TRAPeze® Telomerase Detection Kit

Catalog No. S7700

40 reactions

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada Phone: +1(800) 645-5476
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France: 0825.045.645;
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Introduction

Telomeres are specific structures found at the ends of chromosomes in eukaryotes. In human chromosomes, telomeres consist of thousands of copies of 6 base repeats (TTAGGG) (1-3). It has been suggested that telomeres protect chromosome ends. Damaged chromosomes lacking telomeres may undergo fusion, rearrangement and translocation (2). In somatic cells, telomere length is progressively shortened with each cell division both in vivo and in vitro (4-7), due to the inability of the DNA polymerase complex to replicate the very 5' end of the lagging strand (8, 9).

Telomerase is a ribonucleoprotein that synthesizes and directs the telomeric repeats onto the 3' end of existing telomeres using its RNA component as a template (10-14). Telomerase activity has been shown to be specifically expressed in immortal cells, cancer and germ cells (15, 16), where it compensates for telomere shortening during DNA replication and thus stabilizes telomere length (7, 17). These observations have led to a hypothesis that telomere length may function as a “mitotic clock” to sense cell aging and eventually signal replicative senescence or programmed cell death. Therefore, expression of telomerase activity in cancer cells may be a necessary and essential step for tumor development and progression (16, 18-20). The causal relationship between expression of telomerase and telomere length stabilization and the extension of the life span of the human cell has recently been reported (21).

To develop a sensitive and efficient PCR-based telomerase activity detection method, TRAP (Telomeric Repeat Amplification Protocol) (15, 22), has made possible large scale surveys of telomerase activity in human cells and tissues (15, 23-29). To date, telomerase activity has been detected in over 85% of all tumors tested spanning more than 20 different types of cancers (30, 31).
The TRAP assay was used to detect telomerase in human, mouse, rat, dog, cow, chicken, and Xenopus. However, the implications of positive telomerase activity in species other than human may be more complicated to understand. For example, in mouse, telomerase activity is not restricted to proliferating and/or cancer cells; thus telomerase can be detected in a multitude of tissues. The number of telomeric repeat may vary from the human cells

Principles of the Technique

The methodology utilized in the TRAPEZE® Gel-Based Telomerase Detection Kit is based on an improved version of the original method described by Kim, et al (12). The TRAPEZE® Kit is a sensitive in vitro assay system for detecting telomerase activity. The assay is a one buffer, two enzyme system utilizing the polymerase chain reaction (PCR). In the first step of the reaction (Fig. 1A), telomerase adds an AG plus a number of telomeric repeats (GGTTAG) onto the 3' end of a substrate oligonucleotide (TS). In the second step, the extended products are amplified by PCR using the TS and RP (reverse) primers, generating a ladder of products with 6 base increments starting at 50 nucleotides: 50, 56, 62, 68, etc. TS plus 5 telomeric repeats are normally seen as the smallest TRAPeze® product using this Gel-Based kit.

The TRAPEZE® Gel-Based Telomerase Detection Kit provides substantial improvements to the original TRAP assay, such as a modified reverse primer sequence which (1) eliminates the need for a wax barrier hot start, (2) reduces amplification artifacts and (3) permits better estimation of telomerase processivity. Additionally, each reaction mixture contains a forward primer (K1) and a template (TSK1) for amplification of a 36 bp internal standard (Fig. 1B). Incorporation of this internal positive control makes it possible to telomerase activity more accurately (with a linear range close to 2.5 logs) and to identify false-negative samples that contain Taq polymerase inhibitors. Details of quantitation procedures are discussed in Sec. IV. Data Analysis.

Figure 1A: TRAPEZE® Gel-Based Telomerase Detection Kit Assay Scheme.

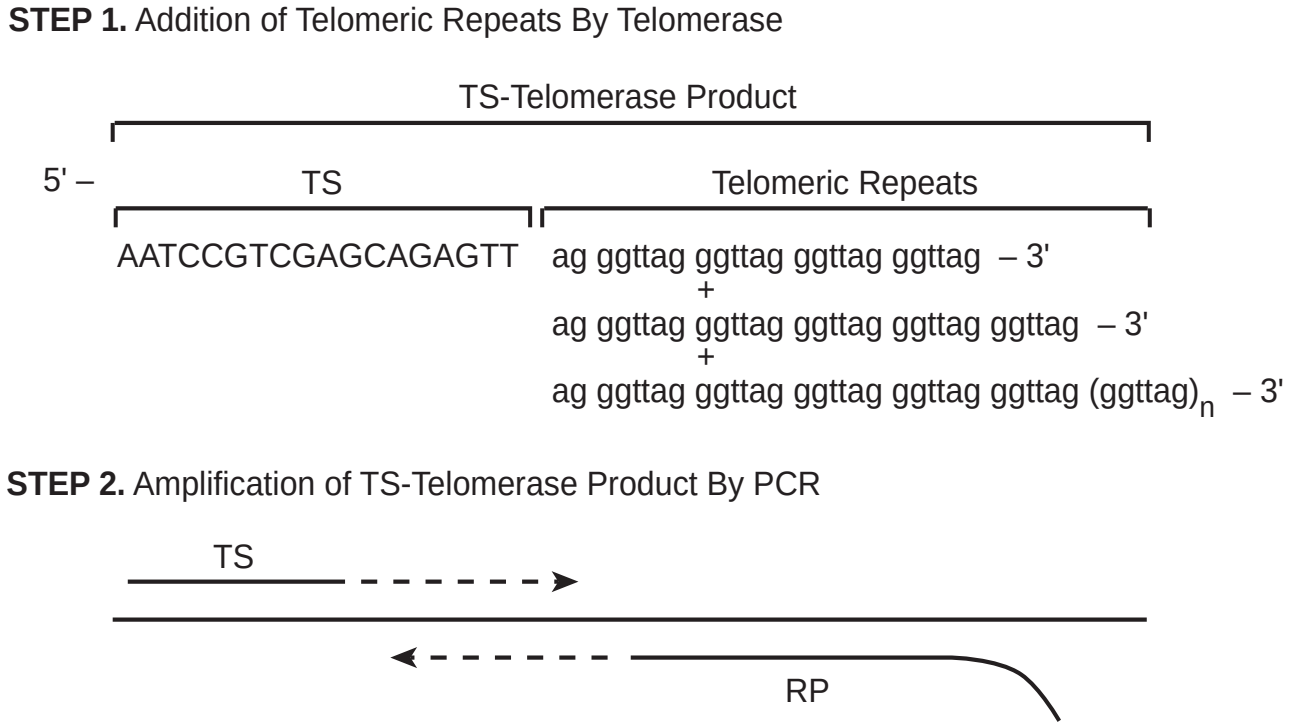
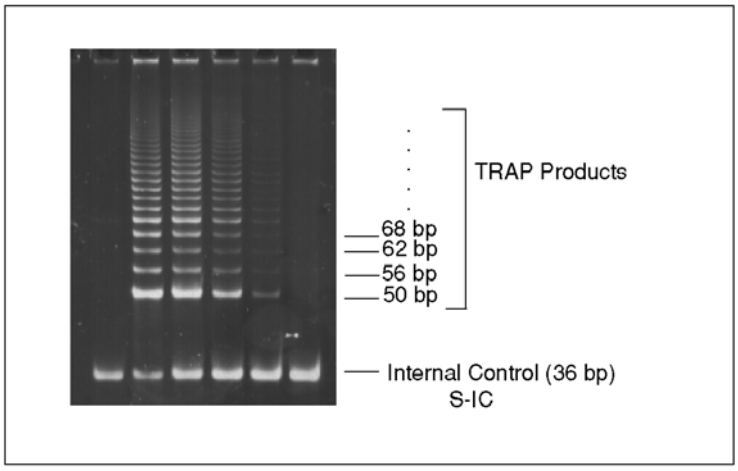


