

## Optimization of competitively differentiated polymerase chain reaction in detection of HBV basal core promoter mutation

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### Abstract

**AIM:** To improve competitively differentiated polymerase chain reaction (CD-PCR) in detection of HBV basal core promoter mutation.

**METHODS:** Recombinant plasmid of double point mutation A1762T/G1764A in basal core promoter of HBV constructed by site-directed mutagenesis was used as mutant control. To reveal the deficiency mechanism of CD-PCR, relationship between the circle number of PCR and the increased speed of products of each competitive primer was comparatively studied. Diversified amount of dNTPs and mutual primer of the competitive primers were tried to optimize CD-PCR. Optimized CD-PCR was evaluated by detecting A1762T/G1764A mutation in recombinant plasmids and clinical sera from patients with HBV infection.

**RESULTS:** The deficiency mechanism of CD-PCR was that the products of mismatched competitive primer grew fast when the amplification of matched primer entered into plateau stage, which led to decrease in or disappearance of the difference in the amount of their products. This phenomenon could be eliminated by reducing dNTPs to 10  $\mu$ mol/L and mutual primer to about 100 nmol/L. Optimized CD-PCR could detect both mutant and wild strain independent of the amount of templates and the number of PCR cycles. Its detection limit was  $10^3$  copies/mL, about 50 copies/reaction. About 10% of mutant DNAs among wild type DNAs could be detected. A1762T/G1764A mutant was detected in 41.8% (51/122) of patients with HBV infection, but not detected in controls with negative HBsAg.

**CONCLUSION:** Optimized CD-PCR can detect mutation independent of the amount of initial templates and the number of PCR cycles.

### INTRODUCTION

Hepatitis B virus (HBV) is a significant cause of life-threatening liver diseases, such as fulminant hepatitis, liver cirrhosis and hepatocellular carcinoma<sup>[1-3]</sup>. HBV point mutations, including precore stop mutation G1896A, immune escape mutations K141E, P142S, D144E and G145R, basal core promoter double point mutation A1762T/G1764A and lamivudine-resistant mutation M552I or M552V, are very common and have important implications in the pathogenesis, treatment and prevention of chronic HBV infection<sup>[4-9]</sup>. Thus, to demonstrate these mutants in time is very important in clinical practice.

Many methods are used in detection of point mutations, especially in laboratory researches. Most methods are based on polymerase chain reaction (PCR). To analyze PCR products using direct sequencing is gold standard, but cannot be used in routine clinical examination. Thus, new methods are still emerging, including fluorometric real time PCR methods<sup>[10,11]</sup>, PCR with elaborately designed primers<sup>[12,13]</sup> and other methods to analyze PCR products using restriction fragment length polymorphism and enzyme linked mini-sequence assay (SMITEST)<sup>[14]</sup>. Fluorometric real time PCR is based on the small differences in hybridization melting curve kinetics of the probes and needs expensive apparatus<sup>[10]</sup>. To analyze PCR products usually needs much additional manipulation. PCR with elaborately designed primers, such as allele specific-PCR (AS-PCR) and sequence specific-PCR or amplification refractory mutation system<sup>[12,13]</sup>, is usually simple and rapid. However, its specificity is influenced by the amount of initial templates<sup>[12]</sup>. Unfortunately, the amount of HBV DNA in sera of patients with HBV infection varies greatly and is uncontrollable.

For these reasons, a novel method of competitively differentiated PCR (CD-PCR) was developed in our laboratory, which can rapidly and effectively demonstrate HBV precore mutation G1986A. CD-PCR was established based on AS-PCR. It employs competitive primers and a differentiation stage to minimize the adverse influence of the amount of initial templates and to stop the cross amplification between

wild and mutant products in subsequent cycles. Theoretically, the difference in PCR products between two competitive primers is independent of the amount of initial templates. However, CD-PCR only has an enlarged distinguishable range ( $10^2$ - $10^8$  copies/mL) as compared with AS-PCR, and clinical samples with high level of HBV DNA have to be diluted before examination. In this study, the deficiency of CD-PCR was investigated and its main components were optimized based on analysis of the PCR products using enzyme immunoassay (EIA).

