Measuring telomerase activity using label-free interaction analysis

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An approach is described using Biacore™ systems to develop a telomeric repeat elongation (TRE) assay for telomerase activity on a sensor surface. Extracts from human cell lines or tissue were assayed for telomerase activity by their capacity to extend biotinylated oligomers containing a telomeric repeat sequence immobilized on a sensor surface. This approach was used to make a comparative study of telomerase activity in normal human fibroblasts, several human cancer cell lines, and a number of normal and tumor tissue samples taken from human cancer patients. The TRE assay results showed that telomerase activity levels were 2- to 10-fold higher in tumor cells than in normal cells. TRE assays were also used to study the effects of two known telomerase inhibitors, demonstrating the potential value of this approach in therapeutic studies focused on telomerase.

Introduction

Telomerase is a specialized reverse transcriptase (RT) enzyme complex that maintains the termini of chromosomes, and has become the focus of growing biomedical research and interest by the pharmaceutical industry. The ends of chromosomes are characterized by G-rich tandem-repeating DNA sequences called telomeric repeats. In humans, these are typically 6 to 12 kb in length and are based on the hexanucleotide sequence, 5´-TTAGGG-3´. These DNA repeats are associated with a number of proteins, forming nucleoprotein structures known as telomeres at the ends of chromosomes. The telomere proteins provide physical capping of the DNA ends of the chromosomes and direct higher order nucleoprotein structure. Telomeres play a vital role in maintaining the integrity of chromosomes, protecting them from degradation and end-to-end fusions during DNA replication events that occur during successive cell divisions. See (1) for a comprehensive review of the biology of telomeres and telomerase.

During the complex process of replication, DNA polymerase is unable to create fully double-stranded ends on daughter chromosomes. This results in a progressive shortening of the telomeric repeats, by around 50 to 200 bp per cell division in somatic cells (2). Once the telomere length reaches a critical lower limit, the cell loses its ability to replicate, entering a state of senescence that usually leads to cell death. The shortening of telomeric repeats is the principal mechanism underlying the limited life span of somatic cells and one of the paramount factors that drives the aging process.

Much of the current interest in telomeres also derives from their key role in cancer biology. In comparison to normal somatic cells, the chromosomes of almost all cancer cells contain relatively short telomeres (typically 3 to 6 kb), but crucially, these are maintained through an indefinite number of cell divisions. Continuous cell division is one of the hallmarks of cancer cells and the resistance to telomeric erosion is likely to be a major factor in the immortalized phenotype of cancer cells.

This fundamentally different behavior of normal and cancer cells derives from the absence of any telomere maintenance function in normal somatic cells. In contrast, approximately 85% of all cancer cells (as well as germ cells and stem cells) express the telomerase ribonucleoprotein complex (3), which is able to repair and maintain the telomeres by replacing telomeric repeats lost during each round of DNA replication. The telomerase complex includes an RNA molecule that contains a sequence complementary to the single-stranded (ss) telomeric repeat sequence left on the lagging strand of the replicating chromosome. The resulting RNA-DNA hybrid then serves as a template for RT, which adds multiple ss telomeric repeats to the lagging strand via an alternating elongation-translocation mechanism (Fig 1). In approximately 15% of tumor cells that do not express telomerase, a less-well understood pathway (known as ALT) maintains the telomeric repeats.

The high level of current interest in the telomerase area reflects the fundamental roles of telomeres in normal cellular function and disease. The implications of this mechanism for the pharmaceutical and biotechnology industries are considerable. The inhibition of telomerase elongation is highly attractive for anti-cancer therapy and may provide a truly broad spectrum approach (4), which unlike anti-angiogenesis strategies, for example, is applicable to both solid tumors and blood cell malignancies. The complexity of the telomerase system presents a number of possible therapeutic targets, including the telomeric DNA-telomerase RNA component interface, the catalytic site of RT and telomeric proteins. Small molecules that stabilize the folding of self-associated G-tetrads into higher-order G-quadruplex structures at the ends of telomeres are also of interest, since it is known that these G-quadruplexes block access of the telomerase and thereby inhibit elongation (5).

