Photometric Enzyme Immunoassay for Quantitative Determination of Telomerase Activity, Utilizing the Telomeric Repeat Amplification Protocol (TRAP)

Cat. No. 12 013 789 001
96 reactions (for up to 31 samples)

Store the kit at −15 to −25°C
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1. Preface

**Caution**

The following reagents used in the assay are toxic or corrosive and should be handled with care:
- Denaturation reagent (bottle 7)
- TMB substrate solution (bottle 13)
- Stop reagent (bottle 14)

1.1 Kit Contents

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Label</th>
<th>Content, including function</th>
</tr>
</thead>
</table>
| 1      | Lysis reagent | • 11 ml  
• Ready-to-use solution  
• For preparation of cell extracts from cell cultures and tissue samples |
| 2      | Reaction mixture, 2x | • 2 x 900 μl  
• Ready-to-use  
• 2x concentrated solution  
• Contains biotinylated telomerase substrate P1-TS, optimized anchor-primer P2, nucleotides, and Taq DNA Polymerase  
• For a one-step telomerase-mediated primer elongation and PCR amplification |
| 3      | Internal Standard (IS) | • 350 μl  
• Ready-to-use solution  
• Contains a 216 bp Internal Standard (IS) DNA, 0.001 amol/μl  
• Used as an internal amplification control  
• Makes evident inhibitors of the amplification process |
| 4      | Control template, low | • 35 μl, 0.001 amol/μl  
• Ready-to-use solution  
• Contains a positive control template DNA with the same sequence as a telomerase product with 8 telomeric repeats (TS8) |
| 5      | Control template, high | • 35 μl, 0.1 amol/μl  
• Ready-to-use solution  
• Contains a positive control template DNA with the same sequence as a telomerase product with 8 telomeric repeats (TS8) |
| 6      | Water, nuclease-free | • 2 x 1.1 ml  
• Contains double-distilled, nuclease-free water |
<table>
<thead>
<tr>
<th>Bottle</th>
<th>Label</th>
<th>Content, including function</th>
</tr>
</thead>
</table>
| 7 violet | Denaturation reagent | • 1.2 ml  
• Ready-to-use solution  
• Contains less than 0.5% sodium hydroxide  
• To denature amplicons |
| 8 white | Hybridization buffer T | • 7 ml  
• Ready-to-use solution  
• Contains a DIG-labeled detection probe complementary to telomeric repeat sequences (P3-T)  
• For specific detection of telomerase-mediated amplification products |
| 9 yellow | Hybridization buffer IS | • 3.5 ml  
• Ready-to-use solution  
• Contains a DIG-labeled detection probe complementary to the Internal standard (P3-IS)  
• For specific detection of amplified Internal Standard (IS) |
| 10 blue 10x | Washing buffer, 10x | • 50 ml  
• 10x concentrated solution |
| 11 red | Anti-DIG-HRP | • 120 mU  
• Polyclonal antibody from sheep, conjugated to horseradish peroxidase (HRP)  
• Lyophilizate, stabilized  
• To prepare conjugate solution |
| 12 red | Conjugate dilution buffer | • 12 ml  
• Ready-to-use solution |
| 13 green | TMB substrate solution | • 12 ml  
• Ready-to-use solution  
• Contains 3,3’,5,5’-tetramethylbenzidine |
| 14 green | Stop reagent | • 12 ml  
• Ready-to-use solution  
• Contains less than 5% sulfuric acid |
| 15 | Microplate | • 1 plate (12 x 8 wells)  
• Ready-to-use  
• Strip frame with 12 modules of 8 wells each, pre-coated with streptavidin and post-coated with blocking reagent |
| 16 | Cover foil | • 3 foils  
• To prevent evaporation during incubation of the MP modules |
1.2 Kit Storage and Stability

- The unopened kit will remain stable at −15 to −25°C until the expiration date printed on the label.
- The kit will be shipped on dry ice.

Storage at −15 to −25°C

The following table lists those components of the Telomerase PCR ELISAPLUS, which remain stable until the expiration date printed on the label, when stored in aliquots at −15 to −25°C. We recommend avoiding repeated freezing and thawing.

<table>
<thead>
<tr>
<th>Bottle/Solution</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lysis reagent</td>
</tr>
<tr>
<td>2</td>
<td>Reaction mixture, 2x conc.</td>
</tr>
<tr>
<td>3</td>
<td>Internal standard</td>
</tr>
<tr>
<td>4</td>
<td>Control template low</td>
</tr>
<tr>
<td>5</td>
<td>Control template high</td>
</tr>
<tr>
<td>6</td>
<td>Water, nuclease free</td>
</tr>
<tr>
<td>8</td>
<td>Hybridization buffer T</td>
</tr>
<tr>
<td>9</td>
<td>Hybridization buffer IS</td>
</tr>
</tbody>
</table>

Storage at +2 to +8°C

The following table lists those components of the Telomerase PCR ELISAPLUS, which if not stated differently remain stable until the expiration date printed on the label when stored in aliquots at +2 to +8°C.

