

Chapter 2

Analysis of Average Telomere Length in Human Telomeric Protein Knockout Cells Generated by CRISPR/Cas9

Jun Xu, Zhou Songyang, Dan Liu, and Hyeung Kim

Abstract

Telomeres play an important role in ensuring the integrity of the genome. Telomere shortening can lead to 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 population. Recent advances in genome editing (such as the development of the CRISPR/Cas9 platform) have further enabled researchers to carry out loss-of-function analysis of how disrupting key players in telomere maintenance affects telomere length regulation. Here we describe the steps to be carried out in order to analyze the average length of telomeres in CRISPR-engineered human knockout (KO) cells (TRF analysis).

Key words Telomere length, TRF, Telomere maintenance, CRISPR, Cas9, Knockout

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]. Different organisms display remarkable variability in the makeup and exact length of the repetitive telomeric elements in their telomere DNA sequences. For example, in yeast, the sequence is 350 ± 75 bps of $C_{1-3}A/TG_{1-3}$ [3], whereas mammalian telomeres contain (TTAGGG) $_n$. Among mammalian species, mouse telomeres can be up to 150 kb, while somatic human cells have telomeres of 5–15 kb in length [4]. Even in a relatively homogenous population such as cultured mammalian cell lines, telomeres exhibit great heterogeneity in length.

Perturbations in the intricate telomere interacting and regulatory network can lead to changes in telomere structure and exposed chromosomal ends [5, 6]. In telomerase-active cells such as cancer cells and during development, such changes in turn impact the length of telomeres and the status of the cell such as its replicative

potential [7, 8]. The advent of new genome-editing tools such as CRISPR/Cas9 has enabled investigators to better understand how inactivation of individual telomere regulators affects the maintenance and status of telomere length.

The CRISPR/Cas9 system, adapted from the acquired immune systems of bacteria and archaea, consists of the Cas9 nuclease and a guide RNA (gRNA). Through RNA-DNA hybridization, the 20 nucleotide sequence at the 5' or 3' end of the gRNA determines the target site, a feature that has simplified the process of targeting endogenous loci for disruption due to the ease with which gRNA sequences can be manipulated. *Streptococcus pyogenes* Cas9 (SpCas9), which has a specific protospacer adjacent motif (PAM) preference of 5'-NGG-3', is the most extensively characterized and widely used in genome editing.

In this chapter, we describe the steps for generating telomeric protein KO cells using the CRISPR/Cas9 platform. These cells are then used to study how inactivation of a telomere regulator impacts telomere length control using terminal restriction fragments (TRF) analysis. 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. The final results reflect the estimation of both the telomeric repeats and sub-telomeric regions that do not contain the particular restriction digest sites.

2 Materials

2.1 For the Generation of KO Cells by CRISPR/Cas9

1. A human cell line of interest and the appropriate medium and supplements for culturing the cell line (*see Note 4.1.1*).
2. The CRISPR/Cas9 vector pSpCas9(BB)-2A-GFP (PX458) from Addgene (Plasmid# 48138) (*see Note 4.1.2*).
3. The restriction enzyme BbsI and its digestion buffer, and calf intestinal alkaline phosphatase (CIP) and its reaction buffer.
4. DNA spin-columns for purification (e.g., QIAquick PCR purification kit from Qiagen).
5. Agarose.
6. Agarose gel electrophoresis apparatus.
7. 50× TAE buffer: Mix 242 g Tris base, 57.1 mL acetic acid, and 18.6 g EDTA in ddH₂O to final volume of 1 L. Make 1× TAE buffer from this stock solution.
8. Gel extraction DNA purification kit (such as the QIAquick Gel Extraction Kit).

9. Two complementary oligonucleotides synthesized based on gRNA design for the target gene locus (*see Note 4.1.3*).
10. T4 polynucleotide kinase used with T4 ligase buffer that contains ATP.
11. T4 DNA ligase and buffer (quick reaction is preferable).
12. Bacterial competent cells for transformation.
13. LB agar plates with appropriate antibiotics (ampicillin for px458) (100 mm/15 mm).
14. 1× LB medium: dissolve 10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 10 g of NaCl in ddH₂O to 1 L. Autoclave and store at room temperature.
15. DNA miniprep and maxiprep kits.
16. Tissue culture dishes (60 and 100 mm) and multi-well plates (6, 24, and 96-well (flat-bottom)).
17. DNase/RNase-free ddH₂O.
18. A set of primers, which can amplify the region encompassing the target site, for Cas9 cleavage efficiency testing and clone genotyping (*see Note 4.1.4*).
19. Filtered sterile pipette tips for PCR amplification.
20. High-fidelity *Taq* polymerase for genomic DNA PCR.
21. QuickExtract™ DNA Extraction Solution (Epicentre).
22. T7 endonuclease I.
23. A thermal cycler.
24. Transfection reagents or an electroporation system (e.g., the Neon® Transfection System from Invitrogen) and the accompanying transfection kit (*see Note 4.1.5*).
25. A 37 °C incubator for bacteria growth.
26. A 37 °C incubator with 5% CO₂ for human cells.

