Bioluminescent Method for Detecting Telomerase Activity

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Background: Telomerase is a promising biomarker in cancer diagnosis and therapy. The elongation of telomeric repeats catalyzed by telomerase is accompanied by release of six PPi for each TTAGGG repeat (1 pmol PPi/310 pg telomeric repeats). We developed a novel method to measure telomerase activity by use of an enzymatic luminometric PPi assay (ELIPA).

Methods: Extracts of cell lines and tissues were incubated with primer at 30 °C for 30 min. Released PPi was converted to ATP by sulfurylase, and ATP was detected by a luciferase bioluminescence system. The ELIPA results were compared with results obtained with the conventional telomeric repeat amplification (TRAP)-ELISA in 42 lung carcinoma tissues and 27 control tissues without malignancy.

Results: The lower detection limits of ELIPA and TRAP-ELISA were 5 and 10 cells, respectively. The within-run imprecision (CV) of ELIPA was <12%. When compared with TRAP-ELISA, the correlation coefficient (r) was 0.79. When we used the cutoff value from ROC analysis to distinguish malignant and nonmalignant tissues, the sensitivity and specificity of ELIPA were 83% and 96%, respectively, whereas the sensitivity and specificity of TRAP-ELISA were 71% and 96%, respectively.

Conclusion: ELIPA is a simple and sensitive homogeneous method to quantify telomerase activity. © 2002 American Association for Clinical Chemistry

Telomerase, which can elongate telomere ends, may contribute to maintenance of cell immortality and to uncontrolled cell growth in cancer (1, 2). Telomerase is a ribonucleoprotein complex that catalyzes the addition of telomeric repeats to the 3' end of chromosomal DNA (3), thereby preventing the loss of telomeric sequences at each cell division (1). Because of its involvement in carcinogenesis, telomerase activity is a promising biomarker in cancer diagnosis and therapy (4, 5) and is becoming an important prognostic tool to determine whether a particular tissue is likely to develop cancer (6). On the other hand, telomerase may be a major target for cancer drugs, which has led to revolutionary new therapies for cancer, such as telomerase inhibitors and antisense therapy (7, 8). Thus, sensitive, accurate, and rapid methods to detect telomerase activity for clinical purposes are needed.

An important step in improving telomerase detection was the development of the telomeric repeat amplification protocol (TRAP)3 assay, which is based on PCR amplification of the in vitro telomerase reaction product (9). The TRAP assay is a very sensitive method by which telomerase activity in a small tissue sample or tumor biopsy can be detected (10, 11), but it is difficult to quantify the telomerase activity because of the logarithmic amplification of the telomerase product. Many adaptations of the conventional TRAP assay have been reported, such as the TRAP scintillation proximity assay (12), TRAP-ELISA (13), the TRAP hybridization protection assay (14), the fluorescent TRAP assay (15), the stretch PCR assay (16), real-time quantitative TRAP (17), the luminometric hybridization assay (18), and bioluminescence linked with TRAP (19). However, because these methods depend on logarithmic amplification of the telomerase product, quantifying enzyme activity has been relatively difficult. To avoid this drawback, a method with a linear dose-response relationship is needed, but such a method has not been available.

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3 Nonstandard abbreviations: TRAP, telomeric repeat amplification protocol; ELIPA, enzymatic luminometric inorganic pyrophosphate assay; dNTP, deoxynucleotide triphosphate; ADA, adenosine deaminase; SNR, signal-to-noise ratio; and RLU, relative light unit(s).
Here we present a novel method that measures telomerase activity with an enzymatic luminometric PPi assay (ELIPA). This method relies on the elongation of telomeric repeats catalyzed by telomerase accompanied by the release of six PPi for each TTAGGG repeat. The PPi is quantitatively converted to ATP by ATP-sulfurylase (20), and ATP is determined by the luciferase luminescence system (21,22). The luciferase luminescence system is based on the measurement of a stable amount of light produced by the luciferase-catalyzed ATP reaction. The emission of light is essentially independent of time and is proportional to the moles of ATP over the range from $1 \times 10^{-17}$ to $1 \times 10^{-9}$ mol.

