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Stretch PCR Assay

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1. Introduction

In 1985, telomerase activity was identified in the macronuclei of the ciliate *Tetrahymena* and was found to add telomeric repeats onto telomeric oligonucleotide primers *(1)*. The radiolabeled elongated products were detected easily as a 6-bp ladder on an electrophoresis gel. In 1989, using the same method, human telomerase activity was identified in a HeLa cell extract *(2)*. However, the telomerase activity of human cells was very weak, and it was difficult to analyze the biochemical properties of telomerase or to investigate the significance of telomerase in various diseases, such as cancer. Therefore, we began to investigate a sensitive polymerase chain reaction (PCR)-based detection method for telomerase activity. Our goal was to develop a method that was not only versatile but also useful for biochemical analyzes, such as the measurement of the level of processing and the kinetics of telomerase.

Telomerase produces telomeric DNA composed of telomeric repeats. There were two major problems to overcome in the amplification of the telomerase product. First, any combination of forward and reverse PCR primers for repetitive sequences would anneal efficiently by themselves. As a result, short PCR products

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from primer dimers are predominantly amplified. Second, these primers can anneal with any repeat unit within the repetitive telomerase product. Therefore, if a short PCR product is formed from a primer annealed with an internal repeat of a template, the length of the PCR product does not represent the original length of the telomerase product. In 1994, the PCR-based telomeric repeat amplification protocol (TRAP) assay was reported, and with this method, telomerase was shown to be active in germ-line cells and most cancer cells, but not in normal somatic cells *(3)*. In the TRAP assay, a non telomeric oligonucleotide primer is used as a substrate for telomerase, and mutations are introduced into the reverse primer to reduce the formation of primer dimers. To determine the activation stage of telomerase during tumorigenesis or to examine the biochemical features of the enzyme complex, it is important to quantify the telomerase activity reproducibly. However, we and other researchers have experienced some difficulty in quantifying the telomerase activity by means of the TRAP assay *(4–6)*.

We have developed a new PCR-based method for measuring telomerase activity *(7)*. To overcome the problems described earlier, primer sequences are carefully designed and the reaction conditions explored. In brief, the primer–primer complex formed between the forward and reverse PCR primers has 1-bp mismatch at the 3' ends, preventing the production of PCR products as primer dimers. Second, unrelated extra tag sequences are introduced onto the 5' end of each primer, maximizing the chance that PCR products represent the length of the original telomerase products. The resulting PCR protocol, designated as stretch PCR assay, is highly suitable for the measurement of relative telomerase activity (*see* **Fig. 1**).

Figures 2 and **3** represent typical experiments with S100 extracts of HeLa cells. When the PCR products were analyzed by denaturing polyacrylamide gel electrophoresis, telomerase activity was represented as a 6-base ladder (**Fig. 2A,B**). This band pattern was not observed when the extract was pretreated with RNase A, which is the standard criterion for telomerase activity (**Fig. 2A**). These signals were also not observed when the extract was treated with proteinase A or heat (data not shown). More importantly, when the

telomerase reaction was sampled at each time point, we observed that the amplified products had increased in length between 0 and 30 min of incubation, indicating that the stretch PCR assay reproduced the original telomerase products faithfully (**Fig. 2B**). As shown in **Figure 1**, the tag sequence of each primer enables the maintainance, i.e., stretch, of the PCR product. This feature permits quantitative analysis of telomerase activity. **Figure 3** shows the analysis of an S100 extract derived from 1×10^5 HeLa cells and five-fold serial dilution. The amount of radioactivity in each lane was measured, and the quantified PCR products were plotted against cell numbers (**Fig. 3B**). A good dose response relationship was observed with extracts containing the equivalent of between 32 and 2×10^4 cells. These results demonstrate that the stretch PCR assay detects the authentic telomerase product and is an extremely powerful tool for the measurement of telomerase activity *(8, 9)*.

