For life science research only.
Not for use in diagnostic procedures.

*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}

Version: 20
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Photometric enzyme immunoassay for quantitative determination of telomerase activity, utilizing the Telomeric Repeat Amplification Protocol (TRAP)

Cat. No. 12 013 789 001 1 kit
96 reactions

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Content
1	colorless	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Lysis reagent	▪ Ready-to-use solution. ▪ For preparation of cell extracts from cell cultures and tissue samples.	1 bottle, 11 ml
2	red	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Reaction mixture, 2x conc.	▪ Ready-to-use solution. ▪ Contains biotinylated telomerase substrate P1-TS, optimized anchor-primer P2, nucleotides, and Taq DNA Polymerase. ▪ For one-step telomerase-mediated primer elongation and PCR amplification.	2 bottles, 900 µl each
3	yellow	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Internal Standard (IS)	▪ 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.	1 bottle, 350 µl
4	white	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Control template, low	▪ Ready-to-use solution. ▪ Contains a positive control template DNA with the same sequence as a telomerase product with 8 telomeric repeats (TS8).	1 bottle, 35 µl, 0.001 amol/µl
5	white	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Control template, high	▪ Ready-to-use solution. ▪ Contains a positive control template DNA with the same sequence as a telomerase product with 8 telomeric repeats (TS8).	1 bottle, 35 µl, 0.1 amol/µL
6	colorless	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Water, nuclease-free	Double-distilled, nuclease-free water.	2 bottles, 1.1 ml each
7	violet	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Denaturation reagent	▪ Ready-to-use solution. ▪ Contains <0.5% sodium hydroxide. ▪ To denature amplicons.	1 bottle, 1.2 ml
8	white	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Hybridization buffer T	▪ 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.	1 bottle, 7 ml
9	yellow	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Hybridization buffer IS	▪ 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)	1 bottle, 3.5 ml
10	blue	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Washing buffer, 10x conc.	For washing steps.	1 bottle, 50 ml
11	red	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Anti-DIG-HRP	▪ Polyclonal antibody from sheep, conjugated to horseradish peroxidase (POD). ▪ Lyophilized, stabilized. ▪ To prepare conjugate solution.	1 bottle, 120 mU

12	red	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Conjugate dilution buffer	Ready-to-use solution.	1 bottle, 12 ml
13	green	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, TMB substrate solution	▪ Ready-to-use solution. ▪ Contains 3,3′,5,5′-tetramethylbenzidine.	1 bottle, 12 ml
14	green	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Stop reagent	▪ Ready-to-use solution. ▪ Contains <5% sulfuric acid.	1 bottle, 12 ml
15	foil bag	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Microplate	▪ Precoated with streptavidin and post-coated with blocking reagent. ▪ Shrink-wrapped with a desiccant capsule (12 × 8 wells).	1 strip frame, 12 modules of 8 wells each
16	–	*Telo**TAGGG*** Telomerase PCR ELISA^{PLUS}, Self-adhesive Plate Cover Foil	Prevents evaporation. ⚠ ***Cover the Microplate modules with the Cover Foils during each incubation step.***	3 foils

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label.

Vial / Bottle	Cap	Label	Storage
1	colorless	Lysis reagent	Store in aliquots at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
2	red	Reaction mixture, 2x conc.	
3	yellow	Internal Standard (IS)	
4	white	Control template, low	
5	white	Control template, high	
6	colorless	Water, nuclease-free	
7	violet	Denaturation reagent	Store in aliquots at +2 to +8°C.
8	white	Hybridization buffer T	Store in aliquots at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing.***
9	yellow	Hybridization buffer IS	
10	blue	Washing buffer, 10x conc.	Store in aliquots at +2 to +8°C.
11	red	Anti-DIG-HRP	Store at −15 to −25°C.
12	red	Conjugate dilution buffer	Store in aliquots at +2 to +8°C for 6 months.
13	green	TMB substrate solution	
14	green	Stop reagent	Store in aliquots at +2 to +8°C.
15	foil bag	Microplate	Store dessicated at +2 to +8°C. Stable for approximately 12 months once the bag has been opened.
16	–	Self-adhesive Plate Cover Foil	Store at +2 to +8°C.

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Sterile aerosol-preventive pipette tips
- Pipettes with disposable positive-displacement tips
- Sterile reaction tubes for preparing dilutions
- Microplate shaker (preferably +37°C)
- Microplate washer (optional)
- Microcentrifuge
- ELISA reader for microplates with 450 nm filter.

