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ORIGINAL PAPER

Real-time PCR detection of telomerase activity using specific molecular beacon probes

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Abstract Telomerase is a potentially important biomarker and a prognostic indicator of cancer. Several techniques for assessing telomerase activity, including the telomeric repeat amplification protocol (TRAP) and its modified versions, have been developed. Of these methods, real-time quantitative TRAP (RTQ-TRAP) is considered the most promising. In this work, a novel RTQ-TRAP method is developed in which a telomeric repeats-specific molecular beacon is used. The use of the molecular beacon can improve the specificity of the RTQ-TRAP assay, making the method suitable for studying the overall processivity results and the turnover rate of telomerase. In addition, the real-time, closed-tube protocol used obviates the need for post-amplification procedures, reduces the risk of carryover contamination, and supports high throughput. Its performance in synthetic telomerase products and cell extracts suggests that the developed molecular beacon assay can further enhance the clinical utility of telomerase activity as a biomarker/indicator in cancer diagnosis and prognosis. The method also provides a novel approach to the specific detection of some particular gene sequences to which sequence-specific fluorogenic probes cannot be applied directly.

Keywords Telomerase activity · Molecular beacon · Real-time PCR · Specific detection

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Introduction

Telomerase is a ribonucleoprotein complex with the functionality of a DNA polymerase. It catalyses the synthesis of telomeric repeats (5′-GGTTAG-3′ in vertebrate) at the 3′-ends of chromosomes, thereby preventing the loss of telomeric sequences at each cell division [1]. Telomerase is repressed in most human somatic cells, but reactivated in almost 85% of more than 3000 human malignant tumor biopsies [2–4]. Therefore, telomerase is a potentially important biomarker and prognostic indicator of cancer [5].

Several techniques to assess telomerase activity have been developed. The most promising one is the telomeric repeat amplification protocol (TRAP) assay [5]. This assay is based on the PCR amplification of in vitro telomerase products, and permits the telomerase activity to be gauged even in limited amounts of cancer tissues or cultured cells. Several modified versions of the TRAP assay have been developed [6–18]. However, most of these methods require complex post-PCR procedures, such as polyacrylamide gel electrophoresis and densitometry, to measure telomerase products. These post-PCR procedures are laborious and time-consuming, and also run the risk of carryover contamination.

The development of real-time quantitative TRAP (RTQ-TRAP) assays enables the procedure to be performed in a closed tube system [19]. It therefore eliminates the risk of

carryover contamination, provides good sensitivity, a wide dynamic range, and high-throughput applicability. To our knowledge, three RTQ-TRAP methods have been reported, which use SYBR Green [20–22], Amplifluor [23] or duplex scropion [24] as fluorogenic probes, respectively. In this work, we describe a novel RTQ-TRAP method obtained by using a specific fluorescent probe-molecular beacon; we term this the molecular beacon method. A molecular beacon [25–27] is an oligonucleotide hairpin with fluorophores and quenchers attached at its 5′- and 3′- termini. In the closed hairpin conformation, the fluorophore and the quencher are in close proximity, and the fluorescence signal is quenched by dissipating the energy as heat. In the presence of the complementary target, the molecular beacon will hybridize to the target and undergo a conformational change. Opening the hairpin separates the fluorophore and quencher, allows fluorescence to occur. In order to incorporate such a specific fluorescent probe into the RTQ-TRAP assay successfully, we adopt a particular PCR amplification protocol in which an extra extension template (EET) is used. Our results demonstrate that this simple molecular beacon assay can provide accurate telomerase activity data with great specificity, and is very effective at monitoring telomerase activity in cultured cells.

Experimental

Materials

The molecular beacon probe, PCR primers and EETs used in this paper are shown in Table 1. A synthetic DNA sequence, MTSR6 (5′-GACAATCCGTCGAACAGAGTTAG[GGTTAG]$_6$-3′), corresponding to the forward primer extended with six telomeric repeats, was used to simulate the telomerase product. The molecular beacon probe was synthesized and purified by Invitrogen Ltd. (Shanghai, China). MTSR6, EETs and PCR primers were synthesized and purified by Sangon Ltd. (Shanghai, China). Other biochemical agents were purchased from Dingguo Ltd. (Beijing, China).

Cell lines and preparation of telomerase extracts

HL-60 (leukemia) cells were kindly provided by Jiatong Chen (Common Lab of Cell Culture, College of Life Science, Nankai University, Tianjin, China). Cell extract containing telomerase was prepared as described previously [5]. Briefly, the cells (1×10^6) were lysed in 200 µL of CHAPS buffer (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% W/V glycerol, 0.5% CHAPS (3- [(3-cholamidopropyl) dimethylammonio] propanesulfonic acid), 0.1 mM PMSF (phenylmethylsulfonyl fluoride)). The mixture was incubated for 30 min in ice, then centrifuged at 14,000 rpm at 4 °C for 30 min. The supernatant was then stored at −70 °C. For the heat pretreatment control, 5 uL of extract was heated at 95 °C for 5 min.

Real-time PCR assay

All PCR reactions were carried out on a Rotor-Gene 3000 (Corbett Research, Sydney, NSW, Australia) with 25 µL of reaction mixture consisting of 1 × reaction buffer for Blend Taq, 5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.4 µM forward primer, 8 nM EET3, 0.4 µM RP1, 80 nM molecular beacon probe, 2 U of Blend Taq polymerase, and 1 µL telomerase extracts. After 30 min of incubation at 37 °C for the telomerase-meditated primer extension, the reaction mixtures were heated to 95 °C for 3 min to inactivate the telomerase activity, and then the samples were subjected to 43 cycles of PCR amplification under the following conditions: the first three cycles consisted of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s; the subsequent forty cycles consisted of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The fluorescence signal was monitored at 55 °C from the fourth cycle.