2. Materials

2.1. Preparation of S100 Extract

1. Phosphate buffered saline (PBS): prepare by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.2 g of KH_2PO_4 in 1 L of distilled H_2O. Autoclave for 15 min using the liquid cycle. Cool and store at 4°C.
2. Cell lysis buffer: 10 m*M* HEPES, pH 8.0, 3 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.5% CHAPS, 1 m*M* DTT, 0.1 m*M* PMSF, 1 µg/mL leupeptin, 2 µg/mL pepstatinA, 10 U/mL RNasin.
3. 5*M* NaCl.
4. Glycerol.
5. Liquid nitrogen.

2.2. Stretch PCR

2.2.1. Primer Extension by Telomerase

1. 0.5 *M* Tris-potassium acetate, pH 8.5: Prepare by dissolving 6.06 g of Tris base and 4.91 g of potassium acetate to 80 mL of sterile H_2O. Adjust the pH 8.5 with glacial acetic acid and bring final volume to 100 mL.
2. 2× reaction mixture: 100 m*M* Tris-potassium acetate, pH 8.5, 1 m*M* dATP, 1 m*M* dGTP, 1m*M* TTP, 10m*M* β–mercaptoethanol, 2 m*M*

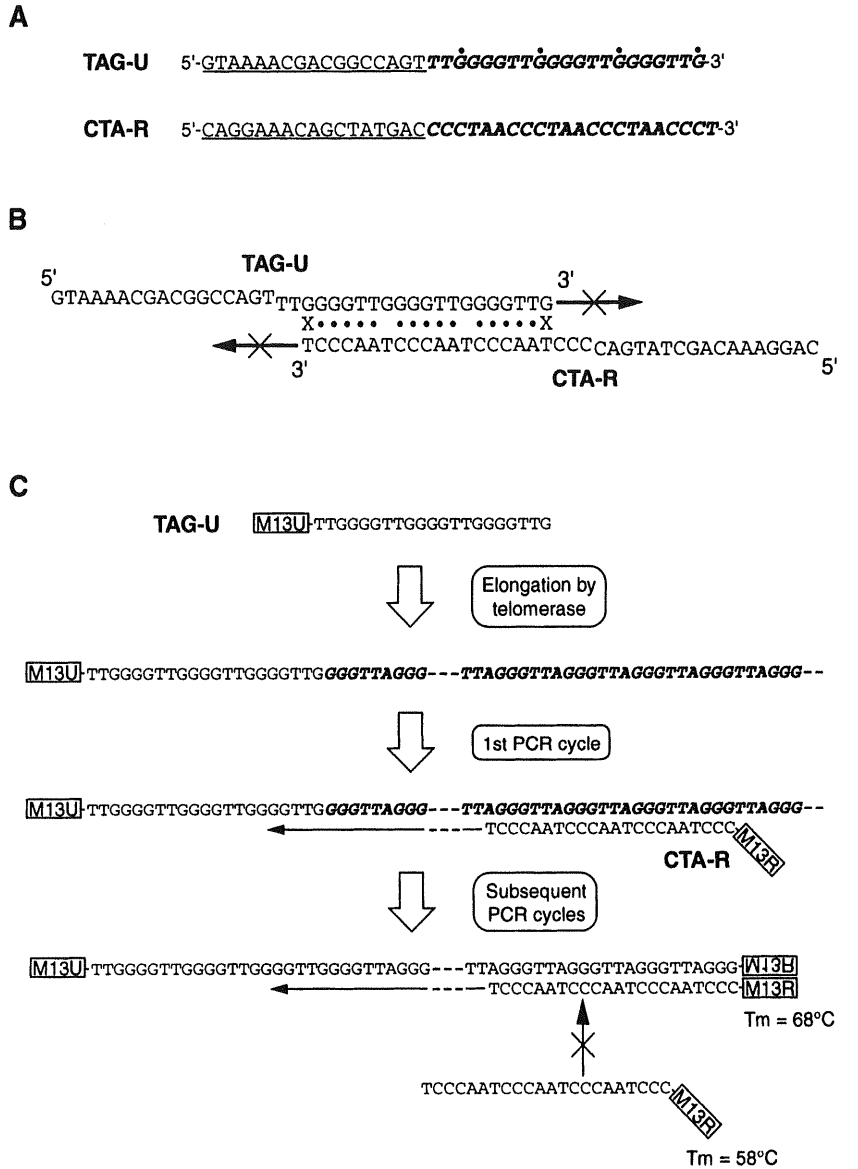

Fig. 1. Schematic diagram of stretch PCR for the detection of telomerase activity. (A) Primer sequences. The 3' half of the substrate oligonucleotide for the telomerase reaction, TAG-U, is a 21-base sequence of *Tetrahymena* telomeric TTGGGG repeats instead of human TTAGGG repeats (italics). The 5' half of the TAG-U oligonucleotide is a 17-base sequence unrelated to the telomeric sequence (M13 universe primer sequence with M13-U underlined). Human telomerase has been shown to initiate the elongation

