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Non-radioisotopic and Semi-quantitative Procedure for Terminal Repeat Amplification Protocol

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We have used fluorescence-labeled primers and an auto-sequencer to detect telomerase activity quickly and easily. The current procedure is superior to the original telomeric repeat amplification protocol in several respects: 1) the result is obtained in real time during electrophoresis, 2) semi-quantitative results are possible without using a photo-capture system, and 3) no radioisotope is needed.

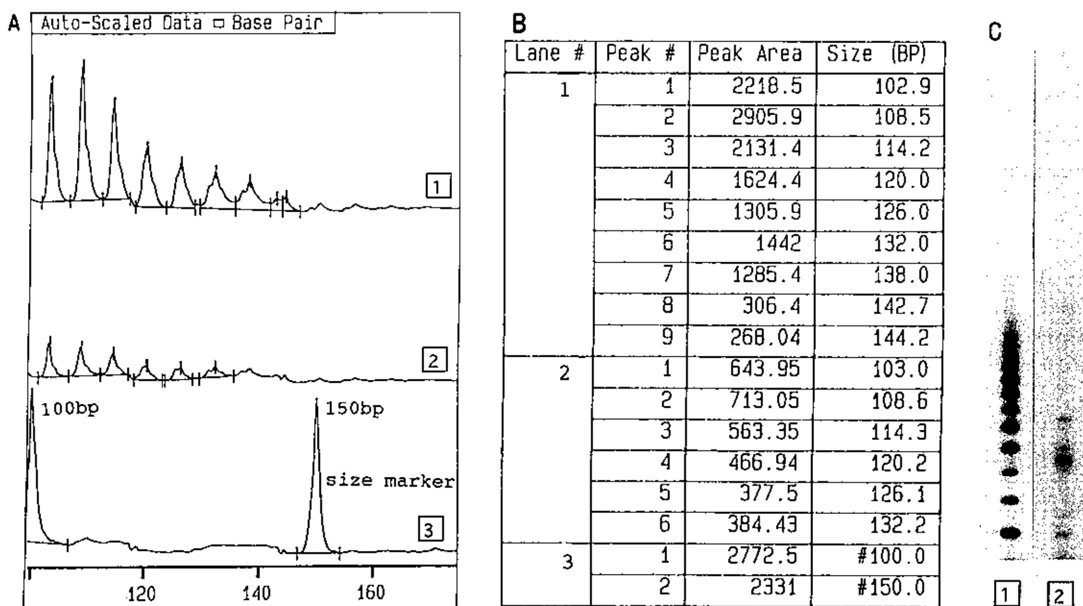
Key words: Telomerase activity — Fluorescence labeling

Detection of telomerase activity using TRAP[3] was first reported in 1994,[1,2] and the method has been widely used to examine the possible role of telomerase in the development of neoplasia.[2–7] However, the original TRAP method[1,2] has a number of limitations: problems include time-consuming procedure using a radio-isotope labeling system and difficulty in quantitating the enzyme activity. Since recent studies have demonstrated low, but distinct, levels of telomerase activity in peripheral blood cells from normal individuals,[3] at least semi-quantitative determination of telomerase activity in samples is needed. To overcome these problems, we have developed a new procedure using fluorescence-labeled primers and an auto-DNA sequencer. The telomerase activity is represented by fluorescence curves, and the peak height and peak area are calculated automatically, so the telomerase activity can be measured in real time.

CHAPS cells extract (from 1×10^7 cells) was prepared according to Kim's procedure.[1,2] To minimize carry-over contamination, we performed extractions, PCR reactions, and gel electrophoresis in physically different areas. We used fluorescence-labeled TS forward-primer (5′-AATCCGTCGAGCCAGAGTT-3′), which is commercially available. Lyophilized CX reverse-primer (5′-CCCTTACCCTTACCCTTACCCTTA-3′) was sealed in a wax (Ampliwax, Perkin-Elmer, Norwalk, CT) barrier just before use.[1,2] TRAP reaction mixture (50 μl) above the wax barrier contained 20 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 63 m*M* KCl, 0.005% Tween-20, 1 m*M* EGTA, 50 m*M* deoxynucleoside triphosphates, 10 pmol of fluorescence-labeled TS forward primer, 1 μg of T4 gene 32 protein (Boehringer Mannheim, IN), bovine serum albumin (0.1 mg/ml), 2 units of Taq DNA polymerase (Takara Shuzo, Kyoto), and 0.5–5 μg of the CHAPS cell extract. Because our fluorescence system is extremely sensitive, overamplified product may give unreliable results; in such cases, we used cell extract of lower protein concentration (0.5–1.0 μg). For RNase treatment, 5 μg of extract was incubated with 1 μg of RNase (Stratagene, La Jolla, CA) for 20 min at 37°C before PCR amplification. The prepared microtube was held at 22°C for 10 min for telomerase-mediated extension of the TS primer. The reaction mixture was heat-inactivated at 90°C for 90 s. Next, PCR was run in a thermal cycler (Perkin Elmer), using 27 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min.

