UNIT 18.6 Analysis of Telomeres and Telomerase

INTRODUCTION

This unit describes techniques to analyze telomeric length (Basic Protocol 1) and telomerase activity (Basic Protocol 2) in human cells. These assays can be used to study the in vitro cellular effects of aging and cancer treatments on telomere biology and telomerase activity.

Telomere length can be determined by a modification of Southern blotting in which the analysis of chromosome terminal restriction fragments (TRFs), as visualized with a radiolabeled telomeric repeat probe (Support Protocols 2 and 3), provides the average lengths of all telomeres in a cell population. A labeled molecular weight marker (Support Protocol 1) is used to determine telomere length.

Telomerase activity can be measured in vitro by a sensitive and efficient polymerase chain reaction (PCR)-based detection method, also known as telomeric repeat amplification protocol (TRAP). This method can use either radiolabeled or fluorescently labeled (Alternate Protocol) primers. Support Protocol 4 describes the preparation of tissue samples for TRAP; the preparation of other reagents that are needed for TRAP are described in Support Protocols 5 and 6.

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and surroundings. Carry out the experiments and dispose of wastes in appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 1D*).

BASIC PROTOCOL 1: TERMINAL RESTRICTION FRAGMENT SIZE DETERMINATION TO MEASURE AVERAGE TELOMERE LENGTHS

The simplified method for isolating DNA described below was developed in the authors' laboratory to efficiently process a large number of samples by eliminating phenol/chloroform extractions and avoiding alcohol precipitation. Because there are no steps where significant losses can occur, DNA concentrations can be calculated based on initial cell numbers, a more reproducible method than optical density measurements, which are frequently distorted by contaminating RNA. The measurement of telomere length relies on the absence of restriction enzyme recognition sites within the TTAGGG tandem repeat sequences. The length of telomeric repeats can be indirectly measured by analyzing the length of the terminal restriction fragment (TRF), the large DNA fragments remaining after the rest of the genomic DNA has been reduced to small sizes

by digestion with frequent cutters (restriction enzymes with recognition sequences of four bases, such as *Hin*fI). There is also a region of mostly repetitive subtelomeric DNA, probably ranging from 2 to 5 kb in length in humans, that lacks functional restriction sites and contributes to the measured size of telomeres on gels. The DNA is probed with a labeled oligonucleotide containing TTAGGG repeats. The measured size of the TRF corresponds to the sum of the sizes of the subtelomeric DNA that lacks functional restriction sites and the length of the telomere repeats. Because small oligonucleotides easily penetrate dried agarose gels, it is more rapid and convenient to perform in-gel hybridization rather than Southern blot transfers.

Materials

Cell pellet from ≥300,000 cells and appropriate medium
Quick-prep lysis buffer (see recipe)
Triton X-100
20 mg/ml proteinase K
TE buffer, pH 7.5 to 8.0 (*APPENDIX 2A*)
Restriction enzyme mix: equal volumes of *Hin*fI, *Rsa*I, *Msp*I, *Cfo*I, *Hae*III, and *Alu*I restriction enzymes (Boehringer Mannheim; 10 U/ul each before mixing, final 1.67 U/ul each)
10× TAE buffer (*APPENDIX 2A*)
10× TBE buffer (*APPENDIX 2A*)
10,000 cpm radiolabeled molecular weight markers mixed with ≥500 ng unlabeled, *Sty*I-digested λ DNA (or other appropriate unlabeled, digested DNA; see Support Protocol 1)
Denaturing solution: 0.5 M NaOH/1.5 M NaCl
Neutralizing solution: 1.5 M NaCl/0.5 M Tris·Cl, pH 8.0 (see *APPENDIX 2A* for Tris·Cl)
Hybridization solution (see recipe)
Radiolabeled telomeric repeat probe (see Support Protocols 2 and 3)
20× SSC (see recipe)
0.1× SSC/0.1% (w/v) SDS

2-ml polypropylene screw-cap tubes (Sarstedt) with an optional extra set of screw caps to be cut vertically with jigsaw to form open screw-cap ring (Fig. 18.6.1A)
37°, 55°, and 70°C water baths heating blocks, or PCR machine
Dialysis membrane sheets or tubing (e.g., Spectra/Por; Spectrum), molecular weight cutoff (MWCO) ≥6000 to 8000
Wide-bore pipet tips
Whatman 3MM filter paper
Hybridization oven, 42°C, and appropriate hybridization containers, or equivalent
PhosphorImager, including phosphor screens and ImageQuant software (Molecular Dynamics)

Additional reagents and equipment for agarose gel electrophoresis (*APPENDIX 3A*)

Lyse cells

1. Resuspend cell pellet from ≥300,000 trypsinized cells in medium and pellet cells in a 2-ml polypropylene screw-cap tube. Remove supernatant and freeze pellet at -80°C or proceed to next step.

It is not necessary to wash cells with phosphate-buffered saline (PBS), as the small amount of medium in the cell pellet does not interfere with subsequent steps. At least 300,000 cells should be used; however, the collection of more cells is desirable (~3 million).

Cell pellets can simply be placed at -80°C; it is not necessary to snap-freeze them.

2. Thoroughly resuspend cell pellet (fresh or just after thawing from -80°C) in quick-prep lysis buffer, using 30 ul per million cells.

100 mM EDTA is used in the lysis buffer to very rapidly inhibit nucleases in these concentrated cell suspensions. The lysis buffer should be added immediately to cells as they are thawing on ice to prevent nucleases from degrading the DNA.

3. Add Triton X-100 to 1% (v/v) final concentration and 20 mg/ml proteinase K to 2 mg/ml final concentration.

Triton X-100 rather than SDS is used so that residual detergent present after dialysis does not inhibit digestion with restriction enzymes.

4. Incubate samples ≥2 hr at 55°C and inactivate proteinase K by heating 30 min at 70°C.

Heat inactivation of proteinase K avoids the need to phenol/chloroform extract the DNA, and the residual protein present after dialysis does not inhibit enzyme digestion.

After digestion with proteinase K, DNA should only be handled using wide-bore tips to prevent shearing of the DNA.

Dialyze sample

5a. *To dialyze sample in screw-cap tube:* Place a 1- to 2-cm^2 piece of dialysis membrane over top of tube and secure with an open screw-cap ring. Invert tube, making sure that sample (and not a bubble) is at the membrane, and place on floating rack in ≥200 vol TE buffer (Fig. 18.6.2). Dialyze 1 to 2 hr at 4°C with constant stirring.

For larger volumes (>0.3 ml for >10 million cells), conventional dialysis (step 5b) can be used.

Dialysis reduces salt and EDTA so that digests will not be inhibited later on.

5b. *To dialyze sample in dialysis tubing:* Wet dialysis tubing with distilled water and clip one end shut. With a wide-bore pipet tip, carefully collect sample and place in tubing without creating bubbles. Smooth any bubbles out and clip the other end shut, leaving room for buffer exchange and volume expansion. Place tubing in ≥200 vol TE buffer and dialyze 1 to 2 hr at 4°C with constant stirring.

Dialysis reduces salt and EDTA so that digests will not be inhibited later on.

