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Optimized for Routine: Highly sensitive fluorescent Telomeric Repeat Amplification Protocol (f-TRAP) V.1

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Abstract

Here we present an optimized protocol for measuring Telomerase activity that combines a fluorescently labeled bait primer and PCR amplification with analytical capillary electrophoresis to achieve a detection limit of one telomerase-positive cell per ten thousand negative cells. Cellular inhibitors of the assay are bypassed by triplicate approaches at different protein concentrations, providing rapid results of telomerase activity with low failure rates. In research laboratories, the simple protocol enables the assessment of immortality in newly generated cell lines or the unambiguous evaluation of iPSC reprogramming. The fast ad-hoc application for single assays up to high throughput of bulk samples should make f-TRAP a promising technique prospect in clinical oncology.

Attachments

PDF

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1MB

Image Attribution

Dirks_protocolio_Fig. 1.pdf

Guidelines

Background: hTERT maintains telomere length of chromosomes in germ line, stem cells and in immortal cell lines. The telomerase ribonucleoprotein is very sensitive to RNAses and temperatures! Precautions are therefore required to prevent PCR and RNase contamination. hTERT activity of cells can be measured by the presented fTRAP assay. Cell lines lacking telomerase activity such as U2OS and cell lines with active hTERT such as HeLa should be used as negative and positive control, respectively.

Materials

- Trapeze 1x CHAPS Lysis Buffer, Merck Cat. No. S7705 (Merck KGaA, Darmstadt, Germany).

- Pierce Coomassie Plus (Thermo Scientific, Cat. No. 23238) for Bradford assay to determine the protein concentration of lysates.

- Oligo Clean & Concentrator, Zymo Research Cat. No. D4061 (Irvine, USA).

- TaKaRa Taq Hot Start Version, Takara Bio Inc. Cat. No. R007A (Shiga, Japan).

- Oligonucleotides by Merck (Darmstadt, Germany).

TS (bait)(D4)5´-AATCCGTCGAGCAGAGTT (D4 is a WellRed flourescent label)
R-oriACX 5´-CTTACCCTTACCCTTACCCTAACC

- Capillary Electrophoresis: All reagents, chemicals, hardware and software from AB Sciex Germany GmbH (Darmstadt, Germany).

Safety warnings

Cellular inhibitors of the assay are bypassed by triplicate approaches at different protein concentrations, providing rapid results of telomerase activity with low failure rates. Pierce Coomassie Plus (Thermo Scientific, Cat. No. 23238) was used in a Bradford assay to determine the protein concentration of lysates. Depending on cell lines used concentrations were generally between 1.0 and 3.5 µg/µl protein. Triplicate aliquots of 10, 5, and 1 µl with equivalents for 9×10^4,4.5×10^4 and 9×10^3 cells, respectively, and a heat-inactivated equivalent of 4.5×10^4 cells were subjected to an extension reaction in a final volume of 50 µl.

Before start

The bait and forward TS primer is labeled at the 5'-end with the fluorophore D4 [WellRED-Oligos, Eurofins, Munich, Germany] in order to achieve a high extinction coefficient, which absorbs at 670 nm. Other fluorophores can also be used for labeling, provided the capillary electrophoresis is equipped with suitable filters.

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1h

1 LYSATE PREPARATION

1h

2 Count and pellet 1×10^6 cells of best viability and place in a 1,5 ml reaction cap.

3 Wash cells 1x using PBS at room temperature.

4 Dissolve pellet thoroughly in 50 µl TRAPeze® 1X CHAPS lysis buffer and incubate for 30 min on wet ice.

CHAPS (3-[(3-Cholamidpropyl)dimethylammonio]-1-propane sulfonate) is anon-denaturing zwitterionic surfactant for the separation of proteins from thenon-soluble parts of the cell including membrane proteins. The use of “classical”Triton-X, Tween, SDS are less efficient, especially with tissue and spheroids,and lead to significantly increased failure rates.

