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Preparation and Characterization of Candidate Reference Materials for Telomerase Assays

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Background: **Telomerase has been measured in body fluids of cancer patients, and clinical tests for telomerase may have utility as noninvasive, cost-effective methods for the early detection of cancer. However, telomerase activity measured by common methods such as the telomerase repeat amplification protocol (TRAP) and telomerase reverse transcriptase catalytic subunit (hTERT) mRNA by reverse transcription-PCR (RT-PCR) varies among laboratories.**

Methods: **We prepared a CHAPS buffer lysate from cultured A549 cells and stored it at −80 °C. Telomerase activity was measured by TRAP/PCR and real-time TRAP/PCR in conjunction with RT-PCR measurements of hTERT mRNA. Activity measured with use of the robot-assisted TRAP (RApidTRAP) multicapillary electrophoresis system was compared with single-capillary and slab-gel measurements in the range 10 to 10 000 cell equivalents.**

Results: **Preparations made after flash freezing and sonication of cells were ∼3-fold more active. Although the slab-gel and capillary instruments detected telomerase activity, the multicapillary instrument was better suited for high-throughput studies. Measurements of telomerase by TRAP/real-time PCR and hTERT mRNA/RT-PCR yielded reproducible titrations in the range 10 to 10 000 cell equivalents (CVs, 1%–8% and 1%–3%, respectively).**

Conclusions: **We have prepared and characterized a candidate reference material that appears to be suitable for use in a wide range of assays of telomerase activity and expression.**

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Cells lose telomeric repeats as they divide in the absence of telomerase *(1)*. Eventually, telomeres become dysfunctional and cause chromosomal instability that drives the multiple genetic changes required for the formation of a carcinoma *(2–4)*. Telomerase activity is a critical factor that increases the replicative potential of tumors and is the most common biomarker of cancer, characterized in ∼90% of all types of cancer *(5)*.

Despite the potential clinical utility of measuring telomerase activity in tumors and body fluids, there is no generally accepted clinical diagnostic method [for a review, see Ref. *(6)*]. Until recently, telomerase protocols have been low throughput and not robust enough to accommodate the statistical power required to validate telomerase activity as a practical cancer biomarker. Moreover, there is currently no consensus on a method for measuring or quantifying telomerase activity. Development of reference materials reflecting the extent of telomerase activity specific to the stages of cancer progression compared with the activity in benign and healthy tissue would facilitate reliable telomerase activity quantification by ensuring that measurement systems maintain precision and limits of detection. Reference materials would also help determine the clinical relevance of measurements of telomerase activity.

The Early Detection Research Network, a scientific consortium for discovering and validating biomarkers for cancer early detection *(7, 8)*, includes 5 biomarker validation laboratories. One of these laboratories has been established between the National Cancer Institute and the National Institute of Standards and Technology for performing and standardizing analytical validation of biomarkers and for developing high-throughput assays and technologies *(9)*. The Early Detection Research Network and the National Institute of Standards and Technology are developing biomarkers and telomerase reference materials that could fulfill needs in the clinical diagnostic community *(7, 9–12)*. An ideal reference material would function across multiple cancer diagnostic platforms to permit normalized and accurate assessment of tumor burden.

Currently, telomerase assays are based on several different measurement technologies. The most commonly

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used system for the detection and quantification of telomerase enzyme activity is the PCR-based telomeric repeat amplification protocol (TRAP)[3] assay *(13)*. Telomerase enzyme 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, determined by the areas under all curves of the ladder, is proportional to the telomerase activity present. Most TRAP assays use slab-gel electrophoresis to size and semiquantify the PCR products *(13, 14)*.

High-throughput measurements are necessary to accommodate potential population screening and to obtain the statistical data required to validate the association of telomerase activity and cancer. However, even with automation, the TRAP assay remains more cumbersome than other detection methods. To help resolve this issue, we have developed a real-time TRAP assay for telomerase activity. This 1-step system reduces the time required for sample analysis and simplifies the data analysis to a single score (if telomerase positive). We have modified the RApidTRAP high-throughput platform for use in real-time technologies such as the Applied Biosystems TaqMan and the Bio-Rad iCycler. Another widely applied method of telomerase measurement is based on the amount of telomerase reverse transcriptase catalytic subunit (hTERT) mRNA; however, this measurement is only an indicator of the expression of telomerase mRNA and is not a direct assay for active telomerase. It is therefore important to correlate this measurement with telomerase activity as measured by the TRAP assay *(15)*. In this study we use reverse transcription (RT)-PCR with the LightCycler to quantify telomerase mRNA. The use of these different methods of analysis in both a high-throughput and non-automated format could ensure maximum flexibility in a wide range of applications.

Materials and Methods[4]

MATERIALS

Enzymes, molecular biology reagents, reagent sets, and chemical reagents were obtained from the following sources: Tris-HCl and CHAPS (Sigma); $MgCl_2$, EGTA, and glycerol (Fisher Scientific); phenylmethylsulfonyl fluoride and 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem); RNA Secure RNase inhibitor (Promega); TeloTAGGG Kit (Roche Molecular Biochemicals); PCR buffer and Taq HotStar polymerase (Qiagen); TRIzol LS Reagent and 2′-deoxynucleoside 5′-triphosphates (dNTPs; Invitrogen); chloroform (Mallinckrodt). Pooled human serum SRM 1951A was from NIST.