<table>
<thead>
<tr>
<th>Bottle/Solution</th>
<th>Contents</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Denaturation reagent</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Washing buffer, 10x</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Conjugate dilution buffer</td>
<td>6 months</td>
</tr>
<tr>
<td>13</td>
<td>TMB substrate solution</td>
<td>6 months</td>
</tr>
<tr>
<td>14</td>
<td>Stop reagent</td>
<td></td>
</tr>
</tbody>
</table>
2. Introduction

2.1 Product Overview

Test Principle

The test principle is shown in the following figure and can be divided into the following steps:

**Fig. 1:** Principle of the TeloTAGGG Telomerase PCR ELISA<sup>PLUS</sup>

**Step 1: Elongation/Amplification:**

In the first step, telomerase adds telomeric repeats (TTAGGG) to the 3'-end of the biotin-labeled synthetic P1-TS-primer. These elongation products, as well as the Internal Standard (IS) included in the same reaction vessel, are amplified by PCR using the primers P1-TS and the anchor-primer P2. PCR products derived from telomerase-mediated elongation products in the first step contain the telomerase-specific 6 nucleotide increments, while the Internal Standard (IS) generates a 216 bp PCR product.

**Step 2: Detection by ELISA:**

The PCR products are split into two aliquots, denatured and hybridized separately to digoxigenin-(DIG)-labeled detection probes, specific for the telomeric repeats (P3-T) and for the Internal Standard (IS) (P3-Std), respectively. The resulting products are immobilized via the biotin label to a streptavidin-coated microplate. Immobilized amplicons are then detected with an antibody against digoxigenin that is conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB.
Applications
The TeloTAGGG Telomerase PCR ELISA\textsuperscript{PLUS}, is designed to be used in the following life science research applications:

- For the highly sensitive semi-quantitative detection of telomerase activity in cell extracts from cell cultures and other biological samples.
- The TeloTAGGG Telomerase PCR ELISA\textsuperscript{PLUS}, utilizes a biotin-labeled primer for immobilization within the microplate. If the typical, telomerase mediated 6-nucleotide ladder is desired, the fragments can be separated by polyacrylamide gel electrophoresis (PAGE), blotted onto a positively-charged membrane, and detected appropriately (see Southern Hybridization Example, section 3.4.2).

Sample Materials
- Cell cultures
- Scientific biopsy material
- Other biological research samples

Assay Time

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part I: Extract preparation</td>
<td>approximately 1 hour</td>
</tr>
<tr>
<td>Part II: Elongation/Amplification</td>
<td>approximately 2.5 hours</td>
</tr>
<tr>
<td>Part III: Hybridization and photometric detection</td>
<td>approximately 2.5 hours</td>
</tr>
<tr>
<td>Total hands-on time</td>
<td>approximately 1 hour</td>
</tr>
</tbody>
</table>

Number of Tests
The kit is designed for 96 reactions.
Up to 31 samples plus control reactions can be performed, depending on the number of samples analyzed per experiment.

Quality Control
The kit is function-tested using varying amounts of a standardized cell extract.
2.2 Product Characteristics

**Sensitivity**
Ddetects 10 cell equivalents (in an exemplary system, using 293 cells)

**Measuring Range**
The linear measuring range of the kit is from 10 to 2,000 cells (in an exemplary system, using 293 cells)

**Advantages**
The TeloTAGGG Telomerase PCR ELISA\(^\text{PLUS}\) offers an extension to the TeloTAGGG Telomerase PCR ELISA, Cat. No. 11 854 666 910, as it combines its features with an approach to control the amplification process and quantify the PCR product formed.

<table>
<thead>
<tr>
<th>Benefits</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of master mixes not required</td>
<td>The kit contains all of the essential compounds as master mixes.</td>
</tr>
<tr>
<td>Reliability</td>
<td>Positive control contained in the kit.</td>
</tr>
<tr>
<td>Specificity</td>
<td>The use of hybridization probes ensures specific detection of the amplified telomerase-reaction product</td>
</tr>
<tr>
<td>Quantitative results</td>
<td>The assay concept allows semi-quantitative determination of telomerase levels.</td>
</tr>
</tbody>
</table>

