TRAPEZE® XL Telomerase Detection Kit

S7707

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I. INTRODUCTION

Using this Manual

This manual accommodates both the novice and the experienced TRAPEZE® XL Telomerase Detection Kit (Cat #S7707) user. These protocols are presented in a streamlined manner. However, users are directed to sections that provide supplemental information by notations in the protocol. The novice user is advised to read the entire manual prior to using the TRAPEZE® XL Kit, particularly Sec. III. *Protocols*, *Experimental Design*. Directions for preparing some of the required reagents can be found in Sec. V. *Appendix*. Should additional questions arise, assistance is available from Millipore Technical Service at (800) 437-7500 or techserv@Millipore.com.

Background

Telomeres are specific structures found at the end of chromosomes in eukaryotes. In human chromosomes, the telomeres consist of thousands of copies of 6 base repeats (TTAGGG)(1-3). It has been suggested that telomeres protect chromosome ends since damaged chromosomes lacking telomeres 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 synthesize 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 the number of cell divisions and eventually signal replicative senescence or programmed cell death when a critical telomere length is achieved. 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).

The development of 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 TRAPEZE® XL Kit is a highly sensitive *in vitro* assay for the fluorometric detection of telomerase activity. It incorporates refinements to the original TRAP assay that were first introduced in the gel-based TRAPEZE® Telomerase Detection Kit (Cat #S7700). As in the original TRAPEZE® Kit, primer sequence modifications that reduce amplification artifacts and an internal PCR control are included. In addition, the TRAPEZE® XL Kit uses fluorescence energy transfer (ET) primers to generate fluorescently labeled TRAP products which permit nonisotopic, quantitative analysis of telomerase activity.

The unique design of these ET primers (Amplifluor® primers) allows detection and quantification of telomerase activity by directly measuring fluorescence emission in the reaction vessels. Since Amplifluor® primers will fluoresce only upon incorporation into the TRAP products or the internal control, post–PCR sample manipulations such as electrophoretic gel or ELISA analyses are eliminated, thereby reducing the the risk of carry-over contamination. Quantitative analysis is not compromised when detection is performed in a high-throughput 96-well format unlike platforms utilizing a qualitative ELISA. In addition, inclusion of an internal control labeled with a second fluorophore serves to both monitor PCR amplification and aid in the quantitation of telomerase activity.

Principles of the Technique

The Amplifluor® Primer System

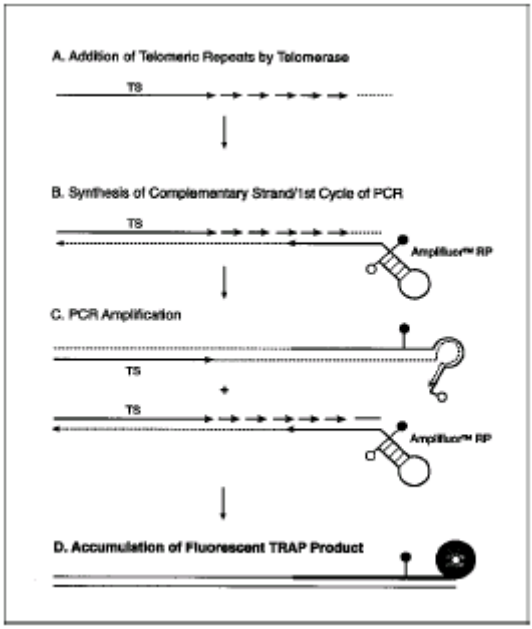
The fluorometric detection of telomerase activity by the TRAPEZE® XL Kit is accomplished through the use of Amplifluor® primers. This patented technology (33) is based on the concept of energy transfer (ET), a process whereby energy shifted from an excited fluorophore to an acceptor moiety results in quenching of the fluorescence emission. In order for this quenching to occur, the donor and acceptor molecules must be in close physical proximity to each other. Amplifluor® primers, which contain energy transfer moieties, are designed to emit a fluorescence signal only when they are incorporated into PCR products. Therefore, the net increase of fluorescence in the reaction vessel directly correlates to the amount of amplified DNA produced in the reaction.

Amplifluor® primers consist of a 3' end sequence complementary to the target sequence and a 5' end hairpin structure. The fluorophore (energy donor) and the quencher DABSYL (4-(dimethylamine)azo benzene sulfonic acid) are in close proximity within the 5’ hairpin (Figure 1, line B). As the primer is incorporated into a double-stranded PCR product, the hairpin is unfolded through the activity of the polymerase (Figure 1, line C). In this extended conformation, the distance between the fluorophore and quencher is increased and a fluorescence signal is generated (Figure 1, line D).

TRAPEZE® XL Telomerase Detection Kit Assay with Amplifluor™ Primers

The TRAPEZE® XL Kit is a one buffer, two enzyme system utilizing polymerase chain reaction (PCR) and Amplifluor® primers (34). In the first step of the reaction (Figure 1, line A), the telomerase enzyme adds a number of telomeric repeats (GGTTAG) onto the 3' end of a substrate oligonucleotide (TS). In the next steps (Figure 1, line B and C), the extended products are amplified by the second enzyme, Taq Polymerase, using PCR with the TS and fluorescein-labeled Amplifluor® RP (reverse) primers. This generates a fluorescent ladder of products with 6 base increments starting at 61 nucleotides: 61, 67, 73, 79, etc. The fluorescence emission produced is directly proportional to the amount of TRAP products generated (Figure 1, line D).

Figure 1: TRAPEZE® XL Telomerase Detection Kit Assay Scheme



Each short arrow on line A symbolizes a 6 nucleotide-long telomeric repeat (GGTTAG). The small filled circles represent DABSYL while the open circles represent the fluorophore quenched by DABSYL. The large circle on line D depicts unquenched fluorescence emission.

In addition to the Amplifluor® RP primer, the 5X TRAPEZE® XL Reaction Mix contains a sulforhodamine-labeled Amplifluor® K2 primer. The K2 primer and the TS primer are involved in the semi-competitive amplification of the internal control template TSK2 which generates a 56 bp product. The excitation/emission wavelengths of sulforhodamine are sufficiently different from those of fluorescein to allow the simultaneous amplification and detection of TRAPEZE® XL assay products (green) and the internal control (red) in a single reaction vessel. The TSK2 control template is used for quantitation of telomerase activity (see Sec. III. *Protocol, Experimental Design*) and as a positive control for PCR amplification.

