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An interference-free fluorescent assay of telomerase for the high-throughput analysis of inhibitors

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Abstract

We have developed a telomerase assay that can quickly and accurately rank the ability of molecules to inhibit telomerase activity. It is based on the method of Orlando and co-workers which utilizes PicoGreen to detect dsDNA formed during the polymerase chain reaction (PCR) amplification of telomerase products. PCR cycles were optimized to give as linear a signal as possible relative to telomerase products; 96-well streptavidin-coated PCR plates were used to isolate the preamplification telomerase products and to wash inhibitors away before the amplification step. The inhibitor removal step is critical to prevent false positives potentially caused by inhibition of *Taq* polymerase during amplification. Use of the streptavidin-coated PCR plate allows this step to be done much more rapidly than use of the liquid/liquid extraction adopted by others. We have demonstrated that this assay can correctly order the ability of four inhibitors to inhibit telomerase and reproduce within a factor of two the absolute IC_{50} values determined by the more time-consuming direct assay. We have shown that the difference in IC_{50} values determined in this assay versus the direct assay can be corrected for by using the standard curve appropriately. Using this method 96 compounds can be assessed in 3–5 h.

Telomerase activity strongly correlates with the presence of cancer cells [1]. In addition to this correlation, there are significant mechanistic reasons that cancer and other immortal cells should require telomerase activity to continually proliferate. For this reason, the assay of telomerase activity is important both for the detection of cancer and for the assessment of the ability of inhibitors to block telomerase [2,3]. There are two main classes of assay methods for accomplishing this, direct assays and PCR-amplified assays [1,4]. Direct assays assess the telomerase-catalyzed incorporation of radiolabeled dNTPs into a telomerase substrate, typically by gel electrophoresis of the extended substrate. When electrophoresed on a gel, these show the classical pattern of clusters separated by six nucleotides. A second class of telomerase assays are PCR-based assays in which telomerase products are amplified using PCR primers complementary to both the substrate oligo, and the extended product (so called TRAP[1] assays) [1].

Recently, Orlando and coworkers showed that the sensitive double-stranded DNA stain PicoGreen could be used to detect telomerase activity in a range of cancer cells, once telomerase products were amplified using a TRAP protocol [5]. As part of our effort to target telomerase via its RNA/DNA duplex, we are combinatorially optimizing lead inhibitors [6,7]. This requires an assay that can quickly and accurately order relative inhibitor efficacies. To this end we have optimized the assay of Orlando et al. for use in analyzing large numbers of inhibitors. We have demonstrated that it can correctly order the inhibition potential of a range of inhibitors and that the absolute values of the inhibition constants are close to those determined by the ''gold standard'' direct assay (which is time consuming and requires significant amounts of radioactivity).

Other researchers have used TRAP-based PCR assays to screen potential inhibitors. To alleviate the problem of false positives due to inhibition of *Taq* polymerase by the tested inhibitors, typically the inhibitors were extracted with organic solvents before assay [8]. This is effective but not practical for the simultaneous screening of 96 compounds or libraries at a time. An alternative approach to correct for false positives due to *Taq* inhibition is to

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[1] *Abbreviations used:* TRAP, telomeric repeat amplification protocol; ITAS, internal telomerase assay standard; DTT, dithiothreitol.

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include an internal telomerase assay standard (ITAS) as originally described by Wright et al. [9]. However, this would require either gel analysis of each sample, to separate the telomerase signal from the ITAS signal, or, if direct fluorescent quantitation of double-stranded products was used, doubling of the number of points needed for each assayed compound. We have previously demonstrated the utility of 96-well streptavidin-coated plates for isolating biotinylated telomerase products [10]. We have now adapted this approach for the PCR-amplified assay and have demonstrated that a 96-well streptavidin-coated PCR plate can isolate telomerase products and effectively wash off inhibitors that could otherwise cause false positives through inhibition of *Taq* in the subsequent amplification step. These products are then directly amplified in the plate and detected using the double-stranded DNA-specific probe PicoGreen, which is analyzed using fluorescence.

We have tested this assay in two ways: by analyzing two known telomerase inhibitors over a range of concentrations and by analyzing four known telomerase inhibitors at a single concentration in triplicate. The results of both analyses show very good correlation with the results of the direct assay and demonstrate the utility of this assay for rapidly assessing telomerase activity in the presence and absence of inhibitors.