2.2 For TRF Analysis

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3.
2. Restriction digestion enzymes RsaI and HinfI and DNase-free RNase.
3. 1× TAE as specified in **item 7** of Subheading [2.1](#).
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 ddH₂O 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 Na₂HPO₄ and 7.9 mL of NaH₂PO₄, 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 γ-³²P ATP (3000 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 Using CRISPR/
Cas9 to Generate
Telomeric Protein KO
Cells

3.1.1 CRISPR Vector
Construction

- 1. Design the gRNA oligos: Identify the genomic DNA region to be targeted for KO (e.g., a 1–2 kb region within the first or second exon) (Fig. 1). Use an online tool (e.g., <http://tools.genome-engineering.org>) to input the genomic DNA sequence as instructed. Select 2–3 potential gRNA sites from the output (see Note 4.1.6).
- 2. Order both the sense and antisense oligos for each site, with the overhang sequence for cloning into the PX458 vector. If the gRNA sequence does not start with a G, add a G to make the guide sequence 21 nt long.

Oligo 1: 5'-CACCGNNNNNNNNNNNNNNNNNNNNNNNNNN-3'
Oligo 2: 3'-CNNNNNNNNNNNNNNNNNNNNNNNNNNNCAAA-5'
- 3. Resuspend each oligo to a final concentration of 100 μM. Phosphorylate and anneal the oligos by mixing them as follows and

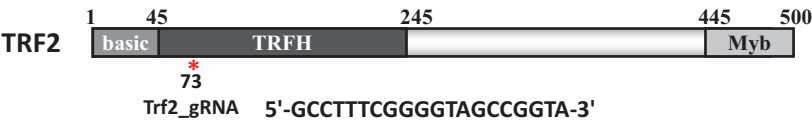


Fig. 1 A CRISPR gRNA is chosen for the telomeric protein TRF2 and predicted to result in truncation of TRF2 at residue 73

incubate the mixture in a thermocycler, 37 °C for 30 min, 95 °C for 5 min, and then cool to 25 °C at 5 °C per min (*see* **Note 4.1.7**).

Component	Amount (μL)
100 μM sense strand oligo (Oligo 1)	1
100 μM antisense strand oligo (Oligo 2)	1
10× T4 ligation buffer (NEB)	1
T4 PNK (NEB)	1
ddH ₂ O	6
Total	10

4. Digest 5 μg of PX458 with *Bbs* I for 1 hr. at 37 °C. Then add 1 μL of CIP and incubate the reaction at 37 °C for another 30 min. quickly purify the digested vector through spin-columns to remove salt and enzymes.
5. Set up a 10 μL ligation reaction with 50 ng of digested vector (from **step 5**), 1 μL diluted oligos (from **step 4**), and T4 DNA ligase, and incubate the mixture at room temperature. The length of incubation time depends on the particular enzyme used.
6. Transform bacterial competent cells with 1 μL of the ligation reaction, and plate the competent cells on an agar plate containing 100 μg/mL ampicillin.
7. Pick a few single bacterial colonies for plasmid DNA extraction using the miniprep kit. Sequence the plasmid DNA using the U6 sequencing primer.

3.1.2 Validate CRISPR/Cas9 gRNA Vector Cleavage Efficiency (*See* *Note 4.1.8*)

1. Transfect the target cell line with the CRISPR/gRNA vector. Alternatively use an easy to transfect cell line (e.g., 293 T cells). Either a transfection reagent or electroporation system may be used. Follow the directions of the manufacturer of the electroporation system for the voltage and pulse for the specific cell type used.
2. Collect and pellet the cells at 48 h after transfection and extract the genomic DNA using the QuickExtract™ DNA Extraction Solution (Epicentre). Follow the manufacturer's directions. Please *see* Fig. 2 for an example of testing the CRISPR gRNA that targets TRF2.
3. Set up a PCR reaction using the genomic DNA from Step 2 above, and the primer set specified in **item 18** of Subheading 2.1, to amplify the genomic region containing the gRNA target site (*see* **Note 4.1.4**). The exact conditions depend on the Taq polymerases used and the length of the predicted products. Follow the manufacturer's directions.