Figure 1B: TRAPEZE® Gel-Based Telomerase Detection Kit Assay Gel.



Serially diluted extracts of telomerase-positive control cells (provided in the TRAPeze® Kit) were subjected to the TRAP assay. Reaction products were run on a native polyacrylamide gel and stained with SYBR® Green. Lane 1: CHAPS Lysis Buffer control; Lanes 2-5: extract from 10,000, 2000, 400 and 80 cells; Lane 6: heat-treated extract from 10,000 cells.

Materials Provided (Kit Components)

The kit provides enough reagents to perform 112 TRAPEZE® Kit assays with 50 µl reaction volume. A 25 µl reaction volume can also be used. With these reagents, 40 samples with appropriate positive and negative controls can be analyzed (for detail of the experimental design, see Sec. III. Protocol: Experimental Design).

Table 1: TRAPEZE® Gel-Based Telomerase Detection Kit (S7700)

Description	Volume/ Quantity	Storage Conditions
1X CHAPS Lysis Buffer (Part Number 90404) 10 mM Tris-HCl, pH 7.5 1 mM $MgCl_2$ 1 mM EGTA 0.1 mM Benzamidine 5 mM β-mercaptoethanol 0.5% CHAPS 10% Glycerol	8200 µL	-25°C to -8°C
10X TRAP Reaction Buffer (Part Number 90405) 200 mM Tris-HCl, pH 8.3 15 mM $MgCl_2$ 630 mM KCl 0.5% Tween 20 10 mM EGTA	560 µL	-15°C to -25°C
50X dNTP Mix (Part Number 90406) 2.5 mM each dATP, dTTP, dGTP and dCTP	112 µL	-15°C to -25°C
TS Primer (Part Number 90407)	112 µL	-15°C to -25°C

Primer Mix (Part Number 90408) RP primer FP (K1) primer TSK1 template	112 µL	-15°C to -25°C
PCR-Grade Water (**Part Number 90411)** protease, DNase, and RNase-free; deionized	8200 µL	-25°C to -8°C
TSR8* (control template) (**Part Number 90409)** 2.0 amole/µL TSR8 template	32 µL	-15°C to -25°C
Human Control Cell Pellet (**Part Number 90410)** Telomerase positive cells	10^6 cells	-75°C to -85°C

Precautions

1. Because the TRAPEZE® Kit detects the activity of telomerase, an RNase-sensitive ribonucleoprotein, and not merely the presence of RNA or protein components of telomerase, the assay requires enzymatically active cell or tissue samples. Furthermore, due to the extremely sensitive nature of the TRAPEZE® Kit assay special laboratory setup and significant precautions are required to prevent PCR carry-over contamination and RNase contamination including DEPC-treated water and UV crosslinking of all laboratory consumables (pipettes, tips etc).

2. Do not combine primers from one lot number of TRAPEZE® Kit with another.

Materials Required But Not Supplied

Equipment and Supplies

a. Thermocycler
b. Water bath incubator or heat block at 85˚C (and 37˚C if the radioisotopic detection method is utilized)
c. Polyacrylamide vertical gel electrophoresis apparatus (Precast gel is preferable)
d. Power Supply (>500 V capacity)
e. If analyzing tissues, homogenization equipment as described in Section III. Protocols.
f. Thin-walled, 0.2 mL PCR tubes or PCR plate
g. Aerosol resistant pipette tips (RNase-free)

Reagents

a. RNase inhibitor
b. Taq polymerase
c. PBS (Mg2+ and Ca2+-free)
d. Reagents for determination of protein concentration
e. Reagents for PAGE
    40% Polyacrylamide/bisacrylamide stock solution (19:1)
    TEMED, 10% Ammonium Persulfate
    10X (or 5X) TBE Solution
f. Radioisotopic Detection (Option 1)
    3MM paper
    Gel dryer
    X-ray film or PhosphorImager™
    γ-32P-ATP (3000 Ci/mmole, 10 mCi/ml)

T4 polynucleotide kinase and 10X reaction buffer

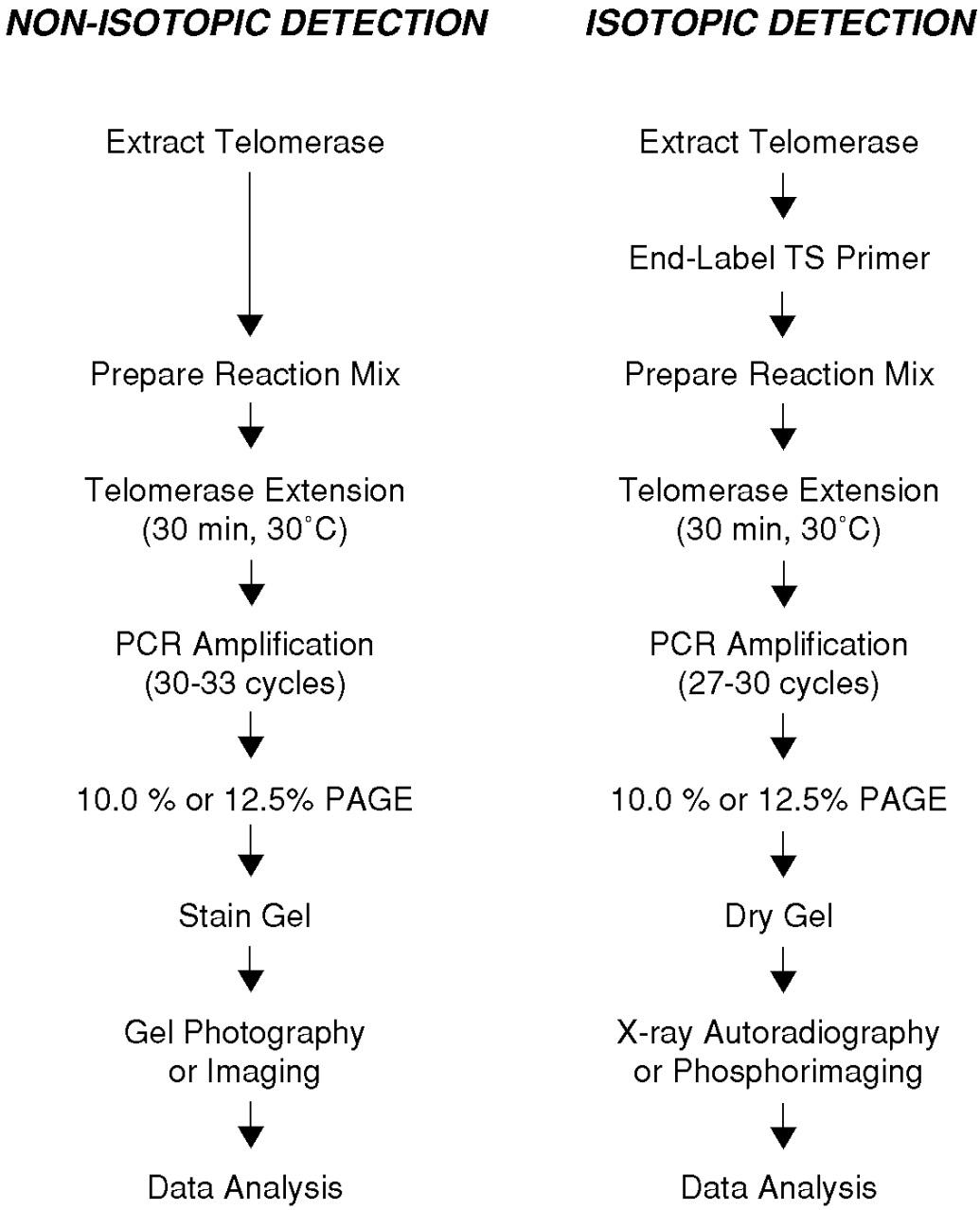
g. Non-Isotopic Detection (Option 2)

SYBR® Green or Ethidium Bromide (EtBr)
UV box: 254 nm or 302 nm for SYBR® Green, 302 nm for EtBr
UV filter: SYBR® Green filter; EtBr (orange filter)
Camera and film or CCD Imaging System

h. Bovine Serum Albumin

Protocol

Figure 2: TRAPEZE® Gel-Based Telomerase Detection Kit Flow Chart.



Extract Preparation

***Note**: The volume of 1X CHAPS Lysis Buffer used is adjusted for the number of cells to be extracted. To determine the volume of 1X CHAPS Lysis Buffer for each sample, establish cell number by counting or extrapolation from tissue weight.*

When preparing extracts from tumor samples, you may add RNase inhibitor to the CHAPS Lysis Buffer prior to the extraction at a final concentration of 100-200 Units/mL.

1. Pellet the cells or tissue, wash 1X with PBS, repellet, and carefully remove all PBS. After removal of PBS, the cells can be aliquoted as 1.0E+06 cell counts and stored at -85˚C to -75˚C. Telomerase in frozen cells or tissues is stable for at least 1 year if stored at -85˚C to -75˚C without frequent freeze-thaw. When thawed for extraction, the cells or tissue should be resuspended immediately in 1X CHAPS Lysis Buffer (step 2).

2a. **Cells**
Resuspend the cell pellet in 200 µL of 1X CHAPS Lysis Buffer/105-106 cells. Resuspend the positive control cell pellet provided in the kit with 200 µL of 1X CHAPS Lysis Buffer. The volume of 1X CHAPS buffer can be adjusted depending on the cell counts. See instructions for use of the positive control cell extract under Sec. III. Protocols: Controls. Proceed to Step 3.

2b. **Tissues**
Prepare the extract according to one of the methods described below. Use 200 µL of 1X CHAPS Lysis Buffer/40-100 mg of tissue.

> ***Soft Tissues:*** Homogenization with Motorized Disposable Pestle: Mince the tissue sample with a sterile blade until a smooth consistency is reached. Transfer the sample to a sterile 1.5 mL microcentrifuge tube, and add 1X CHAPS Lysis Buffer. Keep the sample on ice and homogenize with a motorized pestle (~10 seconds) until a uniform consistency is achieved.
>
> ***Connective Tissues***: Freezing and Grinding: Place the tissue sample in a sterile mortar and freeze by adding liquid nitrogen. Pulverize the sample by grinding with a matching pestle. Transfer the thawed sample to a sterile 1.5 mL microcentrifuge tube, and resuspend in an appropriate amount of 1X CHAPS Lysis Buffer.
>
> Mechanical Homogenizer: Mix the tissue sample with an appropriate volume of 1X CHAPS Lysis Buffer in a sterile 1.5 mL microcentrifuge tube placed on ice. Homogenize with a mechanical homogenizer until a uniform consistency is achieved (~5 seconds). It is critical to keep the sample on ice during homogenization to prevent heat accumulation.

3. Incubate the suspension on ice for 30 minutes.
4. Spin the sample in a microcentrifuge at 12,000 X g for 20 minutes at 4˚C.
5. Transfer all supernatant (normally approximate 160 µL) into a fresh tube. The total protein concentration can be measured as per user's choice in this step.

Table 2: Sample Concentration and Quantity for Assay if cell count is not available.

Sample Type	Concentration	Quantity
Cell Extract	10-750 ng/µL	<1.5 µg per assay
Tissue Extract	10-500 ng/µL	<1.5 µg per assay

6. Aliquot the supernatant into a small volume to avoid frequent freeze-thaw and quick-freeze on dry ice and store at -85˚C to -75˚C. The extract is stable for at least 12 months at -85˚C to -75˚C.