Table 1 Molecular beacon, PCR primers and extra extension templates

Molecular beacon, PCR primers, and EETs		
Molecular beacon	FAM-5′-CCGTGCTAACCCTAACCCTAACCCACGG-3′-DABCYL	
Forward primer	5′-GACAATCCGTCGAACAGAGTT-3′	
Extra extension template (EET)	EET1	5′-TAGAGCACAGCCTGTCCGTG(CTAACC)$_3$GG-3′
	EET2	5′-TAGAGCACAGCCTGTCCGTG(CTAACC)$_3$-3′-phosphorylation
	EET3	5′-TAGAGCACAGCCTGTCCGTGACGATACCGTG(CTAACC)$_3$-3′- phosphorylation
Reverse primer (RP)	RP1	5′-TAGAGCACAGCCTGTCCGTG-3′
	RP2	5′-GGTAGAGCACAGCCTGTCCGTG-3′
	RP3	5′-CGGGTAGAGCACAGCCTGTCCGTG-3′
	RP4	5′-GCCGGGTAGAGCACAGCCTGTCCGTG-3′

Results

Principle of molecular beacon assay

Figure 1 illustrates the principle of the molecular beacon assay. Compared with conventional TRAP assays, the distinctive feature of this method is the use of a telomerase products-specific molecular beacon probe. In order to incorporate the molecular beacon probe successfully, an extra extension template (EET) is used. This EET contains four regions: the 3′-end region (region 1) with complementary sequences of telomeric repeats; the 5′-end region (region 4) with the same sequence as the reverse primer; the region (region 2) with the same sequence as the 5′-arm of the molecular beacon (this region is located at the 5′-end of region 1); the region (region 3) with an arbitrary sequence, which is unrelated to the molecular beacon, the telomerase products, the reverse primer, and to the forward primer (this region is located between region 2 and region 4). During PCR amplification, when region 1 of the EET anneals to the 3′-end of a telomerase product, the telomerase product is extended by the Taq DNA polymerase along the EET. The complementary sequence of regions 2–4 of the EET is added to the 3′-end of the telomerase product, yielding a non-telomeric overhang at this end. In this step, the telomerase product will be extended only when its 3′-end anneals to EET. Otherwise, Taq DNA polymerase cannot extend it because of the mismatch between the 3′-end of the telomerase product and EET (the internal regions of the telomerase product anneal to EET for instance, see the example in parentheses in Fig. 1). In subsequent

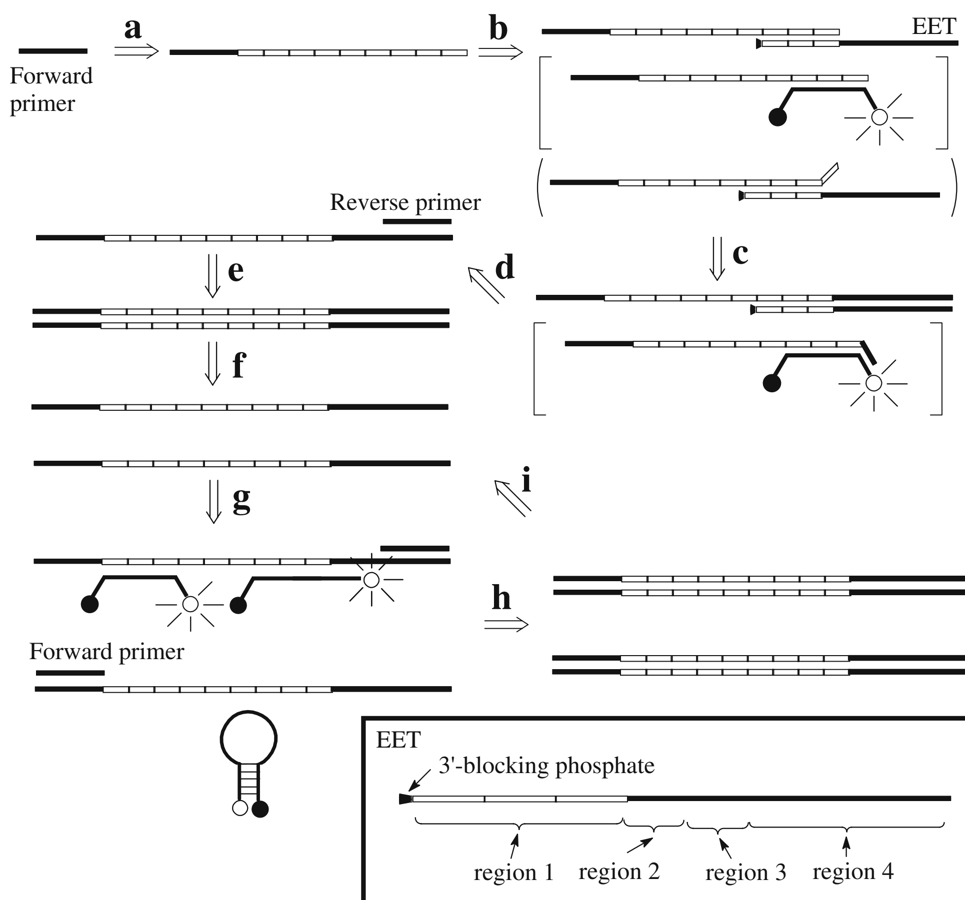

Fig. 1 Assay principle. (*a*) At the initial incubation step (37 °C for 30 min), telomeric repeats are added onto the 3′-end of the forward primer, and telomerase products are generated. (*b*) At the annealing steps of the first few cycles, the 3′-ends of telomerase products anneal to the 3′-end of EET. (*c*) The 3′-ends of the telomerase products are extended along EET in the presence of Taq DNA polymerase, and the resulting products can be used as PCR templates during subsequent cycles. (*d*) During the annealing steps of subsequent cycles, reverse primers anneal to the PCR templates. (*e*) The primers are extended by Taq DNA polymerase, generating double-stranded PCR amplicons. (*f*) The double-stranded PCR amplicons denature at the denaturing step of the next cycle. (*g*) At the annealing step, the loops (or the loop and 5′-arm) of the molecular beacons interact with their corresponding targets, generating fluorescence. Unhybridized molecular beacons maintain the hairpin structure, and no fluorescence occurs. At the same time, the primers (the forward primer and the reverse primer) hybridize with PCR templates. (*h*) The primers are extended by Taq DNA polymerase, and double-stranded PCR amplicons are produced. (*i*) The next cycle starts. *Inset*: diagram of EET

