Analytical Validation of Telomerase Activity for Cancer Early Detection

TRAP/PCR-CE and hTERT mRNA Quantification Assay for High-Throughput Screening of Tumor Cells

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Activation of telomerase plays a critical role in unlimited proliferation and immortalization of cells. Telomerase activity has been shown to correlate with tumor progression, indicating that tumors expressing this enzyme possess aggressive clinical behavior and that telomerase activity may be a useful biomarker for early detection of cancer. However, measurements of telomerase activity by current methods such as telomeric repeat amplification protocol (TRAP)/polymerase chain reaction (PCR) or antibody-based radioimmunoassay (RIA) are low-throughput and not robust enough to easily accommodate the required statistical analysis to determine whether telomerase activity is a practical biomarker. As part of the National Cancer Institute Early Detection Research Network of analytical validation, we have developed a robot assisted TRAP assay (RAPidTRAP) of telomerase, a potential biomarker for cancer early detection. Measurements of human telomerase reverse transcriptase catalytic subunit (hTERT) mRNA were performed in concert with measurement of telomerase activity. For this purpose we determined hTERT mRNA concentration and telomerase activity in human normal (RPE-28) and cancer (A549) cell lines as well as in human serum (SRM 1951A). Telomerase activity measurements were made using the TRAP/PCR capillary electrophoresis (CE) method on (50 to 1000) cells/reaction isolated from cell extracts. Measurement of hTERT mRNA was made using specific primers and probes on a LightCycler in the range of (10 to 7000) cells/reaction. Comparison of high-throughput telomerase activity measurements using the robot and those performed manually were consistent in sensitivity and reproducibility. Using this combination of

telomerase activity and hTERT mRNA measurements, the automated system improved efficiency over traditional TRAP/PCR methods. (*J Mol Diagn* 2004, 6:157-165)

Telomerase activity has been measured in a wide variety of cancerous and non-cancerous tissues and cell lines.¹⁻⁷ The role of telomerase in cancer has been reviewed.^{2,8} Telomerase has repeatedly been described as an expected clinical biomarker for the diagnosis and prognosis of human cancer.¹⁻² Although *in vitro* and *in vivo* studies have reported the absence of a connection between telomerase and cancer and that telomerase activity occurs in normal regenerative cells,⁷⁻¹⁰ the vast majority of clinical studies show a direct association between telomerase activity and cells that are cancerous.¹⁻⁶ Measurable levels of telomerase have also been observed in body fluids of cancer patients,¹¹⁻¹⁵ including the blood sera¹² and in voided urine after prostatic massage in patients with prostate cancer.¹⁵ Such clinical tests for telomerase may have great utility as non-invasive, cost-effective methods for the early detection and monitoring of cancer. However, its use as a diagnostic biomarker has been hindered because of the lack of high-throughput methods essential for large cohort studies required to statistically validate the association between these biomarkers and cancer. To be effective, telomerase assay methods must be optimized for sensitivity, reliability, and throughput.

Currently, telomerase assays are based on several different methods of measurement. The most commonly used system for the detection and quantification of telomerase enzyme activity is the polymerase chain reaction (PCR)-based assay known as the telomeric repeat amplification protocol (TRAP) assay.¹ Telomerase enzyme

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activity can be quantified by synthesis of a telomere sequence ladder complementary to the RNA template inherent in the enzyme. In the TRAP assay, telomerase synthesizes these extension products, which then serve as templates for PCR amplification. The amount of PCR product (area under all curves of the ladder) is proportional to the telomerase present. Most TRAP assays use slab-gel-based electrophoresis matrixes to size and quantify the PCR products. TRAP-ELISA has been developed, but the technique is limited because of variation in enzyme kinetics and the insufficient amount of sample tissue available for protein analysis.¹⁶

Herein, we developed a high-throughput method of analyzing telomerase activity using a robotic platform and capillary electrophoresis. RAPIDTRAP (robot assisted TRAP) uses fluorescent labels and capillaries instead of slab gels. This is of special importance because commercial available multi-capillary systems, when coupled with robotic sample handling, vastly increase throughput. Previously, the advantages of capillary electrophoresis (CE) using non-automated TRAP/PCR techniques were demonstrated.^{17–18} In the current study, we have extended those findings using automated high-throughput methods. This supports the use of capillary electrophoresis in the development of high-throughput automation of the telomerase assay system and sets the stage for automated systems that will accommodate the requirement for large data sets.