2.3 Background Information

**Telomeres**
Telomeres, the specialized DNA/protein structures located at the end of eukaryotic chromosomes, consist of small, tandemly repeated DNA sequences. Numerous telomere sequences have been identified, which display very few sequence variations, even between phylogenetically divergent organisms such as Tetrahymena (sequence: TTGGGG) and human (sequence: TTAGGG). Despite the homology in their sequences, telomeric DNA shows a significant variation with respect to its length, for example, the length of the simple repeat region can range from <50 bp in Euplotes to >100 kbp in mice (1). Telomeres play an essential role in the stable maintenance of the eukaryotic chromosome within a cell by specifically binding to structural proteins. These proteins cap the ends of linear chromosomes, thereby preventing nucleolytic degradation, end-to-end fusion, irregular recombination, and other events that are normally lethal to a cell. Additionally, telomeres are involved in nuclear architecture and interact with other proteins to repress the expression of adjacent genes (2). As DNA polymerase is unable to replicate the very ends of linear DNA, it was suggested that chromosomal ends progressively shorten with each replication cycle (called the “end-replication problem”) (3). This phenomenon, which has been demonstrated \textit{in vitro} and \textit{in vivo}, seems to be linked to the limited proliferative capacity of normal somatic cells (“mitotic clock”). Since germ-line cells, stem cells, and tumor cells all exhibit a prolonged or even infinite life span, it was suggested that these cells must possess a particular mechanism for maintaining telomere length (4).
**Introduction**

**Telomeres**

Maintaining stable telomere length is associated with the activation of telomerase. This enzyme is a ribonucleoprotein that compensates for the loss of telomeric DNA by adding repeat sequences to the chromosome ends using its intrinsic RNA component as a template for DNA synthesis (5, 6). The genes encoding its RNA subunit and the catalytic protein subunit have been cloned from a variety of species, including humans (7 to 9). Both subunits are essential for restoring telomerase activity in vitro, and introduction of these genes into normal human cells can extend the life span of these otherwise mortal cells (10, 11).

Research studies on telomerase expression have consistently demonstrated the presence of telomerase activity in the majority of the various types of cancer- as well as immortalized cells but fail to detect telomerase in most normal tissues (12).

Telomerase has been detected in most neoplastic lesions and appears to be necessary for the sustained proliferation of most advanced cancers (13). Researchers are now attempting to show that telomerase activation is essential to the formation and continued growth of neoplastic cells in vivo. This basic research may provide the framework for the eventual development of telomerase as an effective tumor marker.

**Methods for Detecting Telomerase Activity**

The conventional primer-extension based assay for detecting telomerase activity requires large numbers of cells or ample amounts of tissue and only allows detection of telomerase with limited sensitivity. These disadvantages have been overcome by the Telomeric Repeat Amplification Protocol (TRAP), in which the telomerase-reaction product is amplified by PCR (12). However, until now, the TRAP assay has provided full sensitivity only when used with a radioactive label and when made visible by autoradiography after gel electrophoresis, which is both hazardous and time-consuming. Additionally, unspecific amplification products have been described. Despite of several improvements to the protocol, quantification of telomerase activity remains difficult to be perform and the non-availability of an amplification control may results in production of false-negative results for samples containing PCR inhibitors (14, 15).

The *TeloTAGGG* Telomerase PCR ELISAPLUS kit provides a way to perform a highly sensitive photometric enzyme immunoassay to detect telomerase activity, using nonradioactive techniques.
3. Procedures and Required Materials

3.1 Before You Begin

**General Recommendations**

Determining telomerase activity using the *TeloTAGGG* Telomerase PCR ELISA<sup>PLUS</sup>, requires both the addition of telomeric repeats to a primer by the activity of telomerase (contained in the sample), and their subsequent amplification by PCR.

Consequently, extreme caution is imperative to prevent:

- RNase/DNase contamination that might cause degradation of the internal, telomerase-associated RNA template and the Internal Standard (IS), respectively, as well as
- PCR carry-over contamination resulting in false positive signals.

As opposed to other TRAP assay formats, the *TeloTAGGG* Telomerase PCR ELISA<sup>PLUS</sup> provides all of the compounds required to perform (i) telomerase-mediated primer elongation and (ii) the PCR reaction in a ready-to-use master mix. However, to achieve reliable results, the entire assay procedure must be performed under nuclease-free conditions.

We therefore recommend:

- Using only redistilled water that is nuclease-free (*e.g.*, DEPC or Velcorin<sup>®</sup>-treated) and autoclaved.
- Preparing appropriate aliquots of the kit solutions and keeping them separate from other reagents in the laboratory.
- Using autoclaved or heat-sterilized labware (*e.g.*, pipettes, pipette tips, reactions vials).
- Wearing gloves and a surgical mask during cell extraction and when performing the assay.
- Using fresh aerosol-preventive pipette tips for all pipetting steps is strongly recommended to avoid cross-contamination of samples and reagent.

It is also recommended to separate physically the workplaces for sample preparation, TRAP reaction, and detection of amplicons to minimize the risk of carry-over contamination.

