Use of duplex probes simulating TaqMan to detect hepatitis B virus

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A novel method for duplex probes is designed to simulate the TaqMan probe during polymerase chain reaction (PCR). In this method, two partly complementary single-labelled oligonucleotide probes labelled with a fluorophore or a quencher, respectively, are used. At lower temperature the two probes can bind to each other and form a mismatched duplex, in which the fluorophore and quencher are in close proximity and the same energy transfer mechanism as in molecular beacons may occur between them; thus, a quenching efficiency better than conventional TaqMan probes is acquired. In the anneal-extend step of PCR, one single-labelled probe hybridises to the predetermined target and is cleaved by *Taq* DNA polymerase. Increased fluorescent signal can be observed at lower temperature. The fluorescent data analysis demonstrated that a significantly higher level of fluorescent signal and hence higher sensitivity of detection is obtainable using our duplex probes in place of conventional TaqMan probes. Combined with real-time PCR instruments, the assay can be used to quantify the input target molecules and the dynamic linear range is of at least six orders of magnitude.

Introduction

Hepatitis B virus (HBV) is the most prevalent of all the viral hepatitis agents that can cause death or significant debilitation. Estimates indicate that there are more than 200 million carriers of HBV worldwide and they are the primary reservoir of new infections.[1]

The standard laboratory techniques for the diagnosis of HBV are serological assays. Methods that permit the direct detection of virus in serum can supplement information from serological testing for HBV infection. PCR is a powerful tool for nucleic acid analysis, theoretically enabling the detection of a single copy sequence,[2] and is currently applied for diagnostic purposes. Indeed, detection of HBV DNA sequences in serum for diagnostic purposes is currently performed by PCR in many laboratories.[3,4]

Recently a significant advance in PCR has been the homogenous assay for real-time fluorescence detection of PCR-amplified products in a closed tube. In general, homogeneous detection provides advantages of high sensitivity, specificity, ease of use and multiplexing ability.[5] So far, TaqMan[6–8] and molecular beacons[9–11] are two such robust read-out probes. A few months ago we designed a modified molecular beacon (TaqMan-MB)[12] combining the properties of the molecular beacon and the TaqMan probe. These probes, which are based on fluorescence energy transfer, are characterised by relatively high signal-to-noise ratios and posses a good ability to discriminate between positive and negative reactions. However, for these methods the fluorophore and the fluorescence quencher are dual-labelled on the probe. This seriously complicates the design and the synthesis of the probes[13] and consequently increases the expense of experiments.

We present a novel method for the detection of a specific nucleic acid sequence in a homogeneous solution with two single-labelled probes, relying on the 5′-exonuclease activity of nucleic acid polymerase (Fig. 1). Probe 1 is an oligonucleotide chain matched perfectly to the target sequence and is labelled with a fluorophore, FAM (6-carboxyfluorescence) at the 5′-end, whereas probe 2 is a complementary chain to probe 1 with several bases mismatched and is labelled with a fluorescence quencher, DABCYL [4-(4′-dimethylaminophenylazo)benzoic acid] at the 3′-end. At lower temperature these two probes form a mismatched duplex, which keeps the fluorophore and

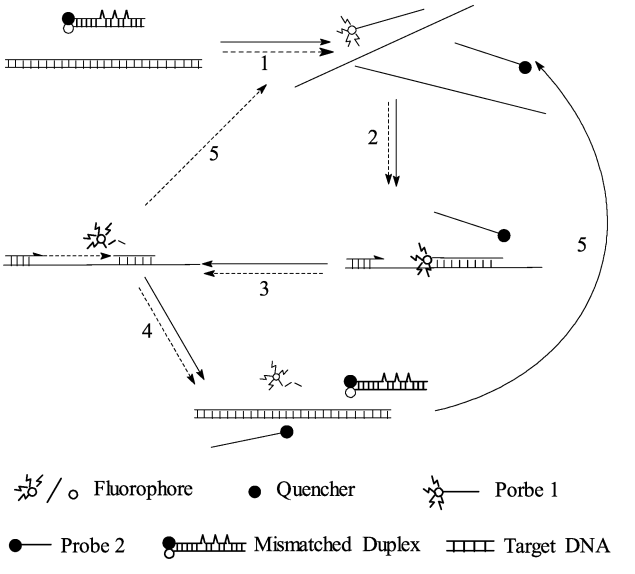

Fig. 1 Assay principle. Step 1: initial denaturation of the target and the mismatched duplex; step 2: annealing of primers and probe 1 to the target; step 3: extension of primers causes the cleavage of probe 1, and fluorescent signal is produced; step 4: on cooling, the mismatched duplex forms again, a change in fluorescence can be observed; step 5: start next cycle. The dashed and solid arrows indicate the routes of end-point and real-time measurement, respectively. For end-point measurements, step 4 is performed at the end of PCR. The bulges in mismatched duplex represent the mismatched bases.