6. Change buffer to ≥200 vol fresh TE buffer and dialyze samples overnight at 4°C with constant stirring.

7. Discard dialysis membrane, replace threaded ring with a closed cap, and save threaded rings for re-use. Alternatively, remove sample from dialysis tubing and place in a fresh tube. Calculate the DNA concentration.

DNA concentration is calculated at 6 ug/10^6 diploid cells or 10 ug/10^6 aneuploid cells divided by the final volume after dialysis. The samples should be viscous and they can now be stored for 1 year at 4°C (or for ≥1 year at -20°C) until they are digested.

Digest and electrophorese DNA

8. Digest 1 ug DNA to completion for 4 hr to overnight at 37°C with ≥1 U/ug restriction enzyme mix.

The six enzymes ensure that genomic DNA, but not telomeric DNA, is digested. The authors generally use 1 ul enzyme mix/1 ug DNA (1.67 U each enzyme/ul) with 1× buffer A (supplied with the enzyme; Boehringer Mannheim).

9. Separate digested DNA on a 0.7% (w/v) agarose gel stained with ethidium bromide (0.5 ug/ml) for 16 hr at 70 V for a 25-cm-long gel in 1× TAE or 0.5× TBE buffer. Load 20,000 cpm radiolabeled molecular weight markers mixed with ≥250 ng unlabeled, *Sty*I-digested λ DNA on either side of the samples.

0.5× TBE can be used to analyze telomeres less than ~8 kb, whereas 1× TAE buffer allows for better separation in the 8- to 20-kb range. The gel should be handled carefully as it may be fragile. Electrophoresis should be stopped temporarily before the <1-kb molecular weight markers migrate off the gel to document complete digestion of the DNA by photographing the gel. Actual time and voltage varies with each power supply and gel apparatus. Digested DNA will run as a smear below the 1-kb molecular weight marker. Anything above this size is incompletely digested. If the sample is not digested completely, it is usually sufficient to use an additional 1 ul enzyme mix for a longer amount of time. If the sample is completely undigested, the viscous sample may have floated up out of the well when the gel was loaded. Electrophoresis (at higher voltage) should be continued until the 1- to 1.5-kb marker is near the bottom of the gel.

Radiolabeled molecular weight markers will allow the markers to be seen after hybridization with the telomere sequence-specific probe. The unlabeled, digested λ DNA is included so the markers can be visualized with ethidium bromide after electrophoresis.

Hybridize DNA

10. Denature gel 20 min in enough denaturing solution to cover the gel, rinse 10 min in distilled water, and dry gel upside down (so that the openings of the wells are against the paper) on two sheets of Whatman 3MM filter paper under vacuum for 1 hr at 50°C.

Denaturing the DNA before rather than after drying the gel increases the signal intensity ~3- to 5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the 0.7% agarose gel than in the very high percentage dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands, which would compete with the probe.

Rinsing with distilled water gets rid of the NaOH and substantially reduces sticking of the gel to the Whatman paper. Some loss of lower-molecular-weight DNA (particularly <400 bp) occurs during drying.

Because most of the DNA samples do not fill the wells and are thus in the bottom half of the gel, the gels are flipped and dried with the upper surface against the filter paper support. This increases the distance between the DNA samples and the filter paper, and significantly reduces the loss of lower-molecular-weight DNA.

11. Neutralize gel 10 to 15 min in enough neutralizing solution to cover the gel, rinse 10 min in distilled water.

12. Prehybridize gel ≥10 min with 10 ml hybridization solution.

13. Hybridize gel overnight at 42°C in hybridization solution containing all of the radiolabeled telomeric repeat probe made in Support Protocol 2.

The volume of the hybridization solution used depends on the gel size and hybridization setup. For a 20 × 25-cm gel rotating inside a glass cylinder in a hybridization oven, 10 ml is sufficient.

Using DNA polymerase fill-in of uracil-containing templates (see Support Protocol 3) results in a probe with higher specific activity.

14. Wash gel 15 min at room temperature in 2× SSC and then one to three times for 10 min each in 0.1× SSC/0.1% SDS at room temperature. Expose gel ≥4 hr to a phosphor screen and then scan with a PhosphorImager and visualize using ImageQuant software.

The number of washes can be adjusted as needed. Two 15-min washes with 0.1× SSC are generally sufficient.

15. Calculate mean TRF lengths from PhosphorImager scans of gels hybridized to the telomeric repeat probe. Position a grid of 30 boxes over each lane and determine the signal intensity and size (kb) corresponding to each box. Calculate the mean TRF length as the weighted mean using the following formula: $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the PhosphorImager output (signal intensity) and L_i is the length of the DNA at position *i*. Calculate sums over the range of the molecular weight marker used in the hybridized gel.

TELORUN, a program that performs these calculations, is available (see Internet Resource).

SUPPORT PROTOCOL 1: MAKING A LABELED MOLECULAR WEIGHT MARKER FOR RUNNING ON AN AGAROSE GEL

Running a radiolabeled molecular weight marker on either side of the gel allows for quantitation of the telomere lengths.

Materials

~250 ng/ul DNA molecular weight marker (e.g., λ DNA digested with *Sty*I)
10× React 2 buffer (Roche)
100 uM dAGT mix: 100 uM each dATP, dGTP, dTTP in H_2O, stored up to 1 year at -20°C
[α-^{32}P]dCTP (3000 Ci/mmol)
2 U/ul Klenow fragment of *E. coli* DNA polymerase I
QIAquick nucleotide removal kit (Qiagen)

1. In a tube, mix the following:

10 ul of ~250 ng/ul DNA molecular weight marker
2 ul of 10× React 2 buffer
5 ul of 100 uM dAGT mix
5 ul [α-^{32}P]dCTP
1 ul of 2 U/ul Klenow fragment

Incubate 30 min at 37°C.

The authors use Roche React 2 buffer because it is the same buffer used to digest the λ DNA with StyI. Any other buffer compatible with the Klenow fragment of DNA polymerase can be used.

2. Remove unincorporated label with QIAquick nucleotide removal kit.

3. Determine the cpm of the radiolabeled marker using a scintillation counter.

SUPPORT PROTOCOL 2: MAKING KINASED RADIOLABELED TELOMERIC REPEAT PROBE

Empirically, the G-rich probe tends to have higher signals, for unknown reasons, but both probes work reasonably well. G-rich probes generally give about 2-fold better signals than C-rich probes (probably due to possible G-quartet formation by the target G-strands which inhibits hybridization), so making a G-rich probe with 3000 Ci/mmol radioisotope is recommended if maximum sensitivity is needed.

Materials

$(T_2AG_3)_4$ or $(C_3TA_2)_4$ oligonucleotide (20 pmol/ul or 0.16 ug/ul)
[γ-^{32}P]ATP (3000 Ci/mmol)
5× forward or exchange reaction buffer (GIBCO/BRL)
10 U/ul T4 polynucleotide kinase (GIBCO/BRL)
QIAquick nucleotide removal kit (Qiagen)

1. In a tube, mix the following:

2 ul $(T_2AG_3)_4$ or $(C_3TA_2)_4$ oligonucleotide
24 ul [γ-^{32}P]ATP
10 ul of 5× forward reaction buffer
12 ul H_2O
2 ul T4 polynucleotide kinase.