**Additional Equipment and Reagents Required**

To perform assays with the *TeloTAGGG* Telomerase PCR ELISA<sup>PLUS</sup>, the following equipment will be needed:

- Microcentrifuge
- PCR thermal cycler, *e.g.*, Perkin-Elmer Cetus Gene Amp 9600
- Tubes for PCR amplification
- Sterile aerosol-preventive pipette tips
- Pipettes with disposable positive-displacement tips
- Sterile reaction tubes for preparing dilutions
- Microplate washer (optional)
- microplate shaker (preferably heatable)
- microplate reader
Preparation of Kit Working Solutions

For maximal recovery of contents, please quick-spin bottles 6 and 10 before opening.

The following table lists protocols for preparing working solutions and the corresponding storage conditions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
<th>Reconstitution/Preparation</th>
<th>Storage/stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Washing buffer, 1x</td>
<td>Dilute an appropriate volume of Washing buffer, 10x conc. (bottle 10) with autoclaved double-distilled water (1:10) and mix thoroughly. Approximately 5 ml of the diluted Washing buffer are needed for one reaction.</td>
<td>Stable at +2 to +8°C for 1 month.</td>
</tr>
<tr>
<td>11</td>
<td>Anti-DIG HRP, bottle 11 stock solution</td>
<td>Reconstitute the lyophilizate in 240 μl autoclaved double-distilled water. This results in an antibody conjugate concentration of 0.5 U/ml.</td>
<td>Stable at +2 to +8°C for 6 months. Do not freeze! Do not add sodium azide!</td>
</tr>
</tbody>
</table>

Anti-DIG HRP, working solution

To prepare the working solution, dilute an appropriate amount of the reconstituted anti-DIG-HRP (solution 11) with Conjugate dilution buffer (solution 12) to a final concentration of 10 mU/ml (e.g., 200 μl antibody solution and 9.8 ml of Conjugate dilution buffer). Prepare immediately before use. Do not store.

Handling of Microplate Modules

The MP modules supplied with the kit are ready to use and need not be rehydrated prior to use.

Use only the MP modules required for the particular experiment. Close the foil bag containing the remaining MP modules and the desiccant capsule tightly with an adhesive tape.

Once the bag has been opened, the microplate modules are stable for approximately 12 months, or until the control date given on the kit if stored desiccated at +2 to +8°C.
3.2 Detection of Telomerase Activity in Cell Extracts of *In Vitro* Cell Cultures and Tissue Samples

**Handling Instructions**

To avoid evaporation during incubation, cover the MP modules with the self-adhesive cover foils during each incubation step of the ELISA procedure. To avoid carry-over contamination, we recommend transferring the required solutions for one experiment into a fresh tube instead of directly pipetting them from stock solutions.

**Internal Standard (IS)**

The TRAP assay is a two-step process in which the telomerase-mediated elongation products are subsequently amplified by PCR to allow highly sensitive detection of telomerase activity. It has been reported that some tissue samples contain inhibitors of Taq DNA Polymerase, thus giving false-negative results when analyzed for telomerase activity (14). These false-negative tumor samples can be identified by including an internal amplification standard (14). Several internal standards have been reported in scientific publications (14, 15), in which a heterologous 36 bp internal standard is most often used (15). However, the use of the 36 bp internal standard has been shown to carry the risk of producing false-negative results: because of its short length, it can be amplified in samples that clearly contain Taq DNA Polymerase inhibitors (16). To overcome this problem, the internal standard provided with the *TeloTAGGG Telomerase PCR ELISA PLUS*, is a 216 bp homologous standard that allows clear detection of Taq DNA Polymerase inhibitors. In addition, as the fragment, it is long enough not to interfere with making the telomerase ladder visible when the products are analyzed after electrophoretic separation (see optional protocol below).

**Negative Control**

As a rule, a negative control with enzyme-inactivated sample material is performed in parallel to the non-inactivated samples to confirm specificity of product formation in the TRAP assay. Two methods used to prepare these negative controls have been described in scientific publications.

| Heat treatment       | Heat treatment of the cell extract for 10 min at +85°C prior to the TRAP reaction may be used to inactivate telomerase protein to produce negative controls. |
Preparation of Extracts from Cells

1. Harvest and count cells using a hemocytometer, Neubauer.
2. Transfer 2 x 10^5 cells per single reaction into a fresh reaction tube.
3. Pellet cells at 3,000 x g for 5 min in a refrigerated centrifuge at +2 to +8°C.
4. Carefully remove the supernatant, resuspend the cells in PBS and repeat the centrifugation step.
5. If the Telomerase PCR ELISAPLUS is not performed immediately after extract preparation, the pelleted cells can be stored at −80°C until use.
6. Resuspend the pelleted cells in 200 μl Lysis reagent (Solution 1), pre-cooled on ice by retropipetting at least 3 times.
7. Incubate on ice for 30 min.
   - If frozen cell pellets were used for extraction, thaw cell pellets on ice before adding Lysis reagent (Solution 1).
8. Centrifuge the lysate at 16,000 x g for 20 min at +2 to +8°C (e.g., in a refrigerated centrifuge).
9. Carefully remove the supernatant and transfer to a fresh tube.
   - To ensure that no cellular debris of the pelleted cells are transferred, we recommend pipetting only 175 μl of the cell extract.
10. Finally, perform the TRAP reaction, as described below.
    - Should the TRAP reaction not be immediately performed, shock freeze the cell extract in aliquots in liquid nitrogen and store the extracts at −80°C.
Preparation of Extracts from Tissues

