

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 ± 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

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 Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3.
2. Restriction digestion enzymes *Rsa*I and *Hinf*I and DNase-free RNase.
3. 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.
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 (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 *Hinf*I, 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 ddH₂O, and soak the gel in denaturation buffer for 30 min with gentle agitation.
6. Discard the solution, rinse the gel in ddH₂O, 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 h (see Note 8).
8. Disassemble the transfer assembly, and UV crosslink 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°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 ($\sim 1\text{--}5\times 10^6\text{ cpm}/\text{mL}$), 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 $6\text{ }\mu\text{g}$ of genomic DNA. For genomic Southern blotting analysis, at least $2\text{ }\mu\text{g}$ of DNA is needed. Typically $5\text{ }\mu\text{g}$ 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 μ L 1 \times PBS.
 - Add 200 μ L of Lysis buffer [0.3 M Tris-HCl (pH 8), 0.15 M EDTA (pH 8), 1.5% SDS] plus 15 μ L 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 μ L of lysis buffer and mix.
 - Add 500 μ L 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 μ L chloroform/isopropanol (1:1). And repeat spinning and transfer step.
 - Add 200 μ L 7.5 M ammonium acetate and 800 μ L 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 μ L 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 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 γ-³²P 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 $\sim 0.5\text{--}1 \times 10^6$ cpm/ μL .
11. The membrane is first washed in low-stringency buffer once at room temperature and once at 37°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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