and Double-Strand Breaks – All's Well that "Ends" Well, Radiat Res 169:1–7, 2008).

**Key words:** Fluorescence in situ hybridization, CO-FISH, ReD-FISH, SKY-CO-FISH, Telomeres

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## 1. Introduction

Chromosome orientation fluorescence in situ hybridization (CO-FISH) differs from standard FISH in its ability to make hybridizations strand-specific. As shown in Fig. 1, the procedure works by culturing cells for a single round of replication in the presence of the thymidine analog 5′-bromo-2′-deoxyuridine (BrdU). During semiconservative DNA synthesis, BrdU incorporates into the newly replicated daughter DNA strands. Cell-cycle progression is blocked in metaphase with a microtubule inhibitor, typically Colcemid, and cells are fixed using standard cytogenetic techniques. The fixed cells are dropped onto microscope slides causing the mitotic cells to spread out and display their condensed chromosomes. The slides are stained with the nucleic acid stain Hoechst 33258 and exposed to long-wave UV light, a process that preferentially nicks bromosubstituted DNA. Next slides are treated with exonuclease III, an enzyme that catalytically degrades one strand of the double helix utilizing nicks as a preferred substrate. Because the newly replicated BrdU-substituted strands are heavily nicked, Exo III selectively and effectively removes these strands, rendering the mitotic chromosomes single-stranded such that each chromatid contains one of the original parental strands. This single-stranded chromosomal DNA serves as an ideal target for probe hybridization. Moreover, because the original parental strands are complementary to one another, a single-stranded probe hybridizes only to its complementary chromosomal target on just one chromatid. Overall the effect is to produce strand-specific, single-sided signals.

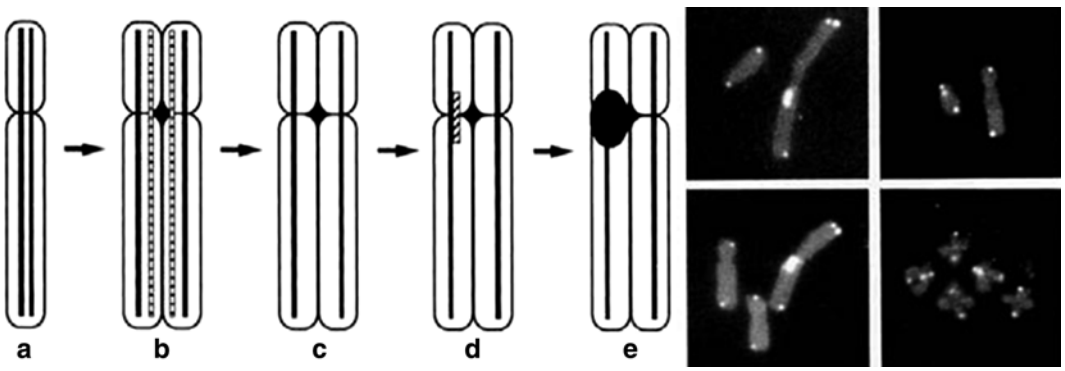


Fig. 1. CO-FISH steps. (**a**) G1 chromosome prior to replication; (**b**) incorporation of BrdU; (**c**) selective removal of newly replicated strands leaving the target parental strands oriented in opposite directions on the two chromatids; (**d**) probe hybridization; (**e**) probe detection. The photographs illustrate single-sided signals following hybridization of probes to repetitive sequences found at telomeres, the centromeres of chromosomes 1 and 18, and the 1p36 region on the short arm of chromosome 1 (from ref. 22, with permission).

The relative orientation of two chromosomal DNA sequences is revealed by unique hybridization patterns. If the sequences are oriented in parallel, probes to both sequences hybridize to and produce visible signals on the same chromatid; signals on opposite chromatids reveal an antiparallel orientation. Repetitive sequences are a special case in which a single probe binds to multiple repeats. Several probes were hybridized to repetitive sequences with results shown in the photographs in Fig. 1. In each case, and as we have found to be generally true of tandem repeats (3–5), these repetitive sequences are oriented head-to-tail (i.e., in parallel) as determined from the CO-FISH hybridization pattern (single-sided signals). In contrast, dispersed repeats such as Alu appear to have no preferential orientation, at least not at the level of resolution of light microscopy.

The CO-FISH concept has been used to provide information not easily obtainable by any other means. For example, it has been modified for use in determining the replication timing of specific mammalian telomeres during S-phase, a technique termed replicative detargeting FISH (ReD-FISH) (11). Replication of a particular probed locus or sequence in the presence of BrdU results in a "switch" from a normal FISH (two-sided; one on each chromatid) signal to a CO-FISH (one-sided; only one chromatid involved) signal as the replication fork passes the region in question. Monitoring such a change during the ensuing metaphase, as in Fig. 2, shows a smooth sigmoid curve

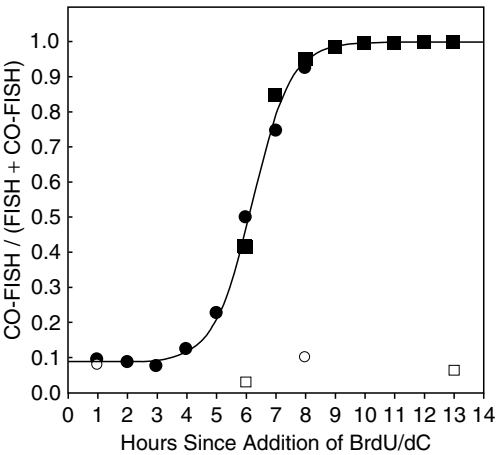


Fig. 2. ReD-FISH in Human Fibroblasts. Two overlapping experiments (*squares* and *circles*). The change in the ratio of "single-sided" CO-FISH signals to those which include conventional "two-sided" FISH hybridization signals accompanies the replication of telomeres. The sigmoid shape of the curve suggests that human telomeres replicate throughout S phase. Controls (*open symbols*) are time-matched controls that were not allowed to incorporate BrdU. (from ref. 22, with permission).

in the ratio of CO-FISH (single-sided) to normal FISH (double-sided) signals, consistent with the proposition that telomeres in normal human cells replicate throughout S-phase. A somewhat more elegant strategy is also possible using a two-color, two-probe approach (i.e., G-rich and C-rich telomere probes labeled with different colors) and monitoring individual (CO-FISH, replicated) vs. overlapping (FISH, not replicated) signals (11).

In addition to the applications described above, CO-FISH studies are providing unique insights into the genetic basis of telomere function. As the significance of strand-specificity has been recognized and better appreciated, the realization came that probes complementary to either G-rich or C-rich telomeric strands could be used to distinguish between telomeres based on their mode of replication. Because replication forks proceed from the most distal origin of replication toward the chromosome ends, the G- and C-rich probes hybridize, respectively, to telomeres that were replicated via leading-strand or lagging-strand DNA synthesis (6). The ability to discriminate leading vs. lagging-strand telomeres has enabled studies that demonstrate important, somewhat unexpected, differences in their postreplicative processing and genetic control.

CO-FISH has also made detection of SCE-like recombination within telomeric DNA itself possible, evidenced by "splitting" of the single-sided CO-FISH signal between the two sister chromatids, often resulting in "sister signals" of unequal intensities, events that have been termed T-SCE (9, 16, 17). T-SCE frequencies, their genetic regulation, as well as their implications for extending proliferative lifespan on one hand and expediting replicative senescence on the other (9, 18) are being recognized and explored. T-SCE analysis is therefore, currently enjoying widespread application, particularly in telomerase-negative (e.g., ALT) backgrounds (19–21). Most studies of telomeres now use probes composed of peptide nucleic acid (PNA) rather than DNA, due to their superior binding properties and consequently brighter signals produced, an especially important attribute for the examination of human telomeres. Prelabeled PNA telomere probes are commercially available and generally give excellent results and improve the sensitivity of T-SCE detection (see Note 1). Hybridization conditions for PNA probes are substantially different from those commonly used for DNA probes. Detailed methods for both types of probe are given below. We also note that it is imperative to be aware of and avoid possible sources of procedural artifact that can result in "false" T-SCE signals (see Note 2); therefore, controls and tests to confirm successful CO-FISH are essential and are also outlined.

## 2. Materials

### 2.1. Incorporation of BrdU, Cell Collection, and Slide Preparation

1. 5′-bromo-2′-deoxyuridine (BrdU): prepare 1 mM stock solution in $H_2O$ and store aliquots at –20°C in the dark.
2. Colcemid.
3. Trypsin–EDTA.
4. 75 mM KCl in $H_2O$.
5. 3:1 methanol: acetic acid fixative (always make up fresh).
6. High-quality glass microscope slides.
7. Glass coverslips (22 × 50 mm, No.1).

### 2.2. Slide Pretreatment

1. RNAse A (100 μg/mL in $H_2O$), prepare 50 mL and store in lidded coplin jar at 4°C. Replace approximately every 2–3 weeks depending on usage.
2. PBS (1×).
3. Formaldehyde (4% w/v) freshly prepared in PBS. Prepare 50 mL and store in lidded coplin jar at 4°C. Replace approximately every 2–3 weeks depending on usage.
4. 75, 85, and 100% ethanol (v/v in $H_2O$). Store in lidded coplin jars at –20°C until immediately prior to use.

### 2.3. Preparation of Single-Stranded DNA/Degradation of Newly Synthesized DNA Strands (CO-FISH)

1. Hoechst 33258 stock (500 μg/mL in $H_2O$). Prepare 50 mL and store in foil-covered lidded coplin jar at 4°C.
2. SSC: prepare 20× stock solution. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of $H_2O$. Adjust the pH to 7.0 with a few drops of a 14N solution of HCl. Adjust the volume to 1 L with $H_2O$. Sterilize by autoclaving and dispense into aliquots. The final concentrations of the ingredients are 3.0 M NaCl and 0.3 M sodium citrate.
3. Exonuclease III (3 U/μL in provided reaction buffer) (Store at –20°C) (Promega).
4. Ultraviolet Light Source, ~365 nm (e.g. Stratalinker 1800 UV Irradiator, Stratagene).
5. Coverglass (22 × 50 mm, No.1).

### 2.4. Denaturation of Chromosomes and Hybridization

1. Formamide (70% in 2× SSC).
2. PNA Probe Cocktail: 0.2 μg/mL fluorochrome-labeled peptide nucleic acid (PNA) probe (Dako USA, Carpinteria, CA), 70% formamide (v/v), 12 mM Tris–HCl pH 7.2, 5 mM KCl, and 1 mM $MgCl_2$.

3. Hybridization chambers (Corning). A homemade chamber consisting of a sealed Tupperware container lined with moist paper towels and pipettes to raise the slides off the towels is suitable.

### 2.5. Posthybridization Washes and Counterstaining

1. PN Buffer: 100 mM $Na_2HPO_4$, 50 mM $NaH_2PO_4$, and 0.1% Triton X-100 (v/v).
2. Vectashield antifade with DAPI (4,6-diamidine-2-phenylindole dihydrochloride) (1.5 μg/mL store at 4°C in dark) (Vector Labs).
3. Fluorescence microscope equipped with appropriate filter sets and objectives.

### 2.6. Anti-BrdU Controls/Tests

1. Anti-BrdU antibody [Invitrogen #B-35132 (red) or #B-35130 (green)].

## 3. Methods

### 3.1. Incorporation of BrdU, Cell Collection, and Slide Preparation

1. Confluent/near confluent cell cultures are subcultured into fresh medium containing 5′-bromo-2′-deoxyuridine (BrdU) at a total final concentration of $1 \times 10^{-5}$ M (see Notes 3–5).
2. Cells are incubated for one cell cycle and Colcemid (0.2 μg/mL, mouse; 0.1 μg/mL human; Gibco) is added for the final 4 h to accumulate mitotic cells (see Notes 6 and 7).
3. Cells, a majority of which are now singly substituted with BrdU, are dislodged with trypsin (5 min at 37°C) and pelleted by centrifugation at $210 \times g$ for 5 min at room temperature (RT). Aspirate the supernatant.
4. Resuspended cell pellet (avoid vortexing) in 4 mL of 75 mM KCl hypotonic solution at 37°C for 15 min.
5. Prefix cells by adding 1 mL of 3:1 methanol/acetic acid and invert tube to mix.
6. Pellet cells by centrifugation at $210 \times g$ for 5 min at RT.
7. Aspirate the supernatant.
8. Fix cells by resuspending cell pellet (avoid vortexing) in 4 mL of fresh 3:1 methanol/acetic acid. Pellets can now be stored at –20°C for years.
9. Prior to dropping slides, re-pellet cells by centrifugation at $210 \times g$ for 5 min at RT and resuspend in fresh 3:1 methanol/acetic acid. Repeat this step for twice for a total of three washes with fresh fixative.
10. Drop onto clean (cold and wet) microscope slides and allow to air-dry. Variation in slide drying time and cell density has a

significant effect on the quality of the metaphase spreads and conditions must be optimized for each case. High-quality metaphase spreads are essential for robust, specific hybridization of the PNA telomere probe.

### 3.2. Slide Pretreatment

We find these steps, though not strictly required for adequate signal, significantly improve the signal strength and reduce nonspecific probe binding.

1. Incubate slides in coplin jar containing 50 mL RNase A (100 μg/mL) prewarmed to 37°C for 10 min, followed by one PBS rinse.
2. "Post-fix" chromosomes in coplin jar containing 50 mL 4% formaldehyde at RT for 10 min, followed by one PBS rinse.
3. Dehydrate slides in 75, 85, and then 100% ice-cold ethanol for 2 min each; allow slides to air-dry after ethanol series.

### 3.3. Preparation of Single-Stranded DNA/Degradation of Newly Synthesized DNA Strands (CO-FISH)

Degradation of newly synthesized, BrdU-substituted strands is the foundation of the CO-FISH procedure. Staining with Hoechst 33258 sensitizes BrdU-substituted strands to the induction of DNA single-strand breaks from exposure to UV light. Acting at the sites of these "nicks", enzymatic digestion with Exonuclease III preferentially removes the BrdU-substituted DNA strands.

1. Slides are stained with 0.5 μg/mL Hoechst 33258 in 2× SSC for 15 min at RT. This step should be performed in the dark due to the light-sensitivity of the Hoechst dye. Briefly, rinse the slides in deionized distilled water and allow to air-dry.
2. Flood the slide with ~50 μL 2× SSC and apply coverslip. Slides are then exposed to 365 nm UV light (see Note 8) for 25–30 min. Do not allow slides to dry out during this process. Remove the coverslip, briefly rinse slides in distilled water and air-dry.
3. Prepare 50 μL of Exonuclease III (3 U/μL) in buffer supplied by the manufacturer (50 mM Tris–HCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol, pH 8.0), apply to slide and coverslip. Allow enzymatic reaction to proceed for 10 min at RT, then remove the coverslip and briefly rinse the slide in distilled water and air-dry. Care should be taken at all times to avoid scratching slide.

### 3.4. Denaturation of Chromosomes and Hybridization

Denaturation of chromosomes is not strictly required because the DNA is rendered single-stranded by the CO-FISH procedure. However, the addition of a denaturation step may be useful to compensate for any incomplete preparation of single-stranded DNA.

1. Denature slides in coplin jar containing 50 mL 70% formamide, 30% 2× SSC solution prewarmed to 72°C for 1–2 min.

Note that adding one slide to a solution in a coplin jar temporarily decreases the temperature 1°C, adjust waterbath temperature accordingly.

2. Immediately dehydrate slides in ice-cold ethanol series (75, 85, and 100% ethanol, 2 min each) and allow slides to air-dry.
3. Prepare hybridization cocktail consisting of 0.2 μg/mL fluorochrome-labeled PNA probe, 70% (v/v) formamide, 12 mM Tris–HCl pH 7.2, 5 mM KCl, 1 mM $MgCl_2$. Excessive light should be avoided from this point forward to prevent bleaching of probe fluorescence. Denature probe for 5 min at 72°C, and move immediately to ice for 5 min (see Note 9).
4. Add 25 μL of the denatured hybridization cocktail to each slide. Apply coverslip to slide, remove any bubbles that may have formed by applying slight pressure to the coverslip, and seal the edges with rubber cement.
5. Place the slides in a dark, moist chamber for 3 h at 37°C.

### 3.5. Posthybridization Washes and Counterstaining

1. After hybridization, carefully remove rubber cement and coverslip.
2. Wash slides in coplin jar containing 50 mL 70% formamide, 30% 2× SSC solution prewarmed to 32°C for 15 min, followed by a second wash in 2× SSC for an additional 15 min at 32°C with shaking (see Note 10).
3. Transfer slide to PN buffer for 5 min at RT.
4. Counterstain chromosomes by adding 18 μL of antifade with DAPI (Vectashield with DAPI) (1.5 μg/mL).
5. Apply coverslip and remove air-bubbles through gentle pressure.
6. Immediate analysis of the slides is recommended as the probe signal and slide quality will deteriorate with time. However, signal should remain stable for a few weeks, if stored in the dark at 4°C.

### 3.6. Anti-BrdU Controls/Tests

To confirm the one cell-cycle in BrdU criterion has been met and to confirm CO-FISH is working properly, we routinely perform and highly recommend the following anti-BrdU controls/tests.

#### 3.6.1. Slides processed for CO-FISH

1. Following step 2, Subheading 3.5, prepare 1:100 dilution of anti-BrdU antibody in PN Buffer; add ~40 μL of antibody solution to slide, coverslip and incubate at 37°C for 30–45 min in moist chamber. Do not let the slide dry out.
2. Rinse two times in PN Buffer for 3–5 min each at RT.
3. Counterstain chromosomes by adding 18 μL of antifade with DAPI (Vectashield with DAPI) (1.5 μg/mL).

4. Dimly fluorescing chromosomes indicate that cells are in the first cycle and that the newly replicated DNA strands have been effectively removed (CO-FISH confirmed). Second-cycle cells will display harlequin staining and are not suitable for CO-FISH analysis.