Another method of telomerase measurement is based on the expression of telomerase hTERT mRNA. However, this measurement is only an indicator of the expression level of telomerase mRNA and is not a direct assay for the presence of active telomerase. In this regard, it is important to correlate this measurement with telomerase activity as measured by the TRAP assay.¹⁹ Common methods used to measure mRNA expression include: Northern blotting, *in situ* hybridization, RNase protection assays, and cDNA arrays. Real-time reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive, accurate, and adaptable RNA quantitative technique. Potentially, RNA from a single cell can be detected under ideal conditions.

In this study we use RT-PCR with the Lightcycler to rapidly quantify mRNA levels in cancer and control cell lines and in human serum. We have used these measurements in conjunction with our high-throughput measurements of telomerase activity. This has enabled us to develop a complete and automated system for high-throughput analysis of telomerase, which will be invaluable for clinical validation studies.

Materials and Methods

Materials

Certain commercial equipment, instruments, materials, or companies are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology (NIST),

nor does it imply that the materials or equipment identified are the best available for the purpose.

Enzymes, molecular biology reagents, kits, and chemical reagents were obtained from the following sources: Tris-HCl, MgCl₂, EGTA, PMSF, CHAPS, glycerol (Fisher Scientific, Pittsburg, PA); RNase inhibitors (Promega, Madison, WI); TeloTAGGG kit (Roche Molecular Biochemicals, Indianapolis, IN); PCR buffer, TS primers, *Taq* HotStar polymerase (Qiagen, Hilden, Germany). TRIzol LS Reagent (Invitrogen, Carlsbad, CA) chloroform (Mallinckrodt, Phillipsburg, NJ). Pooled human serum SRM 1951A was obtained from NIST, Gaithersburg, MD.

Cell Culture

Cells were cultured using standard laboratory techniques. Stock cultures of A549 cells (ATCC, Manassas, VA) were grown in ATCC Vitacell F-12K media supplemented with 10% (v/v) fetal bovine serum (FBS), 5.5 ml of Invitrogen penicillin-streptomycin (containing 100 units/ml, and 100 µg/ml, respectively), 2 mmol/L L-glutamine, and 1.5 g/L sodium bicarbonate at 37°C. Stock cultures of RPE-28 (Coriell, Camden, NJ) cells were grown in Dulbecco's modified Eagle's medium (high glucose) with 2 mmol/L L-glutamine supplemented with 15% (v/v) FBS at 37°C. Cells were harvested using a plastic scraper (Fisher Scientific) and subsequently counted using a Fischer 0.100 mm deep hemacytometer. The number of cells harvested by analysis was $\sim 1 \times 10^6$.

Preparation of Total RNA

For the initial characterization of A549 and RPE-28, cells were seeded at a density of 1×10^4 cells/cm² in their respective media and grown to $\sim 70\%$ confluency in a 75-cm² flask. Total cellular RNA was extracted from A549 and RPE 28 cells via modified phenol procedure. The cells were centrifuged and the resulting pellet was resuspended in 750 µl of TRIzol per 5×10^6 cells. Prior cell wash steps were avoided to decrease the possibility of mRNA degradation. The sample was incubated for 5 minutes at room temperature and subsequently treated with 200 µl of chloroform for 5 minutes at room temperature. The mixture was centrifuged at $10,000 \times g$ for 15 minutes and the resultant aqueous phase containing the RNA was transferred to a clean RNase-free tube. The total RNA was precipitated by adding 0.5 ml of isopropyl alcohol and incubated at room temperature for 10 minutes. Following centrifugation, the RNA pellet was washed with 75% ethanol. Total RNA was separated away from the DNA and protein fractions and re-suspended in CHAPS buffer treated with RNA Secure (Promega). The concentration of total RNA was calculated based on OD₂₆₀ measurements as a means to address RNA yield only. The spectrophotometric value for the total RNA was divided by the number of cells used in the extraction to determine the amount of RNA per cell. The resulting range (3 to 9 pg) is within the range (5 to 30 pg) previously reported.²⁰ The coefficient of variation (CV = 71%) was greater for the measurement of total RNA than

for real-time PCR (CV = 2%). The hTERT mRNA amount per cell was taken directly from the real-time PCR results. In subsequent analyses, the protein fraction was isolated (see below) before RNA extraction. Hence, the same sample could be analyzed by RAPIDTRAP and RT-PCR.