Whereas inhibition of telomere elongation is the primary focus of these anti-cancer strategies, there is also considerable interest in the opposite approach, that is, inducing telomere elongation in somatic cells. This partly derives from the central role of telomere erosion in the general aging process, as well as in relation to specific disorders such as Werner syndrome, which is characterized by premature aging (6). The immortalization of otherwise normal somatic cells by ectopic telomerase expression (7) also has great potential for biomedical research, where the previous lack of maintainable, normal cells for longer term cell culture approaches has seriously impeded progress in many areas.

Because of its commercial potential, this area is of great interest in both academic research and the pharmaceutical and biotechnology industries. One practical problem of common interest to many working in this field is to find a sensitive, reliable and simple assay for telomerase activity. The most commonly used method to date has been a PCR-based method known as the telomeric repeat amplification protocol (TRAP) assay (8). This method has several inherent complications, such as the significant risk of PCR artifacts and cumbersome post-PCR procedures including separation of PCR products by gel electrophoresis and quantitation by densitometry, or phosphorimaging of radiolabeled DNA.

In this Application note, a telomerase assay developed by Dr. Chihaya Maesawa and colleagues is described (9). The TRE assay measures telomerase activity using a Biacore system by monitoring telomere elongation on a sensor surface, on which changes in mass concentration changes are measured, providing real-time, label-free measurement of the elongation of surface-bound oligonucleotides (10). The data show that the TRE assay is capable of measuring telomerase activity from a wide range of cell and tissue extracts, providing a concentration- and time-dependent readout of elongation rates. The results were similar to those from standard TRAP assays and moreover, the TRE assay was shown to have a number of significant advantages over the PCR-based method.

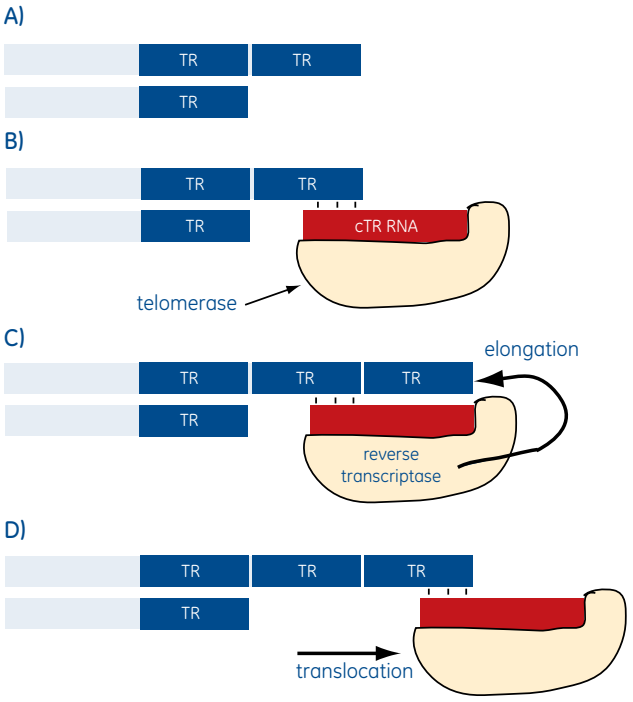


Fig 1. Elongation of telomeric repeats by the telomerase complex. The schematic picture illustrates the principle of telomerase action in maintaining telomere length during lagging strand DNA replication. TR = telomeric repeat sequence 5´- TTAGGG, cTR RNA = telomerase RNA including a complementary sequence to the TR. A) During replication, 3´ overhangs are left following lagging strand DNA synthesis and double-strand synthesis cannot be completed. This leads to shortening of the telomeres during successive rounds of cell division. B) The telomerase complex includes a short RNA sequence that aligns with complementary DNA in the overhanging TR sequence. C) Telomerase elongates the telomere overhangs via the RNA-dependent DNA polymerase action of the reverse transcriptase component of the complex. D) Following elongation, the telomerase complex translocates to align with the newly synthesized TR sequences and the next cycle of elongation occurs. After the requisite number of elongation/ translocation cycles are completed, remaining single strand DNA gaps are filled in by standard lagging strand synthesis.

