PAPER

NJC
www.rsc.org/njc

Real-time detection of telomerase activity utilizing duplex scorpion primers and hairpin-like reverse primers

Yan-Ping Huang,[ab] De-Ming Kong,[a] Qi-Meng Chen,[b] Han-Xi Shen*[a] and Huai-Feng Mi[ac]

[a] *State Key Lab of Functional Polymer Materials for Adsorption and Separation, Chemical School, Nankai University, Tianjin, 300071 P. R. China. E-mail: hxshen@eyou.com; Fax: +86-22-23502458; Tel: +86-22-23505324*
[b] *College of Pharmacy, Tianjin Medical University, Tianjin, 300070 P. R. China*
[c] *Medical School, Nankai University, Tianjin, 300071 P. R. China*

Received (in St. Louis, MO, USA) 23rd May 2004, Accepted 8th September 2004
First published as an Advance Article on the web 15th November 2004

Telomerase, a ribonucleoprotein enzyme, is expected to be a new marker for cancer diagnosis. The development of the telomeric repeat amplification protocol (TRAP) and its modified versions have facilitated the detection of telomerase activity in small tissue and tumour samples. But most of these techniques require complex post-PCR procedures. As for the two real-time quantitative methods (SYBR Green and Amplifluor methods) reported so far, both use fluorogenic probes without specificity. To overcome these problems we developed a new real-time method for the detection of telomerase activity. In this method a duplex scorpion primer and reverse primers with hairpin-like structures were used. The use of duplex scorpion primers shows a series of advantages: the target-specific probe sequence provides higher specificity than the SYBR Green and Amplifluor methods; the unimolecular probing mechanism allow the assay to be conducted under fast cycling conditions and a single operation can be completed in 1.5 h; the closed-tube system reduces the risk of carryover contamination and supports high throughput. This method may be a useful tool to rapidly, specifically and precisely quantify telomerase activity.

Introduction

Telomerase is a ribonucleoprotein enzyme that elongates the telomeres in eukaryotic cells. It catalyses the synthesis of telomeric repeats (5′-TTAGGG-3′ in vertebrates) with the template of its RNA component.[1,2] Telomerase activity is readily detected in almost 85% of more than 3000 human malignant tumour biopsies, encompassing a broad range of cancer types, but is hardly present in premalignant lesions or in normal tissue samples.[3–6] Therefore, telomerase activity is expected to be a new diagnostic and prognostic marker of human cancer. In recent years a telomeric repeat amplification protocol (TRAP)[7] and many modified versions[8–17] have been developed for detection of telomerase activity in a wide variety of human tumours and tumour-derived cell lines. However, most of these techniques require complex post-PCR procedures, which are laborious and time-consuming and furthermore, have the risk of carryover contamination.

The developed real-time quantitative TRAP assays do not require the post-amplification procedures, can provide a closed-tube detection format, reducing the risk of carryover contamination, and support high throughput. Up to now two real-time methods for telomerase activity detection have been reported, using SYBR Green[18,19] or Amplifluor,[20] respectively. SYBR Green is a DNA interrelated dye; the strength of fluorescence will be enhanced by its binding to double-stranded DNA, but it cannot distinguish specific and non-specific PCR products.[21] Amplifluor is a probe system consisting of a molecular beacon with an amplimer attached to the 5′-end.[22] This probe also has no specificity for the target PCR products and the method cannot be accurately described as a specific approach.[21,23]

To overcome the above-mentioned problems, we have developed a new real-time method for detection of telomerase activity. In this method a duplex scorpion primer and reverse primers with a hairpin-like structure are used. The duplex scorpion primer consists of two oligonucleotides with different lengths. The longer one is the primer-probe strand (PPS), a forward primer linked a telomerase product-specific probe sequence to its 5′-end *via* a PCR blocker. A donor fluorophore, FAM (6-carboxyfluorescence), is labelled at the 5′-end of the probe sequence. The shorter one is the quencher strand (QS), which is complementary to the probe sequence of PPS and labelled with a nonfluorescent quenching moiety, DABCYL [4-(4′-dimethylaminophenylazo)benzoic acid] at the 3′-end. In the duplex scorpion primer formed by PPS and QS, FAM and DABCYL are kept in close proximity to each other and the fluorescence of FAM is quenched. After extension of the PPS during the telomerase substrate elongation step and subsequent PCR amplification, the specific probe sequence is able to bind to its complement within the same DNA strand, and a hairpin structure is formed in an intramolecular manner, which is favoured over the intermolecular binding of PPS and QS, leading to production of the fluorescent signal (Fig. 1). The use of the PCR blocker can prevent undesirable read-through of the probe sequence by Taq DNA polymerase,[24] to eliminate the fluorescence produced by unspecific PCR products. The reverse primer with a hairpin-like structure is used to increase the specificity of PCR amplification.