Preparing tissue specimens for determining telomerase activity, requires careful acquisition and storage of clinical research materials since cross-contamination with tumor cells can result in false-positive signals in normal tissues, or negative signals can be observed in tissue samples as a result of inappropriate storage.

Note: Tissue samples should be shock-frozen in small pieces in liquid nitrogen and can be stored at −80°C if not used immediately. However, we recommend that extracts be stored, which have been prepared as described below, rather than storing tissue specimens.

1a Prepare cryostat sections of 10 to 15 μm thickness from frozen tissue samples.

2a Transfer approximately 50 sections into a sterile reaction tube containing 200 μl ice-cold Lysis reagent (solution 1).

Alternative

1b If a microtome is not available, thin slices of frozen tissue specimens may be prepared on sterile disposable petri dishes with surgical disposable knife blades to obtain thin flakes, which are then immediately transferred to homogenization tubes containing 200 μl ice-cold Lysis buffer (solution 1).

2b Homogenize on ice with a motorized pestle until uniform consistency.

3 Incubate on ice for 30 min.

4 Centrifuge the lysate at 16,000 x g for 20 min at +2 to +8°C (e.g., in a refrigerated centrifuge). A refrigerated bench top centrifuge for reaction tubes can be used.

5 Carefully remove the supernatant and transfer to a fresh tube.

To ensure that no debris of the tissue is transferred, we recommend pipetting only 175 μl of the tissue extract

6 Measure the protein concentration by standard methods. Finally, perform the TRAP reaction, as described below.

Should the TRAP reaction not be immediately performed, shock freeze the tissue extract in aliquots in liquid nitrogen and store the extracts at −80°C.
3.3 Telomeric Repeat Amplification Protocol (Trap Reaction)

Preparation of the Master Mix

It is recommended to prepare a master-mix for all samples, negative controls and control templates to be analyzed simultaneously, e.g., if seven samples including controls have to be analyzed, mix 175 μl of Reaction mixture (Solution 2) and 35 μl Internal standard (solution 3).

Protocol

The cycling program mentioned below has been established for use with the GeneAmp4) PCR System 9600 and 9700 Thermal Cycler. Depending on the performance characteristics of other licensed thermal cyclers, minor modifications of the protocol might be required. Before starting the amplification process for some thermal cyclers, it may be necessary to overlay the reaction mixture with mineral oil to prevent water condensation at the top of the tube. It has been shown in a number of experiments that overlaying mineral oil on top of the reaction mixture already for the telomerase-mediated primer elongation does not influence the results.

All pipetting steps should be done on ice.

1. For each sample, negative control and control template to be tested, transfer 25 μl Reaction mixture (Solution 2) and 5 μl of the Internal Standard (IS) (Solution 3) into a tube suitable for PCR amplification. OR Transfer 30 μl of the master mix into tubes suitable for PCR amplification per PCR reaction.

2. Samples
   Add 1 to 3 μl cell extract per tube (corresponding to 1 x 10^3 to 3 x 10^3 cell equivalents or 0.5 to 10 μg total protein, see sample preparation).

   Negative controls
   Add 1 to 3 μl of the corresponding heat treated cell extract (corresponding to 1 x 10^3 to 3 x 10^3 cell equivalents or 0.5 to 10 μg total protein) per tube.

   Control template
   Add 1 μl Control template (use either low or high concentration; Solution 4 or 5) into a separate tube. Add 1 μl Lysis reagent (Solution 1) into another separate tube. This tube serves as blank for the control template.

To obtain valid quantitative analysis of the telomerase products, each sample should contain the same amount of cell equivalents or should have the same protein content.