CELL CULTURE

Cells were cultured by standard laboratory techniques *(10)*. Stock cultures of A549 cells (ATCC) were grown at 37 °C in ATCC Vitacell F-12K medium supplemented with 100 mL/L fetal bovine serum, 10 mL/L penicillin–streptomycin (containing 100 units/mL and 100 μg/mL, respectively; Invitrogen), 2 mmol/ L-glutamine, and 1.5 g/L sodium bicarbonate. Stock cultures of RPE-28 cells (Coriell) were grown at 37 °C in DMEM (high glucose) containing 2 mmol/L L-glutamine and supplemented with 150 mL/L fetal bovine serum. Cells were harvested by use of a plastic scraper (Fisher) and subsequently counted in a Fischer 0.100-mm–deep hemocytometer.

PREPARATION OF TELOMERASE EXTRACTS

We extracted telomerase from the various cell cultures of human lung carcinoma cell line A549 by lysing cells (3×10^7) in 2.0 mL of ice-cold lysis buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L β-mercaptoethanol, 5 g/L CHAPS, and 100 mL/L glycerol. The modified CHAPS detergent was treated with RNA Secure according to the manufacturer's instructions. After centrifugation, the supernatant was stored at −80 °C at concentrations of 2×10^{10} cells/L. This CHAPS preparation method was modified from that described previously *(14)*.

We also extracted telomerase from the A549 cells by lysing 2×10^7 cells (cells were centrifuged for 5 min at 250*g*, the supernatant was discarded, and the cell pellet was flash-frozen and sonicated for 2–5 s on ice) and resuspending the lysed cells in 1.0 mL of heat-inactivated plasma (SRM 1951A). We have shown that this serum SRM does not contain telomerase activity *(10)*; in addition, it was heat inactivated for this purpose under conditions that have previously been shown to inactivate telomerase activity *(12)*. After centrifugation at 10 000*g* for 5 min at 4 °C, the top 500 μL of the supernatant was removed and added to 4.5 mL of ice-cold lysis buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 5 mmol/L β-mercaptoethanol, 0.2 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 g/L CHAPS, 100 mL/L glycerol, and RNase inhibitor (RNA Secure). The mixture (10^9 cells/L) was divided into aliquots, immediately flash frozen, and stored at −80 °C. This previously described method of telomerase extraction, using sonication, yielded ~3-fold higher activity than use of the CHAPS method without sonication *(12)*.

PREPARATION OF TOTAL RNA

A549 cells were seeded at a density of 1×10^4 cells/cm^2 in their respective media and grown to ~60% confluency in

[3] Nonstandard abbreviations: TRAP, telomeric repeat amplification protocol; hTERT, telomerase reverse transcriptase catalytic subunit; RT-PCR, reverse transcription-PCR; dNTP, deoxynucleoside triphosphate; HEX, 4,7,2′,4′,5′,7′,-hexachloro-6-carboxyfluorescein; FAM, 6-carboxyfluorescein; TAM, *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine; and Ct, detection cycle.

[4] Certain commercial equipment, instruments, materials, or companies are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are the best available for the purpose.

a 75-cm^2 flask. Total cellular RNA was extracted from A549 cells by a modified phenol procedure. The cells were centrifuged, and the resulting pellet was resuspended in TRIzol (750 μL per 5×10^6 cells). Previous cell wash steps were avoided to decrease the possibility of mRNA degradation. The sample was incubated for 5 min at room temperature and subsequently treated with 200 μL of chloroform for 5 min at room temperature. The mixture was centrifuged at 10 000*g* for 15 min, and the resulting aqueous phase containing the RNA was transferred to a clean RNase-free tube. The total RNA was precipitated by addition of 0.5 mL of isopropyl alcohol and incubation at room temperature for 10 min. After centrifugation, the RNA pellet was washed with 750 mL/L ethanol. Total RNA was separated from the DNA and protein fractions and resuspended in CHAPS buffer treated with RNA Secure. The concentration of total RNA was calculated based on A_{260} 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 (3–9 pg) is within the range (5–30 pg) reported previously *(16)*. The CV (71%) was greater for the measurement of total RNA than for real-time PCR (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.

THE TRAP ASSAY SYSTEM

Amplified products were generated by the TRAP assay, as described previously *(10–12)*, by use of the following protocol:

Telomerase extension reaction. Cell lysate (2 μL) was added to 23 μL of a solution containing 1× PCR buffer, 200 μmol/L dNTPs (50 μmol/L each), and 200 ng of the telomerase substrate (TS primer). The solution was incubated at 30 °C for 30 min.

PCR amplification step. To the extension reaction we added 25 μL of a solution containing 1× PCR buffer, 200 μmol/L dNTPs, 3.75 U of Taq polymerase, and 100 ng of the reverse CX primer. PCR for the TRAP assay was carried out in a Perkin-Elmer 9600 Thermal Cycler using the following program: 95 °C for 15 min; 36 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min; 4 °C hold.

