Premature termination of telomeric extension-PCR for detection of telomerase activity

Ran Chen, Ji Qian, Lijuan Wang, and Yu-min Mao
Fudan University, Shanghai, P.R. China

BioTechniques 35:158-162 (July 2003)

In this article, we report a simple, rapid, and efficient method to detect telomerase activity: the premature termination of telomeric extension-PCR (PTEP). Similar to the telomeric repeat amplification protocol (TRAP), this method is based on PCR amplification following the in vitro telomerase reaction, while the in vitro telomerase reaction here is prematurely, rather than randomly, terminated. Apart from this, the telomeric extension products are used as initial primers, instead of as templates, to trigger the amplification with a specially constructed plasmid DNA as the template that cannot be directly amplified with the telomerase primer. The end product is a specific 159-bp DNA fragment that reflects telomerase activity. Because its product can be clearly identified with routine agarose gel electrophoresis and ethidium bromide staining, PTEP allows even lesser-equipped laboratories to easily detect telomerase activity.

INTRODUCTION

Telomerase is a reverse transcriptase that catalyzes the addition of telomeric repeats to the 3' end of chromosomal DNA. It plays a key role in the maintenance of chromosome termini (1). Currently, the telomeric repeat amplification protocol (TRAP) is the most effective and sensitive method to detect telomerase activity. The TRAP assay is based on the PCR amplification of the in vitro telomerase reaction (telomeric extension) products. It can reveal telomerase activity, even in a limited quantity of cancer tissues or cultured cells. When TRAP is applied, telomerase is observed as being reactivated in a majority of cancers while remaining inactive in most somatic cells (2–4). Some important modifications have been made on TRAP, including using different primers to improve the specificity of PCR amplification (3,5,6), which enforces an internal standard to improve the linearity and reliability of the result (7–9), and applying nonradioactivated detection (e.g., ELISA, fluorescence, or other bioluminescence methods) to improve sensitivity and operability (7,10–13). With these improvements, the assay becomes sensitive, robust, and quantitative. However, since they require extra cost-generating

reagents and equipment, they are impractical for most laboratories.

Gel electrophoresis is the primary method for the detection of DNA. Agarose gel electrophoresis is much more convenient than polyacrylamide gel electrophoresis (PAGE). Coupled with ethidium bromide staining, it is the simplest and most cost-efficient method thus far to detect PCR products. However, TRAP needs PAGE for the detection of its products. Because TRAP involves the random termination of telomeric extension, the end products are 6-bp DNA ladders, which can hardly be identified in routine agarose gel electrophoresis. Here we report a novel method, premature termination of telomeric extension-PCR (PTEP). The end product of PTEP is a specific DNA fragment that can be identified in routine agarose gel electrophoresis. PTEP was developed around the following characteristics of telomerase and *Taq* DNA polymerase: (i) in vitro telomerase reaction (i.e., telomeric extension) can be prematurely terminated (1,14,15). Three kinds of dNTP (i.e., dATP, dTTP, and dGTP) are necessary for the extension of the telomeric repeat unit “TTAGGG.” Where only dATP and dGTP are used, the telomeric extension will be prematurely terminated at the

third “G.” The products are identical oligonucleotide ssDNA with 3' ends of “AGGG.” (ii) *Taq* DNA polymerase has the ability to bind and extend the matched, but not the mismatched, nucleotides at the 3' end of a primer annealed to a complementary template strand. While the 3' end of the primer that matches the template is necessary for PCR, sporadic mismatches within the primer do not hinder PCR (16–19). Figure 1 shows the schematic diagram of PTEP. Except for two bases of its 3' end, the telomerase primer TS is homologous to the corresponding sites of the specially constructed DNA (SP DNA; the template, a plasmid carrying one pair of reverse repeat sequences: 5'-AATCCGTCGAGCAGAGAAAGGG-3'. See Construction of SP DNA section). However, because its 3' end does not match the template, it does not trigger the amplification of the template. After the telomeric extension is prematurely terminated, with four nucleotides, “AGGG,” linked to the 3' end of TS, the extended oligonucleotide ssDNA (TSG) can be annealed to the template and trigger the amplification. After two cycles, the whole complementary sequences of TSG are synthesized. Thus, a second PCR can be established with TS as the primer and the newly synthesized DNA as the template. In short, there are two PCRs in the system. The first is primed by TSG, and the second is primed by TS. The first is triggered by the telomerase reaction, and the second is triggered by the first. Because TS is prevailing abundant in the system, the second PCR is the dominant one. The end product is a specific DNA fragment (159 bp) that reflects telomerase activity.

