Short Technical Report

A Rapid Direct Telomerase Assay Method Using 96-Well Streptavidin Plates

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ABSTRACT

We have developed a high-throughput direct assay method for the assay of telomerase activity that improves on previous direct telomerase assays in two ways that allow larger numbers of samples to be conveniently processed: (i) 96-well streptavidin coated plates are used to bind and wash biotinylated primer extension products from the telomerase assay, as opposed to tubes containing streptavidin-coated magnetic beads; and (ii) storage phosphor-imaging is used instead of film autoradiography to detect telomerase products after being washed and released from the streptavidin-derivatized matrix. This method improves on previous direct assay methods using magnetic beads by allowing larger numbers of samples to be conveniently assayed. Also, the total activity of the radiolabeled nucleotides used in this procedure is significantly lower than that used in standard direct telomerase assays, lowering costs and exposure to radioactivity. We have validated the assay by repeating, in triplicate, the IC₅₀ determination of rivanol, our previously identified telomerase inhibitor.

INTRODUCTION

The enzyme telomerase is a reverse transcriptase that is able to replace the telomeric portion of the chromosome lost during DNA replication (2,3). Its activity is found in a large majority of cancer cells while being absent in most normal somatic cells (4,9). Inhibition of telomerase activity in cancer cells can revert immortalized cells to the mortal state, eventually resulting in their cell death (6,7,12). For this reason, telomerase is identified as a potential target in cancer treatment (1,10).

We are investigating a novel method of inhibiting telomerase, by targeting the RNA/DNA heteroduplex that forms during its catalytic cycle (5,15). We have shown that molecules that bind to this structure inhibit the enzyme. An essential part of this project is to increase the specificity of inhibitors by using combinatorial chemistry to introduce new interactions between lead molecules and the unique telomerase protein/nucleic acid surfaces. This requires a telomerase assay method that is accurate and reproducible and can be used on large numbers of individual samples.

There are several conventional methods used for the detection of telomerase products. These include (i) PCR-based methods [e.g., telomeric repeat amplification protocol (TRAP) assays (9)], (ii) the direct method as described by Morin (11), and (iii) the direct method using Dynabeads® (Dynal, Lake Success, NY, USA) as described by Sun et al (13). PCR methods, including the TRAP assay, are powerful and conve-

nient methods for the detection of typically small quantities of telomerase reaction products. The main disadvantage in the use of a PCR method stems from the fact that the test compound in the assay mixture may inhibit the *Taq* DNA polymerase used in the PCR procedure. This issue has been noted and commented on previously (14). This potential for interference may be avoided by the use of a direct assay method (i.e., without PCR amplification of the telomerase product).

The direct detection method as described by Morin (11) circumvents the problems associated with PCR amplification mentioned above. However, a disadvantage of the procedure as described by Morin is the large amount of radioactivity required (approximately 30 μ Ci/assay, as described), as well as the potential for interference from other, non-telomerase-generated radioactive nucleic acids in the precipitation step (13).

The direct method using magnetic beads introduced by Sun et al. (13) is an improvement in the efficiency of the isolation of telomerase-generated products. In this method, the telomerase substrate oligonucleotide is biotinylated so that the products of telomerase extension are isolated on magnetic, streptavidin-coated beads, which limits exposure to radiation and reduces background noise by efficiently washing away unincorporated [³²P]dGTP. While this assay is very effective at accurately assaying telomerase activity, it is constrained by the number of samples that can be conveniently handled at any one time. This is due to the physical manipulations in-

Table 1. Analysis of Triplicate Rivanol IC₅₀ Data

Repetition	IC ₅₀ (μ M)	Standard Error	R
A	21.7	6.1	0.94
B	13.0	2.4	0.97
C	15.0	5.8	0.92

volved in washing magnetic beads in tubes and the amount of radioactivity required of each assay point. Our approach improves both of these areas by using 96-well streptavidin-coated plates for product isolation and washing, as well as storage phosphorimager for product detection. These two improvements allow larger numbers of samples to be analyzed, as is required of combinatorially generated libraries. To validate our approach, we have used it to determine, in triplicate, the IC₅₀ value for rivanol, a telomerase inhibitor that we have previously identified.