Primer sequences. The primer sequences were as follows: CX primer, 5′-CCCTTACCCTTACCCTTACCCTAA-3′; TS primer, 5′-AATCCGTCGAGCAGAGTT-3′. Fluorescently labeled TRAP PCR products were produced by use of equivalent amounts of 4,7,2′,4′,5′,7′,-hexachloro-6-carboxyfluorescein (HEX) 5′-end-labeled TS and unlabeled CX primers (PE/Applied Biosystems). This TRAP method was modified from that described previously *(13, 14, 17)*.

CAPILLARY ELECTROPHORESIS MEASUREMENTS

Fluorescently labeled PCR products were prepared *(10–12)* for capillary electrophoresis by combining 1 μL of PCR product with 10.5 μL of deionized formamide and 0.5 μL of ROX-500–labeled internal size standard (PE/Applied Biosystems). The mixture was heated for 5 min at 95 °C and chilled on ice. Separations were performed with the PE/Applied Biosystems Model 3100 multicapillary and the PE/Applied Biosystems Model 310 single-capillary instruments. The Model 3100 measurements were performed with the PE/Applied Biosystems GeneScan capillary array (50 cm × 50 μm) and the POP4 polymer system. The Model 310 measurements were performed with the PE/Applied Biosystems GeneScan capillary (41 cm × 50 μm) and POP4 polymer system. Samples were electrokinetically injected (10 s at 15 kV) and separated at 5.0 kV at a temperature of 60 °C. Data were collected and analyzed by the PE/Applied Biosystems PRISM and GeneScan software (Vers. 2.02 and 3.7, respectively). 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. The means of 8 separate analyses were plotted to determine the SD over the dynamic range by use of Genotyper (PE/Applied Biosystems) and Microsoft Excel software as described previously *(10–12)*. 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/extension products that is proportional to enzyme activity *(14, 17)*. The use of a telomerase assay internal standard was also shown to help normalize variations in the PCR amplification *(14)*.

SLAB-GEL MEASUREMENTS

PCR products were analyzed on a Hoefer SE-600 slab electrophoresis system (Hoefer). Samples (8 μL) including size markers were loaded in a NuPAGE (100 g/L) Bis-Tris polyacrylamide mini gel (Invitrogen) and run according to the manufacturer's recommendations. Gels were run at room temperature for 2 h at a constant voltage of 100 V (7 V/cm, ~50 mA). SYBR Gold (Molecular Probes)–stained gels of unlabeled products were visualized by a charge-coupled device imaging system equipped with a UVP Transilluminator and Alpha Innotech MultiImaging light cabinet (I-Cube).

MEASUREMENT OF hTERT mRNA

The number of hTERT RNA molecules was determined by real-time RT-PCR using the LightCycler and LightCycler TeloTAGGG Kit (Roche Molecular Biochemicals) according to the instructions *(18)*. Alternatively spliced variants were not measured because they do not reconstitute telomerase activity *(19, 20)*. The product was measured during the exponential phase of the reaction. We quantified the product by extrapolating the data against a calibration curve run in triplicate. Human porphobilinogen deaminase (*PBGD*) was used as the housekeeping gene.

Real-time PCR was also performed with a LightCycler. Briefly, each isolate was normalized with respect to the number of cells harvested. Serial dilutions were made to produce various cell concentrations. The RNA was mixed with hTERT reaction buffer and used according to the manufacturer's instructions. The protocol for detection of RNA consisted of 1 cycle of 60 °C for 600 s and 95 °C for 1 s. This was followed by 40 cycles of 95 °C for 1 s, 60 °C for 10 s, and 72 °C for 2 s. This sequence was followed by melting curve analysis at 50–95 °C.

REAL-TIME TRAP ASSAY

TRAP extension reaction. Cell lysate (1 μL) was added to 23 μL of a solution containing 1× PCR buffer (Qiagen), 200 μmol/L dNTPs (50 μmol/L each), and 200 ng of the telomerase substrate (TS primer). The solution is incubated at 30 °C for 30 min.

Real-time PCR. Real-time PCR was performed after the first step of the TRAP extension reaction *(21)*. To the extension reaction we added 5.6 μL of a solution containing 5 μL of IQ Supermix (PCR buffer, 200 μmol/L dNTPs, and 3.75 U of Taq polymerase; Bio-Rad laboratories), 0.1 μL (100 ng) of the reverse CX primer, and 0.5 μL of 30 μmol/L TaqMan 6-carboxyfluorescein/tetramethyl-6-carboxyrhodamine (FAM/TAM)-labeled probe. Fluorescence was detected in real time by the Bio-Rad iCycler iQ Real time PCR Detection System (Bio-Rad Laboratories) using the following program: 95 °C for 2.5 min; 50 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 4 °C hold.

Primer and probe sequences. The TaqMan primers and probes were synthesized by MWG Biotech AG: TS, 5′-AATCCGTCGAGCAGAGTT-3′; CX, 5′-CCCTTACCCTTACCCTTACCCTTA-3′; TaqMan probe, FAM-5′-CCCTTACCCTTACCCTTA-3′-TAM.

