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TRAP-silver staining, a highly sensitive assay for measuring telomerase activity in tumor tissue and cell lines

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Abstract

Measurement of telomerase activity in clinically obtained tumor samples may provide important information for use as both a diagnostic marker and a prognostic indicator for patient outcome. In order to evaluate telomerase activity in tumor tissue without radiolabeling the product, we developed a simple telomeric repeat amplification protocol-silver-staining assay that is less time-consuming, is safe and requires minimal equipment. In addition, we determined the sensitivity of the silver-staining method by using extracts of telomerase-positive thyroid carcinoma cell lines which were serially diluted from 5,000 to 10 cells. Telomerase activity was also assayed in 19 thyroid tumors, 2 normal controls and 27 bone marrow aspirates. The results indicate that the technique permits the detection of telomerase activity from 5000 to as few as 10 cells. We propose that it could be immediately applicable in many laboratories due to the minimal amount of equipment required.

Key words
- Telomeric repeat amplification protocol
- TRAP assay
- Human thyroid cancer
- Leukemia
- Telomerase
- Silver staining

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The measurement of telomerase activity is a useful test for cancer prognosis and management since it is repressed in almost all normal cells and reactivated in immortal cells and many tumor tissues (1). In an effort to better understand the prognostic value of telomerase measurement, new approaches have been proposed. The telomeric repeat amplification protocol (TRAP) assay is considered to be the gold standard. It is performed in a single tube reaction, where the telomerase first synthesizes extension products which work as templates for subsequent amplification by the polymerase chain reaction (PCR). However, the detection of TRAP products involves either radioactive end-labeling of a telomerase primer with [γ-^{32}P]ATP or DNA staining agents such as SYBR green (1,2).

In order to avoid the use of radioactive products or the need for costly laboratory equipment and reagents, we describe herein a simple, low cost and rapid TRAP assay, which, in addition, has higher sensitivity and requires a shorter processing time when compared with previously described methods (3).

As an example of sensitivity and protocol optimization for the detection of telomerase activity, we analyzed extracts prepared from thyroid carcinoma cell lines and fresh tissue from samples obtained from patients at Universidade Federal de São Paulo, São Paulo, SP, Brazil. We analyzed specimens from 21 thyroid tumors (2 normal tissue samples, 10 papillary thyroid carcinomas, 6 follicular thyroid carcinomas and 3 undifferentiated thyroid carcinomas), 1 thyroid papillary carci-

noma cell line (NPA) (4), 1 thyroid follicular thyroid cell line (WRO) (5), 1 undifferentiated thyroid carcinoma cell line (ARO) (4), 3 adjacent normal thyroid tissues, 2 samples from benign thyroid disease, and 27 bone marrow aspirates from 14 patients with acute lymphocytic leukemia, 9 with acute myelocytic leukemia, 3 with chronic myelocytic leukemia, and 1 with myelodysplastic syndrome. The samples obtained from thyroid tissues were rapidly frozen and stored at -80ºC until the time for the TRAP assay. The bone marrow aspirates were frozen in RPMI 1640 medium (Life Technologies®, Rockville, MD, USA) with 5% dimethylsulfoxide and stored at -80ºC (6). The cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (4,5).

We used an extract obtained from ARO and NPA cell lines inactivated by heating at 85ºC for 10 min as a negative control. We demonstrated the absence of primer-dimer and PCR contamination by adding 2 µl 1X CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) lysis buffer to the reaction mixture instead of 2 µl of extracts. Quantification controls were performed by adding 1 and 2 µl of TSR8 (provided by the TRAPeze kit, Oncor, Inc., Gaithersburg, MD, USA) to the reaction mixture.

Telomerase activity was determined using the TRAPeze kit which is based on an improved version of the original method described by Kim et al. (1). Ten to 50 mg of frozen tissue were placed in a sterile mortar, powdered by crushing with a matching pestle, transferred to a sterile 1.5-µl microcentrifuge tube and resuspended in 200 µl of 1X CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM mercaptoethanol, 0.5% CHAPS, and 10% glycerol). To avoid the problems most commonly occurring in the TRAP assay, 20 U of the RNAse inhibitor, 0.4 M dithiothreitol (DTT), was always added to the extract and RNAse-free tips, tubes and solutions were used. The samples from bone marrow aspirates were thawed and centrifuged at 1,250 rpm for 5 min; the supernatant was discarded and the pellet was resuspended in 200 µl 1X CHAPS lysis buffer, 20 U RNAse inhibitor, and 0.4 M DTT. After the samples were homogenized in 1X CHAPS, the suspension was incubated on ice for 30 min, shaken at 5-min intervals and then centrifuged at 14,000 *g* for 20 min at 4ºC. The supernatant was transferred to a sterile tube and protein concentration was determined in all extracts (TRAPeze telomerase detection kit). Two micrograms of protein was used for the tissue sample and 1 µg for the bone marrow aspirate, as recommended by the TRAPeze telomerase detection kit.