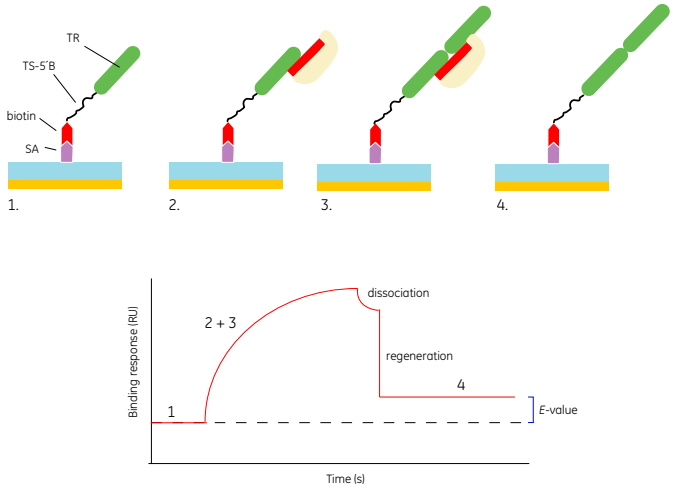


Fig 2. Basic design of the TRE assay. The upper panel shows events occurring on the sensor surface at various stages of the assay (numbered 1 to 4) and the effects of these stages as seen in the interaction profile over time are illustrated in the lower panel. SA = streptavidin, TR = telomeric repeat sequence (TTAGGG) present at the free end of the captured TS-5´B oligonucleotide (for clarity, only one of the tandem repeats is shown). 1) Capture of the oligonucleotide is seen as the baseline binding response. 2) Association of the telomerase complex with the oligomer via complementary RNA: DNA sequences. 3) TR motifs are added to the captured oligomers by the action of the reverse transcriptase component of the telomerase. Both stages 2 and 3 contribute to the binding signal during this period. Dissociation of telomerase is briefly seen at the end of this period during injection of running buffer, after which all protein is rapidly removed by injection of regeneration solution. 4) After regeneration, the binding response still remaining above the baseline is due to the increase in mass of the captured oligomers due to elongation of telomeric repeats (designated as the *E*-value).

Methods

A detailed description of the materials and experimental procedures used (such as cell culture conditions and telomerase extract preparation) can be found elsewhere (9), but some key information is presented below.

TRE assay

Oligomers (44-mers) containing four tandem telomeric repeats (TTAGGG) and biotinylated at either their 5´ (assay oligo TS-5´B) or 3´ (negative control oligo TS-3´B) ends were immobilized by high-affinity capture to the streptavidin surface of Sensor Chip SA. Immobilization was carried out by injecting each oligomer (0.125 µg/ml in 75 µl of 10 mM HEPES buffer pH 7.4, 150 mM NaCl and 10 mM $MgCl_2$) at 5 µl/min at 37°C. After 1500 RU (resonance units) of oligomers were immobilized on the sensor surface, excess DNA was removed by three 100 µl wash pulses with 1% SDS in 10 mM HEPES.

Telomerase extracts from cultured cells and tumor samples were diluted in a customized TRE buffer, consisting of the same buffer used for immobilization, but supplemented with 2.5 mM dNTP's and 10 mM EGTA. A range of telomerase extract dilutions were made and injected over the immobilized oligomer surfaces for various times, at a flow rate of 5 µl/min at 37°C. After the elongation reaction, all protein was removed by regenerating the surface with 1% SDS in 10 mM HEPES (100 µl pulse at 100 µl/min). Following regeneration, the flow rate was reduced to 5 µl/min and the relative response (in RU) between the post-regeneration and pre-telomerase extract injection levels was measured. This "*E*-value" was taken as the measure of telomere repeat elongation from the 3´ends of immobilized TS-5´B oligomers during the telomerase extract injection. TS-3´B served as a negative control for this assay, since elongation is not possible from its free 5´ end. All *E*-values were determined from three to six independent assays, with coefficients of variation below 5%. The strategy of the TRE assay is outlined schematically in Figure 2.

