Chinese Journal of Chemistry, **2004**, *22*, 903—907 **Article**

Detection of Hepatitis B Virus DNA by Duplex Scorpion Primer-based PCR Assay†

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The application of a new fluorogenic probe-based PCR assay (PCR duplex scorpion primer assay) to the detection of Hepatitis B virus (HBV) DNA in human sera was described. Duplex scorpion primer is a modified variant of duplex Amplifluor, and the incorporation of a PCR stopper between probe and primer sequences improve the detection specificity and sensitivity. Combined with PCR amplification, this probe can give unambiguous positive results for the reactions initiated with more than 20 HBV molecules. In addition, the particular unimolecular probing mechanism of this probe makes the use of short target-specific probe sequence possible, which will render this probe applicable in some specific systems.

Keywords duplex scorpion primer, hepatitis B virus (HBV) DNA, fluorogenic probe

Introduction

A significant advance in polymerase chain reactions (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.[1] So far, TaqMan[2,3] and molecular beacons[4-6] are two such robust read-out probes. They have been widely used in pathogenic detection, single nucleotide polymorphism (SNP) genotyping, binding studies of DNA and proteins, and real-time enzymatic cleavage assay. Based on these two probes, several new methods, such as Amplifluor,[7] Scorpion primers[8,9] and TaqMan-MB,[10] have been developed. However, for these methods the fluorophore and the fluorescence quencher are dual-labeled on the probe. This seriously complicates the design and the synthesis of the probes[11] and consequently increases the expense of experiments.

Recently, we developed a new method (duplex Amplifluor) for the detection of PCR-amplified DNA,[12] in that, the use of two single-labeled oligonucleotides provided some advantages, such as relatively low expense and ease for design, synthesis and purification. Duplex Amplifluor was developed by combining Amplifluor and duplex probe methods.[13-15] So, as Amplifluor method, a disadvantage of this method is that specific and non-specific PCR products are indistinguishable,[16,17] and the formation of non-specific PCR product can also displace the quencher strand to bind with the probe-primer strand, leading to an increase in fluorescence. In order to increase reaction specificity, Taq DNA polymerase was added after the temperature of reaction mixture reaching 95 ℃, which increased the complexity of operation and the risk of carry-over contamination in some degree.

In this paper, we report another duplex probe-duplex scorpion primer (DSP), which is a modified version of duplex Amplifluor. A DSP consists of two oligonucleotides with different lengths: the longer one is probe-primer strand (PPS), in which a probe sequence is linked to the 5'-end of a primer sequence via a PCR blocker, and a fluorophore-FAM (6-carboxyfluorescence) is labeled at the 5'-end of the probe sequence. The shorter one is quencher strand (QS), which is complementary to the probe sequence of PPS and labeled with a nonfluorescent quenching moiety-DABCYL (4-[4'-dimethylaminophenylazo]benzoic acid) at the 3'-end. In the duplex 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 PCR amplification, the specific probe sequence of PPS can bind to its complement within the same strand of DNA, and is cleaved by 5'-3' exonuclease activity of Taq DNA polymerase during the next cycle, resulting in a significant increase in emission intensity of the fluorophore (Figure 1). The PCR stopper used 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

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PCR stopper, and the new synthesized strand does not contain the sequence that is complementary to the probe sequence of PPS. That is to say, the formation of non-specific PCR products will not affect the binding of PPS and QS. Thus, the effect of non-specific PCR products is greatly eliminated. In this study, we 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). The electrophoresis assay was conducted on a DF-D slab electrophoretic instrument (Dongfang Teli, Beijing).

Preparation of template DNA

Extraction of HBV DNA from sera was done essentially by using the protease K-phenol method.[18] Serum (200 μL) was mixed with 200 μL of cleavage buffer (50 mmol/L Tris-HCl, pH 7.8, 5 mmol/L EDTA and 1% sodium dodecyl sulfate) containing 1 mg/mL protease K. The mixture was incubated at 60 ℃ for 2 h, then 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 of TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA).