MgCl, 2 m*M* EGTA, 2 m*M* spermidine, 0.2 m*M* spermine, 1 μ*M* denatured TAG-U primer (5'-GTAAAACGACGGCCAGTTTGGGG-TTGGGGTTGGGGTTG-3').

3. RNase mixture: 10 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, 0.1 mg/mL RNase A.
4. Proteinase mixture: 10 m*M* Tris-HCl, pH 8.0, 0.5% SDS, 0.3 mg/mL proteinase K.
5. Phenol/chloroform: 1:1 (v/v).
6. Ethanol.
7. 10*M* ammonium acetate.
8. Yeast tRNA (Boehringer Mannheim).
9. 70% ethanol.

(caption continued)

reaction with a variety of mutant telomeric repeats, including TTGGGG repeats *(2)*. The 3' half of the reverse (deoxycytidine [dC]-rich) primer, CTA-R, is a 22-base tract of dC-rich human telomeric CCCTAA repeats (italics) and the 5' half is a 17-base M13 reverse primer (M13-R underlined). (**B**) As TAG-U and CTA-R are designed to have a 1-bp mismatch at both 3' ends, no product is formed from this primer–dimer complex under the stringent annealing conditions in this assay. (**C**) TAG-U is incubated with S100 cell extract, and telomerase adds telomeric repeats to the 3' end of the oligonucleotide. After the elongation reaction, the DNA is purified and subjected to an amplification reaction. The unreacted TAG-U primer in the reaction mixture is used as the forward primer. A reverse telomeric primer, CTA-R, is added, and PCR is performed in the presence of [α^{32}P]dCTP. The TAG-U primer always recognizes the 3' end of the dC-rich template DNA because of its mutant telomeric sequences. In contrast, in the first cycle of PCR, CTA-R primers anneal at any of the telomeric repeats synthesized by telomerase. However, during subsequent cycles, the 3' end of the deoxyguanosine (dG)-rich template DNA base-pairs with CTA-R over a region of 39 bases, including the M13-R sequence (the calculated melting temperature is 68°C), whereas base-pairing between CTA-R and internal telomeric repeats occurs over a region of 22 bases without the M13-R sequence (the calculated melting temperature is 58°C). Because of the difference in melting temperature between the two possible annealing sites, the CTA-R primer was expected to preferentially anneal to the terminal site at 68°C. Thus, the length of the telomerase product is maintained during the amplification step.