Measurement of hTERT mRNA

The number of hTERT RNA molecules was determined by real-time RT-PCR using the LightCycler and LightCycler TeloTAGGG kit from Roche Molecular Biochemicals according to the instructions.²¹ RNA was converted to cDNA and specific gene primers were used to amplify hTERT mRNA as its full-length product. Alternatively spliced variants were not measured because they do not reconstitute telomerase activity.^{22–23} The product was measured during the exponential phase of the reaction. Quantification of the product was obtained by extrapolating the data against a standard curve run in triplicate. An internal control (a mRNA that does not vary in abundance respective to cell type) was included in each analysis. Human porphobilinogen deaminase (PBGD) mRNA was chosen as the housekeeping gene. Real-time PCR was performed with a LightCycler. Briefly, each isolate was normalized with respect to the number of cells harvested. Serial dilutions were made to produce aliquots of various cell concentrations. The RNA was mixed with hTERT reaction buffer and used according to manufacturer's instructions. The protocol for detection of RNA consisted of 1 cycle of 60°C for 600 seconds; denatured at 95°C for 1 second. This step was followed by 40 cycles of denaturing at 95°C for 1 second; annealing at 60°C for 10 seconds; extending at 72°C for 2 seconds, which was then followed by melting curve analysis of 50°C to 95°C for conformity assessment.

Preparation of Telomerase Extracts

All measurements of TRAP and hTERT reflect variations including extraction of total protein and total RNA from different batches of cultured cells and protein/RNA isolations conducted on different days. There is also variation in the individual extractions (tissue sampling, etc.). To determine this variation in tissue extraction a large number of samples need to be analyzed using automated extraction methods. This was not accomplished in the current study because the extraction process is not part of the current robotic platform.

Telomerase was extracted from the human lung carcinoma cell line A549 (ATCC) by lysing cells (10^6) in 100 μ L ice-cold lysis buffer containing 10 mmol/L Tris-HCl pH 7.5, 1 mmol/L MgCl₂, 1 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L β -mercaptoethanol, 0.5% (w/v) CHAPS (Pierce, Rockford, IL), and 10% (v/v) glycerol. The modified CHAPS detergent was treated with RNA Secure according to manufacturer's instructions. After centrifugation, the supernatant was stored at -80°C at a concentration of 500 cells/ μ L. The telomerase preparation method was modified from that previously described.²⁴ Pooled human serum sample

SRM 1951A was freshly thawed and assayed for telomerase activity without further purification.

TRAP Assay System

Amplified products were manually generated by the TRAP assay as previously described using the following protocol.¹⁷

Telomerase Extension Reaction

Two μ L of cell lysate is added to 23 μ L of a solution containing 1X PCR buffer (Qiagen, Valencia, CA), 200 μ mol/L dNTP's (50 μ mol/L each), and 200 ng of the telomerase substrate (TS primer). The solution is incubated at 30°C for 30 minutes.

PCR Amplification Step

To the extension reaction is added 25 μ L of a solution containing 1X PCR buffer, 200 μ mol/L dNTPs, 3.75 U *Taq* polymerase (Hot Star Qiagen), 100 ng of the reverse CX primer and 10 ng of the Internal TRAP Assay Standard (ITAS). PCR for the TRAP assay was carried out in a Perkin Elmer 9600 Thermal Cycler using the following program: 95°C for 15 minutes; 36 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; 4°C hold.

Primer Sequences

CX Primer: 5'-CCCTTACCCTTACCCTTACCCTAA-3,' TS Primer: 5'-AATCCGTCGAGCAGAGTT-3,' The ITAS is a segment of myogenin cDNA tailed with the CX and TS primer sequences. Amplification with these primers yields a 150-bp (bp) product for the ITAS. Fluorescently labeled TRAP PCR products were produced using equivalent amounts of HEX (4,7,2',4',5',7',-hexachloro-6-carboxyfluorescein) 5' end-labeled TS and unlabeled CX primers (PE-Applied Biosystems). The TRAP method was modified from that previously described.^{1,24}

Automated Production of TRAP Products

Command scripts were written using software provided for the MWG RoboSeq SE 4204. The scripts were linked to make one program that performed all steps of the TRAP assay protocol as described above including equivalent 30-minute extension reaction incubations for each sample to be analyzed. An example of the script is available at <http://jmd.amjpathol.org>. The program used solution volumes sufficient for multiple determinations in the 96-well plate format.