Materials and methods

Cell lines and preparation of telomerase extracts

HL60 (leukaemia) cells were kindly provided by Jiatong Chen (Common Lab of Cell Culture, College of Life Science, Nankai University, Tianjin, P. R. China). The cells (3×10^6) were lysed in 200 μl of CHAPS buffer {10 mM Tris-HCl, pH 7.5, 1 mM

DOI: 10.1039/b407796b

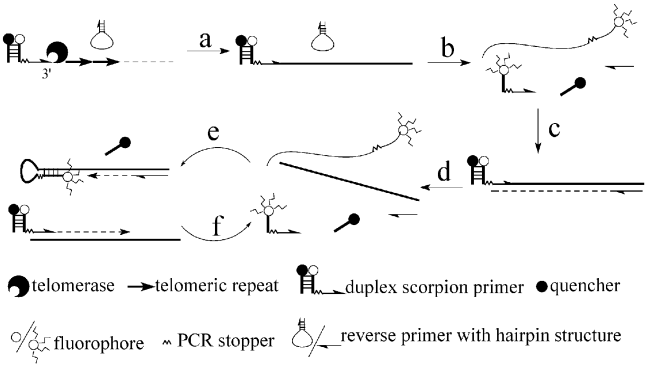

Fig. 1 Working principle of duplex scorpion primers. (a) At the initial incubation step (30 °C for 30 min), telomeric repeats are added onto the 3′-end of duplex scorpion primers by telomerase. (b) At high temperature (95 °C for 3 min), telomerase is inactivated. At the same time, the telomerase product and primers are denatured and the first PCR cycle starts. (c) By reducing the temperature (from 95 to 45 °C), the reverse primer anneals on the telomerase product and the complementary strand is synthesized in the presence of Taq DNA polymerase. The probe sequence of PPS cannot be copied with the presence of the PCR stopper. (d) Increasing the temperature again to start the second PCR cycle, the obtained partly double-stranded PCR products denature at high temperature (94 °C). (e) At low temperature (45 °C), the probe sequence hybridises to its target in an intramolecular manner, the fluorophore and the quencher separate completely, and a fluorescent signal is produced. At the same time the primers (including reverse primer and duplex scorpion primer) are annealed to the template and PCR amplicons are produced. (f) The next cycle starts.

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 was centrifuged at 13 000 rpm at 4 °C for 20 min; the supernatant was stored at −70 °C.[7]

Probe and primers

Two oligonucleotide strands of the duplex scorpion primer were designed as follows: PPS = FAM-5′-$[CCCTAA]_3$-blocker-AATCCGTCGAGCAGAGTT-3′ (PCR blocker is a strand with three carbon atoms); QS = 5′-$[TTAGGG]_3$-3′-DABCYL. Custom oligonucleotides were synthesised and purified by Shanghai Shengyou, Ltd. (Shanghai, P. R. China). A linear reverse primer[25] and three reverse primers with stem-loop structures, whose sequences are shown in Table 1, were synthesised and purified by Sangon, Ltd. (Shanghai, P. R. China). Other biochemical agents were all purchased from Sangon, Ltd. (Shanghai, P. R. China).

Real-time PCR assay

All PCR reactions were carried out on an ABI GenAmp 5700 thermal cycler (Applied Biosystems) with 25 μl of a reaction mixture consisting of 1 × TRAP buffer[7] (20 mM Tris-HCl, pH 8.3, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 0.1 mg ml^{-1} BSA), 1.5 mM $MgCl_2$, 50 μM each dNTP, 2 U of Taq polymerase, 0.5 μM PPS, 1 μM QS, 0.05 μg of reverse primer and 2 μl telomerase extracts. After 30 min incubation at 30 °C for telomerase-meditated primer extension, the reaction mixtures were heated to 95 °C for 3 min to inactivate telomerase activity, then the samples were subjected to 26 cycles for amplification (94 °C for 0 s, 45 °C for 3 s). Telomerase extract aliquots, which were inactivated by heat at 75 °C for 20 min, were used as a negative control.

