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# Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity

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The ends of vertebrate chromosomes (telomeres) are composed of many kilobases of TTAGGG repeats (1–4). In normal somatic cells, some of these repeats are lost during every cell division due to the inability of the lagging-strand to replicate the very 5′ end of a linear DNA molecule (5,6). The ensuing telomere shortening has been shown to limit cellular proliferative capacity (7–10), and is likely to be the molecular measure (clock) that determines *in vitro* cellular senescence (11–13). Germline and most immortal/tumor cells express an enzyme that maintains telomere length and thus prevents cellular senescence. Telomerase is a ribonucleoprotein enzyme that uses its RNA as a template for the synthesis of TTAGGG repeats at the ends of the chromosomes (14–16). This polymerizing activity compensates for the failure to completely replicate the 5′ end of the lagging strands and consequently results in the stabilization of telomere length.

The development of a PCR-based telomerase assay [the telomere repeat amplification protocol; TRAP assay] capable of analyzing small tissue biopsies has permitted a large number of tumor samples to be analyzed (17). Approximately 85% of more than 950 primary tumors have been found to express telomerase (17–21 and unpublished observations), making telomerase a frequent marker for cancer cells. Several weaknesses in the assay have become apparent during these tumor surveys: (i) the assay is non-linear, making statements about relative levels of activity difficult; (ii) some tissue samples contain an inhibitor of *Taq* polymerase, and thus give a false negative results using the standard 6 μg of protein extract per assay but a positive result when diluted 10–100-fold (20) and (iii) the necessity of preparing a cell or tissue extract limits the application of the technique to small but not microscopic samples. We here describe modifications to the original protocol that resolve all of these difficulties.

Telomerase can add TTAGGG repeats to a variety of non-telomeric sequences *in vitro* (16,22). It pauses after adding each repeat, presumably to permit repositioning of its internal RNA template prior to synthesizing the next repeat (15). It thus generates a ladder of 6 bp addition products. These can be visualized following PCR amplification using the telomerase substrate (TS) oligonucleotide as the forward primer and an oligonucleotide able to anneal to the telomeric repeats (CX) as the reverse primer (17). Figure 1A (lanes 1–4) shows that the abundance of product increases with increasing amount of *Taq* polymerase. This demonstrates that the molarity of these multiple amplification products causes *Taq* DNA polymerase to become limiting, even using a radioactive assay.

A 150 bp internal standard, sufficiently long so that it would not interfere with the visualization of the telomerase ladder, was prepared by synthesizing TS and CX oligonucleotides that contained an additional 15 bases at their 3′ ends that overlapped with sequences encoding aa 97–132 of rat myogenin (23):
TS-overlap primer, 5′-**AATCCGTCGAGCAGAGTT**GTGAATGAGGCCTTC-3′ CX-overlap primer, 5′-**CCCTTACCCTTACCCTTACCCTAA**TAGGCGCTCAATGTA-3′ [TS (18 bases) and CX (24 bases) sequences are in bold type, myogenin sequences (15 bases each) are underlined. TS- and CX-overlap primers (30 μM) were only used for the initial amplification after which the 150 bp product was column purified for future use].

Amplification of the myogenin cDNA with these primers generated a 150 bp product which could be reamplified using the same TS and CX primers used to amplify the telomerase ladder in the standard TRAP assay. Figures 1B and C demonstrate that normalizing the intensity of the telomerase ladder to that of the internal standard permits the assay to become highly linear, so that accurate comparisons between samples becomes possible. The inclusion of the internal standard also immediately identifies false-negative tumor samples that contain *Taq* polymerase inhibitors (Fig. 1D).

We have previously shown that the inclusion of up to 50 ng of contaminating genomic DNA did not interfere with the TRAP assay (20). This suggested that whole cells rather than just cell extracts could be analyzed. Aliquots of a dilute cell suspension containing single cells were identified under direct microscopic examination. These aliquots were then diluted with TRAP assay buffer in which the concentration of Tween-20 detergent had been increased to 0.5% to ensure cellular permeabilization, and then transferred to TRAP assay tubes. Increasing the incubation time 2-fold (from 30 min to 1 h), decreasing the concentration of cold precursors from 50 to 25 μM (to increase 2-fold the specific radioactivity of [$^{32}$P]dCTP tracer), and increasing the number of PCR cycles from 31 to 34 allowed the telomerase in single cells to be detected (Fig. 2).

The central role of the derepression of telomerase in the process of cellular immortalization and cancer is becoming increasingly apparent. A multitude of unanswered questions concerning its regulation, prognostic value and utility as a target for therapy remain, and a large number of laboratories are likely to be pursuing these and other questions. The modifications of the TRAP assay described herein should significantly improve the reliability, linearity and sensitivity of these investigations.