## MATERIALS AND METHODS

### Reagents

Recombinant plasmid pTZ19U-HBV containing double copies of HBV DNA (adw) was a present from Professor Zhi-Min Huang, Zhongshan University. T4 DNA ligase and pfu DNA polymerase were purchased from Promega Company (USA). DNA gel extraction, plasmid isolation and serum DNA extraction kits were purchased from Omega Company (USA). Anti-digoxigenin and anti-fluorescein labeled with horseradish peroxidase were purchased from Roche Company (USA).

### Oligonucleotides

Primers and probes shown in Table 1 were designed with the software Omega 2 and synthesized in Bioasia Biological Engineering Company (Shanghai, China). The sequence of BCP-c1 was in concordance with the A1762T/G1764A mutant. BCP1-M and BCP1-W were competitive primers for mutant and wild strain, respectively. PCA was the mutual anti-sense primer of two competitive primers.

### Serum samples and DNA extraction

One hundred serum samples with HBsAg (+), anti-HBe (+) and anti-HBc (+), 60 serum samples with HBsAg (+), HBeAg (+) and anti-HBc (+) and 40 serum samples without HBV serum markers were collected. The serum markers were demonstrated by enzyme-linked immunoadsorbent assay. HBV DNA level in sera was detected using fluorescein quantitative PCR (Taqmen, Roche). Total DNA from 200  $\mu$ L serum was extracted using Omega kits, recovered in 50  $\mu$ L of elution solution and then stored at -70 °C until detection.

### Construction of mutant control using site-directed mutagenesis

To construct A1762T/G1764A mutant controls, pTZ19U-HBV was used as template, BCP-c1 and BCP-c2 were used as primers in PCR to obtain A1762T/G1764A mutant

fragments. The mutant fragments were then cloned into plasmid pUC19 to generate recombinant plasmid pHB-BCP2. After the sequences were confirmed by DNA sequencing, the recombinant plasmid was used as mutant control in CD-PCR.

### Basic CD-PCR for detection of A1762T/G1764A mutant

Principles of competitive primer design and CD-PCR for detection of HBV G1896A mutation were previously described. It was modified for detection of BCP mutation. Briefly, 25  $\mu$ L PCR reaction was performed. The reaction mixture contained 10 mmol/L Tris-HCl, pH 8.5, 50 mmol/L KCl, 1.5 mmol/L MgCl<sub>2</sub>, optimized amount of dNTPs, 2U pfu DNA polymerase, optimized amount of competitive primers (BCP-M, BCP-W), PCA and 5  $\mu$ L plasmid or extracted DNA. The cycling conditions were as follows: two cycles (first set) at 94 °C for 60 s, at 53 °C for 120 s and at 72 °C for 120 s, followed by 38 cycles (second set) at 94 °C for 30 s, at 65 °C for 30 s, and at 72 °C for 60 s. The PCR products were then hybridized with solidified biotin-labeled probe PCP in two different wells of microtiter plate. Color reaction was obtained after captured PCR products were reacted with horseradish peroxidase-labeled anti-DIG or anti-FITC. The optical absorption density ( $A$  value) was measured, and the ratio ( $A_m/A_w$ ) of  $A$  values in two wells with horseradish peroxidase-labeled anti-DIG ( $A_m$ ) or anti-FITC ( $A_w$ ) was used as criteria for the result analysis. The amount and ratio of competitive primers were determined as  $A_m$  for mutant control and  $A_w$  for wild control were almost similar when  $10^5$  copies/mL of recombinant plasmids pHB-BCP2 and pTZ19U-HBV were used as mutant control and wild control, respectively.