Table 1 Primer sequences[a]

Primer name	Oligo sequence
ACX	5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′
hACX1	5′-*GGTTAG*CTTACCCTTACCCTTACC*CTAACC*-3′
hACX2	5′-*GGTTAGG*CTTACCCTTACCCTTAC*CCTAACC*-3′
hACX3	5′-*GGTTAGGG*CTTACCCTTACCCTTA*CCCTAACC*-3′

[a] Italics show the complementary nucleotides.

Results

Preparation of the duplex scorpion primer

A duplex scorpion primer is constructed by two oligonucleotides: PPS and QS. At the fluorescence detection steps of real-time PCR amplification the excess of unextended PPS should form a stable duplex with QS, this duplex keeps the fluorophore and quencher moieties in close proximity to each other. Therefore, the fluorescence of the fluorophore is quenched and the background fluorescence is kept at a low level. The quenching efficiency of light emission from the fluorophore by the quencher is calculated according to the following formula: $E_{ff} = [1 - (F_q - F_b)/(F_{uq} - F_b)] \times 100\%$. Here F_q and F_{uq} are the fluorescence intensities of the PPS/QS duplex and PPS, respectively, F_b is the background fluorescence intensity of buffer only. To obtain maximal quenching efficiency, F_q must be kept at the lowest possible level. We titrated the PPS with the QS to reach the lowest fluorescence. The results show that, when the ratio of QS to PPS exceeds 1.2, the quenching efficiency remains above 97.2%. In order to prevent the formation of inter- or intramolecular secondary structures, the mixture of PPS and QS was denatured in a thermal cycler at 95 °C for 5 min, then cooled to 45 °C and the fluorescence was measured.

Using serial dilutions of telomerase-positive HL60 cell extracts (10^4, 10^3, 10^2, 10 cells) as templates, real-time PCR amplifications with different QS/PPS ratios were compared. All of them gave cell-number-dependent semi-log amplification plots [Fig. 2(A)], and significant and strong linear relationships are confirmed by plotting the threshold cycle C_t values, which represent the PCR cycles at which fluorescence is first detected above the threshold, *versus* the log number of analysed cells (Fig. 3). But the slope values of these calibration curves are not identical [Fig. 2(B)]. With increasing QS/PPS ratio, the slope values of the reaction system are greatly increased; when the QS/PPS ratio exceeds 2.0, the slope values show no further changes. Combining with the experiment mentioned above, we selected 2.0 as the optimal concentration ratio of QS to PPS.

Development of real-time PCR assays

Duplex scorpion primers produce the fluorescent signal by a unimolecular mechanism,[26] and the intramolecular hairpins can be formed in less than a microsecond. Therefore, these probes maybe work well under fast PCR cycling conditions. In this study, a 2-step PCR amplification (94 °C for 0 s, anneal-extend temperature for 3 s) was used. Fluorescent signals were measured at each anneal-extend step.