5 Centrifuge lysate for 20 min at 12.000 g at 4 °C.

6 Save 45 µl supernatant in a fresh cap and add 45 µl sterile water, store at -80°C or use directly.

7 Pierce Coomassie Plus (Thermo Scientific, Cat. No. 23238) is used to determine the protein concentration of lysates (Bradford Assay). Depending on cell lines used concentrations were generally between 1.0 and 3.5 µg/µl protein. Triplicate aliquots of 10, 5, and 1 µl with equivalents for 9×10^4, 4.5×10^4 and 9×10^3 cells, respectively, as well as a heat-inactivated equivalent of 4.5×10^4 cells were subjected to an extension reaction in a final volume of 50 µl).

8 EXTENSION REACTION

1h

Cells from different tissues can differ in the quantity and quality of inhibitors for the TRAP assay. Reliable results are usually obtained with triplicate preparations and 1:10, 1:50 and 1:100 dilutions of the lysate.

9 An aliquot of 15 µl out of 90 µl supernatant is used as negative control by heat inactivation at 85°C for 10 min.

10 A triplicate reaction using 10 µl lysate (corresponds to 90,000 cells), 5 µl lysate (45,000 cells) and 1 µl lysate (9,000 cells) guarantees a reliable result.

11 Pipette 5 µl of 10xTRAP-buffer (200 mM Tris-HCL pH8,3; 15 mM MgCl2, 630 mM KCl2; 0,005% Tween20; 10 mM EGTA) into 0,2 ml PCR caps or cap stripes for each reaction.

12 Add 5 µl of 10 mM dNTP (Takara) and 5 µl of 1 µM flourescent TS primer (bait).

13 Add per triple set 10, 5 and 1 µl lysate and 10 µl of heat-inactivated lysate to the control. Fill up to a final 50 µl volume with sterile water.

14 Mix thoroughly and incubate at 30 °C for 60 min in an PCR device.

15 Inactivate reactions for 5 min at 94°C and keep at 4°C.

16 PURIFICATION OF EXTENSION PRODUCTS 20m

17 Add to each extension reaction 100 µl oligo binding buffer of Oligo Clean & Concentrator Kit (ZymoResearch)

18 Add 400 µl fresh ethanol (98%) to each extension reaction

19 Pipet the total 550 µl to the column and centrifuge at 15 000 g for 30 sec.

20 Wash once using 750 µl wash buffer, centrifuge at 15 000 g for 60 sec.

21 Pipett 25 µl of sterile water to the bound extension products and incubate for 1 min.

22 Eluate extension product by centrifugation at 15 000 g for 30 seconds.

23 Keep the eluates on wet ice.

24 PCR AMPLIFICATION 2h

25 Place 20 µl of eluate into 0,2 PCR caps or cap stripes.

26 Prepare PCR reaction mix for n + 1 sample (2,5 µl 10X Takara buffer, 1 µl dNTP, 1 µl 10µM R-oriACX-Tel primer, 0,2 µl Takara Taq polymerase (1 U), 0,3 µl sterile water.

27 Add 5µl PCR reaction mix to each eluate, mix thoroughly and spin down for 10 sec.

28 f-TRAP PCR PROGRAMM

step1: 60 sec 95°C (1 cycle),
step 2: 30 sec 94 °C, 45 sec 56 °C, 45 sec 70 °C (10 cycles),
step 3: 30 sec 90 °C, 45 sec 56 °C, 45 sec 70 °C (20 cycles),
step 4: 40 min 56 °C, ~ RT.

29 ANALYSIS USING CAPILLARY ELECTRPHORESIS (CE) 1h

30 Prepare 24 µl loading mix for n + 1 sample by placing 23,5 sample loading solution (SLS) and 0,5 µl of a 600 bp size standard (AB Sciex, Darmstadt, Germany).

31 Add 1 µl PCR reaction of sample triplicates to device-specific loading plates for CE.

32 Use device-specific running condition of CE for fragment analysis between 70 and 600 nucleotide length.

33 In general, capillary electrophoreses provide software that enables DNA fragment analysis in order to automatically evaluate the PCR result. Depending on the manufacturer, the analysis parameters are set via dialog boxes that can be saved so that the auto-run function can be activated with a single mouse click. The analysis settings

include options for detecting telomeric fragment sizes of 6 bp lengths and for correcting common chemical artifacts (pull-up, peak saturation, spike removal).

34 For quick access and long-term documentation, evaluated f-TRAP electropherograms can be easily and securely stored in a database.

Protocol references

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