Since the cell lines were used to determine the sensitivity of the silver-staining method, the cell number was first counted using a Neubauer chamber (7) and then centrifuged. The extract was then resuspended in 200 µl 1X CHAPS lysis buffer. Thus, a certain amount of protein from the ARO and NPA telomerase-positive cell lines, which corresponded to 5000, 2500, 1000, 500, 100, 30, and 10 cells, was used.

When telomerase was present in the extracts, it added a number of telomeric repeats to the 3' end of a substrate oligonucleotide telomerase primer (5'-ATTCCGTCGAGCA GAGTT-3'). Telomerase was elongated for 30 min at 37ºC. The extended products were then amplified by PCR using a telomerase primer and a reverse primer. In addition, each reaction mixture contained a primer (K1) and a template (TSK1) for amplification of a 36-bp internal standard. The reaction was subjected to 30 cycles at 94ºC for 30 s and 60ºC for 30 s. Both elongation and PCR were performed in the same tube containing the cell extract with 20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 10 mM EGTA, 0.5 mg BSA, 50 µM each dNTPs, 2 U Taq polymerase (Life Technologies®), and 0.1 µg of each primer in a final volume of 50 µl.

Five microliters of a buffer containing 0.25% bromophenol blue, 0.25% xylenocyanol and 50% glycerol was added to each PCR product. Twenty-five microliters of each sample and controls were loaded onto a 12.5% non-denaturing polyacrylamide gel in 0.5X TBE buffer (1.5 mm spacer and 15 x 17 cm vertical gel, Life Technologies®). Electrophoresis was carried out at 150 V in 0.5X TBE buffer at room temperature until the bromophenol blue just ran off the gel. The gel was transferred to a fixing solution containing 0.5% acetic acid and 10% ethanol with gentle shaking for 15 min. After addition of 0.2% $AgNO_2$, the gel was stained for 10 min followed by washing twice in distilled water and then incubated in developing solution (0.1% formaldehyde and 3% NaOH) for about 10 min (8). The gel was handled after staining, maintained wet and photographed on 677 Polaroid film.

Telomerase activity was considered positive when a ladder of products was observed starting at 50 bp, with 6-bp increments. A 36-bp internal positive control band was detected in every line and was used to identify the non-informative specimen inhibitors of Taq DNA polymerase. Conversely, if the extract was telomerase negative, only the 36-bp internal control was observed. The results revealed that telomerase activity can be detected in extracts from 5,000 to as few as 10 cells; however, different intensities of the telomerase ladder were observed (Figure 1). Telomerase activity was also detected in 6/10 NPA, 3/6 WRO, 2/3 ARO, 3/3 thyroid cell lines, and in all leukemia samples (Figure 2). Telomerase activity was not present in ARO and NPA cell lines after heat inactivation. The samples and controls were analyzed at least twice and fresh material was used each time. Telomerase activity was preserved in the positive extracts stored at -80ºC for several months when a new assay was performed.

Recent studies have demonstrated that most normal human somatic cells exhibit undetectable levels of telomerase activity, whereas the majority of tumor cells express telomerase (3). Telomerase activity is associated with continued proliferation and immortalization, characterizing the advance of

