TRAPEZE® ELISA Telomerase Detection Kit

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Not for use in diagnostic procedures

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I. INTRODUCTION

Using this Manual

The TRAPEZE® ELISA Telomerase Detection Kit provides reagents necessary to perform the Telomeric Repeat Amplification Protocol (TRAP) and ELISA assay for non-quantitative detection of telomerase activity in cells and tissues.

The novice user is advised to read the entire manual prior to using the TRAPEZE® ELISA Kit, particularly Sec. III: *Protocol*. Supplemental protocols can be found in Sec. V. *Appendix*. Should additional questions arise, assistance can be obtained by contacting Chemicon Technical Support at (800) 437-7500 or at techserv@chemicon.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® ELISA Kit provides the reagents necessary for performing the TRAP assay followed by ELISA detection of telomerase activity in cell/tissue samples.

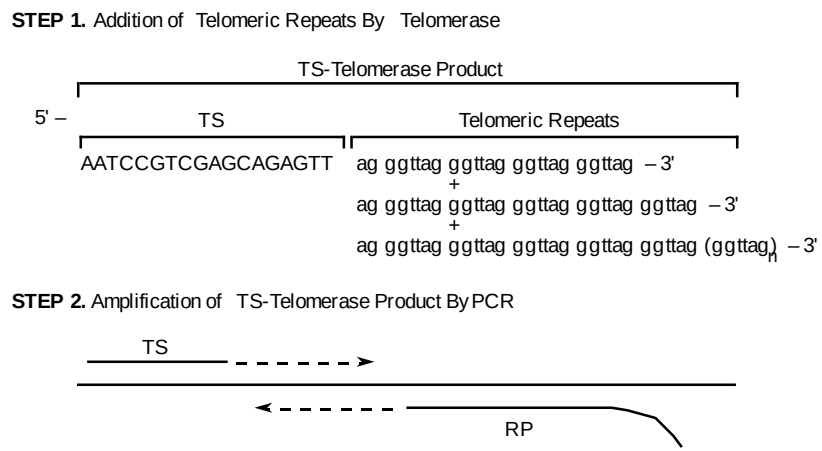
Principles of the Technique

Like the gel electrophoresis-based TRAPEZE® Kit, the methodology utilized in TRAPEZE® ELISA Kit is based on an improved version of the original method described by Kim et al. (12). The TRAPEZE® ELISA Kit is a highly sensitive in vitro assay system for detecting telomerase activity. In addition to those which are necessary for the TRAP assay, the kit includes reagents for non-radioactive detection of the telomerase products by ELISA protocol. The procedures are separated into two steps: TRAP extension/amplification and detection.

Step 1: TRAP extension/amplification

This step is a one buffer, two enzyme system utilizing the polymerase chain reaction (PCR). In the first step of the reaction (Figure 1), telomerase adds a number of telomeric repeats (GGTTAG) onto the 3' end of a biotinylated Telomerase Substrate oligonucleotide (b-TS). In the second step, the extended products are amplified by the polymerase chain reaction (PCR) using Taq polymerase, the b-TS and RP (reverse) primers, and a deoxynucleotide mix containing dCTP labeled with dinitrophenyl (DNP). This extension/amplification reaction generates a ladder of products with 6 base increments starting at 50 nucleotides: 50, 56, 62, 68, etc. The TRAPEZE® ELISA Kit (and the gel-based TRAPEZE® Kit) utilizes a reverse primer (RP) with modified sequence, instead of the CX primer described originally (12). The modified primer RP eliminates the need for a wax barrier hot start and reduces amplification artifacts. This improvement simplifies the procedure and substantially lowers background artifact bands such as primer dimers.

Figure 1: TRAPEZE® ELISA Telomerase Detection Kit Assay Scheme



Step 2: Detection (ELISA)

The TRAP extension/amplification reaction is performed with biotinylated primer and DNP-labeled dCTP. Thus, the TRAP products are tagged with biotin and DNP residues. The labeled products are immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction, and then detected by anti-DNP antibody conjugated to horseradish peroxidase (HRP). The amount of TRAP products is determined by means of the HRP activity using substrate 3,3',5,5'- tetramethylbenzidine (TMB) and subsequent color development. While the method is non-quantitative, telomerase activity in extracts made from 30-1,000 telomerase positive cells can readily be measured. Further, the procedures of TRAPEZE® ELISA kit are optimized for extremely sensitive and specific detection of telomerase activity in clinical research samples. Telomerase activity from as few as 30 telomerase positive cells among 3,000 negative cells can be detected with the TRAPEZE® ELISA (for details of limit of sensitivity and of specificity, see Sec. III. *Protocol, Sensitivity and Specificity of the Assay*).

Detection (PAGE)-optional

As an aid to data interpretation, direct visualization of the TRAP ladder is easily accomplished by polyacrylamide gel electrophoresis and staining with SYBR® Green or ethidium bromide. The presence or absence of the signature telomerase ladder (Figure 3) confirms the ELISA results.

II. KIT COMPONENTS

The kit provides sufficient reagents to perform 96 TRAP reactions and ELISA detections. With these reagents, 28 samples with the appropriate positive and negative controls can be analyzed.

Table 1: TRAPEZE® ELISA Telomerase Detection Kit

Description	Storage Conditions
1. CHAPS XL Lysis Buffer (8.2 mL)	- 25°C to 8°C
2. 5X TRAPEZE® Reaction Mix (1 mL) biotinylated TS primer biotinylated RP primer dA, dC, dG and dTTP internal control primers Tris buffer	- 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) (32 µL) 0.1 amole/µL TSR8 template	-15°C to -25°C
5. Control Cell Pellet Telomerase positive cells (10^6 cells)	-75°C to -85°C
6. Anti-DNP Antibody, HRP-conjugated (22 µL)	-15°C to -25°C
7. Blocking/Dilution Buffer (45 mL)	2°C to 8°C
8. 10X Washing Buffer (45 mL)	2°C to 8°C
9. TMB Solution (10 mL) Tetramethylbenzidine (HRP substrate)	2°C to 8°C
10. Stop Solution (10 mL)	2°C to 8°C
11. Streptavidin-coated Microtiter Plate	2°C to 8°C

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

Materials Required But Not Supplied in the Kit

Equipment and Supplies

1. Thermocycler
2. ELISA plate reader (need 450 nm and 690 nm filters)
5. If analyzing tissues, homogenization equipment as described in Sec. III. *Protocol, Extract Preparation*
6. Tubes for PCR amplification
7. Aerosol resistant pipette tips (RNase-free)
8. Laboratory film

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)

Precautions

1. Because the TRAPEZE® ELISA 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 TRAP 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 *TRAP Station Setup*.

   Before performing a TRAP assay, it is strongly recommended that you read the entire manual. At a minimum, read Sec. V. *Appendix, Laboratory Setup and Precautions* and Sec. III. *Protocol, Experimental Design* which describe all the controls that must be included in each analysis.

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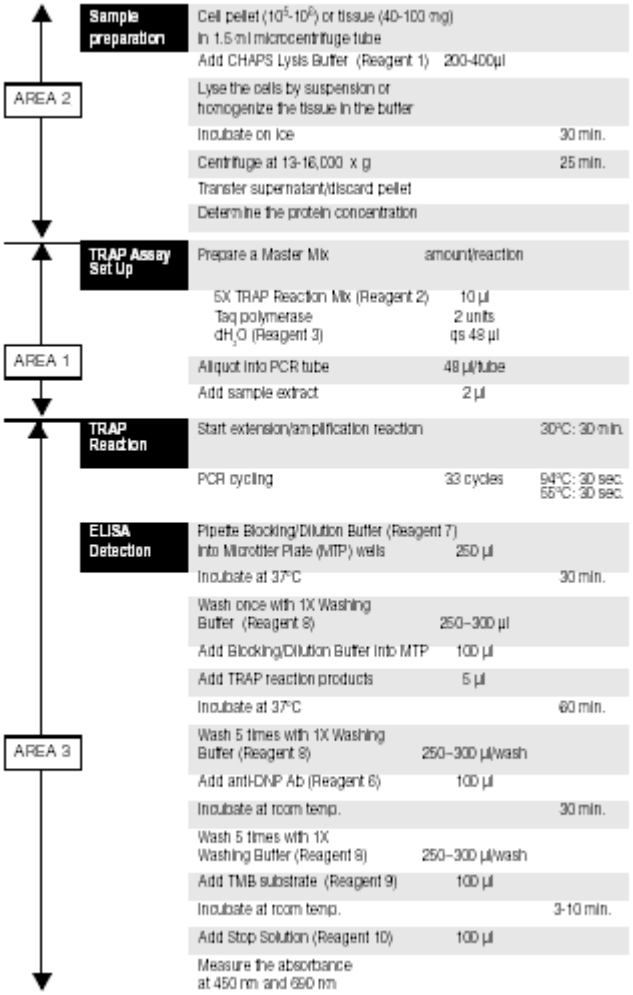
III. PROTOCOL

Figure 2: TRAPEZE® ELISA 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 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 XL Lysis Buffer (step 2).

2a. Cells

Resuspend the cell pellet in 200 µL of 1X CHAPS Lysis Buffer/10^5-10^6 cells. (Use 200 µL of 1X 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 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 obtained. 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 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 1X CHAPS Lysis Buffer.

Connective Tissues - 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 160 µL of the supernatant into a fresh tube and discard the pellet.
6. Set aside 5-10 µL of extract for determination of protein concentration. 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® ELISA 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.*

7. Determine the protein concentration. (See Sec. V. *Appendix, Determination of Protein Concentration*.)

Experimental Design

For a valid analysis of the TRAPEZE® ELISA assay data, three factors need to be considered: (1) the controls to be included in each set of experiments, (2) the amount of cell/tissue extract to be used in each reaction and (3) the option of visual gel analysis of the telomerase ladder.

Controls

For each sample

Telomerase is a heat-sensitive enzyme. As a negative control, every sample extract to be evaluated must 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 TRAP assay to inactivate telomerase.

For each set of TRAP assays:

1. Telomerase Positive Extract Control (control 1)

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

2. PCR/ELISA Positive Control

   Perform the assay using 1-2 µL of TSR8. The TSR8 is a synthetic oligonucleotide with 8 telomeric repeats and thus, it serves as a template of the PCR reaction.

3. Minus Telomerase Control (control 2)

   Perform a TRAP assay with 2 µL 1X CHAPS Lysis Buffer substituted for the cell/tissue extract.

   Primer-dimer PCR artifacts are template-independent PCR products that can be generated just with the input primer(s) in the absence of a template DNA. Potential generation of primer-dimer PCR artifacts and/or PCR product carry-over contamination should be tested for each set of TRAP assays. Detection of a positive signal in the CHAPS Lysis Buffer-only control reaction suggests one or more of the following: 1) the presence of primer-dimer PCR artifacts due to faulty PCR conditions; 2) the presence of PCR contamination (amplified TRAP products) carried over from another assay, or 3) contamination of telomerase positive cell extract into one of the components of the assay. (See Sec IV. *Troubleshooting*)

Assay Design

The TRAPEZE® ELISA Telomerase Detection Kit is designed for the successful analysis of 28 experimental samples in 4 separate experiments. Supposing 7 experimental samples (n) are analyzed at a time, each assay would consist of 24 PCR reactions (3n + 3).

Wells 1-21:	7 experimental samples alternating with 2 test extracts (duplicates or at 2 different concentration of the extracts: for example, use 1.0 µg and 0.2 µg extract) and the heat inactivated controls
Well 22:	Telomerase positive control cell extract – 2 µL control cell extract at concentration of 500 cells/µL.
Well 23:	PCR/ELISA positive control – 1 or 2 µL TSR8
Well 24:	Primer-dimer/PCR contamination control – 2 µL 1X CHAPS Lysis Buffer

Protein Concentration in TRAP Assay

Many samples isolated from human tissues and fluids contain Taq polymerase inhibitor(s). In addition, the presence of large quantities of proteins in the reaction mixture can occasionally cause amplification of non-specific PCR

products. Therefore, it is essential to control the amount of input proteins in the assay. Diluting the samples below the recommended maximum protein concentration minimizes most of these problems. The TRAPEZE® ELISA Kit is designed to achieve maximum sensitivity for samples with Taq polymerase inhibitor(s). Under the conditions described in the manual, detection of telomerase activity in 30 telomerase-positive control cells is feasible (see Figure 3).

Determine the protein concentration of the sample extracts (Sec. V. *Appendix, Determination of Protein Concentration*) and dilute with 1X CHAPS Lysis Buffer prior to using them in the TRAP assay according to the recommendations in Table 2.

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

Note: *The sensitivity can be modified by adjusting several experimental conditions of the assay as described in Sec. V.* Appendix, Enhancing Detection Sensitivity.

Analysis by Polyacrylamide Gel Electrophoresis (PAGE)-Optional

As described above, many clinical research samples contain Taq polymerase inhibitor(s), which may cause potential false-negative results. Dilution of the samples minimizes the effect of inhibitors. However, as an aid to data interpretation, direct visualization of the TRAP ladder is easily accomplished by polyacrylamide gel electrophoresis and staining with SYBR® Green or ethidium bromide . The presence or absence of the signature telomerase ladder (Figure 3B) confirms the ELISA results. (A 36 bp PCR control band should be visible in all lanes including the telomerase negative control lane. Amplification of the TRAP products and the 36 bp control is noncompetitive. Therefore, the 36 bp control shoud be visible regardless of the amount of telomerase present in the sample. The amplification of this internal control band does not affect the ELISA value of each sample, as it is not labeled with biotin.)

For details of PAGE analysis refer to Sec. V. *Appendix, Native PAGE Analysis.*

TRAPEZE® ELISA 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 TRAP 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 TRAP 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, **3n+3** reactions are necessary. Multiply the volume of each reagent listed above (except cell/tissue extract) by **3n+4** and mix them in a sterile tube (this "Master Mix" will contain extra reagent for pipetting variances).

2. Aliquot 48 µL of the Mix into 3n+3 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. Telomerase positive extract control: add 2 µL of positive control cell extract at a concentration of 500 cells/µL.
   d. PCR/ELISA positive control: add 1 µL of TSR8
   e. Primer-dimer/PCR contamination control: add 2 µL of CHAPS Lysis Buffer.

PCR Amplification

1. Place the tubes in the thermocycler block, and incubate at 30°C for 30 minutes.
2. In a thermocycler, perform a 2-step PCR at 94°C/30 seconds, 55°C / 30 seconds **33 cycles.**

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.

ELISA Detection

a. Pretreatment of the microtiter plate (MTP):

Add 250 µL of Blocking/Dilution Buffer (prewarmed to room temperature) into each well.

Cover the wells with laboratory film and incubate at 37°C for 30 minutes.

Remove the Blocking/Dilution Buffer and rinse the well once with 250-300 µL of 1X Washing Buffer.

Note: *Shaking of the MTP module is NOT necessary throughout this ELISA procedure except in the peroxidase reaction step where occasional gentle tapping of the MTP moduleholder provides sufficient mixing.*

b. Binding of the PCR products:

Aliquot 100 µL of the Blocking/Dilution Buffer into a well of pretreated MTP. Add 5 µL of TRAP reaction product and mix throughly by pipetting up-and-down 10-15 times. Be careful not to contaminate the end of the pipettor with TRAP reaction product. (Figure 2 shows an example of sample arrangement for the assay.) Cover the wells with laboratory film and incubate at 37°C for 1 hour. Shaking the MTP during this incubation is not required.

c. Remove the solution completely and rinse each well 5 times with 250-300 µL of 1X Washing Buffer.

d. Dilute the stock Ab solution (Reagent 6) 1:500 with Blocking/Dilution Buffer just prior to the ELISA procedure. Add 100 µL of anti-DNP antibody-HRP conjugate into each well. Cover the wells with Parafilm™.