MATERIALS AND METHODS

Preparations of HeLa Cell Extract

Homogenates of the HeLa cell line, which has been demonstrated to possess telomerase activity, were prepared using a method based on that of Kim et al. (9). Briefly, HeLa S3 cells obtained from the National Cell Culture Center (Minneapolis, MN, USA) were suspended in cold washing buffer (10 mM HEPES-KOH, 1.5 mM MgCl₂, 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 MgCl₂, 1 mM EGTA, 0.1 mM PMSF, 5 mM BME, 1 mM DTT, 0.5% CHAPS, 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 removed and adjusted to 20% glycerol, and aliquoted and stored at -80°C. The cell concentration of the final homogenate preparation is 3.4 × 10⁶ cells per 20 μ L lysis buffer.

Assay Procedure

The assay method used is adapted from the procedure of Sun et al. (13).

All assays were performed in a 96-well Teflon® plate. An IC₅₀ experiment was conducted in triplicate (A, B, and C) with 6 assay points done at 0, 8, 16, 32, 64, and 128 μ M rivanol concentrations. The final composition of the assay mixture is 50 mM Tris-acetate, pH 7.2, 50 mM KCl, 1 mM MgCl₂, 5 mM β -mercaptoethanol, 1 mM Spermidine, 1 mM dATP, 1 mM dTTP, 2.4 μ M [Redivue α -³²P 160 Ci/mmol] dGTP (7.6 μ Ci/assay), 1 μ M biotinylated primer substrate oligonucleotide (5'biotin-(TTAGGG)₃), 3.2 μ L HeLa cell homogenate, and 5 μ L rivanol at the appropriate concentrations, in a final reaction volume of 20 μ L. The telomerase reaction was initiated by the addition of the cell homogenate, and the assay mixture was incubated at 37°C for 1 h. After the reaction period, the mixture was transferred to a pre-washed Reacti-Bind™ NeutrAvidin™ Coated Polystyrene Strip Plate blocked with Blocker™ BSA (Pierce Chemical, Rockford, IL, USA), having 80 μ L reaction termination buffer (10 mM Tris-HCl, pH 7.4, 2 M KCl) in the wells. The reaction product was allowed to bind at room temperature with shaking for 30 min. At the end of the binding period, the solution was removed and discarded. A 200- μ L washing buffer volume (10 mM Tris-HCl, pH 7.5, 1 M NaCl) was added to each well and allowed to shake for 5 min, followed by the removal of the washing buffer solution. The wells were washed a total of five times by this method. After the washing step, 200 μ L 5 M guanidinium HCl were added to the wells, heated at 90°C for 20 min, and the Guanidine solution was removed and transferred to Eppendorf® microcentrifuge tubes. The oligonucleotide product was precipitated by the addition of carrier tRNA and glycogen to the guanidine solution and adjusted to 75% EtOH, chilled for 30 min at -10°C and cen-

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trifuged at 17 500× *g* for 30 min. The supernatant was removed, and the pellet was allowed to air-dry for 20 min at room temperature. The pellet was dissolved in 2.5 µL fresh loading buffer (80% formamide, 1× TBE) and gently vortex mixed for 20 min. The sample was then denatured by heating at 90°C for 10 min and cooled in ice. Samples were loaded onto a CastAway® 8% pre-cast polyacrylamide, 1× TBE, 7 M urea gel (Stratagene, La Jolla, CA, USA) and electrophoresed for 45 min at 2000 V. The gel was then exposed on a storage phosphor screen for four days and subsequently read using a Storm™ phosphorimager (both from Molecular Dynamics, Sunnyvale, CA, USA).

Biochemicals used in the preparation of the buffers were obtained from Sigma (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA). Nucleotides were obtained from Amersham Biosciences (Piscataway, NJ, USA). Biotinylated primer oligonucleotide was obtained from Sigma-Genosys (The Woodlands, TX, USA), and rivanol was obtained from Sigma-Aldrich (St. Louis, MO, USA).

RESULTS AND DISCUSSION

Figure 1 shows the gel image obtained from the triplicate rivanol IC₅₀ experiment, in which the characteristic “ladder” of telomerase extension product is observed. The plot obtained from whole-lane quantitation of the rivanol IC₅₀ gel results give typical IC₅₀ profiles (Figure 2). The activity of telomerase in the presence of inhibitor was expressed as a proportion of the control activity and varied between 0 and 1. The proportions (*p*) were fit by nonlinear regression to the expression $p = 1/(1 + [I]/IC_{50})$, where [*I*] was the experimental inhibitor concentration and IC₅₀ was the fit parameter (concentration of inhibitor required to achieve 50% of the uninhibited activity). Analysis of the individual IC₅₀ fits obtained indicate a close agreement of IC₅₀ values, and the standard error values and correlation coefficients are within acceptable limits (Table 1). The data obtained from this experiment also compare favorably with the results from previously published rivanol IC₅₀ results using the

Dynabeads method (8.2 µM) (5). The amount of variation observed may be due to differences in inhibitor batch preparation. It is also possible that the difference in values is due to inaccuracies in the assay because of relatively lower signal.

