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Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP)

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ABSTRACT

The telomeric repeat amplification protocol (TRAP) assay has been used to test telomerase activity in numerous cancer specimens. We describe primers, controls and quantification methods for the TRAP assay to accurately measure the level of telomerase activity in clinical samples. The assay is reliable and reproducible in routine analyses and can be used to estimate the processivity of telomerase activity.

Telomerase is a ribonucleoprotein complex that plays a critical role in telomere maintenance and cellular immortality (1,2). Previously we have described a highly sensitive PCR-based telomerase activity assay, designated the telomeric repeat amplification protocol (TRAP) (3), that has proved to be an important tool for understanding the role of telomerase in cancer. Since then others have discussed quantification of the level of telomerase activity in primary tumors using the TRAP assay (4,5). In this report we discuss new primers, controls and quantification methods for the TRAP assay to characterize telomerase expression in clinical samples.

Anchored return primers were developed (ACT, 5′-GC-GCGG[CTAACC]$_3$-3′; ACX, 5′-GCGCGG[CTTACC]$_3$CTA-ACC-3′) that have a 6 bp ‘anchor’ at the 5′-end which is neither telomeric nor complementary to telomeric sequences, followed by sequences that hybridize to telomeric repeats. The presence of the 6 bp anchor and new permutations of the telomeric sequence in these primers result in a significant reduction in primer dimer PCR artifact formation as compared with the published CX primer (3), even in the absence of stringent hot start conditions. Primer ACX was more resistant to primer dimer artifact formation when compared with primer ACT and thus was used more frequently for our routine TRAP analysis. Very rarely, primer dimer artifacts may form with primers TS and ACX in the TRAP assay. However, unlike the primer dimer artifacts with primer CX, the artifacts resulting from primers TS and ACX or TS and ACT are different from the typical 6 bp TRAP ladder pattern and are easily distinguished from the genuine telomerase products (Fig. 1), providing an additional confirmation that the products of the TRAP assay represent genuine telomerase activity and are not the result of PCR artifacts.

The processivity of telomerase is defined as the total number of telomeric repeats added to a DNA substrate. For simplicity, we define a ‘processivity measurement’ as the maximum number of observed telomeric repeats added to the TS oligonucleotide substrate in a given *in vitro* reaction. In the TRAP assay a return primer can bind anywhere along the telomeric repeats and produce PCR products of different lengths. PCR-mediated elongation of telomerase products in the TRAP assay with the CX return primer can be observed in Figure 2. When synthetic telomerase products R5–R8 (5′-AATCCGTCGAGCAGAGTT-AG[GGTTAG]$_{4–7}$-3′), corresponding to primer TS extended with five to eight telomeric repeats, were amplified in the TRAP reaction using the CX return primer the resulting bands consist of products that are both shorter and longer than the original starting template.

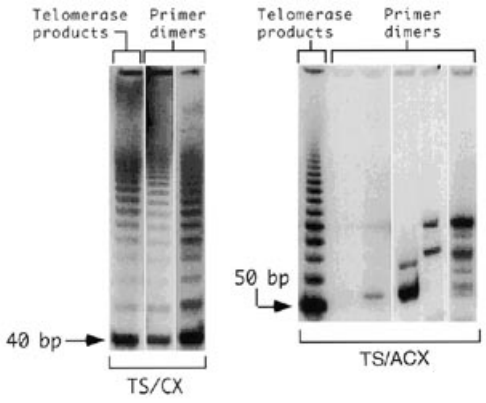

Figure 1. Primer dimer PCR artifacts derived from CX and ACX primers. Products of telomerase activity start at 40 (TS/CX primers) or 50 bp (TS/ACX primers) and display 6 bp periodicity. Primer dimer PCR artifacts derived from TS and CX primers are indistinguishable from the genuine telomerase products. However, the primer dimer PCR artifacts derived from TS and ACX primers lack 6 bp periodicity and can be easily distinguished from the telomerase products.