f. Incubate at room temperature for 30 minutes **protected from light**.

g. Remove the antibody solution completely and rinse each well 5 times with 250-300 µL of 1X Washing Buffer.

h. Add 100 µL of TMB Solution (prewarmed to room temperature) and incubate 3-10 minutes at room temperature. To achieve uniform mixing of the reaction components, gently tap the side of the MTP module holder once every 1-2 minutes.

i. Add 100 µL of Stop Solution into each well. (This changes the color of the peroxidase product from blue to yellow.)

j. Using a microtiter plate reader, measure the absorbance of the samples at 450 nm and at 690 nm within 30 minutes after the termination of the reaction. Compute the value for each sample as:

$$\text{Absorbance (units)} = A_{450} - A_{690}$$

Data Analysis

For a valid TRAP assay, the controls listed below must show the following absorbance measurements.

1) Primer/dimer/PCR contamination control:

   The absorbance units should be **lower than 0.200.**

2) TSR8 PCR/ELISA control:

   If 1 µL of TSR8 (0.1 amole) is used, the absorbance units should be **greater than 0.800.**

3) Heat treated sample extract:

   The absorbance units should be **less than 0.250.**

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

Experimental samples:

If the extract is telomerase-positive:

$\Delta A > 0.150$

ΔA: net increase of absorbance for the sample = $A_{sample} - A_{heat\text{-}treated\ sample}$

When duplicate assays are performed, the ΔA of each of the assays should be greater than 0.150. If only one of them is high, it may be due to potential primer/dimer artifacts or PCR-carry over contamination into that assay tube. In this case, repeat the assay.

Sensitivity and Specificity of the Assay

Sensitivity

The sensitivity of the TRAPEZE® ELISA Kit is shown in Figure 3A. Under the conditions described in this manual, telomerase activity in an extract from as few as 30 telomerase-positive control cells (provided in the kit) can be detected by the TRAPEZE® ELISA assay. The positive ELISA signal correlates with the authentic TRAP ladder visualized by optional PAGE analysis of the samples (Fig. 3B). (The 36 bp band in every sample including the CHAPS control reaction is an internal PCR control, which is not detected by the ELISA assay.)

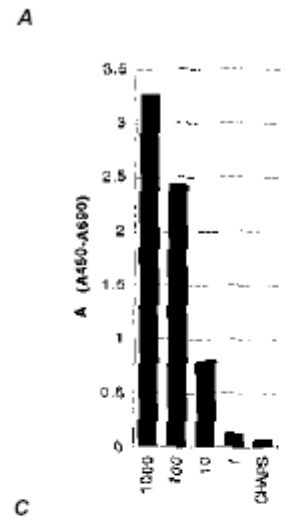
The telomerase activity equivalent to 30 cells is also detectable in clinical research samples where the control cell extract was added prior to the extension/amplification reaction. Thus, assuming the absence or low level of Taq polymerase inhibitors in the samples, telomerase activity expressed in less than 1% of cell population can be detected by the kit (i.e. 30 telomerase positive cells among 3,000 negative cells are detectable by the TRAPEZE® ELISA.)

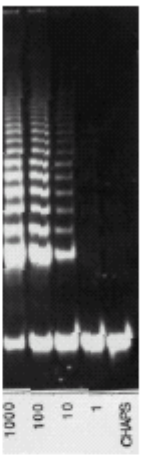
The number of PCR amplification cycles may be increased to enhance the sensitivity, but this may increase the background.

Specificity

No significant telomerase activity was detected using the TRAPEZE® ELISA Kit in samples containing extract equivalent to 10,000 cells from fibroblast cell lines WI-38 and IMR-90 (ATCC) (Figure 3C).

Figure 3





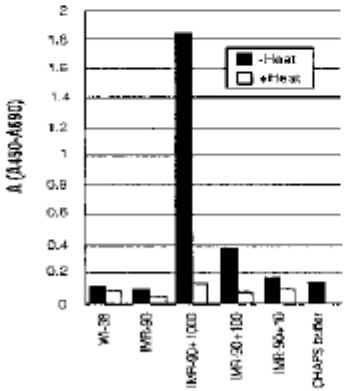


Figure 3 Legend

A. TRAPEZE® ELISA results using extracts from telomerase-positive control cells. The cell number represented by the amount of extract added is indicated under the bar. All procedures are as described in the manual. HRP reaction time is 6 minutes.