quencher in close proximity to each other. Therefore, the fluorescence of the fluorophore is quenched. At the anneal-extend step of PCR, in which the temperature is higher than the melting temperature (T_m) of the mismatched duplex and lower than that of the perfectly complementary duplex formed by probe 1 with the target sequence, probe 1 hybridises with the target sequence and is cleaved by 5′-3′ exonuclease activity of *Taq* DNA polymerase, producing an exponential increase in emission intensity of the reporter dye. Thus, we can deduce the presence of the predetermined target by observing the change of fluorescence at a temperature lower than the T_m of the mismatched duplex. In this study, we will use HBV DNA as a target gene and discuss the advantages of this method over conventional TaqMan probes.

Materials and methods

Preparation of template DNA

Extraction of DNA from sera was done essentially by using the protease K-phenol method. Serum (200 μL) was mixed with 200 μL cleavage buffer (50 mM Tris-HCl, pH 7.8, 5 mM EDTA and 1% sodium dodecyl sulfate) containing 1 mg mL^{-1} protease K. The mixture was incubated at 60 °C for 2 h, then was extracted once with phenol–chloroform and once with chloroform. After precipitation with ethanol, the DNA pellet was washed with 70% ethanol, dried and dissolved in 20 μL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Design of probes

Two single-labelled probes were designed. Probe 1 is FAM-5′-CCGTCTGTGCCTTCTCATCTGCCGG; a 3′-blocking phosphate group is contained to prevent probe extension during PCR cycling. Probe 2 is CCGGCAGATGAC̲AAGC̲CA-G̲AGACGG-3′-DABCYL (underlines identify mismatched bases). The corresponding TaqMan probe is FAM-5′-CCGTCTGTGCCTTCTCATCTGCCGG-3′-TAMRA (tetramethylrhodamine). Shanghai Shenyou Ltd. carried out the coupling reactions and purification of the probes.

Amplification by PCR and fluorescence analyses

For PCR, the primers (primer 1: 5′-ATCCTGCGCGG-GACGTCCTT-3′, primer 2: 5′-CGTTCACGGTGGTCTC-CATG-3′) were designed to amplify a 225 bp region within a HBV gene. PCR conditions were identical for the duplex probe and the TaqMan probe, a 25 μL reaction mixture consisting of 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25 °C, 0.1% Triton X-100), 1 mM $MgCl_2$, 0.2 mM each dNTP, 2.5 U of *Taq* DNA polymerase, 0.4 μM of each primer, 0.3 μM of either probe and 2 μL of template. Cycling was designed with a pre-cycle (95 °C for 8 min), 40 cycles for amplification (94 °C for 30 s, 64 °C for 1 min). After amplification, 25 μL production was diluted to 100 μL with PCR buffer (3.67 mM $MgCl_2$ included) and analysed for fluorescence at ambient temperature. Fluorescence emission was measured at $\lambda_{em} = 516$ nm with $\lambda_{ex} = 490$ nm.

Real-time quantitative PCR

The reaction mixture was prepared as above except that the concentration of Mg^{2+} was changed to 3 mM. Amplification conditions consisted of 8 min at 95 °C to activate the polymerase, followed by 40 cycles of 30 s at 94 °C, 30 s at 64 °C, 90 s at 45 °C. Emitted fluorescence was analysed at the lowest cycling temperature (45 °C), at which uncleaved probe 1 bonded sufficiently to probe 2 to give the minimal fluorescent state.

Agarose gel electrophoresis

Electrophoresis was conducted in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) in a 2% (W/V) agarose gel containing 0.5 μg mL^{-1} of ethidium bromide (EB). Twenty microlitres of final PCR product were loaded on the gel. After electrophoresis, the DNA bands were visualised through a UV transilluminator.