### Optimization of CD-PCR

Two hundred, 50, 10 and 2  $\mu$ mol/L of dNTPs and 500, 250, 100 and 50 nmol/L of primer PCA were comparatively studied. Two hundred microliters PCR reaction was performed with three parallel tubes. The products were analyzed at every five circles.

### DNA sequencing

Fragments of HBV basal core promoter, precore and core from 10 samples (five mutants, and five wild strains of HBV according to CD-PCR) were analyzed by DNA sequencing after they were amplified using primer PCSc and PCAc, and cloned into plasmid pUC19.

**Table 1** Primers and probe

Denomination	Sequences
PCSc	5'-CCCGAATTCACCCGTGAACGCCCATCAG-3'
PCAc	5'-CCCAAGCTTGCAGTATGGTGAGGTGAGCAATG-3'
BCP-c1	5'-CGGAATTCGAGGAGCTGGGGAGGAGATTAGGTTAATGATC-3'
BCP-c2	5'-AGACTGCAGAGGATAGAATCTAGCAGGC-3'
BCP1-W	5'-FITC-CGTCCGTAGTCCGAGGGAGGAGATTAGGTTAAAGG-3'
BCP1-M	5'-DIG -CGCTGAGATGCCACTGGGAGGAGATTAGGTTAATGA-3'
PCA	5'-CTCGCTGCACACTCTAAGGCTTCTCGATACAG-3'
PCP	5'-BIO-GACGGAAGGAAAGAAGTCAGAAGGCAAAA-3'

The underlined nucleotides were loci of restriction endonucleases. The italic characters had no relationship with HBV genome.

**RESULTS**

**Deficiency mechanism of CD-PCR**

The deficiency mechanism of CD-PCR was that the products of mismatched competitive primer grew fast when the amplification of matched primer entered into plateau stage, which led to decrease in or disappearance of difference in the amount of their products (Figure 1).

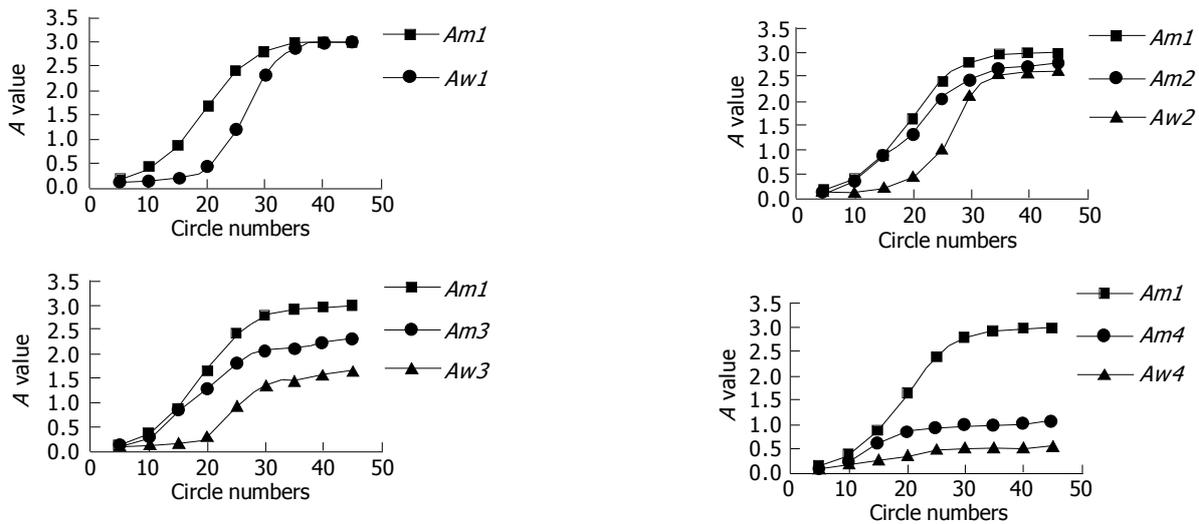
**Optimization of CD-PCR**

When  $10^7$  copies/mL of pHB-BCP2 were used as template,  $A$  value of the products of matched competitive primer BCP-M ( $A_m$ ) decreased, but the difference from the mismatched competitive primer BCP-W ( $A_w$ ) was enlarged independent of the number of cycles as the concentration of dNTPs was gradually reduced (Figure 1). However, both  $A_m$  and  $A_w$  were greatly decreased at 2  $\mu\text{mol/L}$  dNTPs, which was