Incubate 30 min at 37°C.

2. Remove unincorporated label with QIAquick nucleotide removal kit (Qiagen).

3. Determine the cpm of the radiolabeled marker using a scintillation counter (≥5000 cpm/ul). Store <2 weeks at -20°C.

SUPPORT PROTOCOL 3: SYNTHESIS OF A HIGH-SPECIFIC ACTIVITY TELOMERIC REPEAT PROBE

In this protocol, the template oligonucleotide contains uracils so that it can be degraded later with uracil deglycosylase, thus leaving a single-stranded probe. The template also contains a small mismatch at one end (GGGAAA rather than GGGTTA), so that the priming oligonucleotide will preferentially anneal in the correct spot rather than anywhere along the template. The template, GTU4, is an oligonucleotide of four G-rich telomeric repeats with uracil. The oligonucleotide T3C3+9 begins with TTTCCC followed by nine nucleotides of correct telomeric sequence. The annealed product of these two oligonucleotides is shown in Figure 18.6.3. Thus, six cytosines will be incorporated per synthesized product. If 50 uCi of [α-^{32}P]dCTP (3000 Ci/mmol) is used, then one has 16.7 pmol of [α-^{32}P]dCTP available. Assuming the target is to incorporate 10 pmol so that one gets mostly full-length products, then 1.7 pmol of annealed target is needed. In general, the efficiency of incorporation is so high that it should not be necessary to remove unincorporated dCTP. If desired, one can use plug dialysis on 20% (w/v) acrylamide. The authors currently use [α-^{32}P]dCTP as the radioisotope because they routinely have it on hand. G-rich probes generally give about 2-fold better signals than C-rich probes (probably because of possible G-quartet formation by the target G-strands, which inhibits hybridization), so making a G-rich probe with 3000 Ci/mmol radioisotope is recommended if maximum sensitivity is needed.

Materials

10 pmol/ul GTU4 oligonucleotide dissolved in TE buffer: 5′-GGGUUAGGGUUAGGGUUAGGGAAA-3′
100 pmol/ul T3C3+9 oligonucleotide dissolved in TE buffer: 5′-TTTCCCTAACCCTAA-3′
1 M NaCl
10× buffer M: 10 mM Tris·Cl (pH 7.5)/10 mM $MgCl_2$/50 mM NaCl/1 mM dithioerythritol (DTE; Roche)
2 M Tris·Cl, pH 7.4 to 7.6 (*APPENDIX 2A*)
10 mg/ml BSA (e.g., Ambion)
1.25 mM dAdT: 1.25 mM each of dATP and dTTP
[α-^{32}P]dCTP (3000 Ci/mmol)
5 U/ul Klenow large fragment of *E. coli* DNA polymerase I
1 U/ul uracil deglycosylase (UDG)

37°, 95°, and 99°C water baths heating blocks, or PCR machines
PhosphorImager, including phosphor screens (Molecular Dynamics)

Additional reagents and equipment for DNA acrylamide gel electrophoresis (*APPENDIX 3A*)

1. To make 1.7 pmol/ul annealed template oligonucleotide stock, mix the following:

3.4 ul 10 pmol/ul GTU4 oligonucleotide
15.6 ul 100 pmol/ul T3C3+9 oligonucleotide
1 ul 1M NaCl (50 mM final salt concentration).

Incubate 1 min at 99°C, 15 min at 37°C, and 15 min at room temperature.

The template oligonucleotide can be stored indefinitely at -20°C.

2. To make 8× buffer M, mix the following (total volume 625 ul):

500 ul 10× buffer M
100 ul 2 M Tris·Cl, pH 7.4 to 7.6
25 ul 10 mg/ml BSA.

Buffer M is the equivalent of Klenow buffer (50 mM Tris·Cl, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; 50 ug/ml BSA; 50 uM dNTP) without dNTP and with extra salt so that the oligonucleotides do not melt. It is very stable at -20°C.

3. To prepare the reaction mixture, mix together:

3.125 ul of 8× buffer M (step 2)
1 ul annealed template oligo (step 1)
1 ul of 1.25 mM dAdT (final 50 uM)
13.875 ul dH_2O
5 ul [α-^{32}P]dCTP
1 ul Klenow large fragment.

Incubate reaction mixture (25 ul total) 30 min at room temperature and then 5 min at 95°C.

The incubation at 95°C is important because if the Klenow is not inactivated, then the probe is degraded during UDG treatment.

4. Add 0.5 ul of 1 U/ul UDG and incubate 10 min at 37°C and then 10 min at 95°C.

This step is to cleave deglycosylated uracils and release probe as a single-stranded molecule after denaturing the UDG.

5. To check probe, run 30 fmol labeled probe on a 20% (w/v) acrylamide minigel (170 V, 20 min), wrap gel in cellophane (while still attached to one glass plate), and expose 10 min to a phosphor screen. Visualize with a PhosphorImager to confirm that adequate signal is present. Store the probe <2 weeks at -20°C.

The screen is saturated after a 1-hr exposure. A 10-bp ladder can be used for size markers, or a kinased probe or free dCTP can be used for reference, if desired.

Approximately 40% of the [α-^{32}P]dCTP should be incorporated into the full-length

probe.

Either all or half the probe is used for the assay (>20,000 cpm/ul).

BASIC PROTOCOL 2: MEASUREMENT OF TELOMERASE ACTIVITY BY THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL

The telomeric repeat amplification protocol (TRAP) is a highly sensitive in vitro assay used for the detection of telomerase activity by the polymerase chain reaction (PCR) amplification of the products produced by the processive elongation of an oligonucleotide primer (Fig. 18.6.4). The current TRAP is a modification of the assay first described by Kim et al. (1994). This improved version allows for the elimination of the need for a wax barrier hot start, reduction of amplification artifacts, and better estimation of telomerase processivity by using a modified reverse primer sequence. Incorporation of an internal standard control allows for quantitation of telomerase activity. The TS primer is a nontelomeric sequence that telomerase can nonetheless recognize as a substrate. The reverse primer ACX contains several modifications (an anchor sequence at its 5′ end and two mismatches within the telomerase repeats) that both reduce PCR artifacts and more faithfully amplify the distribution of telomerase-elongated products than occurs with unmodified telomeric sequence primers.

Materials

100,000 cells grown in culture or tissue lysate containing 6 ug protein (see Support Protocol 4)
Liquid nitrogen
NP-40 lysis buffer (see recipe), ice cold
RNase
50× dNTP mix: 2.5 mM each dATP, dTTP, dGTP, and dCTP in RNase-free water
10× TRAP buffer (see recipe)
Radiolabeled TS primer (see Support Protocol 5)
Primer mix (see Support Protocol 6)
RNase-free H_2O (DEPC-treated)
50 mg/ml BSA, ultrapure (Ambion)
5 U/ul *Taq* DNA polymerase
Loading dye (see recipe)
0.5 M NaCl/50% (v/v) ethanol/40 mM sodium acetate (pH 4.2), optional
DNase-, RNase-free 0.5-ml microcentrifuge and PCR tubes
Tabletop centrifuge (e.g., 5415D; Eppendorf), room temperature and, optionally, 4°C
37°C water bath or 85°C heating block
Thermal cycler
PhosphorImager with phosphor screens and ImageQuant software (Molecular Dynamics)

Additional reagents and equipment for nondenaturing acrylamide gel electrophoresis (*UNIT 6.5*)

NOTE: Most of the reagents are included in the TRAPeze kit (Intergen).