Materials and methods

HeLa S3 cells were obtained from National Cell Culture Center (Minneapolis, MN, USA). The telomerase assay buffer, reaction termination buffer, cell lysis buffer, and washing buffer components were obtained from Fisher Scientific (Pittsburgh, PA, USA) and Sigma (St. Louis, MO, USA). The oligo primers were synthesized by Sigma–Genosys (St. Louis, MO, USA). dNTPs were supplied by Amersham Pharmacia Biotech (Piscataway, NJ, USA). The *Taq* polymerase was obtained from Promega (Madison, WI, USA). PicoGreen was obtained from Molecular Probes (Eugene, OR, USA). Streptavidin-coated PCR tubes were obtained from Roche Molecular (Mannheim, Germany). Intercalators were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of HeLa cell extract

HeLa cell extract was prepared as previously described [11]. Briefly, HeLa S3 cells were suspended in cold washing buffer (10 mM Hepes–KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, pH 7.5) and pelleted at 10,000*g* for 1 min at 4 °C. The pellet was resuspended in cold lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM BME, 1 mM DTT, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid, 10% glycerol) and lysed for 60 min on ice. The suspension was then centrifuged at 100,000*g* for 1 h at 4 °C, the supernatant was removed, and adjusted to 20% glycerol, aliquoted and stored at −80 °C.

Preparation of intercalators

Approximately 1 mg of intercalator was dissolved in 500 μL H_2O, vortexed 20 min, and centrifuged in a microcentrifuge for 20 min to remove undissolved material. The supernatant was removed and the concentration determined spectrophotometrically.

Assay procedure

In a 96-well teflon plate, the reaction mixture containing assay buffer (50 mM Tris–OAc, 50 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM Spermidine, pH 7.1), 1 mM dATP, 1 mM dTTP, 1 mM dGTP, 1 μM biotinylated TRAP primer substrate oligo (5′ biotin AATCCGTCGAGCAGAGTT), 5 μL intercalator stock, and 3.2 μL HeLa cell extract (10 μg protein/μL) in a final volume of 20 μL was incubated for 1 h at 37 °C. After the reaction period, the mixture was transferred to Streptavidin-coated PCR tubes containing 200 μL reaction termination buffer (10 mM Tris–HCl, 2 M KCl, pH 7.2). The reaction product was allowed to bind at room temperature, with gentle shaking for 30 min. After the binding period the solution was aspirated and discarded. The PCR tubes were washed five times by the addition of 200 μL washing buffer (10 mM Tris–HCl, 1 M NaCl, pH 7.5) and subsequent removal of the washing buffer.

PCR amplification of the isolated telomerase product was carried out by the addition of 50 μL of PCR mixture having a final composition of 50 μM dATP, 50 μM dTTP, 50 μM dGTP, 50 μM dCTP, 0.1 μg biotinylated TRAP forward primer, 0.1 μg reverse primer (5′ CCCTT ACCCTTACCCTTACCCTAA), 1×PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM $MgCl_2$), and 1.25 units *Taq* polymerase. An initial denaturation step of 94 °C for 5 min was followed by 22 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s. At the end of the PCR amplification step, 20 μL of the solution was removed and added to a 96-well plate containing 100 μL of a 1/100 dilution of PicoGreen in 1× TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). The solution was shaken gently for 5 min at room temperature, and quantitated using fluorescence detection (excitation 485 nm, emission 535 nm).

PCR amplification response varying telomerase product concentration

A 5× volume telomerase reaction (i.e. 100 μL) was carried out in an eppendorf tube having the assay

composition as described above for 1 h at 37 °C. The reaction was quenched by the addition of 100 μL reaction termination buffer. Dilutions of the resulting assay mixture having telomerase product were made in reaction termination buffer. The term "100% telomerase products" as used in the text refers to the telomerase products found in 40 μL of this solution, "80% telomerase products" refers to the telomerase products found in 40 μL of a 4/5 dilution of this solution, and so forth; 40 μL volumes of the dilutions were transferred to Streptavidin-coated PCR tubes containing 180 μL reaction termination buffer. The binding, washing, and PCR amplification steps were carried out as described above.