**Materials and Methods**

**CELL AND TISSUES**

A549 lung carcinoma cells were maintained in RPMI-1640 supplemented with 100 mL/L heated-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 5% CO2 in air. The lung tissues were obtained from 42 patients with lung carcinoma and 11 control cases without malignancies at Tongji Hospital. The diagnosis for each tissue sample was made by histologic examination by a trained pathologist. All surgical specimens were frozen in liquid nitrogen immediately after removal and stored at -80 °C until extraction. A549 cells and tissue samples were lysed by the CHAPS detergent method as described previously (9). Cell extracts were serially diluted 10 times or diluted 1:1 with lysis buffer (1 volume of lysis buffer plus 1 volume of extract).

**DETERMINATION OF TELOMERASE ACTIVITY BY ELIPA**

In the ELIPA, each sample required an RNase-treated blank. One primer (5'-TTAGGGTTAGGGTTAGGG-3') was used in the ELIPA procedure. The CHAPS extracts (3 μL) were mixed with 22 μL of elongation reaction mixture [20 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl2, 63 mmol/L KCl, 0.05 mL/L Tween 20, 1 mmol/L EGTA, 0.1 mg/L bovine serum albumin, 4 mmol/L spermidine, 50 μmol/L each deoxynucleotide triphosphate (dNTP), and 0.16 μg of primer], incubated at 30 °C for 30 min, and then heated to 90 °C for 3 min to inactivate the telomerase activity. One microgram of adenosine deaminase (ADA) was then added, and the reaction was incubated at 37 °C for 10 min to remove residual dATP and then heated to 90 °C for 3 min to inactivate ADA activity. Finally, 175 μL of the ELIPA mixture [0.1 mol/L Tris-acetate, 2 mmol/L EDTA, 10 mmol/L magnesium acetate, 1 g/L bovine serum albumin, 1 mmol/L dithiothreitol, 0.4 g/L polyvinylpyrrolidone (360 000), 100 mg/L d-luciferin, 4 mg/L r-luciferin, 5 μmol/L adenosine-5'-phosphosulfate, 0.5 kU/L ATP-sulfurylase, and 0.4 g/L purified luciferase] was added at room temperature. The luminescence output was recorded by a Lumat LB 9507 recorder (EG&G Berthold). The moles of PPi, in the mixture were equal to the ATP detected by the luciferase system. When 1 fmol of TTAGGG repeat was synthesized by telomerase, 6 fmol of PPi were released from the telomerase elongation reaction, i.e., 1 fmol of PPi in the ELIPA corresponded to 0.1667 fmol of telomere repeats elongated. The telomerase activity was expressed in terms of fmol TTAGGG/μg of protein.

The principle of the ELIPA for determining telomerase activity is illustrated in Fig. 1. Because the method does not include PCR amplification, the amount of telomeric repeat formed by telomerase during the ELIPA is much less than that formed during the TRAP assay. However, the luciferase luminescence system is very sensitive, with a detection limit of $1 \times 10^{-17}$ mol of ATP. Thus, the ELIPA should be able to detect $1 \times 10^{-17}$ mol of PPi produced, or $1.67 \times 10^{-18}$ mol of TTAGGG synthesized. To determine whether the ELIPA signals were dependent on telomerase activity, the CHAPS extract of 1000 A549 cells was treated with RNase (0.5 μg/assay) for 30 min at 37 °C to inactivate telomerase.