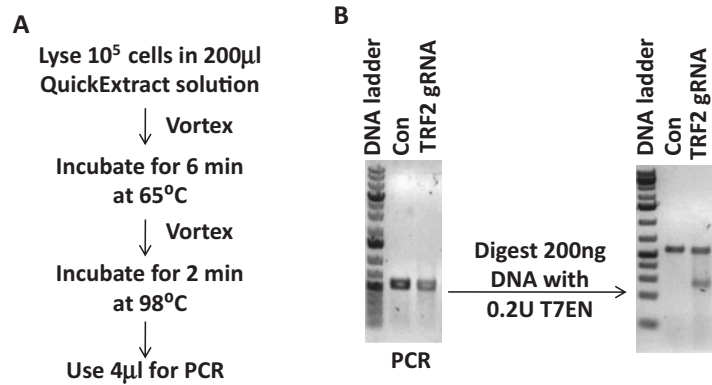


Fig. 2 An example of cleavage efficiency analysis. *Left*, a flowchart for extracting genomic DNA for cleavage analysis. *Right*, the extracted DNA was used to PCR amplify the genomic region encompassing the TRF2 gRNA target site. The PCR products were denatured and then annealed before being incubated with T7 endonuclease I (T7EN). The digested products were resolved on an agarose gel. Parental cells were used as control (Con)

- Once the PCR reaction is done, anneal the reaction products on a thermal cycler using the following conditions:

95 °C, 10 min.
 95–85 °C, –2 °C/s.
 85 °C, 1 min.
 85–75 °C, –0.3 °C/s.
 75 °C, 1 min.
 75–65 °C, –0.3 °C/s.
 65 °C, 1 min.
 65–55 °C, –0.3 °C/s.
 55 °C, 1 min.
 55–45 °C, –0.3 °C/s.
 45 °C, 1 min.
 45–35 °C, –0.3 °C/s.
 35 °C, 1 min.
 35–25 °C, –0.3 °C/s.
 25 °C, 1 min.
 25–4 °C, –0.3 °C/s.

- Digest the annealed PCR products with T7 endonuclease I (*see Note 4.1.9*).
- Separate the digested and undigested PCR products on a 2% agarose 1× TAE gel. Compare cleavage efficiencies of different gRNAs.

3.1.3 *Generation
and Validation of CRISPR/
Cas9 KO Cells*

1. Prepare large quantities of the CRISPR gRNA vector using a DNA maxiprep kit.
2. Transfect your cells of interest as in Subheading 3.1.2. Scale up the amount of DNA for the transfection as more cells need to be transfected.
3. (Optional) At >24 h after transfection, sort the GFP+ cells on a FACS sorter directly into 96-well plates at no more than one cell per well. Alternatively, the cells can be sorted as a pool and then plated into 96-well plates by serial dilution (*see* **Note 4.1.10**).
4. At >24 h after transfection, plate the cells into 96-well plates by serial dilution, with the final dilution at <60 cells/plate. The total number of cells plated depends on the specific cell line and the cleavage efficiencies of the gRNAs tested in Subheading 3.1.2. We recommend at least 600 cells (i.e., ten plates) as a start.
5. Culture the cells and replenish with fresh media as needed. Clones of cells should form around 2–4 weeks after serial dilution plating, depending on the growth rate of the cells (*see* **Note 4.1.11**).
6. Expand the clones as the cells grow back, by transferring the cells to larger dishes or plates as needed. It is normal for different wells to exhibit differences in growth rates.
7. Once there are enough clones that have grown back, determine if the gene of interest has been knocked out by several methods, including PCR, western blotting, and immunofluorescence. Western blotting and immunofluorescence can be quick methods to identify candidate KO clones (Fig. 3). All positive clones should be verified by genotyping. The region encompassing the target site should be PCR amplified using the primer set for cleavage verification and sequenced (*see* **Note 4.1.12**). Because of potential clonal variation, it is also recommended that multiple KO clones be selected and analyzed for TRF below.

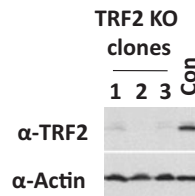


Fig. 3 Western blotting analysis of clones of cells that grew back after single cell cloning, using antibodies against TRF2. Three individual clones were tested here, with parental cells as control. An antibody against actin served as a loading control

3.2 TRF Analysis Using the CRISPR KO Cells

3.2.1 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 $1300 \times g$ in a microcentrifuge at room temperature, wash in $1\times$ PBS and collect the pellet (*see Note 4.2.1*). Cell pellets can be assayed immediately, or directly frozen and stored at $-80\text{ }^{\circ}\text{C}$.
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 4.2.2*). Estimate the amount of DNA based on the number of cells used (*see Note 4.2.2*).
3. Mix $\sim 2\text{--}5\text{ }\mu\text{g}$ of extracted genomic DNA with 15 units each of *RsaI* and *HinfI*, and $1\text{ }\mu\text{g}$ of RNase A. Incubate at $37\text{ }^{\circ}\text{C}$ for $\geq 12\text{ h}$. The digested DNA mixture may be stored at $-20\text{ }^{\circ}\text{C}$ until further use (*see Note 4.2.3*).