***Note**: The extracts for the TRAPEZE® Kit assay should be quick-frozen on dry ice after each use. Aliquots should not be freeze-thawed more than 10 times to avoid loss of telomerase activity. Additionally, aliquoting reduces the risk of contamination.*

Experimental Design

For a valid analysis of the results, two factors need to be considered: (1) controls and (2) detection method.

Controls

For each sample: Telomerase is a heat-sensitive enzyme. As a negative control, every sample must be tested for heat sensitivity. Thus, analysis of each sample consists of two assays: one with and one without a heat-treatment. Heat treat 10 µL of each sample by incubating at 85°C for 10 minutes prior to the TRAP assay to inactivate telomerase.

For each set of TRAP assays: Make a telomerase-positive cell extract using 200 µL of 1X CHAPS Lysis Buffer and the cell pellet (106 cells) provided in the kit. Aliquot the lysate in microcentrifuge tubes and store at -85˚C to -75˚C. **Dilute the stock aliquots 1:20 with 1X CHAPS Lysis Buffer before use and dispense 2 µL per assay (2 µL = 500 cells). Run one positive control reaction for each set of assays.**

PCR Amplification Control (TSK2): An important feature of the TRAPEZE® Gel-Based Telomerase Detection Kit is the inclusion of an internal control to monitor PCR inhibition in every lane. Many cell/tissue extracts contain inhibitors of Taq polymerase and can give potentially false-negative results. To distinguish this from other problems, the TRAPEZE® Primer Mix contains internal control primer K1 and TSK1 oligonucleotide which, together with TS forward primer, produces a 36 bp band (internal control, IC) in every lane except the no Taq control. The IC band also serves as a control for amplification efficiency in each reaction and can be used for semi quantitative analysis of the reaction products. (See Sec. IV. Data Analysis).

Primer-Dimer/PCR Contamination Control: Perform a TRAPEZE® Kit assay with 2 µL 1X CHAPS Lysis Buffer substituted for the cell/tissue extract. Perform a TRAPEZE® Kit assay with 2 µL molecular water substituted for the cell/tissue extract.

Primer-dimer PCR artifacts are template-independent PCR products that can be generated with the input primer(s) in the absence of a template DNA. **Each set of TRAP assays should be tested for the potential generation of primer-dimer PCR artifacts and/or PCR product carry-over contamination.** If the assay worked optimally, except for the 36 bp internal control band, no product should be present in the primer-dimer/PCR contamination control lanes. The presence of products in the primer-dimer/PCR contamination control lanes suggests either: 1) the presence of primer-dimer PCR artifacts due to suboptimal PCR conditions; or 2) the presence of PCR contamination (amplified TRAP products) carried over from another assay or buffer contamination (see Sec. V. Troubleshooting).

No Taq control: Perform a TRAPEZE® Kit assay with no Taq at 1X CHAPS Lysis Buffer sample.

Telomerase Quantitation Control Template – TSR8: Perform the TRAPEZE® Kit assay using 2 µL of TSR8 (Control Template) instead of sample extract. TSR8 is an oligonucleotide with a sequence identical to the TS primer extended with 8 telomeric repeats AG(GGTTAG)7. This control serves as a standard for estimating the amount of TS primers with telomeric repeats extended by telomerase in a given extract. You may see 3-5 different size of ladder PCR products with this control oligonuleotide on the gel depends on the PCR efficiency.

Assay Design

The TRAPEZE® Telomerase Detection Kit is designed for the successful and thorough analysis of 40 or experimental samples using 50 reaction volume (or 80 rxns with 25 µl reaction volume). Below is normal assay design for a single experiment.

Lane 1: DNA molecular weight ladder containing a 25 bp smallest size is preferable
Lane 2-10: 5 experimental samples, each with one heat-treated extract and one without heat treatment. The internal amplification control is included in each sample via the Primer Mix. 1:5 or 1:10 diluted cell extract is also recommended if the telomerase activity is high in the tested sample
Lane 11: 500 cell equivalents of telomerase positive control cell extract with one test extract and one heat-treated extract. 1:5 or 1:10 diluted cell extract can also be used for titration purpose
Lane 12: Primer-dimer/PCR contamination control: Lysis Buffer ONLY control
Lane 13: Quantitation control: 1 µL TSR8 (2.0 amole)
Lane 14: Quantitation control: 2 µL TSR8 (0.2 amole) or 2 µL of 1:10 diluted TSR8
Lane 15: Primer-dimer/PCR contamination control: Water ONLY control
Lane 16: Primer-dimer/PCR contamination control: No Taq control

Detection Methods

There are two options for visualization of reaction products:

Option 1: Detection of radiolabeled product,
Option 2: Staining of products by Ethidium Bromide or SYBR® Green

Each method has different laboratory equipment and reagent requirements (see Sec. II. Kit Components, Materials Required).

Option 1: Detection of Radiolabeled Product
Involves radioactive end-labeling of the TS primer with γ-32P-ATP. Equipment required is X-ray film or the PhosphorImager™. This method is more quantitative than non-radioactive detection.

Option 2: Non-radioactive Detection
Utilizes DNA staining agents such as SYBR® Green or Ethidium Bromide to visualize the reaction products. Equipment required is a camera system or a CCD imaging system.

If using a camera: The staining agent SYBR® Green, a yellow or green filter, and a 254 or 302 nm UV transilluminator must be used. Images produced are less sensitive than those obtained by options described below using a CCD imaging system. We do not recommend Ethidium Bromide detection if using a camera.

If using a CCD imaging system: The best results with SYBR® Green are obtained using (1) a 254 nm UV transilluminator and a SYBR® Green filter or (2) a 302 nm UV transilluminator and an orange UV filter. Ethidium Bromide staining with a 302 nm UV transilluminator and an orange UV filter gives slightly less sensitive results.

Another consideration regarding the choice of detection method is the desired sensitivity, which is defined as the minimal number of telomerase-positive cells required to detect telomerase activity. Under optimum amplification conditions, the sensitivity of the TRAPEZE® Kit utilizing each method is:

Radioactive products: 50 control cells (provided in the kit) after 27 PCR cycles
Non-radioactive products (SYBR® Green Staining): 50 control cells after 30 PCR cycles

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TRAPEZE® Gel-Based Telomerase Detection Kit Assay

Radioisotopic Detection

End-Labeling of the TS Primer
Typical conditions for labeling a sufficient amount of the TS primer for 10 assays:

γ-32P-ATP (3000 Ci/mmol, 10 mCi/mL)	2.5 µL
TS Primer	10.0 µL
10X Kinase Buffer	2.0 µL
T4 Polynucleotide Kinase (10 units/µL)	0.5 µL
dH2O	5.0 µL
	20.0 µL

Incubate 20 minutes at 37˚C, then 5 minutes at 85˚C. Use 2.0 µL per TRAP assay reaction (purification not recommended).