**DETECTION OF TELOMERASE BY TRAP**

Telomerase activities were detected qualitatively by the TRAP electrophoresis assay and then quantitatively by the TRAP-ELISA. TRAP electrophoresis assays were performed as described by Kim et al. (9). In brief, the CHAPS extract was incubated with TRAP mixture at 30 °C for 30 min and then heated to 90 °C for 3 min to inactivate the telomerase activity. The reaction mixture was subjected to 33 PCR cycles amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s). TRAP reaction products were separated by electrophoresis in 10% polyacrylamide gels and detected by SYBR Green I staining (Gene Inc.).

Telomerase activity in the TRAP-ELISA was quantified using a commercially available telomerase PCR ELISA reagent set (Roche) according to the manufacturer's protocol.

**Results**

**VALIDITY OF ELIPA**

As shown in Table 1, the signals in the ELIPA disappeared after RNase treatment. These results confirmed that signals in the ELIPA depended on telomerase activity. To test the linearity of the ELIPA, we assayed serial dilutions

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![Fig. 1. Principle of the ELIPA used in this study.](Image)

**T1** primer (5'-TTAGGGTTAGGGTTAGGG-3') for elongation of telomeric repeats.
of A549 cell extracts (1–1000 cells/extract) with the ELIPA. There was a significant correlation between the signals and the number of cells ($r = 0.987; P < 0.000001$). The relationship between the signal and the number of cells is shown in Fig. 2. To confirm that the ELIPA signals were the result of the telomerase elongation reaction, we assayed telomerase activities by both the TRAP assay and ELIPA. The results (Fig. 3) showed that the ELIPA signals agreed very well with the results of TRAP with SYBR Green staining.

**EFFECTS OF dATP ON LUCIFERASE LUMINESCEENCE SYSTEM**

Effects of dATP on the luciferase luminescence system have been reported (23); luminescence signals will be produced by dATP as well as by ATP. To improve the signal-to-noise ratio (SNR) of the ELIPA, we used ADA to remove residual dATP after the elongation reaction catalyzed by telomerase. As shown in Fig. 4, the SNR for the ELIPA with ADA was 15 times higher than that without ADA. (NOTE: It is very important to use a dNTP solution that is free of PPi, but commercial dNTP mixtures usually contain PPi.)

**DETECTION LIMIT AND REPRODUCIBILITY OF ELIPA**

The detection limit of the ELIPA was assessed using extracts of A549 cells (Table 1). Although the signals produced by 1- or 2-cell extracts were substantially higher than that of the cell-free extract, the SNR [relative light units (RLU) produced by cell extracts divided by the RLU of cell extracts treated with RNase] was $<2$. The RLU for the 5-cell A549 extracts (2897 ± 339) were also substantially higher than those of the cell-free extracts (1240 ± 235), and the SNR for the 5-cell extracts was acceptable (2.3). In the TRAP-ELISA, the SNRs for 1-, 2-, 5-, and 10-cell extracts were 1.03, 1.06, 1.48, and 2.48, respectively; thus, the detection limit of the TRAP-ELISA was 10 cells.

CHAPS extracts from A549 cells and tumor tissues were simultaneously assayed six times in both the ELIPA and TRAP-ELISA, respectively. The CV was 8.9–12% (Table 1) for 5–1000 cells in the ELIPA, and 10–17% (data not shown) for 1–1000 cells in the TRAP-ELISA.

**COMPARISON BETWEEN ELIPA AND CONVENTIONAL TRAP ASSAY**

The telomerase activities in 42 lung carcinoma tissues and 27 control tissues were detected by the ELIPA and TRAP-
ELISA to compare the ELIPA with the TRAP-ELISA method. The results obtained with the ELIPA significantly correlated with those obtained with the TRAP-ELISA ($r = 0.79$; $P < 0.000001$; Fig. 5). Because the telomeric repeats formed by telomerase were not amplified in the ELIPA, the telomerase activities measured by that method were much lower than the values obtained by the TRAP-ELISA. The activities measured by the ELIPA represent the actual telomerase activity because the telomeric repeats produced in the assay are not amplified.