Results

Absorption spectra and quenching efficiency

In the mismatched duplex, the fluorophore and quencher are held sufficiently close to each other that strong dipole-dipole coupling between them may occur. This can be demonstrated by investigating the spectroscopic properties of the duplex probe. From Fig. 2 we can see that the absorption spectrum of the mismatched duplex clearly differed from the spectral summation of equimolecular probe 1 and probe 2, and the changes are very similar to that of molecular beacons (Fig. 2, insert). In addition the absorbance of FAM (496 nm) clearly decreases while the absorption peak of DABCYL (476 nm), which is hardly visible in the spectral summation of probe 1 and probe 2 (and in opened molecular beacons), is relatively increased in the mismatched duplex (and in closed molecular beacons), showing up as a clear shoulder peak around 470 nm. These dramatic changes in the absorption spectra are clearly incompatible with a Förster-type fluorescence resonance energy transfer (FRET) mechanism, since the weak dipolar coupling that governs this mechanism does not induce any change in the absorption spectrum.[14] So, as in molecular beacons, two forms of energy transfer may exist in the mismatched duplex: direct energy transfer, where the donor and acceptor moieties may be in contact, may be dominant,[15] and FRET may also occur over rather longer distances. This mechanism of energy transfer ensures maximal quenching efficiency and minimal background.

The quenching efficiency of the quencher-to-light emission from the fluorophore was 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 mismatched duplex and probe 1, respectively, while F_b is the background fluorescence intensity of the buffer only. For the TaqMan probe, F_q is the fluorescence intensity of the TaqMan probe alone. For the duplex probe the quenching efficiency is 94.4%, which is comparable with that of molecular beacons,[16] and much higher than that of the TaqMan probe: 82.5%. That is to say, our duplex probe has a much better quenching efficiency

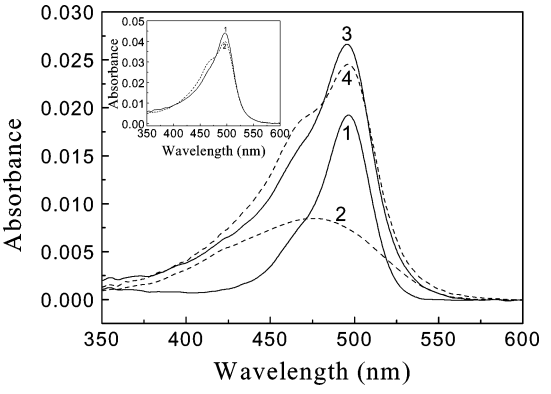

Fig. 2 Absorption spectra of equimolar of (1) probe 1; (2) probe 2; (3) summation of probe 1 and probe; (4) the mismatched duplex. Insert: absorption spectra of the molecular beacon FAM-C̲C̲C̲G̲A̲G̲ATTGAGATCTTCTGCGAC̲T̲C̲G̲G̲G̲-DABCYL (underlines identify the complementary stem sequence) (1) in the presence of 5-fold molar excess of target and (2) without target.

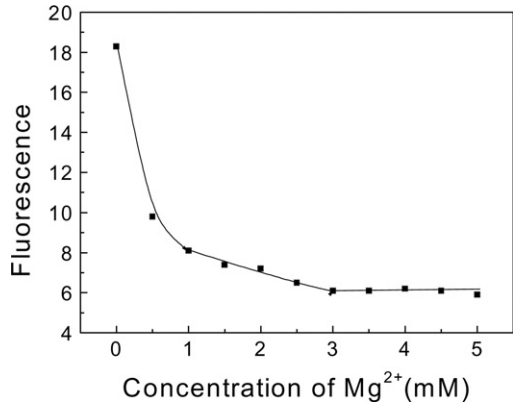

Fig. 3 Effect of the concentration of Mg^{2+} on background fluorescence.

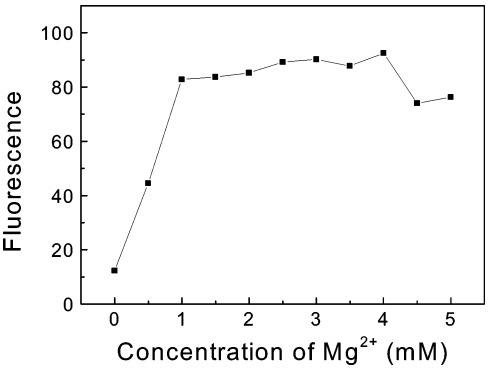

Fig. 4 Effect of the concentration of Mg^{2+} on fluorescence analysis.

than the TaqMan probe and ensures a low level of background signal.