cycles, the non-telomeric overhang on the telomerase product can serve as a primer binding site for the reverse primer. Thus, using this extended telomerase product as a template, and the forward primer and the reverse primer as primer set, the PCR reaction runs in the normal amplification process. At the annealing step of each PCR cycle, molecular beacon probes bind to telomeric repeats to generate fluorescence, permitting the progress of the reaction to be followed in real time. During the linear phase of the reaction, the intensity of the resulting fluorescence correlates with the amount of accumulated telomeric repeats. The telomerase activity can be detected according to the inversely proportional relationship between the log number of the initial target sequence concentration and the threshold cycle (Ct) value, which represents the cycle number at which the emission intensity of the reaction rises above baseline.

Optimization of the method

To obtain optimal detection results, several important factors, such as the molecular beacons, EETs and reverse primers, were studied in detail, as described below.

Design of molecular beacons

In this method, a molecular beacon probe is used. This probe targets telomeric repeats; its loop and 5′-arm have the same sequences as region 1 and region 2 of the EET, respectively. Such a design may be favorable for the conversion of telomerase products to PCR templates. During the initial stage of PCR, even if a telomerase product anneals to the molecular beacon and is extended along it (see the example in square brackets in Fig. 1), the extended telomerase product is still perfectly complementary to the 3′-end of EET. It still has the opportunity to anneal to EET, and to be extended along EET in subsequent cycles. If the 5′-arm sequence of the molecular beacon is unrelated to EET, once the telomerase product anneals to the molecular beacon and is extended along it, the 3′-end of the extended telomerase product is then not complementary to EET. The product cannot then be extended along the EET, and it cannot serve as a PCR template (see the "Electronic supplementary material," Fig. S1). The number of templates available during PCR amplification will decrease and so the efficiency of PCR amplification will also drop.

In order to investigate the competition between the molecular beacon and EET during PCR, especially at the key steps in which telomerase products bind to EET and are extended by Taq DNA polymerase along EET, two sets of PCR amplifications were performed in parallel (Fig. 2). For the first set (reactions 1–3), the molecular beacon probe was added before initiating the reactions. For the second set (reactions 4–6), the molecular beacon probe was added after the first three cycles had completed. In the second set, where no molecular beacons were present at the key steps mentioned above, telomerase products can only bind to EET and be extended along it, and so the resulting products may serve as PCR templates in subsequent cycles. However, in the first set, because of the presence of the molecular beacon, EET has to compete with the molecular beacon when binding to telomerase products. If telomerase products bind to the molecular beacon and are extended along it, the resulting products have no binding sites for the reverse primer, and cannot serve as PCR templates in the next cycle. In this experiment the molecular beacon/EET molar concentration ratio is 10. Theoretically speaking,

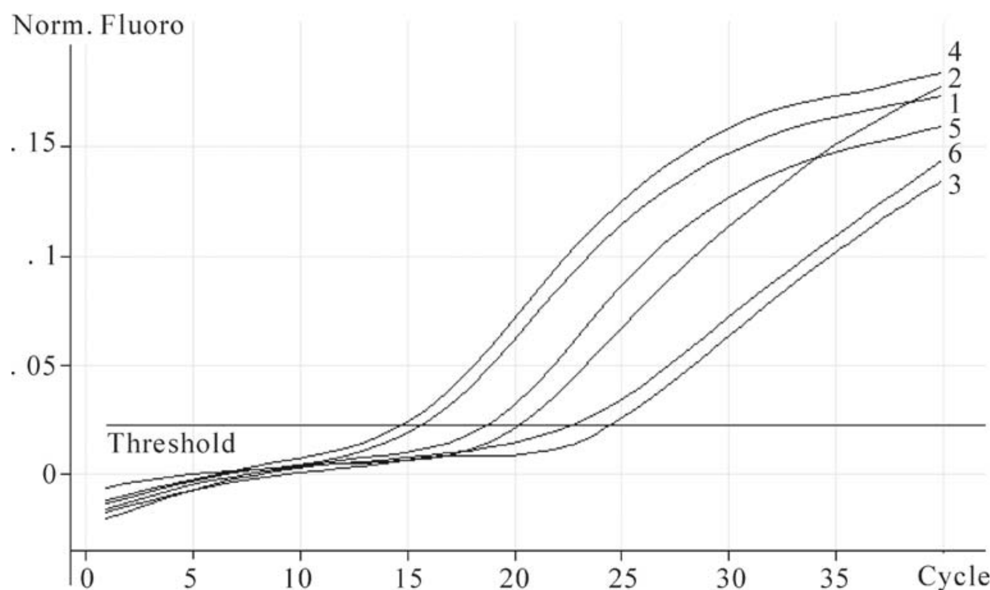

Fig. 2 The influence of the molecular beacon on PCR amplifications. In reactions 1–3, the molecular beacon was added before reactions. In reactions 4–6, the molecular beacon was added after the completion of the first three cycles. The reactions were initiated with different amounts of MSTR6: 10^3 amol (reaction 1 and reaction 4), 10^2 amol (reaction 2 and reaction 5), 10 amol (reaction 3 and reaction 6). The threshold was automatically set by the software of the Rotor-Gene 5. In this experiment, EET3 and RP1 were used

