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## Methods Used in Evaluating Telomerase Activity

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Recent studies have shown that the progression of neoplastic lesions is characterised by the reactivation of telomerase, a ribonucleoprotein complex which stabilizes telomere length by adding hexameric repeat (TTAGGG)<sub>n</sub> to telomeric ends of human chromosomes (1). Telomeres are important for maintaining chromosome structure by protecting the chromosomes from DNA degradation, end to end fusions, rearrangement and chromosome loss (2,3). Telomerase activity has been detected in germline cells and most cancer cells, whereas most normal somatic cells have no clearly detectable telomerase activity (4,5). Because of this difference, telomerase activity is considered to be a diagnostic marker of malignancy (6-8).

### Current Methodologies

A number of methods for determining telomerase activity have been developed. The detection of telomerase activity using the conventional assay, which requires a large amount of cell or tissues, has largely been replaced by the telomeric repeat amplification protocol (TRAP) described by Kim et al. (4). Because of several disadvantages, numerous improvements have been made to the original TRAP protocol, making the assay more sensitive and reliable for potential clinical application.

1. The TRAP assay includes preparation of a protein extract by cell lysis and adding a primer and dNTPs. If telomerase is active in the extract, it elongates the added primer, and the reaction product (templates) is amplified by PCR. This technique is highly sensitive but can provide only qualitative (presence/absence) evaluation. For quantitative analysis, the area or intensity of 6bp ladders appearing in an X-ray film must be measured by densitometry with a computer program, but the result

still depends on conditions of quantitative autoradiography that do not easily produce precise linearity and reproducibility (9). Commercial kits (Trap-eze, TRAP-eze-xl, TRAP-eze-Elisa telomerase detection kits from Oncor (Gaithersburg, MD, USA)) give increased sensitivity with decreased sample processing time, allowing improved detection of telomerase activity in a large number of samples (10).

a. Aldous and Grabill (11) have introduced a fluorescent method of detecting telomerase to alleviate the problems including incorporation of radionucleotides detected by autoradiography and the time required to complete the assay. Telomeric repeats are identified in the fluorescent TRAP (F-TRAP) assay by incorporation of fluorescein labeled primers during amplification and subsequent detection with an automated DNA sequencer.

b. Stretch PCR, a modification of TRAP, has been developed for quantitation of telomerase activity (12). The introduction of steps for purification of telomerase products before PCR reaction and the use of specially designed primers that contain unrelated internal sequences of the templates contributes significantly to the quantitative accuracy of the assay. Because the primers used in the TRAP assay tend to anneal to internal sequences of the templates and shorter products are preferentially amplified, the primer (TAG-U and CTA-R) used in stretch PCR eliminates this shortcoming and also eliminates the possibility of obtaining false negative results caused by the presence of PCR inhibitors in the sample.

c. Gelmini et al. (13) presented a modification of the TRAP method that can provide quantitative information without requiring time-consuming post-PCR procedures

such as gel electrophoresis with radioactive materials and autoradiography. The detection and measurement of telomerase activity is performed by evaluating the amount of double-strand DNA generated in the telomerase reaction and PCR amplification; with the use of the fluorescent dye PicoGreen, which selectively binds double strand DNA. The primers used in stretch PCR are also maintained in this modification, and fluorescence is measured in a spectrofluorophotometer.

d. Specific localization of telomerase activity in tumor cells has been demonstrated using in situ PCR modification of the TRAP assay that can be provide to detect the telomerase activity at the cellular level (14).

II. Hirose et al. (15) described a new quantitative and non-radioactive method, transcription mediated amplification (TMA), in conjunction with the hybridization protection assay (HPA), for telomerase activity. TMA is not based on PCR; it uses an isothermal amplification system that can be performed in a heat block or water bath. In the TMA protocol, amplified telomerase products are RNA and these amplifications are detected using a non-isotopic HPA system. HPA uses an acridinium ester-labeled probe that hybridizes to the junction of the primer and telomeric repeat. Quantitative analysis is based the principle of differential hydrolysis of the bound and free probe. The results are obtained easily by chemiluminescence measurement in a luminometer. The TMA/HPA method is faster than TRAP and as sensitive and reproducible as the TRAP assay. In addition, Hirose et al. showed that TMA/HPA is influenced minimally by TRAP inhibitors that may come from clinical samples.

III. Recent studies have focused on analyzing the expression level of telomerase components after the cloning (16) of genes encoding the components of the

human telomerase complex including the telomerase RNA component (hTERC) that acts as a template to add telomeres to the ends of the chromosomes, telomerase reverse transcriptase (hTERT) and telomerase protein component 1 (hTEP1) (17). These improvements have enabled the analysis of mRNA expression as an alternative assessment of telomerase function by using a quantitative reverse transcription (RT)-PCR assay. hTERT-mRNA expression is closely associated with telomerase activity. Analysis of the hTERT transcript may provide an alternative method for determining the telomerase activity (18-21). Expression levels of telomerase components have also been demonstrated using non-radioactive in situ hybridization (22).

a. The Taq Man fluorogenic detection system has been applied to the quantitative RT-PCR assay (real time PCR) by Yajima et al. (23). This method utilizes the 5' exonuclease activity of taq polimerase to cleave a non-extendable dual-labeled fluorogenic hybridization probe during extension that is quantitatively measured by using a combined thermal cycler-fluorescence detector. This system eliminates the time-consuming process after PCR, and is also relatively resistant to carryover contamination of PCR amplification.

IV. Fletcher et al. (24) used a system in which telomerase in intact nuclei catalyses primer extention. Telomerase activity in isotonicly isolated nuclei from human CEM cells shows low processivity (addition of up to four TTAGGG repeats). Examination of telomerase activity in a more natural nuclear environment may shed new light on the telomerase function and provide a useful system for evaluation of new telomerase inhibitors.

Assessment of the clinical utility of activity measurement for prognostic evaluation awaits more

Methods	Source
Original TRAP assay	Kim et al., 1994
Fluorescent-TRAP	Aldous and Grabill, 1997
Stretch-PCR	Tatematsu et al., 1996
Stretch-PCR and PicoGreen	Gelmini et al., 1998
In situ TRAP	Ohyashiki et al., 1997
TMA/HPA	Hirose et al., 1998
RT-PCR	Kyo et al., 1999; Sumida et al., 1999; Harada et al., 2000
Real-time PCR	Yajima et al., 1998
Telomerase in intact nuclei	Fletcher et al., 1999

Table. Methods used in evaluating telomerase activity.

conclusive studies of large numbers of samples with strictly quantitative methods of telomerase analysis or the development of methods.

### Assessment

If the TRAP method should be used for the determination of telomerase activity, the use of stretch-PCR with PicoGreen must be preferred. The primers contain unrelated tag sequences at 5' termini in this method, and so they do not anneal the internal sequences of the telomerase products. Thus the occurrence of shorter products is prevented (13). In addition, there is no time-consuming post-PCR procedure such as gel

electrophoresis. If the RT-PCR method should be used for the determination of telomerase activity, the level of hTERT expression must be chosen because there is a close correlation between hTERT expression and telomerase activity (19,20).

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