CE Measurements

Fluorescently labeled PCR products were prepared for capillary electrophoresis by combining 1 μ L of PCR product with 10.5 μ L deionized formamide, and 0.5 μ L of

Figure 1. Automated system for high-throughput analysis of telomerase activity. The system incorporates a MWG RoboSeq 4204 for automated sample handling of RNA/protein that has been prepared from cancer patients or normal controls. Telomerase activity is determined using a TRAP/PCR that has been modified for robotic handling of samples and reagents. TRAP/PCR products are then analyzed on the ABI 3100 multi-capillary electrophoresis instrument. Samples prepared on the RoboSeq 4204 are also used for rapid, real-time assay of hTERT mRNA analysis using the Luminex or the Light-Cycler. Part A reprinted from the Weill Medical College of Cornell University Education Center's online resource "PathNotes", entitled "Neoplasia. Biological Characteristics of Benign and Malignant Neoplasms" with permission from Robert C. Mellors, M.D., Ph.D.

TAMRA-500-labeled internal size standard (PE/Applied Biosystems). The mixture was heated for 5 minutes at 95°C and chilled on ice. Separations were performed using the PE/Applied Biosystems PRISM Model 310 Genetic Analyzer and the PE/Applied Biosystems GeneScan capillary (41 cm × 50 μm) and POP4 polymer system. Samples were electrokinetically injected (10 seconds, 15 kV) and separated at 5.0 kV at a temperature of 60°C. Data were collected and analyzed using the PE/Applied Biosystems PRISM and GeneScan software, version 2.0.2. The amount of extension product (incorporated primer) was calculated for each sample as the total summed area of HEX-labeled peaks corresponding to the extension products. This was done using Genotyper (PE/Applied Biosystems) and Microsoft Excel as described previously.¹⁷ The total peak area (sum of the TRAP ladder peak areas) has been shown previously to yield an accurate determination of substrate conversion to elongated products which is proportional to enzyme activity.²⁴ The use of an ITAS standard was also shown to help normalize variations in the PCR amplification.²⁴ In addition, the use of a telomerase standard, such as the A549 cell extract, provides a means to normalize individual assay results and describe the level of telomerase activity in cell equivalents relative to a specific cancer cell line.

Results

An Automated System for High-Throughput Screening

Several automated platforms were linked in tandem to provide high-throughput screening (HTS). Automated sample handling, thermocycling, analysis, and data mining were connected in series as shown in Figure 1. The HTS system incorporates a TRAP PCR assay that has been modified for robotic handling of samples and reagents. The robotic platform can set up the TRAP/PCR

assay of clinical samples at a rate of 96 samples/hour that includes the 30-minute incubation required after the first step of the TRAP assay. The 3100 CE can be used to analyze TRAP/PCR products at the rate of 16 runs/hour. The CE data can then be analyzed using GeneScan Analysis Software with output to Excel at the rate of 16 runs/hour. The HTS capabilities of the RoboSeq and rapid capillary electrophoresis of ABI analytical platforms were used in tandem. Subsequent data analysis was performed using GeneScan software. Hence, one network of systems involving three different platforms provided the basis for high-throughput telomerase screening. To this end, we validated the successful detection of telomerase activity for large numbers of analytes with high sensitivity on as few as 50 cells/reaction. The network was validated with samples that covered the range of telomerase activity from 50 to 1000 cell equivalents.

RT-PCR Measurement of hTERT mRNA

In parallel with the automated system for HTS, cells were trypsinized, collected, and counted. The amount of total RNA was estimated based on OD₂₆₀ measurements. The number of hTERT mRNA molecules was determined using serial dilution of each sample based on the number of cell equivalents used in each real-time RT-PCR. Using the LightCycler, the different cell samples were analyzed by rapid, real-time PCR and subsequent melting curve analysis confirmed the amplification of one pure product. Only splice variants that could be translated into functionally active telomerase were detected. RNA molecules from telomerase-positive cells (A549) were detected from 7000 to a lower limit of nine cells. The percent SD (CV %) for all samples ranged from 0.21 to 4.47, which is a variation of about 2 to 40 RNA molecules per 1000. The interassay CV % ranged from 0.10 to 3.19 and the inter-operator CV % ranged from 1.09 to 4.74. This demonstrates the robust nature of the RT-PCR protocol such that different operators can be expected to obtain variation in the range of 10 to 50 RNA molecules per 1000. The reproducibility increased with increasing numbers of cells/reaction with a variation as low as 1 RNA molecule per 1000. This is well within the criteria for differentiating between cancer and normal cells/tissue.