  Use a reference wavelength of approximately 690 nm.

For preparation of kit working solutions

- Autoclaved, double-distilled water
- DEPC-treated water (optional)

For preparation of negative control

- RNase, DNase-free*

For preparation of extracts from cells

- Neubauer hemocytometer
- Refrigerated centrifuge
- Reaction tubes
- Phosphate-buffered saline (PBS)*

For preparation of extracts from tissue

- Cryostat
- Sterile reaction tubes

For telomeric repeat amplification protocol (TRAP)

- PCR thermal cycler
- Mineral oil
- Tubes for PCR amplification

For nonradioactive detection after transfer to nylon membranes

- 12% nondenaturating acrylamide gel
- Loading dye
- Electrophoresis equipment
- Nylon membranes, positively charged*
- 2% Blocking reagent*
- Streptavidin-alkaline phosphatase (AP) conjugate*
- Chemiluminescence equipment for the visualization of blotted products

The TeloTAGGG Telomerase PCR ELISA^{PLUS} is designed for performing hybridization and detection in combination with a plate shaker at 300 rpm. If a shaker is not available, perform hybridization in reaction tubes as described in section, **Protocols***. The detection steps can be carried out as described in section,* **Protocols***.*

1.4. Application

The *Telo*TAGGG Telomerase PCR ELISA^{PLUS} is designed for the highly sensitive qualitative detection of telomerase activity in cell extracts from cell cultures and other biological samples. The kit utilizes a biotinylated primer for immobilization within the ELISA microplate. Using it together with a biotin-detection system, this biotin label can also serve as a means of detection; If the typical, 6-nucleotide-ladder, resulting from the TRAP assay is desired, the fragments can be separated by polyacrylamide gel electrophoresis (PAGE), blotted onto a positively charged membrane, and detected appropriately.

1.5. Preparation Time

Assay Time

Step	Approximate Duration [hours]
Extract preparation	1
Elongation and amplification	2.5
Hybridization and photometric detection	2.5
Total hands-on time	1
Total assay time	**7**

2. How to Use this Product

2.1. Before you Begin

Sample Materials

The *TeloTAGGG* Telomerase PCR ELISA^{PLUS} can be used with:
- Cell cultures
- Scientific biopsy material
- Other biological research samples.

Control Reactions

Preparation of negative control

Telomerase requires integrity of its internal RNA component as a template for the addition of the telomeric repeat sequences to the telomerase-specific primer. Therefore, preincubation of the cell or tissue extract with RNase, DNase-free* will fully destroy telomerase activity contained in the extract and offers a convenient control of specificity. Alternatively, heat-treatment of the cell extract for 10 minutes at +85°C prior to the TRAP reaction may be used to inactivate telomerase protein for producing negative controls. The following protocol is recommended:

1. Incubate 5 µl of cell extract typically corresponding to 5,000 cell equivalents or 50 µg of protein if extract from tissue samples is used with 1 µl RNase, DNase-free (0.5 µg/µl) for 20 minutes at +37°C.

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2. An aliquot of 1 to 3 µl of the RNase-treated extract is used for the *TeloTAGGG* Telomerase PCR ELISA^{PLUS}.

Incubation of a telomerase-positive extract at +37°C for 20 minutes in the absence of RNase does not affect the telomerase-specific signals. Therefore, any loss of signal is due to specific degradation of the internal RNA component. Exert extreme care to avoid carryover of RNase into samples, resulting in false-negative readings.

With this RNase treatment, the maximum value of absorbance for the negative control should be 0.25 $A_{450\ nm}$ to $A_{690\ nm}$ units. If values are higher, repeat the entire experiment including the TRAP reaction.

General Considerations

Precautions

Determining telomerase activity using the *TeloTAGGG* Telomerase PCR ELISA^{PLUS} 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.
- PCR carryover contamination resulting in false-positive signals.

As opposed to other TRAP assay formats, the *TeloTAGGG* Telomerase PCR ELISA^{PLUS}, provides all of the compounds required to perform telomerase-mediated primer elongation and 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:
- Use only double-distilled water that is nuclease-free, for example, DEPC-treated and autoclaved.
- Prepare appropriate aliquots of the kit solutions and keep them separate from other reagents in the laboratory.
- Use autoclaved or heat-sterilized labware, such as pipettes, pipette tips, and reactions vials.
- Wear gloves and a surgical mask during cell extraction and when performing the assay.
- Use fresh aerosol-preventive pipette tips for all pipetting steps to avoid cross-contamination of samples and reagents.
- Physically separate the workplaces for sample preparation, TRAP reaction, and detection of amplicons to minimize the risk of carryover contamination.