MATERIALS AND METHODS

Samples Collection and Extraction

Lung cancer tissues (14 samples), normal lung tissues (6 samples), and leukemia cells (43 samples) were collected from the Changhai Hospital of the Second Military Medical University (SMMU) in Shanghai. K562 cells were cultured and harvested (2×10^7 cells/flask) in a routine procedure. Cells and tissue extracts were prepared

as previously described (2). The protein concentration of the extract was measured with the bicinchoninic (BCA) protein assay kit (Pierce Chemical, Rockford, IL, USA).

Construction of SP DNA

On each strand of the DnaE (*E. coli* DNA polymerase III α subunit) gene, the sites that are in part homologous to the sequence “5′-AATCCGTCGAGC-AGAGAAAGGG-3′” are searched and paired using the software OMIGA[®] 2.0 (Accelrys, Burlington, MA, USA), and then telomerase-DnaE linker primer pairs (TDLP1 and TDLP2) are designed to amplify a 166-bp fragment. The sequence of TDLP1 is 5′-TGA-AATCCGTCGAGCAGAGAAAGG-GCGCAGGGATT-3′ (corresponding to the region 152–186 of DnaE plus strand with 11 nucleotide differences), and the sequence of TDLP2 is 5′-AGATAATCCGTCGAGCAGAGAA-AGGGCAGATTCTG-3′ (corresponding to the region 317–283 of DnaE minus strand with 11 nucleotide differences); the bases underlined are sequences from the telomerase primer TS, the bases in bold are designed to generate the complementary site that can bind TSG but not TS, and the italicized bases are perfectly complementary to the template to ensure the specific amplification of the DnaE DNA (region 152–317). A standard PCR procedure was applied. The 166-bp fragment was retrieved from the agarose gel with the Gel Extract Kit (Qiagen, Valencia, CA, USA) and cloned into the pGEM[®]-T vector (Promega, Madison, WI, USA). Plasmid thus constructed was amplified in XL1-blue *E. coli* (Stratagene, La Jolla, CA, USA) and then purified. When its sequence was reconfirmed by DNA sequencing, the plasmid was used as the SP DNA.

Telomerase Activity Assay

The telomerase primer, TS (5′-AATCCGTCGAGCAGAGTT-3′) was used, the underlined being the bases that perfectly match the two corresponding sites of SP DNA, and the bold being the 3′ end bases that mismatch the corresponding sites.

Premature termination of telom-

eric extension. The 50- μ L volume of reaction mixture contained 50 mM Tris-HCl, pH 8.2, 150 mM KCl, 2.5 mM MgCl₂, 0.05% Tween[®] 20, 1 mM EGTA, 0.1 mg/mL bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 0.11 mM dATP, 0.11 mM dGTP, 10 U/mL RNasin[®], 2.5 μ M TS, 2 μ L cell extracts (0.5 μ g protein), and 0.05 ng/ μ L SP DNA. The mixture was incubated at 37°C for 20 min to facilitate telomeric extension and then heated at 96°C for 3 min to stop the reaction. This heat treatment can also inactivate potential inhibitors and denature the SP DNA for PCR.

PCR and electrophoresis. PCR additional reagents mixture (5 μ L) was added to the telomeric extension system, and the final concentration of the additional reagents was 0.1 mM dTTP, 0.1 mM dCTP, and 0.04 U/ μ L *Taq* DNA polymerase. This system consisted of 30

PCR cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The products were analyzed using agarose (2%) ethidium bromide (0.5 μ g/mL) gel electrophoresis. The gel was then viewed under UV transillumination and photographed. The existence of 159-bp band indicates a telomerase-positive result.

In contrast, TRAP assays were conducted with the TRAPeze[®] telomerase detection kit (Oncor, Gaithersburg, MD, USA), according to the manufacturer's recommended protocol. The products were resolved on a 12% polyacrylamide gel and detected with silver staining.