Microrecovery of elongated oligomers

In order to independently confirm the telomerase-dependent elongation indicated by the *E*-values, additional experiments were carried out in which oligomers were recovered from the sensor surface after the TRE assay. In these cases, TS-5´B was captured via an anti-biotin antibody immobilized on a sensor surface via standard amine coupling. Approximately 3000 RU of TS-5´B were captured and following the TRE assay, oligomers were recovered. After phenol/chloroform extraction, the DNA was then amplified by PCR and assayed for 6 bp repeats using a fluorescent DNA sequencing system.

Results

Preliminary verification of the TRE assay

Preliminary studies were undertaken to verify the basic assay strategy, using a known telomerase-positive colon cancer cell line. As shown in Figure 3, TRE assays carried out using the colon cancer cell line extract showed binding of extract components (presumably telomerase) to both TS-5´B and TS-3´B, but whereas *E*-values in the region of 100 RU could be seen for TS-5´B, no response above initial baseline levels was seen after regeneration for TS-3´B. These data indicate a telomerase extract-dependent elongation of immobilized telomeric repeat oligomers with a free 3´ end, which does not occur when an otherwise identical oligomer is biotinylated and captured via the 3´ end.

Further control experiments were carried out to verify the TRE assay, in which dNTP's or telomerase extract were omitted from the TRE buffer, or extracts were heat-inactivated prior to the assay. In the absence of dNTP's, some extract binding was observed to TS-5´B and a small, positive *E*-value was obtained, whereas heat inactivation or absence of telomerase extract resulted in no binding to TS-5´B and no apparent elongation. As a control against the possibility of non-telomerase DNA polymerase activities in the extracts, non-telomeric oligomers were also assayed, but did not show any significant *E*-values (see Table 1 for *E*-values for these control experiments).

In order to confirm that the *E*-values from the TRE assays derived from on-surface elongation of the oligomers, post-assay TS-5´B oligomers were recovered from the sensor surface to provide templates for PCR reactions using primers from a commercial TRAP assay kit. These experiments produced DNA fragments with incremental 6 bp differences, ranging in total length from 44 to 80 bp, and consistent with the addition of TTAGGG telomeric repeats to the TS-5´B oligomer (9).

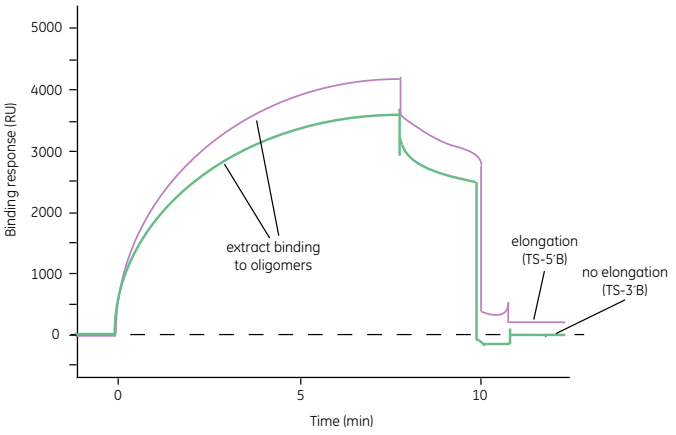


Fig 3. TRE assay using 5´and 3´biotinylated oligomers. The overlay interaction profile shows binding responses using oligomers TS-5´B (purple plot) and TS-3´B (green plot). Telomerase extracts bind to both oligomers, but only TS-5´B, which is biotinylated and captured at its 5´ end, is able to act as a template for telomerase-dependent elongation.