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. These false-negative tumor samples can be identified by including an internal amplification standard. Several internal standards have been reported in scientific publications, in which a heterologous 36 bp internal standard is most often used. 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. To overcome this problem, the Internal Standard provided with the *Telo*TAGGG 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.

Number of tests

Up to 31 samples plus control reactions can be performed, depending on the number of samples analyzed per experiment.

Safety Information

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

Laboratory procedures

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.
- Do not eat, drink or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.

Waste handling

- Discard unused reagents and waste in accordance with country, federal, state, and local regulations.
- Safety Data Sheets (SDS) are available online on dialog.roche.com, or upon request from the local Roche office.

Working Solution

Solution	Content	Reconstitution/Preparation of Working Solution	Storage and Stability	For use in...
10	Washing buffer, 1x conc. (Bottle 10)	▪ Dilute an appropriate volume of Washing buffer, 10x conc. with autoclaved, double-distilled water (1:10) and mix thoroughly. ▪ Approximately 5 ml of the diluted Washing buffer is needed for one reaction.	Store at +2 to +8°C for one month.	Washing steps
11a	Anti-DIG-HRP, stock solution (Bottle 11)	▪ Quickly spin Bottle 11 before opening. ▪ Reconstitute the lyophilizate in 240 µl autoclaved, double-distilled water. ▪ This results in an antibody conjugate concentration of 0.5 U/ml.	Store at +2 to +8°C for 6 months. ⚠ ***Do not freeze.*** ⚠ ***Do not add sodium azide.***	Preparation of Solution 11b.
11b	Anti-DIG-HRP, working solution	▪ Dilute an appropriate amount of the reconstituted Anti-DIG-HRP (Solution 11a) with Conjugate dilution buffer (Bottle 12) to a final concentration of 10 mU/ml, for example, 200 µl antibody solution and 9.8 ml of Conjugate dilution buffer.	Prepare immediately before use.	Labeling reaction
15	Microplate	Use only the Microplate modules required for the particular experiment. Close the foil bag containing the remaining modules and the desiccant capsule tightly with adhesive tape. ⓘ *The Microplate modules are ready-to-use and do not need to be rehydrated before use.*	Once the foil bag is opened, store Microplate modules desiccated at +2 to +8°C for approximately 12 months.	ELISA assay

2.2. Protocols

Preparation of extracts from cells

⚠ ***To avoid carryover contamination, transfer the required volumes of Bottles 1, 2, 3, 4, and 5 for one experiment into a fresh tube instead of directly pipetting from stock solutions.***

1. Harvest and count cells using a hemocytometer.

2. Transfer 2 × 10^5 cells per single reaction into a fresh reaction tube.

3. Pellet cells at 3,000 × *g* for 5 minutes in a refrigerated centrifuge at +2 to +8°C.

4. Carefully remove supernatant and resuspend the cells in PBS; repeat the centrifugation step.

5. Carefully remove the supernatant.
   – If the *TeloTAGGG* Telomerase PCR ELISA^{PLUS} is not performed immediately after the extract preparation, store the pelleted cells at −80°C until use.

6. Resuspend the pelleted cells in 200 µl Lysis reagent (Bottle 1), pre-cooled on ice by reverse pipetting at least 3 times and incubate on ice for 30 minutes.
   If frozen cell pellets were used for extraction, thaw cell pellets on ice before adding Lysis reagent (Bottle 1).

7. Centrifuge the lysate at 16,000 × *g* for 20 minutes in a refrigerated centrifuge at +2 to +8°C.

8. Carefully remove the supernatant and transfer to a fresh tube.
   – To ensure that no cellular debris of the pelleted cells is transferred, pipette only 175 µl of the cell extract.

9. Perform the TRAP reaction as described below.
   – If not immediately performing the TRAP reaction, shock freeze the cell extract in aliquots in liquid nitrogen and store the extracts at −80°C.

Preparation of extracts from tissue

Preparing tissue specimens for determining telomerase activity requires careful acquisition and storage of research materials since cross-contamination with tumor cells can result in false-positive signals in normal tissues or negative signals in tumor samples due to improper storage.