Results and discussion

There are four stages to our assay. First, homogenate containing telomerase is incubated with a biotinylated substrate oligonucleotide. Telomerase acts upon it and extends it through the processive addition of nucleotides. Then, the telomerase extension products are isolated and washed in a streptavidin-coated PCR plate to remove cellular debris and any assayed inhibitors. These products are then amplified using TRAP primers and PCR. Finally, the amplified products are quantitated using the fluorescent dye PicoGreen.

We initially analyzed the effect of PCR cycle numbers on the linearity of signal from PicoGreen-stained telomerase products under our experimental conditions. These experiments were done using biotinylated telomerase substrate oligonucleotide which was isolated on streptavidin-coated magnetic beads after extension by telomerase, as previously described [7,11]. This sample was released from the beads with guanidine–HCl, precipitated, and subjected to the PCR protocol described under Materials and methods. Our aim was to find a number of cycles that gave as close to a linear signal with respect to telomerase products as possible, while still giving enough signal to be accurately measured.

We compared the signal generated from varying dilutions of telomerase products using different numbers of cycles of PCR amplification. As expected the overall signal increases in general with increasing cycle numbers. The shape of the signal versus the telomerase product concentration curve, however, changes, appearing more linear at lower cycle numbers and more steeply curved at higher cycle numbers. This can be seen in Fig. 1 where the fluorescent signals have been normalized to the signal determined at the highest concentration of telomerase products.

In their use of a PicoGreen-based telomerase assay, Orlando and co-workers [5] used 60 PCR cycles, a point at which amplification reaches saturation and no further products are being synthesized with each cycle. We selected 22 PCR cycles, a point at which telomerase amplification products are still increasing with each cycle, in an attempt to provide maximum spread between signal values for a given starting telomerase product amount. Our lower number of PCR cycles compared to that of Orlando et al. may be due in part to the fact that we typically use a higher concentration of cell protein in the assay. We chose a high cell extract concentration so that our results could be directly compared to our previous results using the direct assay, which requires high extract amounts to allow for detectable signal. It should be noted that, for screening inhibitors, a purely linear relationship between signal and telomerase product

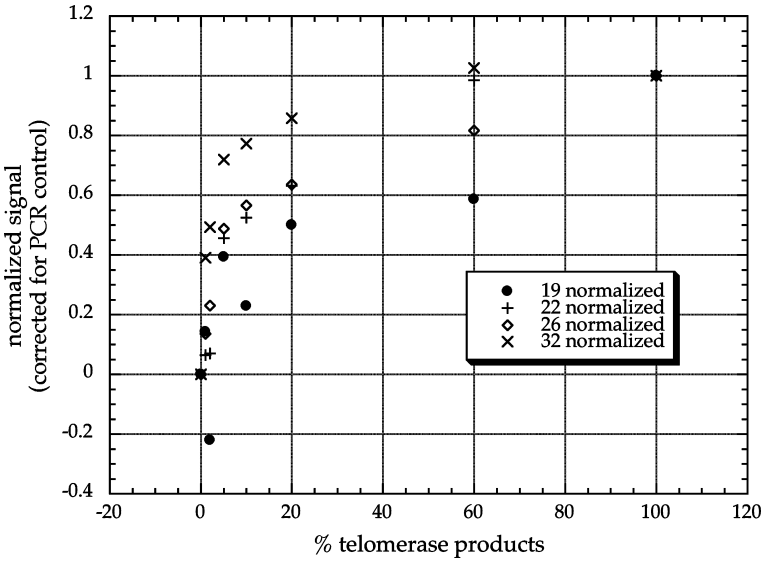

Fig. 1. Analysis of the curvature of normalized signal versus telomerase product graphs. These were performed at different numbers of PCR amplification cycles as indicated. Signals were normalized to the signal at the highest concentration of telomerase products.

amount is not an absolute requirement. The most important thing is that the signal *vary* with telomerase product amount. This allows the detection of inhibition and the correct ordering of equimolar inhibitors. We selected 22 cycles of amplification based on a compromise between signal intensity and signal linearity.