only about 10% of the telomerase products can bind to EET and convert to PCR templates. Thus, reaction 1, which initially has 10^3 amol MTSR6, should have a similar Ct value to reaction 5, which starts with 10^2 amol MTSR6. However, from Fig. 2, we can see that the Ct value of reaction 1 is between those of reaction 5 and reaction 4, which starts with 10^3 amol MTSR6. That is to say, over 10% of the MTSR6 converts to PCR templates in reaction 1. Supposing that the conversion efficiencies of MTSR6 in reactions 4–6 are all 100%, the conversion efficiency in reaction 1 can be calculated, and the result shows that the conversion efficiency is around 60.75%. Similarly, the calculated conversion efficiencies in reactions 2 and 3 are 43.50% and 46.80%, respectively. These results demonstrate the validity of our assumption above—that even if MTSR6 binds to the molecular beacon and is extended along it, the products still have the opportunity to convert to PCR templates. To simplify the manipulation, the molecular beacon probes were added before the reactions were initiated unless otherwise noted.

EET design

Phosphorylation of the 3′-end of EET In this method, EET is only used as an extra extension template for the conversion of telomerase products to PCR templates, so we name it EET. An important characteristic of this oligonucleotide is that a blocking phosphate is added to its 3′-end. This design serves two purposes:

(a) EET can bind to any conterminous three telomeric repeats on telomerase products during PCR amplification. If its 3′-end can be extended, a large number of shorter PCR products can be generated. The amount of binding sites for the molecular beacon will decrease, and the fluorescence signals will not reflect the genuine lengths of telomerase products (“Electronic supplementary material,” Fig. S2).
(b) The 3′-end of EET has the same sequence as the loop and the 5′-arm of the molecular beacon; the primer-dimers and the nonspecific PCR products formed by EET may also open the hairpin structure of the molecular beacon, generating nonspecific fluorescence signals. Depriving EET of its ability to extend by adding a blocking phosphate at its 3′-end will greatly reduce the formation of such primer-dimers and nonspecific PCR products.

To prove this assumption, the background fluorescence levels for three PCR amplifications containing individual EETs (EET1, EET2, and EET3) were compared (“Electronic supplementary material,” Fig. S3). EET2 and EET3 are all end-labeled with a 3′-blocking phosphate. EET1 is not end-labeled, but its 3′-end is extended with two bases of “GG.” In these three reactions, no PCR templates were added. If the specificities are good, no detectable increase in background fluorescence should be seen throughout the reactions. When EET2 or EET3 is used, this result is obtained. However, for the reaction using EET1, the background fluorescence sharply increases at the last stage of PCR. The addition of “GG” to EET1 can incorporate the mismatch between EET1 and telomerase products, preventing PCR-mediated elongation or shortening the telomerase products, and the PCR products reflect the original telomerase products with high fidelity. However, this EET still has the ability to extend during PCR, and the primer-dimer artifacts formed by it will contain binding sites for the molecular beacon, thus increasing the background fluorescence signal greatly. This result demonstrates that the modification of EET with a 3′-blocking phosphate can prevent its extension during reactions, and increase the detection specificity of RTQ-PCR.

Lengthening the EET The EET (EET3) we used consists of four regions. Regions 1 and 4 are prerequisite for the conversion of telomerase products to PCR templates. The addition of region 2 to EET3 depends on two considerations: one is used because it offers sufficient flexibility for the design of the molecular beacon. The sequence of region 2 can be arbitrarily adjusted according to the arm sequence of the molecular beacon, and other regions of EET do not affect the design of the arm sequence. The other is used to eliminate the competition between the molecular beacon and the reverse primer during PCR amplification. The addition of region 3 to EET3 is used to meet the design guideline for molecular beacons; that is, the distance of the 3′-end of the corresponding primer from the 5′-end of the molecular beacon should be greater than six nucleotides.

To demonstrate the advantages of lengthening the EET, two EETs (EET2 and EET3) with different lengths were compared (“Electronic supplementary material,” Fig. S4). EET2 is an end-labeled oligonucleotide with two regions (regions 1 and 4). Because of the absence of region 2, the 3′-end of the reverse primer must contain the same sequence as the 5′-arm of the molecular beacon, and there are some overlaps between their binding sites on PCR templates when EET2 is used in PCR amplification (“Electronic supplementary material,” Fig. S5). The competition between them results in various consequences:

1. The decrease in binding efficiency of the reverse primer to the PCR template leads to larger Ct values (Fig. 3)
2. The decrease in binding efficiency of the molecular beacon to the PCR template leads to lower fluorescence signal levels

In this work, we selected EET3 as the extra extension template.

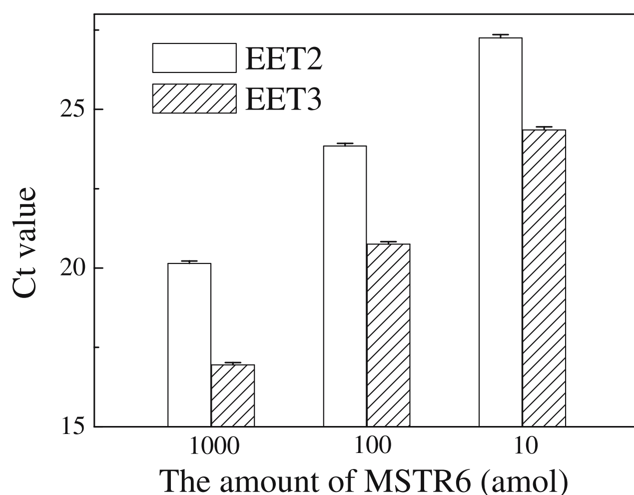

Fig. 3 Comparison of molecular beacon assays with different EETs (EET2 or EET3). Values are means ± SD (n=4)