A typical example of the results is shown in Figure 2. The detection curves correspond to the mRNA copy number. Linearity of the signal was sustained over a range of (9 to 100,000) cells/reaction. The averaged copy number extrapolation decreased from (629 to 5) copies of hTERT mRNA. Negative controls (RPE-28) were analyzed at concentrations ranging from (100 to 1 × 10⁶) cells/ml using the same primer sets as those used on A549 samples. Internal controls verified the assay was optimized with no detectable hTERT mRNA for over 50 cycles at all concentrations for RPE-28. Likewise, NIST Standard Reference Material 1951A, a pre-existing serum standard, was also analyzed at concentrations ranging from freshly thawed undiluted serum to 10⁶ diluted aliquots. After more than 50 cycles of amplification, no hTERT mRNA was detected. To verify that the assay

Figure 2. Real-time amplification and probe-specific detection of hTERT RNA using the LightCycler. The y-axis represents the relative ratio of fluorescence between the reference channel and the sample channel. Curves labeled 1 to 11 represent dilutions of extracted total RNA from fresh cultured A549 cells. For clarity, sample triplicates are not shown. Curve 1, RNA detection from 7000 cells; curve 2, RNA detection from 3500 cells; curve 3, RNA detection from 1750 cells; curve 4, RNA detection from 875 cells; curve 5, detection from 219 cells; curve 6, detection from 55 cells; curve 7, detection from 175 cells; curve 8, detection from 110 cells; curve 9, detection from 70 cells; curve 10, detection from 9 cells; and curve 11, results of the no template control. Each 6.6 cycle numbers represents a 100-fold difference in concentration of PCR product.

reaction mix was optimized, A549 samples were analyzed using the same reaction mix and positive hTERT signal was observed, as expected. No signal was obtained from SRM1951A or from RPE 28 over a concentration range of (9 to 100,000) cells/reaction.

The intra-run and inter-run variation of the hTERT assays was determined by comparing increasing concentrations of mRNA/reaction. The superimposed amplification curves showed an assay variance (CV = 3.1%) when analyzing 1000 copies or less. Copy numbers were calculated from external standard curves that were run in triplicate. Measurements of hTERT mRNA were reproducible with copy numbers less than one per cell. This agrees with reports about other cell lines¹⁹ that harbored less than one copy of functionally active hTERT mRNA per cell. The difference between the samples containing 7000 cells (curve 1) and 70 cells (curve 9), a 100-fold change, is 6.4 cycle numbers (~ 6.6 cycle numbers) due to PCR inefficiency.

In Figure 3, A and B, real-time data from cancer cell lines is displayed. Figure 3A shows the results from different replicates and their reported crossover threshold (Ct) values. The detection of specific RNA was identical at 7000 cells. Even lower concentrations produced reliable data, however, real-time PCR exhibited increased variation when the concentration of cells was below 1×10^3 . Figure 3B shows the detection of RNA from 110 cells. Every 3.3 Cts represents a 10-fold difference in sample concentration. Both Figure 3A and Figure 3B have low Ct variation (CV = 1.4%).

RapidTRAP Analysis of Telomerase Activity

In a previous study, it was shown how capillary electrophoresis could be used to analyze TRAP/PCR products

Figure 3. Real-time data from cancer cell lines. The y-axis represents the relative ratio of fluorescence between the reference channel and the sample channel. **A** and **B**: Typical results from different replicates (R1 and R2) and their crossover threshold (Ct) values. **A**: Quantification of mRNA from 7000 cell equivalents. **B**: Quantification of mRNA from 110 cell equivalents. A549 cell cultures were treated with the same isolation methods and analyzed for their abundance of hTERT mRNA. Ct values were compared for each experiment. As shown, precision in measurement is best at high cell equivalents/reaction (**A**). For practical purposes, cell concentrations between 110 and 9 cells were not significantly different.