RESULTS

To detect telomerase activity effectively, PTEP must not only be an effective amplification system where there is

telomerase activity but also an effective amplification-refractory system where there is no telomerase activity. It has been acknowledged that certain mismatches such as G:T or C:A (primer:template) are not refractory to amplification when PCR conditions are not sufficiently stringent (16,18). Therefore, these mismatches must be avoided in PTEP. To ensure the specificity and operability of PTEP, a three-tier strategy was applied. First, a T:T mismatch was chosen, which has the greatest amplification-refractory power among all T-involved mismatches (16,18). Second, two adjacent bases in the SP DNA were designed to mismatch the 3' end of TS. This design can also increase the amplification-refractory power (16,18). Finally, a double-stranded amplification-refractory system was established. In allele-specific PCR, only one strand extension (downstream) is inhibited, and the upstream strand extension runs well since the upstream primer is perfectly complementary to the template. Therefore, ssDNA products, capable of causing nonspecific amplification, will accumulate in the system. In PTEP, either TS or TSG is used as upstream and downstream primers simultaneously. Both strand extensions primed by TS are inhibited where the SP DNA alone is used as the template, thus resulting in much greater amplification-refractory power than the allele-specific PCR. Under standard PCR conditions (annealing temperature 55°–60°C, 30–35 thermal cycles), the TS-SP DNA PCR system containing telomerase-negative extracts produces nothing except, occasionally, primer dimers. This confirms that it is an effective amplification-refractory system. In contrast, under the same conditions, the TS-SP DNA PCR system containing telomerase-positive extracts produces 159-bp products. This confirms that it is an effective amplification system (Figures 2A and 3A). Meanwhile, factors that significantly affect the result of PTEP are determined, including reagent components and reaction parameters (Table 1).

To assess the sensitivity and specificity of PTEP, telomerase activity in serially diluted K562 cell extracts was tested using the PTEP ethidium bromide staining assay, and the results were compared with those obtained us-

ing the TRAP silver staining assay. As shown in Figure 2A, the telomerase-positive band (159 bp) is clearly present in lanes 3–7, unfolding telomerase activity from 1×10^0 to 1×10^4 cells. In control experiments, the 159-bp band is not present in lanes 1 or 2, where telomerase was inactivated with heat or an RNase A (DNase-free) treatment, indicating that the 159-bp band is correlated to telomerase activity. Similarly, 1×10^0 and 1×10^2 to 1×10^4 cells were found telomerase-positive with the TRAP silver staining assay. However, 1×10^1 cells and the heat-inactivated control are hard to determine via this assay (Figure 2B). These data indicate that telomerase activity in as few as one cell can be detected by PTEP assay. In contrast, the result of a telomerase-weak or -negative sample is unclear in the TRAP silver staining assay.

The sensibility and specificity of PTEP was also confirmed when telom-

erase activities of clinical research samples were detected. The presence of a 159-bp band indicates that the samples of leukemia cells and lung cancer tissues are telomerase-positive. In contrast, there is no 159-bp band from the sample of normal lung tissue, which shows that it is telomerase-negative (Figure 3A). The assay shows that 13 of 14 (92.9%) lung cancer tissues and 40 of 43 (93.0%) leukemia cells are telomerase-positive, and all six normal lung tissues are telomerase-negative. These results are consistent with the previous reports of the TRAP assay that telomerase activity is present in cancer tissues but not in the normal somatic tissue (2–4). In addition, the clear results of the PTEP assay reconfirm the high specificity and sensibility of PTEP. In contrast, although identical results can be obtained using the TRAP silver staining assay of the telomerase-positive samples, the results with the

Figure 2. Detection of K562 cell extracts. (A) PTEP ethidium bromide staining. (B) TRAP silver staining assay. N, telomerase-negative control (heat-treated K562 extract); R, RNase A-treated K562 extract. Lanes 1–5, 10-fold diluted K562 cells extracts (10^0 – 10^4). M, DNA marker (50-bp ladder) (Promega).

Figure 3. Detection of cancer samples. (A) PTEP ethidium bromide staining. (B) TRAP silver staining assay. N, telomerase-negative control (heat-treated K562 extract); P, telomerase-positive control (K562 extract). Lane 1, normal lung tissue; lanes 3 and 4, leukemia; and lanes 5 and 6, lung cancer. M, DNA marker (50-bp ladder).

telomerase-negative samples are unclear with the TRAP silver staining assay (Figure 3B).