For analysis of amplification products, we prepared 8% denaturing gel (Long Ranger, AT Biochem, Malvem, PA) containing 6 *M* urea, according to the supplier's instructions. One-time TBE was used as a running buffer. A mixture of 1 μl of PCR product and 1 μl of loading buffer consisting of 90% formamide and 10% Blue dextran (Pharmacia Biotech, Uppsala, Sweden) was carefully loaded in each well of the denaturing gel. We used sizer 100, sizer 150, and sizer 200 (Pharmacia Biotech) as fluorescent size markers. These sizers were also used as indicators of fluorescence intensity. The fluorescence data from the ALF DNA sequencer II were collected and analyzed automatically by the Fragment Manager program (Pharmacia Biotech). Each fluorescent peak was quantitated in terms of size (in base pairs), peak height, and peak area.

[3] Abbreviations used are: TRAP, telomeric repeat amplification protocol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PCR, polymerase chain reaction; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Tween-20, polyoxyethylene sorbitan monolaurate; TBE, Tris-borate/EDTA; ALF, automated laser fluorescence.



Lane #	Peak #	Peak Area	Size (BP)
1	1	2218.5	102.9
	2	2905.9	108.5
	3	2131.4	114.2
	4	1624.4	120.0
	5	1305.9	126.0
	6	1442	132.0
	7	1285.4	138.0
	8	306.4	142.7
	9	268.04	144.2
2	1	643.95	103.0
	2	713.05	108.6
	3	563.35	114.3
	4	466.94	120.2
	5	377.5	126.1
	6	384.43	132.2
3	1	2772.5	#100.0
	2	2331	#150.0

Fig. 1. Representative fluorescence curves showing telomerase activity (A). Multiple peaks correspond to telomeric repeats that were synthesized by telomerase. Lane 1: human leukemia cell line, HL-60. The protein content was 0.5 μg/assay. Lane 2: mononuclear peripheral blood obtained from a normal subject. The protein content was 5 μg/assay. No peak was observed after RNase treatment (data not shown), indicating that these reactions were inhibited by addition of RNase. Lane 3: size markers (100 bp and 150 bp). The results analyzed by the Fragment Manager system are shown in B. Each peak area was calculated automatically. Multiple peaks show approximately 6 bp periodicity (see size in right column). The same cell lysates were also analyzed by the original TRAP assay (C). The gel was autoradiographed at −70°C for 3 h. Lane 1: HL-60 (0.5 μg/assay). Lane 2: mononuclear peripheral blood obtained from a normal subject (5 μg/assay).

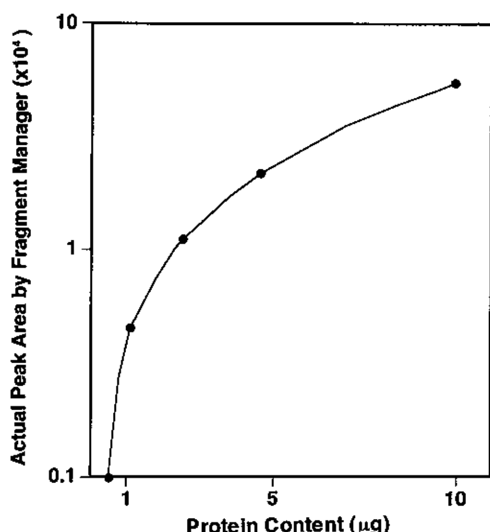


Fig. 2. The relationship between the protein content and relative telomerase activity of human leukemia cell line (HL-60). A positive correlation between protein level and telomerase activity is evident. The vertical axis (logarithmic scale) indicates actual peak area (electron volt) output by the Fragment Manager system, and the horizontal axis shows the amount of the extracted protein of HL-60 cells (μg).

As shown in Fig. 1, telomerase activity was detected in a shark-tooth pattern with a periodicity of about 6 nucleotides, and the telomerase activity was blocked after RNase treatment. In contrast to the original TRAP methodology by Kim *et al.*,[1,2] we calculated the area under the curve, then corrected the result using the level in an immortalized leukemic cell line (HL-60) as a standard telomerase activity (taken as 100%). Because the Fragment Manager system used in this study detects very weak fluorescence signals automatically, it is impossible to judge an extremely low level of telomerase activity accurately. However, it might be possible to detect the presence or absence of telomerase activity at a very low level of telomerase.

In order to compare fluorescence-TRAP assay with the original TRAP assay, the same cell lysates were analyzed using non-fluorescence-labeled primers and [α-^{32}P]dCTP according to Kim's procedure. As shown in Fig. 1C, similar telomerase ladders were observed in both fluorescence-TRAP and the original TRAP.

We next conducted dilution experiments using the protein obtained from leukemia cell line HL-60. A good correlation between the protein amount and the telomerase activity was obtained (Fig. 2), indicating that the

procedure presented here is suitable to evaluate the relative amount of telomerase activity. The telomerase activity can be estimated within 90 min after applying samples, whereas the original TRAP assay needs exposure periods for autoradiography and densitometric analysis.

This protocol using fluorescence-labeled primers will be useful for semi-quantitative, real-time determination of telomerase levels for cancer biology studies, and should help to establish the biological and clinical significance of telomerase activity in cancer cells.[7]

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