The fluorescence generated by the TRAPEZE® XL Kit products may be measured by a variety of methods. Ideally, the reactions are measured directly in PCR tubes using a fluorescence plate reader. The reactions may also be transferred to a 96-well plate or a cuvette for measurement in a plate reader or in a spectrofluorometer, respectively. Alternatively, the reactions can be subjected to non-denaturing gel electrophoresis and then visualized using a fluoroimaging system. Each of these detection methodologies produce informative and quantitative telomerase activity measurements. An additional option involves the visualization of the TRAP ladder following non-denaturing polyacrylamide gel electrophoresis and staining with an intercalating. Although not quantitative, the presence or absence of the signature telomerase ladder confirms the fluorescence measurement results (see Sec. III. *Protocol; Sensitivity, Specificity and Dynamic Range of the Assay*) .

II. KIT COMPONENTS

The kit provides enough reagents to perform 112 TRAPEZE® XL assays. With these reagents, 40 experimental samples and the appropriate controls can be analyzed (for details of the experimental design, see Sec. III. *Protocol, Experimental Design*).

Table 1: TRAPEZE® XL Telomerase Detection Kit

Description	Storage Conditions
1. CHAPS Lysis Buffer (13.5 mL)	- 25°C to 8°C
2. 5X TRAPEZE® XL Reaction Mix (1.12 mL) TS primer RP Amplifluor® primer K2 Amplifluor® primer TSK2 template dA, dC, dG and dTTP diluted in : 100 mM Tris-HCl, pH 8.3 7.5 mM $MgCl_2$ 315 mM KCl 0.25% Tween 20 5 mM EGTA 0.5 mg/mL BSA	- 15°C to -85°C
3. PCR - Grade Water (8.2 mL) protease, DNase, and RNase-free; deionized	2°C to 8°C
4. TSR8* (control template) (45 µL) 0.2 amole/µL TSR8 template	-15°C to -25°C
5. Control Cell Pellet Telomerase positive cells (10^6 cells)	-75°C to -85°C

** Caution – refer to Sec. II.* Kit Components, Precautions.

Materials Required But Not Supplied in the Kit

Equipment and Supplies

1. Thermocycler
2. Spectrofluorometer (Option 1)
3. Fluorescence plate reader (Option 2) with appropriate filters for fluorescein and sulforhodamine detection (See Sec. V. *Appendix, Excitation and Emission Filters*)
4. Optically clear tubes for PCR amplification and detection, a holder for PCR tubes, or a 96-well plate if using a fluorescence plate reader (Option 2)
5. If analyzing tissues, homogenization equipment as described in Sec. III. *Protocol, Extract Preparation*
6. Tubes for PCR amplification and detection
7. Aerosol resistant pipette tips (RNase-free)

Reagents

1. Taq polymerase (cloned, unmodified)
2. PBS (Mg^{2+}- and Ca^{2+}-free)
3. Reagents for protein concentration measurement (See Sec. V. *Appendix, Determination of Protein Concentration*)
4. RNase inhibitor (for extract preparation from tissues)
5. Buffer used with the analytical spectrofluorometer (Option 1) (See Sec. III. *Protocol, TRAPEZE® XL Telomerase Detection Kit Assay*)
6. Bovuminar® Bovine Serum Albumin

Precautions

1. Because the TRAPEZE® XL Telomerase Detection Kit detects the activity of telomerase, a RNase sensitive ribonucleoprotein, and not merely the presence of the RNA or protein components of telomerase, the assay requires enzymatically active cell or tissue samples. Furthermore, due to the sensitivity of the TRAPEZE® XL Kit assay, which can detect telomerase activity in a very small number of cells, a special laboratory setup and significant precautions are required to prevent PCR carry-over contamination and RNase contamination. These precautions are discussed in detail in Sec. V. *Appendix, Laboratory Setup and Precautions* and *TRAPeze® XL Telomerase Detection Kit Station Setup* (Area 1).

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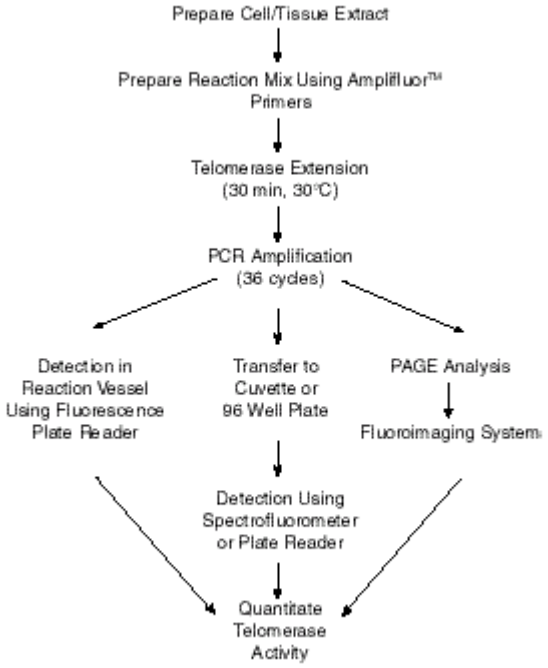
III. PROTOCOL

Figure 2: TRAPEZE® XL Telomerase Detection Kit Flow Chart



Extract Preparation

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

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

1. Pellet the cells or tissue, wash once with PBS, repellet, and carefully remove all PBS. After removal of PBS, the cells or tissue pellet can be stored at -85°C to -75°C or kept on dry ice. Telomerase in frozen cells or tissues is stable for at least 1 year at -85°C to -75°C. When thawed for extraction, the cells or tissue should be resuspended immediately in CHAPS Lysis Buffer (step 2).

2a. Cells

Resuspend the cell pellet in 200 µL of CHAPS Lysis Buffer/10^5-10^6 cells. (Also use 200 µL of CHAPS Lysis Buffer for the preparation of the positive control cell extract in the kit.) Proceed to Step 3.