Prepare sample

1. Place 100,000 cells in a DNase-, RNase-free 0.5 ml microcentrifuge tube. Pellet cells 6 min in a tabletop centrifuge at 6000 rpm (2940 × *g*), room temperature. Discard supernatant.

Care must be taken not to contaminate any step in the TRAP assay with RNAses. RNA-Zap (Ambion) can be used to help eliminate RNAses.

Much lower cell numbers can be used because one generally analyzes only 100 to 1000 cell equivalents.

Washing the pellet is not necessary.

2. Snap freeze cell pellet in liquid nitrogen and store at -80°C until ready to lyse.

Lead shot beads can be used as an alternative to snap freezing in liquid nitrogen. The beads are poured into a container and placed at -80°C. Samples added to the beads are snap-frozen in a couple of minutes.

Alternatively, the cell pellet can be assayed immediately.

3. Resuspend sample (before it is fully thawed) in 100 ul ice-cold NP-40 lysis buffer and incubate 30 min on ice.

4. *Optional:* Centrifuge sample 20 min at 14,000 × *g*, 4°C, after lysis. Collect 80% of the supernatant in a fresh tube, making sure that no traces of cell debris from pellet are withdrawn.

The whole cell lysate can be used instead of pelleting the insoluble material. The authors have found that recovery of telomerase activity is higher and more reproducible when the samples are not centrifuged and the entire lysate is analyzed. Centrifugation is usually needed only when tissues are used (see Support Protocol 4).

5. Snap-freeze lysate in liquid nitrogen and store at -80°C until analyzed by TRAP assay.

Lead shot beads can be used as an alternative to snap freezing in liquid nitrogen. The beads are poured into a container and placed at -80°C. Samples added to the beads are snap-frozen in a couple of minutes.

The TRAP assay can be performed using a radioactive or nonradioactive (see Alternate Protocol) primer.

To prevent contamination with PCR-amplified products, the TRAP assay should be performed in a separate room from which the lysis took place.

Perform primer extension

6. As a negative control, incubate 5 ul lysate with 1 ug RNase for 20 min at 37°C or heat inactivate 10 min at 85°C prior to telomerase assay.

A lysis-buffer-only control should also be included to check for presence of PCR contamination in the lysis buffer.

7. Place 1 to 2 ul lysate (equal to 100 to 1000 cells) in the bottom of an RNase-free PCR tube on ice and add the following:

1 ul of 50× dNTP mix (50 uM final)
5 ul of 10× TRAP Buffer
2 ul radiolabeled TS primer
1 ul primer mix
38.6 ul RNase-free H_2O.

Mix and add 0.4 ul of 50 mg/ml ultrapure BSA (20 ug final) and 0.4 ul of 5 U/ul *Taq* DNA polymerase (2 U final). Incubate 30 min at room temperature.

This recipe is designed for one sample. A stock reaction mixture can be made just before assembling the reaction by combining the appropriate amount of dNTP mix, 10× TRAP buffer, BSA, primer mix, and water. The radiolabled TS primer and Taq DNA polymerase should be added immediately before the reaction mixture is added to each sample.

To prevent contamination by PCR products, the preparation of samples and the assembly of the TRAP assay should take place in a separate room from that in which the PCR and electrophoresis are performed.

During the initial 30-min incubation in the presence of the TS primer, varying numbers of hexameric repeats are added by telomerase. Subsequent amplification will yield a 6-bp incremental ladder.

8. Amplify extension products by polymerase chain reaction (PCR) in a thermal cycler as follows:

Initial step: 90 sec 94C (to inactivate telomerase)
24 to 27 cycles: 30 sec 94C
30 sec 60C.

Analyze extension products

9. Add 5 ul loading dye to 20 ul TRAP reaction mixture and run on a 10% (w/v) nondenaturing acrylamide gel in 0.5× TBE.

10. *Optional:* Fix gel 30 min in 0.5 M NaCl/50% ethanol/40 mM sodium acetate, pH 4.2.

Fixing the gel to prevent diffusion of the bands may be performed when overnight

exposure times are expected.

11. Wrap gel in plastic wrap and expose gel, without drying, to phosphor screens 30 min to overnight and visualize on a PhosphorImager using ImageQuant software.

12. Sum the intensities of the bands in the TRAP ladder and divide by the intensity of the internal standard control band to determine relative TRAP activity. Compare the activity of each sample with the negative and positive controls.

ALTERNATE PROTOCOL: MEASUREMENT OF TELOMERASE ACTIVITY BY THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL USING FLUORESCENT PRIMERS

Measurement of telomerase activity using fluorescent primers rather than radiolabeled primers can be used as an alternative to Basic Protocol 2 to reduce the exposure to radioactivity. This alternative procedure may be slightly less sensitive than the radioactive method.

Additional Materials (also see Basic Protocol 2)

100 ng/ul fluorescently labeled TS primer: 5′-Cy5-AATCCGTCGAGCAGAGTT (Integrated DNA Technologies), HPLC or PAGE purified

1. Prepare cells or tissue lysate as described (see Basic Protocol 2, steps 1 to 5).

2. Set up a negative control and TRAP reaction mixture as described (see Basic Protocol 2, steps 6 and 7), but use 100 ng/ul fluorescently labeled TS primer instead of radiolabeled TS primer in step 7.

3. Amplify extension products by polymerase chain reaction (PCR) in a thermal cycler as follows:

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Initial step:  90 sec  94°C (to inactivate telomerase)
30 cycles:     30 sec  94°C
               30 sec  52°C
               45 sec  72°C.
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4. Electrophorese the TRAP reaction mixture and fix the gel, if desired, as described (see Basic Protocol 2, steps 9 and 10).
Fixing of the gel for nonradioactive TRAP allows for easier handling when the gel is placed on a PhosphorImager.

5. Wrap gel in plastic wrap and scan under Cy5 (Blue) fluorescence with a PhosphorImager using ImageQuant software (Molecular Dynamics). Analyze the TRAP ladder as described (see Basic Protocol 2, step 12).

SUPPORT PROTOCOL 4: LYSIS OF TISSUE SAMPLES FOR THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL

This procedure can be used to obtain lysates to analyze telomerase activity from tissue samples. Because the sample will be used in an RNase-sensitive procedure, standard precautions to avoid RNase contamination should be observed.

Additional Materials (also see Basic Protocol 2)

50 to 100 mg tissue sample, frozen at -80°C
Washing buffer (see recipe), ice cold
BCA protein assay kit (Pierce)

Kontes tubes and disposable pestles (VWR)
Hand-powered drill

1. *Optional:* Wash 50 to 100 mg frozen tissue sample in enough ice-cold washing buffer to cover the tissue. Centrifuge and remove buffer from tissue. Mince tissue with scalpel.

