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Increased sensitivity and reproducibility of TRAP assay by avoiding direct primers interaction

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ABSTRACT

Telomeric Repeat Amplification Protocol (TRAP) is a sensitive procedure to measure telomerase activity in small samples of cell or tissue extracts. Due to the strict correlation between high levels of telomerase activity and neoplastic transformation, TRAP assay could provide an important diagnostic marker of malignancy. Although the original TRAP assay is very sensitive and some improvements have been described, occasional artifacts still persist in the modified procedures. Here we describe how changes in the sequence of the primer used for the amplification step enhance the reproducibility and sensitivity in the TRAP assay.

Telomerase is a ribonucleoprotein complex which adds short TTAGGG repeats to the 3′ end of eukaryotic chromosomes, using its RNA subunit as template (1). In the absence of telomerase activity, telomeres shorten at each cell division, leading to chromosome instability and cell death (2). Differently from most of human somatic cell populations, the vast majority of tumors contain telomerase, conferring to the cell unlimited growth capability. The development of a very sensitive 'Telomeric Repeat Amplification Protocol' (TRAP) (3) for measuring telomerase activity in cell extracts provided an assay as a potentially very useful tumor marker (4). The technique is based on the ability of telomerase to recognize and elongate *in vitro* an artificial oligonucleotide substrate TS, 5′-AATCCGTCGAGCAGAGTT-3′. The resulting products are amplified via PCR using as primers the TS oligonucleotide and a reverse primer, a 24mer oligonucleotide, CX, 5′-CCCTTACCCTTACCCTTACCCTAA-3′. CX is complementary to four telomeric repeats but contains a single base mismatch at the same position in each of three of the telomeric repeats. The PCR leads to the formation of a ladder with a six base stepwise increase which is in part due to the non-uniform length of the products of telomerase and in part results from a artifactual staggered annealing between the elongated TS and CX. This staggered positioning of CX is only partially reduced by the introduction of the three above mentioned mismatches (T/A) between CX and the telomeric repeat (3).

A number of improvements to the original TRAP assay have been described (5–8) which reduce the occasional occurrence of false positives. In particular, a recent study showed that the artifacts in TRAP assay result from primer dimer formation and subsequent slippage during amplification (5). It was found that the addition of three non-complementary nucleotides to the 5′ end of CX (CXext), limits staggered annealing, essentially eliminating the non-specific ladder. However, as for the CX, the CXext could still form primer dimer products due to the two base overlap between the 3′ ends of the TS and CXext oligonucleotides. Since we noticed a reduction of sensitivity with the newly suggested protocol, we reasoned that production of primer dimers may adversely affect the amplification of telomerase products as it leads to the formation of a competing species which is amplified by the same TS and CX (or CXext) primers. In order to address this problem, we designed a new reverse primer CXa, 5′-GTGTAACCCTAACCCTAACCC-3′, which is not complementary to the TS 3′ end and thus should be incapable of primer dimer formation. In addition, the 5′ end of CXa consists of three 'anchoring' nucleotides (5), GTG. These are not complementary to the 3′ end of telomerase product and thus should reduce staggered annealing. The CXa oligonucleotide is otherwise perfectly complementary to the telomerase product which we assumed would enhance its priming efficiency. An example of reactions performed with the new CXa in a classical or a simplified set up, and with the extended CXext oligonucleotides are compared in Figure 1A and B.

If our assumptions were correct, then the TRAP assay should be less efficient at low substrate concentrations. Figure 1B shows that lowering the concentration of substrate (PCR template) by reducing the amount of telomerase containing extract in the first part of the reaction, dramatically decreased the yield of PCR product produced by the TRAP assay performed with CXext as compared to CXa. At low concentrations of substrate only the primer dimer product was seen after PCR with the CXext primer. By contrast there was a linear relationship between the amount of extract and the yield of PCR products generated by the TRAP assay using the CXa oligonucleotide. To test whether the superior performance of the TRAP assay with CXa is in fact due to lack of competition by primer dimer formation, we added a synthetic double strand oligonucleotide, TLa, 5′-AATCCGTCGAGCAGAGTTGGGTAAGGGTTAGGGTTA-CAC-3′, that corresponds to a dimer of TS and CXa, to a CXa-containing reaction. TLa dramatically reduces the formation of telomeric ladder when added to the TRAP assay even at very low concentrations (Fig 1.C). This result would be expected if the primer dimer TLa competed in the PCR reaction with the low number of TS molecules that had been elongated by the telomerase.

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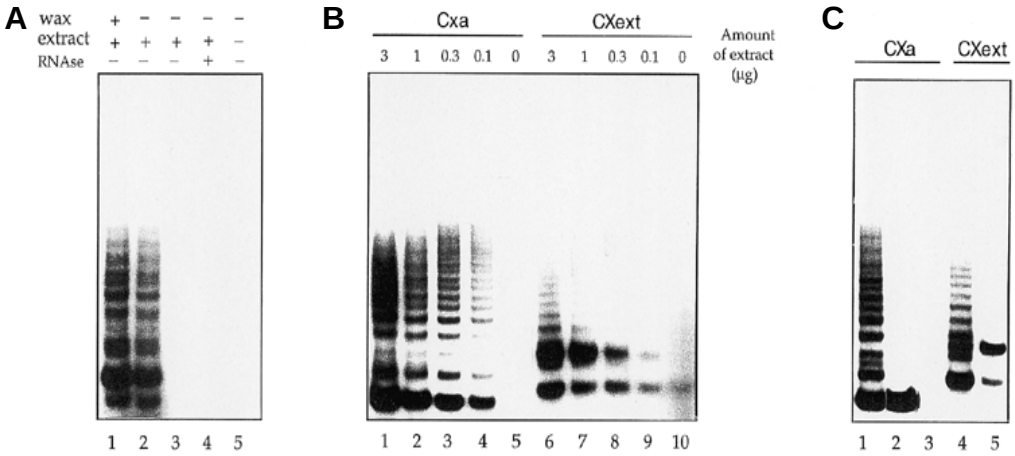