Effect of Mg^{2+} concentration on background fluorescence intensity

The stability of the mismatched duplex has a great influence on the background fluorescence intensity. The more stable the mismatched duplex is, the lower the background signal and the better quenching efficiency will result. Bivalent cations, such as magnesium, have a powerful stabilising influence on nucleic acid hybrids. They can play an important role in decreasing electrostatic repulsion between anionic chains of nucleic acids. We investigated the influence of different Mg^{2+} concentrations on the background fluorescence of the mismatched duplex (Fig. 3). With increasing of Mg^{2+} concentration, the fluorescence is greatly decreased. When the Mg^{2+} concentration exceeds 3 mM, the fluorescence reaches a plateau and the screening of the electrostatic charges reaches saturation.

Development of PCR assays

The ability of this duplex probe for detection of HBV DNA in PCR assay was investigated. HBV DNA isolated from patient serum was used as the template. A 2-step PCR with a denature temperature at 94 °C and an anneal-extend temperature at 64 °C was used for end-point measurements. The anneal-extend temperature is higher than the T_m of the mismatched duplex and lower than that of the perfectly complimentary duplex formed by probe 1 with the target sequence. So at the anneal-extend step of PCR, probe 1 cannot bind to probe 2 but can hybridise to the complementary target sequence within the template and can be cleaved by *Taq* DNA polymerase in the process of primer extension. Fluorescence emission is measured at ambient temperature (Fig. 1, dashed arrow).

We also optimised PCR assays by adjusting the concentration of Mg^{2+}. The increasing of Mg^{2+} concentration has a great influence on the fluorescence in the low concentration range. When the Mg^{2+} concentration exceeded 1 mM, the fluorescence showed no further changes (Fig. 4). But the results of agarose gel electrophoresis (Fig. 5) show that the specificity of PCR decreases dramatically when the concentration of Mg^{2+} exceeds 1 mM. Therefore, we selected 1 mM as the concentration of Mg^{2+} in PCR. According to the experiment above, 1 mM is not the best concentration for optimising quenching efficiency, so in the end-point measurement, we added 75 μL PCR buffer (3.67 mM Mg^{2+} included) to the PCR mixture to adjust the ultimate Mg^{2+} concentration to 3 mM.

The effects of duplex probes on PCR

The duplex probe did not interfere with PCR amplification. For the same template (HBV-infected or not), agarose gel electrophoresis-EB staining gave the identical results whether the duplex probe was present in the PCR mixture or not (Fig. 6). So the duplex probe can be added directly to the PCR mixture before amplification. Thus, on one hand the manipulation will be simplified, on the other hand the duplex probe can be used in real-time PCR detection if specific instruments are introduced.

Monitoring duplex probes assay during PCR

Using HBV DNA extracted from identical serum samples as templates, we monitored three different polymerase chain reactions (Fig. 7). In reaction 1, using our duplex probes, the probes were added directly to the PCR mixture before reaction. This reaction reflects the typical characteristics of PCR. At the initial stage, a small amount of PCR product is synthesised and the fluorescence of the duplex probe remains at a low level. With increasing number of cycles, the PCR product accumulates exponentially and the fluorescent signal rapidly increases simultaneously. At the last stage of amplification, the accumulation of PCR product stops due to depletion of reaction components and the increase of the fluorescent signal slows down. Here the magnitude of fluorescence correlates strongly with the amount of synthesised PCR product.

Our duplex probe possesses some properties of molecular beacons. The fraction of probe 1, which exactly hybridises to amplicon, can also provide a fluorescent signal before cleavage. In order to investigate the hybridisation contribution, we designed reaction 2. In this reaction probe 1 and probe 2 were added to the PCR mixture after amplification. Thus, the fluorescent signal only came from the probe 1 fraction that hybridised to the target. To ensure sufficient hybridisation between probe 1 and amplicon, the solutions were denatured at 95 °C for 5 min, annealed at 50 °C for 15 min and allowed

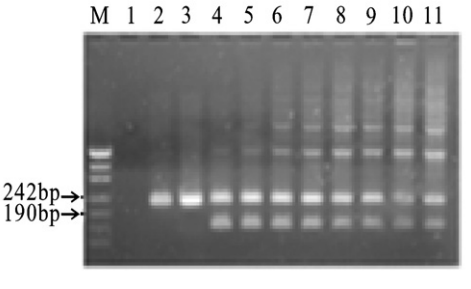

Fig. 5 Effect of the concentration of Mg^{2+} on PCR amplification. The concentration of Mg^{2+} was increased from 0 to 5 mM in 0.5 nm increments.