Anchored primers, such as ACX, prevent 3′ elongation of telomerase products by capping the 3′-end of a telomerase product after the first PCR cycle. In subsequent PCR cycles this 3′-cap, or anchor, prevents 3′ elongation of telomerase products even when primer ACX is bound in a staggered manner to the 3′-end of the telomerase product. Thus the length of products produced in a TRAP assay utilizing primer ACX accurately reflects the processivity of the telomerase activity being tested. As seen in Figure 2, a TRAP assay performed with primer ACX on the synthetic telomerase products results in products whose lengths are representative of the length of the starting template. In

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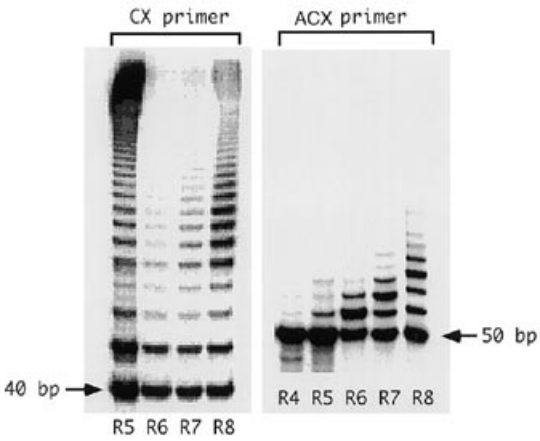

Figure 2. TRAP assay of synthetic telomerase products using primer CX or ACX are shown. One tenth of an attomole of synthetic telomerase products comprising TS primer extended with four (R4, 5′-AATCCGTCGAGCAGAGTTAG[GGTTAG]$_3$-3′) to eight (R8, 5′-AATCCGTCGAGCAGAGTTAG[GGTTAG]$_7$-3′) telomeric repeats were used in the TRAP assay. With primer CX the resulting products are not representative of the length of input products, while primer ACX results in products that reflect the original length of the input product. The additional one to four faint bands visible above the main bands are PCR artifacts which are often present when reaction condition do not include a hot start.

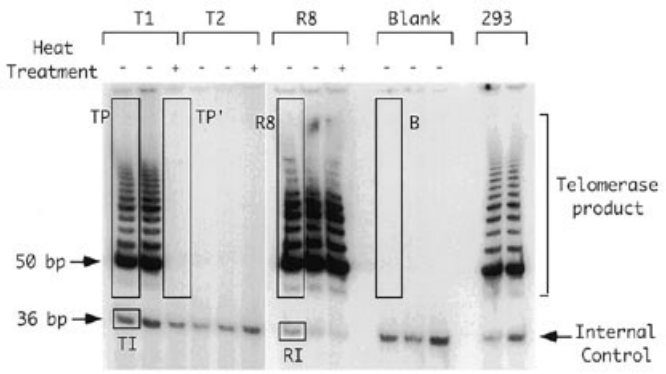

Figure 3. The results of quantitative TRAP assays are shown. The TRAP assay was performed as previously described (3,6,7) on two tumor specimens (T1, telomerase positive; T2, telomerase negative), R8 synthetic telomerase product (0.1 amol in CHAPS lysis buffer), lysis buffer only blank and 293 positive control extract. An aliquot of 1 µg of TS substrate primer (5′-AATCCGTCGAGCAGAGTT-3′) was end-labeled in 10 µl reaction mix [10 µCi [γ-^{32}P]ATP (3000 Ci/mmol), 1× One Phor All™ buffer (Pharmacia) and 5 U T4 polynucleotide kinase] incubated for 20 min at 37°C and then 5 min at 95°C. Fifty microlitre TRAP reactions contained 1× TRAP buffer (3,6,7), 50 mM dNTPs, 1 µl TS end-labeling reaction (0.1 mg TS primer), 0.1 µg ACX return primer, 0.1 µg NT internal control primer, 0.01 amol TSNT internal control, 2 U *Taq* DNA polymerase (Perkin Elmer) and 2 µl CHAPS cell extract (2 µg protein). Each TRAP reaction mix was placed in a thermocycler block preheated to 30°C and incubated at 30°C for 10 min for one cycle, then at 94°C for 30 s, 60°C for 30 s for 27 cycles. Typical products of telomerase activity with primers TS and ACX are shown by the bands starting from 50 bp; the internal control results in a single band of 36 bp. Heat inactivation was performed by heating 10 µl extract at 75°C for 10 min prior to assaying 2 µl by TRAP analysis. The boxed areas show integration of radioactive counts from telomerase products from the test extract (TP), telomerase products from heat-inactivated control (TP′), internal control of the test extract (TI), telomerase product R8 quantification standard (R8), telomerase products from lysis buffer only blank (B) and internal control of the R8 quantification standard (RI). These values were used to quantify the level of telomerase activity using the formula $\{[(TP - TP')/TI]/[(R8–B)/RI]\} \times 100 = TPG$.