*3.6.2. Slides Not Processed for CO-FISH*

1. Following step 3, Subheading 3.2, denature chromosomes in coplin jar containing 50 mL 70% formamide, 30% 2× SSC solution prewarmed to 72°C for 1–2 min.
2. Immediately dehydrate slides in ice-cold ethanol series (75, 85, and 100% ethanol, 2 min each) and allow slides to air-dry.
3. Prepare 1:100 dilution of anti-BrdU antibody in PN Buffer; add ~40 µL of antibody solution to slide, coverslip and incubate at 37°C for 30–45 min in moist chamber. Do not let the slide dry out.
4. Rinse two times in PN Buffer for 3–5 min each at RT.
5. Counterstain chromosomes by adding 18 µL of antifade with DAPI (Vectashield with DAPI) (1.5 µg/mL).
6. Cells that have been through a complete S-phase in BrdU will be very brightly stained on both chromatids, whereas a banding pattern indicates cells have replicated for a partial S-phase in BrdU and are not suitable for CO-FISH analysis.

## 4. Notes

1. PNA vs. DNA probes: We have observed more T-SCE with a PNA probe in comparison to a DNA probe when both are applied to slides prepared from the same sample. Why this occurs is not known. We hypothesize that the brighter PNA probe may visualize smaller T-SCE. Alternatively, the shorter PNA probe, typically 18 bases long compared with a 42-mer DNA probe, may bind short segments in subtelomeric regions that by chance have at least 18 bases of canonical telomere sequence.
2. Multiple T-SCE: As far as we know, there is nothing to prevent more than one exchange from taking place in the same telomere. Light microscopy does not have the resolution to resolve multiple events in a single telomere; therefore, the correct interpretation of an observed T-SCE pattern is that one or more exchanges have taken place. Counts of observed T-SCE underestimate the true frequency, but the underestimate will be significant only when exchanges are exceptionally high. If desired the true T-SCE frequency can be estimated

by assuming exchanges occur randomly and therefore follow a Poisson distribution. Moreover, observations of sister telomeres of unequal brightness do not necessarily imply exchanges of unequal lengths of telomere segments. Rather, the point of exchange may have been someplace other than the center of the telomere.

3. Adequate BrdU substitution: BrdU is converted to a monophosphate primarily by thymidine kinase (TK1) and to a lesser extent by the mitochondrial TK2 kinase. In our experience, $10^{-5}$ M BrdU has produced adequate results in all normal cell lines we have encountered. Some transformed cell lines have required higher (sometimes much higher) BrdU concentrations. In such cases, altered nucleotide metabolism, such as a mutation in the TK1 gene, is suspected. Medium supplemented with nucleic acids may also require higher BrdU concentrations, therefore we routinely avoid these formulations. In general, a level of BrdU that yields good sister chromatid differentiation in the fluorescence-plus-Giemsa method of SCE detection will also produce good CO-FISH results.
4. Bromodeoxycytidine (BrdC): The $(TTAGGG)n$ vertebrate telomere sequence has twice as many T bases in one strand as in the other. In some cases, we have used a 3:1 mixture of BrdU + BrdC reasoning that it might help to equalize bromosubstitution in the two newly replicated strands. However, for the highly repetitive telomere sequence, we find perfectly adequate results can be obtained without the addition of BrdC. In contrast, BrdC should never be used alone because there would be no bromosubstitution when the $(CCCTAA)n$ strand is used as a template.
5. Irradiation studies: Confluent cultures are held for 24 h after exposure before subculturing to ensure that cellular DNA is not bromosubstituted either at the time of irradiation or during the subsequent time interval when most DNA damage was repaired.
6. The "one-cycle" rule: CO-FISH requires that cells progress through one, and only one, S phase in the presence of BrdU. Difficulties in obtaining good cell synchrony, or in maintaining it, can lead to violations of the one-cycle rule and produce misleading results and erroneous conclusions. For example, if some cells are midway through S phase at the time of BrdU addition, those telomeres that had completed replication will not be bromosubstituted. Without bromosubstitution, a PNA probe hybridizes to both sister telomeres producing a pattern that could be mistaken for a T-SCE. Fortunately, there are simple tests for adherence to the one-cycle rule. One such test is to process slides as for SCE detection by the fluorescence-plus-Giemsa method. Cells progressing through exactly one cycle in BrdU will not show differential staining.

Another test relies on detecting BrdU incorporation with a labeled anti-BrdU antibody. The newly replicated strands should not be removed, and chromosomal DNA should be denatured to allow the antibody to gain access to incorporated BrdU. In cells having undergone a single S phase in BrdU, sister chromatids labeled with the antibody will fluoresce with equal intensity along their lengths (the mouse major satellite, being T-rich in one strand, is an exception).

7. Other than for ReD-FISH, the necessity for a single round of replication in BrdU is a strict requirement. When BrdU is added to exponentially growing cultures, this requirement will not be met for all cells, thus the importance of the anti-BrdU tests. Whenever possible, we suggest allowing a culture to become confluent so the cells are synchronized in a $G_0$ state, then subculturing into fresh medium containing BrdU and blocking cell-cycle progression in metaphase with Colcemid.
8. Wavelength of UV light: Long-wave (including BLB or "black light") bulbs have a peak in their energy emission spectrum at 365 nm, which we recommend for CO-FISH. Sunlight "tanning" bulbs (313 nm) have been successfully used for CO-FISH, but emit some photons sufficiently energetic to damage unsubstituted DNA, so we caution against their use. Short-wave (254 nm) "germicidal" UV bulbs should never be used, as their high-energy photons are capable of inducing pyrimidine dimers, random strand breaks, and other undesired photoproducts.
9. DNA telomere probe: A probe to telomeric DNA can be prepared by synthesizing an oligomer having either the sequence $(TTAGGG)_7$ or $(CCCTAA)_7$ and labeled by terminal deoxynucleotidal transferase tailing (Boehringer Mannheim) with, for example, Cy3-dCTP according to the manufacturer's instructions (Amersham). A hybridization mixture containing 0.4 μg/mL probe DNA in 30% formamide, and 2× SSC is applied to slides that have been prepared for CO-FISH. Following an overnight hybridization at 37°C in a moist chamber, slides are washed in 2× SSC at 42°C (five times, 15 min each), placed in PN Buffer (Phosphate NP-40) at room temperature for 5 min and mounted in a glycerol solution containing 1 mg/mL of the antifade compound *p*-phenylenediamine HCl and 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield; Vector Labs). Cells are examined with a microscope outfitted for fluorescence and image analysis.
10. The protocols presented above were developed for specific telomere probes. Other probes may have different melting temperatures and will require adjusting hybridization and wash temperatures accordingly.

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# Chapter 12

# Visualization of Human Telomerase Localization by Fluorescence Microscopy Techniques

**Eladio Abreu, Rebecca M. Terns, and Michael P. Terns**

## Abstract

Human telomerase is a ribonucleoprotein (RNP) that synthesizes DNA repeats at the ends of chromosomes and maintains telomere length and genome stability. The enzyme comprises telomerase RNA (hTR) (which provides the template for telomere addition) and several protein subunits, including telomerase reverse transcriptase (hTERT) (the catalytic component). Intracellular trafficking of the enzyme has emerged as an important factor in the regulation of telomerase activity. Telomerase trafficking between nuclear Cajal bodies (proposed sites of telomerase biogenesis and regulation) and telomeres (sites of action) is regulated by the cell cycle in concordance with telomere synthesis during S phase. Here, we describe fluorescence microscopy approaches to visualize the subcellular localization of the essential RNA component of hTR relative to Cajal bodies and telomeres in cultured human cells. These methods include fluorescence in situ hybridization (to detect hTR and telomeric DNA) and immunofluorescence (IF) (to detect Cajal bodies and telomere-binding proteins). Because telomerase localization to telomeres is normally restricted to S phase, we also describe methods to synchronize and analyze cells within this phase of the cell cycle.

**Key words:** Telomerase, Telomere, Telomerase RNA, Telomerase reverse transcriptase, Fluorescence in situ hybridization, Immunofluorescence, Cajal body

## 1. Introduction

Telomerase plays a critical role in genome stability and tissue renewal through maintenance of telomere length in the face of inevitable replication-dependent shortening. Human telomerase activity can be reconstituted from just two essential components, telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT) (1, 2); however, activity in vivo requires several additional proteins. There is evidence that fully assembled, functionally mature telomerase (often referred to as telomerase holoenzyme complex) also includes the dyskerin complex (dyskerin, Nop10, Nhp2, and Gar1) (3–5), hEST1A (6, 7), and the most recently

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identified telomerase component, TCAB1, which is critical for proper intracellular trafficking and function of telomerase (8, 9). hTERT is normally a rate-limiting determinant of cellular telomerase activity (10, 11). hTERT is undetectable (absent or present at very low levels) in normal adult cells, but is detectable in cancer cells and primary tumors (10, 11). In contrast, hTR is present in both normal and cancer cells (12). Ectopic expression of hTERT in normal cells is sufficient to reinstate telomerase activity, to lengthen telomeres, and to immortalize cells (13, 14).

The low natural abundance of telomerase components has made it especially challenging to visualize their subcellular localization by traditional fluorescence microscopy approaches. However, sensitive and specific procedures were recently established, enabling the localization of key telomerase components to be investigated (9, 15–20). The recent telomerase localization studies have made significant contributions to our understanding of the regulation of telomerase activity. We now know that telomerase localization is regulated by the cell cycle and that the enzyme transiently localizes to its site of action, telomeres, specifically during S phase (17, 19). At other times during the cell cycle, telomerase accumulates in conserved intranuclear structures called Cajal bodies (16, 20). (Cajal bodies are generally implicated in the biogenesis and trafficking of RNPs (21, 22).) Evidence indicates that Cajal body localization is important for telomerase recruitment to telomeres and function (telomere elongation) (9, 15). Furthermore, holoenzyme assembly is a requirement for localization of hTR to both Cajal bodies and telomeres (9, 18). Thus, the relatively recently acquired ability to localize telomerase components in cells has revealed that telomerase activity is controlled in part by regulated trafficking of essential components of the enzyme.

In this chapter, we primarily describe a sensitive and specific fluorescence in situ hybridization (FISH) procedure that we have established to track the subcellular localization of endogenous hTR. We also describe how the hTR FISH approach can be combined with immunofluorescence (using antibodies against proteins markers of Cajal bodies and telomeres) to evaluate the localization of telomerase relative to Cajal bodies and telomeres. Finally, we describe a simple procedure to enrich for cells in S phase – the window in the cell cycle when telomerase can be found at telomeres (17–19).

## 2. Materials

### 2.1. hTR FISH

1. 1× PBS (100 mM $Na_2HPO_4$, 20 mM $KH_2HPO_4$, 137 mM NaCl, 27 mM KCl, pH 7.4). Sterilize the solution by autoclaving, and store at room temperature. Alternatively, make a 10× stock solution and dilute to 1× with water.

2. 37% Formaldehyde liquid stock. Alternatively, prepare fresh formaldehyde from paraformaldehyde. Store at room temperature shielded from light.
3. 100% Ethanol. Store at –20°C.
4. 20× SSC (3 M NaCl, 300 mM sodium citrate). Store at room temperature.
5. Vanadyl–ribonucleoside complex (RNase inhibitor, Fisher). Store at 20°C.
6. Dextran sulfate (Sigma). Store at room temperature.
7. Deionized formamide. Store at –20°C.
8. 20 mg/mL Nuclease-free bovine serum albumin.
9. *Escherichia coli* tRNA (Roche). Store at –20°C.
10. N-50 DNA (50 bp synthetic DNA where N is all nucleotides, MWG). Store at –20°C.
11. Three aminoallyl (C6 amino-modified dT) DNA probes complementary to hTR synthesized and PAGE purified by IDT (Integrated DNA Technologies) or Qiagen. The probes used are as follows (where * is a C6 amino-modified dT):

    Probe 1 (against nts 128–189) 5′ to 3′: GC*GACATTTT*T GTTTGCTC*AGAATGAACGG*GGAAGGCGGCAG GCCGAGGC*T

    Probe 2 (against nts 331–383) 5′ to 3′: C*CCGTTCCTCTT CC*GCGGCCTGAAAGGCC*GAACCTCGCCC*CG CCCCCGAG*G

    Probe 3 (against nts 393–449) 5′ to 3′: A*GTGTGAGCCG AG*CCTGGGTGCACG*CCCACAGCTCAGGGAA* CGCGCCGCGC*C
12. Glass coverslips (square no.1, 22 mm). Treat in pure HCl for 10 min. Rinse thoroughly with $H_2O$ and store in pure ethanol at room temperature. Flame just before use.
13. Glass slides (3 × 1, 0.9 mm plain precleaned Gold Seal).
14. 6-Well cell culture plates, tissue culture treated.
15. Prolong Gold anti-fade mounting media (Molecular Probes). Store at –20°C.
16. Dulbecco's modified eagle's medium (DMEM) plus 10% fetal calf serum.
17. Cy3 monoreactive dye (Amersham). Store at 4°C.
18. Centri-spin columns (dye removal columns, Princeton separations).

### 2.2. Combined hTR FISH/Immunofluorescence

1. Primary antibodies: Rabbit polyclonal PB124 anti-coilin antibody (gift from Greg Matera, University of North Carolina), mouse monoclonal anti-TRF2 antibody (Imgenex catalog #IMG-124A). Store at −80°C.
2. Fluorescently conjugated secondary antibodies: Cy2 goat α-mouse IgG (H + L), Cy5 goat α-rabbit IgG (H + L), (Jackson Immunoresearch catalog #115-225-003, 115-225-020). Store at −20°C.
3. 1× PBS-T:PBS with 0.05% Tween-20. Store at room temperature.

### 2.3. Cell Cycle Analysis

1. 100 mM thymidine (Sigma). Dilute in water, filter sterilize and store at −20°C.
2. 1 mM BrdU. Dissolve in water, filter sterilize and store at −20°C.
3. Sheep polyclonal α-BrdU antibody (Abcam catalog #ab1893). AMCA donkey α-sheep IgG (H + L) (Jackson Immunoresearch catalog #713-155-147). Store at −20°C.
4. 1 mg/mL Propidium iodide in $H_2O$ (Sigma catalog #P4170-25MG). Store at 4°C. Protect from light.
5. 100% Ethanol. Store at −20°C.
6. RNase A (lyophilized powder form). Store at −20°C.

## 3. Methods

### 3.1. hTR FISH

hTR is of low abundance; our Northern blot estimates suggest that there are ~800–1,000 hTR molecules in a HeLa cell. We have found that optimal detection of endogenous hTR by FISH requires the use of a combination of three probes directed against distinct regions of the hTR molecule, each containing up to five individual fluorophore molecules. The protocol described below for visualization of hTR in cancer cells is closely adapted from the general procedure described by Robert Singer's lab (http://www.singerlab.org/protocols) and utilizes short (~50 nt) synthetic DNA probes that contain aminoallyl-modified bases that permit coupling of a variety of fluorescent dyes.

1. Probe labeling and purification: We use three aminoallyl-modified DNA probes against distinct regions of hTR for optimal FISH signal. The sequences of these probes are provided in the materials section. The probes are routinely labeled with Cy3 fluorescent dye according to the dye manufacturer's instructions. Probes can be labeled with other fluorescent dyes if desired (see Note 1). Labeled probes are purified away from unincorporated Cy3 dye via centri-spin columns using the manufacturer's instructions. Be sure to keep separate

samples of probe from both before and after purification for analysis and verification later. From this point on, ensure that all probes and solutions containing probes are kept in the dark (e.g., by covering with foil). Failure to do so results in photobleaching of the probes and decline or loss of signals. Once labeled, the probes must be tested to ensure that they are intact and well labeled. The relative success of the FISH procedure is highly dependent on the quality and degree of labeling (DOL) of all three probes. Before and after purification samples can be run on a 15% native polyacrylamide gel to verify the presence of intact probes. Following ethidium bromide staining of the gel (to detect total DNA), fluorescently labeled DNA/dye bands can be visualized on a gel imager equipped with filters corresponding to the specific fluor(s). Concentrations of probes can be estimated using a standardized DNA marker of choice, but a spectrometric estimation is the preferred and most accurate method of quantitation. To determine the DOL, purified probes are loaded into a UV spectrophotometer for analysis. The spectrophotometer is blanked with deionized water, and a small volume of probe is loaded and quantified. The absorptions at 260, 280, and 550 nm ($A_{max}$ of Cy3, this wavelength varies by fluorochrome) are recorded. These readings are then entered into the Base-Dye calculator (derived from DOL formulas) at Invitrogen's Web site (http://www.invitrogen.com), along with the wavelength, extinction coefficient, and correction factor for the fluorophore to calculate the number of dye molecules per 100 bases. The wavelength, extinction coefficient, and correction factor are provided for standard fluorochromes (or can be found on the compound datasheets); the properties of Cy3 are equivalent to Alexa fluor 555. The DOL is a critical measure of the quality of a probe. Labeling between 2–4 dye molecules per 100 bases is acceptable for detecting hTR; a DOL below this ratio results in poor FISH signals.

2. Cell culture: HeLa and a HeLa cell line that stably overexpresses both hTR and hTERT (23) have been used most extensively to study telomerase in vivo. HeLa cells are cancer cells that express relatively high levels of hTR and have readily detectable Cajal bodies and telomeres. Other cell lines can be assayed as well (see Note 2). Cells are grown to 80% confluency on glass coverslips in 6-well plates overnight (see Note 3). (For HeLa, typically plate 150,000 cells per well and grow overnight).
3. Fixation and permeabilization: The process of fixation covalently links cellular components in position and helps preserve the morphology of the cells to be studied. After an initial 1× PBS wash, cells are incubated in 4% formaldehyde (fixative)

in 1× PBS at room temperature for 10 min. Next, cells are rinsed twice with 1× PBS. Permeabilization facilitates penetration of FISH (DNA probes) and IF (antibody) reagents. Typically, cells are permeabilized in 70% ethanol in $H_2O$ overnight at 4°C. (Cells can be stored for several days in this solution at 4°C.). Alternatively, cells can be permeabilized by incubating in 1× PBS supplemented with 0.1% Triton X-100 for 5 min at 4°C.