Add Water, nuclease-free (Solution 6) for a total volume of 50 μl.
Transfer tubes to a thermal cycler and perform a combined primer elongation/amplification reaction by the following protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp.</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer elongation</td>
<td>10 to 30 min</td>
<td>25°C</td>
<td>1</td>
</tr>
<tr>
<td>Telomerase inactivation</td>
<td>5 min</td>
<td>94°C</td>
<td>1</td>
</tr>
<tr>
<td>Amplification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 sec</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>30 sec</td>
<td>50°C</td>
<td>1 to 30</td>
</tr>
<tr>
<td>Polymerization</td>
<td>90 sec</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td></td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

**Pipetting Scheme for the MP**

**Fig. 2:** Pipetting scheme
Hybridization buffer T (T): Columns 1 + 2, 4 + 5, 7 + 8, 10 + 11
Hybridization buffer IS (S): Columns 3, 6, 9, and 12
Standard curve:
- TS8: TS8, Control template, telomerase–specific hybridization buffer
- TS8,0: Lysis buffer, telomerase–specific hybridization buffer
- TS8,IS: TS8, Control template, Internal Standard (IS)–specific hybridization buffer

Samples:
- S1,....: Samples, telomerase–specific hybridization buffer
- S1,0,...: Heat-treated samples, telomerase–specific hybridization buffer
- S1,IS,..: Samples, Internal Standard (IS)–specific hybridization buffer:

Hybridization and ELISA

1. For each sample, pipette 10 µl of Denaturation reagent (solution 7) into 2 separate reaction tubes.
   △ For large numbers of samples, use of a nuclease-free uncoated MP is recommended.

2. To each tube, add 2.5 µl of the amplification product; incubate all tubes at +15 to +25°C for 10 min.

3. • For each pair of sample tubes, add 100 µl Hybridization buffer T (solution 8) to one tube and 100 µl Hybridization buffer IS (solution 9) to the other tube.
   • Mix thoroughly by vortexing briefly.
   △ Negative controls are only treated with Hybridization buffer T; see pipetting scheme (previous page).

4. Do ONE of the following, depending on whether an appropriate MP shaker is available for Step 5 below:
   • IF an MP shaker is available, THEN: According to the pipetting scheme on the previous page, transfer 100 µl of each reaction mixture into one well of a precoated MP module (supplied with the kit). Cover the MP modules with the self-adhesive cover foil. Proceed to Step 5a below.
   • IF an MP shaker is not available, THEN: Leave the reaction mixtures in their reaction tubes. Proceed to Step 5b below.
Procedures and Required Materials

**5a** IF you transferred the samples to a precoated MP module in Step 4, THEN do the following:
- Place the MP module on an appropriate MP shaker.
- Incubate the MP modules for 2 hours at +37°C with shaking (300 rpm).
  
  This step allows the samples to hybridize and to bind to the wells of the MP module.

**5b** IF you left the samples in their reaction tubes in Step 4, THEN do the following:
- Place the reaction tubes into a conventional tube rack and place the rack on a conventional shaker.
- Incubate the tubes for 2 hours at +37°C with shaking (300 rpm).
  
  This step allows the samples to hybridize.
- After shaking the tubes for 2 hours, transfer 100 µl from each tube into one well of a precoated MP module (supplied with the kit).
- Cover the MP modules with the self-adhesive cover foil and incubate for another 1 hour at +37°C (without shaking).
  
  This step allows the samples to bind to the wells of the MP module.

**6** Remove the Hybridization solutions completely from the wells of the MP module.

**7** Wash each well 3 times with 250 µl of Washing buffer, 1x (solution 10) for a minimum of 30 sec each. Remove the Washing buffer carefully after each wash.

**8** Add 100 µl Anti-DIG-HRP working solution (solution 11) to each well.
- Cover the MP modules with a cover foil and incubate at +15 to +25°C for 30 min while the plate is shaking at 300 rpm.

**9** Remove the solution completely from the wells.
- Rinse each well 5 times with 250 µl of Washing buffer (solution 10) for a minimum of 30 sec each. Remove the Washing buffer carefully after each wash.

**10** Warm the TMB substrate solution (Solution 13) to +15 to +25°C.
- Add 100 µl prewarmed TMB substrate solution to each well.
- Cover the MP modules with foil and incubate for color development at +15 to +25°C for 10 to 20 min while the plates are shaking at 300 rpm.

**11** Without removing the reacted substrate, add 100 µl Stop reagent (Solution 14) into each well to stop color development.
  
  Addition of the stop reagent causes the reacted HRP substrate to change color from blue to yellow and is required for maximum sensitivity.

**12** Using a Microplate (ELISA) reader, measure the absorbance of the samples at 450 nm (with a reference wavelength of approximately 690 nm) within 30 min after adding the stop reagent.
3.4 Non-Radioactive Detection of Telomerase-Mediated DNA-Ladder after Transfer to Nylon Membranes

General
The biotin-labeled P1-TS Primer used in the 
Telomerase PCR ELISA<sup>PLUS</sup>, allows easy analysis of the amplification products obtained by Southern hybridization, if proof is desired for the presence of the typical, telomerase-mediated 6-nucleotide ladder.

Additional Reagents Required
Nylon Membranes, positively charged*

Preparation of Samples
Prepare cell extracts and perform the TRAP reaction as described above.

Protocol
Please refer to the following table.