B. 12.5% PAGE analysis of the samples shown in Fig.3A.

C. TRAPEZE® ELISA results using extracts equivalent to 10,000 cells containing either telomerase-negative cells (WI-38 and IMR-90) or a mixture of IMR-90 cells and telomerase-positive cells. The spiked extracts used in each assay contained 1000, 100 or 10 telomerase-positive cells. Black bars: no heat treatment White bars: heat treatment

IV. TROUBLESHOOTING

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

? Signals of TSR8 are too low or absent.

1. Potential problem: PCR amplification is not initiated or amplification conditon is not optimum.

Recommendations:

a. Check the TRAP assay reagents. Was the TRAP Reaction Mix diluted correctly? Was Taq polymerase included? Was the Taq polymerase active?
b. Check the thermocycler for proper temperature and time settings. Is the thermocycler cycling at 94°C/30 seconds and 55°C/30 seconds for 33 cycles?
c. The optimal annealing temperature may need to be tested empirically (53°C to 58°C) for each thermocycler.

2. Potential problem: Difficulty with the ELISA procedure.

Recommendation:

Check the ELISA protocol. Was the anti-DNP antibody diluted correctly? Was the antibody active? Was the antibody stored properly? Was the antibody incubation for the appropriate length of time? Was the incubation with the substrate for the appropriate length of time?

? Signals of telomerase control cell extract are too low or absent.

1. Potential problem: Telomerase activity is not initiated. Possible RNase contamination.

Recommendations:

a. If positive ELISA signal is observed in TSR8 control, the problem is likely caused by RNase contamination. The TSR8 is a synthetic oligomer with 8 telomeric repeats and therefore, its amplification is telomerase independent.
b. Check the TRAP Reaction Mix and dH_2O.
c. Always use RNase free tips, tubes and solutions.
d. Use a fresh aliquot of TRAP Reaction Mix, taking extra precautions to prevent RNase contamination.
e. Add RNase inhibitor into the 1X CHAPS Lysis Buffer (see Sec. III. *Protocol, Extract Preparation*), taking extra precautions to prevent RNase contamination.

f. Always use a clean labcoat and gloves. Keep the TRAP Station and telomerase extraction areas clean with bleach and alcohol.

? The signals of negative controls are too high.

1. Potential problems: PCR carry-over contamination.

Recommendations:

a. Use fresh aliquots of every component of the assay (TRAP 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.

2. Potential problems: Difficulty with the ELISA procedure.

Recommendations:

c. Increase the number of wash steps.
d. Reduce the incubation time with the TMB substrate.
e. Check that the microtiter plate was blocked prior to sample application.

3. Potential problems: PCR artifacts.

Recommendations:

a. Check the PCR parameters.
b. Decrease the number of PCR cycles to 30-32 cycles.

? Signals of heat-treated extracts are too high. Extracts are not heat sensitive.

1. Potential problem: Insufficient heat inactivation of the extracts, primer-dimer PCR artifacts, or contamination of TRAP products into the test extracts or PCR artifacts due to high protein concentration in the assay.

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 contamination of TRAP products from a prior TRAPEZE® ELISA assay.
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. Dilute the extracts and repeat the analysis.

? Only one of the duplicated samples shows positive signal. The data on duplicate samples are inconsistent.

1. Potential problem: Primer-dimer PCR artifacts in the tube with high signal.

As is anticipated with a PCR-based assay, some unavoidable PCR artifacts are expected even when the optimal assay conditions are employed. Though at a very low frequency, this artifact is occasionally observed in the reaction tube with low or no telomerase activity.

Recommendations:

a. Recheck the PCR parameters and repeat the assay.
b. Analyze the TRAPEZE® ELISA 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 on the nondenaturing PAGE procedure, see Sec. V. *Appendix, PAGE Analysis (Optional)*.