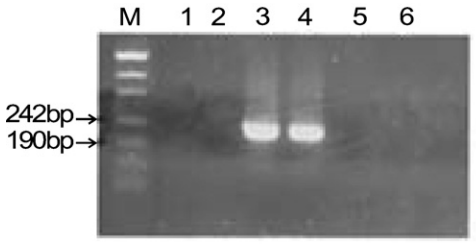

Fig. 6 Effect of the duplex probe on PCR amplification. Lane 1, PCR amplification of healthy serum without probes; lane 2, PCR amplification of healthy serum in the presence of the two probes; lane 3, PCR amplification of HBV-infected serum without probes; lane 4, PCR amplification of HBV-infected serum in the presence of the two probes; lane 5, negative control without probes; lane 6, negative control in the presence of the two probes.

to cool to ambient temperature. Fluorescent data show that reaction 2 has a much weaker fluorescent signal change than reaction 1. This means that hybridisation only made a very small fluorescence contribution in the PCR/duplex probes assay and the fluorescent signal mostly comes from the cleavage of probe 1. As a comparison, we also monitored the fluorescence change of the TaqMan probe (reaction 3). TaqMan has a relatively higher background signal and its change of signal-to-noise ratio is not so remarkable as in reaction 1.

From these three reactions, we can find that our duplex probe actually acts as a TaqMan probe during PCR amplification and, with its better quenching efficiency, it can work much better than conventional TaqMan probes.

Specificity of PCR/duplex probe assays

With the competition of probe 2 our duplex probe is endowed with a much better specificity than conventional TaqMan probes. Four human serum specimens were analysed by PCR/duplex probe assay. The results show that only HBV-infected serum (10^6 copies ml^{-1}) gives an intense fluorescent signal above background (Fig. 8). For healthy serum, HCV-infected serum (5×10^6 copies ml^{-1}) and HEV-infected serum (5×10^6 copies ml^{-1}), the fluorescence intensities are very low and can be considered as negative results.

Some patients may be simultaneously infected by several kinds of viruses such as HBV, HCV and HEV. To determine whether our duplex probe can specifically recognise HBV DNA in mix-infected sera, four mix-infected sera were prepared by mixing the HBV, HCV or/and HEV-infected sera in equal amounts for the PCR/duplex probe assay. As expected, HCV/HEV mix-infected serum has a low fluorescent signal intensity, all the other three sera (HBV/HCV, HBV/HEV and HBV/HCV/HEV) give positive results and the fluorescence intensities are comparable with that of solely HBV-infected serum. This result shows that the duplex probe can specifically detect HBV in mix-infected sera; other concurrent viruses did not interfere with the detection.

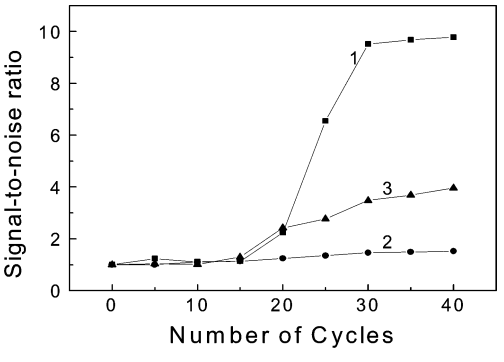

Fig. 7 Monitoring duplex probes assay during PCR: (1) using duplex probe with the probes added directly to the PCR mixture before reaction; (2) using duplex probe but the probes were added to the PCR mixture after reaction; (3) using TaqMan probe with the probe added to the PCR mixture before reaction.

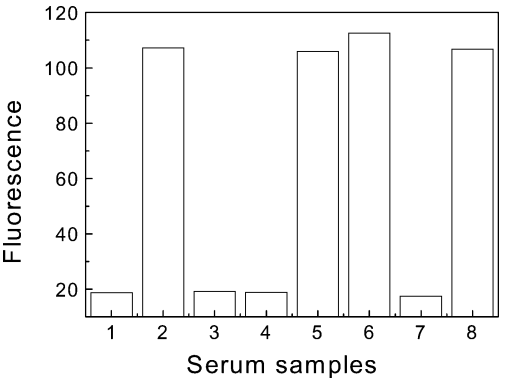

Fig. 8 Fluorescence analysis of different serum samples: (1) healthy, (2) HBV-infected, (3) HCV-infected, (4) HEV-infected, (5) HBV/HCV-mixed infected, (6) HBV/HEV-mixed infected, (7) HCV/HEV-mixed infected, (8) HBV/HCV/HEV-mixed infected.