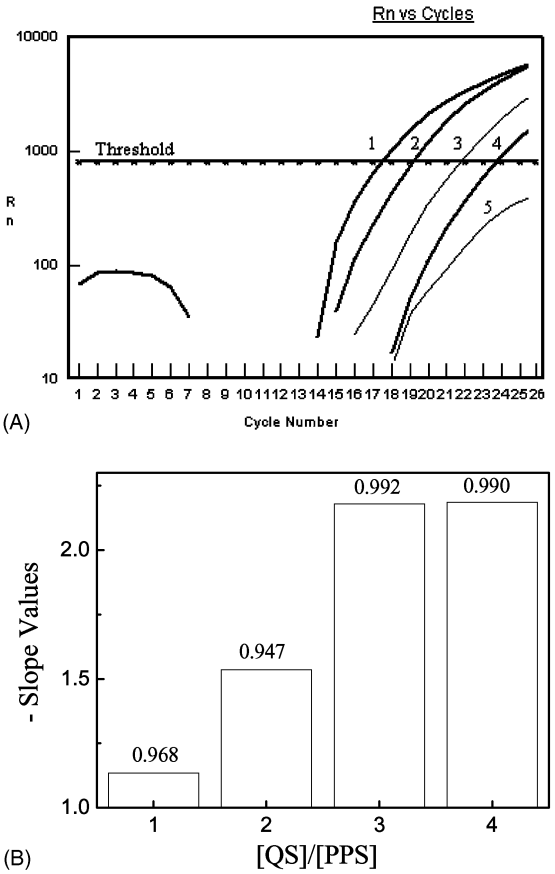

Fig. 2 (A) Semi-log amplification plots and (B) slope values of the corresponding calculation curves of the real-time PCR assays. The serial dilutions of cell extract from HL60 expressing telomerase activity were analysed using duplex scorpion primers. The assay was performed on a GenAmp 5700 Sequence Detection System as described in the Experimental. (A) shows a represent experiment in which the concentration ratio of QS/PPS is 2.0. The log change in fluorescence intensity (Rn), shown on the *y* axis, is plotted against the cycle number. The threshold was set by the user, generally above the negative control. Curves 1–4 represent cell numbers of 10^4, 10^3, 10^2, and 10, respectively. Curve 5 is a negative control. (B) shows the slope values of the corresponding calculation curves, which were obtained by plotting the C_t values *vs.* the log of the number of cell molecules. The data above the bars represent the linear regression values (R^2) of the corresponding calculation curves.

To assure that excess unextended PPS can sufficiently bind to QS at the fluorescence-monitoring step, the temperature of the anneal-extend step must be selected very carefully. Santalucia's HyTher software (http://ozone2.chem.wayne.edu/Hyther/hytherm1main.html) was used for calculating the melting temperature (T_m) of the duplex formed by PPS and QS (concentration ratio 2.0). In 63 mmol L^{-1} NaCl and 1.5 mmol L^{-1} $MgCl_2$ the predicted T_m is 58.7 °C. Thus, the anneal-extend step must be conducted at a temperature lower than 58.7 °C.

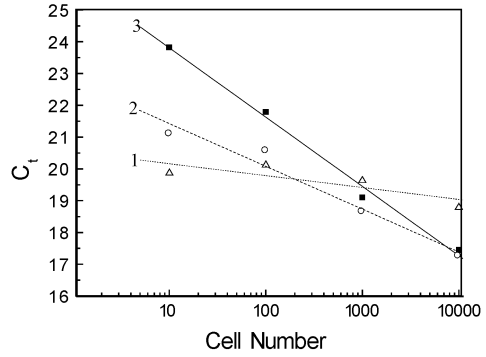

Fig. 3 The calculation curves for the real-time assays by the anneal-extend temperature at (curve 1) 53 °C (open triangles), (curve 2) 50 °C (open squares), (curve 3) 45 °C (black squares). Curve 3 was derived from Fig. 2(A).

Three different anneal-extend temperatures (53, 50 and 45 °C) were tested to set up calibration curves for serial dilutions of telomerase-positive HL60 cell extracts. From Fig. 3, we can see that the linearity of the calibration curve is very poor when the anneal-extend temperature is set at 53 °C, and the value of ΔC_t between the reactions initiated with 10^4 and 10 template molecules is smaller than 1.5 cycles. This may be caused by the poor stability of the PPS/QS duplex at higher temperature. Most of the excess unextended PPS cannot bind to QS at the fluorescence-monitoring step, and this causes the undesired high fluorescence background. But for the reactions with an anneal-extend temperature of 50 or 45 °C, the calibration curves give good linear relationships between C_t values and the log number of HL60 cells; the ΔC_t value between adjacent cell concentrations seems larger for the reaction at 45 °C. So we adjusted the temperature of the anneal-extend step to 45 °C, which provided satisfactory quantitative results without drastically compromising the yield and specificity of PCR.

Effect of reverse primers

To increase the specificity of PCR amplification, hot-start oligonucleotide primers with a hairpin-like structure[27] were used as reverse primers. The performance of three such primers (with 5, 6 or 7-basepair stems, respectively, Table 1) were test in real-time PCR assays. The results show that these three hairpin-like primers give comparable results, and their corresponding slope values are all larger than that of the ACX-a linear reverse primer that is commonly used in TRAP assays (date not shown). For other experiments in this paper, hACX1 was used as the reverse primer. By combination of the above-mentioned experimental results, the optimal experimental conditions obtained are listed in Table 2.