Design of probes

Two single-labeled oligonucleotide probes were designed. PPS (5'-FAM-CAAAGC CACCCAAG-Blocker-GTTCAAGCCTCCAAGCTGTG-3') was constructed by linking an FAM-labeled probe sequence to the 5'-end of upstream primer via a PCR blocker (a strand with three carbons). An oligonucleotide, which was labeled with DABCYL at the 3'-end was used as QS: 5'-CTTGGGTGGCTTTG-DABCYL-3'. Custom oligonucleotides were synthesized and purified by Shanghai Shenyou Ltd. (Shanghai China).

Amplification by PCR and fluorescence analysis

For PCR, the primers (PPS and downstream primer: 5'-TCAGAAGGCAAAAAAG AGAGTAACT-3') were designed to amplify a 104 bp region within an HBV

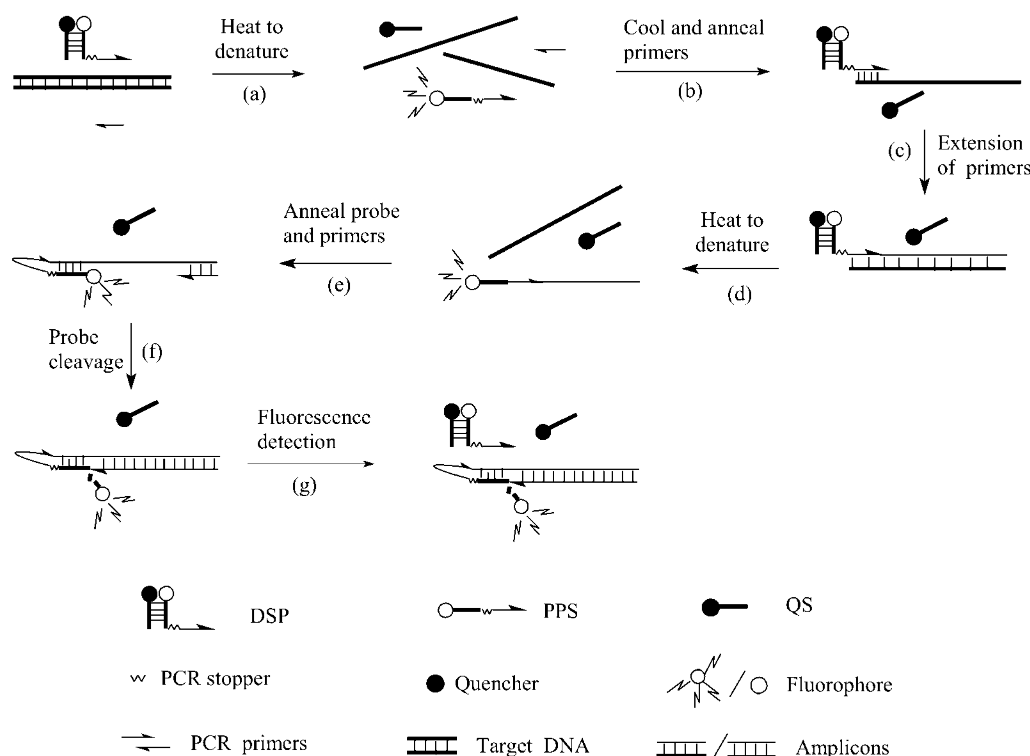

Figure 1 Working principle of duplex scorpion primers. (a) Initial denaturation of double-stranded template DNA and duplex scorpion imer. (b) Annealing of duplex scorpion primer to target. (c) Extension of primer in the presence of Taq DNA polymerase, and double-stranded DNA being produced. (d) The obtained double-strand PCR product denatured at high temperature. (e) The probe sequence of duplex scorpion primer hybridized to its target in an intramolecular manner, and the downstream primer annealed at its binding site. (f) In the presence of the Taq DNA polymerase, extension of downstream primer caused the cleavage of hairpin stem, and fluorophore and quencher moieties were separated completely. (g) On cooling, unextended duplex scorpion primer was formed again, and fluorescent signal was detected at this step.

gene. PCR amplification consisted of 25 μL of reaction mixtures containing 1×PCR buffer (10 mmol/L KCl, 8 mmol/L $(NH_4)_2SO_4$, 10 mmol/L Tris-HCl, pH 9.0, NP-40), 3.5 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 2.5 U of Taq DNA polymerase, 0.4 μmol/L PPS, 0.4 μmol/L downstream primer, 0.64 μmol/L QS, and 2 μL of template. Cycling was designed with a pre-cycle (95 ℃ for 5 min) and 45 cycles with amplification (94 ℃ for 30 s, 60 ℃ for 45 s). After amplification, 25 μL of product was diluted to 100 μL with 1×PCR buffer (3.5 mmol/L $MgCl_2$ included) and analyzed with fluorescence. Fluorescence emission was measured at λ_{ex}=490 nm and λ_{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 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

Preparation of DSP

A DSP was constructed by two oligonucleotides: PPS and QS. At the fluorescence detection steps of PCR amplification, excess unextended PPS would form a stable duplex with QS, which keeps the fluorophore and quencher moiety in close proximity to each other. Therefore, the fluorescence of the fluorophore was quenched, and background fluorescence was kept at a low level. In this paper, DSP was made by titrating the PPS with QS to reach the lowest fluorescence. It was noticed that, when the QS/PPS ratio exceeded 1.2, the background fluorescence was kept at a low level, and the quenching efficiency was kept higher than 97% (Figure 2). In this study, we selected 1.6 as the concentration ratio of QS to PPS. In order to prevent possible inter- or intra-molecular secondary structure, the mixture of PPS and QS was denatured in a thermal cycler at 95 ℃ for 5 min, annealed at 45 ℃ for 15 min and allowed to cool to room temperature.

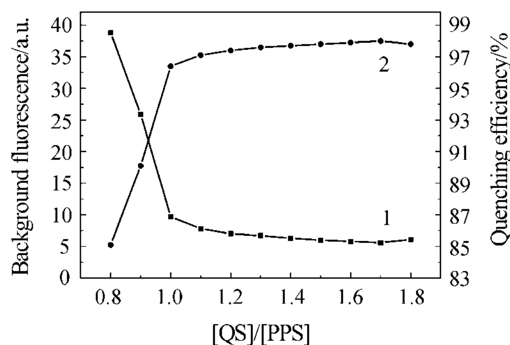

Figure 2 Effect of different concentration ratios of QS to PPS on (1) background fluorescence and (2) quenching efficiency. Quenching efficiency was calculated according to the following formula: $E_{ff}=[1-(F_q-F_b)/(F_{uq}-F_b)]\times 100\%$. Here F_q and F_{uq} are the fluorescence intensities of the PPS/QS duplex and the PPS, respectively. F_b is the background fluorescence intensity of buffer only.

Development of PCR assays

The ability of this DSP 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 ℃ and an anneal-extend temperature at 60 ℃ was used for end-point measurements. Combined with a specific PCR instrument, this DSP can also be used in real-time PCR, in which fluorescent signal detection is concurrent with target amplification. In either end-point or real-time detection format, fluorescence detection must be conducted at a relative low temperature, so that PPS can hybridize to QS, and a stable duplex is formed. John Santalucia's 'Hyther' software (http://ozone2.chem.wayne.edu/Hyther/hythe/main.html) was used to calculate the melting temperature (m.p.) of the duplex formed by PPS and QS. In 100 mmol/L NaCl and 3.5 mmol/L $MgCl_2$ the predicted m.p. is 57.0 ℃. For end-point detection format, fluorescence emission can be measured at ambient temperature. In real-time detection format, an anneal-extend temperature lower than 57.0 ℃ should be used, or a fluorescence detection step with a temperature lower than 57.0 ℃ could be added at the end of each PCR cycle. In this paper, the use of DSP in the end-point format was studied.

PCR assays were also optimized by adjusting the concentration of Mg^{2+}. The results show that the increase of Mg^{2+} concentration has great influence on the fluorescence at low concentration range. When Mg^{2+} concentration exceeds 3.5 mmol/L, the fluorescence shows no further change and is kept at high levels (Figure 3). So the concentration of Mg^{2+} was adjusted to 3.5 mmol/L, which provided the maximum fluorescence signal without drastically compromising the yield and specificity of PCR (data not shown).

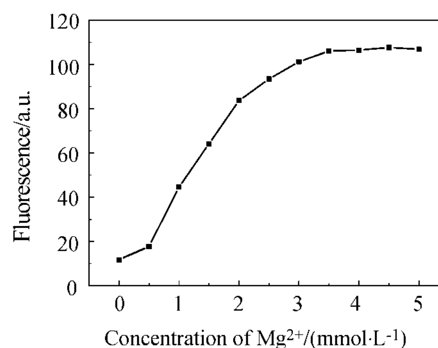

Figure 3 Effect of the concentration of Mg^{2+} on fluorescence analysis.

Monitoring of PCR in real time

To demonstrate that the fluorescence produced dur-

ing PCR can be used to monitor the reaction, two PCR amplifications were performed in parallel (Figure 4): reaction 1 used HBV DNA as template but reaction 2 did not contain any template DNA. The intensities of the fluorescent signal were plotted as a function of the number of thermal cycles completed. Reaction 1 gave a fluorescence plot with typical exponential rates followed by a plateau phase. The exponential rate is consistent with a cyclic phase of PCR reaction in which the products of amplification function as templates. The plateau phase is consistent with the stage of PCR amplification when the amplicon stops accumulating exponentially, due to depletion of reaction components. But in reaction 2, no significant fluorescence was developed as the increase of PCR cycles. The results show that the magnitude of fluorescence is correlated strongly with the amount of amplification product that has been synthesized, and DSP can be used to monitor the process of PCR amplification.

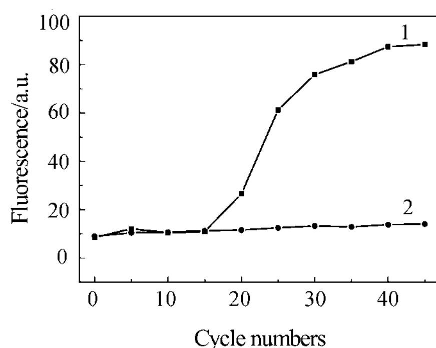

Figure 4 Monitoring PCR amplification in real time. (1) PCR amplification using HBV DNA as template. (2) PCR amplification without adding template molecules.

Sensitivity of PCR/DSP assay

Ten-fold dilution series of serum samples were prepared by diluting HBV-infected serum (containing 10^8 HBV copies per mL) with HBV-negative serum, and HBV DNA was tested in triplicate by PCR/DSP, PCR/duplex Amplifour and PCR/agarose gel electrophoresis methods, respectively (Table 1). The last dilution (10^{-4}) of serum sample found to be positive in triplicate by these three assays contained 10^4 HBV copies in one milliliter serum. That is to say, all of these methods can produce consistent positive results when they are initiated with 200 copies of template DNA molecules. When a 10-fold higher dilution containing approximate 10^3 HBV copies/mL serum was tested, two of three replicas were positive by PCR/DSP assay and none was positive by PCR/duplex Amplifluor and PCR/agarose gel electrophoresis assay. When the dilution reached 10^{-6}, all assays gave negative results for three replicas. From these results it can be found that duplex Amplifluor and electrophoresis assays have compatible sensitivities, and the sensitivity of DSP is higher than that of those two methods. Compared with duplex Amplifluor, the higher sensitivity of DSP can be attributed to its better specificity, thus, its cut-off value, which is calculated by adding the mean fluorescence to 4 standard deviations of ten healthy serum templates, is lower than that of duplex Amplifluor.

Application to human serum sample detection

A blinded panel of serum samples was tested by PCR/DSP assay (Figure 5A), and the results were compared to those obtained from PCR-agarose gel electrophoresis method (Figure 5B). The concordance between these two methods was 100%. Compared with agarose gel electrophoresis method, DSP method greatly simplified the post-PCR operation. Combined with special PCR instruments, the real-time detection format of DSP can abrogate the need for post-PCR operation, and the risk of carry-over contamination can be reduced.

DSP was developed as a modified version of duplex Amplifluor and scorpion primers. Some advantages of duplex Amplifluor, such as ease for design, synthesis

Table 1 Comparison of the analytical sensitivities of the three methods

Dilution	The copies of HBV virus in 1 mL of human sera	HBV DNA copies in PCR mixtures[a]	Result		
			DSP method	Duplex Amplifluor method	Electrophoresis method[b]
1	10^8	2×10^6	+, +, +	+, +, +	+, +, +
10^{-1}	10^7	2×10^5	+, +, +	+, +, +	+, +, +
10^{-2}	10^6	2×10^4	+, +, +	+, +, +	+, +, +
10^{-3}	10^5	2×10^3	+, +, +	+, +, +	+, +, +
10^{-4}	10^4	2×10^2	+, +, +	+, +, +	+, +, +
10^{-5}	10^3	20	+, +, −	−, −, −	−, −, −
10^{-6}	10^2	2	−, −, −	−, −, −	−, −, −

[a] The HBV DNA copies in PCR mixtures were calculated according to the extraction of DNA with an efficiency of 100%. [b] In PCR-electrophoresis method, an upstream primer: 5'-GTTCAAGCCTCCAAGCTGT G-3' was used in stead of PPS.

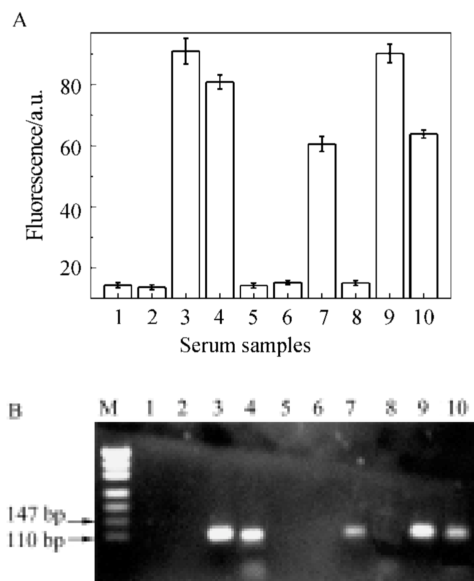

Figure 5 HBV detection in clinical human serum samples by (A) PCR/DSP method or (B) PCR-agarose gel electrophoresis method. Each sample was assayed five times in duplicate.

and purification, and low background signal, are also fit for DSP. The use of PCR stopper in DSP makes it workable as an internal probe, and hybridization of an internal probe provides an additional level of specificity of detection over that by duplex Amplifluor and agarose gel electrophoresis analysis. The higher specificity of DSP not only increases the sensitivity of this method in some degree, but also can help to reduce the false positive reactions. In the PCR/duplex Amplifluor method, PCR amplifications should be started by manual "hot start" operation, namely, Taq DNA polymerase is added after the temperature of reaction mixture reaching 95 ℃. Otherwise, the sensitivity of this method will decrease, and false positive results may appear. The use of DSP eliminates the need of "hot start" operation, and no false positive reactions were found for the samples tested.

In our DSP method, the probe sequence binds to its target in an intramolecular manner, and an intramolecular hairpin structure is formed. Compared with bimolecular hybridization mechanism, unimolecular probing mechnism is kinetically favorable and highly efficient.[9,19] For the two forms of DNA duplex: intramolecular hairpins and intermolecular hybrids, if the double-stranded stem of intramolecular hairpin has identical length and sequence with intermolecular hybrids, the stability of the former will be much higher than the latter. Therefore, a short target-specific probe sequence can be used in DSP, making DSP much applicable to the detection system in which long probe sequence can not be used. But to assure that the PPS/QS duplex keeps sufficient stability at the fluorecence-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 probe sequence (Figure 6). In conclusion, the development of DSP may provide a new method for the gene diagnosis of human diseases.

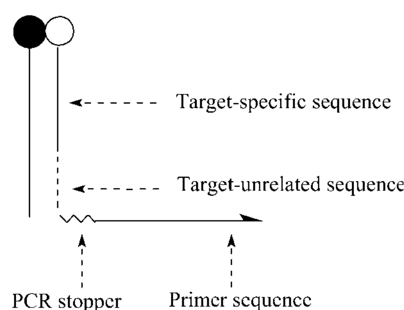

Figure 6 Diagram of a DSP with short target-specific probe sequence.

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