with higher reproducibility than the slab-gel-based methods that are reported to have a coefficient of variation as high as 35%.¹⁷⁻¹⁸ The RoboSeq 4204 robotic platform was used for the telomerase activity analysis to automate the TRAP/PCR procedure and to ensure low systematic error. Up to 80 samples per plate or 40 samples in replicate can be processed per run using this method. Figure 4A shows capillary electrophoresis of TRAP/PCR products generated using the MWG robot (RAPID-TRAP). The TRAP/PCR assay was performed using fluorescent-labeled TS primer and analyzed on the Applied Biosystems 310 CE as previously described.¹⁷ Electropherograms were produced from separate TRAP/PCR reactions using increasing concentrations of telomerase (A549 cell extract). The extension products range in size from 40 bp to about 200 bp in 6-bp intervals as expected.

CE Analysis of SRM 1951A is shown in Figure 4B. Electropherograms from TRAP/PCR analysis of SRM 1951A (undiluted) are shown in comparison to the blank (no telomerase) and the positive control (A549 cells). Primer and primer-dimer peaks appear in the range of (40 to 70) bp for all measurements including the blank. SRM 1951A exhibits no telomerase activity as evidenced by the absence of extension products in the range of (75 to 190) bp.

Figure 5 is a plot that compares the TRAP/PCR titration data obtained using the MWR robot to titration data obtained manually. The total extension product peak areas were calculated from the electropherograms shown in Figure 4 using Genotyper and Excel (see Materials and Methods). Data obtained from manual TRAP/PCR is shown for comparison. The data from both the robot and the manual method could be fit by a single titration curve

Figure 4. Capillary electrophoresis of TRAP/PCR products generated using the MWG robot. The TRAP/PCR assay was performed using fluorescently-labeled TS primer as described previously for the manual method and analyzed on the Applied Biosystems 310 CE. **A:** Electropherograms produced from separate TRAP/PCR reactions using increasing concentrations of telomerase (A549 cell extract). The extension products range from about 40 bp to about 200 bp with 6 bp repeats as previously described.¹⁷ In our capillary electrophoresis method we have used the fluorescently labeled TS primer. The traditional slab-gel method instead uses a non-covalent stain such as Sybr Green I. The non-covalent dye does not bind the primer-dimer with enough affinity to produce the high intensity primer-dimer bands/peaks which appear in the CE method using the covalent-labeled primer. We correct the total peak area of the TRAP ladder peaks by subtracting or not including the contribution of these primer-dimer peaks. **B:** TRAP/PCR CE analysis of SRM 1951A. Electropherograms from SRM 1951A are shown in comparison to the blank (no telomerase) and the positive control (A549 cells). SRM 1951A (undiluted) shows no telomerase activity as evidenced by the absence of extension products.

within the SD (error bars) estimated from repeatability measurements of each method. The error is in the expected range (CV = 20%) for individual TRAP/PCR reactions. This showed that the two methods were comparable in sensitivity and variability.

The relationship between the telomerase activity and the amount of cellular hTERT mRNA is shown in Figure 6. The plot shows the amount of cellular hTERT mRNA measured in the A549 cells in comparison to the telomerase activity obtained from automated TRAP/PCR measurements. Both measurements show an increase with cell equivalents, as expected. The ratio of telomerase (total peak area) and mRNA (copy number) when normalized

Figure 5. Plot of the total extension product peak areas as a function of telomerase concentration. Areas were calculated from the electropherograms shown in Figure 4A (filled symbols). Data obtained from manual TRAP/PCR (open symbols) is shown for comparison. Error bars (± 1 SD) are based on repeatability measurements for each method. Analysis was performed using GenoTyper and Excel as previously described.¹⁷

in terms of cell equivalents is expected to vary with different cell types. In addition, if the structure of the telomerase has been modified by genetic mutation, the inherent activity and this ratio would be expected to vary. However, the release of a reference standard (SRM) containing both the A549 cell extract and total RNA would normalize such data.