To improve potential throughput, we did all subsequent experiments with streptavidin-coated 96-well PCR plates instead of streptavidin-coated magnetic beads. A washing step is absolutely required when applying a PCR-based assay to the analysis of inhibitors, as these inhibitors may cause false positives by inhibiting the *Taq* polymerase used in amplification. Some authors have circumvented this problem by using organic extraction to eliminate inhibitors from the mixture before amplification [8]. This was shown to be effective but it is not easily applicable to a high-throughput method. We have previously used streptavidin-coated 96-well plates for product isolation and washing in our rapid direct assay and we applied that approach to the fluorescent PCR-based assay [10].

In a typical experiment, biotinylated telomerase substrate oligonucleotide was incubated with dNTPs and telomerase-containing cell extracts, in the presence and absence of varying concentration of inhibitors. After 1-h incubation, these assay solutions were transferred to a 96-well streptavidin-coated PCR plate where the telomerase extension products were allowed to bind for 30 min. After this, the wells were washed five times to get rid of competing nucleic acids which can lead to interference and inhibitors, if present, which can lead to false positives. To these wells was added the standard TRAP PCR primers, dNTPs, and *Taq* polymerase to amplify the telomerase product. After 22 cycles of amplification, the amount of double-stranded DNA produced was quantitated in a scanning fluorescent plate reader using PicoGreen. Rather than using a forward primer different from the telomerase substrate, we use the biotinylated substrate oligonucleotide as the forward primer. This is essentially done for convenience, as it allows a single oligonucleotide stock to perform two functions. The possibility that the biotinylated primer may bind to the streptavidin plate and affect the results was not a significant concern, as the heat of the PCR cycles likely denatures the streptavidin, releasing the fraction of bound biotinylated oligonucleotides. If this were not the case, we would not observe signal above background or signal varying with cycle numbers. It is possible that using a nonbiotinylated forward primer for the PCR step would increase the signal observed.

Fig. 2 shows the fluorescent signal generated by varying the concentrations of telomerase products used in the PCR amplification and fluorescent labeling. This shows the increase in signal with increasing telomerase product concentration. Because the amount of telomerase products at the end of a telomerase assay is difficult to determine absolutely, we form the standard curve by making a series of dilutions of telomerase products formed under our standard conditions. These standard conditions are listed under Materials and methods. "100% telomerase" products thus refers to the amount of telomerase product formed in 40 μL of assay volume without any inhibitor present, "80% telomerase products" is a 4/5th dilution of this assay volume, and so

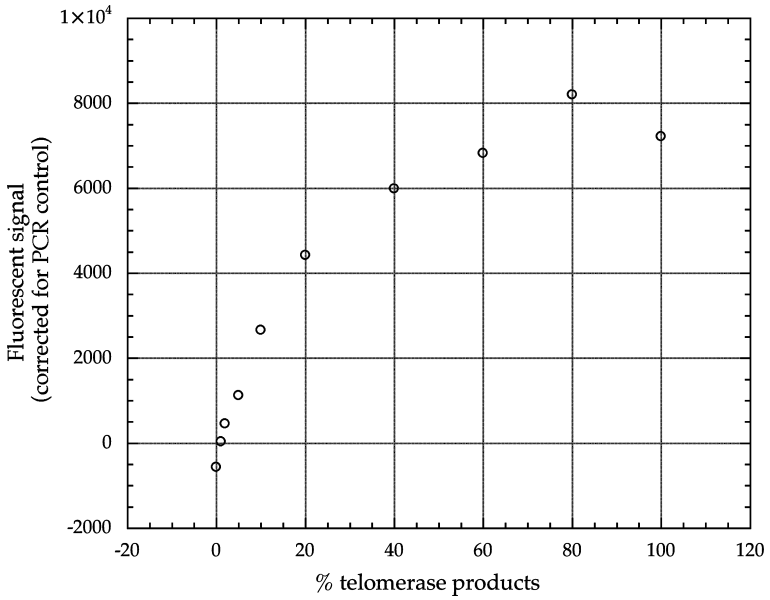

Fig. 2. Standard curve of fluorescent signal versus % telomerase products using streptavidin-coated PCR plates to isolate/wash telomerase products; 22 cycles of PCR amplification were used and the total fluorescent signal was corrected for the signal due to binding of PicoGreen to PCR primers.

forth. These diluted products were then isolated, amplified, and stained as described under Materials and methods. The standard curve in Fig. 2 indicates that the fluorescent signal is not linear with amount of telomerase products, which may be expected due to the nonlinear nature of the amplification process.