2. Homogenize tissue with 200 ul ice-cold NP-40 lysis buffer in a Kontes tube on ice using a disposable pestle rotating at 450 rpm with a hand-powered drill. Use short (<10- to 15-sec) pulses.
Care must be taken not to overheat the samples and destroy the telomerase activity in the tissue.

3. Incubate sample 25 min on ice, centrifuge lysate 20 min at 16,000 × *g*, 4°C. Collect 80% of the supernatant in a fresh tube. Save an aliquot for protein assay. Rapidly freeze the supernatant in liquid nitrogen. Store up to ~1 year at -80°C.

4. Measure the concentration of protein in the lysate using a BCA protein assay kit (*APPENDIX 3H*).
An aliquot of the extract containing 6 ug of protein will be used for each telomerase assay (see Basic Protocol 2, step 7).

SUPPORT PROTOCOL 5: MAKING RADIOLABELED TS PRIMER FOR TELOMERIC REPEAT AMPLIFICATION PROTOCOL

For the radioactive TRAP assay (see Basic Protocol 2), the TS primer (5′-AATCCGTCGAGCAGAGTT-3′) is end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The following protocol makes enough primer for ten samples, and the amounts can be adjusted appropriately for varying number of samples.

Materials

100 ng/ul TS primer (5′-AATCCGTCGAGCAGAGTT-3′)
[γ-^{32}P]ATP (3000 Ci/mmol), sterile
10 U/ul T4 polynucleotide kinase and 5× forward kinase buffer (GIBCO/BRL)
Sterile, RNase-free H_2O

RNase-free 1.5-ml microcentrifuge tubes
37° and 85°C water baths or heating block, or PCR machine

1. Mix together the following in a RNase-free 1.5-ml microcentrifuge or PCR tube:

10 ul of 100 ng/ul TS primer
2.5 ul [γ-^{32}P]ATP
4 ul of 5× forward kinase buffer
3 ul sterile, RNase-free H_2O
0.5 ul of 10 U/ul T4 polynucleotide kinase.

Incubate 20 min at 37°C.

2. Heat inactivate enough kinase for one assay 5 min at 85°C and use immediately or freeze up to 2 weeks at -20°C. Do not freeze and thaw more than once.

SUPPORT PROTOCOL 6: MAKING THE PRIMER MIX FOR TELOMERIC REPEAT AMPLIFICATION PROTOCOL

The primer mix includes the reverse primer (ACX), the substrate for the 36-bp internal standard control (TSNT), and the reverse primer for the internal standard (NT). Preparation of the stock TSNT primer should be done in a separate room from the other TRAP steps and prepared with separate micropipettors. The TSNT primer should be purchased from a different company from the other two primers because it is possible to contaminate them at the production facilities with the TSNT oligonucleotide. This protocol makes enough primer mix for 100 sample reactions.

Materials

TSNT oligonucleotide: 5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′
RNase-free H_2O
1 ug/ul ACX primer: 5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′
1 ug/ul NT primer: 5′-ATCGCTTCTCGGCCTTTT-3′
10% (v/v) bleach
RNase-free 0.5-ml PCR tubes, including some that are siliconized

1. Dilute dry TSNT oligonucleotide in RNase-free water to 100 uM (1.0×10^{-10} mol/ul) in a siliconized, RNase-free PCR tube. Perform three dilutions (1:100, 1:1000, and 1:1000) in siliconized tubes so that the final concentration of the TSNT stock is 1.0×10^{-18} mol/ul.

This step should be done in a separate room with separate micropipettors and tips from the other steps. Because extremely dilute solutions of the TSNT substrate are required, siliconized tubes should be used for all dilutions. The actual amount added to the primer mix below is empirically adjusted to compensate for experimenter error.

2. Mix 10 ul each of the 1 ug/ul ACX and NT primers (100 ng/ul final) and 79.0 ul RNase-free water together in an RNase-free tube.

3. Move to the area where the TSNT was prepared and add 1 ml dilute TSNT oligonucleotide (0.01 × 10-18 mol/ml final) to the mix. Clean the outside of the tube with 10% bleach.

4. Return to the nonTSNT work area and divide the primer mix into 25-ul aliquots. Use aliquots immediately or store up to 1 year at -20°C. Do not freeze and thaw more than once.

REAGENTS AND SOLUTIONS

Note

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

Denhardt solution, 100×

10 g Ficoll 400
10 g polyvinylpyrrolidone
10 g BSA (Pentax Fraction V; Miles Laboratories)
H_2O to 500 ml
Filter sterilize and store at -20°C in 25-ml aliquots

Hybridization solution

5× SSC buffer (see recipe)
5× Denhardt solution (see recipe)
10 mM Na_2HPO_4
1 mM $Na_2H_2P_2O_7$
Store up to 6 months at -20°C

Loading dye

0.25% (w/v) each of bromophenol blue and xylene cyanol in 50% (v/v) glycerol/50 mM EDTA. Store up to 1 year at 4°C.

For nonradioactive TRAPs, the xylene cyanol should be removed, as this shows up while imaging the gel.

NP-40 lysis buffer

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
1 mM $MgCl_2$
1 mM EDTA
1% (v/v) NP-40
0.25 mM sodium deoxycholate
10% (v/v) glycerol
150 mM NaCl (*APPENDIX 2A*)
5 mM 2-mercaptoethanol
0.1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride)
Store up to 6 months at -20°C

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Store up to 1 month at room temperature

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)
0.3 M Na_3citrate·$2H_2O$ (88 g/liter)
Adjust pH to 7.0 with 1 M HCl (*APPENDIX 2A*)
Store up to 6 months at room temperature

TRAP buffer, 10×

200 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
15 mM $MgCl_2$
630 mM KCl
0.5% (v/v) Tween 20
10 mM EGTA
Store up to 6 months at -20°C

Washing buffer

10 mM HEPES (adjusted to pH 7.5 with KOH)
1.5 mM $MgCl_2$
10 mM KCl
1 mM DTT (*APPENDIX 2A*)
Store up to 6 months at 4°C

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COMMENTARY

Background Information

Cells contain repeated TTAGGG DNA sequences, called telomeres, at the ends of chromosomes to provide genomic stability. Telomeres provide a source of expendable DNA, which alleviates the end-replication problem in which one DNA strand cannot complete copying all the way to its end during cell division (reviewed in Collins, 2000). An in-gel Southern hybridization analysis of the chromosome terminal (i.e., telomeres) restriction fragments (TRFs) allows for the determination of all telomere lengths in a cell population (Harley et al., 1990; Ouellette et al., 2000). Telomere length has been shown to decrease with time and with increasing age (Allsopp et al., 1992). Most normal human cells lack telomerase activity, and their telomeres shorten with each cell division until they enter replicative senescence, where cells cease to proliferate (Harley et al., 1990; Wright et al., 1996). Cells are mortal unless a rare survivor activates a mechanism to maintain its telomeres.