Tissue samples should be shock frozen in small pieces in liquid nitrogen, and can be stored at −80°C if not used immediately in the TeloTAGGG Telomerase PCR ELISA^{PLUS}. However, we recommend that extracts be stored, which have been prepared as described below, rather than storing tissue specimens.

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

2. Transfer approximately 50 sections into a sterile reaction tube containing 200 µl ice-cold Lysis reagent (Bottle 1).
   Alternatively, 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 (Bottle 1). Homogenize on ice with a motorized pestle until uniform consistency.

3. Incubate on ice for 30 minutes.

4. Centrifuge the lysate at 16,000 × *g* for 20 minutes in a refrigerated centrifuge at +2 to +8°C.
   – A refrigerated benchtop centrifuge using reaction tubes can also be used.

5 Carefully remove the supernatant and transfer to a fresh tube.
– To ensure that no debris of the tissue is transferred, pipette only 175 µl of the tissue extract.

6 Measure the protein concentration by standard methods; shock freeze the tissue extract in aliquots in liquid nitrogen and store the extracts at −80°C.

Telomeric repeat amplification protocol (TRAP reaction)

Preparation of the master mix

Prepare a master mix for all samples, negative controls, and control templates to be analyzed simultaneously. For example, if 7 samples including controls have to be analyzed, mix 175 µl of Reaction mixture (Bottle 2) and 35 µl Internal Standard (Bottle 3).

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.

1 For each sample to be tested and the controls, transfer 25 µl Reaction mixture (Bottle 2) and 5 µl of the Internal Standard (IS) (Bottle 3) into a tube suitable for PCR amplification.
– Alternatively, transfer 30 µl of the master mix into tubes suitable for PCR amplification per PCR reaction.

2 Add the following volumes as mentioned in the table.
i *Perform all pipetting steps on ice.*

Samples/Control	Volume
Samples	1 to 3 µl cell extract per tube, corresponding to 1 × 10^3 to 3 × 10^3 cell equivalents, or 0.5 to 10 µg total protein, see section, **Preparation of extracts from cells**.
Negative controls	1 to 3 µl of the corresponding RNase treated or heat-treated cell extract, corresponding to 1 × 10^3 to 3 × 10^3 cell equivalents, or 0.5 to 10 µg total protein.
Control template	1 µl Control template (use either low or high concentration, Bottles 4 or 5) into a separate tube. Add 1 µl Lysis reagent (Bottle 1) into another separate tube to serve as a blank for the Control template.

i *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 (Bottle 6) to a final volume of 50 µl.

3 Transfer tubes to a thermal cycler and perform a combined primer elongation/amplification reaction:

Step	Time	Temperature [°C]	Cycles
Primer elongation	10 – 30 min	25	1
Telomerase inactivation	5 min	94	1
Amplification: Denaturation Annealing Polymerization	 30 sec 30 sec 90 sec	 94 50 72	 1 – 30
Final extension	10 min	72	1
Hold	–	4	–