? No signals are observed in samples in which the presence of telomerase activity is strongly anticipated.

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

Recommendations:

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.

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

Recommendation:

Refer to Recommendations for Problem “Signals of telomerase control cell extract are too low or absent”.

IV. APPENDIX

Laboratory Setup and Precautions

One of the most important considerations when performing the TRAP 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:

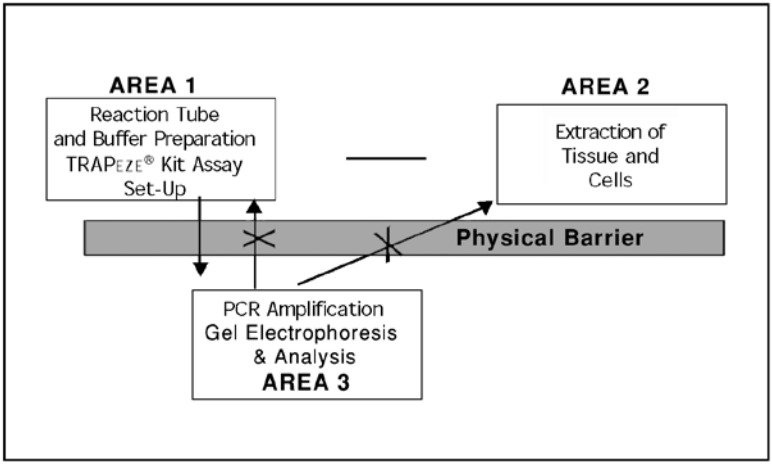
1. solutions and tubes not treated with an RNase inhibitor
2. any equipment handled without gloves.

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

1. Always wear gloves.
2. Always use a designated set of pipettes exclusively for the assay, and always use aerosol resistant tips (RNase free).
3. 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.
4. Keep the assay solutions (TRAP Reaction Mix, 1X CHAPS Lysis Buffer, dH_2O, Taq polymerase, etc.) separate from other reagents in the lab.
5. Post amplification TRAP procedures should never be carried out near the TRAPEZE® ELISA Kit assay preparative areas.

6. Decontaminate the PCR tube racks with 10% bleach and UV irradiation after each use.

Figure 4: TRAP Station Setup



The optimal working environment partitions TRAP procedures into three areas. 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 4). The ideal setup employs separate rooms. If the same room must be used, then a TRAP 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 TRAP 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.

TRAP 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.

Determination of Protein Concentration

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

Materials Required

Coomassie Protein Assay Reagent

BSA standards: 0.1 mg/mL and 1 mg/mL (diluted in CHAPS XL 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 XL 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. 23).

1. PCR amplification cycles:

   Increase the number to 34-36 cycles.

2. Thermocycling conditions:

   Evaluate the annealing/extension temperature and time.

   Add a 72°C extension step to perform PCR at 3 temperatures per cycle. (Depending on the thermocycler used, the addition of an extra step may increase amplification efficiency of the TRAP products.)

3. 1X 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

When desired, TRAP products can be analyzed on a 10-12% native polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel is stained with ethidium bromide or SYBR® Green to visualize the TRAP ladder (Figure 3B).

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.)*

2. Electrophoresis should proceed for 1.5-2 hours at 400 volts for a 10% acrylamide, 10-12 cm vertical gel. The bromophenol blue dye front should just run off the bottom of the gel.

3. After electrophoresis, stain the gel with ethidium bromide or SYBR® Green 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. The smallest telomerase product band is 50 bp (the 36 bp band is the internal amplification product).
4. Record the PAGE results by UV transilluminator (254 or 302 nm for SYBR® Green or 302 nm for EtBr) and camera system. Alternatively, a CCd imaging system can be used to record and analyze the data, permitting the data to be saved to disk for densitometric analysis.

If TRAP assay is performed under optimum amplification conditions, TRAP products of 30 telomerase-positive control cells (provided in the kit) will be visible with SYBR® Green staining (see Figure 3B).

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 a 36 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

V. REFERENCES

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