inconvenient for the method to be used further. The optimized concentration of dNTPs was about 10  $\mu\text{mol/L}$ . Similar results were obtained when wild control pTZ19U-HBV was used as template. The concentration of primer PCA had an effect on CD-PCR similar to that of dNTPs and its optimized concentration was about 100 nmol/L.

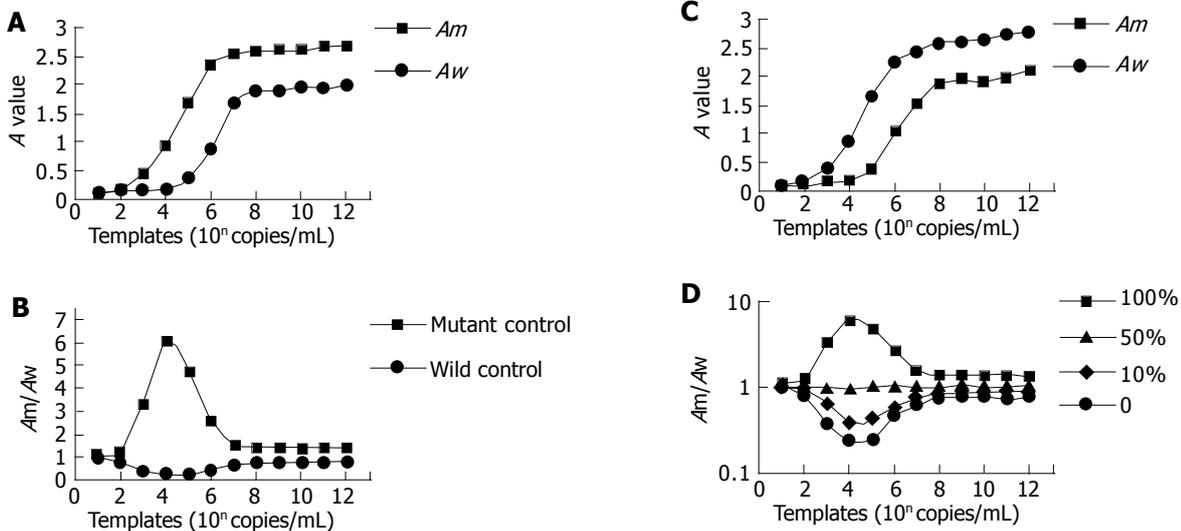
**Detection of BCP mutation in recombinant plasmid DNA using optimized CD-PCR**

The CD-PCR results of 10-fold dilution ( $10^1$ - $10^{12}$ /mL) of pHB-BCP2 and pTZ19U-HBV are shown in Figure 2. Both mutant and wild DNA could be detected using optimized CD-PCR independent of the amount of templates up to  $10^{12}$  copies/mL, which was higher than the serum level of HBV DNA in patients with chronic HBV infection. Its limit of detection was  $10^3$  copies/mL, about 50 copies/reaction.



**Figure 1** Optimization of the concentration of dNTPs. A: optical absorption density,  $A_m$  and  $A_w$ : A values of wells with horseradish peroxidase-labeled anti-DIG

( $A_m$ ) and anti-FITC ( $A_w$ ), respectively, 1: 200  $\mu\text{mol/L}$  of dNTPs, 2: 50  $\mu\text{mol/L}$  of dNTPs, 3: 10  $\mu\text{mol/L}$  of dNTPs, 4: 2  $\mu\text{mol/L}$  of dNTPs.



**Figure 2** Detection of BCP mutation in recombinant plasmid DNA using optimized CD-PCR. A: pHB-BCP2 as template, B: pTZ19U-HBV as template, C:  $A_m/A_w$  ratio as criteria for the result analysis, D: detection BCP mutation among mixture of pHB-

BCP2 and pTZ19U-HBV. A: optical absorption density,  $A_m$  and  $A_w$ : A values of wells with horseradish peroxidase-labeled anti-DIG ( $A_m$ ) and anti-FITC ( $A_w$ ), respectively, 0-100%: copy ratio of pHB-BCP2 with total recombinant plasmid DNA in mixture.

In order to demonstrate co-infections of mutant and wild HBV strains, the  $A_m/A_w$  ratio was employed in this study (Figure 2). About 10% of mutant DNA among wild DNA could be detected. The preferable range for co-infection detection was  $10^{3-7}$  copies/mL.

### Detection of BCP mutant in clinical samples

HBV DNA was positive in 122 of 200 serum samples for fluorescein quantitative PCR. The results of A1762T/G1764A mutant are shown in Table 2. A1762T/G1764A mutant was detected in 41.8% (51/122) of patients with HBV infection, but not detected in controls with negative HBsAg or in patients with negative HBV DNA. The DNA sequencing results of 10 selected samples were in concordance with those of CD-PCR.

**Table 2** A1762T/G1764A mutant detection using optimized CD-PCR in clinical sera

	Cases(n)	HBV DNA n (%)	A1762T/G1764A n (%)
HBsAg(+)/HBeAg(+)	60	60 (100.0)	19 (31.7)
HBsAg(+)/HBeAg(-)	100	62 (62.0) <sup>1</sup>	32 (51.6) <sup>2</sup>
HBsAg(-)/HBeAg(-)	40	0	0

<sup>1</sup> $\chi^2 = 29.90$ ,  $P < 0.01$ , <sup>2</sup> $\chi^2 = 4.99$ ,  $P < 0.05$ , compared with the group of HBsAg (+)/HBeAg (+).

## DISCUSSION

A simple and rapid method is urgently needed to meet massive patients and growing types of HBV mutants<sup>[15,16]</sup>. CD-PCR may be one of those methods. However, clinical samples with high level of HBV DNA have to be diluted before examination, suggesting that CD-PCR still depends on the amount of initial templates.

The reason why CD-PCR is still dependent on the amount of initial templates is unclear. In our study, the products of mismatched competitive primer grew very fast when the amplification of matched primer came into the plateau stage, which led to the decrease in or disappearance of difference in the amount of their products, suggesting that the mismatched competitive primer is preponderant in competition in the later stage of PCR because of its dominance in number due to less consumption. To let the reaction stop before the formation of plateau like AS-PCR by reducing the cycles with the introduction of basic amplification previously<sup>[17]</sup> or employing real time technique may be the right way to solve the problem. However, the former needs additional manipulation and increases the chance of contamination, and the later needs expensive apparatus. Optimized CD-PCR is independent of the number of cycles, suggesting optimized CD-PCR is sensitive since the templates at low level can be thoroughly amplified by increasing the number of cycles.

HBV A1762T/G1764A mutant is very common and usually accompanied with increase in HBV replication and decrease in HBeAg secretion, and may be related to liver deterioration<sup>[18-23]</sup>, or does not affect HBV DNA level<sup>[24]</sup>. This mutant occurs in patients with positive HBeAg, and may be predictive of seroconversion<sup>[25]</sup>, or a favorable factor

for interferon-induced anti-HBe seroconversion<sup>[26]</sup>.

In conclusion, HBV A1762T/G1764A mutant may be responsible for HBeAg negative hepatitis, and should be detected routinely in clinical practice. Optimized CD-PCR can detect HBV mutation.

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