Microplate pipetting scheme

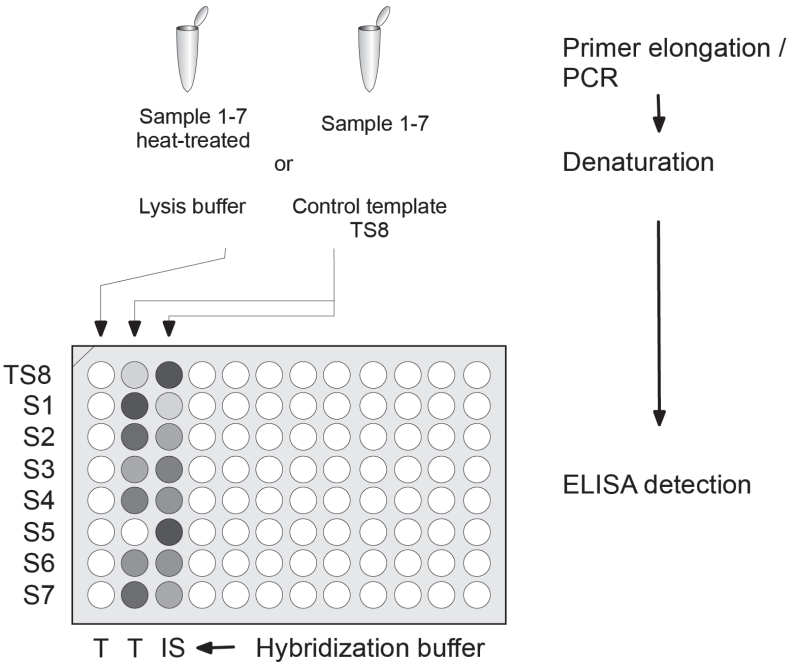

Fig. 1: Pipetting scheme
Hybridization buffer T (T): Columns 1 + 2, 4 + 5, 7 + 8, 10 + 11.
Hybridization buffer IS (S): Columns 3, 6, 9, and 12.
Set up the pipetting scheme according to the following table.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TS8,0	TS8	TS8,IS	–	–	–	–	–	–	–	–	–
B	S1,0	S1	S1,IS	–	–	–	–	–	–	–	–	–
C	S2,0	S2	S2,IS	–	–	–	–	–	–	–	–	–
D	S3,0	S3	S3,IS	–	–	–	–	–	–	–	–	–
E	S4,0	S4	S4,IS	–	–	–	–	–	–	–	–	–
F	S5,0	S5	S5,IS	–	–	–	–	–	–	–	–	–
G	S6,0	S6	S6,IS	–	–	–	–	–	–	–	–	–
H	S7,0	S7	S7,IS	–	–	–	–	–	–	S31,0	S31	S31,IS
–	**T**	**T**	**S**	–	–	–	–	–	–	–	–	–

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. Per sample, transfer 10 µl of Denaturation reagent (Bottle 7) into 2 suitable reaction tubes.
   i *For a large numbers of samples, use a nuclease-free, uncoated microplate.*

2. Add 2.5 µl of the amplification product to each tube and incubate at +15 to +25°C for 10 minutes.

3. Add 100 µl Hybridization buffer T (Bottle 8) to one tube and 100 µl Hybridization buffer IS (Bottle 9) to the other tube and mix thoroughly by vortexing briefly.
   i *Negative controls are only treated with Hybridization buffer T; see section,* **Microplate pipetting scheme**.

4. Transfer 100 µl of the mixture per well of the precoated Microplate modules supplied with the kit and cover the wells with the Self-adhesive Cover Foil to prevent evaporation.

5. Incubate the Microplate modules at +37°C on a shaker at 300 rpm for 2 hours.
   – This step allows the samples to hybridize and to bind to the wells of the Microplate module.
   i *If an appropriate microplate shaker is not available, perform the hybridization reaction in a reaction tube, and after shaking for 2 hours at +37°C at 300 rpm, transfer 100 µl of the hybridization mixture per well of the precoated Microplate modules.*
   – Cover the wells with the Self-adhesive Cover Foil and incubate for an additional 1 hour at +37°C. This step allows the samples to bind to the wells of the Microplate modules.

6. Remove the Hybridization solution completely.
   – Wash 3 times with 250 µl of Washing buffer (Solution 10) per well for a minimum of 30 seconds each; carefully remove Washing buffer.

7. Add 100 µl Anti-DIG-HRP working solution (Solution 11b) per well.
   – Cover the Microplate modules with a Cover Foil and incubate at +15 to +25°C for 30 minutes while shaking at 300 rpm.

8. Remove the solution completely.
   – Rinse 5 times with 250 µl of Washing buffer (Solution 10) per well for a minimum of 30 seconds each; carefully remove Washing buffer.

9. Add 100 µl TMB substrate solution (Bottle 13) prewarmed at +15 to +25°C per well.
   – Cover the wells with a Cover Foil and incubate for color development at +15 to +25°C for 10 to 20 minutes while shaking at 300 rpm.

10. Without removing the reacted substrate, add 100 µl Stop reagent (Bottle 14) per well to stop color development.
    i *Addition of the Stop reagent causes the reacted POD substrate to change in color from blue to yellow, and is required to achieve maximal sensitivity.*

11. Using a Microplate (ELISA) reader, measure the absorbance of the samples at 450 nm, using a reference wavelength of approximately 690 nm within 30 minutes after addition of the Stop reagent.

Nonradioactive detection of telomerase-mediated DNA ladder in extracts of cultured cells after transfer to nylon membranes

The biotin-labeled P1-TS Primer used in the *Telo*TAGGG Telomerase PCR ELISA^{PLUS} enables easy analysis of the amplification products, see section, **Telomeric repeat amplification protocol** by Southern hybridization, if proof for the presence of the typical, telomerase-mediated 6-nucleotide-ladder is desired.