2b. Tissues

Prepare the extract according to one of the methods described below. Use 200 µL of 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 obtained. Transfer the sample to a sterile 1.5 mL microcentrifuge tube, and add 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 it in an appropriate amount of CHAPS Lysis Buffer.

Connective Tissues - Mechanical Homogenizer: Mix the tissue sample with an appropriate volume of 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 160 µL of the supernatant into a fresh tube and determine the protein concentration. See Sec. V. *Appendix, Determination of Protein Concentration.*

Table 2: Sample Concentration and Quantity for Assay

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 and quick-freeze the remaining extract on dry ice*, and store at -85°C to -75°C. The extract is stable for at least 12 months when stored at -85°C to -75°C.

***Note:** *The extracts for the TRAPEZE® XL Kit 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. In addition, aliquoting reduces the risk of contamination.*

Experimental Design

For a valid analysis of the results, two factors need to be considered: (1) appropriate controls and (2) the amount of cell/tissue extract to be used in each reaction.

Controls

For each sample

Telomerase is a heat-sensitive enzyme. As a negative control, every sample extract to be evaluated should also be tested for heat sensitivity. Thus, analysis of each sample consists of two assays: one with a test extract and one with a heat-treated test extract. Heat treat 10 µL of each sample by incubating at 85°C for 10 minutes prior to the TRAPEZE® XL Kit assay to inactivate telomerase.

For each set of TRAPEZE® XL Kit assays:

1. Generation of TSR8 Standard Curve:

   Perform the TRAPEZE® XL Kit assay using dilutions of TSR8 (control template) instead of the sample extract to generate a standard curve. TSR8 is an oligonucleotide with a sequence identical to the TS primer extended with 8 telomeric repeats AG(GGTTAG)$_7$. This standard curve permits the calculation of the amount of TS primers with telomeric repeats extended by telomerase in a given extract.

   The stock TSR8 (control template) concentration provided within the kit is 0.2 amoles/µL. Prepare 1:5 serial dilutions of the stock concentration with CHAPS Lysis Buffer to obtain TSR8 concentrations of 0.04 amoles/µL, 0.008 amoles/µL and 0.0016 amoles/µL. Perform the TRAPEZE® XL Kit assay using 2 µL of each TSR8 dilution including the 0.2 amoles/µL stock concentration. The TSR8 dilutions can be stored at 4°C for at least 2 weeks.

   Table 3: Dilution of Control Template TSR8

TSR8 Concentration	Volume of TSR8 Stock	Volume of Diluent CHAPS Lysis Buffer
1. 0.2 amoles/µL	No dilution	-
2. 0.04 amoles/µL	5 µL of #1	20 µL
3. 0.008 amoles/µL	5 µL of #2	20 µL
4. 0.0016 amoles	5 µL of #3	20 µL

2. Telomerase Positive Extract Control (control 1)

   Make a telomerase-positive cell extract using 200 µL of CHAPS Lysis Buffer and the control cell pellet (10^6 cells) provided in the kit. Aliquot the lysate and store at -75°C to -85°C. Dilute the stock aliquots 1:10 with CHAPS Lysis Buffer before use and dispense 2 µL per TRAPEZE® XL Kit assay (2 µL = 1000 cells). Run one positive control reaction for each set of TRAPEZE® XL Kit assays.

3. Minus Telomerase Control (control 2)

   Perform a TRAPEZE® XL Kit assay with 2 µL CHAPS Lysis Buffer substituted for the cell/tissue extract. The fluorescein emission in this control is subtracted from the fluorescein signal in the sample when determining the net fluorescence increase of each sample (see Sec. III. *Protocol, Data Analysis*).

If the assay worked optimally, only a sulforhodamine signal should be observed from the 56 bp internal control in the minus telomerase control reaction. The detection of fluorescein labeled products in the minus telomerase control reaction suggests either: 1) the presence of primer-dimer PCR artifacts due to suboptimal PCR conditions; 2) the presence of PCR contamination (amplified TRAPEZE® XL products) carried over from another assay; or 3) the contamination of an assay component with the telomerase positive cell extract (see Sec. IV. *Troubleshooting*).

4. Minus Taq Polymerase Control (control 3)

   Perform the TRAPEZE® XL Kit assay excluding the Taq polymerase. The sulforhodamine emission signal in this control is subtracted from the sulforhodamine signal from the sample when determining the net fluorescence increase of each sample (see Sec. III. *Protocol, Data Analysis*).

5. PCR Amplification control (Internal control - included in each assay by default)

   An important feature of the TRAPEZE® XL Kit is the inclusion in each assay of an internal standard to monitor PCR inhibition in every reaction. Many cell/tissue extracts contain inhibitors of Taq polymerase, and thus give potentially false-negative results. To distinguish this from other possibilities, the 5X TRAPEZE® XL Reaction Mix contains the K2 Amplifluor® primer (red), the TS primer and the TSK2 template. These primers and template produce a detectable 56 bp sulforhodamine amplification product that serves as a control for amplification efficiency in each reaction and is used for quantitative analysis of the TRAPEZE® XL products (see Sec. III. *Protocol, Data Analysis*).

Assay Design

The TRAPEZE® XL Telomerase Detection Kit is designed for the successful analysis of 40 experimental samples in 4 separate experiments. Supposing 10 experimental samples (n) are analyzed at a time, each assay would consist of 27 PCR reactions (2n+7).

Tubes 1-20:	10 experimental samples alternating test extracts and the heat inactivated controls
Tubes 21-24:	4 TSR8 quantitation controls
Tube 25:	**1000 cell equivalents** of telomerase positive extract control (Control 1)
Tube 26:	The minus telomerase control (Control 2)
Tube 27:	The minus Taq polymerase control (Control 3)

TRAPEZE® XL Telomerase Detection Kit Assay

Assay Setup

1. Prepare a "Master Mix" by mixing the reagents outlined below **except** for the extract. Thaw all reagents, mix well and store on ice.