4. Rehydration: Cells are rehydrated in a solution containing 2× SSC and 50% formamide for 5 min at room temperature.
5. Prehybridization: Each coverslip of rehydrated cells is incubated at 37°C for at least 1 h in 40 µL of 1× hybridization solution consisting of 2× SSC, 50% deionized formamide, 10% dextran sulfate, 2 mM vanadyl ribonucleotide complex, 0.002 mg/mL nuclease-free bovine serum albumin, 1 mg/mL *E. coli* tRNA and 250 µg/mL of N-50 DNA (see Note 4). Coverslips are placed cell-side-down on this solution (spotted on parafilm) in a humidified chamber for incubation.
6. Hybridization: The cells are incubated at 37°C overnight in 40 µL of 1× hybridization solution (described in step 5 above) containing 25 ng of each of the three fluorescently labeled hTR probes (total of 75 ng of DNA). Coverslips are incubated cell-side-down on this solution (spotted on parafilm) in a humidified chamber.
7. Washes: Wash the coverslips two times with a solution containing 2× SSC and 50% formamide at 37°C for 30 min per wash.
8. Mounting and sealing: Place coverslips cell-side-down on slides with one drop of Prolong Gold (available supplemented with DAPI to stain the nuclear DNA or without this dye) being careful to eliminate all air bubbles. Seal the coverslip with clear nail polish and slides are ready for microscopy or can be stored at –20°C and images acquired at a later time.

### 3.2. Combined hTR FISH/Immunofluorescence

An antibody-based approach is used to observe telomerase trafficking relative to the primary nuclear structures where the enzyme resides: Cajal bodies and telomeres. In telomerase-positive cancer cells, telomerase localizes to Cajal bodies throughout most of the cell cycle (16, 20). However, during S phase, telomerase mobilizes to telomeres (17–19). Cajal bodies can be localized using antibodies against coilin, a Cajal body marker protein, and telomeres can be localized with an antibody against a core telomere-binding protein, such as TRF2. Other antibodies or FISH probes can also be used to reliably mark telomeres or Cajal bodies (see Note 5). The protocol below describes simultaneous detection of hTR, Cajal bodies, and telomeres. An example is shown in Fig. 1. Combination of the FISH and immunofluorescence approaches requires care to avoid signal diminution or loss.

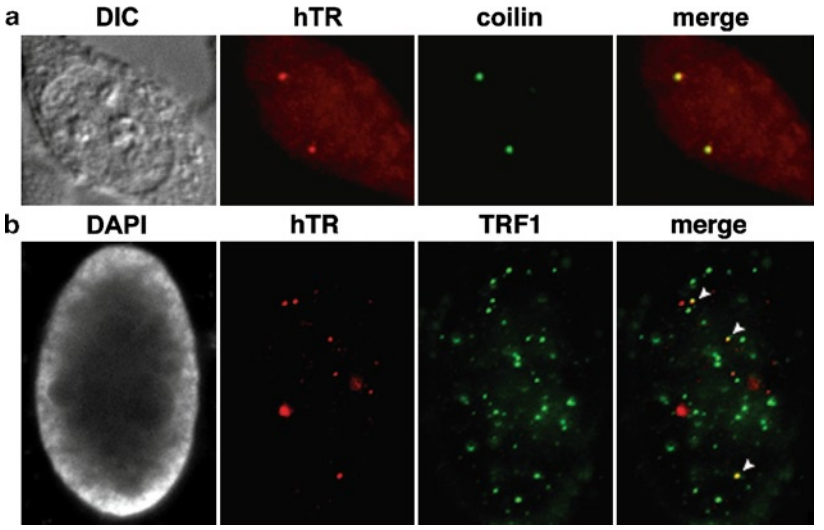


Fig. 1. Subcellular localization of telomerase RNA relative to Cajal bodies and telomeres. (**a**) Fluorescence in-situ hybridization (FISH) and immunofluorescence (IF) were used to detect hTR and coilin (Cajal body marker) in HeLa cells. Differential interference contrast (DIC) panel shows the outline of the cell and cell nucleus. Merge panel shows superimposition of hTR and coilin signals (indicating colocalization of hTR and Cajal bodies). (**b**) FISH and IF were used to detect hTR and TRF1 (telomere marker) in S phase HeLa cells. DAPI panel (DNA staining) defines the cell nucleus. Merge panel shows superimposition of hTR and telomere signals and *arrows* indicate hTR/telomere associations. This figure was reproduced (19, 20) with permission from Molecular Biology of the Cell.

1. Wash away formamide: Following the final 50% formamide wash of the FISH procedure (see Subheading 3.1 above), wash coverslips five times in 1× PBS to remove all excess formamide.
2. Block coverslips: Incubate coverslips cell-side-down in 1× PBS-T solution containing 3% bovine serum albumin at room temperature in a humidified chamber for an hour (see Note 6).
3. Primary antibody incubation: We routinely use mouse and rabbit primary antibodies simultaneously in immunofluorescence without any detectable cross reactivity. Primary antibodies from a third or fourth distinct species (e.g., sheep or goat) allow further flexibility in the number of antigens that can be detected simultaneously (each primary antibody must be detected with a secondary antibody that contains a distinct fluorophore to distinguish each unique signal). While blocking, centrifuge primary antibodies (anti-coilin rabbit polyclonal and anti-TRF2 mouse monoclonal) at 18,000 × *g* in a microcentrifuge at 4°C for 10 min to eliminate any antibody aggregates. Use the supernatant. Dilute the antibodies (rabbit polyclonal anti-coilin antibody 1:500 and mouse monoclonal anti-TRF2 antibody 1:1,000; see Note 7) in a suitable volume (e.g., 50 µL per coverslip) of 1× PBS-T containing 3% bovine serum albumin to make the combined primary antibody solution. After the block is completed, incubate coverslips cell-side-down

in primary antibody solution at room temperature in a humidified chamber for an hour.

4. Secondary antibody incubation: Wash coverslips three times with 1× PBS. Centrifuge secondary antibodies [Cy2 (green) labeled antimouse and Cy5 (near infrared) labeled antirabbit] at 14,000 rpm at 4°C for 10 min. Dilute both antibodies 1:100 in a suitable volume (e.g., 50 µL per coverslip) of 1× PBS-T to make the combined secondary antibody solution. Incubate coverslips cell-side-down in this solution at room temperature for an hour in a humidified chamber. Wash three times with 1× PBS and then mount and seal coverslips to slides as described in the FISH procedure (Subheading 3.1). The use of different primary antibodies may call for some variations in this procedure when combining FISH and IF (see Note 8).

### 3.3. Cell Cycle Analysis

Previous work has shown that the trafficking of telomerase is linked to the cell cycle (17–19). Subsequently, many telomerase trafficking studies include cell cycle status assessment and/or synchronization, with the primary focus on S phase when telomere localization is observed. Cells can be synchronized using a double thymidine block to arrest the cells at the G1/S border, and then released to obtain a population of cells proceeding through the cell cycle synchronously. The protocol provided here includes a simple method to evaluate the extent of synchronization [fluorescence-activated cell sorting (FACS) analysis following propidium iodide staining of DNA]. Additionally, pulse-labeling of newly replicated DNA with 5′-bromo-2′deoxyuridine (BrdU) can be employed in either asynchronous or synchronized cells to identify individual cells in S phase.

1. Synchronizing cells using double thymidine (G1/S phase) arrest: Plate cells on to glass coverslips and allow to grow to desired confluency (20–25% is optimal to allow for two population doublings during synchronization) overnight in a 37°C $CO_2$ incubator. (For example, for HeLa cells, plate 100,000 cells per well in a 6-well plate.) Add 2 mM thymidine to culture medium in each well and incubate an additional 18 h. Release by washing twice with 1× PBS, replacing medium, and returning the cells to 37°C incubator. When washing and replacing media, be sure to add media to the sides of each well (not directly to the top of the coverslips) to avoid the dislodging cells by the force of the liquid exiting the pipette. After 9 h, repeat 2 mM thymidine treatment. After 18–19 h of treatment, release cells by washing twice with 1× PBS and replace cell culture medium.
2. Cell cycle time course: Fix cells at desired time points following release from double thymidine block. A 0 h control sample

(cells arrested at G1/S border) is prepared by fixing the cells prior to release from the block. The majority of HeLa cells is likely in early S phase 2 h post-release, in mid S phase 4 h post-release and in late S phase 6 h post-release (S phase typically lasts for 6–8 h in HeLa cells). Phasing for individual cell lines may vary. Proceed with FISH or IF analysis as desired.

3. FACS analysis to evaluate cell cycle progression: The fraction of cells that are in S phase (and other phases such as G1, G2, and M) can be determined through FACS. Propidium iodide staining and flow cytometry are used to quantitatively assess DNA content/ploidy, allowing one to estimate the percentage of cells in a population in G1, S, and G2/M phases. Cells are trypsinized at desired time points post-release. The cells are washed once with 1× PBS and then counted using a hemocytometer. An optimal number of cells (e.g., $10^6$–$10^7$ cells) are fixed in 70% ethanol in a 15 mL conical tube. Shortly before FACS analysis, cells are washed with 1× PBS and stained with a 0.02 mg/mL propidium iodide solution containing 0.1% Triton X-100 and 0.2 mg/mL RNase A (to eliminate RNA which would otherwise also bind propidium iodide) in 1× PBS for 30 min at 37°C. Cell samples are then protected from light (e.g., in foil) and analyzed by flow cytometry (e.g., with a Dako CyAn Flow cytometer and FlowJo software).
4. Identification of S phase cells by BrdU pulse-labeling: BrdU is a thymine analog that is incorporated into the DNA during replication. Indirect immunofluorescence can be used to recognize cells that have incorporated BrdU (and are in S phase). Cells are incubated with 100 μM BrdU in the culture medium for 30 min (at 37°C in $CO_2$ incubator). The cells are fixed in 4% formaldehyde as described above. Immediately following fixation and prior to FISH, heat denaturation is performed to expose the BrdU epitope. FISH follows heat denaturation and BrdU detection is incorporated into the IF procedure immediately following FISH. Primary antibodies against BrdU and appropriate secondary antibodies are included in the combined antibody solutions. Distinct BrDU staining patterns (24) allow for discrimination of cells in early, mid, and late S phase.

### 3.4. Fluorescence Microscopy

Fluorescence signals in the cells are detected using a fluorescence light microscope. While other suitable microscope systems are available, we use a Zeiss Axioskop 2 Mot Plus fluorescence microscope (Carl Zeiss Microimaging, Thornwood, NY) fitted with the following band pass filters from Chroma to separate fluorescence signals: DAPI/Hoechst/AMCA #31000, Cy5 #41008, HQ: CY3 #41007A, and FITC/Bodipy/Fluo3/DIO #4101 (see Note 9). Images are acquired at 63× (Plan Apochromat objective,

numerical aperture 1.4) using a cooled charge-coupled device ORCA-ER digital camera (Hamamatsu Photonics, Bridgewater, NJ) and IPLab Spectrum software (BioVision Technologies, Inc., Exton, PA).

## 4. Notes

1. We have successfully labeled probes with various fluorescent dyes, including Cy3 (red), Oregon green (green), AMCA (blue), and Cy5 (near infrared) to provide multiple labeling options (e.g., for simultaneous detection of telomeres and hTR by FISH). Note that some dyes are water soluble and others are soluble in DMSO, which must be removed from the labeled probe by ethanol precipitation.
2. We have detected hTR localization in every telomerase-positive cancer cell line that we have assayed and HeLa cells are just one possible choice for telomerase localization studies. On the other hand, hTR is apparently diffusely localized within the nucleus in most primary cells lines that we have examined (i.e., hTR does not localize to specific structures, e.g., Cajal bodies or telomeres in these cell lines which typically express hTR but not hTERT or active telomerase activity). The telomerase-negative, WI-38 VA-13 subline 2RA (ATCC catalog #CCL-75.1) is an excellent negative control cell line since it does not express any detectable hTR (i.e., any hTR FISH signals observed in these cells are artifactual).
3. Growing cells to optimal confluency is important for imaging and analysis. Suboptimal confluency leads to few cells per microscopy field making imaging and analysis laborious. High-density growth can result in poor cell health and morphology.
4. A master mix of hybridization and prehybridization solutions can be made to accommodate multiple coverslips. Simply increase each reagent amount according to the number of coverslips, and mix thoroughly by vortexing. Also, successful RNA FISH signals require that care must be taken that all solutions and reagents used are RNase-free.
5. While coilin remains the most commonly used Cajal body marker protein, other components such as small Cajal body (sca) RNAs (e.g., U85) are reliable indicators of Cajal bodies in cells. Specific antibodies against any of the core telomere-binding proteins (TRF1, TRF2, Rap1, TIN2, TPP1, and POT1) successfully mark telomeres. Alternatively, DNA FISH can be utilized to label the TTAGGG telomeric DNA repeats. An example of a DNA probe typically used in our lab with good results is: 5′ to 3′ (where * is a C6 amino-modified dT):

C*AACCCTAACCC*AACCCTAACCC*AACCCTAACCC*AACCCTAACCC*A. This probe can be labeled with any chosen fluorescent dye. Alternatively, DNA or peptide nucleic acid (PNA) probes of the same sequence but with just one fluorescent dye conjugated to the 5′ end can be purchased commercially and are perfectly suitable to detect telomeres. Telomere FISH requires a denaturation step to expose the telomere DNA strand to the FISH probes. This is done immediately prior to the rehydration step and is accomplished by incubating the fixed cells at 85°C for 10 min in a solution of 70% formamide and 2× SSC. Special care must be taken to ensure that the temperature does not drop below 85°C or rise above 90°C. After rehydrating and prehybridization steps are performed as described above, the hybridization solution is supplemented with 20 ng of telomere probe per coverslip.

6. A blocking step is used to minimize nonspecific binding of primary and/or secondary antibodies. We typically use 1× PBS-T solution containing 3% bovine serum albumin but other blocking agents, such as 1–10% gelatin or normal serum from the same species from which the secondary antibody was raised can also be tested if nonspecific background signals become an issue. The effectiveness of any blocking agent must be determined empirically by comparing signals plus and minus the treatment and assessing signals resulting from the treatment of primary and secondary antibodies vs. secondary antibodies alone.
7. Correct antibody dilutions are needed to achieve optimal antibody signals and are specific to each antibody used. Manufacturers suggest ranges of optimal dilutions for their antibodies, but empirical testing of various dilutions is usually needed to find the best results. When combining antibodies in a single IF experiment, each antibody is diluted to optimal concentration in the volume needed to accommodate the desired number of coverslips.
8. The activity of antibodies used for IF can be sensitive to some components of the FISH procedure. The formamide and/or heat denaturation steps are capable of inhibiting antibody signal when performed after IF. One way to avoid signal loss is to do FISH before IF. This means that if denaturation is needed (e.g., for telomeric DNA FISH or BrdU labeling), it is performed immediately prior to FISH, followed by FISH and then IF the following day. Several 1× PBS washes are required before IF to wash away excess formamide. In some cases, antibodies do not work well when IF is performed after FISH. Moreover, the researcher may want to perform both FISH and IF in 1 day due to time constraints. In these cases, performing IF then FISH is a great option. When done in this order, the procedure can be performed in one day (saving

the final washes and mounting for the following day). With this alternative approach, it is important to remember that the heat denaturation step (if needed) must be done prior to IF. Additionally, IF antibodies must be fixed to the treated cells prior to FISH. The process is outlined with key steps underlined (1) fixation, (2) permeabilization, (3) denaturation (if needed), (4) five 1× PBS washes, (5) IF (concludes three 1× PBS washes), (6) repeat fixation (includes two 1× PBS washes), (7) FISH (begins with rehydration and concludes with two 30-min formamide washes), and (8) coverslip mounting and sealing.

9. In multicolor imaging (use of two or more fluorescent labels), it is critical to rule out potential fluorescence bleed-through artifacts that can complicate interpretation of results (e.g., bleed-through artifacts could be misinterpreted as true colocalization of signals if not controlled for). Thus, it is both imperative to use high-quality band pass filters and importantly, to confirm that there is no spillover of fluorescence from one probe into the channel configured for another probe. One approach to determine if fluorescence bleed-through occurring is to perform a single labeling experiment (label one target with one fluorochrome) and check to see that the signal is visible only under the appropriate filter and not other filter combinations designed to exclude that signal. It is best to use a target that produces a strong, distinct signal. Repeat this test with each fluorochrome.

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# Chapter 13

# Cytogenetic Analysis of Telomere Dysfunction

**Asha S. Multani and Sandy Chang**

## Abstract

Dysfunctional telomeres arising either through natural attrition due to telomerase deficiency or by the removal of telomere binding proteins are recognized as double stranded breaks (DSBs). Repair of DSBs is crucial for the maintenance of genome stability. In mammals, DSBs are repaired by either error prone nonhomologous end joining (NHEJ) or error free homologous recombination (HR) and can be visualized as chromosomal fusions.

**Key words:** Telomere protection, Telomere FISH, PNA, Chromosomal fusion

## 1. Introduction

Telomeres are nucleoprotein structures that provide both end-protection and a mechanism for the maintenance of chromosomal ends. In mammals, telomeres consist of TTAGGG repetitive sequences that associate with and are protected by shelterin, a core complex of telomere binding proteins that includes the double-stranded DNA binding proteins TRF1 and TRF2 and Protection of Telomeres 1 (POT1) that interacts with its binding partner TPP1 to protect single-stranded G-rich overhangs (1). Telomeres are maintained by the enzyme telomerase which is limiting in human somatic cells, resulting in progressive telomere shortening. Dysfunctional telomeres that can no longer exert end-protective functions are recognized as DSBs by the DNA damage repair (DDR) pathway and are ultimately repaired. In mammals, dysfunctional telomeres are repaired primarily by the nonhomologous end joining (NHEJ) pathway, resulting in chromosomal fusions which can readily be visualized under the microscope.