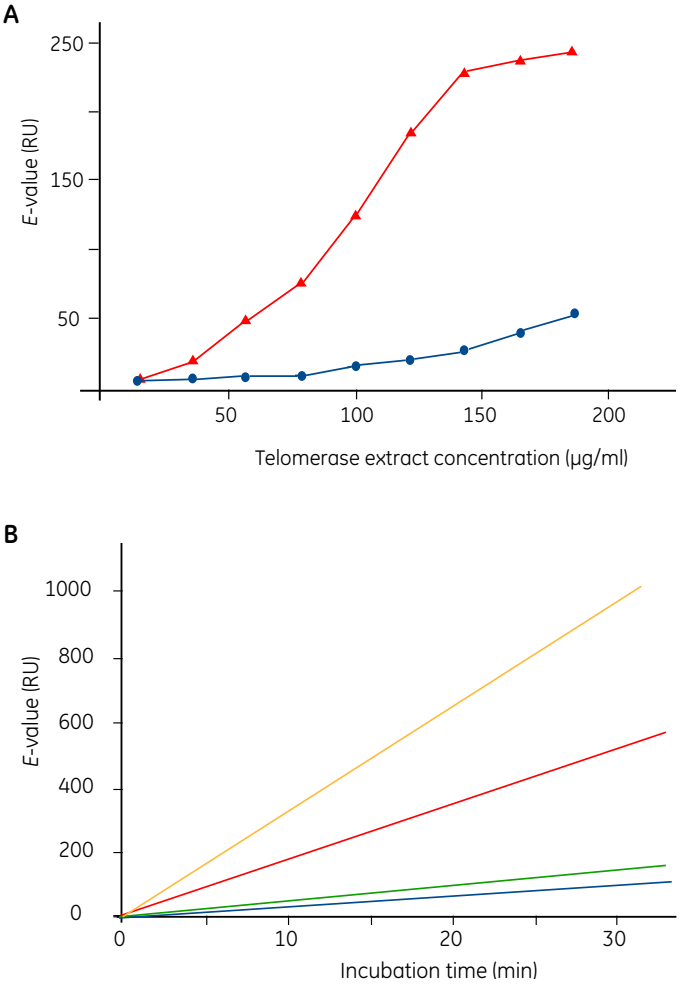


Fig 4. Dependence of *E*-values on telomerase concentration and incubation time in TRE assays. A) *E*-values after 10 min injections of telomerase extracts over a range of concentrations, from a normal fibroblast cell line (blue circles) and a colon cancer cell line (red triangles). B) Time-dependence of elongation using a fixed telomerase extract concentration of 100 µg/ml. Linear responses were observed using extracts from several sources. The data include extracts from two different normal fibroblast lines (blue and green plots), a colon cancer line (red plot) and a breast cancer line (yellow plot).

Table 1. Standard and control TRE assay results from a human colon cancer cell line. The SW 480 cell line was selected as a known high-level telomerase-expressing source for the preliminary experiments. The nontelomeric oligomer sequence was derived from the hTERT promoter and used as a control for general DNA polymerase activity in the telomerase extracts. In these studies, background levels were designated as 5 RU or less.

TRE assay conditions	Oligomer immobilized	*E*-value (RU)
Standard	TS-5´B	118
No dNTPs	TS-5´B	12
heat-treated extract	TS-5´B	Background
No extract	TS-5´B	Background
Standard	Nontelomeric	Background

Table 2. Telomerase elongation rates in different human samples. The sources derived from cultured cell lines (denoted*). In the case of tissue samples taken from patients with different types of cancer (denoted#), both tumor and normal mucosa samples were taken from each patient. The mean elongation rates are shown for the tumor samples only. Telomerase elongation rates in different human samples. For each of these cancer types, the T/N ratio range describes the range of relative elongation rates calculated for the matched tumor/normal mucosa samples from each patient. Abbreviations: n/a = not appropriate, n/s = not shown.

Compound	Elongation rate (mer/min)			
Source	Mean	(n)	Range	T/N ratio range
Breast cancer*	0.759	(3)	0.684–0.872	n/a
Esophageal cancer*	0.406	(5)	0.255–0.691	n/a
Gastric cancer*	0.407	(5)	0.206–0.739	n/a
Colon cancer*	0.411	(5)	0.231– 0.632	n/a
Fibroblasts*	0.102	(3)	0.086–0.113	n/a
Esophageal cancer#	0.464	(5)	n/s	6.6–37.7
Gastric cancer#	0.550	(5)	n/s	6.5–14.5
Colon cancer#	0.687	(5)	n/s	10.6–12.7

Quantitative aspects of the TRE assay

Given that the TRE assay was developed to be able to compare telomerase activities from different cells and tissues and to monitor the effects of telomerase inhibitors, studies were also carried out to establish the relationship of *E*-values with parameters such as telomerase concentration and effective incubation time. As shown in Figure 4A, *E*-values increased in a telomerase extract concentration-dependent manner for both the colon cancer and normal fibroblast cell lines (i.e. moderate-to-high and low-level telomerase-expressing cells) used in the preliminary verification experiments. These results indicate that the TRE assay should be applicable to a wide range of cells with different telomerase levels, but that the minimum extract concentration required to give an *E*-value above background levels (taken here as 5 RU) must be established for each cell type.