Fluorescence analysis of duplex and TaqMan probes

HBV DNAs were extracted from healthy serum and serially diluted HBV-infected serum by using the protease K-phenol method. We performed PCR with a template of these HBV DNAs as described above (Fig. 9). The results show that the intensity of the fluorescent signal of the duplex probe was significantly higher than that of the TaqMan probe for each dilution (up to 10^{-6} dilution). Thus, as a nucleic acid probe the optimised duplex probe is preferable to the conventional TaqMan probes with its increased signal intensity and its increased sensitivity in detection of fluorescence analysis by performing PCR.

Real-time quantitative PCR

Combined with a specific PCR instrument (GeneAmp 5700 sequence detection system), duplex probes can be used as suitable tools for the real-time monitoring of DNA amplification during PCR. For this purpose, a recording step should be added at the end of each PCR cycle and the fluorescent signal is monitored at this step. This step should be processed at a temperature that is lower than the T_m of the mismatch duplex. At this temperature sufficient binding of uncleaved probe 1 to probe 2 can be ensured. In this experiment, we selected 45 °C as the recording temperature.

In assays that use PCR to exponentially amplify a target sequence, there is an inverse linear relationship between the

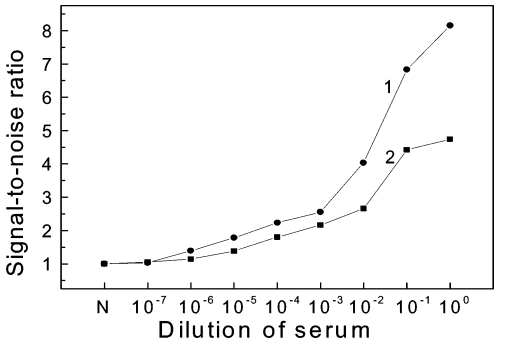

Fig. 9 Relative fluorescent signal from the duplex probe (1) and the TaqMan probe (2) in the presence of different concentrations of HBV.

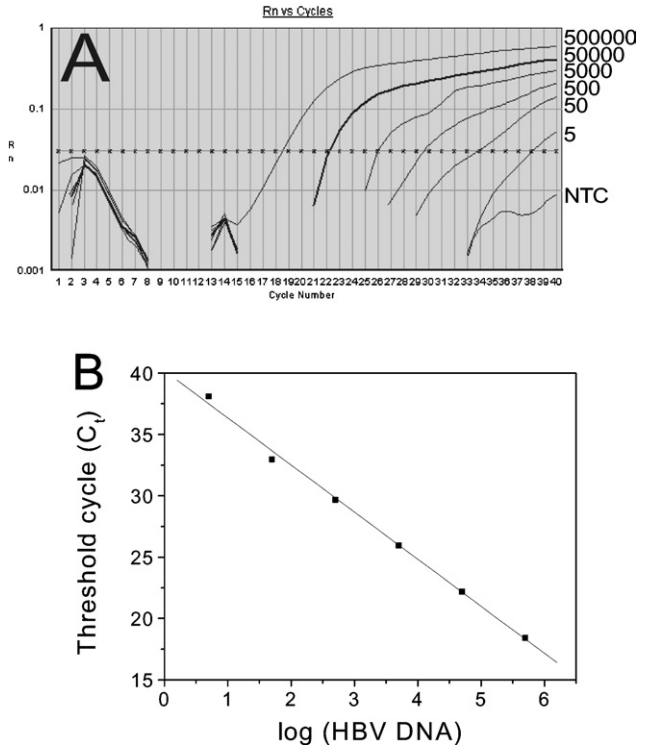

Fig. 10 Amplification plots (A) and standard curve (B) of the real-time PCR assay. Serial 10-fold dilutions of HBV DNA template were used in each reaction.

number of thermal cycles it takes for enough amplicons to be synthesised for a fluorescent signal to appear and the logarithm of the number of target molecules that were present initially.[17,18] To see whether our PCR/duplex probe assay display the same quantitative relationship, seven reactions, each initiated with a different number of HBV DNA copies, were carried out in parallel. As shown in Fig. 10, a perfectly linear relationship was obtained as expected and the dynamic linear range was at least six orders of magnitude from 5×10^5 to 5 HBV DNA copy numbers. The correlation coefficient of the calibration curve was consistently 0.99 or greater. The assay was specific for target DNA as shown by the high threshold cycle (C_t values) of the NTCs, which was consistently larger than 40. These results demonstrate that the duplex probe assay can be used to quantify the input target concentration.