Results for IC_{50} dose response curves are shown in Figs. 3 and 4. These were determined by varying concentrations of ethidium and rivanol, two inhibitors that we have previously identified [7]. The fluorescent signal normalized to signal in the absence of inhibitor (p) was plotted versus inhibitor concentration and fit to the equation $p = 1/(1 + [I]/IC_{50})$. The IC_{50} values 7.7 and 20.5 μM, respectively, show good correlation to values determined by the direct assay (3.3 μM and 8.2 μM). The signal values plotted are the direct fluorescence signals as generated by the fluorometer, corrected for the PicoGreen signal that arises due to binding to single-stranded oligonucleotide PCR primers. The single-stranded oligonucleotide primers used in the PCR amplification step are in excess to the amount of amplified double-stranded product. In addition, PicoGreen can generate a signal when bound to single-stranded DNA, albeit less than that upon binding to double-stranded DNA. Because of this, even with no telomerase product present, there is a significant null signal (approximately half of the total signal). We correct for this by having a PCR control point, which contains an amount of single-stranded PCR primers identical to that used in the amplification, but no telomerase product. This sample is analyzed with the other samples using PicoGreen, and its fluorescent signal is subtracted from the total signal. Because the PCR primers are at a higher concentration than the final amplified double-stranded DNA concentration, this correction is close to the true zero signal point, although at very low signal values it can overcorrect.

In addition to IC_{50} response curves, we analyzed individual compounds at single concentrations (50 μM) in triplicate, to assess the ability of the assay to correctly and reproducibly order the efficacy of a range of inhibitors under conditions typical of a library screening. Table 1 shows the values for IC_{50} estimated from the single point signal. Single point signal s was converted to IC_{50} using the expression $IC_{50} = [I]/(1/[(s-c)/(t-c) - 1])$, where s is the fluorescent signal observed with inhibitor present at concentration $[I]$, c is the PCR control signal as described above, and t is the fluorescent signal observed with no inhibitor present. The values so determined exactly match the ordering of efficacies observed using the more time-consuming direct assay (Table 1). In addition, the values match well the IC_{50} values determined for ethidium and rivanol using the full range of concentrations in Figs. 3 and 4. Although the values estimated do not exactly match the numbers generated from the direct assay, they are within a factor of 2 and correctly order the efficacies. It is this correct ordering that is crucial for the assessment of combinatorially generated libraries.

Upon examination, the reason why the measured IC_{50} values differed from the direct results by approximately two to threefold becomes clear. It is because of the curved nature of the fluorescent signal versus the telomerase product standard curve (Fig. 2). To reach a

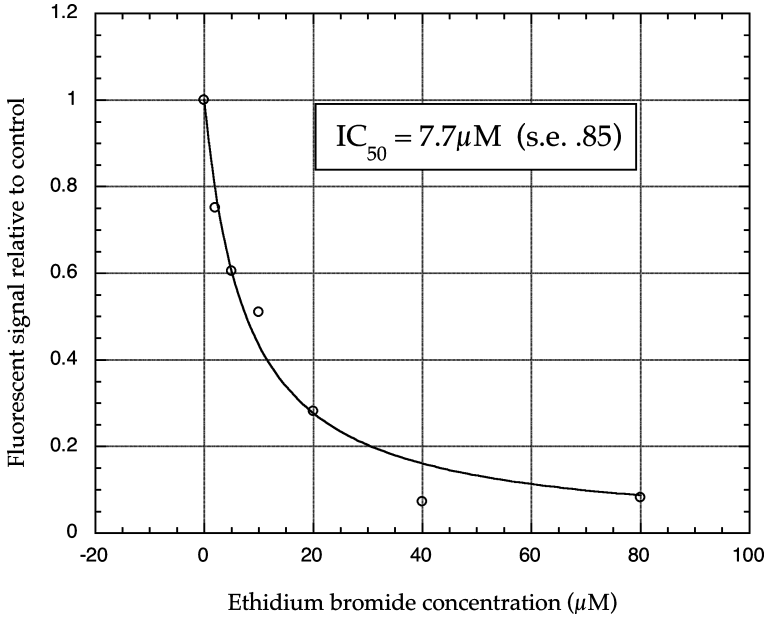

Fig. 3. IC_{50} plot of ethidium inhibition of telomerase, using the fluorescent signal due to stained, amplified telomerase products in the presence of varying concentrations of ethidium. Signals were corrected for nonspecific signal due to binding of PicoGreen to PCR primers and then normalized to the signal in the presence of no inhibitor. The data were fit as described in the text.