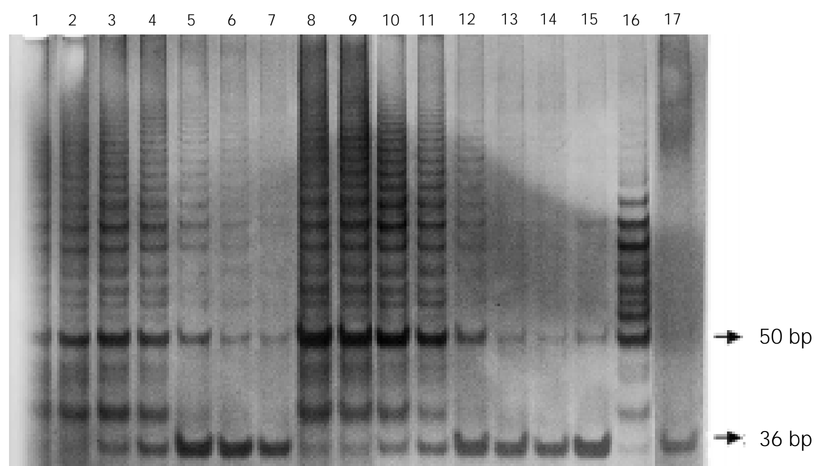

Figure 1. Detection of telomerase activity by the silver-staining method in a papillary thyroid carcinoma cell line (NPA) and in a follicular thyroid carcinoma cell line (ARO). Lanes 1-7, ARO cell line; lanes 8-14, NPA. Telomerase activity was detected in extracts from 5,000 cells (lanes 1, 8), 2,500 cells (lanes 2, 9), 1,000 cells (lanes 3, 10), 500 cells (lanes 4, 11), 100 cells (lanes 5, 12), 30 cells (lanes 6, 13), 10 cells (lanes 7, 14), in the primer-dimer control (lane 15) and TSR8 control (lane 16), and in ARO cells inactivated by heating (lane 17) (15.4 x 9.8 cm; 1:100).

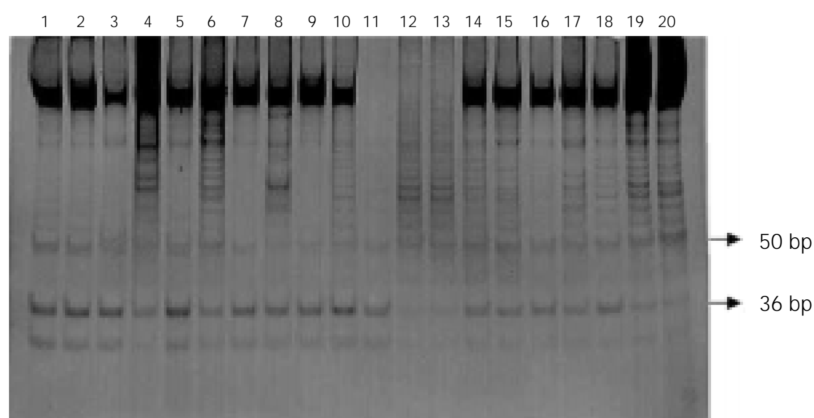

Figure 2. Detection of telomerase activity by the silver-staining method in papillary thyroid carcinomas (NPA), follicular thyroid carcinomas (WRO), undifferentiated thyroid carcinomas (ARO), and all leukemia samples. NPA cell line (lane 20); WRO cell line (lane 19); undifferentiated thyroid carcinoma (lanes 17, 18); mixed thyroid carcinoma (lane 16); mixed thyroid carcinoma (papillary/medullar thyroid carcinoma) (lane 15); papillary thyroid carcinoma (lanes 4, 5, 14); TSR8 control (lanes 12, 13); primer-dimer control (lane 11); mixed thyroid carcinoma (follicular/medullar thyroid carcinoma) (lane 10); normal control (lanes 7, 9); Goiter (lane 8); follicular thyroid carcinoma (lanes 1, 3, 6); lymph node metastases from papillary thyroid carcinoma (lane 2) (9.8 x 4.6 cm; 1:100).

tumor cells to immortality and their potential for malignancy (9). In addition, many studies have investigated the correlation between telomerase activity and clinical-pathological factors such as tumor size, histopathological grade, stage, clinical outcome and genetic alterations (10,11). These studies found that an increase in telomerase activity may be associated with tumor progression and that its level may be of prognostic value. Thus, the TRAP assay has become an important additional procedure for the identification of a marker associated with the acquisition of immortality and malignancy. However, a common problem in many laboratories is the equipment and reagents required to detect telomerase activity. Since the isotopic labeling for the TRAP assay is not a common routine procedure, we have developed a highly sensitive method to detect telomerase activity by a non-isotopic silver-staining method. Even though an instrument for detecting telomerase activity is available, this procedure is less sensitive and quite different from ours. We are able to detect telomerase activity in an extract with as few as 10 cells while the alternative method mentioned requires 1,000 cells (3). Presumably, our optimized protocol allowed the detection of increased band intensity. We used different pre-staining times and solutions, decreased staining time with a higher concentration of silver nitrate in the staining solution, and different developing solutions and times.

Since it can detect telomerase activity in a very small number of cells, the procedure could be helpful for the analysis of anything from extremely small samples obtained by minimally invasive fine-needle aspiration to surgical biopsies and specimens. The availability of this technique may help in the characterization of tumor tissues to reveal the character of the phenotype, which is clinically very valuable. The methodology described here is a procedure of very high sensitivity that reduces exposure to radioactivity, requires minimal equipment and can be used immediately in many laboratories.

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