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Nonradioactive, Agarose Minigel Procedure for Telomeric Repeat Amplification Protocol

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Telomeres, the ends of chromosomes, are composed of many kilobases of TTAGGG repeats in vertebrates. These repeats are added by the enzyme telomerase, a ribonucleoprotein DNA polymerase that synthesizes the repeats based on its RNA template. A sensitive assay combining telomerase activity and the polymerase chain reaction (PCR) known as the telomeric repeat amplification protocol (TRAP) has been developed (6,10). In this assay, telomerase adds TTAGGG repeats to a telomerase substrate (TS) oligonucleotide that has no telomeric sequence; the enzyme pauses after adding each repeat, presumably to permit repositioning of its RNA template prior to synthesizing the next repeat. This generates a DNA ladder of 6-bp addition products that is then sensitively detected by polyacrylamide gel electrophoresis (PAGE) after PCR amplification (using a second CX oligonucleotide as reverse primer).

Telomerase activity has been detected in tumor, germ and stem cells but not in adult somatic cells. Thus, the enzyme may play a significant role in aging and in the pathogenesis of cancer

(2,6). Because of this widespread interest, improvements to TRAP are continually being developed, e.g., internal standard, fluorescent TS primer and improved CX-ext primer (7,9,12). We report fluorometric detection of TRAP DNA ladders by high-resolution agarose gel electrophoresis.

The human embryonic kidney (HEK) 293T cell line and the human monocytic macrophage Mono Mac 6 cell line were cultured as described (3, 5). Telomerase extracts were obtained from cells by the procedure of Kim et al. (6) with minor modifications (1,10, 11). Cells were counted, harvested and treated with ice-cold TRAP lysis buffer at 10^6 cells/100 μ L buffer. The TRAP lysis buffer contained 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, 25 mM 2-mercaptoethanol, 1 ng/mL leupeptin and 50% glycerol in diethyl pyrocarbonate (DEPC)-treated water. The lysate was kept on ice for 30 min and then centrifuged at $16000\times g$ for 20 min at 4°C in a Model 5415 Eppendorf® Microcentrifuge (Brinkmann Instruments, Westbury, NY, USA). Afterwards, 80 μ L of supernatant were collected, flash-frozen in liquid nitrogen and stored at -75°C until used.

Detection of telomerase activity from cell extracts was performed in a two-step process: (i) telomerase-mediated extension of the TS nucleotide and (ii) PCR amplification of the resultant product with both TS and the reverse CX primers (1,10,12). The oligonucleotides TS (5'-AATCCGTCGAGCAGAGTT-3') and CX (5'-CCCTTACCC-TTACCCTTACCCTAA-3') (both from BioSynthesis, Lewisville, TX, USA) were each dissolved in DEPC-treated water at 0.1 μ g/ μ L. Each thin-wall PCR tube contained 20 μ L of a reaction mixture that included 16.5 μ L of TRAP reaction buffer (20 mM Tris-HCl, pH 8.2, 63 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mg/mL 1 $\times$ bovine serum albumin [BSA] and 0.05% Tween® 20), 1 μ L of TS (0.1 μ g), 0.5 μ L of dNTPs (10 mM stock) and 2 μ L cell extract (from 1000 cells). The telomerase extension reaction was completed in a Model DB 66925 Temp.Tronic® Thermal Cycler (Barnstead/Thermolyne,

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Dubuque, IA, USA) after 30 min incubation at 25°C; the temperature was then raised to 90°C for 2 min to inactivate the telomerase. During this time, 5 µL of PCR mixture (containing 3.5 µL of TRAP reaction buffer, 1 µL of CX [0.1 µg] and 0.5 µL of *Taq* DNA polymerase [2.5 U]) were added to each tube together with 30 µL of mineral oil for hot-start PCR. A 34-cycle PCR was performed at 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, with a final extension at 72°C for 2 min. TRAP products were analyzed by high-resolution agarose gel electrophoresis.

For gel analysis, a horizontal minigel apparatus (Mini-Sub® Electrophoresis Cell; Bio-Rad, Hercules, CA, USA) and the TBE buffer system were used. The gel was composed of 4.5% MetaPhor® agarose (FMC BioProducts, Rockland, ME, USA). For a 3-mm gel, 1.125 g agarose were stirred in 26 mL TBE in a beaker, weighed and

dissolved using a microwave oven (with care not to cause spilling) and reweighed (for loss during microwave treatment), and warm water was added to obtain the original weight before cooling and pouring. After setting at room temperature, the gel was chilled for 30 min at 4°C before use. For each lane, 21 µL (18 µL TRAP products and 3 µL gel-loading buffer [6× dye: 0.25% bromophenol blue, 0.25% xylene cyanol and 15% Ficoll® Type 400 in water]) were loaded. For molecular weight standard, 0.5 µL of the 123-bp ladder (Life Technologies, Gaithersburg, MD, USA) was also loaded onto a separate gel lane. The agarose gel was run at 100 V for 2 h in a 4°C coldroom. After gel electrophoresis, DNA bands in the gel were detected after a 30-min staining with SYBR® Green I fluorescent dye (FMC BioProducts or Molecular Probes, Eugene, OR, USA) in a tinfoil-covered polypropylene contain-