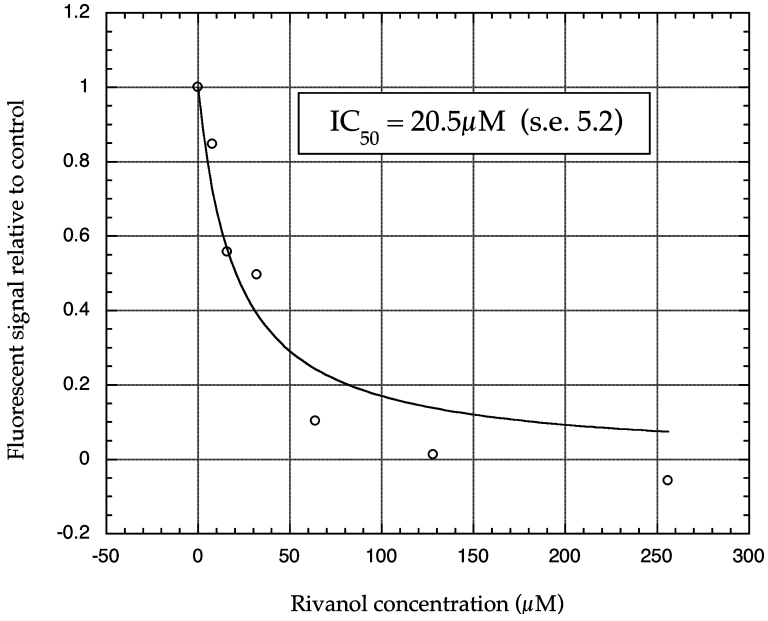

Fig. 4. IC_{50} plot of rivanol inhibition of telomerase, as described in the legend to Fig. 3.

Table 1
IC_{50} values estimated from single-point fluorescent PCR assays done in triplicate, compared with the IC_{50} values previously determined using the direct assay and full dose response curves [7]

Compound	PCR assay		Direct assay	
	IC_{50} (μM)	Standard error	IC_{50} (μM)	Standard error
Ethidium bromide	6.5	0.7	3.3	0.84
Rivanol	20.8	1.5	8.2	0.75
Acridine orange	39.6	7.7	12.2	1.4
Doxorubicin	251.0	45.2	> 100	—

signal that is approximately half of the maximum signal requires the presence of approximately one *quarter* of the telomerase product. To achieve this in turn requires a concentration of inhibitor roughly twice that of the true IC_{50}. Curved signal versus telomerase product plots have been observed by a range of groups [5,12]. To more accurately assess the true IC_{50} for an inhibitor, it is more appropriate to use in the analysis the actual amount of telomerase products as opposed to the fluorescent signal alone. To effectively do this, it is necessary to use the standard curve (Fig. 2) to convert from fluorescent signal to telomerase product concentration. These values can then be replotted to give the true concentration of telomerase products at each inhibitor concentration, which provides a more accurate assessment of telomerase activity.

We have done this reanalysis using the data found in Figs. 3 and 4. First the standard curve data in Fig. 2 were replotted, putting "% telomerase products" (our measure of telomerase product quantity) on the *y* axis and fluorescent signal on the *x* axis. This allows easier subsequent computation of % telomerase products from a fit equation. Signal was normalized, so that each value was taken as a proportion to the signal observed at maximum telomerase products. We then fit these data to a variety of functions. We have no a priori mechanistic reason to select one function over another. Our only criterion was that the function fit the data well. This allows the conversion of an arbitrary signal to % telomerase products using the fit equation. We found that a quadratic equation fit the data well (Fig. 5). Using the fit equation we then converted normalized fluorescent signals into telomerase product amount (so called "% telomerase products").