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## 2. Materials

### 2.1. Cell Culture and Harvesting

1. Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone), supplemented with 10% fetal bovine serum (FBS, Sigma).
2. Penicillin–Streptomycin (100 U/mL Penicillin G and 0.1 mg/mL Streptomycin (Gemini Bio-products).
3. Mouse embryonic fibroblasts (MEFs).
4. Trypsin (0.25%) and Ethylenediamine tetra acetic acid (EDTA) (1 mM) solution (Gibco).
5. Colcemid (0.05 μg/mL) from Gibco.
6. A 0.075 M solution of KCl in water.
7. Freshly prepared solution of methanol:acetic acid (3:1) (v/v).
8. 15 mL Conical centrifuge tubes.
9. Glass Pasteur pipettes.
10. Glass slides with frosted ends.

### 2.2. Giemsa Staining

1. 4% Giemsa stain diluted in 0.01 M phosphate buffer, 0.469 g $NaH_2PO_4$ + 0.937 g $Na_2HPO_4$/L $ddH_2O$ (Gibco).

### 2.3. PNA Telomere Fluorescence In Situ Hybridization

1. PNA telomere probe [Tam-OO-$(CCCTAA)_4$] Panagene Inc.
2. DAPI (Vectashield).
3. Triton-X 100 (Sigma).
4. Formaldehyde (Sigma).
5. PNA probe in hybridization buffer: 2.85 μL $ddH_2O$, 0.15 μL tRNA (100 μg/mL), 1.5 μL 2% bovine serum albumin, 21 μL formamide, 3 μL 0.6× SSC, 1.5–3.0 μL PNA probe (10 ng/μL).

## 3. Methods

### 3.1. Harvesting the Cultures for Chromosomal Preparations

1. Plate about one million MEF cells in 100 mm Petridishes and add 10 mL culture medium supplemented with 10% FBS and Penicillin–Streptomycin. Incubate the cultures at 37°C overnight (see Note 1).
2. Next day, examine the cultures for the presence of doublets indicating the presence of dividing cells and at the time such doublets are present add colcemid (0.05 μg/mL) to the cultures and incubate them at 37°C for 45 min to 1 h (see Note 2).
3. Aspirate the medium and rinse the cells with (phosphate buffered saline) PBS.

4. Detach the cells using 0.025% trypsin and transfer them to 15 mL conical centrifuge tubes.
5. Spin at $450 \times g$ for 6–8 min.
6. Remove the supernatant leaving about 150–200 µL of medium in the tube. Gently pipette up and down to break the clumps.
7. Add 5–7 mL of 0.075 M KCl solution and gently mix with a Pasteur pipette.
8. Incubate at room temperature for 15 min.
9. Add about 1 mL of methanol:acetic acid (3:1) solution to the tube and mix slowly.
10. Centrifuge at $450 \times g$ for 6–8 min and remove the supernatant.
11. Break the pellet by tapping the bottom of the tube and slowly add about 1 mL of fixative. Mix with a Pasteur pipette to break any clumps and add 5–6 mL more fixative.
12. Spin at $450 \times g$ for 6 min and remove the supernatant.
13. Repeat steps 11 and 12 thrice.
14. Resuspend the final pellet in small volume of fixative so that the cell suspension looks slightly milky.
15. Take clean slide and pour distilled water on it. Drain the slide, hold it at an angle and drop 2–3 drops of cell suspension on the slide.
16. Either air dry the slide or dry it on a hot plate heated to about 60°C.
17. The slides can be stored at room temperature for about 2 weeks or for longer period at –80°C.

### 3.2. Giemsa Staining

1. Giemsa staining of the slides prepared from cell culture harvest is a useful tool in the study of chromosomes. Slides are stained in a 4% Giemsa stain diluted in 0.01 M phosphate buffer (0.469 g $NaH_2PO_4$ + 0.937 g $Na_2HPO_4$/L $ddH_2O$) for 5 min, washed in double distilled water and air dried. Observe the stained slide under a bright field microscope using 60× oil immersion objective.

### 3.3. PNA Telomere Fluorescence In Situ Hybridization

1. Immerse the slide in PBS for 2 min.
2. Fix the slide in 4% formaldehyde in PBS for 2 min.
3. Wash in PBS three times (5 min each).
4. Treat slides with pepsin (1 mg/mL in 10 mM HCl) for 10 min at 37°C, wash with PBS.
5. Fix the slide in 4% formaldehyde in PBS for 2 min.
6. Dehydrate the slide in 70, 85, and 100% ethanol 2 min each and air dry.

7. Apply 30 µL Peptide Nucleic Acid (PNA) telomere probe in hybridization buffer to each slide.
8. Cover slide with coverslip and seal with rubber cement.
9. Denature the slide for 3 min at 80°C on a heat block and then incubate for 2–3 h in dark at room temperature.
10. Remove the coverslip gently with forceps and wash the slide in 70% formamide/0.06× SSC (Saline-Sodium Citrate) (pH 7.2) 2× 15 min.
11. Wash with PBS containing 0.05% Triton-X 100 three times, 5 min each.
12. Apply a drop of mounting medium with DAPI to the target area of the slide.
13. Cover with a coverslip and allow the solution to spread evenly under the coverslip.
14. Observe the stained slide under a fluorescence microscope using 60× oil immersion objective and appropriate filters.

### 3.4. Analysis of Chromosomal Fusions

In laboratory mouse, Mus musculus, the diploid chromosome number is $2n=40$. All mouse chromosomes, including the sex chromosomes, are acrocentric in morphology. Translocations involving total chromosomes or whole chromosome arms can be classified into three basic types, centromere–centromere (C–C), centromere–telomere (C–T), and telomere–telomere (T–T) fusions. Depending on the way the fusion has occurred, the resulting translocated chromosome may exhibit different morphology (2).

1. In centromere–centromere type fusion, one chromosome has a break in the long arm immediately below the centromere and the other has a break in the telomeric region above the centromere. This results in an asymmetrical biarmed chromosome and a "bare" centromere which is subsequently lost. If the fusion occurs between the two mouse chromosomes retaining both centromeres, then one centromere may be rendered inactive.
2. In the centromere–telomere type fusion, two chromosomes fuse, one at the centromeric end and other at the telomeric end. This type of translocation can result in either a long acrocentric chromosome or a biarmed chromosome depending on which centromere is lost or inactivated. It is also called a tandem translocation.
3. In telomere–telomere fusion two chromosomes fuse at the telomeric ends. Here again, the morphology of the resulting chromosome could either be dicentric with one centromere at each end or a long acrocentric chromosome if one of the centromere is lost or inactivated.

4. Ring chromosomes may be formed by:
   (a) Telomere–telomere fusion between telomeres present at both ends of a single chromosome.
   (b) Sister chromatid fusion at one end of the same chromosome leading to the formation of a ring-like structure.
   (c) Fusion at the telomeric regions of the long arms of two acrocentric chromosomes.

## 4. Notes

1. To get a good mitotic yield, cells should be growing in exponential condition and the cells should be freshly fed with the medium a day before the harvest.
2. Colcemid treatment should be monitored to obtain an optimum length of the chromosomes.

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# Chapter 14

# Probing the Telomere Damage Response

Rekha Rai and Sandy Chang

## Abstract

Telomere dysfunctions, rendered through replicative attrition of telomeric DNA or due to the inhibition of shelterin components, are recognized as DNA double-stranded breaks (DSBs) by the DNA damage repair (DDR) pathway. This leads to the activation of DNA damage checkpoint sensors, including the Mre11-Rad50-Nbs1 (MRN) complex, γ-H2AX and 53BP1, the ATM and ATR signal-transducing kinases and downstream effectors, including Chk1, Chk2, and p53. Robust DNA damage response signals at dysfunctional telomeres, achieved by the complete deletion of TRF2 or by expressing dominant negative mutant TPP1$^{\Delta RD}$, can be detected by their association with γ-H2AX and 53BP1 forming "telomere dysfunction induced foci (TIFs)." Induction of TIFs at telomeres provides an opportunity to quantify the extent of telomere dysfunction and monitor the signaling pathways.

**Key words:** DNA damage, Telomere dysfunction, Telomere-induced foci, Telomere-FISH

## 1. Introduction

Telomeres are nucleoprotein complexes at chromosome ends, consisting of TTAGGG repetitive sequences, a 3′ single-strand G-overhang, and a set of telomere binding proteins. In mammals, telomere binding proteins form a highly organized functional unit known as the shelterin complex, composed of six telomeric core proteins: telomeric-repeat-binding factor 1 (TRF1) and 2 (TRF2), TRF1-interacting protein 2 (TIN2), transcriptional repressor/activator protein RAP1, protection of telomeres 1 (POT1), and POT1- and TIN2-organizing protein (TPP1) (1). The shelterin complex contributes to the formation of protective telomere loop (t-loop) structures, where the single-stranded 3′-overhang is postulated to invade the telomeric duplex in order to prevent chromosome ends from being recognized as DSBs by the DDR pathway (2). Failure of the protective features of

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telomeres due to natural replicative attrition or due to genetic alteration of shelterin components leads to "dysfunction" or "uncapped" telomeres (3). Uncapped telomeres are sensed by Mre11/Rad50/Nbs1 (MRN) complex activates ATM/ATR mediated DNA damage-signaling cascade, leading to the recruitment of DNA damage response factors, such as 53BP1, γ-H2AX, MDC1, and initiation of inappropriate DNA repair pathways (nonhomologous end joining or homologous recombination) at the chromosome ends. The recruitment of DNA damage response factors that co-localize with dysfunctional telomeres are referred as telomere-induced foci (TIF) (4, 5). TIFs can be efficiently visualized in the interphase nuclei using the telomere FISH (Fluorescent In Situ Hybridization) technique outlined below.

## 2. Materials

### 2.1. Cell Culture

1. Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone SH30022.01) supplemented with 10% fetal bovine serum (FBS, Sigma F-6178), OPTI-MEM (Gibco 31985).
2. 293T (ATCC) and mouse embryonic fibroblast (MEF) cells.
3. 0.25% Trypsin (Gibco 25200).
4. Phosphate buffer saline (PBS) (Hyclone SH30028.02).

### 2.2. Retroviral Production and Infection

1. pCL Eco, Empty pBabe, p-Babe shTRF2, Empty pQCXIP, pQCXIP TPP1$^{\Delta RD}$.
2. Polybrene Stock 6 mg/ml. Filter through a 0.2 μm filter, aliquot, and store at −20°C.
3. Lipofectamine Plus Reagent (Invitrogen), Fugene 6 (Roche).
4. 0.45 μm Syringe filters (Millipore).

### 2.3. Immunofluorescence Telomere FISH

1. Nunc Lab-Tek 8-well slide chambers (Nalgene Nunc, 12-565-1), Microscope coverslips 18×18-1 (Fisher-12-548-A), Microscope Slides (Fisher 12-544-3).
2. PBS: Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM $Na_2HPO_4$, 18 mM $KH_2PO_4$ (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part with nine parts water.
3. Fix solution: 2% paraformaldehyde (USB 199431)/2% sucrose (Sigma S-1888).
4. Blocking Solution PBG: 0.2% fish gelatin (Sigma G-7765), 0.5% BSA (Sigma A-2153). Aliquot PBG and store at −20°C.

5. Permeabilization Buffer: 0.5% (v/v) Nonidet-P40 (USB 19628) in PBS.
6. TBST: 1× PBS with 0.1% Triton (Sigma T9284).
7. Primary antibodies: 53BP1 (Novus 100-304) and γ-H2AX antibodies (Upstate 05-636).
8. Secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG {H + L} A-11008 and Alexa Fluor 488 goat anti-mouse IgG {H + L} A-11001.
9. 20× SSC (Invitrogen 15557-036).
10. Formamide (Fisher BP227-500).
11. TelC-TAMRA, TAMRA-OO-$(CCCTAA)_3$ PNA Probe (Pan agene-F2002). Stock solution 1 μg/μl in $H_2O$.
12. Yeast tRNA (Invitrogen 15401-011). Stock solution 1 mg/ml in $H_2O$.
13. Wash Solution I: (70% formamide, 0.1% Tween 20 Sigma BP337 500, 0.1% BSA, 10 mM Tris–HCl, pH 7.5).
14. Wash Solution II: (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% BSA, 0.1% Tween-20).
15. Mounting Medium with Antifate: DAPI (Vectashield H-1200).

## 3. Methods

Robust TIFs are monitored by disrupting the protective function of telomere through inhibition of TRF2 or through expression of dominant negative mutant $TPP1^{\Delta RD}$, which efficiently removes the endogenous TPP1-POT1a complex from telomeres (6, 7). TIFs can also be visualized with a dominant negative allele of TRF2 ($TRF2^{\Delta B\Delta M}$) (3) and siRNA-mediated inhibition of TRF2 or TPP1 in Osteosarcoma U2OS or Hela cells. To accomplish efficient induction of TIFs in MEFs, it is recommended to perform retroviral infections for the complete removal of TRF2 using shRNA against TRF2 or expression of $TPP1^{\Delta RD}$. For infecting MEFs, use pCL-ECO, and for infecting human cells, use VSV-G/Gag-Pol packaging constructs for generating high titer shTRF2 or $TPP1^{\Delta RD}$ retroviral particles. To achieve high titer retroviral particles, use early passage 293T cells and high-quality DNA preparations. Digital images can be acquired and analyzed using a Nikon Eclipse 800 microscope, X63 apo-plan objectives and a CCD camera with a 5 megapixel, 9×9 micron chip. Cells ≥5 53BP1 or γ-H2AX signals co-localized with telomere signals considered as TIFs positive cells. Score at least 100–200 TIFs positive cells to reach the statistical significance.

### 3.1. Retrovirus Production in 293T Cells

1. *Day 0*: On the day before transfection, plate 0.5–1 × $10^6$ 293T cells into 6 cm dish in 4.0 ml DMEM supplemented 10% FBS (see Note 1). The cells are ready for transfection after 18–20 h, or when they are about 60–70% confluent. Plate slightly more cells when making VSV-G pseudotyped viruses.
2. *Day 1*: Aspirate the medium and replace the cells with 2.0 ml DMEM without FBS and any antibiotic. Transfect 293T cells with 2.0 μg pCL-ECO and 4.0 μg of transfer vectors (p-Babe shTRF2, PQXCIP TPP1$^{\Delta RD}$) using Lipofectamine Plus Reagent following the manufacturer's protocol. For making VSV-G retroviruses by triple transfection use 0.9 μg Gag/Pol expression vector, 0.1 μg VSV-G expression vector, and 1.0 μg transfer vector. For making VSV-G pseudotype viruses, use Fugene 6 following the manufactures protocol.
3. Change the medium 5–7 h after transfection with 4.0 ml of fresh medium (see Note 2).
4. *Day 3*: Harvest the viral supernatants at 48 h post-transfection. Filter the viral supernatant with 0.45 μm syringe filter. Add 10 ml of fresh DMEM medium supplemented with 10% FBS to the cells.
5. *Day 4*: Harvest the viral supernatant at 72 h of postinfection as above.

### 3.2. Retroviral Infection

1. *Day 2*: Grow 20% confluent target cells either on coverslips in 6-well plates or in 8-well slide chambers (see Note 3).
2. *Day 3*: Infect the cells with 48 h harvested retroviral particles. Add 2.0 ml viral particles (1:1 diluted in DMEM/10% FBS) in 6-well plate and 500 μl in 8-well chambers to infect MEFs/Human cells. Add 6 μg/ml polybrene for infecting MEFs and 4 μg/ml polybrene for infecting Hela or U2OS cells (see Note 4).
3. *Day 4*: After 24 h of first infection, reinfect the cells with 72 h harvested retrovirus particles.
4. *Day 5*: Replace the cells with fresh medium (see Note 5).

### 3.3. Immuno-fluorescence-Telomere FISH

1. *Day 6*: Aspirate the medium and wash the cells twice for 5 min each with 1× PBS at RT.
2. Fix the cells with 2% paraformaldehyde/2% sucrose for 10 min at RT.
3. Wash the fixed cells twice for 5 min each with 1× PBS.
4. Permeabilize the cells with 0.5% Nonidet-P40 for 10 min at RT.
5. Wash the cells thrice for 5 min with 1× PBS.
6. Incubate the cells with PBG for 1 h to block the nonspecific binding.

7. Incubate the cells with the primary antibody diluted in PBG (1:2,000 53BP1 or γ-H2AX) for overnight at 4°C in humidifier chamber (see Note 6). Add 60 µl antibody for 8-well chambers, 30–50 µl for cells on coverslips, place cells face down on antibody drop over paraffin film.
8. *Day 7*: Wash the cells thrice for 5 min each with PBST followed by 5 min blocking with PBG.
9. Incubate the cells with appropriate secondary antibody diluted in PBG (1:2,000) for 1 h at RT. Incubation should be performed in dark.
10. Wash the cells thrice for 5 min each with 1× PBST.
11. Postfix the secondary antibody to primary antibody by incubating the cells in 4% paraformaldehyde for 10 min at room temperature (see Note 7).
12. Wash the cells twice for 5 min each with PBS, RT.
13. Add freshly prepared PNA–FISH hybridization mix: (30 µl/coverslip, face down on paraffinized slides; 60 µl per 8-chamber slide).

| | |
|---|---|
| $H_2O$ | 2.85 µl |
| 2% BSA | 1.5 µl |
| 100 µg/ml tRNA | 0.15 µl |
| 0.6× SSC | 3.0 µl |
| 100% Formamide | 21.0 µl |
| 10 ng/µl PNA probe | 1.5 µl |
| Total | 30.0 µl |

14. Denature the slide at 85°C on a hot plate for 3 min; place the slide in the dark for overnight in a humidified chamber (see Note 8).
15. *Day 8*: Wash twice for 15 min each in Wash Solution I. For slides use coplin jar, for coverslips use 6-well plate.
16. Wash thrice for 5 min each in Wash Solution II.
17. Ethanol dehydrate the slides for 2 min each with 70, 85, and 95% ethanol.
18. Counterstain with DAPI and seal with nail vanish. Cover slip should be carefully inverted to a drop of mounting medium on a microscope slides. For 8-well chambers, carefully remove the gasket and place few drops of mounting medium and cover with a coverslips (see Note 9). The slides can be viewed immediately when the varnish is dried or be stored in the dark at 4°C for up to a month.
19. Image on a fluorescent microscope.