Design of reverse primers

Because of possible competition between EET3 and the reverse primer, the PCR amplification efficiency can drop, especially during the initial stage of the PCR reaction. In order to increase the priming efficiency of the reverse primer, we designed four reverse primers (RP1-RP4). RP1 has the same sequence as region 4 of EET. In RP2–RP4, two-, four- or six-nucleotide “anchors” are added to the 5′-end of RP1, respectively. When a long reverse primer (for example RP4) is used in PCR, it can bind to a PCR template and be extended along this template. At the same time, the PCR template may also be extended along the 5′-anchor of RP4, yielding a six-nucleotide overhang at its 3′-end. Thus, in subsequent cycles, the RP4 binding site on the template is elongated. Because the “anchor” has no relationship with EET3, the EET3 binding site on the template does not change. Therefore, the competitive ability of RP4 can improve, producing an increase in the priming efficiency (“Electronic supplementary material,” Fig. S6).

To prove this assumption, four molecular beacon assays were performed, using RP1–RP4, respectively (“Electronic supplementary material,” Fig. S7). If our assumption is correct, the longer the reverse primer, the smaller the Ct value. However, the experiment shows a different result. The reaction using RP1 gives the smallest Ct value, while the reactions using RP3 or RP4 give similar Ct values that are larger than that of the reaction using RP2 (Fig. 4). This result suggests that when the annealing temperature of PCR is low enough, the key factor that affects the priming efficiencies of the reverse primers is the binding kinetics, not the binding stability. A shorter reverse primer can bind to the PCR template with higher speed, and more reverse primers can anneal to PCR templates when the temperature is reduced rapidly from the denaturing temperature to the annealing temperature. As soon as the annealing of the reverse primer to PCR templates occurs, the reverse primer is extended, and its binding stability increases gradually. However, the reverse primer should not be made too short, otherwise its melting temperature will be lower than the annealing temperature of PCR, and it will not be able to anneal to the PCR templates. In this work we selected RP1 as the reverse primer for use in subsequent experiments.

The performance of the molecular beacon assay in MTSR6

To investigate the linearity of our molecular beacon assay, eight reactions, each initiated with different numbers of copies of MTSR6, were carried out in parallel. A strong linear relationship between the Ct values and the log(number of copies of MTSR6) is found, and the linear relationship holds over at least seven orders of magnitude, from 10^4 amol to 10^{-2} amol of MTSR6. The correlation coefficient of the calibration curve is consistently 0.99 or greater. The assay is specific for this synthetic telomerase product, MTSR6, as shown by the high Ct values of the no-template control (NTC), which is consistently larger than 40 (Fig. 5). This result demonstrates that the molecular beacon assay developed here can be used to quantify synthetic telomerase products over a broad dynamic linear range.

The performance of the molecular beacon assay in cell extracts

In order to investigate the performance of the molecular beacon assays in cell extracts, HL-60 cell extracts was prepared and used in a molecular beacon assay (Fig. 6). The amplification plots reveal that the amplification depends on the cell number. There is a perfectly linear

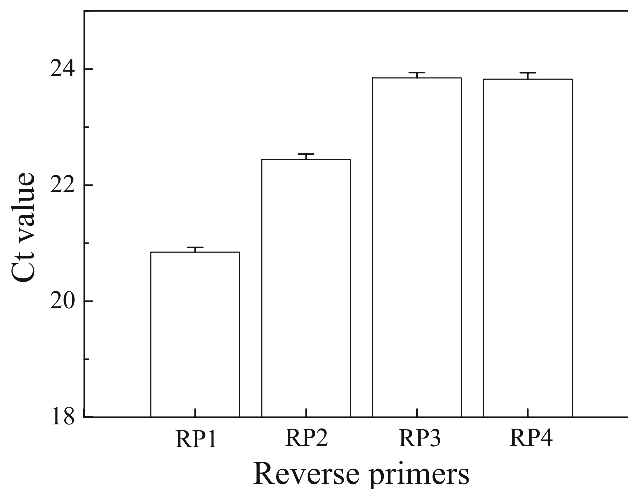

Fig. 4 Comparison of the results from molecular beacon assays with different reverse primers. All reactions were initiated with 10^2 amol MSTR6. Values are the mean ± SD (n=4)