Full dose response curves for rivanol and ethidium were plotted, using the ratio of telomerase products in the presence and absence of specific concentrations of inhibitors as determined by the standard curve in Fig. 5. Fig. 6 shows the full dose response curve for ethidium, corrected as described above. The inhibition constants so determined were 2.6 μM (SE 0.22) for ethidium and 9.9 μM (SE 2.2) for rivanol. These fit values are now identical (within the standard errors) to those determined using the direct assay. The difficulty of lack of linearity in the signal versus telomerase product curves can thus be remediated by using the nonlinear standard

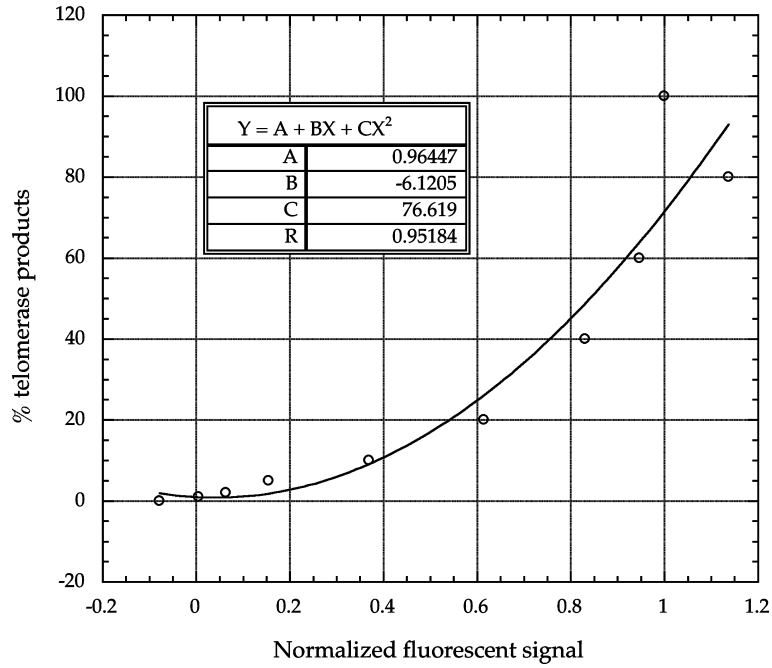

Fig. 5. Replot of standard curve from Fig. 2. Fluorescent signal is now taken as a proportion to the signal found at the highest telomerase product concentration. The data were fit to a quadratic equation. The parameters as fit are shown above. The axes were interchanged to allow easy conversion of signal to telomerase product concentration using the fit equation.

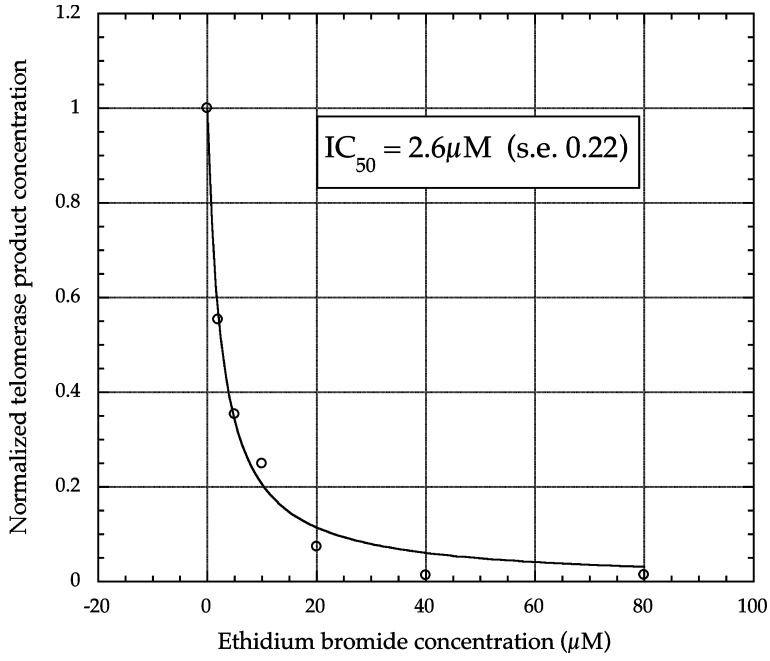

Fig. 6. IC_{50} plot of ethidium inhibition of telomerase, using fluorescent signal converted into telomerase product concentrations using the standard curve in Fig. 5.