Figure 1. Efficiency of TRAP assays performed with different reverse primers. (**A**) TRAP products obtained with primer CXa following a standard or a one-step protocol. After 10 min incubation at room temperature for telomerase-mediated elongation and 2 min at 96°C to inactivate telomerase activity, 30 cycles of PCR were performed in a 50 µl solution (20 mM Tris–HCl pH 8.0, 50 µM dNTPs, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.05% Tween 20, 15 pmol of TS and CXa primers, 0.2 µl of [α-^{32}P]dCTP, 10 µCi/µl, 3000 Ci/mmol, 2 U *Taq* polymerase) with the following settings: 94°C for 30 min, 50°C for 30 min, 73°C for 30 min. In lanes 1–4, 1 µg of cell extract was assayed. In lane 1 the primers were separated by a wax barrier. In lane 2 extension and amplification steps were carried out with a single mastermix, containing both TS and CXa primers, with no wax barrier. In lane 3 the extension step was performed substituting the TS substrate with CXa primer. In lane 4 the extract was pre-treated with RNAse (10 µg of extract plus 0.5 µg of RNAse for 30 min at 37°C). In lane 5 no extract was added to the reaction mix. (**B**) Comparison of TRAP assay testing different amounts of U937 cell extract by CXa or CXext. The left side of the figure (lanes 1–5) shows ladders obtained using serial 3-fold dilutions of cell extract. The samples were assayed in a single reaction mix with TS and CXa primers not separated. The right side of the figure (lanes 6–10) shows reaction products obtained by CXext following the two-step protocol. In lanes 5 and 10 no extract was added. Since PCR reaction with CXext primer was very inefficient using an annealing temperature of 50°C, it was lowered to 48°C. (**C**) The effect of a potential primer dimer on TRAP. All the samples were assayed in a single reaction mix with the primer pairs, TS+CXa and TS+CXext respectively, not separated by the wax barrier. Lane 1 shows typical telomeric ladder obtained with 1 µg of U937 cell extract using CXa as reverse primer. The same amount of cell extract is analyzed using CXext (lane 4). In lanes 3 and 5, the reaction was performed without extract. Lane 2 shows that the addition of the hypothetical dimer, TLa, abolishes the telomeric ladder generated by CXa.

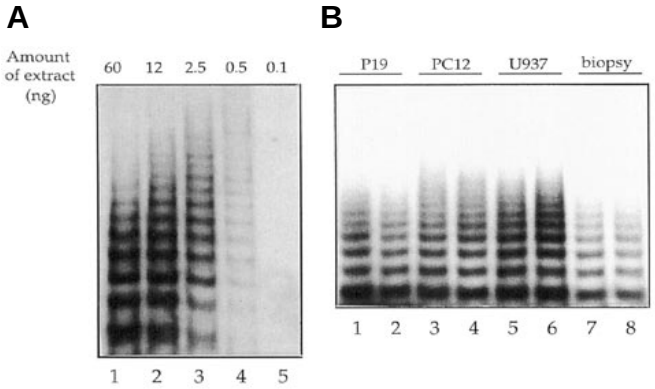

Figure 2. Sensitivity and reproducibility of TRAP assay with CXa. (**A**) The TRAP assay was performed on 5-fold dilutions of U937 cell extract, ranging from 60 to 0.1 ng. (**B**) The TRAP assay performed with 50 ng of different cell lines: P19 (lanes 1 and 2), PC12 (lanes 3 and 4), U937 (lanes 5 and 6). Lanes 7 and 8 show telomerase activity detected in a bioptic sample of malignant meningioma.

If the TRAP assay is to be useful as diagnostic marker for cancer it should be both sensitive and reproducible. We could routinely detect telomerase activity from <0.5 ng of cell extract from a variety of cell lines using our standard conditions (Fig. 2A). An important factor in the increased sensitivity using our assay is the lack of primer dimer formation by the CXa primer even after a large number of PCR cycles. As we demonstrate in Figure 1C, primer dimer can abolish PCR amplification of the telomerase product. Figure 2B shows that our assay produced very reproducible results with both biopsy material and a variety of cell lines: human, murine and rat.

In conclusion, the use of CXa as reverse primer allows a more accurate and sensitive measurement of telomerase activity. In addition, the elimination of primer dimer formation provides a number of practical advantages. The TS and CXa primers do not need to be separated by a wax barrier (as in the original procedure) nor is a hot start required. All these features will greatly simplify the development of automated TRAP assays with no need of separating the TRAP reaction products by acrylamide gel electrophoresis.

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