Linearity and accuracy of the assay and its comparison with the SYBR Green method

From Fig. 2 we can find that the duplex scorpion primer is suitable for the detection of telomerase activity in real time. A semi-log amplification plot reveals a cell-number-dependent amplification. There is a perfectly linear relationship between the C_t values and the log number of analysed cells, but not for the control of inactivated cells. The good dynamic linear range extends from 10^4 to 10 cells and the correlation coefficient of the calibration cure is consistently over 0.99. The coefficient of variation (CV) was also determined for each dilution as an indicator of interassay variation. With increasing degree of dilution, the accuracies of this assay decrease gradually: the CVs are 9.2%, 9.5%, 11.4% and 14.5% for dilutions with 10^4, 10^3, 10^2 and 10 cells per assay, respectively. Telomerase activities can also be frequently detected in single-cell dilutions, but the results are less reproducible. Therefore, telomerase activity measurements using the duplex scorpion methods should be performed on extracts with relatively higher cell equivalents.

Under the same conditions the real-time PCR assay using SYBR Green gives a very poor result. The semi-log amplification plots of different dilutions of cell extracts often intersect or overlap, and the corresponding calibration curves cannot give satisfactory linear relationships (data not shown). As a double-strand DNA interacting dye, SYBR Green has no ability to discriminate specific and non-specific PCR products. Otherwise, when using SYBR Green as the reporter dye the expensive hot-start polymerase (*e.g.*, AmpliTaq Gold polymerase) should be used, which will increase the cost of the experiments.

Table 2 Optimal experimental conditions of the real-time PCR assay using duplex scorpion primers

PCR conditions			Primers used			
Initial incubation	Telomerase inactivation	Cycle reaction	Forward	Reverse	Product quantification	Internal control
30 °C for 30 min	95 °C for 3 min	94 °C for 0 s, 45 °C for 3 s, 26 cycles	Duplex scorpion primer	Hairpin-like primer hACX1	Fluorescent analysis using real-time PCR	No internal control

With duplex scorpion primers the use of hot-start polymerase can be avoided and the PCR stopper in PPS can prevent undesirable read-through of the probe sequence by Taq DNA polymerase. Even if non-specific PCR products are produced, the extension of downstream primers will be terminated at the site of the PCR stopper, the new synthesised strand does not contain the sequence that is complimentary to the probe sequence of PPS, the binding of PPS to QS will not be affected and therefore, no undesired fluorescent signal will be found. Thus, the effect of non-specific PCR products can be eliminated.

Discussion

The specificity of PCR amplification is dependent on the binding specificity of primers to templates. To increase the specificities of real-time PCR assays, another target-specific element could be added. Fluorogenic probes, such as TaqMan probes,[28,29] molecular beacons[30–33] and Lightcycler probes,[34,35] are often regarded as good choices. But it is difficult to directly incorporate such probes into the detection of telomerase activity on account of the particularity of the telomerase product (short repeated sequence of the hexanucleotide 5′-TTAGGG-3′). When these probes are used, their sequences must be either complementary to or the same as the repeat sequence of the telomerase product. If they are complementary, the probe sequences must be the same as or similar to the reverse primers. At the anneal step of PCR amplification, the probes may competitively bind to the template (the repeat sequence of the telomerase product) and it will interfere with the PCR amplification. This effect may be prominent in the initial stage. If they are the same, the probe sequences will be complementary to that of the reverse primers. At the anneal step of real-time PCR, the probes may preferentially bind to the reverse primers to produce an undesired fluorescent signal.

In our duplex scorpion primer method, the probe sequence binds to its target in an intramolecular manner to form an intramolecular hairpin structure. In this structure the probe sequence only occupies a limited repeat sequence of the telomerase product. This is fundamentally different from molecular beacons and TaqMan probes, which may occupy all of the repeat sequence, leaving no space for the binding of reverse primers. By using of the "DNA folding" software (http://www.bioinfo.rpi.edu/applications/mfold/old/dna) for predicting the formation of the intramolecular hairpin structure, two hairpins are given (Table 3). From these two structures we can find that the probe sequences tend to bind with the repeat sequence nearby, forming stem-loop structures with a short loop sequence. That is, the binding of the probe sequence to its target will not interfere with the binding sites of reverse primers, and its effect on PCR amplification is largely eliminated.