The mechanism predominately used to maintain human telomeres is the addition of telomeric sequences by telomerase. Human telomerase is an RNA-dependent DNA polymerase complex consisting of a reverse transcriptase catalytic subunit (hTERT), which uses the integral RNA component (hTR) of the complex as a template for adding TTAGGG repeats to the end of the chromosome (Fig. 18.6.4; reviewed in Greider and Blackburn, 1985; Cong et al., 2002). Telomerase is expressed in only a small number of normal proliferating cell types such as germline and somatic stem cells, but it is found in ~90% of human cancer cells. Once telomerase is activated, it stabilizes telomere length and permits continued cell division (Counter et al., 1992).

The development of a highly sensitive assay to detect telomerase activity in cells and tissues enhanced the evaluation of a wide variety of human tissue and tumor cells (Kim et al., 1994). The telomeric repeat amplification protocol (TRAP) measures in vitro telomerase activity by a primer extension assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers (Fig. 18.6.5; Kim et al., 1994). The products can then be amplified by the polymerase chain reaction (PCR) and run on an acrylamide gel. There are several publications describing modifications and alternative methods of extracting and detecting telomerase activity in human samples using the TRAP assay (Piatyszek et al., 1995; Wright et al., 1995; Holt et al., 1996; Norton et al., 1998; Gollahon and Holt, 2000). The protocol described in this unit incorporates the modifications suggested in these reports. Together with the TRF analysis, telomerase has been shown to be directly involved in telomere maintenance and has been linked with immortality (Kim et al., 1994).

Critical Parameters

Terminal restriction fragments

It is desirable to obtain ≥300,000 cells for the determination of telomere length so that 1 to 2 ug DNA can be assayed for each TRF analysis. The amount of DNA is estimated at 6 ug/10^6 diploid cells or 10 ug/10^6 aneuploid cells; therefore, 300,000 cells yields 1.8 ug

DNA. Triton X-100 rather than SDS is used in the lysis so that the residual detergent present after dialysis does not inhibit subsequent digestion by restriction endonucleases. High concentrations of EDTA (100 mM) are present to inhibit nucleases very rapidly in these concentrated cell suspensions. Frozen cell pellets are first suspended in lysis buffer to quench nuclease activity and obtain a uniform distribution of cells before adding Triton X-100. Including Triton X-100 in the initial suspension buffer leads to a rapid clumping of cells. Lysis buffer should be added before cell pellets are fully thawed to prevent nuclease digestion. Heat inactivation of the proteinase K avoids the need to phenol/chloroform extract the DNA, and the residual salt, EDTA, and peptides present after dialysis do not inhibit enzyme digestion. After digestion with proteinase K, DNA should be handled using only wide-bore pipet tips to prevent shearing of the DNA. The final DNA solution should be viscous. Lack of viscosity suggests nonspecific nuclease degradation of the DNA, which is easily tested by running 100 ng undigested DNA on a 0.7% (w/v) agarose gel. Degraded DNA is frequently seen the first time an investigator attempts this DNA purification procedure, but generally it is rarely present thereafter. If the DNA concentration is too low after dialysis to load a sufficient quantity on a gel, then concentration on a 30,000-molecular-weight spin-filter or ethanol precipitation of the DNA may be necessary. This is rarely necessary when starting with $\geq 1 \times 106$ cells.

Digested DNA should run as a smear below the 1-kb molecular weight marker. Anything above this size is incompletely digested. If the sample is not digested completely, an additional microliter of enzyme mix can be added to the sample and allowed to digest for a longer amount of time. It is essential to have the genomic DNA completely digested to have interpretable results after hybridization with the telomeric probe. If digestion problems persist, completion of the restriction digest can be checked ahead of time by electrophoresing 250 ng DNA on a small agarose gel stained with ethidium bromide.

TRF gels should be handled carefully as they are fragile until they are dried. The dried gel can be hybridized directly without the need to transfer the DNA to a membrane. Denaturing the DNA in the gel before rather than after drying the gel increases the signal intensity ~3- to 5-fold, presumably by permitting a much greater diffusion of the denatured DNA strands in the agarose gel than in the very-high-percentage dried and rehydrated gel, and thus inhibiting the reannealing of the parental strands, which would compete with the probe. Rinsing the denatured gel with distilled water gets rid of the NaOH used in the denaturing solution and substantially reduces sticking of the gel to the Whatman 3MM paper. Because most of the DNA samples do not fill the wells and are thus in the bottom half of the gel, the gels are flipped and dried with the upper surface against the filter paper support. This increases the distance between the DNA samples and the filter paper and significantly reduces the loss of lower-molecular-weight DNA. Some loss of lower-molecular-weight DNA (particularly <400 bp) occurs during drying, and this results in the lower limit of detection in the TRF analysis.

Telomeric repeat amplification protocol

Because the TRAP assay is a highly sensitive PCR-based assay, precautions must be taken to eliminate RNases and PCR product contamination. The following guidelines ensure that contamination is kept to a minimum. Gloves must be worn at all times and

each step of the protocol should be done in separate rooms or at least in areas separated by barriers. DEPC-treated water must be used for all solutions, solutions should be kept from other solutions in the laboratory, and small aliquots of the solutions should be made and used only once. Separate micropipettors should be designated to be used only for the TRAP assay, and only aerosol-resistant pipet tips should be used with these micropipettors. Samples and solutions should also be kept on ice or snap-frozen, unless otherwise specified, to prevent degradation of telomerase.

Troubleshooting

The most common problem associated with the TRF assay is that the genomic DNA is not completely digested. This results in a smear or tight band >1 kb as visualized by ethidium bromide staining, and this interferes with the interpretation of telomere lengths after hybridizing the gel with a telomeric probe. A test gel using 200 ng of DNA digested with the enzyme mix can show whether the samples have been completely digested. If the DNA is not completely digested, the usual cause is that the sample contains too much EDTA, which interferes with the restriction enzymes. This usually results from dialyzing too many samples against an insufficient volume, or from a failure to ensure that all the liquid rests against the membrane, resulting in a few microliters of 100 mM EDTA failing to get dialyzed. Other occasional failures result from using too much DNA because of an inaccurate measurement of the viscous DNA. Another problem is that the sample may not contain any detectable DNA because of a tear in the dialysis membrane. Weak signals after scanning the hybridized gel may be due to too stringent washing or insufficiently labeled probe. Use a Geiger counter to ensure that the background signal of the gel is low but that the areas expected to have telomeres contain a higher signal before exposing the gel. Check to make sure that the probe is specific for telomeric sequences and that the radioisotope and other components are fresh.

Table 18.6.1 lists some common problems and solutions associated with the TRAP assay. The most common problems associated with the TRAP assay are RNase and PCR contamination or forgetting to add one of the components in the reaction, such as the *Taq* polymerase. Careful handling of the samples and reagents in the TRAP assay ensures successful detection of telomerase activity.

Anticipated Results

The results of an in-gel Southern hybridization of digested genomic DNA with a telomeric probe yields a smear that corresponds to the distribution of all telomere engths within the population of cells collected (Fig. 18.6.6). Including a labeled molecular weight marker on the hybridized gel allows for calculation of the average telomere length as well as the longest and shortest telomere lengths.