The effect of increasing the time of the telomerase injection (effectively increasing the incubation time for RT activity) was also investigated for a number of cancer and normal fibroblast cell lines (Fig 4B). In all cases examined, the relationship between extract injection time and *E*-value was linear from 1 to 30 min. Based on previous studies of on-surface RT-dependent elongation of oligomers using Biacore systems (10), a 1500 RU immobilization response was calculated to be equivalent to 1.8 ng (1.276×10^{-13} mol) of TS-5´B. The estimated binding response of a single nucleotide extension to all of the immobilized oligomers was therefore in the order of 33.8 RU, which is well above background level and readily observable in TRE assays. Since the results shown in Figure 4B establish that elongation rates are constant for a given telomerase extract over the course of the injection times used, this enables *E*-values from a TRE assay to be converted to an elongation rate (average increase in oligomer length per min).

The initial TRE assays were performed exclusively with established cell lines, but practical applications of a telomerase assay may well involve the analysis of heterogeneous tissue samples to try to identify telomerase-expressing tumor cells. Further experiments were therefore performed in which tumor cells were diluted into normal fibroblast cell suspensions prior to TRE analysis. These experiments established that the lower limit of detection was around one tumor cell per thousand normal cells (data not shown).

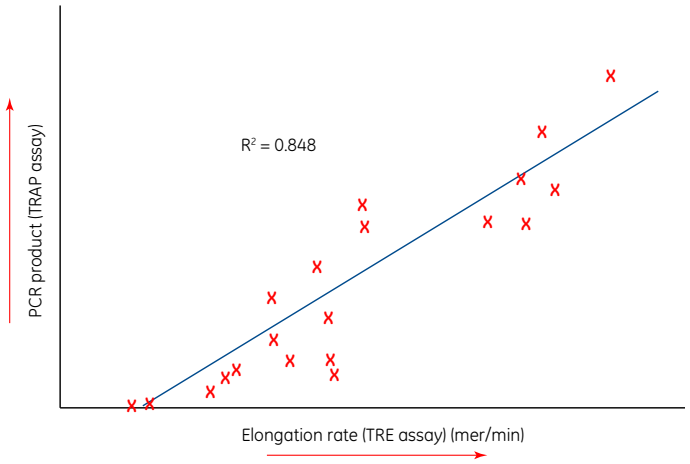


Fig 5. Comparison of results from TRAP and TRE assays. Telomerase extracts from the cancer cell lines used in the studies presented in Table 2 were also examined using a standard TRAP assay. Results from the TRAP assay (measured as total product generated) were plotted against the elongation rates derived from the TRE assays.