The results showed that the ELIPA has a lower detection limit (5 cells) than conventional TRAP-ELISA (10 cells), and the reproducibility of ELIPA was better than that of TRAP-ELISA. In addition, the cost of ELIPA (approximately US $1 for one sample) was much lower than that of TRAP-ELISA (approximately US $5 for one sample).

**Detection of Telomerase Activity in Tissues**

Different tissues from patients with lung cancer were assayed by both the TRAP and ELIPA methods. The telomerase activities in healthy lung tissue, adenocarcinoma, and squamous epithelial carcinoma are shown in Fig. 6. The telomerase activities in the adenocarcinomas and squamous epithelial carcinomas were significantly higher than that in healthy lung tissue. When the cutoff value was calculated using ROC analysis, the calculated cutoff values of the TRAP-ELISA and ELIPA were 0.095 ($A_{450} - A_{690}$)/µg protein and 1.45 fmol TTAGGG/µg protein, respectively. At these cutoff values, the sensitivity and specificity of the ELIPA were 83% and 96%, respectively, and of the TRAP-ELISA were 71% and 96%, respectively.

**Discussion**

Telomerase is a promising biomarker for the diagnosis and prognosis of cancer. TRAP is a popular method to detect telomerase activity, and most methods that quantitatively determine telomerase activity are based on TRAP. However, TRAP has some disadvantages, particularly for clinical use: it is time-consuming, and it depends on PCR, which is susceptible to inhibition from extracts of clinical samples (24).

In this study, we developed a novel quantitative method to determine telomerase activity based on ELIPA. ELIPA can easily detect 1 fmol of PPi in an endpoint analysis and has been used to monitor DNA polymerase activity and DNA sequencing (25, 26). Practically, ELIPA could detect the telomerase of 5 cell-equivalents in an extract of A549 cells.

Because it does not require PCR, the telomerase activity determined by ELIPA is directly related to actual telomerase activity. Under the same conditions, there were no significant differences among results obtained for the same sample at different dilutions. The method provides unique markers for the diagnosis and prognosis of cancer and other diseases. In addition, ELIPA is a homogeneous method that does not require PCR or gel electrophoresis, and it can be carried out in one tube. Finally, Kim et al. (9) used a primer (5'-AATCCGTCGAGCA-GAGTT-3') containing mismatches to authentic sequences as the telomerase substrate to minimize the formation of primer-dimers in the PCR step of the TRAP assay. A sequence with complete homology to the human telomere sequence (5'-TTAGGGTTAGGTTAGG-3') was used as the telomerase substrate in the ELIPA. We hypothesize that the authentic sequence is recognized more faithfully.

![Fig. 5. Comparison of the telomerase activity measured using both TRAP-ELISA (x axis) and ELIPA (y axis) in 27 healthy tissues and 42 carcinoma tissues. $r^2 = 0.79$; $P < 0.000001$.](image1)

![Fig. 6. Telomerase activity from different tissues.](image2)
than the mismatched sequence, with a potential gain in assay sensitivity.

It is important to improve the SNR because dATP can affect ELIPA signals. ADA was used to remove the residual dATP after the telomerase elongation reaction. The SNR for the ELIPA with ADA treatment was much better than that without ADA treatment (Fig. 4). In the present study, a suitable reagent was prepared by mixing the individual components of the ATP monitoring reagent without PPi. The DNA polymerase in a few tissues can catalyze the synthesis of DNA using only a primer and four dNTPs without a template. SNR, but excessive concentrations of inhibitors of DNA polymerase may be useful to improve the SNR. In the present method, no additional inhibitors were used to improve the SNR. The results showed that the background signal obtained with a negative control was satisfactory.

In general, ELIPA is a homogeneous and very rapid assay that relies on a PPi assay other than TRAP, and it can be completed in 3 h. Because the results are automatically expressed quantitatively in RLU by a luminometer, ELIPA is applicable to high-throughput formats and can be applied to large numbers of clinical samples at the same time.

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