Results

RApidTRAP ANALYSIS OF TELOMERASE ACTIVITY: COMPARISON WITH SLAB-GEL AND SINGLE-CAPILLARY MEASUREMENTS

The RApidTRAP system is designed for automated sample handling of prepared samples from cancer patients or healthy controls *(10)*. The MWG RoboSeq 4204 robotic platform can set up the TRAP/PCR assay of clinical samples at a rate of 96 samples/h, which includes the 30-min incubation required after the first step of the TRAP assay. The Applied Biosystems Model 3100 multicapillary (16 capillary) instrument with GeneScan Analysis Software can be used to analyze TRAP/PCR products at the rate of 16 samples (injections)/h.

Capillary electrophoresis results for the TRAP/PCR products generated by the MWG robot (RApidTRAP) are shown in Fig. 1A. The TRAP/PCR assay was performed with fluorescently labeled TS primer and analyzed on the Model 3100 multicapillary instrument. Electropherograms were produced from separate TRAP/PCR reactions using increasing concentrations of the candidate reference material (A549 cell extract using CHAPS method). The extension products ranged in size from ~40 bp to ~140 bp in 6-bp intervals as expected *(10)*. Fig. 1B shows comparable electropherograms of the same TRAP/PCR products described above but separated by use of the Applied Biosystems Model 310 single-capillary instrument. The lower number of extension products, which ranged in size from ~40 bp to ~100 bp in 6-bp intervals, indicates a lower instrument sensitivity than that observed with the Model 3100. Electrophoretic separation of the TRAP/PCR products was also performed in parallel with 10% acrylamide slab gels. The gels produced the characteristic TRAP ladder with the usual variations in the number of bands and band intensities attributable to changes in sample loading, gel composition, and staining, as described previously *(11, 12)* (data not shown). The slab-gel analysis also required a much larger amount of sample for each PCR product (~20%) compared with capillary analysis, which used only 0.01%.[5]

The data from 8 separate TRAP/PCR titrations, measured as shown in Fig. 1A, are plotted in Fig. 2. The total peak areas of the TRAP extension products for each concentration of the candidate reference material are plotted as a function of cell equivalents. These peak areas were calculated from the electropherograms by Genotyper and Excel programs (see the *Materials and Methods* section). The titrations are similar to those described previously, using the Model 310 single-capillary instrument, except that the present measurements were obtained over a much wider range of telomerase concentrations *(10)*. The inset in Fig. 2 shows the titration at a one-tenth concentration scale. In this range of 10–1000 cell equivalents, the interassay CV was ≤20%, with linearity as observed previously with the Model 310 *(10)*. In the range 1000–10 000 cell equivalents, the interassay CV was also ≤20%, but the results exhibited reduced linearity. These results show that the candidate reference material can be reliably used with the multicapillary instrument in a wide range of telomerase activity, which is important in the analysis of clinical cancer samples *(12)*.

RT-PCR MEASUREMENT OF hTERT mRNA

In parallel with the RApidTRAP measurements, cells were trypsinized, collected, and counted for measurement of hTERT mRNA. The amount of total RNA was estimated based on A_{260} measurements. The number of hTERT mRNA molecules was determined by use of serial

[5] In our capillary electrophoresis method, we used the fluorescently labeled TS primer. The traditional slab-gel method instead uses a noncovalent stain such as SYBR Green I. The noncovalent dye does not bind the primer-dimer with sufficient affinity to produce the high-intensity primer-dimer bands/peaks that appear in the capillary electrophoresis method using the covalent labeled primer. We corrected the total peak area of the TRAP ladder peaks by subtracting or not including the contribution of these primer-dimer peaks.