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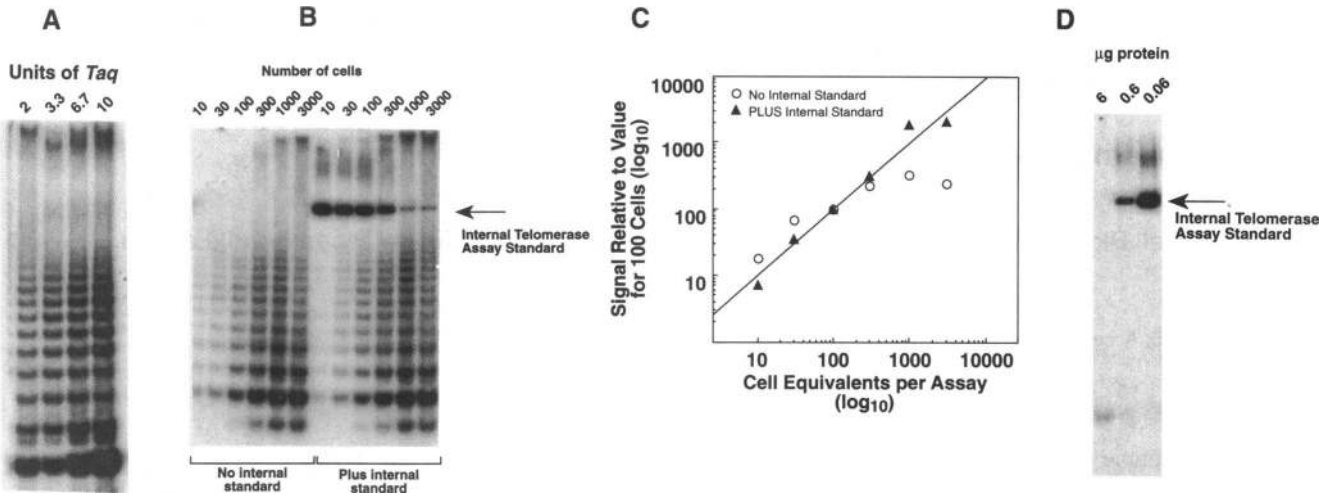


**Figure 1.** An internal standard permits the linearization of the TRAP assay. (**A**) *Taq* polymerase is limiting for the amplification of telomerase ladders. Extracts containing 1000 cells were amplified for 31 cycles under standard TRAP assay conditions (17,20 ) with increasing amounts of *Taq* polymerase. The strength of the telomerase ladder signal is roughly proportional to the number of units of *Taq* polymerase. (**B**) Internal standards indicate when *Taq* becomes limiting. Serial 3-fold dilutions of cell extracts were assayed in the absence (left) or the presence (right) of a 150 bp internal standard (15 attograms/reaction) added to the PCR mix above the wax barrier. The decreased signal from the internal standard at higher telomerase activity levels indicates when insufficient *Taq* polymerase is present. (**C**) Quantitation of telomerase ladders. The lanes in Figure 1B were scanned and quantitated using a PhosphoImager. All of the peaks were summed to yield a net incorporation per assay. The data for the first six lanes (open circles) are expressed relative to the 100-cell signal. The data for the last six lanes (solid triangles) were first normalized to the signal from the internal standard, and then expressed as relative to the normalized 100-cell signal. The solid line was computer fitted to the data normalized to the internal standard. The divergence of the standard data (without the internal standard) from this line at higher activity levels demonstrates the value of the internal standard. (**D**) The identification of false negatives using the internal standard. Occasional biopsies contain inhibitors of *Taq* polymerase. These samples can be identified by the failure to amplify the internal standard (lane containing 6 µg of protein extract from a primary human brain tumor), which only become amplifiable once the original extract is diluted (lanes containing 0.6 and 0.06 µg of protein).

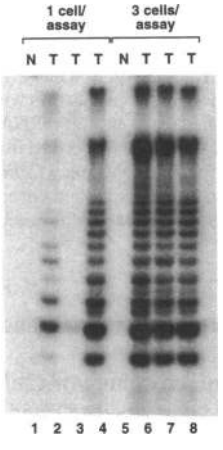


**Figure 2.** Telomerase activity can be detected in single cells. Immortal breast tumor cells (SCC70 labeled T, lanes 2–4 and 6–8) expressing telomerase activity were diluted to 500 or 1500 cells/ml, and then 2 µl aliquots were placed on a glass slide. Individual drops containing one or three cells were identified under the microscope, and then the visually confirmed aliquots were solubilized in 48 µl of complete TRAP assay buffer containing 0.5% Tween-20. The sensitivity of the assay was further increased by performing 34 rather than 31 cycles of PCR amplification and increasing 2-fold the specific radioactivity of dCTP. As a negative control normal human mammary epithelial cells (HME31 cells, labelled N, lanes 1 and 5) without detectable telomerase activity were used (lanes 1 and 5). Telomerase activity was detected in all three samples containing 3 tumor cells (lanes 6–8) while activity could be detected in two of three samples containing single cells (lanes 2–4). Occasionally additional minor bands appear between the 6 bp ladders (lanes 4,6,7,8) which may be do to inaccurate priming of TS primers by telomerase. When single cells are analyzed, the amount of telomerase is likely to be close to the limits of detection under the conditions described. This can result in band drop out (lane 2) and may reflect low abundance of telomerase activity products which are subjected to PCR.

## ACKNOWLEDGEMENTS

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