The driving force behind developing this approach was to increase the number of compounds that can be conveniently assayed, while generating the results that only a direct assay can provide. The direct assay as described by Sun et al. (13) presents two problems

Figure 1. Triplicate rivanol IC₅₀ experiment gel image.

Figure 2. Plot of telomerase activity in the presence of varying rivanol concentrations (whole-lane quantitation). Results of three separate experiments are shown.

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when dealing with large numbers of samples (>8): (i) each sample processed uses 38.4 μCi labeled dGTP, which leads to unacceptably large amounts of radioactivity when large numbers of samples are processed (e.g., 1843 μCi for 48 samples); and (ii) isolating the telomerase products on magnetic beads becomes cumbersome when dealing with more than approximately eight samples, because each of 5–10 washing steps requires the physical manipulation of an individual Eppendorf tube containing the magnetic beads and isolated telomerase products.

We have modified this method by incorporating two improvements that allow the simultaneous processing of multiple samples (e.g., 20–100): (i) the reduction of the specific activity of the dGTP used, and therefore the total radioactivity, by 80%, which requires the use of the more sensitive detection

method of storage phosphorimager, as opposed to film autoradiography used in the previous publication (13); and (ii) the use of streptavidin-coated 96-well plates instead of magnetic beads to isolate and wash the telomerase products. These plates allow for fewer manipulations to wash the telomerase products and significantly reduce the amount of time for this step of the assay. We estimate an approximate 50%–75% savings in time by using this approach, which could be further increased by use of an automatic 96-well plate washing station.

We identified two factors necessary to improve the speed of the direct assay: (i) decrease the amount of physical manipulation required of the person doing the assay and (ii) decrease the amount of time needed for the signal to develop. The use of streptavidin-coated plates as opposed to beads addressed

this first factor. The second factor was addressed by using phosphorimager. The amount of exposure time needed to generate quantifiable signal was reduced from seven (13) to four days. Ideally, eliminating this exposure period completely would further accelerate the assessment of telomerase activity. This may prove challenging to do with direct assays, as their signal is, by their very nature, not amplified.

A separate series of experiments was conducted with the objective to avoid the gel analysis step completely by directly exposing and reading neutravidin wells having bound ^{32}P -labeled oligonucleotide products. However, the expected trend in activity was not observed because of high background signal attributable to nonspecific binding of $[^{32}\text{P}]\text{dGTP}$ to the well surface. Several blocking agents were used to reduce this nonspecific binding

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effect, as well as more thorough washing of the wells (>10 times) to allow direct reading of the wells, but these efforts were not successful. In addition to a range of pre-blocked plates, we also used a range of washing buffers to attempt to remove background counts. These included (i) standard assay buffer, (ii) 1% SDS in 0.5 M NaCl, and (iii) 25 mM Tris, 150 mM NaCl, 0.05% Tween® 20. Sonication was also attempted using these buffers, as well as gentle agitation. Unfortunately, none of these was able to remove sufficient background counts to allow direct visualization of the plates without the need for the gel analysis step. It should be noted that the oligonucleotides bound to these plates, when released using guanidinium HCl and quantitated using gel electrophoresis and phosphorimager, showed the typical trends as depicted in Figures 1 and 2.

This paper demonstrates the robustness of the streptavidin 96-well plate format for use in a high-throughput telomerase assay, as well as the option to use a lower amount of radioactivity in the assay procedure without affecting the quality of the data obtained. The use of 80% less radioactivity is made possible by using storage phosphorimager as opposed to film autoradiography, an inherently less sensitive technique (8). The reduction in the amount of radioactivity used effectively increases by a factor of five the number of samples that can effectively be processed. The second improvement that we have introduced is the use of streptavidin-coated plates for the isolation and washing steps. These allow for a more straightforward washing process, with no need for repeated cycles of magnetic bead separation and washing. The efficiency of the assay can potentially be further improved by the use of equipment to increase handling speed such as multi-channel pipettors and an automatic washing station for the rapid washing of the wells. In our laboratory, we have found that this assay combines the information on inhibition provided by direct assays with the potential for high-throughput provided by PCR-based methods. As such, it has become a key tool in our refinement of inhibitors against the anticancer target telomerase.

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