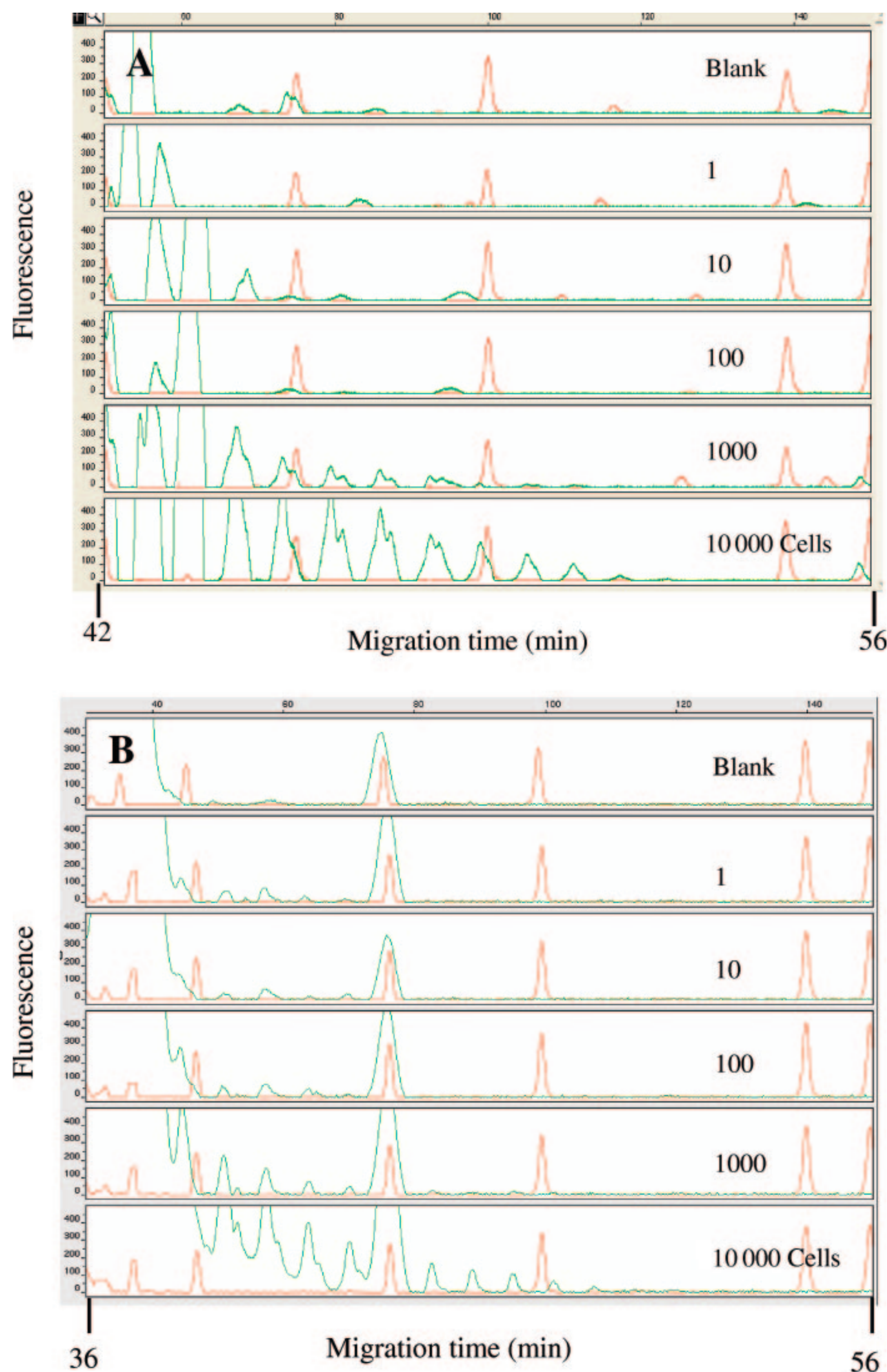

Fig. 1. Comparison of capillary electrophoresis of TRAP/PCR products generated by use of the MWG robot.

The TRAP/PCR assay was performed with fluorescently labeled TS primer as described previously. (*A*), electropherograms produced from separate TRAP/PCR reactions using increasing concentrations of the candidate reference material extracted from cultured A549 cells (0–10 000 cell equivalents) and analyzed by the Model 3100 multicapillary instrument. Extension products, ranging from ~40 bp to ~140 bp with 6-bp repeats, are shown in *green*. Internal size standards (75, 100, and 139 bp) are shown in *red*. (*B*), electropherograms of TRAP/PCR reactions analyzed by the Model 310 single-capillary instrument.

dilutions of each sample based on the number of cell equivalents used in each real-time RT-PCR. Using the LightCycler, we analyzed different cell samples by rapid, real-time PCR, and subsequent melting curve analysis confirmed the amplification of one pure product. RNA molecules from telomerase-positive cells (A549) were detected from 100 000 to a lower limit of 10 cells. A dilution to 1 cell equivalent did not contain a detectable product.

A typical example of the results is shown in Fig. 3. The detection curves correspond to the mRNA copy numbers. The signal was linear over a range of 10–10 000 cells per reaction. The averaged copy number extrapolation decreased from 12 650 to 11 copies of hTERT mRNA. Negative controls (RPE-28) were analyzed previously at concentrations ranging from 1×10^5 to 1×10^9 cells/L, using the same primer sets as those used on A549 samples *(10)*. After more than 50 cycles of amplification, no hTERT

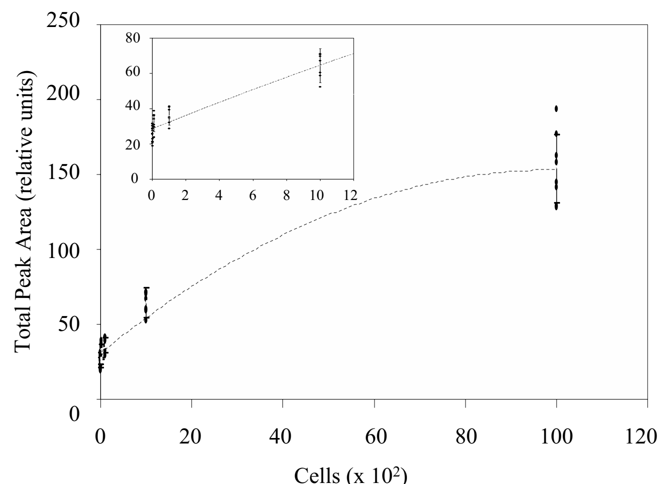

Fig. 2. Plot of the total extension product peak areas as a function of telomerase concentration (n = 8 for each concentration).

Areas were calculated from the electropherograms as shown in Fig. 1A for the Model 3100. The *inset* shows the same plot on a one-tenth scale in concentration (1000 cell equivalents). Measurements in this lower concentration range were linear within ±1 SD, as indicated by the *dashed line*.

mRNA was detected. To verify that the assay reaction mixture was optimized, we analyzed A549 samples, using the same reaction mixture, and obtained positive hTERT mRNA signals, as expected.