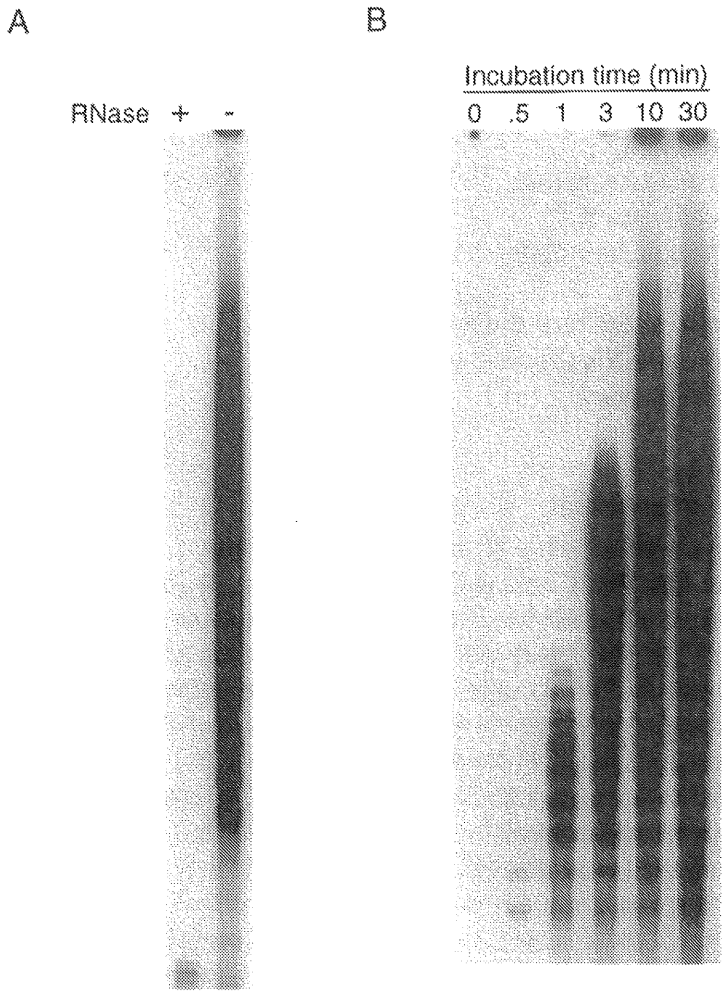

Fig. 2. Detection of telomerase products by stretch PCR. A S100 extract fom 1×10^4 HeLa cells was used for the assay. The amplified products were analyzed by electrophoresis on a 7% denaturing polyacrylamide gel. S100 extract was pretreated with RNase A (left lane, +) or mock treated (right lane, –). Since telomerase contains an RNA component as a template, the reaction is sensitive to RNase A treatment. The length of the PCR product varies after various incubation times with telomerase. S100 samples derived from 2×10^4 HeLa cells were used in this assay. The reaction was stopped at each time point and then subjected to a further amplification step. The time course of primer elongation by telomerase was between 0 and 30 min.