   The amount of reagents required for each assay reaction is:

5X TRAPEZE® XL Reaction Mix*	10.0 µL
Taq Polymerase (5 units/µL)	0.4 µL (2 Units)
dH_20	37.6 µL
	48.0 µL
Cell Extract (10 - 750 ng/µL) OR Tissue Extract (10 - 500 ng/µL)	2.0 µL (of either)
TOTAL VOLUME	50.0 µL

**Upon first use, make aliquots of 5X TRAPEZE® XL Reaction Mix, which can be freeze-thawed no more than 5 times.*

To determine the total number of reactions to be run in the assay, refer to Sec. III. *Protocol, Experimental Design*. Typically, for analysis of **n** number of sample extracts, **2n+7** reactions are necessary. Multiply the volume of each reagent listed above (except cell/tissue extract) by **2n+8** and mix them in a sterile tube (this "Master Mix" will contain extra reagent for pipetting variances). **Before adding Taq polymerase to the "Master Mix," aliquot 47.6 µL for the Minus Taq Polymerase Control.**

2. Aliquot 48 µL of the Mix containing Taq polymerase into 2n+6 RNase-free PCR tubes.
3. Add 2 µL of test extracts, heat-inactivated extracts or controls into each tube:
   a. Sample extracts: add 2 µL to each of the sample tubes.
   b. Heat-inactivated controls: incubate 10 µL of each sample extract at 85°C for 10 minutes. Add 2 µL into each of the heat-inactivation control tubes.
   c. TSR8 quantitation controls: add 2 µL of each TSR8 dilution including the stock concentration.

d. Telomerase positive extract control: add 2 µL of positive control cell extract at a concentration of 500 cells/µL.

e. Minus telomerase control: add 2 µL of CHAPS Lysis Buffer.

f. Minus Taq polymerase control: add 2.4 µL of CHAPS Lysis Buffer to the "Master Mix" not containing Taq polymerase.

Note: *The order of steps b and c may be reversed.*

PCR Amplification

1. Place the tubes in the thermocycler block, and incubate at 30°C for 30 minutes.

2. In a thermocycler, perform a 4-step PCR at 94°C / 30 seconds, 59°C / 30 seconds, 72°C / 1 minute for **36 cycles** followed by a 72°C / 3 minute extension step and then at 55°C / 25 minutes, concluding with a 4°C incubation**.**

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

Fluorescence Measurements

Option 1 - Spectrofluorometer

The yield of the PCR reaction may be determined by placing a diluted aliquot of the completed reaction in a cuvette and measuring the fluorescence in a spectrofluorometer.

1. Prior to measurement in the spectrofluorometer, 20 µL of each reaction mix is diluted to 600 µL with 10 mM Tris-HCl pH 7.4, 0.15 M NaCl and 2 mM $MgCl_2$.

2. The fluorescence of each sample is measured in a 10 x 3 mm cuvette after setting the excitation/emission parameters for fluorescein (495 nm/516 nm) and sulforhodamine (600 nm/620 nm).

Option 2 - Fluorescence Plate Readers

The PCR reactions may be performed in tubes designed to fit into holders that are compatible with fluorescent plate readers. It is essential that optically clear PCR tubes be used when the PCR reactions will be measured directly in the tube. Also, the dimensions of the chamber that holds the plate in the plate reader must be sufficient to accommodate capped tubes placed in the microtube tray/retainer.

1. Place the PCR reaction tubes in the plate reader holder.
2. Measure the fluorescein and sulforhodamine emission utilizing appropriate emission and excitation filters (see Sec. V. *Appendix, Excitation and Emission Filters*).

   Note: *If direct measurement in the PCR tube is not possible, the reaction may be transferred to an appropriate 96-well plate. If this is necessary, transfer all of the reaction into a well containing 150 µL of the buffer described above in Option 1. A completely black 96-well plate gives optimum performance.*

Data Analysis

The following is an example of telomerase activity quantification using fluorometric detection. To obtain valid quantitative results using the TRAPEZE® XL Kit, the production of a standard curve using the TSR8 template and the inclusion of the assay controls are necessary (see Sec. III. *Protocol, Experimental Design*).

Table 4: Data for the Generation of the TSR8 Standard Curves (Figures 3A and 3B)

1	2	3	4	5	6	7	8	9
R8 (amole/Rx)	**R8 (TPG Unit)**	**log[TPG]**	**FL (RFU)**	**ΔFL**	**R (RFU)**	**ΔR**	**ΔFL/ΔR**	**log [ΔFL/ΔR]**
0.4	400	2.602	54228	45691	17301	3838	11.905	1.076
0.4	400	2.602	54010	45475	18193	4730	9.614	0.983
0.08	80	1.903	42024	33489	21438	7975	4.199	0.623
0.08	80	1.903	47155	38620	23456	9993	3.865	0.587
0.016	16	1.204	23562	15027	30588	17125	0.877	-0.057
0.016	16	1.204	25675	17140	30750	17287	0.991	-0.004
0.0032	3.2	0.505	14485	5950	39415	25952	0.229	-0.640
0.0032	3.2	0.505	16391	7856	40558	27095	0.290	-0.538
Control 2 (-telomerase)			9792	0	47853	34390		
			7278		41930	28467		
	mean		**8535**					
Control 3 (-Taq pol)			7702		13098	0		
			9740		13828			
	mean				**13463**			

Fluorescein (FL) and sulforhodamine (R) emission data used for the generation of the TSR8 standard curves (Figures 3A and 3B). The relative fluorescence units (RFU) were determined by direct measurement of the TRAPeze® XL reaction in the PCR tubes using a fluorescence plate reader.

1. Calculate the net increase of fluorescein (ΔFL) and sulforhodamine (ΔR) emission in each reaction by subtracting the fluorescein emission from control 2 (minus telomerase control) and sulforhodamine emission from control 3 (minus Taq polymerase), respectively. (Refer to Table 1: Columns 5 and 7).

Option 1 (Linear Plot)

a. Calculate the $\log_{10}$ of the relative ratio of net fluorescence increase (ΔFL/ΔR) for each reaction. (Refer to Table 1: Column 9)

b. Generate a standard curve based on the measurements of the reactions that were performed with the TSR8 dilutions. Plot the $\log_{10}$ (ΔFL/ΔR) on the Y axis against the $\log_{10}$ value of the corresponding concentration of TSR8 (expressed as TPG units; refer to Table 4: Column 2) on the X axis. (See Figure 3A).