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, using a 12% nondenaturing acrylamide gel.
   – Apply current until Bromophenol blue runs off the gel following standard procedures.
   The smallest telomerase product band should be 52 bp.

2. After electrophoresis, transfer PCR products by vacuum blotting onto a positively charged nylon membrane* using standard techniques.

3. Block the membrane with a solution of 2% Blocking reagent* to prevent nonspecific binding for 30 minutes at +15 to +25°C.

4. Discard Blocking solution and incubate the membrane with a streptavidin-alkaline phosphatase (AP) conjugate*, diluted appropriately.

5. Rinse membrane 2 times in Washing buffer for 15 minutes at +15 to +25°C.

6. After rinsing the membrane, visualize the blotted products by chemiluminescence.

   The band derived from the standard template DNA is at 216 bp.

2.3. Parameters

Detection range

Measuring range

Linear measuring range is from 10 to 2,000 cells in a model system using 293 cells.

Sensitivity

Detects <10 cell equivalents in a model system, using 293 cells.

3. Results

Interpretation of the results

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

Negative control

As described in section, **Control Reactions**, an appropriate negative control for checking the specificity of the telomerase reaction, is degrading the telomerase-associated RNA by preincubating the cell extract with DNase-free RNase. Alternatively, heat-treatment may be used to prepare negative controls. 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 <0.1 $A_{450\ nm}$ to $A_{690\ nm}$ units. If values are higher, repeat the entire experiment including the TRAP reaction.

Positive control

The values (ATS8–ATS8,0)/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 minutes substrate reaction.

Samples

Subtract the mean of the absorbance readings of the negative controls from absorbance readings of the samples (AS – AS,0). Samples are to be considered as telomerase-positive if the difference in absorbance (ΔA) 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 (low and high) provided with the *Telo*TAGGG Telomerase PCR ELISA^{PLUS} 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 analyzing samples with very high telomerase activity. Therefore, 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:

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

Fig. 2: Calculation of relative telomerase activities.
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)

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).

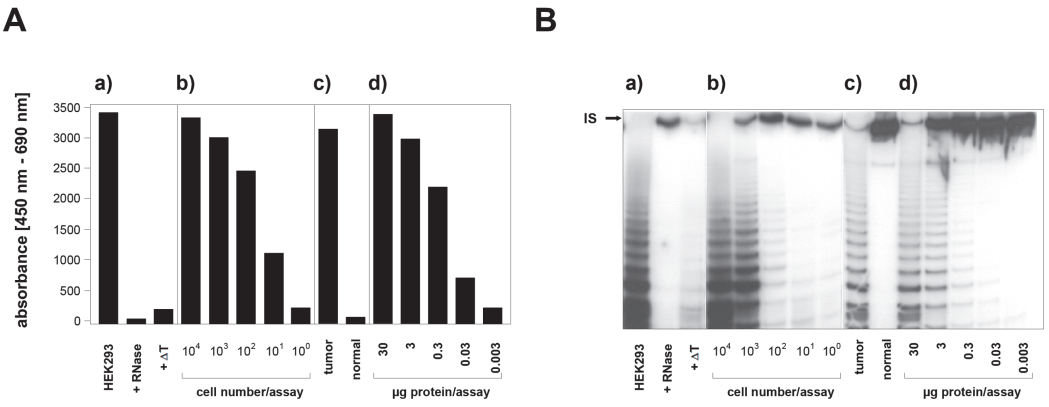

Fig. 3: Specificity and sensitivity of the *Telo*TAGGG Telomerase PCR ELISA^{PLUS}. Immortalized cell lines and biopsies from healthy donors and tumor subjects were analyzed for telomerase activity using the *Telo*TAGGG Telomerase PCR ELISA^{PLUS} (A) and the isotopic gel-based TRAP assay (B) parallel to one another.
a) and c): Specificity of telomerase detection.
b) and d): Sensitivity of telomerase detection using serially diluted extracts from HEK-293 cells and biopsy material (bladder carcinoma).