We determined the variation of the hTERT mRNA assays by comparing increasing concentrations of mRNA/reaction. The superimposed amplification curves showed an assay CV of 0.6%–1% when 1000–100 000 cell equivalents were analyzed. The assay CV was 2%–3% when <1000 cell equivalents were analyzed. Copy numbers were calculated from external calibration curves run in triplicate. Measurements of hTERT mRNA were reproducible with copy numbers that ranged from 1.1 to 0.13 copies/cell. This result agrees with reports of other cell lines that harbored, on average, <1 copy of functionally active hTERT mRNA per cell *(15)*. The difference between the samples containing 1000 cells (curve 8; Fig. 3) and 100 cells (curve 11; Fig. 3), a 10-fold change, was 3.2 cycle numbers (~3.3 cycle numbers) and was caused by PCR inefficiency.

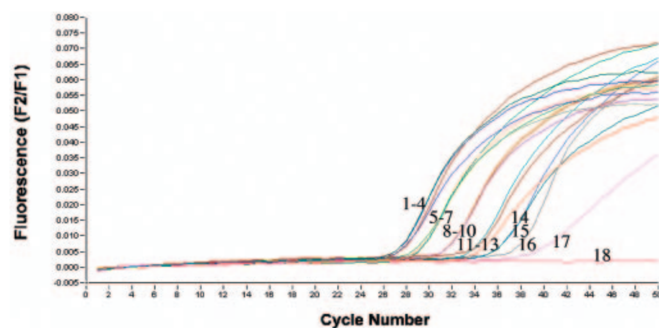

Fig. 3. Real-time amplification and probe-specific detection of hTERT mRNA by the LightCycler.

The *y axis* represents the relative ratio of fluorescence between the reference channel and the sample channel. *Curves 1–17* represent dilutions of the candidate reference material extracted from cultured A549 cells. *Curves 1–4* represent RNA detection from 100 000 cells (Ct = 26.9); *curve 5–7* represent RNA detection from 10 000 cells (Ct = 28.6); *curves 8–10* represent RNA detection from 1000 cells (Ct = 31.1); *curves 11–13* represent RNA detection from 100 cells (Ct = 34.3); *curves 14–17* represent detection from 10 cells (Ct = 38.1); *curve 18* represents the no-template control, which does not cross the threshold. *F2/F1* is the fluorescence ratio of the bound (F2) and unbound (F1) probes.

TELOMERASE REAL-TIME TRAP ASSAY

RT-PCR measurements of telomerase activity by the Bio-Rad iCycler iQ are shown in Fig. 4. In this assay, a specially designed fluorescent probe is activated when it binds to the (T_2AG_3) sequence moiety of PCR products as they are generated in the second stage of the TRAP reaction. This yields a timecourse in real time that is proportional to the concentration of telomerase enzyme *(21)*. The curves show a titration from 190 to 5600 cell equivalents, which yield detection cycle (Ct) values from 17.5 to 11.5, respectively.

COMPARISON OF TELOMERASE ASSAYS (TRAP, REAL-TIME TRAP, AND hTERT mRNA)

The relationship between the telomerase activity and the amount of cellular hTERT mRNA is shown in Table 1, which also shows the telomerase activity of the candidate reference material as measured by the real-time TRAP assay (Ct is inversely proportional to activity). Both the TRAP and the real-time TRAP show an increase with cell equivalents as expected. The cellular hTERT mRNA also increases with the number of cell equivalents as shown by the high correlation of activity and mRNA abundance in Fig. 5. The ratio of telomerase activity and mRNA (copy number) when normalized 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, a telomerase refer-

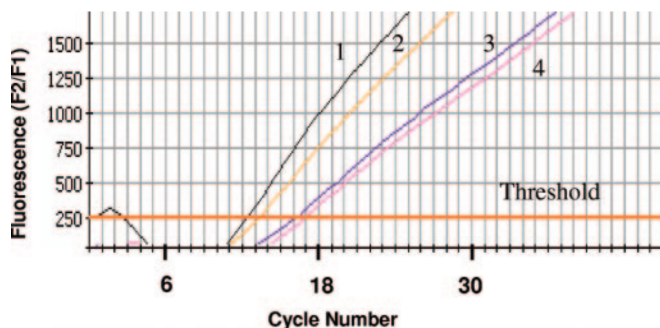

Fig. 4. Real-time TRAP measurement of telomerase activity by the iCycler.

The *y axis* represents the relative ratio of fluorescence between the reference channel and the sample channel. *Curves 1–4* represent dilutions of the candidate reference material extracted from cultured A549 cells. *Curve 1* represents the detection of telomeric repeats (T_2AG_3) from 5600 cells (Ct = 11.5); *curve 2* represents (T_2AG_3) detection from 1900 cells (Ct = 13.2); *curve 3* represents (T_2AG_3) detection from 740 cells (Ct = 17.1); *curve 4* represents (T_2AG_3) detection from 190 cells (Ct = 17.5). *F2/F1* is the fluorescence ratio of the bound (F2) and unbound (F1) probes. *Threshold* is the background obtained with the no-template control.

Table 1. Comparison of telomerase assays using TRAP/PCR, TRAP/real-time PCR, and hTERT mRNA/RT-PCR.

