Poly-linker primer system for detection of single nucleotide polymorphisms and its application in genotyping hepatitis B virus

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Single nucleotide polymorphisms (SNPs) and single-base mismatch in genome are closely associated with disease development, progress, treatment, and prognosis [1]. Many methods have been developed to detect SNPs. Some assays, such as Taqman MGB probe and high resolution melting, amplify and detect both wild-type and mutant genomic DNA by specific primers and probes [2], while some other assays, such as ARMS and PNA-LNA clamp PCR, only amplify and detect mutant templates [2,3]. Amplification Refractory Mutation System is widely used to detect SNP and mutations; however, the detection limit is ~1% to 5% of mutants in wild-type DNA content [4]. PNA-LNA clamp PCR is very sensitive and specific, but it is based on nest PCR and just a lab assay [5]. For the detection of drug-resistant genotypes of virus, such as hepatitis B virus (HBV), it is necessary to detect a few mutant templates at high level of wild-type DNA background. Recently, a dual priming oligonucleotide system (DPO) has been reported that poly-linker inserted into a primer formed a bubble-like structure, which would block the extension of non-specifically primed templates, and generate consistently high PCR specificity [6]. It greatly improves the distinguishing ability of primers for the detection of SNPs.

In this study, we reported a new real-time PCR-based assay for the detection of SNPs and single-base mismatch. The method is based on poly-linker primer system (PLPS) which is an improvement of DPO. The poly-linker primer contains four parts: (i) 5’-segment tail: 2–3 nt natural bases added to 5’-segment terminal, and not complementary with target sequence at the same position; (ii) anchor: 5’-segment 16–20 nt in length; (iii) 3 nt poly-linker (5’-III-3’), and (iv) distinguishing part: 3’-segment 6–7 nt in length. We hypothesize that the inserted 3 nt deoxyinosines (dI) which locates at primer 3’-segment n–6–7 position without bases mapping in the target sequence endows the SNP distinguishing ability of the primers. We used this improved assay for genotyping of HBV, which validated the ability of the assay to distinguish SNPs or single-base mismatch.

Serum samples from 159 patients were randomly retrieved from the Third People’s Hospital of Changzhou. This study was approved by the Ethics Committee of The Third People’s Hospital of Changzhou. All participants provided their written informed consent to participate in this study. HBV viral DNA was isolated from 200 μl of serum samples using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) and eluted into 50 μl of buffer according to manufacturer’s instructions. Full-length HBV genotype B and C genomes were cloned according to the method of Parekh et al. [7]. The plasmids were serially diluted from 1 × 109 to 1 × 102 IU/ml by using NanoDrop 2000 (Thermal Fisher Scientific, New Hampshire, USA) and used as control. Poly-linker primers and TaqMan probes for genotyping HBV are listed in Table 1. The RT-PLPS was performed in 50 μl of reaction mixture containing 0.15 μM PLPS primers, 0.1 μM probe, 2.5 mM Mg2+, 0.2 μM dNTP, 20 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50 mM KCl, 50% glycerol, and 2U Hotstart Taq DNA polymerase (BiOSCI, Shanghai, China). The amplification conditions were as follows: 94°C for 10 min, 10 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 30 s, and then 30 cycles of 95°C for 20 s, 58°C for 40 s, 72°C for 20 s. The PCR was carried out on a 7500 real-time PCR (Applied Biosystems, Foster City, USA). DNA sequencing and analysis of HBV genotypes were described as previously reported [8]. PCR products were separated by electrophoresis, purified by using a Qiagen gel extraction kit, and sequencing was done with the BigDye terminator cycle-sequencing reaction kit and Prism 3730 DNA analyzer (Applied Biosystems).

RT-PLPS is an improvement of DPO system. Three nt dI instead of 5 nt in DPO are inserted into the natural base primer, located at 3’-segment n–6–7 position. Due to weak hydrogen bonding, 3 nt poly-linker easily forms a ