Application of Single-labelled Probe-primer in PCR Amplification to the Detection of Hepatitis B Virus DNA

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A new method based on the incorporation of a single-labelled probe-primer into polymerase chain reaction (PCR) for the detection of PCR-amplified DNA in a closed system is reported. The probe-primer consists of a specific probe sequence on the 5′-end and a primer sequence on the 3′-end. A fluorophore is located at the 5′-end. The primer-quencher is an oligonucleotide, which is complementary to the probe sequence of probe-primer and labelled with a quencher at the 3′-end. In the duplex formed by probe-primer and primer-quencher, the fluorophore and quencher are kept in close proximity to each other. Therefore the fluorescence is quenched. During PCR amplification, the specific probe sequence of probe-primer binds to its complement within the same strand of DNA, and is cleaved by Taq DNA polymerase, resulting in the restoration of fluorescence. This system has the same energy transfer mechanism as molecular beacons, and a good quenching efficiency can be ensured. Following optimization of PCR conditions, this method was used to detect hepatitis B virus (HBV) DNA in patient sera. This technology eliminates the risk of carry-over contamination, simplifies the amplification assay and opens up new possibilities for the real-time detection of the amplified DNA.

Keywords probe-primer, polymerase chain reaction (PCR), hepatitis B virus (HBV) DNA

Introduction

On the post-genome era, quantitative studies of genomic information for disease diagnosis and prevention and drug discovery will be fast-growing areas of research and development. Growth in these areas has already produced a strong demand for advanced biomolecular recognition probes, which have high sensitivity and excellent specificity.[1]

TaqMan[2-4] and molecular beacon[5,6] are the two such promising probes. TaqMan uses a linear probe that has a fluorophore at its 5′-end and a quencher at the 3′-end. When the probe is single stranded, the three-dimensional conformation of the probe brings the two labels close enough to each other for the quencher to quench the fluorophore fluorescence. During polymerase chain reaction (PCR), this probe is cleaved by the 5′-3′ exonuclease activity of Taq DNA polymerase if, and only if it hybridizes to the DNA segment being amplified. Cleavage of the probe between the fluorophore and the quencher generates an increase in the fluorescence intensity of the fluorophore. The difference between TaqMan probes and molecular beacons is that the optimized stem-loop structure of molecular beacons brings the two labels as close together as possible when the probe is not hybridized to a target sequence. This ensures maximal quenching efficiency. Specific binding to a PCR product opens the hairpin, separating the fluorophore and quencher, thus producing fluorescence.

Amplifluor[7] is a new beacon variant that is a PCR primer with a hairpin structure at the 5′-end containing a fluorophore and quencher. When the primer is incorporated into a PCR product, the fluorophore and quencher moieties are separated and fluorescence is thus increased. Scorpion primer[8,9] is a modified Amplifluor with a PCR stopper between the hairpin loop and the 5′-end of a primer. Under fast cycling condition, scorpion primers may outperform TaqMan or beacon probes.

All of these methods based on fluorescence energy transfer are characterized by relatively high signal-to-noise ratios and a good ability to discriminate between positive and negative reactions. However, they are all limited in the sense that a dual-labelled probe has to be used. This seriously complicates design and synthesis of the probe,[10] consequently increases the expense of experiments.

The method reported in this paper is designed for the detection of the amplified DNA by incorporation of a single-labelled primer into PCR amplification. In this method, two complementary oligonucleotides of different length are used. The longer one is probe-primer strand, which is a PCR primer with a specific probe sequence attached at the 5′-end. A donor fluorophore-FAM (6-carboxyfluorescence) is labelled at the 5′-end of probe sequence. The short one is primer-quencher strand, which is complementary to the probe sequence of probe-primer and labelled with a nonfluorescent quenching moiety-DABCYL (4-[4′-dimethylaminophenylazo] benzoic acid) at the 3′-end. In the duplex formed by probe-primer and primer-quencher, FAM and DABCYL are kept in close proximity to each other. Therefore the fluorescence of FAM is quenched. After extension of the probe-primer during PCR amplification, the specific probe sequence is able to

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bind to its complement within the same strand of DNA, and is cleaved by 5′-3′ exonuclease activity of Taq DNA polymerase in the process of extension during the next cycle, resulting in a significant increase in emission intensity of the fluorophore (Fig. 1). In this study, we will use hepatitis B virus (HBV) DNA as a target gene and discuss the feasibility of this method.

Experimental

Apparatus

PCR was performed in a Perkin Elmer 480 DNA Thermal Cycler (USA). Fluorescence intensity was obtained in a microquartz cuvette (16.40-F, Starna Brand, England) using a Shimadzu Model RF-540 spectrofluorometer (Kyoto, Japan). A UV-VIS TU-1901 spectrophotometer (Beijing Purkije General Instrument Co.) was used for recording absorption spectra. The electrophoresis assay was conducted on a DF-D slab electrophoretic instrument (Dongfang Teli, Beijing).

Design of primers and probes

The primers (upstream primer: 5′-GTTCAAGCCTCCAAGCTGTG-3′, downstream primer: 5′-TCAGAAGGCAAAAAAGAGAGTAACT-3′) were designed to amplify a 104bp region within a HBV gene. A probe-primer (5′-FAM-CAAAGCCACCCAAGGTTCAAGCC TCCAAGCTGTG-3′) was constructed by linking a FAM-labelled probe sequence (underlined) to the 5′-end of upstream primer. An oligonucleotide, which is labelled with DABCYL at the 3′-end was used as primer-quencher: 5′-CTTGGGTGGCTTTG-DABCYL-3′. Shanghai Shenyou Ltd. carried out the coupling reactions and purification of the probes.

PCR *condition and fluorescence analyses*

25 μL of reaction mixture consisted of 1 × PCR buffer (10 mmol/L KCl, 8 mmol/L $(NH_4)_2SO_4$, 10 mmol/L Tris-HCl, pH 9.0, NP-40), 2 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 2.5 U of Taq DNA polymerase, 0.4 μmol/L probe-primer or upstream primer, 0.4 μmol/L downstream primer, 0.6 μmol/L primer-quencher, and 2 μL of template. Cycling was designed with a pre-cycle (95 ℃ for 5 min), 40 cycles for amplification (94 ℃ for 30 s, 60 ℃ for 45 s). In order to increase reaction specificity, Taq DNA polymerase was added after the temperature of reaction mixture reaching 95 ℃. After amplification, 25 μL of product was diluted to 100 μL with PCR buffer (2 mmol/L $MgCl_2$ included) and analyzed for fluorescence. Fluorescence emission was measured at $\lambda_{ex} = 490$ nm, $\lambda_{em} = 516$ nm. Biochemical agents were all purchased from Sangon (Shanghai).

Agarose gel electrophoresis

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

Results and discussion

Absorption spectra

DABCYL is a nonfluorescent quenching moiety that can serve as a universal quencher in molecular beacons.[11,12] It

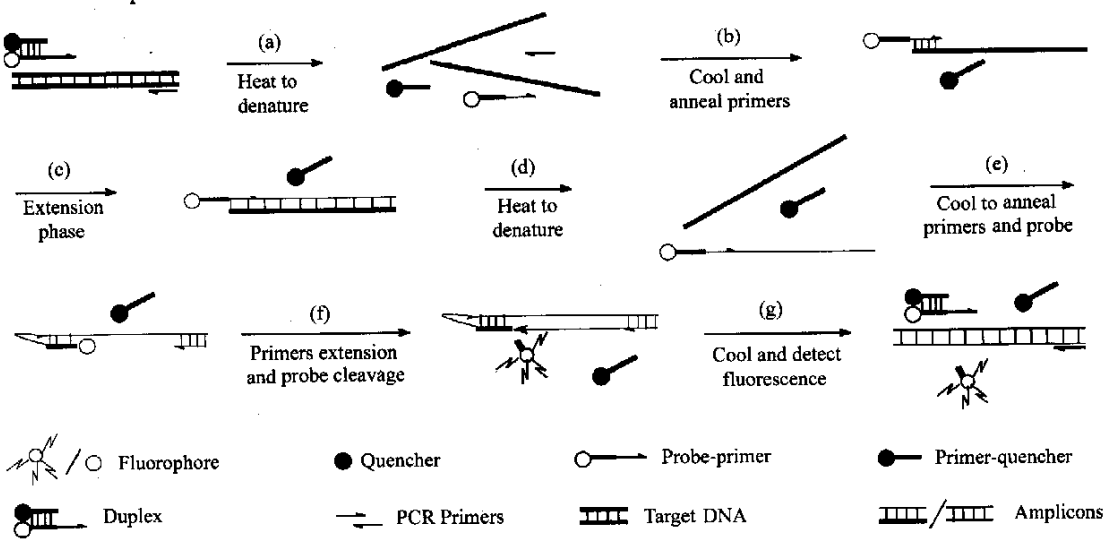

Fig. 1 Assay principle. (a) Initial denaturation of target and the duplex formed by probe-primer and primer-quencher; (b) annealing of primers to target; (c) extension of probe-primer produces double-stranded DNA; (d) denaturation of double-standed DNA produced in step (c), this gives a single-stranded target molecule with the probe-primer attached; (e) on cooling, the probe sequence of probe-primer binds to its target in an intramolecular manner; (f) extension of downstream primer causes the cleavage of hairpin stem; (g) on cooling, primer-quencher binds to probe-primer again, and fluorescence signal is produced.

can not quench the fluorescence of some fluorophores successfully via fluorescence resonance energy transfer (FRET), because its absorption spectrum has little overlap with the emission spectra of those fluorophores. But for molecular beacons, the closed conformation brings the fluorophore and quencher sufficiently close to one another. Thus two forms of energy transfer may exist: direct energy transfer and FRET, and the first one may be dominant.[1] This mechanism of energy transfer ensures maximal quenching efficiency and minimal background. In the duplex formed by probe-primer and primer-quencher, the fluorophore and quencher are also held close together. In this state the same energy transfer mechanism as molecular beacons may occur between them. It is confirmed by comparing the visible absorption spectra of the probe-primer system (Fig. 2) and molecular beacons (Fig. 3). In Fig. 2, trace 1 and trace 2 are the absorption spectra of probe-primer and primer-quencher, respectively. Trace 3 is the spectral summation of them, this case is very similar to molecular beacons in opened conformation (Fig. 3, trace 1), in which the fluorophore and quencher separate away from each other, no interaction occurs between them. Trace 4 is the absorption spectrum of the duplex formed by probe-primer and primer-quencher, as closed molecular beacons, in which the fluorophore and quencher are held sufficiently close to each other to perturb their electronic structure,[12,13] causing the substantial change of absorption spectra.

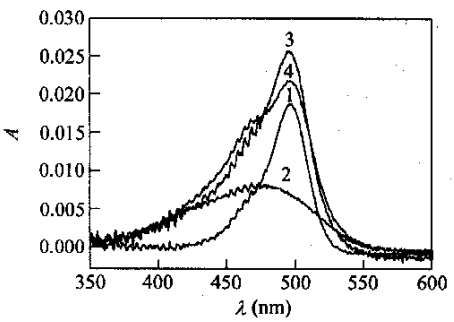

Fig. 2 Absorptin spectra of equal mole of (1) probe-primer; (2) primer-quencher; (3) summation of (1) and (2); (4) duplex formed by probe-primer and primer-quencher.

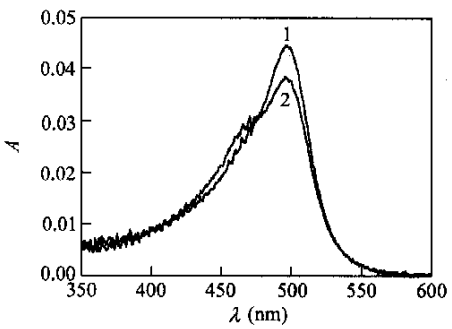

Fig. 3 Absorption spectra of a molecular beacon: FAM-CCCGAGATTGAGATCTTCTGCGACTCGGG-DABCYL (underlines identify the complementary stem sequence) (1) in the presence of equivalent target; (2) without target.

Effects of primer-quencher concentration on quenching efficiency

Quenching efficiency is a very important factor in determining the quality of fluorogenic probes. The higher the quenching efficiencies are, the better the probes can work. The quenching efficiency of light emission from the fluorophore by the quencher was calculated according to the following formula: $E_{ff} = [1 - (F_q - F_b)/(F_{uq} - F_b)] \times 100\%$. Here F_{uq} is the fluorescence intensity of probe-primer in the absence of primer-quencher and F_q is its fluorescence intensity when bound to primer-quencher, F_b is the background fluorescence intensity of buffer only. In order to acquire maximal quenching efficiency, F_q must be kept at the lowest level. Therefore, the probe-primer should sufficiently bind to primer-quencher. Fig. 4 shows the influence of different concentration ratios of primer-quencher to probe-primer on background fluorescence and quenching efficiency. With the increasing of concentration ratios, the background fluorescence will be greatly decreased, and the quenching efficiency increases correspondingly. When the ratio exceeds 1.5, the background reaches a plateau, and the quenching efficiency keeps at 97.3%, which is compatible with that of molecular beacons,[14] and much higher than that of TaqMan probes. In this study, we selected 1.5 as the concentration ratio of primer quencher to probe-primer in PCR.

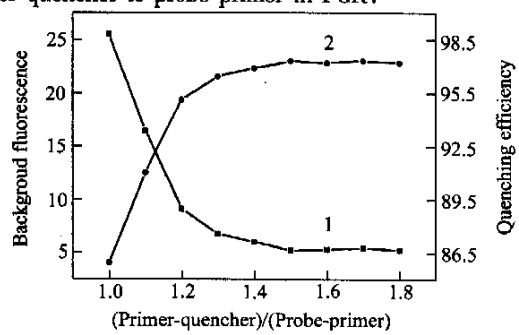

Fig. 4 Effect of different concentration ratios of primer-quencher to probe-primer on (1) background fluorescence and (2) quenching efficiency.

PCR *condition optimization*

We optimized PCR assays by adjusting the concentration of Mg^{2+}. Fig. 5 shows the fluorescence intensities of PCR assays at different Mg^{2+} concentration. The increasing of Mg^{2+} concentration has great influence with the fluorescence at low concentration range. When Mg^{2+} concentration exceeded 1.5 mmol/L, the fluorescence had nearly no change with it. So we adjusted the concentration of Mg^{2+} to 2 mmol/L, which provided the maximum fluorescence signal without drastically compromising the yield and specificity of PCR.

Effect of probe-primer on PCR

In order to investigate the influence of probe-primer on

product amplification, PCR amplifications of a 104bp product from HBV DNA directed by downstream primer and upstream primer (or probe-primer) were carried out. Agarose gel electrophoresis-EB staining results show that probe-primer has no effect on the amount of amplified product generated. Samples using upstream primer or probe-primer show a comparable level of specific product. But for the reaction with probe-primer, the product has a little larger size, and the electrophoretic band is a little wider (Fig. 6). After PCR extension of the probe-primer, the resultant amplicon contains a sequence that is complementary to the probe. At the anneal-extend step of next cycle, the probe sequence of probe-primer binds to its target in an intramolecular manner and a hairpin stem forms. In this case, when the DNA polymerase with 5′-3′ exonuclease activity reaches the 5′-end of the hairpin stem, it will displace and cleave it into mono- and oligonucleotide. Thus the stability of hairpin structure decreases gradually till it opens. Then primer extension continues and a part of probe sequence is also copied. As a result, the product has larger size than that formed by upstream primer. And due to the uncertainty of the sizes of released fragments during cleavage, PCR products with different length can be produced, and its electrophoretic band is a little wider. For the PCR amplification of healthy serum or negative control, no intended products are produced no matter whether upstream primer or probe-primer is used.

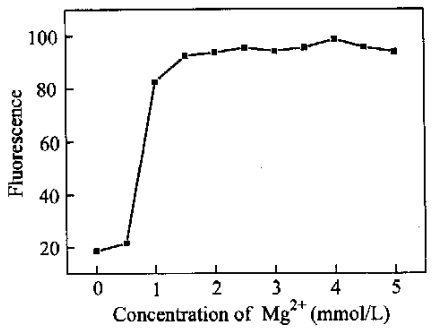

Fig. 5 Effect of the concentration of Mg^{2+} on fluorescence analysis.

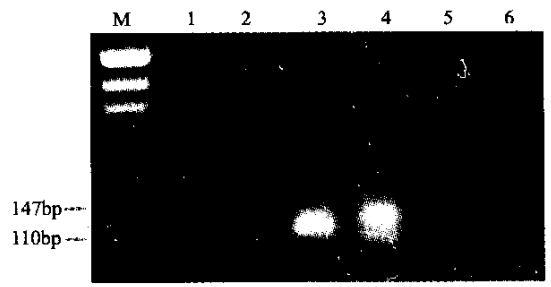

Fig. 6 Effect of probe-primer on PCR amplification. Lane 1, PCR amplification of healthy serum with upstream primer; Lane 2, PCR amplification of healthy serum with probe-primer; Lane 3, PCR amplification of HBV-infected serum with upstream primer; Lane 4, PCR amplification of HBV-infected serum with probe-primer; Lane 5, negative control with upstream primer; Lane 6, negative control with probe-primer.

Monitoring of PCR *in real time*

To demonstrate that the fluorescence produced during PCR can be used to monitor the reaction, HBV DNA extracted from patient serum was amplified with the probe-primer (primer-quencher included). The fluorescence intensity was determined after different numbers of cycles using a spectrofluorephotometer. This reaction reflects the typical characteristics of PCR (Fig. 7). At the initial stage, a small amount of PCR product is synthesized, and the fluorescence keeps at a low level. With the increasing of cycling numbers, PCR product accumulates enormously and fluorescence signal increases rapidly simultaneously. At the last stage of amplification, PCR product stops accumulating exponentially due to depletion of reaction components, and the increasing of fluorescence signal is slowing down. The magnitude of fluorescence correlates strongly with the amount of amplification product that has been synthesized.

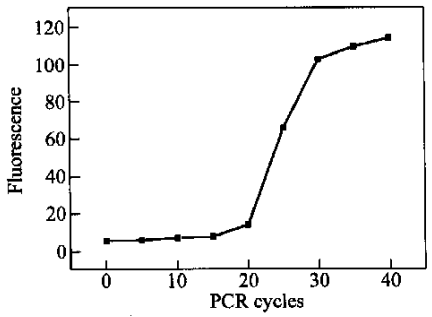

Fig. 7 Monitoring polymerase chain reaction on real time.

Specificity of PCR/*probe-primer assays*

Four human serum specimens were analyzed by probe-primer method (Fig. 8). The results show that only HBV-infected serum gives an intense fluorescence signal above background. For healthy serum, HCV-infected serum and HEV-infected serum, the fluorescence intensities are very low and can be considered as negative.

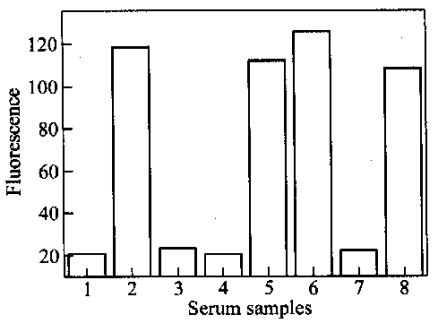

Fig. 8 Fluorescence analysis of different serum samples. 1, Healthy; 2, HBV-infected; 3, HCV-infected; 4, HEV-infected; 5, HBV, HCV-mixed infected; 6, HBV, HEV-mixed infected; 7, HCV, HEV-mixed infected; 8, HBV, HCV, HEV-mixed infected.

Some patients may be simultaneously infected by several kinds of viruses such as HBV, HCV and HEV. To determine whether our probe-primer can specifically recognize HBV DNA in mix-infected sera, four mix-infected sera were prepared by mixing HBV, HCV or/and HEV-infected sera equivalently for PCR/probe-primer assay (Fig. 8). As expected, HCV, HEV mix-infected serum has low fluorescence signal intensity, all the other three sera give positive results and the fluorescence intensities are compatible with that of alone HBV-infected serum. This result shows that this method can specifically detect HBV in mix-infected sera, other concurrent viruses did not interfere with the detection.

Sensitivity of PCR/*probe-primer assay*

HBV DNAs were extracted from healthy serum diluted HBV-infected serum by using protease K-phenol method. We performed PCR with a template of these HBV DNAs as described above. The results show that PCR/probe-primer assay was able to detect the target up to 10^4 HBV copies in one millilitre serum unambiguously (Fig. 9). It is to say that this assay can produce consistent positive results when it is initiated with 200 copies of template DNA molecules. This sensitivity is compatible with that of agarose gel electrophoresis-EB staining. If it is combined with real-time PCR instrument, the detection sensitivity will be further improved.