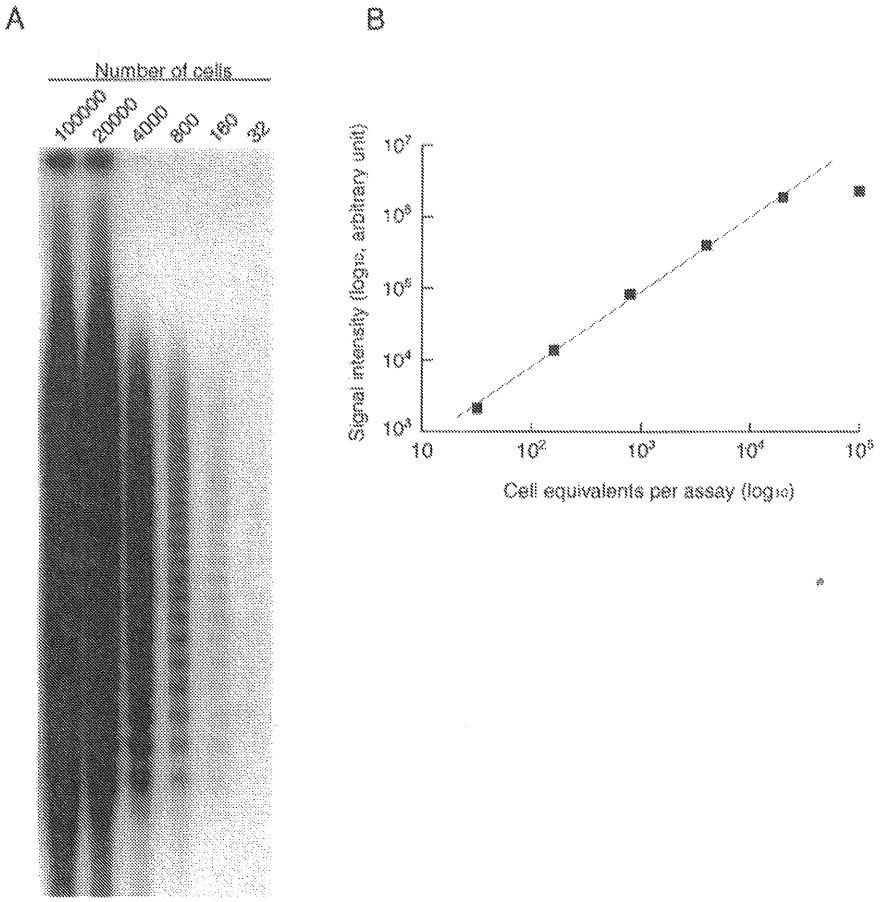

Fig. 3. Quantitative detection of telomerase activity by stretch PCR. (**A**) A serial 5-fold dilution of S100 extract, ranging from the equivalent of 32 to 1×10^5 cells, was analyzed by stretch PCR assay. The amount of PCR product was quantified using a PhosphorImager (BAS200) and plotted (**B**) as arbitrary units (solid square). The graph was linear up to a cell equivalent of 2×10^4 cells.

2.2.2. Stretch PCR Reaction

1. 10× PCR buffer: 200 m*M* Tris-HCl, pH 8.3, 750 m*M* KCl.
2. 50 m*M* $MgCl_2$.
3. 1% (w/v) polyoxyethylene ether W-1 (Sigma).

4. 2.5 μ*M* CTA-R primer (5'-CAGGAAACAGCTATGACCCCTAA-CCCTAACCCTAACCCT-3').
5. dNTP mixture: 10 m*M* dATP, 10 m*M* dGTP, 10 m*M* TTP, 1 m*M* dCTP.
6. *Taq* DNA polymerase (5 U/mL).
7. [α-^{32}P]dCTP: specific activity of 3000 Ci/mmol.
8. Mineral oil.
9. Thermal cycler (MJ Research, PTC-100).
10. Chloroform.
11. 3*M* sodium acetate, pH 5.2.
12. Ethanol.
13. 70% ethanol.

2.2.3. Gel Electrophoresis and Analysis of PCR Products.

1. Formamide loading buffer: 50% formamide, 5 m*M* EDTA, pH 8.0, 0.025% bromophenol blue, 0.025% xylene cyanol.
2. 10× TBE: 0.89 *M* Tris base, 0.89*M* boric acid, 20 m*M* EDTA, pH 8.0.
3. 40% acrylamide stock: acrylamide/bisacrylamide, 19:1.
4. Urea.
5. 10% ammonium sulfate, stored in the dark at 4°C (stable for 1 wk).
6. TEMED.
7. Whatman 3MM paper.
8. BAS2000 (Fuji Film).

3. Methods

3.1. Preparation of S100 Extract (see Note 1)

1. Harvest the cultured cells by centrifugation for 10 min at 2000 rpm (700*g* max).
2. Wash twice with ice-cold PBS.
3. Resuspend the pelleted cells in cell lysis buffer at a concentration of 2×10^7 cells/mL (**Notes 1** and **2**).
4. Store on ice for 20 min.
5. Centrifuge the suspension at 10,000 rpm (12,000*g* max.) for 10 min at 4°C.

6. Collect the supernatant and add 0.02 vol of 5*M* NaCl.
7. Incubate the extract at 4°C for 20 min with gentle shaking.
8. Centrifuge the extract at 40,000 rpm (100,000*g* max.) for 60 min at 4°C.
9. Collect the supernatant and add 0.25 vol of glycerol.
10. Divide into aliquots, freeze in liquid nitrogen and store at −80°C.

3.2. Stretch PCR

3.2.1 Primer Extension by Telomerase

1. Add 20μL of 2× reaction mixture (*see* **Note 3**) to 20 μL of S100 extract.
2. Incubate the mixture at 30°C for 60 min.
3. Stop the reaction by adding 50 μL of RNase mixture. Incubate at 37°C for 10 min.
4. Add 50 μL of proteinase mixture and incubate further at 37°C for 10 min.
5. Extract with an equal volume of phenol/choroform.
6. Add 45 μL of 10*M* ammonium acetate, 5 μg of yeast tRNA, and 540 μL of ethanol (*see* **Notes 4–6**).
7. Store at −20°C for 15 min and centrifuge at 15,000 rpm (19,000*g*) for 15 min at 4°C.
8. Wash pellets with 70% ethanol and dry in a centrifugal evaporator.