1. Mix loading dye containing bromophenol blue and xylene cyanol with 20 μl of the PCR product.
   - Perform polyacrylamide gel electrophoresis according to standard protocols (17), using a 12% non-denaturing acrylamide gel.
   - Stop the procedure when xylene cyanol exits the gel.
   - The smallest telomerase product band should be at 52 bp.

2. After electrophoresis, transfer PCR products by vacuum blotting onto a positively charged nylon membrane.
   - Block the membrane with a solution of 2% Blocking reagent* for 30 min at +15 to +25°C to prevent unspecific binding.

3. Discard blocking solution and incubate the membrane with a streptavidin alkaline phosphatase (AP) conjugate or an anti-biotin-AP conjugate, diluted appropriately.
   - Rinse membrane 2 times in a Washing buffer for 15 min at +15 to +25°C.
   - Make the blotted products visible with chemiluminescence techniques as described for the detection reagents.
   - The band derived from the standard template DNA is at 216 bp.
4. Results

4.1 Data Analysis

Interpretation of Results

Absorbance values are reported as the $A_{450\,\text{nm}}$ reading against blank (reference wavelength $A_{690\,\text{nm}}$).

Negative Control

As described above, an appropriate negative control for checking the specificity of the telomerase reaction is prepared by heat-treatment of the samples. Alternatively, degrading the telomerase-associated RNA by preincubating the cell extract with DNase-free RNase can be used to prepare a negative control. For negative controls, the absorbance readings when analyzed with the telomerase specific detection probe (P3-T) are dependent on the effectiveness of telomerase inactivation. Values routinely found are less than 0.1 $A_{450\,\text{nm}}$ to $A_{690\,\text{nm}}$ units.

If the telomerase-specific values are higher, the entire experiment—including the TRAP reaction—should be repeated with prolonged heat- or RNase-treatment.

Positive Control

The values $(\text{ATS8–ATS8,0})/\text{ATS8, IS}$ obtained with 1 µl of the Control template, low, and 1 µl of the Control template, high, should be in the range of 0.3 to 0.8 and 0.9 to 4.0, respectively, after 10 min substrate reaction.

Samples

Subtract the mean of the absorbance readings of the negative controls from absorbance readings of the samples $(A - A_{0})$. Samples are to be considered as telomerase-positive if the difference in absorbance ($\Delta A$) is higher than the twofold background activity (background activity is the value of negative control or heat-treated sample).

Quantification of Telomerase Activity

The level of telomerase activity in a given sample is determined by comparing the signal from the sample to the signal obtained using a known amount of a Control template (TS8; solutions 4 or 5). The control templates (Control templates, low and high) provided with the TeloTAGGG Telomerase PCR ELISAPLUS are ready to use solutions that contain TS8 at a concentration of 0.001 amol/ml and 0.1 amol/ml respectively.

The Control template used (TS8; solutions 4 and 5) are identical to a telomerase elongation product with 8 telomeric repeats.

For most applications it has been shown that using the lower concentrated Control template (TS8; solution 4) provides reliable results. However, because amplification of the TRAP products and the Internal Standard (IS) are competitive, the signal of the internal control may be near background level when analysing samples with very high telomerase activity. Therefore, in such cases using the higher concentrated Control template (TS8; solution 5) is recommended.
### Calculation

Relative telomerase activities (RTA) within different samples in an experiment are obtained using the following formula:

\[
RTA = \frac{(AS - AS,0)/AS,IS \times 100}{(ATS8 - ATS8,0)/ATS8,IS}
\]

- \(AS\): absorbance of sample
- \(AS,0\): absorbance of heat- or RNase-treated sample
- \(AS,IS\): absorbance of Internal Standard (IS) of the sample
- \(ATS8\): absorbance of Control template (TS8)
- \(ATS8,0\): absorbance of Lysis buffer
- \(ATS8,IS\): absorbance of Internal Standard (IS) of the Control template (TS8)

### 4.2 Typical Results

**Note**

Absorbance values are reported as the A450 reading against blank (reference wavelength \(A_{690\text{ nm}}\)).

**Typical Results**

The following figures show typical results regarding the specificity and sensitivity of telomerase detection (Fig. 3), and quantification of telomerase activity (Fig. 4). In addition, they show the effects of Taq DNA Polymerase inhibitors on the results of telomerase detection (Fig. 5).

**Fig. 3:** Specificity and Sensitivity of the TeloTAGGG Telomerase PCR ELISA\textsuperscript{PLUS}. Immortalized cell lines and biopsies from healthy donors and tumor subjects were analysed for telomerase activity using the TeloTAGGG Telomerase PCR ELISA\textsuperscript{PLUS} (A) and the isotopic gel-based TRAP assay (B) parallel to one another.