3.2.2 Electrophoresis and Transfer

1. Prepare a large agarose gel (0.7%, 20–25 cm long) (roughly 300 mL) in $1\times$ TAE buffer containing ethidium bromide ($2\text{--}3\text{ }\mu\text{L}$) (*see Note 4.2.4*).
2. Load $1\text{--}2\text{ }\mu\text{g}$ of digested genomic DNA per lane. Load DNA molecular weight markers (preferably mixed with $1\text{--}3 \times 10^5$ cpm radiolabeled DNA marker) to aid visualization under UV and to facilitate quantification steps (*see Note 4.2.5*). Run the gel at 1.5 volts/cm until the 1 kb marker is at the bottom of the gel (*see Note 4.2.6*).
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 4.2.7*).
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 $2\times$ SSC for 5–10 min, and wet the Hybond N^+ nylon membrane in $2\times$ SSC. Mark the gel and membrane for easy orientation during hybridization and analysis. Set up capillary transfer in $2\times$ SSC for $>12\text{ h}$ (*see Note 4.2.8*).
8. Disassemble the transfer assembly, and UV cross-link the DNA to the membrane ($120\text{ mJ}/\text{cm}^2$) with the DNA side facing up. The membrane can be stored in a sealed plastic bag with support at $-20\text{ }^{\circ}\text{C}$ until ready to use (*see Note 4.2.9*).

3.2.3 Hybridization and Analysis

1. Pre-hybridize 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 **item 13** of Subheading 2.2. Determine the specific activity of the probe using a liquid scintillation counter (*see* **Note 4.2.10**).
3. Discard the prehybridization solution, add fresh Hybridization solution (10–20 mL) along with the labeled probe ($\sim 1\text{--}5 \times 10^6$ 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 4.2.11** 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 PhosphoImager cassette for visualization and quantification on a PhosphoImager (*see* **Note 4.2.12**).
6. Use the Telorun spreadsheet to calculate average telomere length, which can be found at the homepage of the Shay and Wright laboratory (<http://www.utsouthwestern.edu/labs/shay-wright/methods/>).

4 Notes

4.1 CRISPR/Cas9 KO Cells

1. The particular cell line of choice will be dictated by factors such as readout assays, biological questions asked, and the ease with which CRISPR reagents can be introduced. The method described here requires transfection of CRISPR plasmids. Cell lines that are less amenable to transfection may be more difficult to use for KO purposes.
2. There are many different CRISPR/Cas9 vectors, with different designs, tags, and markers. We use PX458 because of the GFP marker in the construct that enables FACS sorting of the transfected cells.
3. The oligo sequences depend on the gRNA site chosen and the specific vector to be used.
4. The gRNA vectors should be tested for cleavage efficiency. And PCR-based genotyping is needed for clone analysis. Therefore, a set of primers that robustly and specifically amplify

the genomic region encompassing the target site is needed for each gRNA. Use the NCBI/Primer-Blast to design genomic PCR primers. The ideal PCR product size is from 300 bp to 1 kb. Longer products are less efficient to amplify and smaller products may be difficult to detect on an agarose gel.