Discussion

Compared with dual-labelled probes, single-labelled ones have the advantages of lower expense and ease of design, synthesis and purification. During the synthesis of molecular beacons and TaqMan probes, at least two times purifications are performed step-by-step.[19] This will greatly affect the productivity of probes. In this paper, we introduced two single-labelled probes, which only need one purification step each. The process of probe preparation is simplified and less costly. On the other hand, the presence of oligonucleotides, which are only labelled with fluorophore, will significantly increase the background signal and hence affect detection effects. This problem will not occur in our probes. In addition, we can optimise the quenching efficiency by adjusting the ratio of the two probes.

The most critical problem associated with TaqMan technology is the distance between the dye and the quencher.[20] If they are close together, the likelihood of cleaving the dye from the quencher drastically decreases, even though a lower background fluorescence is observed. On the other hand, placement of the quencher at the 3′-end of the probe increases the signal during PCR assays, but at the expense of a much higher background.[21] Our duplex probes have a similar energy transfer mechanism as molecular beacons and their quenching efficiency is much better than conventional TaqMan probes. Consequently, a better detective sensitivity can be acquired.

For molecular beacon technology, the most critical problem is that the molecular beacon is an internal probe, which must compete with the opposite strand of the amplicon for binding to its complementary target. Only the probe fraction that will successfully compete against the strand complementary to the target strand in the hybridisation reaction will emit fluorescence.[22] Therefore, the detection signal and/or the sensitivity of detection of a molecular beacon based PCR assay is expected to be restricted. In our method, duplex probes can work as TaqMan probes during PCR amplification and fluorescent signal does not come mainly from the hybridisation of the probes but from their cleavage. So, competing hybridisation is insignificant in this method. On the other hand, in the opened conformation of molecular beacons, the quencher and fluorophore are in the same oligonucleotide and the quencher remains close enough to partly quench the fluorophore by a non-collisional Föster mechanism. This must place a limitation on the fluorescence intensity. This limitation is not present with duplex probes, which are enzymically cleaved during PCR, thus separating the fluorophore from the quencher.

In recent years, several homogeneous assay formats based on single-labelled probes (adjacent hybridisation probes, double-stranded probes, duplex scorpion primers) have been established. Adjacent hybridisation probe[23,24] technology uses two adjacent probes that are labelled such that when both probes are hybridised to a target, the labels are brought close to each other and a FERT occurs between them. The sensitised acceptor emission is measured instead of the donor fluorescence. Double-stranded probes[25] consist of two complementary oligodeoxyribonucleotides of different lengths labelled with a fluorophore and a quencher in close proximity in the duplex. The probes on their own are quenched, but they become fluorescent upon displacement hybridisation with the target. As molecular beacons, the fluorescent signal level in these two systems mainly depends on the hybridisation between probes and amplicons; they also suffer from the drawback of competing hybridisation. In duplex scorpion primer format,[26] the primary probing mechanism is intramolecular. Unimolecular probing is kinetically favourable and highly efficient and the effect of competing hybridisation is reduced. But adding a PCR stopper between probe and primer sequence will increase the difficulty and expense of probe preparation.

The aim of our experiments is to design a duplex probes system that can work as TaqMan probes during PCR amplification. So probe 1 must bind to the target before primer extension, which will occlude the probe-binding site. This can be achieved by manipulating the sequence and length of the probe.[6] In our experiments, probe 1 hybridises to a predetermined target sequence at the downstream of primer 1, and there are 127 nucleotides between the 3′-end of primer 1 and the 5′-end of the probe 1 binding site. Santalucia's 'Hyther' software (http://ozone.chem.wayne.edu) was used to calculate T_ms of primer 1 and probe 1. In 100 mM NaCl and 2 mM $MgCl_2$ the predicted T_m of primer 1 is 71.6 °C and that of probe 1 is 73.4 °C. From the two points mentioned above, we can see that primer 1 and probe 1 possess similar binding abilities and the distance of 127 nucleotides is enough for probe 1 to bind to amplicon before primer 1 extension reaches the binding site. Thus, cleavage of probe 1 can be ensured.