curve to determine true telomerase product concentrations. Full dose response curves using these corrected data then give IC_{50} values that are virtually the same as the true values as determined from direct assays. In the absence of this standard curve, the *relative* values of IC_{50} are correct, but for the *absolute* values to be correct, a standard curve needs to be applied. Because of variations in homogenates, it is recommended that a new standard curve be generated with each set of assays on a given day.

There is a possibility that inhibitors present during the telomerase assay can persist in the streptavidin-coated plate even after the extensive washing step designed to remove them. If they do persist, they can potentially cause false positives in the assay by inhibiting the *Taq* polymerase used for amplification. We have tested for this possibility and demonstrated that the inhibited telomerase signal observed for tested compounds is due to reduced telomerase products and not simply to the inhibition of *Taq* polymerase in the subsequent amplification step. Specifically, we performed a telomerase assay without any inhibitor present and then quenched it with high salt buffer. To this was added either ethidium or rivanol at a concentration equal to their IC_{50} values or nothing. These three samples were washed as described and then analyzed. Telomerase activity was found to be identical in the presence and absence of late-addition inhibitor, showing that these molecules, known to inhibit polymerases, had no effect on *Taq* after the extensive washing step. This suggests that this washing step will be sufficient to prevent false positives in future screens.

An additional advantage of using a streptavidin-coated PCR plate to wash inhibitors away from the telomerase product is the elimination of an additional DNA precipitation step in the assay. Either the heat of the first PCR cycles is sufficient to release the biotinylated telomerase extension product or this plate-bound product can still be acted upon by the *Taq* polymerase. Because of this, the guanidine release procedure followed by precipitation required in streptavidin-coated magnetic bead assays or the precipitation step needed in conventional assays is not necessary. The varying efficiency of precipitation is therefore eliminated as an extra confounding factor in the assay, eliminating the need for an internal control as used in other assays.

Finally, we examined the possibility that these assays could be performed *directly* in the streptavidin-coated 96-well PCR plate. This would require that the telomerase be able to add telomeric repeats to telomerase substrates directly bound to the plate surface through the biotin–streptavidin linkage. We performed the assay of telomerase in triplicate, in the presence and absence of a single concentration of ethidium directly in the PCR plate. After the assay, the plate was washed and analyzed and the IC_{50} value determined as above. The value so determined (6.9 μM SE 1.0) was virtually identical to that determined using the more time-consuming approach of using a separate plate for the assay and the washing steps. This increases the potential utility of this assay, as it removes a plate-transfer step requiring the manipulation of 96 samples.

In conclusion, we have adapted the telomerase assay method of Orlando et al using PicoGreen and applied it for the rapid screening of potential telomerase inhibitors. This required the use of a high-efficiency washing method, streptavidin-coated 96-well PCR plates, which allows the removal of inhibitors before the PCR amplification step, to prevent false positives. We demonstrated with appropriate controls that the washing step does effectively remove inhibitors. We found that the assay required the optimization of the number of PCR cycles to give the best linearity of signal with telomerase product concentration. In addition, we found that a PCR control point was needed to correct for fluorescent signal due to single-stranded PCR primers that are in excess. By careful processing of the data, we were able to correct for the nonlinearity in signal versus product graphs. The inhibition constants determined using this processed data were identical to those determined by the direct assay. Finally, we showed that this assay can be completely performed in the 96-well streptavidin-coated PCR plate, without having to transfer from an assay plate. The use of the 96-well streptavidin-coated PCR plate therefore eliminates or accelerates two very time-consuming steps: the elimination of inhibitors and the isolation of telomerase products. Currently, the assay takes approximately 3–5 h to assess a complete plate of 96 compounds, including 1 h for telomerase extension, 1 h for PCR amplification and 1–3 h for operator manipulations. Use of a 96-well washing station and a quantitative PCR machine will further reduce operator time, giving even greater utility to this rapid and accurate method of analyzing telomerase inhibitors.

Acknowledgments

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