Assay Setup

1. Prepare a "Master Mix" by mixing all of the reagents outlined below except for the cell extract. Thaw all reagents and store on ice. Do not use the buffer provided with the Taq enzyme. Use the 10X TRAP reaction buffer contained in this kit.

The amount of reagents required in each assay is:

10X TRAP Reaction Buffer	5.0 µL
50X dNTP Mix*	1.0 µL
^{32}P-TS Primer (from step 1)	2.0 µL
TRAP Primer Mix	1.0 µL
Taq Polymerase (5 units/µL)	0.4 µL (2 Units)
dH2O	38.6 µL
	48.0 µL
Cell Extract (10 - 750 ng/µL)	2.0 µL (of either)
OR	
Tissue Extract (10 -500 ng/µL)	
TOTAL VOLUME	50.0 µL

**Upon first use, make aliquots of 50X dNTP Mix, which can be freeze-thawed no more than 5 times.*

2. Aliquot 48 µL of prepared "Master Mix" into RNase-free PCR tubes (without the samples).
3. Add 2 µL of test extracts, heat-inactivated extracts or controls into each tube:

- *Sample extracts*: add 2 µL to each of the sample tubes with two different concentration at 1:0 and 1:5 dilution if cell number is enough.
- *Heat-inactivated controls*: incubate 4-5 µL of each sample extract at 85˚C for 10 minutes. Add 2 µL into each of the heat-inactivation control tubes.
- *Telomerase-positive cell control*: add 2 µL of positive control cell extract at two different concentration of 5,000 cells/ µL and 1,000 cells/ µL.
- *TSR8 controls*: add 2 µL of two different concentration at 2.0 amole and 0.2 amole
- *Primer-dimer/PCR contamination control*: add 2 µL of 1X CHAPS Lysis Buffer.
- *Primer-dimer/PCR contamination control*: add 2 µL of H2O
- *Primer-dimer/PCR contamination control*: No Taq control

PCR Amplification

1. Place tubes in the thermocycler block, and incubate at 30˚C for 30 minutes.
2. Perform 2-step PCR at 94˚C/30 seconds and 59˚C/30 seconds for 27-30 cycles in a thermocycler.

***Note**: These PCR conditions should work on most thermocyclers, but may need to be tested empirically for the specific machine that is being used. See Sec. V. Troubleshooting.*

Electrophoresis and Data Analysis

1. Add 5 µL of loading dye containing bromophenol blue and xylene cyanol (0.25% each in 50% glycerol/50 mM EDTA) into each reaction tube. Load and run 25 µL of this mixture on a 10.0% or 12.5% non-denaturing PAGE (no urea) in 0.5X TBE buffer. Run PCR products (after the addition of the loading dye) by electrophoresis using 2% agarose gels. Load a low molecular weight size marker as well.

Loading: Use extreme care to prevent sample carry-over into adjacent wells, which may produce false-positive results. For optimal interpretation of results, load heat-treated and non-heat-treated samples in alternating lanes (i.e., extract 1+heat, extract 1–heat, extract 2+heat, etc.) Load the TSR8 quantitation control on the last lanes of the gel.

2. Run time: 1.5 hours at 400 volts for a 10-12 cm vertical gel, or until the xylene cyanol runs 70-75% of the gel length. The smallest telomerase product band should be 50 bp and the S-IC internal control band is 36 bp.
3. Dry the gel and visualize reaction products with the PhosphorImager™ or by autoradiography.

Non-radioactive Detection By Staining

Assay Set-Up

1. Prepare a "Master Mix" using unlabeled TS primer and all of the reagents outlined below except for the cell extract. Thaw all reagents and store them on ice. Do not use the buffer provided with the Taq enzyme. Use the 10X TRAP reaction buffer contained in this kit.

The amount of reagents required in each assay is:

10X TRAP Reaction Buffer	5.0 µL
50X dNTP Mix*	1.0 µL
TS Primer	1.0 µL
TRAP Primer Mix	1.0 µL
Taq Polymerase (5 units/µL)	0.4 µL (2 Units)
dH2O	39.6 µL
	48.0 µL
Cell Extract (10-750 ng/µL)	
OR	2.0 µL (of either)
TissueExtract (10-500 ng/µL)	
TOTAL VOLUME	50.0 µL

**Upon first use, make aliquots of 50X dNTP Mix, which can be freeze-thawed no more than 5 times.*

2. Aliquot 48 µL of the prepared "Master Mix" into RNase-free PCR tubes (without samples).

3. Add 2 µL of test extracts, heat-inactivated extracts or controls into each tube:

- *Sample extracts*: add 2 µL to each of the sample tubes.
- *Heat-inactivated controls:* incubate 4-5 µL of each sample extract at 85˚C for 10 minutes. Add 2 µL into each of the heat-inactivation control tubes.
- *Telomerase-positive control:* add 2 µL of positive control cell extract at a concentration of 250 cells/µL.
- *Primer-dimer/PCR contamination control*: add 2 µL of 1X CHAPS Lysis Buffer.
- *Quantitation control*: add 1 µL TSR8 (Control Template) +1 µL H2O into one tube and 2 µL of TSR8 (Control Template) into the other control tube.

PCR Amplification

Place the tubes or plate in the thermocycler block.

Cycle 1:	30°C	30 min	1 cycle
Cycle 2:	95°C	2.0 min	1 cycle*
Cycle 3:	94°C	15 sec	
	59°C	30 sec	
	72ºC	1.0 min	30-34 cycles
	4°C	∞	1 cycle

* when hot-start Taq polymerase is used.

A short PCR program can also be used if the telomerase activity is very strong

Cycle 1:	30°C	30 min	1 cycle
Cycle 2:	95°C	2.0 min	1 cycle*
Cycle 3:	94°C	15 sec	
	59°C	30 sec	26 cycles
	4°C	∞	1 cycle

* when hot-start Taq polymerase is used.