***Note:** 1 amole of TSR8 = 1,000 TPG units*

Option 2 (Logarithmic Plot)

a. Calculate the relative ratio of net fluorescence increase (ΔFL/ΔR) for each reaction. (Refer to Table 4: Column 8)

b. Generate a standard curve based on the measurements of the reactions that were performed with the TSR8 dilutions. Plot the (ΔFL/ΔR) on the Y axis against the corresponding concentration of TSR8 (expressed as TPG units) on the X axis. Use a logarithmic scale for both the X and Y axes. (See Figure 3B).

c. Obtain the TPG value for each sample from the standard curve using the respective ΔFL/ΔTR ratio.

Each unit of TPG (Total Product Generated) corresponds to the number of TS primers (in 1 x 10_{-3} amoles or 600 molecules) extended with at least 3 telomeric repeats by telomerase in the extract in a 30 minute incubation at 30°C. The assay has a linear range of 2 to 400 TPG, which is equivalent to telomerase activity from approximately 30 to 6,000 control cells.

Figure 3A: Linear Plot of the TSR8 Standard Curve

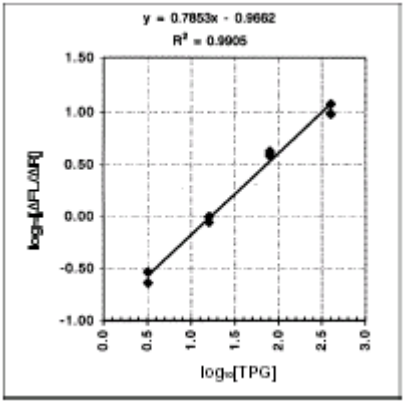
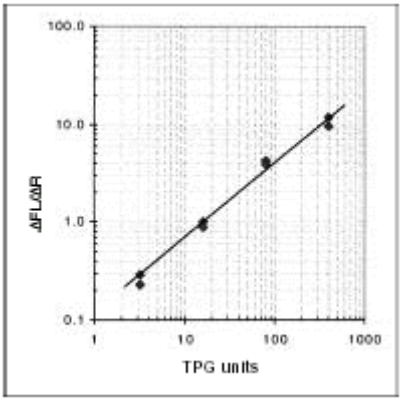


Figure 3B: Logarithmic Plot of the TSR8 Standard Curve



Sensitivity, Specificity and Dynamic Range of the Assay

Sensitivity

Under the conditions described in this manual, telomerase activity in an extract from 100 telomerase-positive control cells can be detected (Figure 4A) using a spectrofluorometer (see Sec. III. *Protocol, TRAPEZE® XL Telomerase Detection Kit Assay*). The positive fluorescence signal correlates with the signature TRAP ladder visualized by non-denaturing PAGE analysis of the samples (Figure 4B). The 56 bp band internal control is observed in every sample including the minus telomerase control.

Notes: *The assay sensitivity may be reduced depending on the instrument utilized. When a 96-well format fluorescence plate reader is used for direct measurement of the reaction mix, the background fluctuation between PCR tubes, and therefore samples, may be higher than that obtained when measuring samples using a cuvette in a spectrofluorometer. This may reduce the sensitivity of the assay since the minimum sensitivity strictly depends on the signal/noise ratio.*

The number of PCR amplification cycles may be increased to enhance the sensitivity, but this may increase the background and alter the profile of the standard curve.

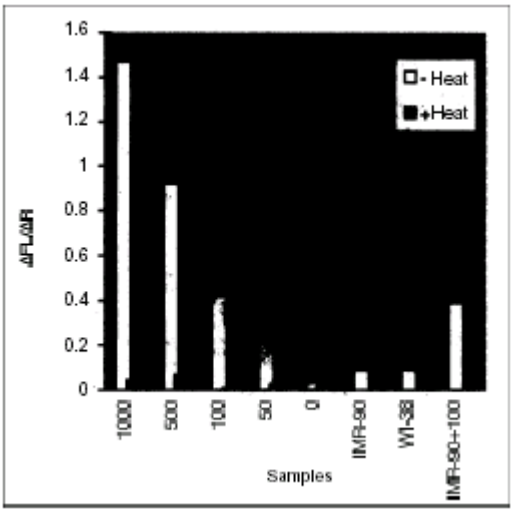
Specificity

No significant telomerase activity was detected using the TRAPEZE® XL Kit in samples containing extract equivalent to 10,000 cells from fibroblast cell lines WI-38 and IMR-90 (ATCC) (Figures 4A and 4B).

Dynamic Range

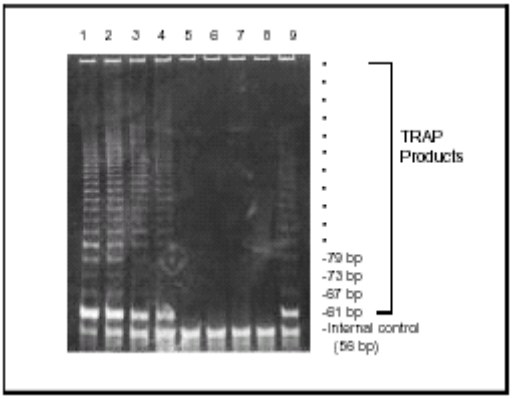
Under optimal assay conditions, a linear standard curve extending over two orders of magnitude can be obtained. (See Figures 3A and 3B).

Figure 4A: Comparison of Telomerase Activity in Various Cell Extracts



The TRAPEZE® XL Kit assay was performed using varying amounts of extract made from telomerase-positive or telomerase-negative cells. Fluorescence was measured using a spectrofluorometer. Columns 1000 through 0 represent the results obtained with telomerase positive cell extracts equivalent to 1000, 500, 100, 50, and 0 cells, respectively. With telomerase-negative cell lines, IMR-90 and WI-38, extracts equivalent to 10,000 cells were used. The ΔFL/ΔR values obtained for IMR-90 or WI-38 indicates that these cell lines express less than 0.5% of the telomerase activity detected with the equivalent number of telomerase-positive cells. The final column (IMR-90 + 100) represents the results obtained from IMR-90 cell extract spiked with telomerase positive extract. The absence of PCR inhibition in the spiked IMR-90 cell extract indicates that the lack of telomerase activity in the extract was not caused by PCR inhibition.

Figure 4B: PAGE Analysis of TRAPEZE® XL Telomerase Detection Kit Products



Aliquots (20 µL) of the TRAPEZE® XL reactions described previously were subjected to electrophoresis on a non-denaturing 10% polyacrylamide gel. Lanes 1-4: reaction products using cell extracts equivalent to 1000, 500, 100 and 50 telomerase-positive cells respectively. Lane 5: minus telomerase control. Lane 6: heat-treated cell extract (1000 cells). Lane 7: IMR-90 Lane 8: WI-38 Lane 9: IMR-90 spiked with telomerase-positive cell extract (100 cell equivalent). After electrophoresis, the gel was stained with SYBR® Green I and the image was recorded with a CCD camera imaging system. The 56 bp band present in every lane is the internal control. Upon excitation with a transilluminator, the internal control band appears pink due to the combination of the red fluor and the green stain. The bands of the signature TRAP ladder are green. The length of the shortest band in the ladder is 61 bp.

IV. TROUBLESHOOTING

The following are the most commonly encountered problems with the TRAPEZE® XL Kit assay.

? No significant increase in fluorescence signal (fluorescein/sulforhodamine; ΔFL/ΔR) is observed for the TSR8 quantitation controls and telomerase-positive samples.

1. Potential problem: PCR amplification is not initiated.

Recommendations:

a. Check the TRAPEZE® XL Kit assay components. Were the 5X TRAPEZE® XL Reaction Mix and the Taq polymerase included in the appropriate amounts?
b. Check the thermocycler for proper temperature and time settings. Is the thermocycler cycling at 94°C / 30 seconds, 59°C / 30 seconds and 72°C / 1 minute for 36 cycles?
c. The optimal annealing temperature may need to be tested empirically (53°C to 59°C) for each thermocycler.
d. Check the Taq polymerase to see if it is active. Confirm that a cloned, unmodified Taq polymerase was used.

2. Potential problem: Measurement parameters chosen for either the spectrofluorometer or the fluorescent plate reader are incorrect.

Recommendations:

a. Check the wavelength settings for the spectrofluorometer. Consult the instruction manual of the instrument for the required settings of additional assay parameters.
b. Check the excitation and emission filters for the plate reader. Are the filters appropriate for measuring fluorescein and sulforhodamine? Are all the necessary machine parameters set? Is the aperture set at the appropriate width? For recommendations on emission and excitation filters see Sec. V. *Appendix, Excitation and Emission Filters*.

3. Potential Problem: Optically clear tubes were not used for measurements with the fluorescent plate reader.

Recommendations:

a. Perform the TRAPEZE® XL Kit assay with optically clear amplification tubes and caps.
b. Transfer PCR reactions to optically clear tubes or to a 96-well microtiter plate. (see Sec. III. *Protocol, TRAPEZE® XL Telomerase Detection Kit Assay).*

? Fluorescein signal of the telomerase-positive control cell extract is low but sulforhodamine emission is acceptable.

1. Potential problem: Telomerase activity is not initiated. Possible RNase contamination exists if a positive fluorescence signal is only observed with the telomerase-independent TSR8 quantitation controls.

Recommendations:

a. Always use RNase- free tips, tubes and solutions.
b. Use a fresh aliquot of 5X TRAPEZE® XL Reaction Mix, taking extra precautions to prevent RNase contamination.
c. Telomerase is heat-sensitive; make sure that the extraction and TRAPEZE® XL reaction setup is performed at a temperature below 25°C.
d. Add RNase inhibitor into the CHAPS Lysis Buffer (see Sec. III. *Protocol, Extract Preparation*), taking extra precautions to prevent RNase contamination.
e. Always use a clean labcoat and gloves. Keep the TRAPEZE® XL Telomerase Detection Kit Station Setup (Area 1) and telomerase extraction areas clean with bleach and alcohol. (See Sec. V. *Appendix, TRAPEZE® XL Telomerase Detection Kit Station Setup (Area 1)*).

? All reaction samples and the minus telomerase control (control 2) show a positive fluorescein signal.

1. Potential problems: PCR carry-over contamination.

Recommendations:

a. Use fresh aliquots of every component of the assay (5X TRAPEZE® XL Reaction Mix, Taq polymerase and PCR grade water).
b. Follow the recommendations described in Problem B above and in Sec. V. *Appendix, Laboratory Setup and Precautions*. The PCR tube racks are the most likely source of PCR carry-over contamination. Decontaminate the racks as described in Sec. V. *Appendix, TRAPEZE® XL Telomerase Detection Kit Station Setup (Area1).*

? A high fluorescein signal is observed with heat-treated extracts. Extracts are not heat sensitive.

Potential problems: Insufficient heat inactivation of the extracts, primer-dimer PCR artifacts, or contamination of the extract with TRAPEZE® XL 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 a PCR carry-over contamination.
c. Make and test the new extract, taking extra precaution to prevent PCR carryover contamination.
d. Tumor extracts containing a relatively high protein concentration may produce PCR artifacts. The presence of excess proteins in the reaction mixture may result in non-specific PCR amplification products. Dilute the extracts and repeat the analysis.

? The data on duplicate samples are inconsistent.

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 reactions with minimal telomerase activity. Inconsistent reactions are readily distinguishable with high signals observed for both fluorescein and sulforhodamine.

Recommendations:

a. Recheck the PCR parameters and repeat the assay.
b. Decrease the number of PCR cycles to 33-34 cycles.
c. Analyze the TRAPEZE® XL reaction products by non-denaturing PAGE (optional). Primer/dimer artifacts are easily distinguishable from genuine telomerase products after samples have undergone electrophoresis through a polyacrylamide gel. For details of the nondenaturing PAGE procedure, see Sec. V. *Appendix, PAGE Analysis (Optional).*
d. Tumor extracts containing a relatively high protein concentration may produce PCR artifacts. The presence of excess proteins in the reaction mixture may result in non-specific PCR amplification products. Dilute the extracts and repeat the analysis.