3.2.2. Stretch PCR Reaction

1. Prepare PCR reaction buffer as follows: 5.0 μL of 10× reaction buffer, 1.5 μL of 50 m*M* $MgCl_2$, and 2.5 μL of 1% W-1. Add distilled water to a final volume of 47 μL.
2. Dissolve the pelleted samples in 38 μL of PCR reaction buffer and transfer to new 0.5-mL microtubes (template mixture).
3. Add 2 μL of 2.5 μ*M* CTA-R primer to the template mixture and overlay 20 μL of mineral oil (*see* **Note 7**).
4. Prepare *Taq* mixture as follows: 9.0 μ L of PCR reaction buffer, 0.2 μL of dNTP mixture, 0.3 μL of *Taq* polymerase (5 U/μL), and 0.5 μL of [α-^{32}P]dCTP (3000 Ci/mmol).
5. Place the tubes of template mixture into the thermal cycler, incubate at 95°C for 5 min, and cool to 80°C.

6. Incubate the *Taq* mixture at 80°C for 1 min and add to template mixture (*see* **Note 8**).
7. Start the amplification cycle as follows: 25 main cycles of 93°C for 1 min, 68°C for 1 min, and 72°C for 10 min, followed by a final extension of 72°C for 10 min.
8. Extract with 50 µL of choroform.
9. Add 0.1 vol of 3*M* sodium acetate (pH 5.2) and 2.5 vol of ethanol.
10. Store at –20°C for 15 min and centrifuge at 15,000 rpm (19,000*g*) for 15 min at 4°C.
11. Rinse pellets with 70% ethanol and air-dry.

3.2.3. Gel Electrophoresis and Analysis of PCR Products

1. Dissolve pellets in 2 µL of formamide loading buffer.
2. Heat samples to 95°C for 2 min prior to loading.
3. Use a gel that is 0.35 mm thick and 20 × 40 cm in size, consisting of 7% polyacrylamide and 7*M* urea in 1× TBE. Prepare the gel by mixing 8.75 mL of 40% acrylamide stock, 21.02 g of urea, and 5mL of 10× TBE.Add distilled water to a final volume of 50 mL. Filter through a 0.45-µm filter, degas, and add 200 µL of 10% ammonium sulfate and 40 µL of TEMED.
4. Prerun and run the gel at a constant current of 30 mA until the bromophenol blue reaches the bottom.
5. After the run, transfer the gel to Whatman 3MM paper that has been cut to the same size as the gel, cover with Saran Wrap,™ and dry on a gel dryer.
6. Expose the gel to X-ray film or analyze using a PhosphoImager BAS2000.

4. Notes

1. As telomerase is a large ribonucleoprotein complex that contains an RNA component as a template, the isolation of the cell extract should be carried out at low temperature (0–4 °C) and all reagents should be free of RNase contamination. The S100 extract is stable for at least 6 mo.
2. The procedure for preparing S100 extract is suitable for a small quantity of cultured cells or for tissue samples of up to 1×10^8 cells. A

Dounce homogenizer is useful for disrupting the cells in scaled-up experiments.

3. The TAG-U primer used as a substrate for telomerase is denatured at 72°C for 5 min prior to use. This step prevents the G-rich oligonucleotides from forming the tertiary G-quartet structure.
4. The ethanol precipitation of the elongated product before the PCR amplification step is important for quantitative analysis because it has been reported that there are inhibitors of *Taq* polymerase in the S100 extract.
5. Ammonium acetate precipitation removes unincorporated nucleotides.
6. Artificial bands of high molecular weight can be amplified in the PCR step when *Escherichia coli* tRNA is used in place of yeast tRNA. Yeast tRNA is recommended in this step.
7. Although 5 pmol of CTA-R is used as the reverse primer in this PCR reaction, one-tenth of this amount of primer is sufficient to detect strong telomerase activity, such as that in cell lines or tumor samples. Increased amounts of CTA-R increases the sensitivity of this assay; however, the length of the amplified products becomes shorter at the same time.
8. To reduce the amplification of unrelated products, we recommend the use of a hot-start PCR protocol by adding *Taq* polymerase at 80°C.

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