## 4. Notes

1. It is very important to have single cells suspensions (trypsinize well) and evenly distributed cells.
2. 293T cells detach easily, be careful with all medium changes.
3. Cover slips must be autoclave or sterilized using flame with 95% alcohol. Place the cover slips in 6-well plates to cool down.
4. Polybrene enhances the rate of infection.
5. Visualize at any GFP control plates under the fluorescent microscope to be sure the cells are expressing GFP. If they are, you can assume that the cells have taken in the DNA and are producing virus.
6. Make sure that cells are covered and do not dry out.
7. It is critical to postfix the secondary antibody to primary antibody using paraformaldehyde in order to retain the primary antibody signals.
8. Make absolutely sure that the temperature is exactly 85°C using a thermometer.
9. Air bubbles are undesirable in the mounting medium.

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# Chapter 15

# Analysis of Telomere Proteins by Chromatin Immunoprecipitation (ChIP)

**Wenbin Ma**

## Abstract

Telomere Chromatin Immunoprecipitation (ChIP) is an experimental method used to determine whether proteins are associated with telomere DNA inside the nucleus of cells or tissues. Telomere-associated proteins are covalently cross-linked to telomere DNA, and then immunoprecipitated using a specific antibody for the protein. This method has become one of the most indispensable tools for unraveling the protein complexes that associate with the telomeres.

**Key words:** Telomere Chromatin immunoprecipitation, ChIP, Telomere-associated proteins

## 1. Introduction

Telomeres are protected by a multitude of proteins, several of which exhibit specific-binding activity for telomere DNA sequences. Among the telomere DNA-binding proteins identified in the last 20 years, TRF1 and TRF2 associate with double-stranded telomere DNA (1), whereas POT1 binds single-stranded telomere overhangs (2). These proteins in turn associate with other signaling proteins to achieve telomere-end protection and length control (3–5). Telomere targeting can be assessed in a variety of assays, and telomere chromatin immunoprecipitation (ChIP) has emerged as an indispensable tool for examining the localization of telomere-recruited factors (6).

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## 2. Materials

### 2.1. Cell Preparation

1. 10× Phosphate buffered saline (PBS): Dissolving 2 g KCl, 80 g NaCl, 17.8 g $Na_2HPO_4$*$2H_2O$, and 2.4 g $KH_2PO_4$ in 800 mL distilled water. Adjust pH to 7.4 with HCl if necessary, and make final volume to 1 L with distilled water. Autoclave and store at room temperature.
2. 37% formaldehyde solution (Fisher Scientific). A working solution of 1% (v/v) is freshly prepared in PBS or medium not supplemented with serum.
3. Stop solution: Prepare 2 M Glycine stock solution, filter through 0.22 μm filter and store at room temperature. Working concentration is 0.125 M.
4. Complete protease inhibitors (Roche Molecular Biochemicals).
5. Solution A: 10 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, and freshly added complete protease inhibitors.
6. Solution B: 10 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, and freshly added complete protease inhibitors.
7. Solution C: 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Hepes (pH 7.5), 0.1% SDS, 1% Triton X-100, 2 mM PMSF, and freshly added complete protease inhibitors.
8. 20 mg/mL Proteinase K solution (Invitrogen).
9. VIRSONIC 600 Sonicator (The Virtis Company, Gardiner, NY).
10. Thermo Scientific Labquake Tube Shaker/Rotators (Barnstead/Thermolyne Corporation, Dubuque, IA).
11. 20% SDS (w/v).

### 2.2. Preclearing

1. *Escherichia coli* DNA preparation. Dissolve *E. coli* DNA (Sigma D-2001, type VIII) in 10 mM Tris–HCL (pH7.5) at a final concentration of 2.5 mg/mL. Sonicate the DNA solution to obtain a fragment pool of ~500 bp (see Subheading 3.1). Purify the fragmented DNA by phenol/chloroform/isoamylalcohol (25:24:1) extraction once and chloroform extraction once. Collect the aqueous phase and add 1/10 volume of 3 M sodium acetate solution. Mix well and add 2 volumes absolute ethanol. Centrifuge the mix at top speed to pellet the DNA. Wash the pellet once with 70% ethanol. Resuspend *E. coli* DNA in 1× TE and measure DNA concentration at OD 260. Make 1 mg/mL *E. coli* DNA working stock and store at −20°C.

2. 5% BSA (w/v). Dissolve 5 g of Bovine serum albumin Fraction V (Roche) in 100 mL of PBS. Sterilize through a 0.22 μm filter and store at 4°C.
3. Preclearing protein A/G-agarose bead slurry prepared the day before immunoprecipitation. Mix 5 μg sheared *E. coli* DNA and 10 μL 5% BSA with 50 μL protein A-agarose beads (Thermo scientific) in a microcentrifuge tube. Shake/Mix overnight at 4°C for use the next day.

### 2.3. Immuno-precipitations

1. Control immunoglobulins such as IgG.
2. 1 M dithiothreitol (DTT). Dissolve 1.54 g of DTT in $H_2O$ plus 33.33 μL of 3 M NaOAc (pH 5.2) to make final volume of 10 mL. Sterile filter the solution, aliquot, and freeze at –20°C. Working concentration is 1 mM.
3. CHIP I buffer: 0.1% sodium deoxycholate, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 50 mM Hepes (pH7.5). Add complete protease inhibitors and DTT right before use.
4. CHIP II buffer: 0.1% sodium deoxycholate, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 50 mM Hepes (pH 7.5) Add complete protease inhibitors and DTT right before use.
5. CHIP III buffer: 0.5% sodium deoxycholate, 0.25 M LiCl, 0.5% NP-40, 1 mM EDTA, and 10 mM Tris–HCl (pH 8.0). Add complete protease inhibitors and DTT right before use.
6. TE/DTT buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA pH 8.0, and 1 mM DTT. Add DTT right before use.
7. Elution Buffer: 0.5% SDS and 0.1 M $NaHCO_3$. Make fresh from 10% SDS and 1 M $NaHCO_3$ before use.
8. 5 M NaCl.

### 2.4. Telomere DNA Purification

1. 0.5 M EDTA, pH 8.0.
2. 1 M Tris–HCl, pH 6.5.
3. DNase-free RNase A, 10 mg/mL.
4. Glycogen (Roche, 20 mg/mL stock).

### 2.5. Dot Blot

1. Hybond-N+ membrane (GE Heathcare, Buckinghamshire, UK) and 3 M filter paper (Whatman, Maidstone, UK).
2. Bio-Dot microfiltration apparatus (Bio-Rad).
3. 20× SSC: Dissolve 175.3 g NaCl and 88.2 g sodium citrate in $ddH_2O$ to final volume of 1 L. Adjust pH to 7.0 with 10 N NaOH and autoclave before storage at room temperature.
4. Denaturing Solution: 1.5 M NaCl, 0.5 M NaOH.
5. Neutralizing Solution: 3 M NaCl, 0.5 M Tris–HCl pH 7.0.
6. UV cross-linker (*Stratagene*).

7. T4 Polynucleotide Kinase (New England Biolabs).
8. 1 M sodium phosphate buffer pH 7.2: 134 g $Na_2HPO_4.7H_2O$ in 1 L $ddH_2O$. Stir until dissolved. Adjust pH to 7.2 with $H_3PO_4$. Store at room temperature.
9. Hybridization buffer: Mix 250 mL 1 M sodium phosphate buffer (pH 7.2), 1 mL 0.5 mM EDTA (pH 8.0), 175 mL 20% SDS, and $ddH_2O$ to make final volume of 500 mL. Filter sterilize and store at –20°C. Heat to 55°C to dissolve any precipitates before use.

## 3. Methods

### *3.1. Cell Lysis and Chromatin Shearing*

1. Grow cells to confluence (on three 15 cm dishes) (see Note 1).
2. Remove medium and replace with 10–15 mL of freshly prepared 1% formaldehyde working solution. Rock gently for 10 min at room temperature (see Note 2).
3. Remove the cross-linking solution, and wash twice with ice-cold 1× PBS.
4. Add 10 mL of 1× PBS, and 625 µl of 2 M Glycine (to a final concentration 0.125 M) to each dish. Mix gently and incubate at RT for 15 min to quench the formaldehyde and stop the cross-linking reaction.
5. Wash the cells twice with 20 mL ice-cold 1× PBS. Aspirate off the PBS after each wash.
6. Scrape cells into a 50 mL conical tube with a cell scraper.
7. Pellet at 1,000×*g* for 5 min at 4°C and discard the supernatant.
8. Add 1 mL of Solution A to the pellet, vortex, and centrifuge on a microcentrifuge at 1,500×*g* for 5 min at 4°C, discard the supernatant.
9. Repeat step 7 with 1 mL of Solution B.
10. Add 1 mL of Solution C. Vortex to mix well.
11. Sonicate the samples on ice until the lysate clears. Sonication should be carried out in bursts with intervals of cooling on ice. In general, 8 cycles of 10 s sonication (at power setting 4) followed by 30 s of cooling on ice will suffice. The lysate should remain ice-cold throughout the sonication step (see Note 3).
12. Transfer the sonicated samples to fresh 1.5 mL microcentrifuge tubes. Centrifuge at 18,400×*g* for 15 min at 4°C. Transfer the supernatant, which contains sheared chromatin, to fresh 1.5 mL microcentrifuge tubes. The samples can be used directly or flash frozen in liquid $N_2$ and stored at –80°C.

### 3.2. Preclearing and Immunoprecipitation

1. For every 900 µL of sonicated lysate, add 50 µL of the preclearing protein A/G-agarose bead slurry from Subheading 2.2 and 2 µg control immunoglobulin (e.g., IgG). If frozen samples are used, thaw the lysate on ice and then centrifuge at 18,400×*g* for 15 min at 4°C. Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube (see Note 4).
2. Incubate at 4°C with rocking (or on a rotator) for 2–4 h. Centrifuge the sample at 850×*g* for 5 min at 4°C.
3. Transfer the supernatant to a new microcentrifuge tube. Remove ~40 µL and save as the input sample. Divide the remaining lysate equally for each IP (~400 µL), and add 3 µg of the desired antibody or appropriate IgG, together with 25 µg of sheared *E. coli* DNA plus 1 µL of 5% BSA. Rock or rotate the samples at 4°C for 6 h to overnight (see Note 5).
4. Prepare fresh protein A/G beads just before use. For each IP, 20 µL of 50% slurry is needed. Aliquot appropriate amount of the bead slurry into a clean microcentrifuge tube. Add 0.5–1 mL of Solution C. Spin down the beads at 850×*g* for 1 min at 4°C. Resuspend with Solution C to ~50% slurry.
5. Add 20 µL of the slurry to each IP tube. Rotate for another 1–2 h at 4°C.
6. Centrifuge at 850×*g* for 1 min at 4°C. Gently aspirate the supernatant. Add 400 µL of ChIP I buffer. Rotate for 10 min at 4°C.
7. Repeat step 6 once with ChIP II.
8. Repeat step 6 once with ChIP III.
9. Repeat step 6 once with TE/DTT.
10. Centrifuge at 850×*g* for 1 min at 4°C. Discard the supernatant.

### 3.3. Elution and DNA Purification

1. Add 100 µL of freshly prepared Elution buffer to each sample.
2. Incubate for 10 min at room temperature, vortex every 2 min. Centrifuge at 850×*g* for 1 min at room temperature, and transfer the supernatant to a new microcentrifuge tube.
3. Steps 1 and 2 may be repeated 1–2× to increase total DNA recovered. Combine the eluate.
4. Add 1/25 (v:v) of 5 M NaCl. Mix and incubate overnight at 65°C.
5. Briefly spin the tubes. For every 300 µL of eluted DNA sample, add 6 µL of 0.5 M EDTA, 12 µL 1 M Tris–HCl (pH 6.5), and 1.2 µL DNase-free RNase A (from 10 mg/mL stock). Incubate at 37°C for 30 min.

6. To every 300 µL of eluted DNA sample, add 1 µL Proteinase K (from 20 mg/mL stock). Incubate at 37°C for 1 h.
7. Purify the DNA using the QIAquick PCR purification kit (Qiagen). Mix the sample with PB buffer supplied with the kit and agitate for 30 min before loading the sample onto the spin column. Elute DNA in 50~100 µL Qiagen elution buffer (see Note 6).

### 3.4. Dot Blot

1. Moisten Hybond-N+ membrane (12×9 cm, mark edge for orientation) with $ddH_2O$, and then soak it in 2× SSC for 10 min (see Note 7).
2. Clean, dry, and assemble the Bio-Dot Microfiltration Apparatus according to the instruction manual. Load 100 µL 2× SSC and switch on the vacuum until all liquid drains from the wells. Switch the vacuum off until ready to load samples.
3. Boil the DNA samples for 5 min, cool on ice, and spin briefly in a microcentrifuge to collect all samples.
4. Load 200 µL of 2× SSC in each well. Then, load the desired amount of DNA samples (30–100 µL). Mix well with the 2× SSC (see Note 8).
5. Switch on the vacuum to gently remove buffer.
6. Disassemble Bio-Dot apparatus. Place the membrane on a piece of 3 M Whatman paper saturated with Denaturing solution for 10 min with the DNA side up.
7. Place the membrane on another piece of 3 M Whatman paper saturated with Neutralizing solution for 10 min with the DNA side up.
8. Gently blot the membrane on dry filter paper to remove excess liquid.
9. Cross-link the DNA to the membrane immediately in the Stratagene UV cross-linker (120 mJ/cm$^2$). Rinse in 2× SSC. Allow the membrane to air dry.
10. Prehybridize the membrane with 10 mL hybridization buffer in sealed bag or roller bottle for 2 h at 50°C.
11. Prepare radiolabeled $(TTAGGG)_3$ probe.
    (a) Mix 10 pmol oligonucleotide, 2 µL $T_4$PNK buffer (10×), 7 µL $^{32}$P-γ-ATP (3,000 Ci/mmol), 2 µL T4 Polynucleotide Kinase, and 8 µL $ddH_2O$. Incubate at 37°C for 1 h. Add 80 µL TE (pH 8.0) and incubate for 5 min at 70°C to stop the reaction.
    (b) Purify the probe using QIAquick nucleotide removal kit (QIAGEN). Check the radioactivity by liquid scintillation counter. Specific activity should be around $0.5–1 \times 10^6$ cpm/µL (see Note 9).

12. Discard prehybridization solution. Add 10 mL fresh hybridization buffer together with the labeled probe ($\geq 1 \times 10^6$ cpm/mL) and hybridize overnight at 50°C.
13. Wash the membrane in 4× SSC, 0.1%SDS for 10 min once at room temperature, and once at 37°C.
14. Wash the membrane in 2× SSC, 0.1%SDS for 10 min at 50°C (see Note 10).
15. Wash the membrane in 2× SSC for 5 min at room temperature.
16. Wrap the membrane with plastic wrap for autoradiography. Expose either to films (10–24 h) or in a phosphoImager cassette (1.5–3 h).

## 4. Notes

1. Different cell types yield different numbers of cells. For cells such as Hela, each confluent 15 cm dish contains $1–2 \times 10^7$ cells, enough for 2 IPs, whereas one 15 cm dish of primary cells may be enough for 1 IP. A 0.5 mL cell suspension ($1 \times 10^7$ cells/mL) is desirable for each IP experiment. However, we have obtained excellent results with fewer cells when obtaining large numbers of cells is impossible.
2. The length of cross-linking and the concentration of formaldehyde can be adjusted according to the protein of interest. The extent of cross-linking is critical, too much cross-linking may mask epitopes while too little may result in incomplete fixation.
3. DNA should be sheared to an average fragment size of ~500–1,000 bp. Optimal sonication conditions are important for ChIP efficiency. Sonication conditions differ for each cell type, fixation protocol, and sonicator apparatus. To check the sonication result, mix 10 µL of the sonicated sample with 2 µL Protease K (20 mg/mL) and incubate at 37°C for 20 min. Add one drop of phenol, vortex, and centrifuge at $18{,}400 \times g$ for 1 min. Use the supernatant to run DNA agarose gels to determine fragment size.
4. Ideally, the control immunoglobulin should be species and isotype matched to the primary antibody of interest. It is also advisable to save a small aliquot of the lysate for western blot analysis.
5. It is important to include all necessary controls, including controls for primary antibodies and positive controls for the experiment. Plan ahead so there is enough material for all the samples.

6. Alternatively, DNA can also purified using the following method.
   (a) Add 24 µg of Proteinase K, incubate for 1 h at 37°C.
   (b) Cool the samples to room temperature, add 400 µL phenol/chloroform/isoamyl alcohol (25:24:1), vigorously vortex for 5 s.
   (c) Centrifuge for 2 min at 16,000×*g* at RT. Transfer the aqueous phase to a microcentrifuge tube.
   (d) Add 12 µg of glycogen (from 20 mg/mL stock) and 2.5× volumes of ice-cold 100% ethanol, mix well and precipitate the DNA overnight at –20°C.
   (e) Centrifuge the samples at 18,400×*g* for 20 min at 4°C.
   (f) Carefully remove the supernatant and add 500 µL of ice-cold 70% ethanol to the pellet.
   (g) Centrifuge the samples at 18,400×*g* for 20 min at 4°C. Carefully remove the supernatant, air dry the pellet until all traces of ethanol have evaporated (>30 min).
   (h) Resuspend pellet in 100 µL of $ddH_2O$ or 10 mM Tris–HCl (pH 7.5). The DNA is now ready for downstream applications or being aliquoted and stored at –80°C.
7. Always use forceps or wear gloves when handling membranes and 3 M paper.
8. Mixing 2× SSC with DNA samples ensures even distribution of the sample on the membrane. The apparatus has different vacuum settings. When loading the DNA sample, vacuuming should be on the gentle setting.
9. The probe can also be purified by adding 30 µL of 10 M ammonium acetate, 2 µL of 10 mg/mL tRNA, and 400 µL ethanol. Mix by votexing, centrifuge samples at 18,400×*g* for 10 min at 4°C. Wash the pellet with 70% ethanol, and then dissolve the pellet in 100 µL TE (pH8.0).
10. The blot should be carefully monitored using a Geiger counter throughout the wash. The membrane should be ready for exposure once the edges or areas without samples no longer set off the Geiger counter. The number or temperature of washes can be adjusted accordingly. Usually, the membrane needs to be stripped and hybridized to a control probe (e.g., the Alu probe). It is critical that the membrane should stay moist throughout the experiment.