Note: These PCR conditions should work on most thermocyclers, but may need to be tested empirically for the specific machine that is being used. See Sec. V. Troubleshooting.

Electrophoresis and Data Analysis

1) Run PCR products (after the addition of loading dye) by eletrophoresis using 2% agarose gel. Load a low molecular weight size marker as well.

Loading: Use extreme care to prevent sample carry-over into adjacent wells, which may produce false-positive results. For optimal interpretation of results, load heat-treated and non-heat-treated samples in alternating lanes (i.e., extract 1+heat, extract 1–heat, extract 2+heat, etc.) Load the TSR8 quantitation control on the last lanes of the gel.

2) Run time: 1.5 hours at 400 volts for a 10-12 cm vertical gel, or until the xylene cyanol runs 70-75% the length of the gel. The smallest telomerase product band should be 50 bp and the S-IC internal control band is 36 bp.

3) After electrophoresis, stain the gel with Ethidium Bromide or SYBR® Green according to the manufacturers instructions (see also Ref. 17). For Ethidium Bromide, dilute the 10 mg/ml stock solution 1:10,000 in deionized water. Stain for 30 minutes and destain for 20-30 minutes in deionized water at room temperature.

IV. DATA ANALYSIS

Visual Analysis

For a valid TRAPEZE® Kit assay, all of the conditions below should be met:

1) Primer-dimer/PCR contamination control lane: No product should be visible except the 36 bp internal control band (S-IC).
2) Telomerase-positive control lane: Exhibits the 36 bp internal control band and a ladder of products with 6 base increments starting at 50 nucleotides (i.e. 50, 56, 62, 68, etc.).
3) Heat-treated sample extract lane: No product should be visible except the 36 bp internal control band (S-IC).

If all 3 controls have produced the desired results, analysis of experimental extracts can proceed.

Note: *The internal control band may not be visible in a sample with excessively high telomerase activity because amplification of the TRAP products and the S-IC control band are semi-competitive. When the radiolabeling detection method is utilized, several faint bands may be observed at the lower end of the gel (close to the bromophenol blue) on an overexposed image or film. These bands correspond to the oligonucleotide synthesis by-products and do not hamper overall analysis.*

Experimental Samples

If extract is telomerase positive: A ladder of products with 6 base increments starting at 50 nucleotides (i.e. 50, 56, 62, 68, etc.) and a 36 bp internal control band should be seen. An identical pattern should be seen in the telomerase-positive control lane.

If extract is telomerase negative: Only the 36 bp internal control band (S-IC) is seen.

Quantitation

The following is an example of quantitative analysis of 32P-labeled samples using the PhosphorImager™. To obtain valid quantitative values of telomerase activity using the TRAPEZE® Kit, inclusion of all the assay controls outlined in Sec. III. Protocols, Experimental Design is necessary. Perform a heat-inactivation telomerase control for each test extract. Also include a primer-dimer/PCR contamination control and a TSR8 quantitation control for each set of assays. The PCR amplification control is automatically incorporated into each reaction mixture.

1) Measure the signal of the region of the gel lane corresponding to the TRAP product ladder bands from all samples including non-heat-treated (x) and heat-treated sample extracts (xo), 1X CHAPS Lysis Buffer only control (primer-dimer/PCR contamination control), and TSR8 quantitation control (r) (Fig. 3B).
2) Measure the signal from the internal standard (S-IC) in non-heat-treated samples (c) and TSR8 quantitation control (cR).
3) Quantitate the amount of telomerase product using the following formula:

$$\text{TPG (units)} = \frac{(x - x_o) / c}{(r - r_o) / c_R} \times 100 \quad \text{(if 0.1 amole of TSR8 is used)}$$

Each unit of TPG (Total Product Generated) corresponds to the number of TS primers (in 1 x 10-3 amole or 600 molecules) extended with at least 4 telomeric repeats by telomerase in the extract in a 30 minute incubation at 30˚C. The assay has a linear range of 1 to 300 TPG, which is equivalent to telomerase activity from approximately 30 to 10,000 control cells. Results shown in Fig. 3A and 3B demonstrate application of this quantitation methodology.

When the staining methodology is utilized and densitometric evaluation is desired, use a CCD imaging system and image analysis program. The same quantification methodology detailed above can be applied on data obtained by densitometry of the digitized image.

Estimation of Processivity

The special design of the anchored reverse primer (RP) allows assessment of the processivity of telomerase in the sample extracts. The largest band of the ladder observed in the gel corresponds to the largest TS-extended product generated during the initial telomerase reaction. For a telomerase-extended product to be amplified by TS and RP primers, it must have at least 3 telomeric repeats (18). Therefore, the presence of n number of ladder bands on the gel indicates that the largest telomerase-extended products have n+3 telomeric repeats. For example, the 11 ladder bands observed in lanes 3 and 4 in Figure 3B, indicate that the largest extended product has at least 14 telomeric repeats.

Figure 3A: Quantitation Curve.

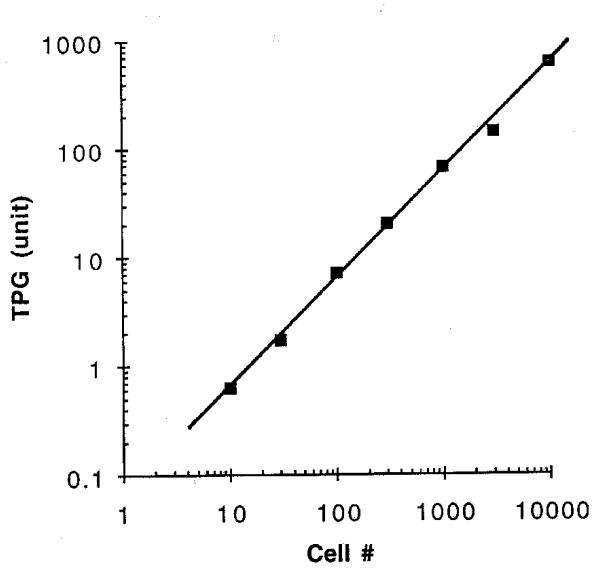
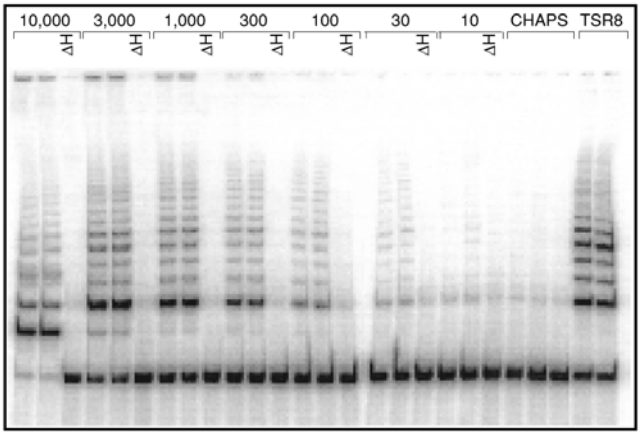


Figure 3B: Quantitation of Telomerase Activity.