a routine TRAP assay using primer ACX, processivity can be described as $x = y + 3$, where x is the number of telomeric repeats on a telomerase product and y is the observed number of telomerase product bands on the gel. Depending on the purity of primers and the stringency of PCR conditions, a few additional faint bands can sometimes appear in the TRAP analysis. Thus, in most cases, the accuracy of the processivity measurement in a quantitative TRAP assay without hot start is $x = (y + 3) \pm 2$.

Unlike most PCR applications, which normally measure a fixed amount of nucleic acid target in a sample, the TRAP assay measures an enzymatic activity where the amount of target is dependent upon the biochemical activity of the enzyme. When quantifying telomerase activity there are two aspects of consideration: first, how many telomeric repeats the enzyme adds to the substrate; second, how many substrate molecules are extended by telomerase. The first aspect relates to the processivity and the second relates to the turnover rate and the number of active telomerase complexes. The quantification method described here specifically evaluates turnover and is based on the use of an end-labeled primer in the TRAP assay. End-labeling the substrate primer ensures that the measured total signal strength corresponds to the number of substrate primers extended by telomerase and is independent of the length of each extended product. It is possible to measure the total number of repeats telomerase adds to the substrate by directly incorporating labeled nucleotides during the TRAP assay. However, the end-labeling method was emphasized because in the TRAP assay the return primer can bind anywhere along the telomeric repeat sequence of the telomerase product, resulting in amplified products whose number of telomeric repeats may not directly correspond to the number of telomeric repeats present in the starting telomerase products.

In a typical TRAP analysis, each sample extract was tested in duplicate and a negative control for each test extract was performed with heat-inactivated extract (Fig. 3). Telomerase activity was quantified by integrating the radioactive signal from the sample lane and comparing it with the radioactive signal from a known amount of quantification standard (R8). Utilization of the quantification standard provides a means to describe the level of telomerase activity as an absolute value. From this quantification scheme the level of telomerase activity is expressed as a TPG (total product generated) value, where one unit of TPG is defined as 0.001 amol, or 600 molecules, of primer TS extended for at least three telomeric repeats by telomerase present in the extract. One TPG corresponds approximately to telomerase activity from one immortal cell. The near linear range of this assay is 1 (0.001 amol) to 10 000 TPG (10 amol) using dilutions of R8 synthetic product (Fig. 4). The products generated from dilution of 293 cell extracts are nearly linear from ~1 to 1000 TPG. This range encompasses the minimum and maximum levels of telomerase activity in most tumor samples tested. Thus quantification of telomerase activity in most tumor samples can be measured using identical assay conditions.

The TRAP internal control utilized in the assay, TSNT (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′), is amplified by primer TS and its own dedicated return primer, NT (5′-ATCGCTTCTCGGCCTTTT-3′), which is not a substrate for telomerase. Thus, this TRAP internal control is a semi-competitive internal control in that it shares only one of the