Cell equivalents	TRAP/PCR total peak area[a]	Ct: TRAP/real-time PCR[b]	Ct: hTERT mRNA/RT-PCR[c]	mRNA copy no.[d]
0	27.1 (4.1)			
10	31.6 (5.7)	16.7 (1.3)	38.1 (1.1)	11 (0.3)
100	36.0 (6.7)	17.5 (2.7)	34.3 (0.9)	171 (4)
1000	64.3 (7.2)	14.3 (0.5)	31.0 (0.2)	926 (6)
10 000	154.1 (22.9)	11.0 (0.1)	28.6 (0.2)	4454 (31)
100 000			26.9 (0.3)	12 650 (139)

[a] Total peak area of extension products in arbitrary fluorescence units. Values are the mean (SD); n = 8.
[b] Ct inversely proportional to TRAP extension products. Values are the mean (SD); n = 6.
[c] Ct inversely proportional to mRNA. Values are the mean (SD); n = 6.
[d] Values are the mean (SD); n = 6.

ence standard containing both the A549 cell extract and total RNA could be used to normalize such data.

Discussion

At the National Institute of Standards and Technology, our role in biomarker validation for early detection of cancer differs from that of research laboratories *(7–9)*. Diagnostic assay systems are systematically evaluated so that a standardized, reproducible, high-throughput format for clinical applications is developed. Once laboratory performance is established, traceable standards are developed as reference materials to help define clinical applications.

Biomarker validation proceeds through systematic phases *(7, 8)*. In the early phases of clinical assay development, standardization and analytical validation by measurement with a high-throughput system are particularly important. We have developed the working model of a high-throughput assay for telomerase activity in normal cell controls and human serum. This assay provides technology for additional large-scale validation studies, which are required for telomerase activity to become a useful clinical biomarker. Automated systems with lower cost would also be necessary for large-scale studies.

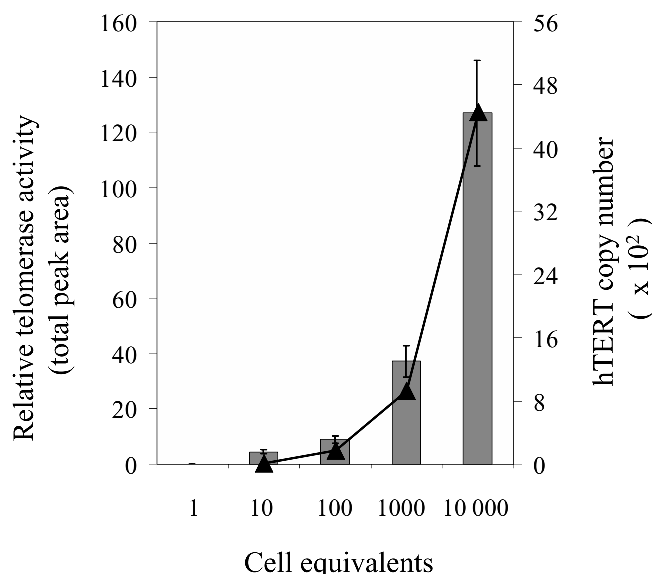

Fig. 5. Comparison of hTERT mRNA and telomerase activity.

Relationship between the relative telomerase activity (*columns*) and the amount of cellular hTERT mRNA (▲). The amount of cellular hTERT mRNA (copy number) was measured in the A549 cells and compared with the telomerase activity (total peak area) obtained from automated TRAP/PCR measurements (Fig. 1A). Activities with standard errors (*error bars*) are plotted with the blank subtracted.

One reason for the lack of widespread use of telomerase activity as a clinical biomarker for cancer detection or prognosis determination is the lack of a reference material to enable regulatory agencies to assess the quality of submitted clinical data. Our candidate reference method enabled us to determine the relationship between telomerase activity and message abundance, an important step in evaluating whether changes observed in telomerase activity are attributable to physical changes in the telomerase molecule or changes in mRNA expression, as evidenced in a report of alternative spliced telomerase variants that did not express telomerase telomeric repeat activity *(15)*. Although these variants did not contribute to the activity, as measured by the production of telomeric T_2AG_3 formation, they may have alternative functions *(22)*.

Our characterization of the A549 cell line by measuring the number of molecules of hTERT mRNA at various concentrations of our candidate standard produced results consistent with previous RApidTRAP analyses, in which a correlation was observed between hTERT mRNA and telomerase activity *(10)*. The sensitivity and efficiency of RT-PCR methods are advantageous for measuring the predicted amount of telomerase activity. The ratio of telomerase activity to hTERT mRNA abundance is expected to vary among tissue types. This ratio, a measure of the status of telomerase in the cell and at low telomerase concentrations, helps to confirm its presence. We use these measurements in conjunction with our measurements of telomerase activity to evaluate whether observed changes in telomerase activity are the result of changes in the enzyme or changes in mRNA expression. A standard preparation of telomerase with a fixed ratio of activity to hTERT mRNA would be helpful for

this measurement. The extent to which this ratio is important in cancer diagnostics has yet to be determined.