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# Chapter 16

# Studying of Telomeric Protein–Protein Interactions by Bi-Molecular Fluorescence Complementation (BiFC) and Peptide Array-Based Assays

**Wenbin Ma, Hyeung Kim, and Zhou Songyang**

## Abstract

Studying protein–protein interactions is critical to our understanding of the signaling pathways. The Telomere Interactome is assembled around the telomeres and consists of proteins and factors from diverse pathways. Dissecting how this protein network contributes to telomere protection and length regulation requires the elucidation of the complex and dynamic interactions between the proteins within the interactome. Here, we focus on the Bi-molecular fluorescence complementation (BiFC) and peptide array methods that have proven vital in our studies of telomere protein interaction networks.

**Key words:** Telomere Interactome, Protein–protein interactions, BiFC, Peptide array

## 1. Introduction

Within the cell, signals are often relayed through protein–protein interaction networks. For example, the dynamic and regulated assembly of various protein complexes on the telomere chromatin makes up the Telomere Interactome (1). Within this interactome, one major protein interaction hub is TRF2. It binds to telomeres through the myb domain, and recruits proteins and enzymes from diverse signaling pathways through other domains, such as the peptide-binding TRFH domain (2, 3). The complexity and dynamic nature of these associations serve to underline the importance of tools and methodologies in analyzing protein–protein interactions. Here, we describe two important methods that seek to analyze protein interactions under drastically different conditions.

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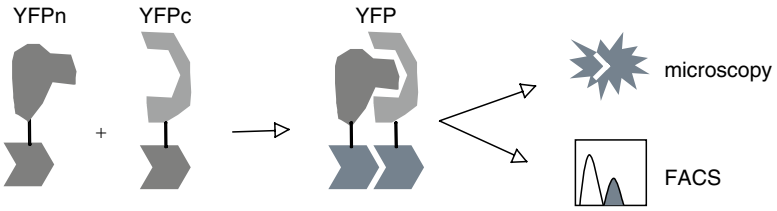


Fig. 1. BiFC strategy.

The Bi-molecular fluorescence complementation (BiFC) method was developed to investigate protein–protein interactions in vivo (4). Here, potential interacting protein pairs are respectively tagged with complementary halves of a fluorescent protein (FP). GFP, CFP, BFP, or RFP have all been successfully used (5–8). When the two proteins do interact, the two FP halves are brought together to co-fold (irreversibly) into a functional fluorophore (Fig. 1). Fluorescence can be analyzed by fluorescence microscopy or flow cytometry. The major advantage of BiFC is the ability to investigate interactions in live cells and to capture transient and low affinity interactions. Our lab has successfully utilized the BiFC method to analyze interactions among telomere proteins and their binding partners (9). We have used YFP extensively in our studies. Specifically, we used the first 155 amino acids of Venus YFP and the C-terminal 156–239 fragment of GFP. Tagging can occur either at the N- or C-terminus of the protein of interest. In this chapter, we focus on using our pBabe-based retroviral vectors to stably express YFP fragment-tagged proteins in human cell lines to investigate protein–protein interactions.

Protein–protein interactions are often mediated by modular domains. A particular domain may bind to several targets, each with its unique sequence. The binding regions of the shared targets, however, may also exhibit similarities in sequence. In such cases, a consensus motif can be derived, where positions critical for the interaction are frequently conserved between different targets. We developed the OPAL technique (based on SPOT array) to determine consensus binding motifs of antibody-antigen and phospho-dependent interactions (10). This strategy was subsequently adapted to probe the interactions between telomere proteins (11). In this method, we synthesize the entire domain or protein as overlapping peptides on membranes. The resulting membranes can then be "probed" with the interacting partner, to reveal the residues that are critical for the interaction (Fig. 2). This technique is particularly useful for generating an otherwise "functional" full-length protein with a specific disruption in its target binding capacity because point mutations should leave intact the overall folding and structure of the protein.

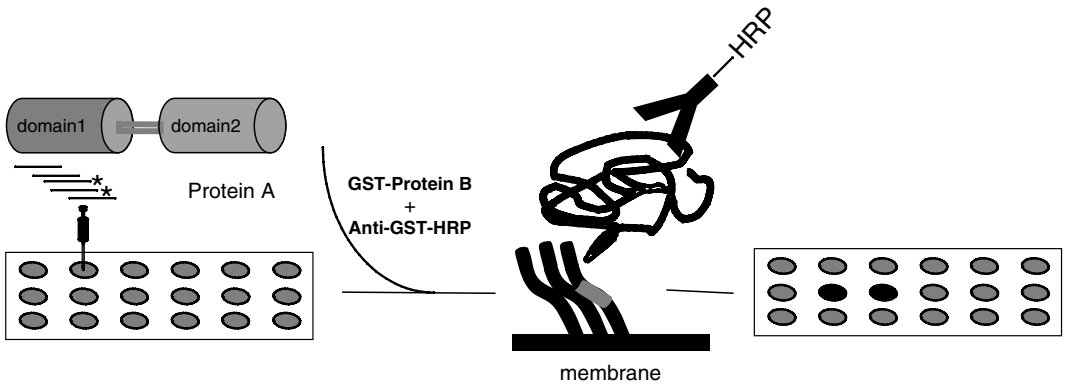


Fig. 2. Peptide array analysis of protein–protein interactions.

## 2. Materials

### *2.1. Studying Protein–Protein Interactions Using BiFC*

1. Plasmid expression vectors encoding the proteins of interest (bait and prey) fused to either the N- or C-terminal fragments of an FP, with different selection markers (see Note 1). We use the N-terminal 1-155 amino acids of Venus YFP and the C-terminal 156–239 of YFP.
2. Retroviral packaging cell lines: 293T cells or their derivatives (e.g., Phoenix-Ampho cells).
3. Target cells: cells of interest that are used for BiFC assay. The BiFC assay can be performed in many mammalian cell lines, including HTC75, COS-1, HEK293, HeLa, U2OS, Hep3B, aTN4, and NIH3T3 cells.
4. Transfection reagents of choice, examples include Lipofectamine from Invitrogen (see Note 2).
5. PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH 7.3.
6. 10 mg/mL polybrene stock solution. Aliquot and store at –20°C.
7. Trypsin-EDTA solution.
8. Hoechst 33342 stock solution (10 mg/ml). Dilute the stock 1,000-fold for the experiment.
9. Inverted fluorescence microscope equipped with filters for visualization of YFP (excitation 500 ± 10 nm; emission 535 ± 15 nm).
10. A flow cytometer such as LSRII (BD Bioscience, San Jose, CA).

### 2.2. Peptide Array Mapping of Specific Interactions

1. LB medium: dissolve 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl in dd$H_2O$ to 1 L. Autoclave and store at room temperature.
2. Prepare 1 M IPTG stock solution, filter through 0.22 μm filter, aliquot, and store at –20°C.
3. NETN buffer: 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA.
4. Prepare 0.1 M PMSF stock solution in isopropanol, aliquot, and store at –20°C.
5. Lysozyme: used to digest the polysaccharide component of bacterial cell walls.
6. Sonicator.
7. Glycerol.
8. Glutathione agarose beads.
9. 10% (w:v) sodium azide solution.
10. 100 mM Glutathione (GSH) solution. Adjust pH to 8, aliquot, and store at –20°C.
11. SDS-PAGE apparatus.
12. Anti-GST-HRP antibody (Amersham).
13. TBST: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20.
14. Bovine serum albumin (BSA), Fraction V.

## 3. Methods

### 3.1. Using the BiFC Approach to Visualize Protein–Protein Interactions in Live Cells

1. Prepare plasmids encoding BiFC-tagged bait and prey fusion proteins (e.g., retrovirus based) (see Note 1).
2. Prepare for retrovirus packaging. Seed packaging cells the day before transfection at an appropriate density depending on the cells used (see Note 3).
3. Transfect packaging cells with the retroviral vector encoding BiFC-tagged *bait* protein together with the appropriate packaging vector plasmid (if necessary) using the desired transfection reagent (see Note 4).
4. Replace with fresh media 6 h to overnight after transfection. And seed target cells at the appropriate density the day before infection (see Note 5).
5. At 48 h after transfection, collect the supernatant and filter it through a 0.45 μm syringe filter. This prevents carry-over of packaging cells. The harvested virus is now ready to use (see Note 6). It may also be aliquoted and stored at –80°C.

6. Add fresh media to the virus plate. The supernatant may be harvested again at 72 h after transfection, and stored frozen at –80°C.
7. Mix the harvested bait viruses with fresh media at 1:1 (v:v) ratio, and add to the target cells. Add polybrene to a final concentration of 4 μg/mL (see Note 7). Suspension cells may be spin-infected at 1,000× *g* for 1–2 h at room temperature (see Note 8).
8. Replace with fresh medium 16–24 h after infection.
9. At 48 h after infection, add the appropriate selection antibiotic to the target cells. Replace the selection medium every 2 days until cells achieve complete selection (see Note 9).
10. While the *bait*-expressing cells are undergoing selection, repeat steps 2–6 to generate viruses encoding the *prey* protein.
11. Ensure that *bait*-expressing target cells are actively dividing once they come out of selection. Seed these cells for infection with *prey* viruses.
12. Repeat steps 7–9 to generate target cells stably expressing both bait and prey proteins (see Note 10).
13. Prepare an aliquot of the cells for fluorescence complementation analysis (see Note 11).
    (a) For fluorescence microscopy, suspension cells may be collected, washed twice with 1× PBS, resuspended in serum supplemented phenol-red free media, and spun onto slides for analysis under the microscope. For adherent cells, aspirate the media, wash twice with 1× PBS, replace with serum containing phenol-red free media, and observe under the microscope (see Note 12). A DNA-staining dye (e.g., Hoechst 33342) may be added to the cells (1 μg/mL) to aid the analysis.
    (b) For flow cytometry measurements, trypsinize the cells (if adherent), collect and wash the cells with 1× PBS, and resuspend in phenol-red free media (see Note 13).

### 3.2. Peptide Array Mapping of Specific Interactions

#### 3.2.1. Preparation of GST-Fusion Proteins

1. Inoculate a single *E. coli* colony expressing the desired GST-tagging plasmid in 5–10 mL of LB media plus the appropriate antibiotics and shake overnight at 37°C (see Note 14).
2. Dilute the overnight culture 20-fold with additional LB media and antibiotics. And shake at 37°C for >2 h (see Note 15).
3. Check the turbidity and O.D. of the culture. An O.D. between 0.5 and 1 is ideal.
4. Add IPTG to a final concentration of 1 mM (1,000-fold dilution). Grow the cells for 4 more hours (see Note 16).

5. Spin down the bacterial culture and collect the final pellet in 50 ml conical tubes (see Note 17).
6. Resuspend the pellet in 30 ml of ice-cold 1× NETN plus 1 mM PMSF (from 0.1 M stock solution). Vortex to ensure complete resuspension.
7. Add lysozyme to 200 μg/mL and mix vigorously and thoroughly. Incubate on ice for >20 min (see Note 18).
8. Sonicate the sample on ice until viscosity of the lysate is significantly reduced. Sonication (50% power) should be carried out in short 10 s bursts followed by 30 s intervals of cooling on ice. Check between cycles to make sure that the lysate remains ice-cold (see Note 19). Typically, 6–8 rounds of sonication are sufficient.
9. Spin down the lysate at 17,000× *g* for 30 min at 4°C.
10. Transfer the supernatant to a fresh tube. If the lysate does not become clear, repeat step 9. Add glycerol to the sample to a final concentration of 15%, and then freeze the tube in –80°C for future use. Otherwise, proceed to the following GST pull-down steps.
11. Wash appropriate amount of GSH-agarose beads twice with 1× NETN buffer. Resuspend in 1× NETN to achieve 50% slurry. In general, ~200 μL of beads can be used for 20 ml of total supernatant.
12. Add the bead slurry to the cleared lysate. Shake at 4°C for >2 h.
13. Spin down the mixture at 1,000× *g* at 4°C for 5 min. Carefully remove the supernatant (see Note 20).
14. Add ice-cold 1× NETN buffer plus PMSF to the pelleted beads. Volume of the buffer should equal volume of lysate used for thorough washing. Invert to mix.
15. Repeat step 13. Discard the supernatant.
16. Repeat step 14 and 15 once.
17. Repeat step 14 and 15 with half as much 1× NETN buffer.
18. Add ice-cold 1× PBS to the pelleted beads. Invert to mix. Spin at 1,000× *g* at 4°C for 5 min.
19. Carefully remove and discard supernatant. Resuspend beads in 1× PBS (e.g., 100 μL) plus 0.2% sodium azide. Transfer the bead slurry to a fresh microcentrifuge tube.
20. Add 1× volume (100 μL) of GSH solution to the beads. Mix gently and incubate on ice for 10 min.
21. Centrifuge the tube in a microcentrifuge at 2,000× *g* for 1 min, transfer the supernatant to a fresh tube and store on ice.

22. Repeat steps 20–21 three times and combine all eluate. The purified proteins are now ready for further analysis or storage at 4°C.
23. Save an aliquot for analysis with SDS-PAGE to verify protein expression and pulldown efficiency (see Note 21).

*3.2.2. Peptide Array Synthesis and Analysis*

1. Synthesize peptide mixtures on cellulose membranes by FMOC-solid phase synthesis chemistry using the ASP222 SPOT robot (see Note 22).
2. Block the peptide array membrane in 3–5 ml of 3% BSA/1× TBST for 2 h at room temperature.
3. Preincubate the purified GST-fusion proteins from step 22 in Subheading 3.2.1 with an anti-GST-HRP antibody (5:1) in a very small volume (<10 μL) at room temperature for 30 min (see Note 23).
4. Incubate the blocked membrane with the preincubated GST-fusion protein mixture from step 3 at a concentration of 1 μg/ml in 1× TBST (1 mL of total reaction volume) at room temperature for 30 min.
5. Wash the membrane with 1× TBST for 10 min, changing the buffer every 3 min.
6. Analyze the membrane by ECL to visualize the peptides bound by GST-fusion proteins.

## 4. Notes

1. An expression vector that can accommodate both transient and stable expression is desirable. We use a retroviral pBabe-based vector for all our studies. Because proteins may be tagged at either the N- or C-terminus with either FP fragment, a total of eight configurations are possible. The best configuration for any interaction pair should be determined empirically. It is advisable to test all configurations, since protein folding and function may be adversely affected by tagging at a particular end. Ideally, a control protein that does not interact with the bait protein should be included as the negative control. In our hands, there is practically no background fluorescence when the two FP fragments were co-expressed by themselves. Because two different proteins are co-expressed in the same cell, having different selection markers in the two constructs helps to ensure simultaneous expression of both proteins.
2. BiFC assays may be carried out in cells transiently co-expressing the protein pair. This is especially useful for quickly testing

different tagging configurations. In general, better signal intensity coupled with a higher noise/signal ratio is observed in transient assays compared to stable expression cells.

3. HEK 293T cells are widely used as the packaging cell line for generating retroviruses. In our case, an amphotropic packaging plasmid was cotransfected with the split YFP plasmids at a 1:1 ratio. Optimal cell density at transfection depends on a number of factors, including cell type, transfection reagent, and culture dish used. We normally package retroviruses using 293T cells grown in 6-well plates, and seed ~$0.5 \times 10^6$ cells/well the day before transfection. Alternatively, Phoenix-Ampho cells may be used, in which case the packaging plasmid would not be needed. It is highly recommended that cells of low passage numbers be used. Extensive culturing can lead to a loss of the cells' ability to generate high-titer viruses.
4. Various retrovirus packaging vectors and systems are available. Amphotropic packaging plasmids, such as pCL-Ampho allows for packaged viruses to infect most mammalian cells except for hamster cells, whereas ecotropic packaging plasmids, such as pCL-Eco allows for the infection of mouse and rat cells but not human cells. Follow the manufacturer's directions for the transfection reagent.
5. Because only cycling cells can be infected by retroviruses, it is critical that the target cells do not reach confluence for retroviral transduction. In general, ~30–50% confluence at the day of infection yields good results.
6. Do not use 0.22 μm filters, it will significantly decrease viral titer. In our hands, we typically generate viral titers in the range of 0.5–1 × 10^6 infectious units/ml. Titering is desirable but not absolutely required in these experiments. It is also helpful to generate viruses for each protein pair using the same batch of cells so that the viral titers for the pair are similar.
7. The multiplicity of infection (MOI) may be estimated here if viruses are not tittered. For generating stable lines, MOI of 5–10 is often well tolerated by cells and yield good results, although some cell types may be sensitive to infection of high MOI. Mix the virus supernatant with equal volume of fresh medium may maximize infection efficiency. Depending on the cell type used, optimal polybrene concentrations can vary (1–10 μg/mL). Some cells are transducible in the absence of polybrene, although addition of polybrene can drastically enhance efficiency. Polybrene can be toxic to some cells. Protamine sulfate has been used as an alternative in certain cases.