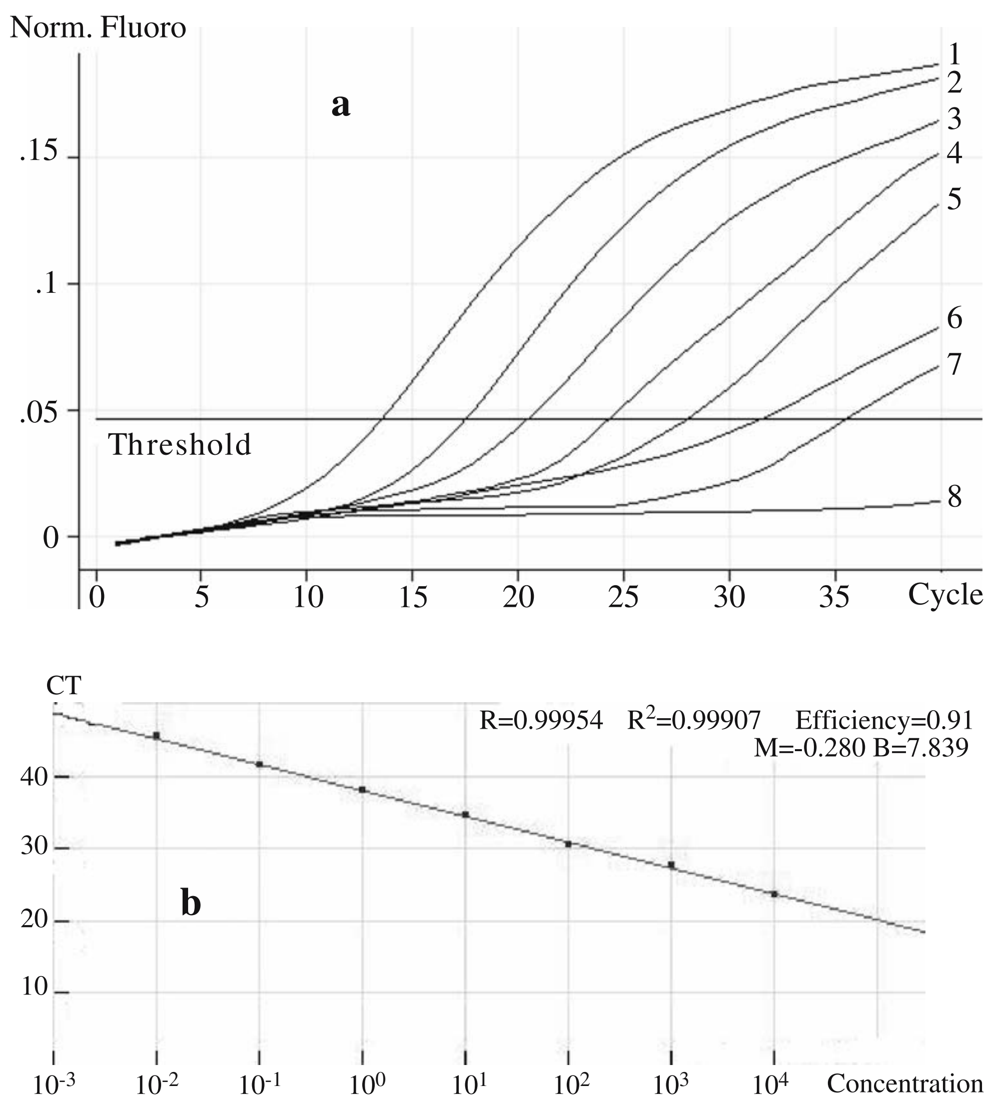

Fig. 5 Amplification plots (**a**) and standard curve (**b**) of the molecular beacon assay for the synthetic telomerase product MTSR6. Serial ten-fold dilution of MTSR6 from 10^4 amol to 10^{-2} amol (traces *1*–*7*) was used in each reaction. Trace *8* was a no-template control in which 1 μL water was used instead of MTSR6. The threshold was set automatically by the software

relationship between the Ct value and the log number of analyzed cells. The good dynamic linear range extends from 10^4 to five cells, and the correlation coefficient of the calibration curve is consistently over 0.99. Correspondingly, the Ct value is consistently larger than 40 for the reactions initiated with heat pretreatment control. In Fig. 6, trace 7 represents a positive control in which 10^2 amol of MTSR6 and 1 μL of heat pretreatment cell extract were added. We can see that its Ct value is larger than that of the reaction initiated with the same amount of MTSR6 alone (Fig. 5, trace 3), which may be attributed to the presence of Taq DNA polymerase inhibitors in the cell extracts. The coefficient of variation (CV) was also determined for each dilution as an indicator of the interassay variation of our method. As the degree of dilution increased, the accuracy of this assay gradually decreased: the CVs were 7.5%, 9.2%, 11.0%, 13.1%, and 14.9% for dilutions with 10^4, 10^3, 10^2, ten and five cells per assay, respectively. Telomerase activity is also frequently detected in single-cell dilutions, but the results are less reproducible. These results demonstrate that the molecular beacon assay developed here can be used to quantify the telomerase activity in cell extracts with a sensitivity comparable to that obtained using the SYBR Green method reported previously [20].

In order to investigate the suitability of the molecular beacon assay for telomerase activity quantification, a series of HL-60 cell line samples with well-known cell numbers were analyzed as unknowns. As shown in Fig. 7, the molecular beacon protocol was validated due to the good agreement between the actual cell numbers and the estimated cell numbers over a wide range of cell numbers, and the agreement is better for higher cell numbers than for lower cell numbers. This, combined with the results mentioned above, shows that telomerase activity measurements should be performed using the molecular beacon method on extracts with relatively high cell equivalents.