? Minimal TRAPEZE® XL Kit assay signals observed for both fluorescein and sulforhodamine with samples where telomerase activity is anticipated.

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

Recommendations:

a. Dilute the extract 5-, 25- and 125- fold with CHAPS Lysis Buffer, then reanalyze. Sometimes, positive telomerase activity can be detected in the diluted extract that cannot be detected in more concentrated extracts.
b. If higher sensitivity is required with diluted extract refer to Sec. V. *Appendix, Enhancing Detection Sensitivity*.
c. 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® XL Kit assay. If inhibitor(s) of Taq polymerase are present in the sample extracts, the signal observed in the "mixed sample" will be decreased substantially compared to those in the "telomerase-positive cell extract only" sample.

2. Potential Problem: Possible presence of RNase in the sample extracts.

Recommendations:

a. Refer to Problem #2 above.

V. APPENDIX

Laboratory Setup and Precautions

One of the most important considerations when performing the TRAPEZE® XL Kit assay is the environment where the initial reaction mixtures are set up. The ideal environment is free of contaminating ribonucleases and amplified PCR DNA products, which can cause false-negative and false-positive results, respectively.

Some sources of PCR product contamination are:

1. gel box and buffer
2. contaminated pipettes and tips
3. tube racks
4. notebooks
5. lab coats
6. any other item exposed to amplified PCR products.

Some sources of RNase contamination are:

1. solutions and tubes not treated with an RNase inhibitor
2. any equipment handled without gloves.

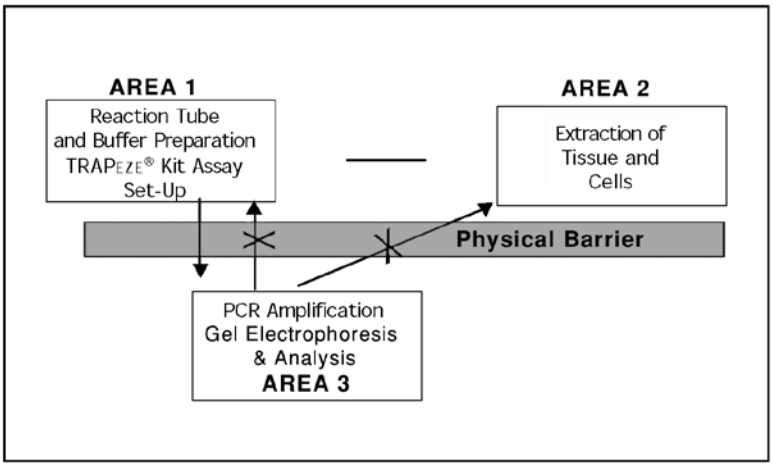
The following precautions should be followed in all steps of the assay protocol including the **telomerase extraction** and the **TRAPEZE® XL Kit assay setup**.

1. Always wear gloves.
2. Use the H_2O provided in the kit or DEPC treated H_2O for all solutions, aliquot the solutions in small amounts, and use fresh aliquots as "working" solutions which are discarded after use.
3. Keep the assay solutions (5X TRAPEZE® XL Reaction Mix, CHAPS Lysis Buffer, dH_2O, Taq polymerase, etc.) separate from other reagents in the lab.
4. Always use a designated set of pipettes exclusively for the assay, and always use aerosol resistant tips (RNase free).

5. Post amplification TRAP procedures should never be carried out near the TRAPEZE® XL Kit assay preparative areas.
6. Decontaminate the PCR tube racks with 10% bleach and UV irradiation after each use.

The optimal working environment partitions TRAPEZE® XL Kit procedures into three areas.

Figure 5: TRAPEZE® XL Telomerase Detection Kit Station Setup



To minimize the potential for carryover contamination, there should be a physical separation of the preparative areas (Areas 1 and 2) from the PCR amplification and detection area (Area 3, see Figure 5). The ideal setup employs separate rooms. If the same room must be used, then a TRAPEZE® XL Telomerase Detection Kit assay station setup should be adopted with a clear division between the preparation area and the PCR amplification/ detection area. Another option is to separate the tasks between personnel: one individual carries out the preparation of the extract and TRAPEZE® XL Kit assay set up, and another performs the analysis of the amplified products. It is mandatory that no amplified products or equipment exposed to the amplified products (Area 3) enter the preparative areas (Area 1 and 2). Optimally, Areas 1 and 2 should be in separate rooms or spaces. However, this is not as critical as separating Areas 1 and 2 from Area 3. Usually, preparation of tissues and cell extracts are performed in a laminar flow hood with appropriate sterile protocols, so the division between Area 1 and Area 2 (tissue culture hood) is convenient.

TRAPEZE® XL Telomerase Detection Kit Station Setup (Area 1)

Laboratory personnel can easily be contaminated with PCR products when carrying out routine manipulations such as opening tubes, pipetting PCR products, or discarding gel buffer. DNA may remain on the person for many days. To avoid this source of contamination, a positive air displacement hood that blows in filtered air over the workspace toward the investigator works well. Separate solutions, pipettes, tubes, and tips should always be used and kept inside the hood. The work space should be wiped with 10% bleach prior to set up of the reaction, and the hood should be routinely UV irradiated (shortwave) when not in use.

Once every 1-3 weeks, the barrels of pipettes should be soaked in 10% bleach, even if aerosol resistant tips are used. The investigator should always wear gloves and use clean lab coats or disposable sleeves which should be changed every week.

Excitation and Emission Filters

For the direct detection of fluorescein and sulforhodamine, it is essential to utilize the appropriate excitation and emission filters with the fluorescence plate reader. It is recommended that >20 nm should separate the upper limit (maximum λ) of the excitation filter bandwidth and the lower limit (minimum λ) of the emission filter bandwidth (λ = wavele
excitation light will contribute to the emission signal (all crosstalk will be eliminated).