Once the conditions for the TRAP assay have been optimized and RNase contamination is eliminated, the amplified telomerase-extended products can be seen as an incremental ladder (Fig. 18.6.7). Because human telomerase is processive, the 30-min incubation yields a TS primer extended by a variable number of hexameric repeats. When the resulting 10% (w/v) acrylamide gel is visualized by a PhosphorImager, a 6-bp DNA incremental ladder is seen as well as the internal

standard control band. The lysis buffer only and the heat-treated sample lanes should contain only the internal standard control band. The intensities of each of the bands in the TRAP ladder can be summed together and divided by the internal standard control band to determine relative TRAP activity. Too much telomerase activity will result in intense ladders and a failure to amplify the internal standard. The actual amount of the TSNT primer used is empirically adjusted so that one gets an internal standard signal roughly equivalent to the most intense first band of the telomerase ladder using 100 to 1000 positive tumor cells as a control. The activity of each sample can then be compared with the negative and positive controls.

Time Considerations

The entire TRF procedure can be performed in 4 days (day 1: harvest and digest cells with proteinase K and dialyze overnight; day 2: restriction digest DNA and run gel overnight; day 3: dry gel and hybridize overnight; day 4: wash and expose gel). Alternatively, isolated genomic DNA can be stored at 4°C (or at -20°C for longer storage) until it is digested. The TRAP assay can be performed in 1 day or separated into a 3-day procedure: one day for lysing the samples, a second day for the PCR assay, and a third day for running and exposing the gel.

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Internet Resource

http://www.swmed.edu/home_pages/cellbio/shay-wright/

Includes TELORUN for calculating mean TRF.

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Figure 18.6.1 Construction of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1).

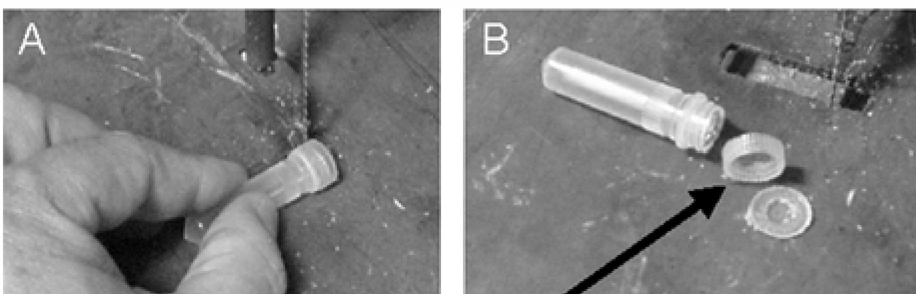

Figure 18.6.1 Construction of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1). Large numbers of these open-ended caps can easily be prepared as follows. (**A**) Screw the caps onto a tube so that the tube functions as a handle and cut off the end of the cap using a jigsaw. (**B**) The threads of the cap are attached as a ring on the tube (arrow). These rings are reusable as tube closures.

Figure 18.6.2 Assembly of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1).

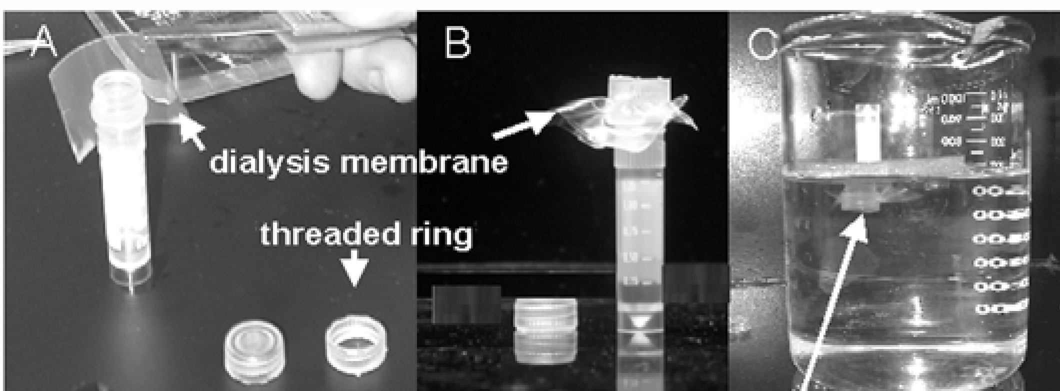

Figure 18.6.2 Assembly of the dialysis tube for the terminal restriction fragment (TRF) protocol (see Basic Protocol 1). (**A**) A wet 1- to 2-cm^2 piece of dialysis membrane is placed on the tube containing the sample (save the original screw cap). (**B**) An open-end screw cap (threaded rings) is carefully threaded on the tube over the membrane without tearing. (**C**) The tube (arrow) is inverted and placed in a floating rack in 1× TE buffer. There should be no droplets remaining on the walls of the tube and no bubbles on the membrane when inverted, as this will prevent dialysis.

Figure 18.6.3 Annealed priming and template oligonucleotides for synthesis of a high-specific activity telomeric repeat probe.

5′- TTTCCCTAACCCTAA
3′- AAAGGGAUUGGGAUUGGGAUUGGG

Figure 18.6.3 Annealed priming and template oligonucleotides for synthesis of a high-specific activity telomeric repeat probe. The template contains a small mismatch at its 3′ end (GGGAAA rather than GGGTTA) so the priming oligonucleotide will anneal in the correct spot.

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Figure 18.6.4 Telomere elongation by telomerase.

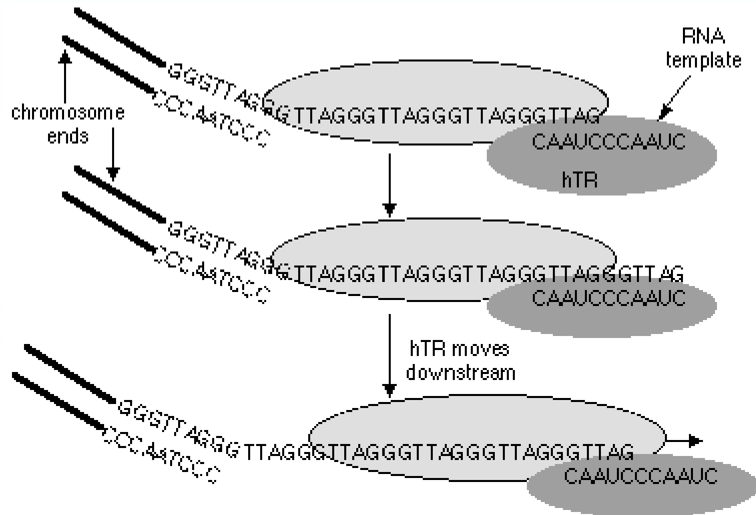

Figure 18.6.4 Telomere elongation by telomerase. Telomerase acts on the ends of chromosomes to add telomeric repeat sequences. Telomerase (hTERT) adds hexameric TTAGGG repeats by using its own RNA template (hTR) as a primer to add nucleotides. Telomerase then repositions its template RNA downstream for the addition of more telomeric repeats.

Figure 18.6.5 The telomeric repeat amplification protocol (TRAP; see Basic Protocol 2).