At high concentrations of Mg^{2+}, non-specific amplified products will emerge, so we selected 1 mM as the Mg^{2+} concentration in PCR. In fact, the hybridisation between probe 1 and its target introduces extra specificity to the detection. If the non-specific amplified products do not contain the target sequence

of probe 1, no hybridisation will occur between them and *Taq* DNA polymerase cannot cleave probe 1, thus, no fluorescence increase will be observed. That is to say, non-specific amplification will not affect the detection of predetermined targets. So we can also perform the PCR/duplex probe assay with a Mg^{2+} concentration above 1 mM and an optimised quenching efficiency can be guaranteed. This point is especially important for real-time assays.

Compared with TaqMan probes, another advantage of our duplex probes is that they possess better recognition specificity and this specificity can be adjusted by altering the numbers of mismatched bases between the two probes. For example, if a point mutation is to be detected, we can design a duplex probe with only one base mismatch between the two probes. Thus, in an appreciate temperature range, probe 1 can specifically hybridise to a perfectly matched target. The presence of larger amounts of probe 2 will prevent probe 1 from binding to even one base mismatched templates. If we want to detect a predetermined sequence that can tolerant one base mismatch, two base mismatched duplex probes can be designed. This makes the experiment more flexible.

Acknowledgements

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References

1 M. A. Winkler, N. Xu, H. Wu and H. Aboleneen, *Anal. Chem.*, 1999, **71**, 664A.
2 K. B. Mullis and F. A. Faloona, *Methods Enzymol.*, 1987, **155**, 335.
3 S. Kaneko, R. H. Miller, S. M. Feinstone, M. Unoura, K. Kobayashi, N. Hattori and R. H. Purcell, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 312.
4 P. Paterlini, G. Gerken, E. Nakajima, S. Terre, A. D. Errico, W. Grigioni, B. Nalpas, D. Franco, J. Wands, M. Kew, E. Pisi, P. Tiollais and C. Brechot, *New Engl. J. Med.*, 1990, **323**, 80.
5 B. Schwetzer and S. Kingsmore, *Curr. Opin. Biotech.*, 2001, **12**, 21.
6 P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 7276.
7 C. A. Heid, J. Stevens, K. J. Livak and P. M. Williams, *Genome Res.*, 1996, **6**, 986.
8 K. M. Weinberger, E. Wiedenmann, S. Böhm and W. Jilg, *J. Virol. Methods*, 2000, **85**, 75.
9 S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303.
10 L. G. Kostrikis, S. Tyagi, M. M. Mhlanga, D. D. Ho and F. R. Kramer, *Science*, 1998, **279**, 1228.
11 S. Tyagi, D. P. Bratu and F. R. Kramer, *Nat. Biotechnol.*, 1998, **16**, 49.
12 D. M. Kong, L. Gu, H. X. Shen and H. F. Mi, *Chem. Commun.*, 2002, **21**, 854.
13 J. Nurmi, A. Ylikoski, T. Soukka and M. Karp, *Nucleic Acids Res.*, 2000, **28**, e28.
14 S. Bernacchi and Y. Mély, *Nucleic Acids Res.*, 2001, **29**, e62.
15 X. H. Fang, J. J. Li, J. Perlette, W. H. Tan and K. M. Wang, *Anal. Chem.*, 2000, **72**, 747A.
16 S. K. Poddar, *J. Virol. Methods*, 1999, **82**, 19.
17 R. Higuchi, C. Fockler, G. Dollinger and R. Waston, *Biotechnology*, 1993, **11**, 1026.
18 J. A. M. Vet, A. R. Majithia, S. A. E. Marras, S. Tyagi, S. Dube, B. J. Poiesz and F. R. Kramer, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 6394.
19 F. R. Kramer, S. Tyagi, J. A. M. Vet and S. A.E. Marras, http://www.molecular-beacons.org.
20 K. J. Livak, S. J. A. Flood, J. Marmaro and W. Giusti, *PCR Methods Appl.*, 1995, **4**, 357.
21 W. Chen, G. Martinez and A. MulChandani, *Anal. Biochem.*, 2000, **28**, 166.
22 S. K. Poddar, *Mol. Cell. Probes*, 2000, **14**, 25.
23 C. T. Witter, M. G. Herrmann, A. A. Moss and R. P. Rasmussen, *Biotechniques*, 1997, **22**, 130.
24 M. J. Lay and C. T. Wittwer, *Clin. Chem. (Washington, D.C.)*, 1997, **43**, 2262.
25 Q. G. Li, G. Y. Luan, Q. P. Guo and J. X. Liang, *Nucleic Acids Res.*, 2002, **30**, e5.
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**, e26.