TPG units for each reaction were calculated as described above (Section IV. Data Analysis: Quantitation). The TRAPEZE® Gel-Based Telomerase Detection Kit assay was performed with ^{32}P-ATP labeled TS primer using the following PCR conditions: 94˚C/30 seconds and 59˚C/30 seconds for 30 cycles. The amount of extract (expressed as cell number) present in telomerase-positive sample (duplicates), heat-treated controls (□H) and other controls is shown above each lane. The intensity of the TRAP product band and S-IC bands was determined using a PhosphorImager™.

Sensitivity and Specificity of the Assay

Sensitivity

Under the conditions described in this manual, the telomerase activity in an extract from 50 telomerase-positive control cells can be readily detected by either staining or radioisotopic detection methods. The number of PCR amplification cycles may be adjusted to further increase the sensitivity. However, this may increase the background.

Specificity

No telomerase activity was detected using the TRAPEZE® Kit in samples containing extract equivalent to 10,000 cells from the telomerase negative cell lines WI–38 and IMR–90 (not provided in the kit). See Ref. 15.

V. TROUBLESHOOTING

1) No visible products in any of the lanes, including S-IC internal control band and TSR8 PCR positive control lane.

Potential Problem: PCR amplification is not initiated.
Recommendations:

a. Check all the assay components. Were Taq Polymerase, dNTPs, all primers, proper buffer and efficiently-labeled TS primer included? Check the efficiency of end-labeling of TS primer.
b. Check the thermocycler for proper temperature and time settings. Is the thermocycler cycling at 94°C/30 seconds and 59°C/30 seconds for 27-33 cycles?
c. The optimal annealing temperature may need to be tested empirically (50°C to 60°C) for each temperature cycler.
d. Check Taq Polymerase to see if it is active.

2) Few (or no) TRAP-ladder bands are visible (poor processivity) in the sample lanes. The 36 base-long S-IC internal control band in these lanes shows reduced intensity. TRAP products and internal control are present in the telomerase-positive control lane (See Sec. III. Protocols, Experimental Design and Figure 4B).

Potential Problem: The cell/tissue extract contains an inhibitor of Taq polymerase.
Recommendations:

a. Dilute the extract 5-, 25-, and 125-fold with 1X CHAPS Lysis Buffer, then reperform the PCR. Sometimes, positive telomerase activity can be detected in the diluted extract that cannot be detected in more concentrated extracts.
b. Following the telomerase extension reaction, extract the mixture with phenol/chloroform/isoamyl alcohol (50/48/2) and then perform PCR.
c. Column purify the extract (32), then reanalyze.
d. To check for the presence of inhibitor(s), create a “mixed sample” by adding the telomerase-positive cell extract (prepared from cells provided in the kit) to the sample extracts and then perform the TRAPEZE® Kit assay. If inhibitor(s) of Taq polymerase are present in the sample extracts, the telomerase activity and/or processivity in the “mixed sample” will be decreased substantially as compared to those in the “telomerase-positive cell extract only” sample (Figure 4C).

3) No visible TRAP products in any of the sample lanes, including telomerase- positive control, but S-IC internal control band is present (Sec. III. Protocols, Experimental Design).

Potential Problem: Telomerase activity is not initiated. Possible presence of RNase contamination.
Recommendations:

a. Check for addition of all the assay components.
b. Always use RNase-free tips, tubes and solutions.
c. Use a fresh aliquot of dNTPs (no more than 5 freeze-thaw cycles), 10X TRAP Reaction Buffer, and DEPC-treated water.
d. Use a fresh aliquot of primers, taking extra precautions to prevent RNase contamination.

e. Make and use new 1X CHAPS Lysis Buffer, taking extra precautions to prevent RNase contamination.
f. Telomerase is extremely heat-sensitive; make sure that the extraction and TRAP reaction setup is performed below 25˚C.
g. Add RNase inhibitor into the 1X CHAPS Lysis Buffer (See Sec. III. Protocols: Extract Preparation).
h. Always use a clean labcoat and gloves. Clean the TRAPEZE® Kit station and telomerase extraction areas with bleach and alcohol.

4) TRAP products visible in all the lanes, including the primer-dimer/PCR contamination control (See Section III. Protocols: Experimental Design and Fig. 4.A).

Potential Problem: PCR carry-over contamination.
Recommendations:
a. Use fresh aliquots of every component of the assay (dNTPs, 10X TRAP Reaction Buffer, Taq, primers, and DEPC-treated water).
b. Follow the recommendations described in Problem C above. The PCR tube racks are a likely source of PCR carryover contamination.
c. Sterilize laboratory consumables used in TRAPEZE PCR set up, including pipettes, tips, and PCR tubes by UV cross linking. It is also advisable to use filter pipet tips.

5) Several bands (or a smear of multiple bands) are visible around 50-100 bp region in CHAPS Lysis Buffer only control lane and/or heat-inactivated sample lanes. See Fig. 4.D.

Potential Problem: Primer-dimer PCR artifacts. As is anticipated with a PCR-based assay, some unavoidable PCR artifacts are expected even when the optimal assay conditions are employed. Though occurring at a low frequency, these artifacts are most often observed in the primer-dimer/PCR contamination control lane (CHAPS Lysis Buffer-only) and/or occasionally in heat treated samples.
Recommendations:

a. Usually, the primer-dimer products that occur with TS and RP primers have a characteristic pattern with an intense first and second band around 50 bases, followed by weak 3rd to 6th bands, with the number of bands usually not exceeding 6. Occasionally, a smear of primer-dimer PCR artifacts may also occur around the 50-100 bp region. The band spacing of primer-dimer artifacts is not even as it is with genuine telomerase products. By observing the banding pattern, one can distinguish primer-dimer PCR artifacts from other problems such as PCR carry-over contamination. Therefore, even if PCR artifacts are observed in the primer-dimer/PCR control lane, valid interpretation of the TRAPEZE® Kit assay is still feasible. Follow the instructions in Sec. IV. Data Analysis.
b. Tumor extracts containing a relatively high protein concentration may produce PCR artifacts (Figure 4D). Dilute the extracts and repeat the analysis or, following the telomerase extension reaction, extract with phenol/chloroform/isoamyl alcohol.
c. Tumor extracts containing a relatively low protein concentration may also produce PCR artifacts. Supplement the 10X TRAP Reaction Buffer by adding BSA to a final concentration of 0.5 - 1.0 µg/mL.
d. A “hot start” may be incorporated into the PCR reaction. This may be accomplished by assembling the reaction mixture as directed, except that the Taq polymerase is excluded. After the 30 minute telomerase extension incubation, the reaction is heated at 94˚C for 2 minutes

The Taq is added after the 2 minute incubation. The procedure continues with the normal PCR cycling conditions.