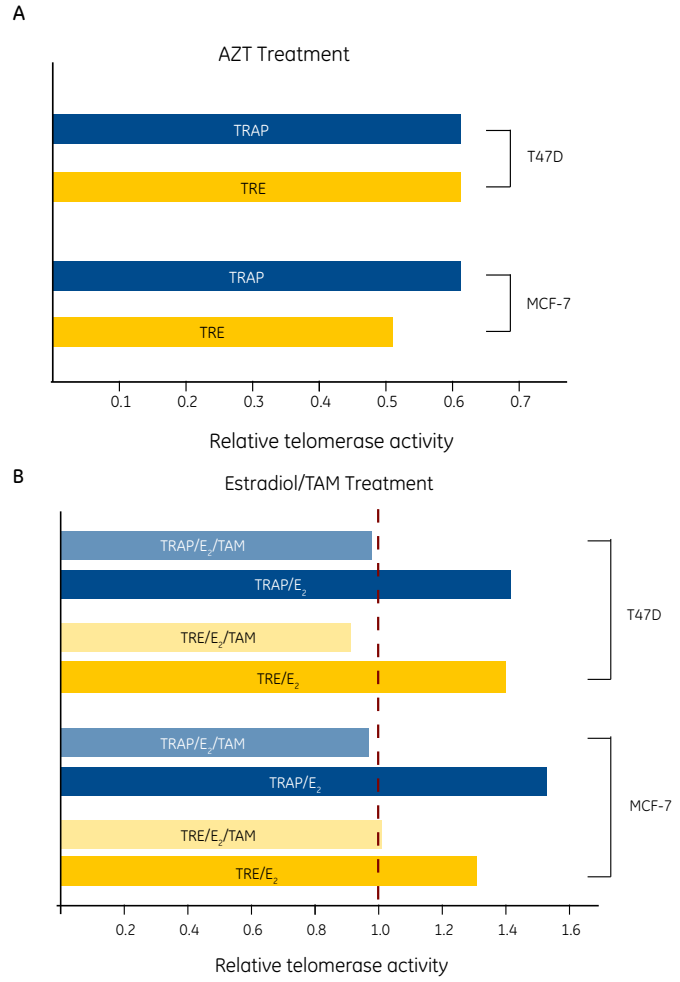


Fig 6. Inhibition of telomerase activity in breast cancer cells by AZT and TAM. The bar charts show the effects of telomerase inhibitors, expressed as relative telomerase activities, where a value of 1 = activity from untreated control cells. Inhibitor action was studied in the T47D and MCF-7 breast cancer cell lines, using both TRE (yellow bars) and TRAP (blue bars) assays. A) Effects of 0.1 nM AZT. B) Effects of TAM on estrogen-induced telomerase activity. Cells were treated with 10 nM estradiol, or with a combination of 10 nM estradiol and 10 nM TAM.

Comparative analysis of telomerase activity in multiple cell and tissue samples

Extensive TRE assays were performed using telomerase extracts prepared from three breast cancer cell lines, five examples each of esophageal, gastric and colon cancer cell lines, three normal fibroblast cell lines and a number of paired normal mucosa and tumor tissue samples taken from patients with esophageal, gastric and colon cancer. The elongation rates calculated from the TRE assays (Table 2) showed significantly higher telomerase activity in the cancer cell lines compared to the fibroblast cells; an average of eight-fold higher in the breast cancer lines and around four-fold higher in the other cancer cell types. Although the elongation rates varied within the limited numbers of individual cell lines derived from each type of cancer, the general patterns of cancer versus fibroblast cell line telomerase activity levels were as expected. Table 2 also shows the results from normal and tumor samples taken from cancer patients and in all cases examined, the elongation rate was significantly higher in the tumor samples compared to matched normal mucosa samples, with ratios ranging from 6.5-fold to almost 38-fold.

The TRE results from cancer and fibroblast cell lines were repeated using a standard TRAP telomerase assay (TRAPeze kit, Intergen, NY). For the cancer cell lines, the two assays delivered similar results (Fig 5). Interestingly, although telomerase activity could be readily quantified by the TRE assay using the fibroblast cell lines, these extracts failed to produce measurable telomerase activity in the TRAP assays. This may be due to the fact that visualization by TRAP laddering requires a minimum elongation length of six nucleotides and that the average elongation per oligomer derived from the fibroblast extracts was simply too low.

TRE assay analysis of telomerase inhibitors

One interesting generic approach to cancer therapy involves the use of telomerase inhibitors to induce senescence in rapidly proliferating tumor cells. The ability of TRE assays to monitor the effects of telomerase inhibitors was therefore examined, and compared directly to a standard TRAP assay. Two estrogen receptor (ER)-positive breast cancer cell lines were used for these studies, which included two known telomerase inhibitors. Azidothymidine (AZT) is an RT inhibitor and tamoxifen (TAM) is an estrogen antagonist used in breast cancer therapy. TAM functions as an indirect inhibitor of telomerase in ER-positive cells, where the action of estrogens otherwise stimulates telomerase activity. As shown in Figure 6, AZT inhibited telomerase activity by 50% to 70% in the two cell lines, as determined by TRE or TRAP assays. The two methods also yielded similar data in the TAM experiments, where stimulation of telomerase activity with estradiol (E_2) was inhibited to prestimulation levels using combined E_2/TAM treatment. These data show that the TRE assay is well-suited to studies of telomerase inhibitors.