5. The CRISPR plasmids can certainly be transfected into cells using transfection reagents, especially for cells that are easy to transfect. We have found that electroporation routinely achieves higher efficiency without sacrificing viability. Multiple rounds of transfection can be done to increase the efficiency. Higher transfection efficiency should help improve KO frequencies.
6. There are many free online tools for selecting gRNA sequences. To minimize off-target effects, the 20 nt gRNA sequence (followed by the NGG PAM) should have little homology to other genomic sites. To achieve complete KO of a gene and avoid the expression of a truncated protein, the gRNA target site should locate within the first few exons of the coding sequence. In cases of the existence of alternative splicing products, the common region of all splicing isoforms should be targeted, whenever possible.
7. The T4 ligation buffer should be fresh. Aliquot and freeze if necessary. Avoid frequent freeze–thaw cycles, which degrade the ATP in the buffer. Alternatively, T4 polynucleotide kinase buffer and fresh ATP solution can be used instead of the T4 Ligation buffer.
8. Cas9 cleaves at the position 3 nt 5' of the PAM sequence, but the efficiency of this cleavage remains impossible to predict. We recommend making three different gRNA vectors and test them in cells. If none works well, more gRNAs need to be screened.
9. Use no more than 0.2U of T7 endonuclease I (NEB) per 20 μ L of digestion reaction. Cas9 cleavage results in indels in one or both strands of genomic DNA. Since not all of the cells will have been cleaved by Cas9, some PCR products will be from wildtype cells and some from mutant cells. Annealed duplexes that are not precisely complementary to each other will create mismatches that can be recognized and cut by T7 endonuclease I. Instead of T7 endonuclease I, the SURVEYOR nuclease can be used as well.
10. If the CRISPR vector has a fluorescent marker (e.g., GFP for PX458), the transfected cells can be sorted by flow cytometry based on GFP expression. Take care to avoid contaminating the cells during sorting. Cells with high enough transfection efficiency (>80%) do not need to be sorted. Sorting can also help separate cells into single-cell suspensions. For some cell

types, sorting offers no increased advantage over serial dilution plating in terms of cost and excessive handling of the cells.

11. Fast growing cells such as 293 T and Hela form colonies in 2 weeks, which are clearly visible under the microscope. Slow dividing cells may take much longer to form visible colonies. In fact, some cells may not be easily cloned from low-density plating or single cells. It is highly recommended to test whether the cell line of interest is clonable from single cells, if not already known.
12. If a good antibody is not available for easy western blotting or immunostaining, we recommend designing two CRISPR gRNAs so as to delete an entire exon. The two CRISPR gRNA target regions should be in the intron regions on each side of the exon. Deletion of the exon should result in frameshift mutations and premature stop codons. Deletion of the exon can then be more easily screened by genomic DNA PCR and sequencing.

4.2 TRF Analysis

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 \times PBS.
 - Add 200 μL of Lysis buffer (0.3 M Tris (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 $^{\circ}\text{C}$ for 2–12 h. Heat the sample to 70 $^{\circ}\text{C}$ 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 4⁴ bps. 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 \times TBE buffer (1 \times 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 2.14. The 1 kb DNA ladder should first be dephosphorylated using calf intestinal phosphatase (CIP, NEB) (1 μg of DNA ladder, 0.5 unit CIP, 1 \times NEB buffer, 60 min at 37 °C). The dephosphorylated DNA should be purified through 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 \times kinase

reaction buffer (NEB), 3 μL $\gamma\text{-}^{32}\text{P}$ ATP (3000 Ci/mmol), and 0.5–120 μg of DNA ladder for 30–60 min at 37 $^{\circ}\text{C}$. 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 \times 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 \times SSC before cross-linking.
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 $\sim 0.5\text{--}1 \times 10^6$ cpm/ μL .
11. The membrane is first washed in low stringency buffer once at room temperature and once at 37 $^{\circ}\text{C}$, 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 $^{\circ}\text{C}$ or 50 $^{\circ}\text{C}$ 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.

Acknowledgment

The authors thank Drs. Matthew O’Conner and Dong Yang for technical input and advice, and the generous support from the Dan L. Duncan Cancer Center and program grant P30CA125123.

References

1. de Lange T (2002) Protection of mammalian telomeres. *Oncogene* 21:532–540
2. Xin H, Liu D, Songyang Z (2008) The telosome/shelterin complex and its functions. *Genome Biol* 9:232
3. Wang SS, Pluta AF, Zakian VA (1989) DNA sequence analysis of newly formed telomeres in yeast. *Prog Clin Biol Res* 318:81–89
4. Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR (1988) A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A* 85:6622–6626
5. Deng Y, Chan SS, Chang S (2008) Telomere dysfunction and tumour suppression: the senescence connection. *Nat Rev Cancer* 8:450–458
6. Raynaud CM, Sabatier L, Philipot O, Olausson KA, Soria JC (2008) Telomere length, telomeric proteins and genomic instability during the multistep carcinogenic process. *Crit Rev Oncol Hematol* 66:99–117
7. Artandi SE, DePinho RA (2010) Telomeres and telomerase in cancer. *Carcinogenesis* 31:9–18
8. Svenson U, Roos G (2009) Telomere length as a biological marker in malignancy. *Biochim Biophys Acta* 1792:317–323

Telomeres and Telomerase

Methods and Protocols

SONGYANG, Z. (Ed.)

2017, XI, 218 p. 37 illus., 18 illus. in color., Hardcover

ISBN: 978-1-4939-6891-6

A product of Humana Press