DISCUSSION

The TRAP silver staining assay has been reported to be a sensitive nonisotopic method for the detection of telomerase activity. The lower equipment and cost requirements make this assay practical for most laboratories. However, it has also been reported that it is difficult to recover clear TRAP ladders via this assay when the protein concentration of extracts is very high (20,21). A possible explanation is that silver staining is very sensible and able to stain minimal nonspecific products and other contaminated polymers such as proteins, lipids, and polysaccharides (22). Another possible explanation is when the protein concentration is very high, some contaminant inhibitors, such as nuclease and proteinase, may become active and inhibit the telomeric extension and PCR. This problem can be solved via dilution; however, the telomerase activity will be diluted at the same time. Our data show that the results with telomerase-weak or -negative samples are unclear with the TRAP silver staining assay. Thus, more reliable methods should be introduced to the low-budget laboratories. Here we report that the PTEP assay is simpler and more reliable than the TRAP silver staining assay because it takes less time and is cost-efficient.

Basically, PTEP is a simpler telomerase-PCR system than TRAP. This method generates a specific PCR product, rather than a ladder as in the TRAP assay, and detects the telomerase product on agarose gels with ethidium bromide. Because the product is specific, PCR artifacts are less likely to confound results. In addition, it has been reported that human telomerase seldom exhibits high activity and processivity simultaneously in vitro, and some mammalian telomerase such as Chinese hamster ovary (CHO) telomerase tends to exhibit nonprocessivity in vitro (1,14,15). While the results of TRAP depend on both the activity and processivity of telomerase, the results of PTEP depend only on telomerase activity. Thus, PTEP

Table 1. Summary of Important Features of PTEP Conditions

Features	Basic Rules	Optimum	Comments
MgCl ₂	1.5–2.5 mM	2.5 mM	Too high: reduces specificity; too low: reduces efficiency.
KCl	100–250 mM	150 mM	Too high or low: reduces efficiency.
dNTP	0.05–0.2 mM	0.1 mM	Too high or low: reduces efficiency.
TS	2.0–4.0 μM	2.5 μM	Too high: reduces specificity; too low: reduces efficiency.
SP DNA	0.001–0.01 ng/μL	0.01 ng/μL	Too high: reduces specificity; too low: reduces efficiency.
Telomeric extension	35°–37°C, 20 min	37°C	Too high or low: reduces efficiency.
Protein concentration	up to 0.12 μg/μL	(6 μg in 50 μL)	Too high: may cause false-negative results.
PCR annealing	58°–62°C, 30 s	60°C	Too high: reduces efficiency; too low: reduces specificity.

SP DNA, specially constructed DNA. See text for details.

is more suitable to detect other mammalian telomerase activities. Moreover, unlike the TRAP assay, the PTEP assay can be performed at higher temperatures

(37°C) and higher concentrations of Mg⁺⁺, K⁺, and primer, which can significantly increase telomerase activity but decrease the processivity (1,14,15).

Figure 1. The schematic diagram of premature termination of telomeric extension-PCR (PTEP). N, telomerase-negative; P, telomerase-positive; I, complementary; and X, mismatched.

PTEP shares the same drawback as all simple PCR systems, in that it scarcely provides quantitative information about the effective activity of telomerase because the efficiency of PCR is flexible. This limitation makes it currently unsuitable for clinical applications (5). Fortunately, in principle, PTEP can be improved to be quantitative, and all quantitative methods for TRAP should work well for PTEP. Further, TRAP can hardly adopt the TaqMan® system (Applied Biosystems, Foster City, CA, USA), the most convenient and popular quantitative PCR system, because its initial template is so short that TS will interfere with the hybridization between the template and the intermediate probe (23). In contrast, PTEP can because its template is long enough. Although such a quantitative system and the clinical application need further experiments to test, one can conclude that PTEP is a simple and convenient method for the detection of telomerase activity in general. It can replace the TRAP silver staining method in most laboratories.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NSFC) grant no. 39700082 (to R.C.). We thank Dr. Fang Xia and Dr. You-zhong Yuan at the Changhai Hospital of the SMMU in Shanghai for providing clinical samples.