6) Products in the heat-treated extracts.

Potential Problem: Insufficient heat inactivation of the extracts, primer-dimer PCR artifacts or contamination of the extract with TRAP products.
Recommendations:

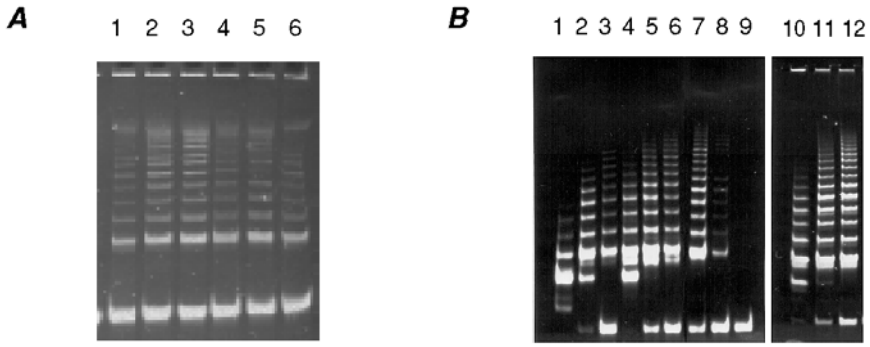
a. Check the temperature of the heat block or water bath used for heat inactivation of the extract.
b. Repeat the assay. If the problem persists, it is likely that the extract has PCR carry-over contamination.
c. Make and test the new extract, taking extra precaution to prevent PCR carry-over contamination.
d. A possible source of PCR contamination is the TSR8 quantitation control. Take extreme precautions when pipetting from the stock tube.
e. Avoid sample carry-over while loading adjacent lanes. If an unheated positive extract spills into the adjacent lane containing heat-treated extract, telomerase products will be visible.
f. Tumor extracts containing a relatively high protein concentration may produce PCR artifacts. Either dilute the extract and repeat or following the telomerase extension reaction, extract with phenol/chloroform/isoamyl alcohol.

7) Extra bands are visible between the 36 bp internal control band and the TRAP ladder bands in the sample lanes. These bands are not observed in the telomerase-positive control lane (assuming the reaction is performed using extract from less than 500 cells). The intensity of S-IC internal control band is very weak.

Potential Problem: Telomerase activity in the sample extracts is too high.
Recommendations:

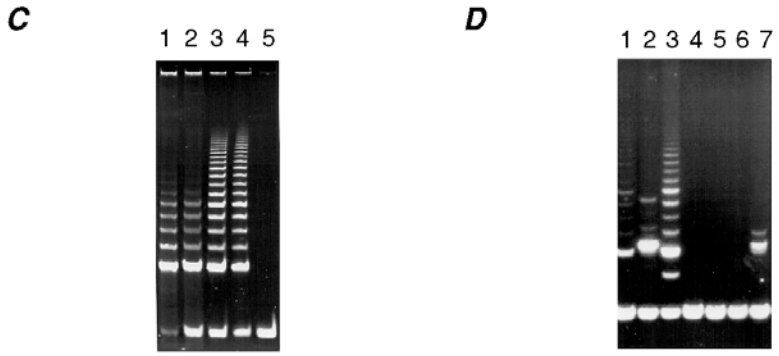
a. Dilute the sample extracts (eg. 1:10) and reanalyze.
b. The extra band does not affect overall detection of telomerase activity.

Figure 4: Troubleshooting Examples



A. PCR contamination.
Lane 1: 1X CHAPS Lysis Buffer; Lanes 2-5: extract equivalent to 1,000 (duplicate), 100 (duplicate) telomerase positive cells; Lane 6: heat-treated extract equivalent 1,000 cells. Note the appearance of the TRAP ladder in lanes 1 and 6, the negative control lanes.

B. TRAPEZE® Kit assay with samples that contain Taq polymerase inhibitors.
Lanes 1-3: 1 µg, 0.2 µg and 0.04 µg of mouse spleen extract; Lanes 4-6: 1 µg, 0.2 µg and 0.04 µg of mouse testes extract; Lanes 7-8: telomerase-positive control cell extract equivalent to 100 and 10 cells; Lane 9: CHAPS Lysis Buffer control; Lanes 10-12: 4 µg, 1 µg and 0.25 µg of colon cancer extract. The 36 bp internal control (S-IC) is not visible in lanes 1, 5, and 10, indicating the presence of PCR inhibitors. In all three types of extract, the processivity and intensity of the TRAP products increase as the more dilute samples are used in the assay (lanes 1-3, 4-6, 10-12).



C. Decreased processivity due to Taq polymerase inhibitor(s) in a "mixed" sample.
Lane 1: Inhibited "mixed" sample consisting of extract equivalent to 1000 telomerase positive cells plus 5 µg of mouse testes extract; Lane 2: Inhibited "mixed" sample consisting of extract equivalent to 1000 telomerase positive cells plus 5 µg of heat-treated mouse testes extract; Lanes 3-4: extract equivalent to 1000 telomerase positive cells; Lane 5: CHAPS Lysis Buffer control. Note that the addition of extract containing PCR inhibitors to an extract made from telomerase positive cells results in a reduction both in the processivity of TRAP ladders and the amplification of the internal control (lanes 1 and 2).

D. Primer-dimer PCR artifacts.
Lanes 1 and 3: telomerase positive cell extract: Lanes 2, 4-7: CHAPS Lysis Buffer controls. Lanes 2 and 7 contain primer-dimer artifacts, which are clearly distinguishable from the laddering pattern resulting from genuine telomerase activity (lanes 1 and 3).

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