# Tissue Quality is an Important Determinant of Telomerase Activity as Measured by TRAP Assay

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Telomerase is a ribonucleoprotein with the function of a DNA polymerase, in which a segment of the RNA component operates as an internal template. It adds hexameric (TTAGGG) repeats to the telomeric ends of the chromosomes, thus compensating for the continued erosion of the telomeres that occurs in its absence (3,7). Reactivation of telomerase might be a necessary event for the sustained growth of most human tumors (9). The development of a polymerase chain reaction (PCR)-based telomerase assay, the telomeric repeat amplification protocol (TRAP), has permitted a large number of tumor samples to be analyzed (5).

Telomerase activity has been detected in almost all human immortal cell lines and in most human cancer tissues (2,5). However, some human tumor tissues seem not to express telomerase. A review that compiled several studies of human tumors indicated that telomerase activity occurred in human cancer tissues in 75%–100% of cases, with an average of 84.8% (8). Furthermore, different studies on histologically similar tumors revealed different proportions of tumors with telomerase activity. For example, in colorectal cancer, telomerase activity was detected in 32 of 35 cases (92%) by Kim et al. (5), in 40 of 50 cases (80%) by Li et al. (6) and in 8 of 8 cases (100%) by Yoshida et al. (11). An explanation for these discrepancies could be heterogeneity of human tumor tissues that invariably consist of mixtures of tumor cells and surrounding stromal tissue. However, the TRAP assay has been shown to detect telomerase activity in as few as 1 positive cell per $10^4$ cells (5). We propose that some of the apparently negative tumors yield RNA of insufficient quality and are therefore false negative.

A second reason for false-negative results might be the presence of *Taq* DNA polymerase inhibitors. To recognize this type of problem, the TRAP assay should be performed using an internal control (10). The TRAP-eze™ Telomerase Detection Kit (Oncor, Gaithersburg, MD, USA) includes the amplification of an internal control of 36 bp in each assay, and a false-negative result is concluded when the 36-bp amplified product is not observed. This control, however, does not account for all apparently negative tumor tissues.

Telomerase is a ribonucleoprotein enzyme that uses its RNA as a template for the synthesis of TTAGGG repeats at the ends of the chromosomes (4). For this reason, telomerase activity necessitates an active protein and a nondegraded RNA. These two conditions indicate that the quality of the tissue is of vital importance for the success of the telomerase detection assay. Strikingly, RNA quality control has never been proposed to date. We therefore included a control for RNA quality in our TRAP assay protocol.

We measured telomerase activity in 50 colorectal cancer tissues derived from 50 patients using the TRAP assay. About 10 mg of frozen tissue were homogenized in 150 µL of 1× CHAPS lysis buffer (5). The whole-tissue lysate was rapidly frozen and stored at -80°C. The protein concentrations of the extract were measured using the BCA™ Protein Assay Kit (Pierce Chemical, Rockford, IL, USA). Approximately 0.1 µg of protein extracted from tissue was used for each telomerase assay (25 µL reaction). Forty-two samples (84%) were found to be positive in this assay, while 8 samples were telomerase-negative. All 50 samples were taken from the tissue bank of the Institute of Pathology (Lausanne, Switzerland). Some samples were not frozen immediately. This lapse of time that occurred sometimes between surgery and freezing in liquid nitrogen is relatively important. Warm ischemia might account for tissue autolysis including RNA degradation, which can affect the telomerase activity results. To test this hypothesis, total RNA from these 50 colon cancer tissues was extracted using TRIZOL® Reagent (Life Technologies, Gaithersburg, MD, USA). For each case, approximately 50 mg of frozen tissue were used to extract RNA. The quality of RNA was controlled on agarose gels (Figure 1A). In 25 out of 50 colon cancer tissues, the RNA showed no or partial 28S and 18S ribosomal RNA degradation (Figure 1A, lanes 3–6). In the other 25 cases, significant to complete RNA degradation was observed (Figure 1A, lanes 7–9). All of the 25 cases with only partially degraded or intact RNA showed telomerase activity (Figure 1, A and C, lanes 3–6). Only 17 of the 25 cases (68%) with strongly or completely degraded RNA were positive for telomerase activity (Figure 1, A and C, lanes 7–9). This difference is statistically significant ($P$ <0.0001). Various levels of telomerase activity were observed. All of the 25 cases with only weak RNA degradation showed strong telomerase activity (Figure 1C, lanes

3–6), whereas most cases with strongly degraded RNA had either weak (Figure 1C, lanes 7 and 8) or even no (Figure 1C, lanes 9 and 10) telomerase activity. Most cases with complete RNA degradation had no detectable telomerase activity (Figure 1, A and C, lanes 9 and 10). The fact that telomerase activity was observed in some cases with apparent RNA degradation can be explained on the assumption that the full length of the RNA component of telomerase is not necessary for telomerase activity (1). Another explanation for the presence of telomerase activity in partially degraded samples could be the sensitivity of the TRAP assay. Even a significant reduction in the number of active telomerase molecules due to tissue degradation might not totally abolish detectable telomerase activity in the sample.