8. Transduction efficiency may be enhanced by multiple factors, including higher MOI and addition of agents, such as polybrene. Cells may also be spin-infected. In addition, cells may be infected again 16 h after the initial transduction, for example, with viruses collected at 72 h after transfection.
9. The concentration of the drugs used should be determined empirically for the cell type used. Commonly used selection antibiotics, such as G418 and puromycin, have distinct kill curves, which should be obtained for optimal treatment concentration and length of time. For example, selection in 500 μg/mL (active) G418 for 7–10 days is sufficient for HT1080 cells.
10. Western blotting analysis may be carried out at this point to ensure co-expression of the bait and prey proteins before going further with the experiments.
11. For most interactions, cells exhibit fluorescence at 24–48 h after infection with the prey viruses. Incubating the cells at 30°C with 5% $CO_2$ may promote maturation of certain fluorophores. If stable interactions between the bait-prey pair have adverse effects on cell growth, continued culturing of the cells will lead to diminished fluorescence over time.
12. It is helpful to plate adherent cells in thin-bottom plates or chamber slides for analyzing under fluorescence microscopes. The bottom of regular tissue culture plates is generally too thick for high-resolution imaging.
13. For quantitative analysis by flow cytometry, it is critical to include all the negative and positive controls for proper gating of the data. Even for the same fluorophore, the intensity of fluorescence varies considerably between different protein interaction pairs, possibly a reflection of affinity.
14. Before carrying out large-scale purification of GST proteins, a scaled-down pilot pull down (following the same steps) should be performed to confirm expression.
15. A 1:10 dilution may be used if pressed for time.
16. The working concentration of IPTG may need to empirically determine. A range of 0.1–1 mM is generally used.
17. Multiple spin steps may be needed if large cultures are used. Alternatively, cultures may be aliquoted into multiple tubes. Conical tubes make subsequent steps easier. At this point, the pellets may be stored at –20°C.
18. The incubation step can be longer. Depending on the size of the starting bacterial culture, there may be a need to scale up or down for the amount of NETN added, and consequently, the type of tubes to be used for sonication. For example, 15 ml tubes may be used for smaller volumes.

19. Ultrasonic treatment must be applied in multiple short bursts to prevent excessive heating. And the sample needs to be immersed in an ice bath. Sonication is usually best suited for volumes <100 mL. If viscosity is not significantly reduced, DNase (5 μg/mL) may be added and the sample incubated on ice for 10–15 min.
20. The used lysate may be transferred to a fresh tube, frozen and reused. This works especially well if the protein is highly expressed.
21. During SDS-PAGE, include serially diluted protein samples with known concentration (e.g., BSA) to estimate the amount of proteins eluted from the preparations.
22. This section is beyond the scope of this chapter. Please follow the detailed steps as described (12). Major steps include membrane preparation, peptide synthesis cycles, Fmoc deprotection, and side-chain deprotection. In our case, an extra-fine needle tip was used to achieve a density of 1,536 peptides per SPOT membrane (8×12 cm). Each spot contained ~5 nmols of peptides. For the interaction studies described here, residues from a particular protein domain can be synthesized as a series of overlapping (at 2 amino acids intervals) and continuous 12-mer peptides.
23. The actual volume depends on the concentration of the purified proteins. If the GST-fusion proteins are too diluted, they may be concentrated first before proceeding. It is critical to maintain the volume of the reaction at <10 μL for the assay to work well.

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# Chapter 17

# Human Telomere POT1-TPP1 Complex and Its Role in Telomerase Activity Regulation

**Feng Wang and Ming Lei**

## Abstract

Telomeres, the specialized DNA-protein complexes found at the termini of all linear eukaryotic chromosomes, protect chromosomes from degradation and end-to-end fusion. The protection of telomeres 1 (POT1) protein binds the single-stranded overhang at the ends of chromosomes in diverse eukaryotes. It is essential for chromosome end-protection and involved in telomere length regulation. TPP1 is a previously identified binding partner of POT1 that has been proposed to form part of a six-protein shelterin complex at telomeres. Through structural and biochemical studies, we have demonstrated that human TPP1 is the missing human homolog of the β subunit of protozoan telomere end-binding-protein-complex (TEBPα-TEBPβ). Therefore, capping of telomeres by a TEBPα-TEBPβ/POT1-TPP1 dimer is more evolutionarily conserved than that had been expected. In addition, we also discovered that the human POT1-TPP1 complex is a processivity factor for telomerase.

**Key words:** Telomere, Telomerase, Telomeric proteins, POT1, TPP1, Protein purification, In vitro telomerase activity

## 1. Introduction

Telomeres, the natural ends of linear eukaryotic chromosomes, are essential for cell viability and genome integrity (1). Aberrations in telomere length have been implicated in cancer and aging. In most eukaryotes, telomere length is maintained by telomerase, a specialized reverse transcriptase, which adds telomeric DNA to the 3′ ends of chromosomes to ensure complete genome replication. Telomerase activation is one of the factors necessary for cell immortalization and transformation, and thus telomerase is an attractive anticancer target (2). A six-protein complex, called shelterin, protects the telomeres of human chromosomes (3). TRF1 and TRF2 bind double-stranded telomeric DNA (4–6),

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POT1 binds the single-stranded 3′-G-overhang (7–14), and these are bridged through protein–protein interactions involving RAP1, TIN2, and TPP1 (15–17). Although much information has been accumulated about the protein components of human telomeres, the molecular mechanisms by which shelterin mediate the maintenance and protection of human telomeres remains unclear. We have demonstrated that human TPP1 is the missing human homolog of the β subunit of protozoan telomere end-binding-protein-complex (TEBPα-TEBPβ) (18). Therefore, capping of telomeres by a TEBPα-TEBPβ/POT1-TPP1 dimer is more evolutionarily conserved than that had been expected. In addition, we also discovered that the human POT1-TPP1 complex is a processivity factor for telomerase (18).

The full length POT1 protein was expressed and purified in baculovirus insect cell expression system while the N-terminal OB-fold of TPP1 was expressed and purified from *Escherichia coli*. Both proteins were subsequently further purified in homogeneity by gel filtration chromatography and then subjected to structural and functional studies. By using an in vitro telomerase activity assay, we revealed that POT1-TPP1 complex could stimulate the activity and processivity of the in vitro immunopurified human telomerase complex.

## 2. Materials

### 2.1. Insect Cell Culture

1. *Spodoptera frugiperda* (Sf)-900 II SFM (Invitrogen, Carlsbad, CA) supplemented with penicillin-streptomycin (50–100 U of penicillin, 50–100 μg of streptomycin) (Invitrogen, Carlsbad, CA). Sf-900 II SFM is used for Sf9 cell culture.
2. 10% (v/v) fetal bovine serum (FBS, Hyclone, Ogden, UT) is added into Sf-900 II SFM when necessary.
3. Insect-XPRESS medium (Lonza, Walkersville, MD) supplemented with penicillin-streptomycin. Insect-XPRESS is used for Hi Five™ cell culture.
4. Trypan blue stain.

### 2.2. E. coli Culture

1. Lysogeny broth (LB) medium (Invitrogen, Carlsbad, CA) supplemented with ampicillin (Fisher Scientific, Pittsburgh, PA) or kanamycin (Invitrogen, Carlsbad, CA).
2. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Fisher Scientific, Pittsburgh, PA) is dissolved in water at 1 M, filter sterilized, stored in aliquots at −20°C, and then added to *E. coli* culture at certain concentration as indicated in Subheading 3.

### 2.3. Recombinant Protein Purification

1. Lysis buffer for cell lysis: 50 mM Tris–HCl pH 8.0, 50 mM $NaH_2PO_4$ pH 8.0, 400 mM NaCl, 10% (v/v) glycerol, 3 mM imidazole, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/mL lysozyme, 2 mM 2-mercaptoethanol and complete protease inhibitor cocktail tablets (Roche, Indianapolis, IN) (one tablet for 50 mL lysis buffer), and stored at 4°C (see Notes 1 and 2).
2. Washing buffer: 10 mM imidazole in lysis buffer, and stored at 4°C.
3. Elution buffer: 250 mM imidazole in lysis buffer, and stored at 4°C.
4. FPLC buffer: 25 mM Tris–HCl pH 8.0, 150 mM NaCl, and stored at 4°C.
5. DNase I (Roche, Indianapolis, IN).
6. Ni-NTA agarose beads (Qiagen, Valencia, CA).
7. Glutathione sepharose™ 4B beads (GE Healthcare, Piscataway, NJ).
8. Econo-Pac chromatography columns (Bio-Rad, Hercules, CA).
9. The protease HRV 3C (Novagen, Madison, WI).
10. Hiload Superdex 200 (GE Healthcare, Piscataway, NJ).

### 2.4. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4×): 1.5 M Tris–HCl pH 8.8, 0.4% (w/v) SDS.
2. Stacking buffer: (4×): 1.5 M Tris–HCl pH 6.8, 0.4% (w/v) SDS.
3. 30% Acrylamide/Bis solution (37.5:1) (this is a neurotoxin when unpolymerized and so care should be taken not to receive exposure) and N,N,N,N′-Tetramethyl-enthylenediamine (TEMED, Invitrogen, Carlsbad, CA) (see Note 3).
4. Ammonium persulfate (Sigma-Aldrich, San Francisco, CA): prepare 10% solution in water and immediately freeze in single use (200 µL) aliquots at –20°C.
5. Running buffer (10×): 30 g tris (hydroxymethyl) aminomethane (Tris), 144 g glycine, and 10 g SDS powder in 1 L water.
6. PageRuler™ plus prestained protein ladder (Fermentas Inc., Glen Burnie, MD).
7. 4× SDS sample buffer: 4 mL glycerol, 0.8 g SDS powder, 2.5 mL 1 M Tris–HCl pH 6.8, 80 µL bromophenol blue slurry (5 mg/mL in water; vortex well before use), and 20% 2-mercaptoethanol.
8. Staining buffer: 8 g coomassie blue, 10% (v/v) glacial acetic acid, 20% (v/v) ethanol in 1 L water.

9. Destaining buffer: 10 %(v/v) glacial acetic acid, 20% (v/v) ethanol in 1 L water.

### 2.5. Western Blotting for Confirming Recombinant Protein Expression

1. Equilibration buffer: 25 mM Tris (do not adjust pH), 190 mM glycine, 20% (v/v) methanol, store at room temperature.
2. Transfer buffer: add 0.05 % (w/v) SDS to equilibration buffer, store at room temperature.
3. Supported nitrocellulose membrane (Millipore, Bedford, MA) and extra thick blot paper (Bio-Rad, Hercules, CA).
4. 10× Tris-buffered saline (TBS): 1.37 M NaCl, 27 mM KCl, 250 mM Tris–HCl pH 7.4.
5. TBS-Tween (TBS-T): Dilute 100 mL 10× TBS with 900 mL water, add 0.1% (v/v) Tween-20 (Fisher Scientific, Pittsburgh, PA).
6. Blocking buffer: 5% (w/v) nonfat milk in TBS-T.
7. Primary antibody dilution buffer: TBS-T supplemented with 2% (w/v) fraction V bovine serum albumen.
8. Anti-GST HRP conjugate antibody (available from GE Healthcare, Piscataway, NJ, see Note 4).
9. Enhanced chemiluminescence (ECL) reagents (Bio-Rad, Hercules, CA) and Bio-Max ML film (Kodak, Rochester, NY).

### 2.6. In Vitro Transcription/ Translation and Purification of Human Telomerase

1. TnT® Quick Coupled Transcription/Translation System (Promega, Madison, WI).
2. $^{35}$S-methionine (Perkin-Elmer, Waltham, MA) (this is radioactive and so care should be taken).
3. Anti-HA F7 agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA).

### 2.7. In Vitro Telomerase Activity Assay

1. 10× Telomerase activity assay buffer: 500 mM Tris–HCl pH8.0, 500 mM NaCl, 10 mM $MgCl_2$, 10 mM spermidine, and 50 mM 2-mercaptoethanol.
2. 20× dNTPs: 10 mM dATP, 10 mM dTTP, 0.04 mM dGTP (Sigma-Aldrich, San Francisco, CA) in water.
3. Stopping solution: 3.6 M $NH_4OAc$, 20 mg/mL glycogen (Roche, Indianapolis, IN).
4. $^{32}$P-dGTP (0.25 μM stock, 800 Ci/mmole, 1Ci = 37 GBq) (this is radioactive and so care should be taken).
5. Single-stranded (ss) telomeric DNA primer (1 μM stock in water).
6. Phosphorimager (GE Healthcare, Piscataway, NJ).

### 2.8. Electrophoresis for In Vitro Telomerase Activity Assay

1. Gel solution I: 20% acrylamide/Bis solution (37.5:1), 7 M urea in 1× Tris-Borate-EDTA (TBE) buffer and stored at 4°C.
2. Gel solution II: 7 M Urea in 1× TBE buffer and stored at 4°C.
3. 2× loading dye: 94% formamide, 0.1× TBE, 0.1% bromophenol blue, 0.1% xylene cyanol.

## 3. Methods

### 3.1. Human POT1 Expression in Baculovirus Insect Cell System

1. The gene of full length human POT1 is cloned into the modified pFASTBac-GST vector. The recombinant bacmid was obtained by following the Invitrogen Bac-to-Bac® Expression System Handbook.
2. The recombinant baculovirus of pFASTBac-GST-hPOT1 ($1\times10^8$~$1\times10^9$ plaque forming units (pfu/mL)) is prepared and amplified in Sf9 cells by following the Invitrogen Bac-to-Bac® Expression System Handbook. For bacmid transfection in Sf9 cells, Sf-900 II SFM without Penicillin-Streptomycin and FBS should be used. Sf9 cells should be in excellent health, low passage (5–20), log-phase growth, at density of $2\times10^6$ cells/mL, and have >95% viability.
3. Hi Five™ cells are used for virus infection and recombinant protein expression. Prepare 1 L Hi Five™ cells that are in excellent health, low passage (5–20), log-phase growth, at density of $2\times10^6$ cells/mL, and have >95% viability. Infect Hi Five™ cells by pFASTBac-GST-hPOT1 baculovirus at a multiplicity of infection (MOI) of 1–5. The inoculum required (mL) is calculated based on the following equation:

   Inoculum required (mL) = [MOI (pfu/cell) × number of cells]/titer of viral stock (pfu/mL). For example: we wish to infect a 1 L culture at $2\times10^6$ cells/mL using an MOI = 5. The titer of our viral stock is $1\times10^8$ pfu/mL. The inoculum required (mL) is 100 mL.
4. Incubate the cells at 27°C shaker at 125 rpm until viability reduced to 80–85% (approximately 48 h postinfection). Cell viability can be calculated by counting Trypan Blue stained cell samples in a cytometer under a reverse optical microscope.
5. Harvest cells by centrifuging at $3{,}600\times g$ at 4°C. Discard the supernatant and store the cell pellet at –80°C. Protein expression can be detected and confirmed via SDS-PAGE or western blotting by using anti-GST antibody.

### 3.2. Human TPP1 Expression in E. coli

1. The gene of amino-terminal of human TPP1 (residue 89–334) is cloned into the modified pET28b vector with a SUMO protein fused at the N-terminus after the 6× His tag. The plasmid is then transformed into *E. coli*. BL21(DE3) strain.
2. Inoculate a fresh transformant into 5 mL LB media supplemented with Kanamycin (50 μg/mL); incubate at 37°C shaker at 250 rpm for overnight.
3. Transfer the overnight culture into 1 L LB media supplemented with Kanamycin (50 μg/mL); incubate at 37°C shaker at 250 rpm until $OD_{600}$ reaches 0.55 (approximately 4 h).
4. Cool down the cells by ice-water bath for approximately 30 min.
5. Induce the cells by adding 0.1 mM IPTG and then incubate the cells at 22°C shaker at 250 rpm for 16 h.
6. Harvest cells by centrifuging at 3,600 × *g* at 4°C. Discard the supernatant and store the cell pellet at −80°C.

### 3.3. Protein Purification of GST-hPOT1

1. Resuspend the cell pellets in cold lysis buffer (50 mL lysis buffer for 1 L cell pellet), and then lyse the cells by sonication (80% power, 1 min pulse three times with 1 min rest interval) on ice. After sonication, add DNase I (10 μg/mL) and 10 mM $MgCl_2$, incubate on ice for 30 min.
2. Pellet the cell debris by ultracentrifugation at 180,000 × *g* at 4°C for 2 h.
3. During the ultracentrifugation, equilibrate 2 mL Glutathione Sepharose™ 4B beads by 20 mL cold lysis buffer.
4. Mix the supernatant from step 2 with the Glutathione Sepharose™ 4B beads from step 3 and rock the mixture at 4°C for 4–6 h.
5. Load the mixture into the Econo-Pac chromatography column and let it flow through by gravity in cold room.
6. Repeat step 5 once.
7. Wash the Glutathione Sepharose™ 4B beads with 20 column volume cold lysis buffer (e.g., 40 mL lysis buffer for 2 mL beads).
8. Elute the protein by 10 mM reduced glutathione in lysis buffer.
9. The eluted proteins can be analyzed by SDS-PAGE.
10. The protease HRV 3C was added (protease:target protein ration (unit/μg) of 1:50, rotate in cold room for overnight cleavage) to remove the GST-tag.
11. The protein is further purified by gel-filtration chromatography on Hiload Superdex 200 equilibrated with FPLC buffer with 5 mM dithiothreitol (DTT).