One problem we must face is that the probe sequence in unextended duplex scorpion primers may anneal to the repeat sequence in the template in an intermolecular manner. This problem can be resolved by using a short target-specific probe sequence. In this study, we used a duplex scorpion primer with an 18-nucleotide probe sequence. The "DNA folding" software was used to calculate the melting points (T_m) of the intramolecular hairpin structures. In the presence of 63 mmol L^{-1} NaCl and 1.5 mmol L^{-1} $MgCl_2$, the predicted T_m of a hairpin structure with a 7-basepair stem is 57.6 °C (Table 4). Therefore, the length of the probe sequence can be further shortened; the shorter the probe sequence, the weaker will be the probe's effect on the reverse primers. But to assure that the PPS/QS duplex retains sufficient stability at the fluorescence-monitoring step, the probe sequence should have enough length. This can be resolved by adding several nucleotides, which have no relationship with the target, into the probe sequence (Fig. 4).

The use of target-specific probe sequences in duplex scorpion primers can improve the specificity of real-time PCR assays and reduce the effect of non-specific PCR products. If the non-specific amplified products do not contain the target sequence of the probe strand, no intramolecular hairpin structure will be formed and no fluorescent signal will be produced. A nefarious effect may come from the primer dimer formed by PPS and reverse primers; this effect can be weakened by using hot-start polymerase or by the design of reverse primers. The reverse primers we used are not perfectly complementary to the repeat sequence of telomerase products as there are several bases mismatched between them. If a mismatched intramolecular hairpin structure is formed, its stability must be weaker than that of the perfect hairpin, and the shorter the probe sequence is, the more evident this effect of mismatching will be. In this paper, we have revealed that the use of reverse primers with hairpin-like structures can reduce the formation of primer dimers to some degree. The suitable length of the target-specific sequence of the probe will be studied in further research.

The primary probing mechanism of duplex scorpion primers is intramolecular. The unimolecular probing is kinetically

Table 3 The formation of hairpin structures predicted by the "DNA folding" software

Predicted hairpin structure	Length of loop sequence (nucleotides)	Predicted T_m/°C
	16	74.7
	22	73.3

Table 4 The predicted T_m of intramolecular hairpin structures with different stem length

Intramolecular hairpin structures	Stem length (basepairs)	Predicted T_m/°C
	11	73.4
	10	72.9
	9	68.0
	8	61.6
	7	57.6
	6	49.6

favourable and highly efficient.[36] Thus, duplex scorpion primers can work very well under fast cycling conditions. This single technique can be performed in 1.5 h, and enables robust analysis of up to 96 samples at the same time. This makes it suitable for high-throughput analysis, which is a considerable improvement in comparison with TRAP assays and its modified versions. Moreover, duplex scorpion primers can be synthesised with different colours of fluorophores and different probe sequences, enabling assays to be designed for detection of different targets in a single reaction simultaneously. This greatly facilitates the use of internal standards in the detection of telomerase activity. The use of internal standards can be profitable not only for providing more accurate quantitative information, but also for reducing the appearance of false-positive or false-negative results.

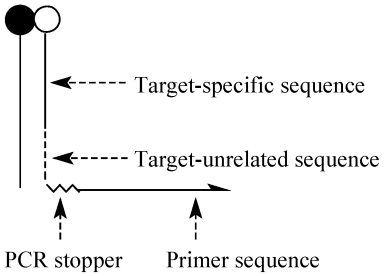

Fig. 4 Diagram of a duplex scorpion primer with a short target-specific probe sequence.

In conclusion, the use of duplex scorpion primers can provide rapid, specific real-time quantitative PCR assays for telomerase activity. It has potential application in cancer molecular diagnosis.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (Grant No. 20075012 and No. 20405007), the Outstanding Scholar Program of Nankai University and the Outstanding Scholar Program of Tianjin Medical University.