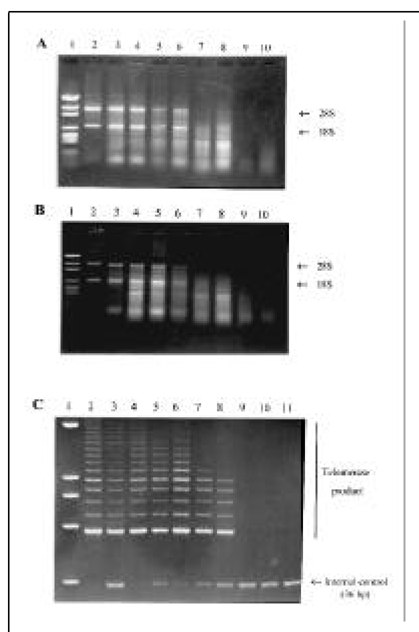

**Figure 1. RNA quality and telomerase activity in colorectal cancer tissues.** (A and B) Total RNA extracted from frozen colon cancer tissues (A) and from tissue sections (B) was subjected to 1% agarose gel electphroresis. Lanes 2–10 contained about 0.6–1.0 µg of total RNA. (C) Telomerase activity was detected using the TRAP assay. About 0.1 µg of protein extracted from frozen colon cancer tissues was placed in 25 µL of PCR mixture for each assay. One-fifth of the PCR amplification product was applied to a 12.5% nondenaturing polyacrylamide gel and visualized by SYBR® Green I (Molecular Probes, Eugene, OR, USA) staining. Lane 1: pGEM® DNA Marker (Promega, Madison, WI, USA). RNA analysis (A and B) or telomerase assay (C) from SW480 colorectal cancer cell line (lane 2) or from frozen colorectal tumor tissues (lanes 3–10). Panel C, lane 11: negative control where no protein was added.

**Table 1. Telomerase Activity and RNA Degradation in Colorectal Cancer Tissues**

| | RNAi | RNAd |
|---|---|---|
| Telomerase+ | 25 | 17 |
| Telomerase- | 0 | 8 |

RNAi: RNA intact or only partially degraded

RNAd: high RNA degradation (28S and 18S rRNA species completely missing)

A limitation of this approach is that the amount of tissue required to perform an rRNA measurement is too high for small biopsies. To overcome this limitation, different approaches have been tested. First, RNA quality was determined directly from the RNA in tissue extracts obtained from CHAPS lysis buffer. Using TRIZOL, 5–10 µg of total RNA were obtained from tissue extracts (60 µg of protein extract). Some rRNA degradation was observed by agarose gel analysis, and in most cases, no 28S and 18S rRNA bands were observed. The same RNAs were also analyzed by reverse transcription (RT)-PCR using β-actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as markers. Almost all cases gave an amplification of β-actin and *GAPDH*, even those in which telomerase activity was undetectable. RT-PCR gave many false-positive results and is therefore not a suitable method to determine tissue quality. In another approach, total RNA was extracted from consecutive tissue sections. Depending on the size of the tissue sample to analyze, 1–5 sections of 10 µM are enough to extract cells in 30 µL of CHAPS lysis buffer for telomerase analysis, whereas 4–10 sections are necessary for extraction of a sufficient amount of RNA (total RNA dissolved in 20 µL water). Using this approach, the results for telomerase activity and rRNA measurements are perfectly superimposable with those obtained with large amount of tissues (see Figure 1, A and B, for rRNA quality). Furthermore, sections for histological analysis can be prepared just before and after the

section necessary for telomerase and RNA analysis. Thus, histological characteristics of the analyzed lesions can be correlated directly to telomerase activity, and false-negative results due to sampling error can be avoided.

These results indicate that if RNA is not strongly or completely degraded (28S and 18S rRNA species not completely missing), more reliable results will be obtained. If RNA is strongly degraded, telomerase activity is not always observed (Table 1). Our results clearly indicate that for a valid TRAP assay, the control of RNA quality is essential to reduce the number of false-negative results. This control is simple and rapid and can be done in less than 2 h.

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