Table 5: Middle λ of Filter Bandwidth

	Excitation	Emission
Fluorescein	485 nm	535 nm
Sulforhodamine	585 nm	620 nm

Determination of Protein Concentration

A variety of protein determination methods and reagents are available. The following procedure is rapid and reliable.

Materials Required

1. Coomassie Protein Assay Reagent
2. BSA standards: 0.1 mg/mL and 1 mg/mL (diluted in CHAPS Lysis Buffer to the appropriate concentration)

Table 6: Set-up for BSA Standards

Cuvette	BSA (µg) Final Amt.	BSA Std.-Vol. 0.1 mg/mL stock	BSA Std.-Vol. 1.0 mg/mL stock	Lysis Buffer Vol.
1	0	0	0	50 µL
2	1	10 µL	0	40 µL
3	2	20 µL	0	30 µL
4	5	50 µL	0	0
5	5	0	5 µL	45 µL
6	10	0	10 µL	40 µL
7	15	0	15 µL	35 µL
8	20	0	20 µL	30 µL

Procedure

1. Prepare BSA standard dilutions.
2. Using CHAPS Lysis Buffer, prepare a dilution series of the extract in a total volume of 50 µL. A typical range is 1 to 5 µL of extract.
3. Add 1 mL of Protein Assay Reagent to each standard or samples.
4. Mix well, and incubate for 5 minutes at room temperature (try to achieve equal incubation time for all tubes by appropriate staggering of reagents).
5. Read at OD_{595} (with tube #1 as blank).
6. Determine extract protein concentration from BSA standard plot of OD_{595} versus µg BSA.

Enhancing Detection Sensitivity

The following factors may be considered if higher detection sensitivity is required (also see Ref. 18):

1. PCR amplification cycles:
   Increase the number to 37-38 cycles.
2. Thermocycling conditions:
   Optimize the annealing/extension temperature and time.
   Eliminate the 72°C extension step for 2 temperature cycling.
   (Depending on the thermocycler used, removal of the extension step may increase amplification efficiency of the TRAPEZE® XL products and reduce the TSK2 internal control product.)
3. CHAPS Lysis Buffer:
   Other detergents have been successfully used for extraction of telomerase. For example, 1% NP40 and 0.25 mM Deoxycholate (DOC) were utilized (J. Norton, AACR abstract, 1997).
4. Amount of extract:
   If Taq polymerase inhibitors are not present, the amount of extract used in the assay may be increased.

   Note: Many tumor extracts contain Taq polymerase inhibitors. It is advisable to optimize the other variables described above first.

PAGE Analysis (Optional)

PAGE and Data Analysis

The TRAPEZE® XL Kit assay products can be visualized following polyacrylamide gel electrophoresis (PAGE) on a 10% non-denaturing gel. After electrophoresis, the gel is stained with ethidium bromide or SYBR® Green I to visualize the TRAP ladder (Figure 3B). Alternatively, the TRAPEZE® XL Kit assay products may be detected using a fluoroimaging system, eliminating the need to stain the gel after electrophoresis.

1. Add 2.5 µL of loading dye containing bromophenol blue and xylene cyanol (0.25% each in 50% glycerol/50 mM EDTA) to 20 µL of the PCR reaction. Load the samples on a 10% non-denaturing polyacrylamide gel (no urea) in 0.5X TBE buffer.

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

2. Electrophoresis should proceed for 1.5 hours at 400 volts for a 10% acrylamide 10-12 cm vertical gel, or until the xylene cyanol dye runs 1-2 cm from the bottom of the gel. The smallest telomerase product band should be 61 bp and the internal control band is 56 bp.

3. After electrophoresis, view the fluorescent products directly using a fluoroimaging system or stain the gel with ethidium bromide or SYBR® Green I according to the manufacturer's instructions. For ethidium bromide staining, dilute a 10 mg/mL stock solution 1:10,000 in deionized water. Stain for 20 minutes and destain 20 minutes in deionized water at room temperature.

4. There are two options for recording the PAGE results after staining. Each method has different requirements for laboratory equipment.

   If using a camera: The staining agent SYBR® Green I, a yellow or green filter, and a 254 nm 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. Ethidium bromide detection is not recommended if using a camera.

   If using a CCD imaging system: The best results with SYBR® Green I are obtained using (1) a 254 nm or a 302 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. A CCD imaging system allows data storage for densitometric analysis.

   Experimental samples:

   If extract is telomerase positive: A ladder of products with 6 base increments starting at 61 nucleotides (i.e. 61, 67, 73, 79, etc.) and a 56 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 a 56 bp internal control band is seen.

Materials Required but Not Supplied for PAGE Analysis

PAGE Equipment and Reagents

1. Polyacrylamide vertical gel electrophoresis apparatus
2. Power Supply (>500 V capacity)
3. 40% Polyacrylamide/bisacrylamide stock solution (19:1)
4. TEMED

5. 10% Ammonium Persulfate
6. 10X (or 5X) TBE Solution
7. Loading Dye Solution

Visualization Equipment and Reagents

1. SYBR® Green I or ethidium bromide
2. UV transilluminator: 254 nm or 302 nm for SYBR® Green I, 302 nm for ethidium bromide
3. UV filter: SYBR® Green I or yellow filter, ethidium bromide (orange filter) Camera and film or CCD Imaging System

Reagent and Buffer Preparation

5X TBE Buffer

To make 1 liter:

54 g	Tris Base
27.5 g	Boric Acid
20 mL	0.5M EDTA, pH 8.0

pH should be 8.1-8.5, adjust if necessary.

10% Non-denaturing Polyacrylamide Gel

To make 50 mL:

49.5 mL	10% Polyacrylamide (mono/bis=19:1) Stock in 0.5X TBE
0.5 mL	10% Ammonium Persulfate
0.05 mL	TEMED

Loading Dye Solution (Non-Denaturing Gel)

To make 5 mL:

2.5 mL	Glycerol
1.0 mL	1.25% bromophenol blue (in Deionized Water)
1.0 mL	1.25% xylene cyanol (in Deionized Water)
0.5 mL	0.5 M EDTA, pH 8.0

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August 2016
Version 8: 41420