Discussion

The Early Detection Research Network (EDRN) is a scientific consortium for discovering and validating biomarkers for cancer early detection.^{25–26} It consists of 18 Biomarker Discovering Laboratories (BDLs), nine Clinical and Epidemiological Centers (CECs), three Biomarkers Validation Laboratories (BVLs), and a Data Management and Coordinating Center (DMCC). One of these BVLs has been established between the National Cancer Institute (NCI) and the National Institute of Standard and Technology (NIST) for performing and standardizing analytical validation of biomarkers and for developing high-throughput assays/technologies.²⁷ The research programs are divided into three integrated components: 1) the discovery of biomarkers; 2) the validation of methods and biomarkers; 3) and testing biomarkers as diagnostic assays for preclinical and early-stage disease. Herein, we have identified a promising direction for biomarker development (component 2), the validation of methods for telomerase activity and human telomerase reverse transcriptase catalytic subunit (hTERT) mRNA. To achieve these goals, we have developed a high-throughput assay for telomerase activity as the first step toward clinical validation and evaluation of telomerase and hTERT mRNA as biomarkers for cancer early detection.

Clinical decisions are highly dependent on the precision of laboratory diagnostic data. The role of the National Institute of Standards and Technology in biomarker validation for early detection of cancer differs from that of research laboratories.^{25–27} Diagnostic assay systems are systematically evaluated with the goal of developing a standardized, reproducible, high-throughput format for

Figure 6. Comparison of hTERT mRNA and telomerase activity. The relationship between the relative telomerase activity (**bars**) and the amount of cellular hTERT mRNA (**filled symbols**). The amount of cellular hTERT mRNA (copy number) was measured in the A549 cells in comparison to the telomerase activity (total peak area) obtained from automated TRAP/PCR measurements (Figure 5).

clinical applications. Once laboratory performance is established, traceable standards are developed as standard reference materials (SRMs) to help define clinical applications.

In this study, we determined the relationship between telomerase activity and message abundance. For this purpose we measured the number of molecules of hTERT RNA in SRM 1951A (pooled human serum), A549 (human cancer cell line), and RPE-28 (human cancer negative-control cell line). The analysis was reproducible among different users of the protocol. We were able to detect hTERT transcripts with as few as nine cell equivalents. The variation (CV) ranged from 3% at 110 cell equivalents to only 1% at 7000 cell equivalents. SRM 1951A contained no detectable hTERT transcripts. The standard reference material SRM1951A is available through NIST for use as quality control to normalize data across different technological platforms, so that data can be readily shared and easily compared between different diagnostic laboratories.

More importantly, we successfully transferred the TRAP assay from the manual procedure to a robotic platform, with results that were consistent in sensitivity and reproducibility with previous methods using slab-gel and single capillary platforms. Analysis by RAPidTRAP requires only a few cells to measure telomerase activity.

Table 1. Comparison of Slab-Gel, CE, and Rapid-TRAP Analysis of Telomerase

	Slab-gel	CE	RAPidTRAP
Number of samples per run	16	16	96
Time (hours)			
TRAP/PCR	4	4	2
Electrophoresis	4	2	2
Total	8	6	4
Precision (CV%)			
TRAP/PCR	15*	15*	15
Electrophoresis	20*	5*	5
Total	35*	20*	20
Cost reagents (\$)/sample [†]			
TRAP/PCR	4	4	4
Electrophoresis	2	1	0.5
Total	6	5	4.5
Cost labor (\$)/sample [‡]			
TRAP/PCR	10	10	1
Electrophoresis	10	5	1
Total	20	15	2
Total cost (\$)/sample [§]	26	20	6.5

*Based on published data.¹⁸

[†]Estimated cost, in US dollars, based on time per run and number of samples per run.

[‡]Estimated cost based on time per run, number of samples per run and labor at rate of \$40/hour.

[§]Estimated total cost based on sum of reagent and labor costs per sample.

For both the manual and the robotic platforms, telomerase activity was observed with as few as 50 cells (A549). Hence, small volumes of cells, collected with minimally invasive techniques from cancers such as bladder, breast, colon, skin, and prostate, could have immediate diagnostic utility. In addition, the NIST SRM 1951A was evaluated for telomerase activity by RAPidTRAP and had no detectable activity. This material is being developed for a negative control in this assay.