Conclusions

Maesawa *et al*. have developed an assay to measure telomerase activity from cell or tissue extracts, using a Biacore system. Current developments in the fields of cell biology, cancer and aging mean that effective assays for telomerase activity and the effects of telomerase inhibitors are likely to become increasingly valuable tools for academic biomedical research and the pharmaceutical and biotechnology industries. Biacore based TRE assay has significant advantages over current PCR-based methods such as TRAP, allowing:

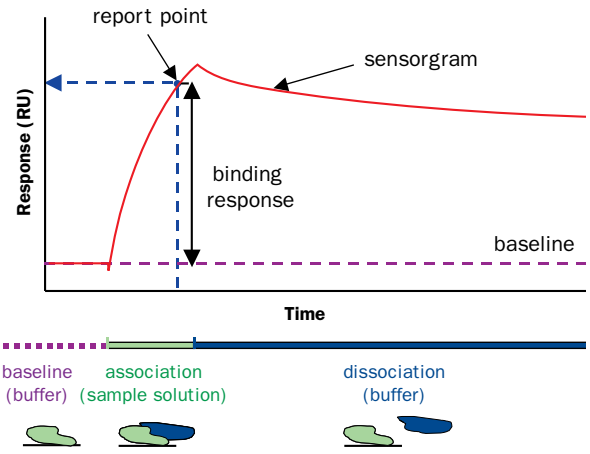
- Direct on-surface measurement of elongation rate (avoids post-PCR procedures and potential PCR artifacts)
- Label-free, automated, one-step analysis, providing a rapid, reproducible readout with real-time monitoring of the process
- Quantitative evaluation of telomerase activity from a range of sources, including both cancer and normal somatic cells

Although PCR is theoretically an extremely sensitive technology (TRAP has been reported to be able to measure telomerase activity from as few as 10 high telomerase-expressing cells), the TRE assay approach using Biacore systems is frequently more sensitive. This is particularly true for the analysis of somatic cells with very low telomerase activity. The TRE assay was able to provide reproducible elongation rate data from non-tumor cell samples, whereas the TRAP assay was unable to detect telomerase activity at these low elongation rates. The PCR approach may face additional potential problems in attempting to detect telomerase activity in somatic cells, since the concentration of the extracts would have to be increased significantly compared to those from cancer cells, and high protein concentrations inhibit the activity of thermostable DNA polymerases required for PCR (*Taq* in the case of standard TRAP assays).

The wide range of telomerase-related applications currently under investigation involves strategies to both inhibit and stimulate telomerase activity in many diverse cell types. As these applications develop, the requirement to accurately and reliably make quantitative comparisons of telomerase activities among a wide range of tissue and cell sources under diverse treatment conditions is likely to become increasingly important. The TRE assay developed by Maesawa *et al*., based on label-free interaction analysis using Biacore systems is well-suited to this role.

Monitoring protein interactions with Biacore systems

Biacore systems monitor protein interactions in real-time using a label-free detection method. One of the interacting molecules is immobilized onto a sensor surface, while the other is injected in solution and flows over the sensor surface. As molecules from the injected sample bind to the immobilized molecules, this results in an alteration in refractive index at the sensor surface that is proportional to the change in mass concentration. Using the phenomenon of surface plasmon resonance (SPR), these changes are detected in real-time and data are presented as a sensorgram (SPR response plotted against time). Sensorgrams display the formation and dissociation of complexes over the entire course of an interaction, with the kinetics (association and dissociation rates) revealed by the shape of the binding curve.



The sensorgram provides real-time information about the entire interaction, with binding responses measured in resonance units (RU). Binding responses at specific times during the interaction can also be selected as report points. More information can be found at **www.gelifesciences.com/biacore**.

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