- a) and c): Specificity of telomerase detection.
- b) and d): Sensitivity of telomerase detection using serially diluted extracts from HEK293 cells and biopsy material (bladder carcinoma).
Results

Fig. 4: Dynamic Range of the TeloTAGGG Telomerase PCR ELISA<sup>PLUS</sup>, is shown. The indicated number of 293 cells (10, 100, 1,000, 2,500, and 10,000 cell equivalents), ranging from 10 to 10,000 cells, were analysed for telomerase activity and the Internal Standard (IS) according to the kit protocol. The findings of three independent experiments have been included. RTA values were calculated as described in Section 4.1.

Fig. 5: Effect of PCR inhibition on amplification of telomerase and Internal Standard (IS) -specific signals. Extracts from 293 cells (293) and a biopsy sample (sample #137) were prepared. The indicated amounts of extract were analysed for telomerase activity and the internal standard template as described in the protocol. Sample #137 shows an increase of both the telomerase- and the Internal Standard (IS)-specific signals with decreasing amounts of extract, which indicates presence of PCR inhibitors in the sample.
5. References


## 6. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signals of negative controls are too high</td>
<td>Insufficient heat-inactivation or RNase pretreatment</td>
<td>Repeat the entire experiment with prolonged heat- or RNase-treatment.</td>
</tr>
<tr>
<td></td>
<td>Incubation with TMB substrate solution was too long.</td>
<td>Shorten incubation time with TMB substrate solution.</td>
</tr>
<tr>
<td></td>
<td>Cross-contamination of negative controls with active samples, Positive control template or amplification products from previous experiments.</td>
<td>Repeat the entire assay with fresh aliquots of the reagents. Check reagents for cross-contaminations.</td>
</tr>
<tr>
<td></td>
<td>Washing, when performing the detection step, was not sufficient.</td>
<td>Increase the number of washing steps.</td>
</tr>
<tr>
<td>Signals of positive controls are too low</td>
<td>Thermal cycling program is not appropriate for the type of thermal cycler used.</td>
<td>Optimize cycling conditions.</td>
</tr>
<tr>
<td></td>
<td>Anti-DIG-HRP, working solution is inactive.</td>
<td>Use only freshly prepared Anti-DIG-HRP, working solution.</td>
</tr>
<tr>
<td></td>
<td>Reagents are contaminated with nucleases.</td>
<td>Repeat the entire assay with fresh aliquots of the reagents. Check reagents for the presence of DNase and RNase contaminations.</td>
</tr>
<tr>
<td></td>
<td>Inadequate storage of Positive control templates and/or other kit components.</td>
<td>Check storage conditions. See section 3.2.</td>
</tr>
</tbody>
</table>
7. Supplementary Information

7.1 Conventions

7.1.1 Text Conventions

To make information consistent and memorable, the following text conventions are used in this document:

<table>
<thead>
<tr>
<th>Text Convention</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbered stages labeled ( 1, 2 ) etc.</td>
<td>Stages in a process that usually occur in the order listed.</td>
</tr>
<tr>
<td>Numbered instructions labeled ( 1, 2 ) etc.</td>
<td>Steps in a procedure that must be performed in the order listed.</td>
</tr>
<tr>
<td>Asterisk *</td>
<td>Denotes a product available from Roche Diagnostics.</td>
</tr>
</tbody>
</table>

7.1.2 Symbols

In this document, the following symbols are used to highlight important information:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>❓</td>
<td>Information Note: Additional information about the current topic or procedure.</td>
</tr>
<tr>
<td>⚠</td>
<td>Important Note: Information critical to the success of the procedure or use of the product.</td>
</tr>
</tbody>
</table>

7.2 Changes to Previous Version

- Editorial changes

7.3 Ordering Information

<table>
<thead>
<tr>
<th>Product</th>
<th>Pack Size</th>
<th>Cat. No.</th>
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</thead>
<tbody>
<tr>
<td>TeloTAGGG Telomerase PCR ELISA</td>
<td>96 reactions</td>
<td>11 854 666 910</td>
</tr>
<tr>
<td>TeloTAGGG Telomere Length Assay</td>
<td>1 kit</td>
<td>12 209 136 001</td>
</tr>
<tr>
<td>RNase, DNase-free</td>
<td>500 ( \mu )g (1 ml)</td>
<td>11 119 915 001</td>
</tr>
<tr>
<td>Buffers in a Box</td>
<td>Premixed PBS, 10x</td>
<td>11 666 789 001</td>
</tr>
<tr>
<td>Nylon Membrane, positively charged</td>
<td>10 sheets, 20 x 30 cm</td>
<td>11 209 272 001</td>
</tr>
<tr>
<td></td>
<td>20 sheets, 10 x 15 cm</td>
<td>11 209 299 001</td>
</tr>
<tr>
<td></td>
<td>1 roll, 0.3 x 3 m</td>
<td>11 417 240 001</td>
</tr>
</tbody>
</table>
7.4 Disclaimer of License

Notice to Purchasers

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