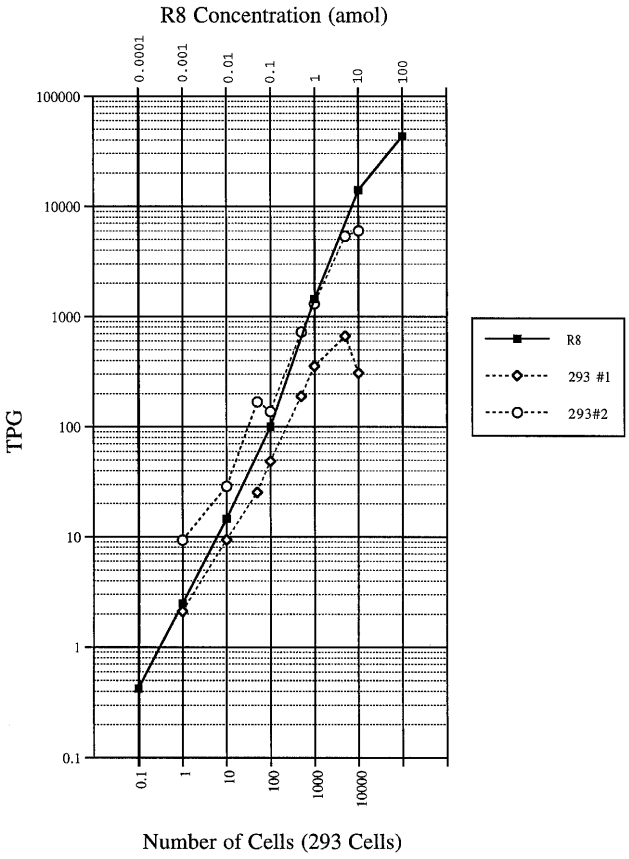

Figure 4. Dynamic range of the quantitative TRAP assay is shown. Dilutions of R8 synthetic products from 100 to 0.0001 amol and dilutions of two different 293 extracts (293 no. 1 and no. 2) of from 1 to 10 000 cells were used in the TRAP assay. The TPG values for the R8 and 293 dilutions are given on the *y*-axis. On this log–log plot the slope of the linear region between 0.001 and 10 amol R8 is ~0.87 and between 1 and 1000 cells is ~0.74. The reduction in product formation for the 293 dilutions at 10 000 cells is due to an endogenous PCR inhibitor present in the highly concentrated 293 extracts.

primers (TS) used to amplify the telomerase products. Since only one of the primers is shared between the telomerase products and the internal control, competition between the target and the internal control for the primers is alleviated, resulting in a wider dynamic range for the assay.

Incorporation of the TRAP internal control is useful in measuring telomerase activity levels in clinical samples that may contain inhibitors of *Taq* polymerase. With incorporation of the internal control, false negative results can be easily identified by the disappearance of the internal control band (Fig. 5, lane 1). As input inhibitors are diluted, both telomerase and internal control products are co-amplified proportionately to their respective PCR efficiency. This characteristic allows for more accurate quantification of telomerase activity and comparisons of telomerase activity between different extracts can be made without the need for dilution of the test extracts, even when different amounts of PCR inhibitors are present in the extracts. If the assay is performed within the linear range with an appropriate amount of TSNT internal control, amplification of both the telomerase products and the internal control should be comparable and the chance of a false negative result for a telomerase-positive sample,

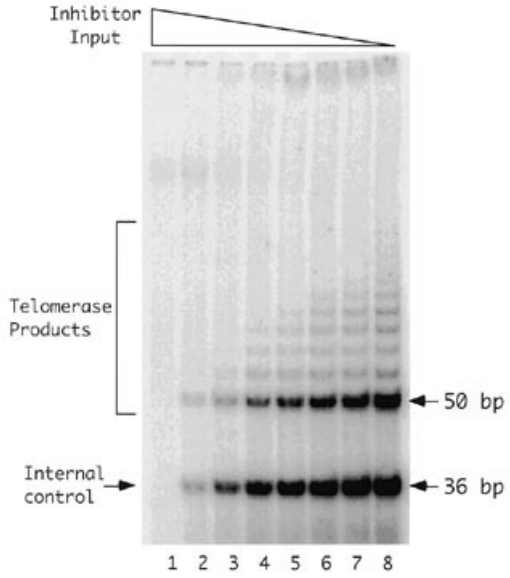

Figure 5. Effect of PCR inhibitor on the TRAP assay is shown. Three-fold dilutions of a telomerase-negative colon extract that is inhibitory for *Taq* DNA polymerase were mixed with equal amounts of 293 cell extracts (extracts from 100 cells) and analyzed for telomerase activity. Undiluted colon extract completely inhibits the PCR reaction (lane 1, 20 μg/μl total protein). As the amount of inhibitory extract is reduced, telomerase products from 293 extracts and the internal control increase proportionally with respect to their PCR efficiencies.

if the TSNT internal control is amplified in the reaction, is extremely low.

Utilization of the anchored primer ACX, the TSNT internal control and the R8 quantification standard streamlines the TRAP assay and provides an accurate measurement of telomerase activity. Furthermore, the new primer combination allows estimation of the processivity of telomerase, which may be useful for characterization of telomerase. Similar primer design and quantification methods were incorporated into the TRAPeze™ kit (Oncor Inc.), which is now commercially available.

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