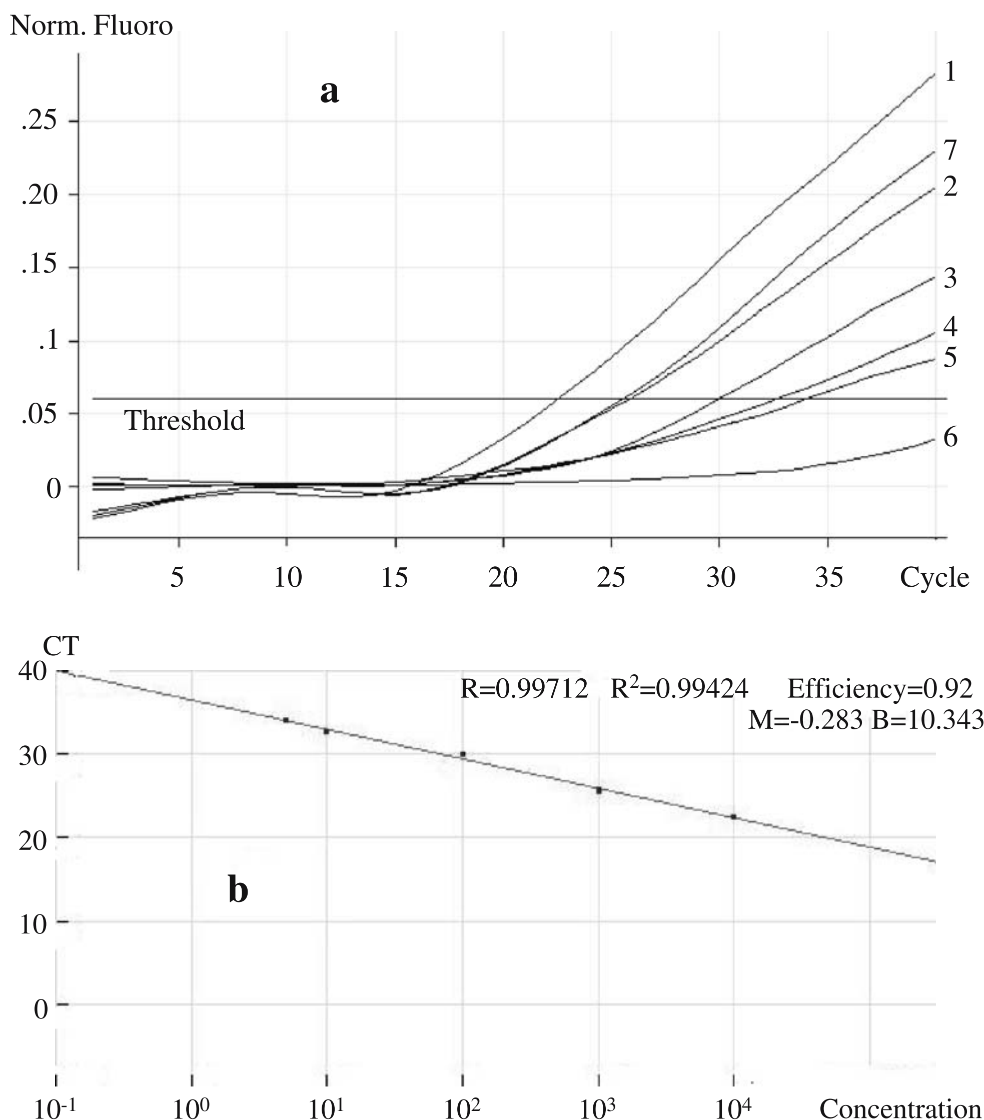

Fig. 6 Amplification plots (**a**) and standard curve (**b**) of the molecular beacon assay for cell extracts. Traces *1–5* represent cell numbers of 10^4, 10^3, 10^2, 10 and 5, respectively. Trace *6* is a negative control in which 1 μL of heat pretreatment cell extract was used. Trace *7* is the positive control, in which 10^2 amol MTSR6 and 1 μL of heat pretreatment cell extract were added. The threshold was set automatically by the software

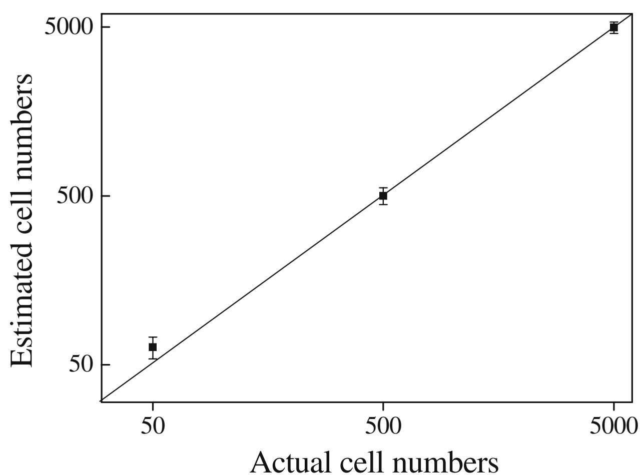

Fig. 7 Correlation between true and estimated HL-60 cell numbers (as determined by the molecular beacon assay). *Line* represents perfect correlation. Five replicates were determined for each point

Discussion

A robust, valid, and easy-to-use method of detecting and quantifying telomerase activity in cell culture samples and tissue biopsies would aid research into telomere biology and carcinogenesis greatly. RTQ-TRAP methods are believed to be the most promising ones for this purpose. RTQ-TRAP is a quantitative telomerase activity assay method based on the PCR amplification of in vitro telomerase products. As for PCR, its specificity is mainly dependent on the binding specificity of the primers to the templates. However, if primer-dimers and other nonspecific reaction products are formed in significant amounts, these spurious amplifications may affect its detection sensitivity. RTQ-TRAP is based on a PCR system in which the formation of primer-dimers is nearly unavoidable. The two RTQ-TRAP methods commonly used today use SYBR Green or Amplifluor as fluorogenic probes. SYBR Green is a

double-stranded DNA interrelated dye which will bind to any double-stranded DNA in the reaction, including primer-dimers and other nonspecific reaction products. Amplifluor is a PCR primer with a hairpin structure at the 5′-end containing a fluorophore and quencher [28]. This probe also has no specificity for the target PCR products [29, 30]. In these two methods, the detection specificity only depends on the binding specificities of PCR primers to templates. Therefore, a possible concern when using these two methods could be the false-positive signals caused by the primer-dimer artefacts. To address this problem, another target-specific element can be added. Fluorogenic probes such as TaqMan probes [31, 32] and molecular beacons are often regarded as good choices. However, because of the sequence's particular telomerase reaction products, it is difficult to directly incorporate such specific fluorogenic probes into TRAP assays. If such a specific probe is used, it should either target telomeric repeats or use telomeric repeats as a probe sequence. When the probe targets telomeric repeats, its probe sequence must be same as or similar to the reverse primers. During the annealing step of the PCR, the priming efficiencies of the reverse primers will drop because of the competition between the probe and the reverse primers. This effect may be more serious during the initial stage. In addition, the primer-dimers formed by the reverse primers will cause a significant increase in the background fluorescence. When the probe uses telomeric repeats as the probe sequence, the probe sequence will be complementary to that of the reverse primers. At the annealing step of the PCR, the probe may preferentially bind to the reverse primers, generating an undesired fluorescence signal.