References

1 C. Greider and E. Blackburn, *Cell*, 1987, **51**, 887.
2 G. B. Morin, *Cell*, 1989, **59**, 521.
3 P. Matthews and C. J. Jones, *Histopathology*, 2001, **38**, 485.
4 M. P. Granger, W. E. Wringt and J. W. Shay, *Crit. Rev. Oncol./Hematol.*, 2002, **41**, 29.
5 D. J. Argyle and L. Nasir, *Vet. Pathol.*, 2003, **40**, 1.
6 E. Hiyama and K. Hiyama, *Cancer Lett.*, 2003, **194**, 221.
7 N. W. Kim, M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. C. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich and J. W. Shay, *Science*, 1994, **266**, 2011.
8 M. L. Falchetti, A. Levi, P. Molinari, R. Verna and E. D'Ambrosio, *Nucleic Acids Res.*, 1998, **26**, 862.
9 G. Krupp, K. Kuhne, S. Tamm, W. Klapper, K. Heidron, A. Rott and R. Parwaresch, *Nucleic Acids Res.*, 1997, **25**, 919.
10 I. Szatmari and J. Aradi, *Nucleic Acids Res.*, 2001, **29**, e3.
11 E. Savoysky, K.-I. Akamatsu, M. Tsuchiya and T. Yamazki, *Nucleic Acids Res.*, 1996, **24**, 1175.

12 Y.-Y. Wu, A. M. Hruszkewye, R. M. Delgado, A. Yang, O. A. Vortmeyer, Y.-W. Moon, R. J. Weil, Z. Zhuang and A. T. Remaley, *Clin. Chem. Acta*, 2000, **293**, 199.
13 M. Hirose, J. Abei-Hashimoto, K. Ogura, H. Tahara, T. Ide and T. Yoshimura, *J. Cancer Res. Clin. Oncol.*, 1997, **123**, 337.
14 M. Hirose, J. Abei-Hashimoto, H. Tahara, T. Ide and T. Yoshimura, *Clin. Chem.*, 1998, **44**, 2446.
15 M. Kolioliou, M. Talieri and E. S. Lianidou, *Clin. Biochem.*, 2001, **34**, 277.
16 D. B. Lackely, *Anal. Biochem.*, 1998, **263**, 57.
17 S. N. Saldanha, L. G. Andrews and T. O. Tollefsbol, *Anal. Biochem.*, 2003, **315**, 1.
18 H. Wege, M. S. Chui, H. T. Le, J. M. Tran and M. A. Zern, *Nucleic Acids Res.*, 2003, **31**, e3.
19 M. Hou, D. Xu, M. Bjorkholm and A. Gruber, *Clin. Chem.*, 2001, **47**, 519.
20 H. Uehara, G. Nardone, I. Nazarenko and R. J. Hohman, *BioTechniques*, 1999, **26**, 552.
21 M. A. Lee, A. L. Siddle and R. H. Page, *Anal. Chim. Acta*, 2002, **457**, 61.
22 I. A. Nararenko, S. K. Bhatnagar and R. J. Hohman, *Nucleic Acids Res.*, 1997, **25**, 2516.
23 B. Schweitzer and S. Kingsmore, *Curr. Opin. Biotechnol.*, 2001, **12**, 21.
24 D. Whitcombe, J. Theaker, S. Guy, T. Brown and S. Little, *Nat. Biotechnol.*, 1999, **17**, 804.
25 N. W. Kim and F. Wu, *Nucleic Acids Res.*, 1997, **25**, 2595.
26 A. Solinas, L. J. Brown, C. McKeen, J. M. Mellor, J. T. G. Nicol, N. Thelwell and T. Brown, *Nucleic Acids Res.*, 2001, **29**, e96.
27 O. K. Kaboev, L. A. Luchkina, A. N. Tret'iakov and A. R. Bahrmand, *Nucleic Acids Res.*, 2000, **28**, e94.
28 P. M. Holland, R. D. Abramson and R. Watson, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 7276.
29 Y. S. Lie and C. J. Petropoulos, *Curr. Opin. Biotechnol.*, 1998, **9**, 48.
30 S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303.
31 L. G. Kostrikis, S. Tyagi, M. M. Mhlanga, D. D. Ho and F. R. Kramer, *Science*, 1998, **279**, 1228.
32 X. H. Fang, J. J. Li, J. Perlette, W. H. Tan and K. M. Wang, *Anal. Chem.*, 2000, **72**, 747A.
33 D. M. Kong, L. Gu, H. X. Shen and H. F. Mi, *Chem. Commun.*, 2002, **21**, 854.
34 C. T. Wittwer, M. G. Herrmann, A. A. Moss and R. P. Rasmussen, *Biotechniques*, 1997, **22**, 130–134.
35 M. J. Lay and C. T. Wittwer, *Clin. Chem.*, 1997, **43**, 2262.
36 N. Thelwell, S. Millington, A. Solinas, J. Booth and T. Brown, *Nucleic Acids Res.*, 2000, **28**, 3752.