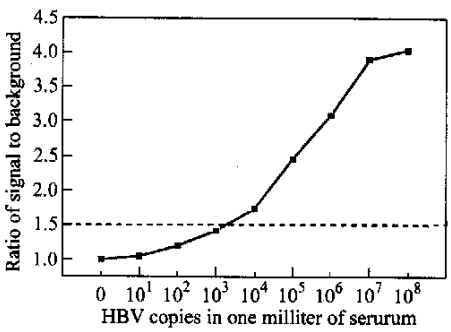

Fig. 9 Sensitivity of probe-primer method. The dash line indicates the cut-off value, which is calculated by adding the mean fluorescence to 4 standard deviatons of ten healthy sera templates.

Recently, the definition in a retrospective study is that the asymptomatic carrier and chronic hepatitis states can be distinguished by a serum HBV DNA concentration of 10^5 copies/mL.[15] If after termination of treatment serum HBV DNA concentration is found to be less than 10^4 copies/mL, hepatitis does not occur thereafter.[16] Our method can fully content with this detection.

The goal of the studies described herein is to design a probe-primer that can act as substrates for the exonuclease activity of Taq DNA polymerase and so function by a TaqMan-like mechanism. So the probe sequence of probe-primer must bind to target before primer extension occludes the probe-binding site. In our experiments, the probe sequence of probe-primer binds to its target in an intramolecular manner, which is much faster than the intermolecular binding of the complementary target strand, such as the binding of primers to template. John Santalucia's 'Nucleic Acid Hybridization' software (http://ozone2.chem.wayne.edu) was used to calculate the melting temperature (T_m) of the hairpin structure formed by the probe sequence and its target. In NaCl (100 mmol/L) and $MgCl_2$ (2 mmol/L) the predicted T_m is 79.6 ℃. That is to say, the hairpin structure is very stable at the anneal-extend temperature of PCR. Thus, the cleavage of hairpin stem can be ensured.

Compared with dual-labelled probes, single-labelled ones have the advantages of lower expense and ease for designing, synthesis and purification. During the synthesis of dual-labelled probes (molecular beacons, TaqMan, amplifluor and scorpion primer) at least twice purifications are needed.[17] 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 single-labelled ones. In addition, we can acquire optimised quenching efficiency by adjusting the ratio of probe-primer to primer-quencher.

The most critical problem associated with TaqMan technology is the distance between the dye and the quencher.[18] 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 will increase the signal during PCR assays, but at the expense of a much higher background.[19] Our probe-primer system has the similar energy transfer mechanism as molecular beacons, it should give a lower background and its quenching efficiency is much better than conventional TaqMan probes. Thus, a better detective effect 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. And only the probe fraction that will successfully compete against the strand complementary to the target strand in the hybridization reaction, will emit fluorescence.[20] 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, probe-primer can work as TaqMan probes during PCR amplification, and fluorescence signal does not mainly come from hybridisation of the probes, but come from the cleavage of them. So, competing hybridization is insignificant for 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 (Förster) mechanism. This must place a limitation of the intensity of fluorescence. This is also a limitation with Amplifluor and scorpion primers but not with probe-primers, which are enzymically cleaved during PCR, thus distantly separating the fluorophore from the quencher.

A disadvantage of this method is that non-specific PCR products (*e. g.* primer dimmers or mispriming events) will

affect the detection of specific products. In this study, PCR hot start is used to minimize this effect. Solinas[21] resolved this problem by adding a PCR stopper into probe-primers between the probe and primer sequence, better results were obtained but the cost increased correspondingly.

Conclusion

A new method for the detection of PCR-amplified DNA was designed, in which two single-labelled oligonucleotides (a probe-primer and a primer-quencher) were used. Compared with dual-labelled probe detection technology, this probe-primer method has some advantage, such as lower expense, ease for design, synthesis and purification, low background signal and hence good detection effects. The emergence of probe-primers may provide a better nucleic acid probe that can be used in homogenous assay of PCR-amplified products in a closed-tube format.

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