To address this problem, a duplex scorpion method has been reported by our research group [33]. Huang et al. [24] further optimized the method and termed it DS/TP-TRAP. In this work, we present a novel specific RTQ-TRAP method. Using a particular PCR protocol which makes use of an additional EET, a telomerase products-specific molecular beacon probe is successfully incorporated into the RTQ-TRAP assay. Use of the molecular beacon can improve the specificity of the RTQ-TRAP assay by reducing the interference from nonspecific PCR products. If the nonspecific products do not contain the target sequence of the probe, no hybridization will occur between them and no fluorescence signal will be produced. The 3′-end of EET has the same sequence as the loop of the molecular beacon, and the primer-dimers formed by it can interact with the molecular beacon, producing nonspecific fluorescence signals. This effect can be reduced by adding a blocking phosphate to the 3′-end of EET. EET is only used as an extra extension template to convert telomerase products to PCR templates. A relatively low concentration of it is needed to do this. In this work, the concentration is only 2% of that of the reverse primer (RP1); such a low concentration of EET3 can further reduce the formation of corresponding primer-dimers. When RP1 is used as the reverse primer, it can be extended during PCR and is present in reaction solutions at relatively high concentrations. However, its sequence has no relationship with that of the molecular beacon, and the primer-dimers formed by it cannot open the hairpin structure of the molecular beacon. The results from assays for synthetic telomerase products and cell extracts demonstrate that our molecular beacon assay permits the rapid and reliable quantification of telomerase activity without primer-dimer interference. The specificity of the detection can be partly quantified by the corresponding PCR efficiency (E), which is calculated according to the formula: $E = \left[10^{(-1/g)}\right] - 1$, where g=1/m, and m is the slope of the corresponding calibration curve. In the ideal case, E=1. However, in practice, the value of E cannot reach 1, because the binding efficiencies of PCR primers to templates are generally below 1. If the value of E exceeds 1, the most probable reason for this is that the formation of nonspecific PCR products contributes to an increase in the fluorescence signal. In our work, the values of E are 0.91 (Fig. 5b) and 0.92 (Fig. 6b) for synthetic telomerase products and cell extracts, respectively. This result demonstrates that the effects of nonspecific PCR products has been reduced to low levels, which is an improvement over the SYBR Green method that reported previously [20].

The molecular beacon we used targets telomeric repeats; any conterminous three telomeric repeat can open its hairpin structure, thus enabling the probe to emit a fluorescence signal. During the initial incubation step of the assay, telomerase added telomeric repeats to the 3′-end of the forward primer. If the number telomeric repeats added exceeds six, the resulting PCR templates may contain more than two molecular beacon binding sites. In other words, one PCR template may open more than two molecular beacon probes, generating a stronger fluorescence signal. This may provide better sensitivity than conventional molecular beacon assays.

There is the possibility that two molecular beacons may be located next to each other on the templates. In this case, the quencher of one probe can be located in close proximity to the fluorescent dye of the other probe. Although primary experiments show that this possibility hardly affects results, this approach may be further improved by replacing the molecular beacon with a TaqMan-MB probe that we have reported [34].

When quantifying telomerase activity, there are two aspects that need to be considered: the first is the processivity of the telomerase (how many telomeric repeats the enzyme adds to the substrate); the second is the turnover rate of telomerase (how many substrate molecules are extended by telomerase) [35]. Based on these considerations, our molecular beacon method, together with other

three RTQ-TRAPs, can be classified into two groups. The first group includes our molecular beacon method and the SYBR Green method. In these two methods, the fluorescence signals are directly correlated with the telomeric repeats the enzyme adds to all substrates. In other words, the fluorescence signals reflect the overall processivity results and the turnover rate of telomerase. The other group consists of the Amplifluor method and the DS/TP-TRAP method. In these two methods, the fluorescence signals can only reflect the turnover rate of telomerase; in other words, the fluorescence signals can only reflect the number of the substrates that are extended by telomerase, no matter how many telomeric repeats are added to these substrates.

An important advantage of the Amplifluor system is the convenience of incorporating an internal standard into the detection system [36]; here, the primers used to amplify the internal standard can be replaced by amplifluors labeled with other dyes, which emit fluorescence at wavelengths other than those produced by the telomerase products. It is well known that multiplex detection ability is an attractive characteristic of molecular beacons that has been widely used in allele discrimination [37, 38] and the simultaneous detection of several targets [39]. Therefore, an internal standard can also be easily incorporated into our molecular beacon method. The internal standard could consist of two oligonucleotides, one an internal standard template, the other an internal standard probe. The internal standard template can be amplified by the same primers that recognize the PCR templates converted by telomerase products. The internal standard probe is another molecular beacon that targets the internal standard template and it could be labeled with different dyes (e.g., TAMRA, JOE, Texas red). Thus, any inhibitors in the sample that affect the amplification of the telomerase products can be detected. This may be an advantage of the molecular beacon method over the SYBR Green method.

The performances of the molecular beacon assay in synthetic telomerase products and cell extracts were investigated. The data obtained demonstrate that our molecular beacon assay permits the rapid and reliable quantification of telomerase activity without primer-dimer interference. The real-time, closed-tube protocol obviates the need for post-amplification procedures, reduces the risk of carryover contamination, and supports high throughput. It can be used as a good alternative method for qualifying telomerase activity, especially when the overall processivity results and the turnover rate of telomerase are required. Such an improved and optimized TRAP assay further enhances the clinical utility of telomerase activity as a biomarker/indicator in cancer diagnosis and prognosis over a wide range.

In addition, this method also provides a novel approach to the specific detection of some particular gene sequences to which sequence-specific fluorogenic probes cannot be applied directly.

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