Step 1: Addition of telomeric repeats by telomerase

TS-telomerase products

TS

telomeric repeats

5′- AATCCGTCGAGCAGAGTT ag ggttag ggttag ggttag ggttag – 3′

ag ggttag ggttag ggttag ggttag ggttag – 3′

ag ggttag ggttag ggttag ggttag ggttag (ggttag)$_n$ – 3′

Step 2: Amplification of TS-telomerase product by PCR

TS

5′- TS-telomerase products

- 5′

ACX reverse primer

Figure 18.6.5 The telomeric repeat amplification protocol (TRAP; see Basic Protocol 2). In the first step, telomerase-mediated extension occurs on a nontelomeric oligonucleotide that serves as a substrate for telomerase (TS). In the second step, the TS-telomerase products are specifically amplified by the polymerase chain reaction (PCR) using the TS as an upstream primer and ACX as the reverse, downstream primer. The addition of telomeric repeats in the first step results in a TRAP ladder of 6-bp increments as seen on a 10% (w/v) acrylamide gel. Adapted from the manual for the TRAPeze Telomerase Detection Kit (Intergen).

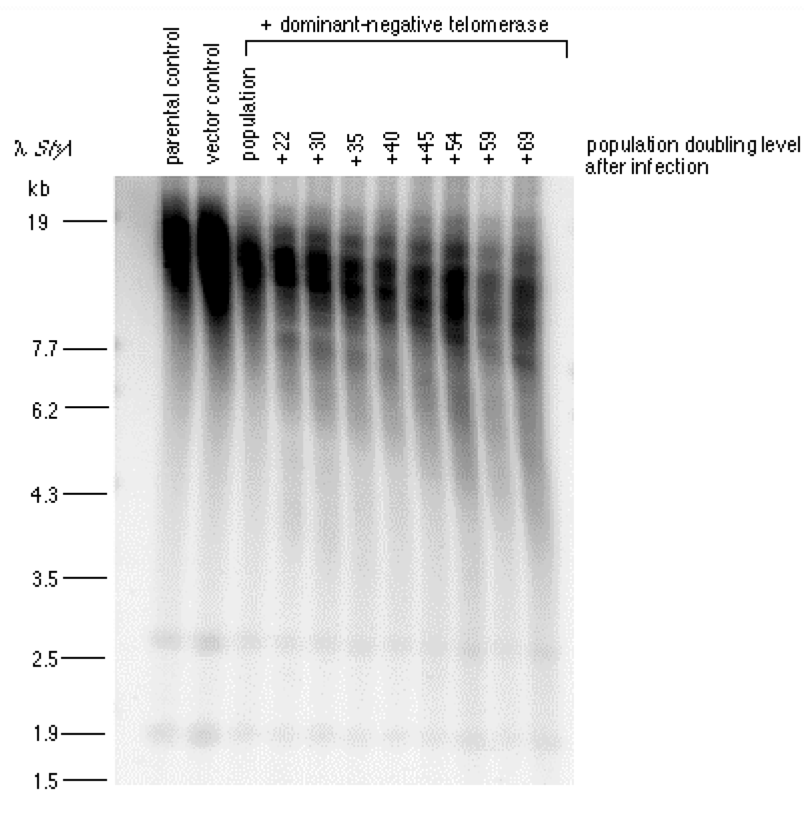

Figure 18.6.6 Determination of telomere length using the terminal restriction fragment (TRF) protocol in sequentially passaged (indicated by population doubling level, or the number of doublings the population has accrued, after infection) H1299 lung carcinoma cells infected with a dominant-negative mutant of human telomerase to inhibit telomerase and induce telomere shortening. Telomere length was determined by digesting genomic DNA with a mixture of six restriction enzymes having four-base recognition sites (see Basic Protocol 1), and then analyzing the DNA on a 0.7% (w/v) agarose gel. λ DNA cut with *StyI* was used as a molecular weight marker.

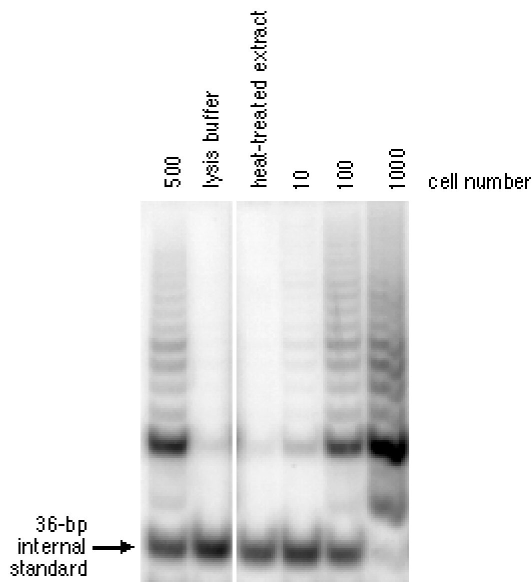

Figure 18.6.7 Analysis of telomerase activity using the telomeric repeat amplification protocol (TRAP; see Basic Protocol 2). Human cancer cell extracts (500 cell equivalents of H1299 lung carcinoma cells) are positive for telomerase activity as evidenced by the 6-bp incremental TRAP ladder. Treating the extract with heat inactivates the telomerase activity as evidenced by no TRAP ladder. Lysis buffer only serves as a negative control. Each TRAP reaction includes a 36-bp internal standard control. Ten-fold increases of H1299 cell equivalents (10 to 1000 cell equivalents) results in increased intensity of the TRAP ladder. The 1000 cell sample in this gel cannot be readily quantitated because of the faint signal in the internal standard (due to competition by excess telomerase products).

Table 18.6.1 TRAP Troubleshooting Guide[a]

Problem	Possible cause	Solution
No visible products in any lane, including the internal standard control band	PCR not initiated	Check that all assay components were added, especially *Taq* DNA polymerase. Check thermal cycler for proper settings and operation.
Few visible TRAP ladder bands and weak internal control band	Cell or tissue extract contains *Taq* inhibitors	Dilute sample with lysis buffer.
No visible TRAP products in any lane but internal standard control band is present	Telomerase activity not initiated Possible presence of RNase contamination	Always use RNase-free tips, tubes, and solutions. Use fresh aliquots of solutions. Do not let pipet tips touch anything except the solutions. Keep samples on ice and reactions below 25 C. Add RNase inhibitor to lysis buffer.
Visible TRAP product in all lanes including lysis-buffer-only control	PCR carryover contamination	Use fresh aliquots of each component of the assay. Perform each step in a separate room with separate micropipettors and tips.
Several bands in the lysis-buffer-only control lane	Primer-dimer artifacts	Incorporate hot start into the PCR reaction: Assemble reaction mixture as described, except eliminate *Taq* polymerase, heat reaction 2 min at 94 C, add *Taq*, and continue PCR procedure as described.
Products in the heat-treated extracts	Insufficient heat inactivation, primer-dimer artifacts, or contamination of extract with TRAP products	Check temperature of heating block. Make and test new extract and check for PCR carryover contamination. Extreme care must be taken to prevent pipetting into multiple samples or solutions.
Extra bands between internal standard control band and TRAP ladder bands		
Weak intensity of internal standard control band	Telomerase activity too high	Dilute samples.

[a] Adapted from the manual for the TRAPeze Telomerase Detection Kit (Intergen).

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