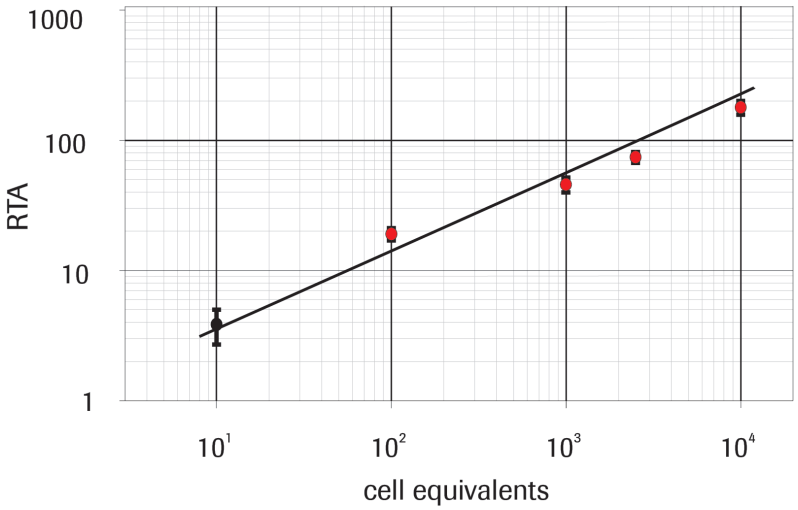

Fig. 4: Dynamic Range of the *Telo*TAGGG Telomerase PCR ELISA^{PLUS} is shown. The indicated number of 293 cells (10, 100, 1,000, 2,500, and 10,000 cell equivalents), ranging from 10 to 10,000 cells, were analyzed for telomerase activity and the Internal Standard (IS) according to the kit protocol. The findings of three independent experiments have been included. RTA values were calculated as described under **Calculation**.

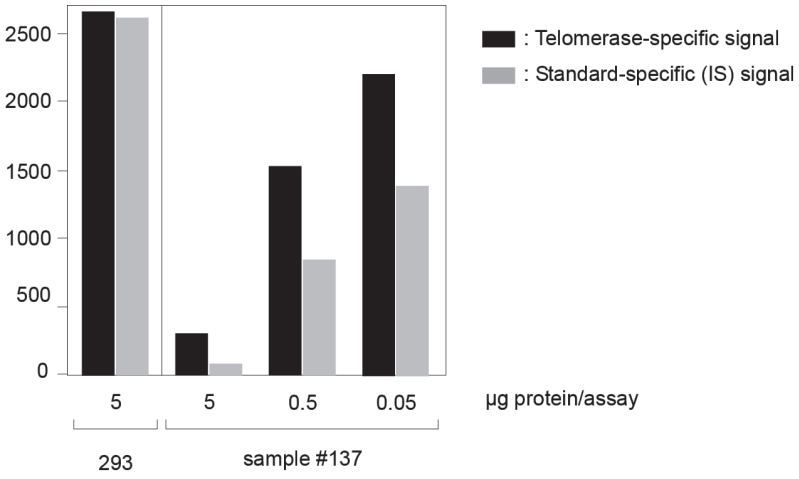

Fig. 5: Effect of PCR inhibition on amplification of telomerase and Internal Standard (IS)-specific signals. Extracts from 293 cells (293) and a biopsy sample (sample 137) were prepared. The indicated amounts of extract were analyzed for telomerase activity and the internal standard template as described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

4. Troubleshooting

Observation	Possible cause	Recommendation
Negative control signals too high.	Negative control, Lysis reagent, or Reaction mixture may be contaminated with a telomerase-positive cell extract.	Inactivate an aliquot of the negative control by RNase-treatment or heating for 10 minutes at +85°C; repeat the entire experiment including amplification.
	Negative control, Lysis reagent, Reaction mixture, or the other reagents may be contaminated with amplification products from previous experiments.	Prepare new negative control and fresh reagents.
	Washing when performing the detection step was not sufficient.	Increase number of washing steps.
	Incubation with TMB substrate solution was too long.	Stop substrate reaction within 20 minutes of adding the TMB substrate solution.
Positive control signals too low or absent.	Thermal cycling program is not appropriate for the type of thermocycler used.	Optimize cycling conditions.
	Water used for the combined primer elongation/amplification was not free of nucleases.	Use only PCR-grade water or double-distilled water treated with DEPC and autoclaved for reconstitution of kit components and preparation of buffers.
	Reagents were contaminated with nucleases.	Repeat the entire assay with fresh aliquots of Positive control cell extract and samples.
		Check reagents for the presence of DNase and RNase contaminations.
	Incubation steps were performed without shaking at 300 rpm.	Absolute levels of absorbance mentioned above were obtained when a microplate shaker was used.
	Anti-DIG-HRP working solution is inactive.	Use only freshly prepared Anti-DIG-HRP working solution.
		Check enzyme activity of the Anti-DIG-HRP working solution and prepare fresh working solution.
	Inadequate storage of Positive control templates and/or other kit components.	Check storage conditions, see section, **Storage and Stability**.