er with gentle shaking (5 µL dye in 50 mL TBE, 10 000× stock). The bands were seen under a Foto/Prep I UV Transilluminator (Fotodyne, Hartland, WI, USA), and the stained agarose gel was analyzed using the Storm™ Fluorescence Scanning System (Molecular Dynamics, Sunnyvale, CA, USA) (4, 10). Although fluorescence scanning was used here, it should be noted that photographic documentation is also possible with the UV transilluminator and a SYBR Green gel stain photographic filter.

By this approach, we were successful in using an agarose minigel system to visualize the TRAP DNA ladders (Figure 1). The reaction was specific because the ladder was absent if the reverse CX primer had been omitted (Figure 1A, lane 2) or if the cell extract had been pretreated with RNase [Figure 1A, lanes 4 and 6, and 1B, lane 4; 5 µL of cell extract were incubated with

Figure 1. Analysis of TRAP products after 4.5% MetaPhor agarose gel electrophoresis and SYBR Green I staining. (A) Lane 1: 123-bp marker; lane 2: extract from 10 000 293T cells, CX primer omitted; lane 3: extract as in lane 2, with all components; lane 4: extract as in lane 2, after RNase treatment; lane 5: extract from 1000 293T cells; lane 6: extract as in lane 5, after RNase treatment. (B) Lanes 1–6: extract from 4000 293T cells; lane 7: extract from 8000 Mono Mac 6 cells. Lane 1: TRAP buffer with RNase inhibitor and T4 gene 32 protein; lane 2: TRAP buffer with T4 gene 32 protein; lane 3: TRAP buffer alone; lane 4: as in lane 3 but cell extract pretreated with RNase; lane 5: TRAP buffer without BSA; lanes 6 and 7: TRAP buffer with 1 mg/mL BSA; lane 8: 123-bp marker.

1 μ L of 1-mg/mL RNase A at 37°C for 20 min, and then 2 μ L of the product were used for the reaction; however, non-telomerase-mediated DNA bands were still seen, as also observed by others (10,11)]. Under the TBE electrophoretic condition, the dye xylene cyanol moved to about 1/3 and bromophenol blue to about 2/3 down the 4.5% MetaPhor agarose gel. Since the majority of the discernible DNA ladder was below the 123-bp band (arrow, Figure 1A, lane 1, and 1B, lane 8), the observed 6-bp DNA ladders seemed to distribute mostly between 40 and about 150 bp in this gel system. Similar to this finding with the high-resolution agarose gel, a 3% MetaPhor agarose gel has previously been used to detect a 4-bp deletion (from 170 to 166 bp) in prenatal diagnosis of β thalassemia in some Chinese patients (8). Because acrylamide monomers are known to be toxic, the use of agarose may be more

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advantageous than polyacrylamide.

Attempts were made to streamline the TRAP conditions and components. For the HEK 293T cells, an extract from 1000 cells gave a better DNA ladder than one from 10000 cells (Figure 1A, lane 5 vs. 3). Individual components were also tested, and several were found not to be essential. Since telomerase is a ribonucleoprotein and we were concerned with RNase contamination, an RNase inhibitor (30 U RNasin®; Promega, Madison, WI, USA) was initially included. This was not needed as long as DEPC-treated water was used (Figure 1B, lane 1 vs. 3). The use of T4 gene 32 protein (2.5 µg; Boehringer Mannheim, Indianapolis, IN, USA) was also not needed (Figure 1B,

lane 2 vs. 3). BSA had no effect (Figure 1B, lanes 1–4, 1× BSA; lane 5, no BSA; lanes 6 and 7, 10× BSA). In addition to HEK 293T cells, others such as Mono Mac 6 cells (Figure 1B, lane 7) and human prostate LNCaP cells (data not shown) all showed the 6-bp DNA ladders in agarose minigels after staining. Furthermore, we have found that DNA ladder patterns were also observed when using the modified CX-ext primer (5'-GTGCCCTTACCCTTACCCTTACCCTAA-3'), one that may be free of some TRAP artifacts (7). Thus, it is possible to combine CX-ext, MetaPhor agarose minigel and SYBR Green I stain in a nonradioactive analysis of TRAP products.

We used the fluorescent dye SYBR Green I for the staining of TRAP products in agarose gels. Previously, the stain had been used to visualize TRAP DNA ladders after PAGE (10). Thus, we are in agreement on the suitability of using fluorescent DNA dye for TRAP instead of radioisotopes. This minimizes potential health hazards and decreases the amount of radioactive waste materials.

In summary, we have reported a convenient telomerase assay that can be done completely, from cell to gel, within a day. To our knowledge, this is the first report on the use of high-resolution agarose gel electrophoresis for the analysis of DNA products from TRAP.

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