The process of biomarker validation requires systematic phases.^{25–26} Standardization and analytical validation of potential markers, combined with a high-throughput system for making these measurements, is particularly important in the early phases of clinical assay development. We have developed the working model of high-throughput assay for telomerase activity in human tumor and normal cell controls and human serum, which provides a technology for further clinical validation of telomerase. Large-scale validation studies are required before telomerase activity becomes a practical biomarker for use in clinical decisions regarding patient management. Such large-scale studies will only be possible using automated systems with lower cost. In Table 1 the cost of slab-gel, CE, and RAPidTRAP are compared. The table illustrates the advantages of the RAPidTRAP method. The use of RAPidTRAP results in at least a fourfold improvement in throughput and cost. If preliminary clinical validation shows promising results, the validation of telomerase with standard assays among multi-site trials will confirm the utility of telomerase activity and/or hTERT mRNA as biomarkers for cancer early detection.

Activation of telomerase plays a critical role in immortalization of cells and unlimited proliferation. However, much remains to be understood about its role during

tumorigenesis. Our combination of RAPid TRAP and real-time RCR methods helps answer these questions. The catalytic protein (hTERT) is absent in telomerase-repressed cells. Repression of telomerase is at the level of transcription, but post-transcriptional and assembly processes are likely cofactors which regulate telomerase activity in telomerase-positive cells. However, there are only a few studies concerning the relative levels of hTERT RNA in different cancer cells.¹⁵ Surprisingly, the number of full-length hTERT mRNA molecules was less than one per cell. Similarly, telomerase-positive cell lines have been reported to harbor between 1 molecule/2 cells to 1 molecule/6 cells.¹⁵ These results are consistent with RAPidTRAP analyses where a correlation was observed between hTERT mRNA and telomerase activity. Since real-time RT-PCR methods are very sensitive and efficient they can be used to advantage in measuring the predicted amount of telomerase activity. The ratio of telomerase activity to hTERT abundance is expected to vary between different tissue types. This ratio is a measure of the status of telomerase in the cell and at low telomerase levels, helps to confirm its presence. The extent to which this ratio is important in cancer diagnostics has yet to be determined.

Telomerase activity has been shown to correlate with poor clinical outcome in late-stage disease, whereas few studies have been conducted in early-stage disease.⁷ In such circumstances, telomerase activity may be used to identify patients with poor prognosis and to select patients with early-stage cancer(s) who might benefit from adjuvant treatment.⁷ However, the therapeutic target would not be telomerase because of the long lag between cancer phenotype and loss of telomerase activity. Hence, telomerase remains a controversial cancer biomarker and further studies necessitate proper evaluation of its usefulness via a HTS method for early detection.

It is widely anticipated that early detection increases the success of treatment and survival.²⁹ Obviously, the earliest known events in cancer are at the chromosomal level.³⁰ Understanding the cancer mechanism at the molecular level could result in a new generation of rational design cancer drugs. Unfortunately, the relationship between the mutation and resultant gene expression and its association with cancer etiology are unclear.⁷ Biomarkers of somatic mutations such as telomerase remain contentious early detection indicators for patients with stage I or II cancers. One study reported telomerase activity measurements in lung cancer patients to show an average 40% detection rate in stage II to IV cancers but was undetectable in stage I.³¹ Standardized sample collection, processing, storage, and automated detection systems are needed to conduct the large-scale studies necessary to make correct associations between biomarkers and disease.³² One component of automated detection systems involves the use of robot-assisted TRAP-CE (RAPidTRAP) and real-time PCR. Although telomerase shows great promise as a biomarker for cancer detection, its use has been associated with a number of potential limitations and problems. The initial TRAP assays were sensitive enough to detect telomerase, but the results were qualitative and analysis in cancers (GI tissues)

have led to conflicting results.³³ These include 1) Different patterns of gene expression occur in different tissues. Therefore, it is critical to determine which cancers possess early robust telomerase activity and which manifest little to no telomerase activity even in the most advanced malignant tumors by performing large-scale studies facilitated by automated assay systems. 2) The observed telomerase activity will vary depending on the method of sample collection, the sample handling, and the assay system used. 3) Statistical analysis, using large numbers of clinical samples, will be required before FDA approval certifies these methods.

Our results show that standardized automation for early detection biomarkers is possible with a small number of cells. Subsequent large-scale validations, made possible by high-throughput systems, such as described, are important to explore the relevance of these biomarkers to cancer and disease course. Our system provides the means to address the quantitative relationship between telomerase expression and activity in a HTS method. This will indicate whether changes in expression/activity occur during tumor progression and determine at what time point the progression can be detected. Hence, it will be possible to confirm the usefulness of these biomarkers for early detection, prognosis, and in rational drug design for individual clinical treatment.³⁴

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