We characterized our candidate reference material by use of 3 different telomerase assay methods representative of 5 different analysis methods: TRAP/PCR and real-time TRAP to measure telomerase activity and RT-PCR to measure hTERT mRNA. These methods were selected primarily because of their utility in high-throughput screening, but we have also shown that these materials can be used in low-throughput applications such as slab-gel electrophoresis and single-capillary electrophoresis. The candidate reference material can be used in variations of the TRAP assay, such as RApidTRAP and real-time TRAP, as well as well as RT-PCR determination of hTERT mRNA. The candidate reference material can be used to normalize results from these different platforms and components in the assays such as occurs with different instrument manufacturers and conditions (e.g., volumes, temperatures, and timing). The fact that the candidate reference material can perform across different platforms and conditions strengthens its value as a standard. Coupled with these and other telomerase assay methods, a telomerase standard would provide the means to reliably address a wide range of quantitative issues with respect to telomerase expression and activity.

We developed an assay in which PCR products produced from the TRAP reaction are monitored in real time *(21)*, thus reducing sample analysis time and simplifying data analysis. We also modified the RApidTRAP high-throughput platform for use in real-time technologies such as the Applied Biosystems TaqMan and the Bio-Rad iCycler. With this method, the same material analyzed by capillary and slab-gel methods can be analyzed in real time in a fraction of the time required for traditional TRAP assays.

Concerns regarding the specificity of telomerase as a biomarker have been raised because some tissues have been reported to contain subsets of cells that express telomerase while in a noncancerous state, even if only weakly or episodically. On the other hand, when standards have been incorporated, reliably assessed telomerase activity has demonstrated its clinical utility. It would therefore be worthwhile to develop a standard telomerase preparation with a fixed activity and hTERT mRNA concentration to address methodologic concerns. With promising preliminary results, further validation by multisite trials would confirm the utility of telomerase activity and/or hTERT mRNA as biomarkers for early detection of cancer.

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References

1. Watson JD. Origin of concatemeric T7 DNA. Nat New Biol 1972; 239:197–201.
2. Muller HJ. The remaking of chromosomes. Collecting Net 1938; 8:182–98.
3. McClintock B. The stability of broken ends of chromosomes: *Zea mays*. Genetics 1941;26:234–82.
4. Blackburn E, Bhattacharyya A, Gilley D, Kirk K, Krauskopf A, McEachem M, et al. The telomere and telomerase: how do they interact? Ciba Found Symp 1997;211:2–13, discussion 15–9.
5. Shay JW, Bacchetti S: A survey of telomerase activity in human cancer. Eur J Cancer 1997;33:787–91.
6. Hess JL, Highsmith WE. Telomerase detection in body fluids. Clin Chem 2002;48:18–24.
7. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, et al. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst 2001;93:1054–61.
8. Srivastava S, Kramer BS. Early detection cancer research network. Lab Invest 2000;80:1147–8.
9. Barker PE. Cancer biomarker validation: standards and process: roles for the National Institute of Standards and Technology (NIST). Ann N Y Acad Sci 2003;983:142–50.
10. Jakupciak JP, Wang W, Barker PE, Srivastava S, Atha DH. Analytical validation of telomerase activity for cancer early detection: Trap/PCR-CE and hTERT mRNA quantification assay for high-throughput screening of tumor cells. J Mol Diagn 2004;6:157–65.
11. Atha DH, Miller K, Sanow AD, Xu J, Hess JL, Wu OC, et al. High-throughput analysis of telomerase by capillary electrophoresis. Electrophoresis 2003;24:109–14.
12. Hess JL, Atha DH, Xu J, Highsmith WE. Telomerase activity measurement in magnetically captured epithelial cells: comparison of slab-gel and capillary electrophoresis. Electrophoresis 2004;25:1852–9.
13. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.
14. Obyashiki JH, Obyashiki K, Toyama K, Shay JW. A nonradioactive, fluorescence-based telomeric repeat amplification protocol to detect and quantitate telomerase activity. Trends Genet (TIG) 1996;12:395–6.
15. Yi X, Shay JW, Wright WE. Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. Nucleic Acids Res 2001;29:4818–25.
16. Brandhorst BP, McConkey EH. Relationship between nuclear and cytoplasmic poly(adenylic acid). Proc Natl Acad Sci U S A 1975; 72:3580–4.
17. Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE, et al. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci 1995;17:1–15.
18. Emrich T, Chang SY, Karl G, Panzinger B, Santini C. Quantitative detection of telomerase components by real-time on-line PCR analysis with the LightCycler. Methods Mol Biol 2002;191:99–108.
19. Yi X, White DM, Aisner DL, Baur JA, Wright W, Shay JW. An alternate splicing variant of the telomerase catalytic subunit inhibits telomerase activity. Neoplasia 2000;2:433–40.
20. Colgin LM, Wilkinson C, Englezou A, Kilian A, Robinson MO, Reddel RR. The hTERT a splice variant is a dominant negative inhibitor of telomerase activity. Neoplasia 2000;2:426–32.
21. Elmore LW, Forsythe HL, Ferreira-Gonzalez A, Garrett CT, Clark GM, Holt SE. Real-time quantitative analysis of telomerase activity in breast tumor specimens using a highly specific and sensitive fluorescent-based assay. Diagn Mol Path 2002;11:177–85.
22. Smith LL, Coller HA, Roberts JM. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat Cell Biol 2003;5:474–9.