REFERENCES

1. Morin, G.B. 1989. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* 59:521-529.
2. Kim, N.W., M.A. Piatyszek, K.R. Prowse, C.B. Harley, M.D. West, P.L.C. Ho, G.M. Coviello, W.E. Wright, et al. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science* 266:2011-2015.
3. Krupp, G., G. Bonatz, and R. Parwaresch. 1997. Telomerase, immortality and cancer. 2000. *Biotechnol. Annu. Rev.* 6:103-140.
4. Shay, J.W. 1998. Telomerase in cancer: diagnostic, prognostic, and therapeutic implications. *Cancer J.* 4:S26-S34.
5. Braunschweig, R., I. Guilleret, F. Delacretaz, F.T. Bosman, A. Mihaescu, and J. Benhattar. 2001. Pitfalls in TRAP assay in routine detection of malignancy in effusions. *Diagn. Cytopathol.* 25:225-230.
6. Krupp, G., K. Kuhne, S. Tamm, W. Klappper, K. Heidorn, A. Rott, and R. Parwaresch. 1997. Molecular basis of artifacts in the detection of telomerase activity and a modified primer for a more robust TRAP assay. *Nucleic Acids Res.* 25:919-921.
7. Hou, M., D. Xu, M. Bjorkholm, and A. Gruber. 2001. Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. *Clin. Chem.* 47:519-524.
8. Kim, N.W. and F. Wu. 1997. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res.* 25:2595-2597.
9. Wright, W.E., J.W. Shay, and M.A. Piatyszek. 1995. Modification of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res.* 23:3794-3895.
10. Wen, J.M., L.B. Sun, M. Zhang, and M.H. Zheng. 1998. A non-isotopic method for the detection of telomerase activity in tumor tissues: TRAP-silver staining assay. *Mol. Pathol.* 51:110-112.
11. Aldous, W.K. and N.R. Grabill. 1997. A fluorescent method for detection of telomerase activity. *Diagn. Mol. Pathol.* 6:102-110.
12. Uehara, H., G. Nardone, I. Nazarenko, and R.J. Hohman. 1999. Detection of telomerase activity utilizing energy transfer primers: comparison with gel- and ELISA-based detection. *BioTechniques* 26:552-558.
13. Xu, S., M. He, H. Yu, X. Cai, X. Tan, B. Lu, and B. Shu. 2001. A quantitative method to measure telomerase activity by bioluminescence connected with telomeric repeat amplification protocol. *Anal. Biochem.* 299:188-193.
14. Maine, I.P., S.-F. Chen, and B. Windle. 1999. Effect of dGTP Concentration on human and CHO telomerase. *Biochemistry* 38:15325-15332.
15. Sun, D., C.C. Lopez-Guajardo, J. Quada, L.H. Hurley, and D.V. Hoff. 1999. Regulation of catalytic activity and processivity of human telomerase. *Biochemistry* 38:4037-4044.
16. Ayyadevara, S., J.J. Thaden, and R.J.S. Reis. 2000. Discrimination of primer 3'-nucleotide mismatch by *Taq* DNA polymerase during polymerase chain reaction. *Anal. Biochem.* 284:11-18.
17. Sommer, R. and D. Tautz. 1989. Minimal homology requirements for PCR primers. *Nucleic Acids Res.* 17:6749.
18. Tsai, M.Y., N.Q. Hanson, K.R. Copeland, I. Beheshti, and U. Garg. 1994. Determination of a T/G polymorphism at nucleotide 3206 of the apolipoprotein C III gene by amplification refractory mutation system. *Clin. Chem.* 40:2235-2239.
19. Baumann, R.E. and A.H. Henschen. 1993. Human fibrinogen polymorphic site analysis by restriction endonuclease digestion and allele-specific polymerase chain reaction amplification: identification of polymorphisms at positions A α 312 and B β 448. *Blood* 82:2117-2124.
20. Fujita, M., S. Tomita, Y. Ueda, and T. Fujimori. 1998. Gel staining methods for detection of telomerase activity with the telomeric repeat amplification protocol (TRAP) assay. *Mol. Pathol.* 51:342.
21. Falchetti, M.L., A. Levi, P. Molinari, R. Verna, and E. D'Ambrosio. 1998. Increased sensitivity and reproducibility of TRAP assay by avoiding direct primers interaction. *Nucleic Acids Res.* 26:862-863.
22. Merrill, C.R., D. Goldman, S.A. Sedman, and M.H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211:1437-1438.
23. Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 88:7276-7280.

Received 16 September 2002; accepted 13 May 2003.

Address correspondence to Ran Chen or Ji Qian, State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, 220 Handan Road, Shanghai 200433, P.R. China. e-mails: rachen1970@yahoo.com (R.C.) or jqian@fudan.edu.cn (J.Q.)