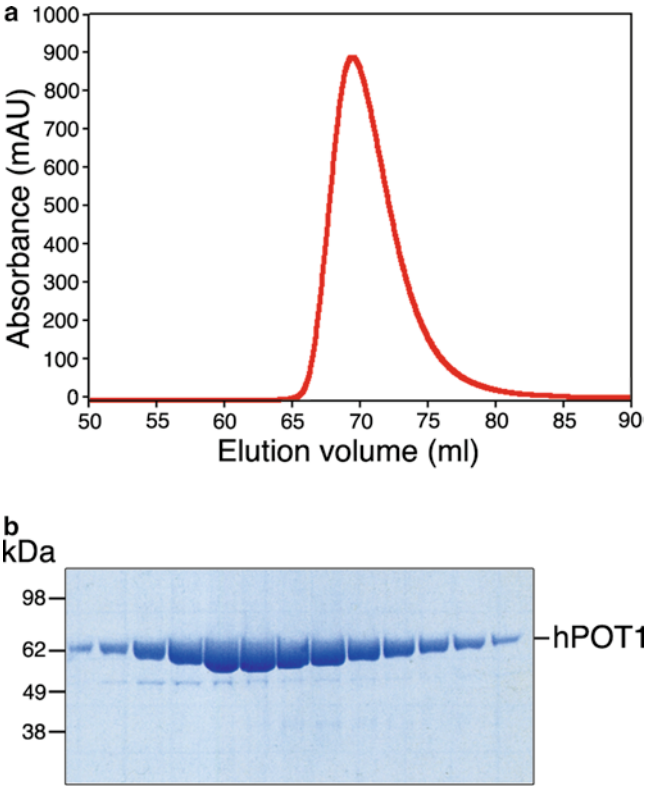


Fig. 1. Purification of GST-hPOT1. (**a**) Gel filtration chromatography profile (Hiload Superdex 200) of GST-hPOT1. (**b**) SDS-PAGE of fraction samples from the gel filtration profile peak in (**a**).

12. The fractions can be analyzed by SDS-PAGE (Fig. 1).
13. Pool the pure fractions and concentrate up to 20 mg/mL and immediately flash freeze in single (25 µL) aliquots by liquid nitrogen and store at −80°C.

### *3.4. Protein Purification of Hix6-Sumo-TPP1*

1. Resuspend the cell pellets in cold lysis buffer (50 mL lysis buffer for 1 L cell pellet), and then lysed by sonication (80% power, 1 min pulse three times with 1 min rest interval) on ice. After sonication, add DNase I (10 µg/mL) and 10 mM $MgCl_2$, incubate on ice for 30 min.
2. Pellet the cell debris by ultracentrifugation at 180,000 × *g* at 4°C for 2 h.
3. During the ultracentrifugation, equilibrate 2 mL Ni-NTA agarose beads by 20 mL cold lysis buffer.
4. Mix the supernatant from step 2 with the Ni-NTA agarose beads from step 3 and rock the mixture at 4°C for 4–6 h.
5. Load the mixture into the Econo-Pac chromatography column and let it flow through by gravity in cold room.

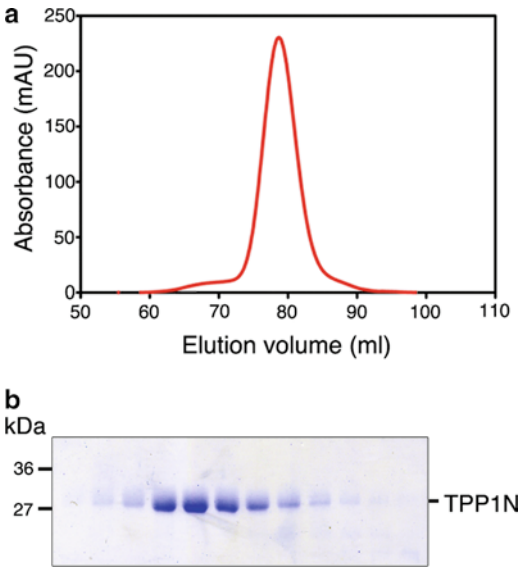


Fig. 2. Purification of His6-Sumo-TPP1N (residue: 89–334). (**a**) Gel filtration chromatography profile (Hiload Superdex 200) of His6-Sumo-TPP1 (residue: 89–334). (**b**) SDS-PAGE of fraction samples from the gel filtration profile peak in (**a**).

6. Repeat step 5 once.
7. Wash the Ni-NTA agarose beads with ten column volume cold lysis buffer (e.g., 20 mL lysis buffer for 2 mL beads).
8. Wash the Ni-NTA agarose beads with ten column volume cold wash buffer (e.g., 20 mL wash buffer for 2 mL beads).
9. Elute the protein by cold elution buffer.
10. The eluted proteins can be analyzed by SDS-PAGE.
11. The protease ULP1 was added (protease:target protein ration (unit/μg) of 1:50, rotate in cold room for overnight cleavage) to remove the His6-Sumo-tag.
12. The protein is further purified by gel-filtration chromatography on Hiload Superdex 200 equilibrated with FPLC buffer with 5 mM DTT.
13. The fractions can be analyzed by SDS-PAGE (Fig. 2).
14. Pool the pure fractions and concentrate up to 20 mg/mL and immediately flash freeze in single (25 μL) aliquots by liquid nitrogen and store at −80°C.

### *3.5. SDS-PAGE*

1. The following instructions are based on the use of a Bio-Rad Mini-PROTEAN Tetra gel system. It is critical that the glass plates for the gels are scrubbed clean with a rinsable detergent after use (e.g., Alconox, Alconox, New York, NY) and rinsed extensively with distilled water.

2. Prepare a 1.5-mm thick, 15% separating gel by mixing 2 ml water, 2 ml separating buffer, 4 ml 30% acrylamide/bis solution (37.5:1), 40 µl 10% ammonium persulfate solution, and 6 µl TEMED. Pour the separating gel, leaving enough space (approximately 1.5 cm) for a stacking gel, and overlay with water. The separating gel should be polymerized in about 30 min.
3. Pour off the water after the separating gel is polymerized.
4. Prepare the stacking gel by mixing 2 ml water, 0.7 ml separating buffer, 0.35 ml 30% acrylamide/bis solution (37.5:1), 25 µl 10% ammonium persulfate solution, and 3 µl TEMED. Quickly pour the stacking gel and insert the comb without generating any bubbles. The stacking gel should be polymerized in about 30 min.
5. Prepare 1× running buffer by mixing 100 ml of the 10× running buffer with 900 ml water.
6. After the stacking gel is polymerized, carefully remove the comb and wash the wells by pipetting running buffer.
7. Pour the running buffer to the inside and outside chambers of the gel box and load the 10 µl of protein sample in a well, including one well for 5 µl of PageRuler™ Plus Prestained Protein Ladder.
8. Complete assembling of the gel box and connect it to a power supply. The gel can be run at 180 V for about 1 h. The dye fronts (blue) can be run off the gel if necessary. The prestained protein ladder can be used to monitor the proper running time.
9. Once the gel is finished, carefully remove the gel from glass plates. Rinse the gel by 50 ml water. Soak the gel in a tray containing 20 ml Staining Buffer. Rock the tray on a rocking platform for about 1 h.
10. Discard the staining buffer, and rinse the gel by 100 ml water. Soak the gel in a tray containing 40 ml destaining buffer. Rock the tray on a rocking platform until the background of the gel becomes clear.

### 3.6. Western Blotting for Confirming Protein Expression

1. The protein samples are subject to SDS-PAGE without staining or destaining as described in Subheading 3.5. The samples that have been separated by SDS-PAGE are transferred to nitrocellulose membranes electrophoretically. The following instructions are based on the use of a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer system.
2. Carefully remove the gel from glass plates, rinse the gel by 50 ml water. Soak the gel in a tray containing 50 ml equilibration buffer. Rock the tray on a rocking platform for about

10 min. Meanwhile, prepare one piece of nitrocellulose membrane and two pieces of Extra Thick Blot paper. Cut the membrane and paper just larger than the size of the separating gel. Soak the nitrocellulose membrane and blot paper in a separate tray containing 50 ml equilibration buffer. Rock the tray on a rocking platform for about 10 min.

3. Disconnect the transfer unit from the power supply. Remove the safety cover and prepare gel sandwich as follows:
   (a) To bottom platinum anode place (see Note 5)
   (b) Prewet blot paper
   (c) Prewet nitrocellulose membrane
   (d) Equilibrated gel
   (e) Prewet blot paper
4. Secure top stainless steel cathode and safety cover.
5. Connect the transfer unit to the power supply and run blot. Optimized conditions may vary for different proteins. Generally, for a minigel, we run the blot at 10 V for 30 min or at 15 V for 15 min.
6. Once the transfer is completed, turn off the power supply, unplug electrodes, and carefully remove blot. The gel and blot papers can be discarded. The colored protein ladders should be clearly visible on the nitrocellulose membrane.
7. Incubate the nitrocellulose membrane in 50 ml blocking buffer for 1 h at room temperature on a rocking platform.
8. Discard the blocking buffer, and quickly rinse the membrane with 100 ml water. Wash the membrane with 50 ml TBS-T for 5 min, three times in total.
9. Prepare the primary (e.g., anti-GST) antibody at 1:1,000-fold dilution in TBS-T/2% BSA. Incubate the membrane with the primary antibody for 1 h at cold room on a rocking platform.
10. Carefully remove the primary antibody (see Note 6). Wash the membrane with 50 ml TBS-T for 5 min, three times in total.
11. Freshly prepare the secondary antibody for each experiment at 1:2,000-fold dilution in blocking buffer. Incubate the membrane with the secondary antibody for 1 h at room temperature on a rocking platform.
12. Discard the secondary antibody and rinse the membrane with 100 ml water. Wash the membrane with 50 ml TBS-T for 5 min, five times in total.
13. Prepare the ECL mixture by mixing 1 ml aliquots of each portion of the ECL reagents. Once the final wash is removed from the membrane, lay the membrane on a piece of

Saran-Wrap, and add the ECL mixture evenly onto the membrane. Incubate for 5 min. Remove the access ECL mixture with Kim-Wipes and carefully wrap the membrane with Saran-Wrap.

14. In a dark room, place the membrane with an X-ray film into an X-ray film cassette for a suitable exposure time.

### 3.7. In Vitro Transcription/ Translation and Purification of Human Telomerase

1. 20 μg HA-hTERT and 20 μg hTR (FokI digested) plasmid are added into the in vitro transcription/translation reaction mixture by following the Promega TnT® Quick Coupled Transcription/Translation System Manual (see Note 7).
2. Equilibrate 500 μL anti-HA F7 agarose beads with 1 mL cold 1× telomerase activity assay buffer.
3. Resuspend anti-HA F7 agarose beads in 250 μL cold 1× telomerase activity assay buffer and add the beads slurry into the in vitro transcription/translation reaction mixture when the reaction is finished. Rotate the beads-reaction-mixture at 4°C for overnight.
4. Next day, pellet the beads by centrifuging at 3,000×*g* for 1 min at room temperature. Let the tube that contains the beads sit for 1 min at 4°C. Carefully remove the supernatant.
5. Wash the beads five times by using 1 mL cold 1× telomerase activity assay buffer with 30% (v/v) glycerol (see Note 7).
6. Resuspend the beads with cold 1× telomerase activity assay buffer with 30% (v/v) glycerol to make 1:1 slurry and stored at −80°C.

### 3.8. In Vitro Telomerase Activity Assay

1. Prepare the reaction mixture (20 μL reaction system) containing 1× telomerase activity assay buffer, 10 μM telomeric DNA primer, 0.5 mM dATP, 0.5 mM dTTP, 2.0 μM dGTP, and 0.025 μM $^{32}$P-dGTP (800 Ci/mmole, 1 Ci = 37GBq) with 5 μL of immunopurified human telomerase complex (~10 nM). The molar ratio of DNA primer and human telomerase complex used here is ~2,000 so that the repeat addition processivity of telomerase can be measured (19).
2. Incubate the reaction mixture at 30°C water bath for 1 h.
3. Stop the reaction by adding 102 μL cold stop solution, 450 μL cold 100% ethanol, and store at −80°C for at least 1.5 h to overnight.
4. Pellet the sample by centrifugation at 4°C at maximum speed for 15 min.
5. Discard the supernatant and invert the tube on paper towel to remove the leftover liquid. The supernatant is radioactive and should be discarded into special container.
6. Wash the pellets by 1 mL cold 70% ethanol.
7. Repeat steps 4 and 5 once.

8. Dry the pellets in speed-vacuum for 10 min.
9. Resuspend the pellets by adding 10 μL water followed by adding 10 μL of 2× Loading Dye.
10. Heat-denature the sample at 95°C for 5 min and spin the samples at maximum speed at room temperature for 5 min.
11. Load the samples (10 μL per well) onto a 10% polyacrylamide/7 M Urea/1× TBE denaturing gel for electrophoresis. (The 10% gel is made by mixing 50 mL of Gel solution I and 50 mL of Gel solution II. 500 μL 10% ammonium persulfate and 15 μL TEMED are added into 100 mL gel solution. The electrophoresis is carried out at room temperature at constant power of 90 W (~3,000 V) for 80 min.
12. After electrophoresis, the gel is dried by heated vacuum gel drier, scanned and quantified by using Phosphorimager (Fig. 3a).

### 3.9. Calculation of Telomerase Activity and Processivity

In telomerase activity assay gels, the signal at round $n$ measures the amount of DNA substrates that disassociate from telomerase after $n$ rounds of processive telomere extension. Since human telomere repeat (TTAGGG) contains three Gs, the signal intensity at round $n$ is proportional to the total amount of $^{32}$P-dGTP incorporated into the DNA substrates from round *1* to round $n$. For simplicity of calculation, we defined the normalized activity (intensity) of round $n$ as

normalized activity (round $n$) = intensity (round $n$) / (total number of labeled G incorporated from round 1 to round $n$).

For instance, if DNA primer a5 (5′-TTAGGGTTAGCG TTAGGG-3′) is used in the assay, the numbers of G incorporated in rounds 1, 2, 3, …, $n$ are 1, 4, 7, …, $3n-2$, respectively. Therefore, the normalized activity (round $n$) is intensity (round $n$)/$(3n-2)$. Total telomerase activity is the total number of telomere repeats synthesized and therefore defined as

total telomerase activity
$= \Sigma_n$ [normalized intensity (round $n$) X $n$] (Fig. 3b)

In contrast, telomere repeats addition processivity (RAP) is the average number of telomeric repeats processively added to the extended primer, which is determined from the slope ($k$) of a plot of the logarithm of normalized activity of round $n$ as a function of $n$ (Fig. 3c). We define telomerase RAP as

$$\text{telomerase RAP} = R_{1/2} = -\ln 2/(2.303k),$$

where $k$ is the slope and $R_{1/2}$ is the number of repeats synthesized before half of the DNA substrates dissociate from telomerase, analogous to $t_{1/2}$ in radioactive decay (Fig. 3c).

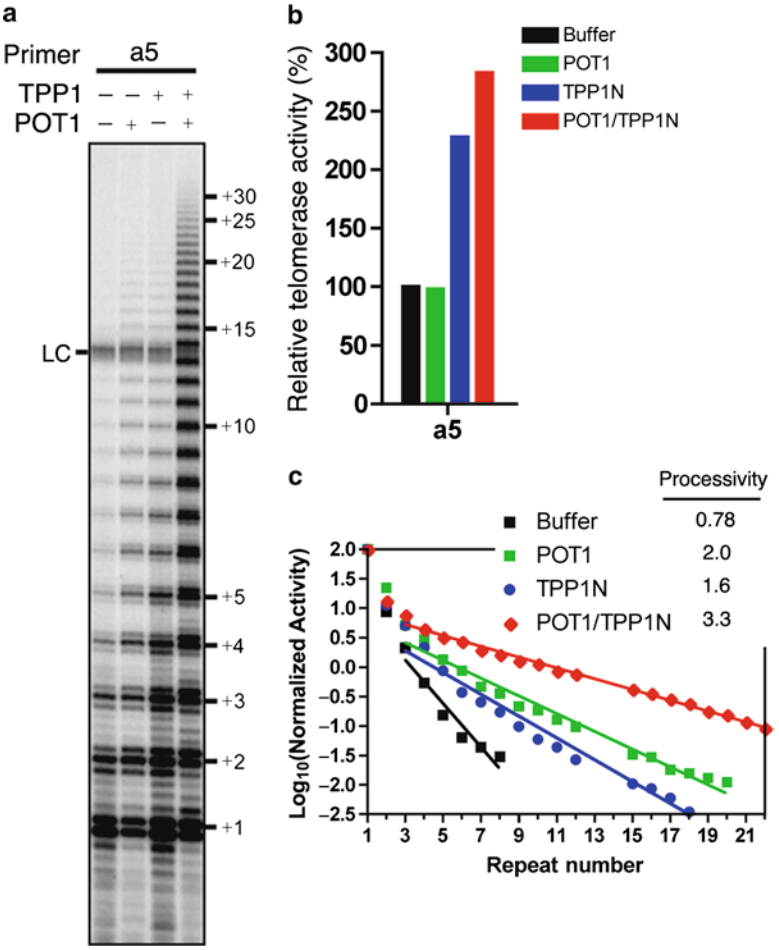


Fig. 3. The POT1–TPP1 complex functions as a telomerase processivity factor. (**a**) In vitro telomerase activity assays were performed with 100 nM DNA primer a5* in the presence of a saturating concentration of POT1, TPP1, or POT1–TPP1. Reaction products were then analyzed by gel electrophoresis (LC, loading control). (*Primer a5: 5′-TTAGGG TTAGCGTTAGGG-3′) (**b**) Quantification of total telomerase activity relative to activity in the presence of protein buffer alone. (**c**) Quantification of telomerase repeat addition processivity $R_{1/2}$.

## 4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as "water" in this text.
2. PMSF is not soluble in water and is dissolved in ethanol or methanol. PMSF is poisonous and should be handled carefully. PMSF, lysozyme, 2-mercaptoethanol and Complete

Protease Inhibitor Cocktail Tablets should be added to cold lysis buffer right before usage.

3. TEMED should be fresh and stored in room temperature. Use small bottles as it may decline in quality after opening.
4. We have found this antibody to be excellent for Western blotting. There are some other equivalent products from other commercial sources.
5. Roll out air bubbles between layers.
6. The primary antibody can be recovered and stored at 4°C. The primary antibody can be used for up to ten times with increased length of exposure time.
7. Radioactive materials are included in this step and so care should be taken for personal safety protection. Special protocols should be followed for proper solid and liquid waste disposal.

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