5. Additional Information on this Product

5.1. Test Principle

The *Telo*TAGGG Telomerase PCR ELISA^{PLUS} enables highly specific amplification of telomerase-mediated elongation products combined with nonradioactive detection following an ELISA protocol (Fig. 6).

1. During elongation, telomerase adds telomeric repeats (TTAGGG) to the 3′ end of the biotin-labeled synthetic P1-TS-primer.

2. 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 product.

3. 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.

4. The resulting products are immobilized via the biotin label to a streptavidin-coated microplate.

5. Immobilized amplicons are then detected with an antibody against digoxigenin, which is conjugated to horseradish peroxidase (Anti-DIG-HRP) and the sensitive peroxidase substrate TMB.

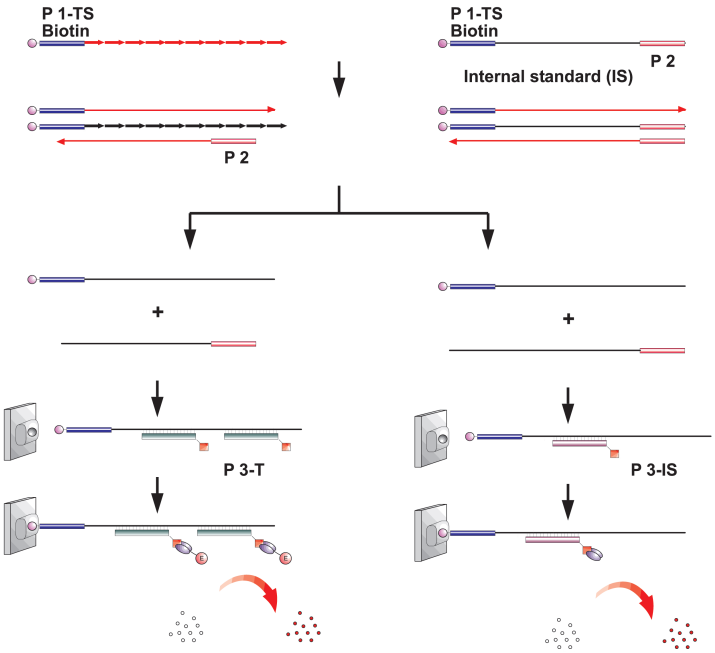

Fig. 6: Principle of the *Telo*TAGGG Telomerase PCR ELISA^{PLUS}.

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. 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. 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". This phenomenon, which has been demonstrated *in vitro* and *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.

Telomerase

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. The genes encoding its RNA subunit and the catalytic protein subunit have been cloned from a variety of species, including humans. 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.
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.
Telomerase has been detected in most neoplastic lesions and appears to be necessary for the sustained proliferation of most advanced cancers. 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. 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, nonspecific amplification products have been described. Despite several improvements to the protocol, quantification of telomerase activity remains difficult to perform and the non-availability of an amplification control may result in production of false-negative results for samples containing PCR inhibitors. The *Telo*TAGGG Telomerase PCR ELISA^{PLUS} provides a way to perform a highly sensitive photometric enzyme immunoassay to detect telomerase activity using nonradioactive techniques.

5.2. Quality Control

For lot-specific certificates of analysis, see section **Contact and Support**.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Layout changes.
Editorial changes.
Update to include new safety Information to ensure handling according controlled conditions.

6.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Buffers in a Box, Premixed PBS Buffer, 10x	4 l	11 666 789 001
Nylon Membranes, positively charged	10 sheets, 20 x 30 cm	11 209 272 001
	20 sheets, 10 x 15 cm	11 209 299 001
	1 roll, 0.3 x 3 m	11 417 240 001
RNase, DNase-free	500 µg, 1 ml	11 119 915 001
Streptavidin Conjugates	Streptavidin-AP Conjugate, 1,000 U	11 089 161 001
	Streptavidin-β-Gal Conjugate, 500 U, *Not available in US*	11 112 481 001
	Streptavidin-POD Conjugate, 500 U	11 089 153 001
Blocking Reagent	50 g	11 096 176 001

6.4. Trademarks

All product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

6.6. Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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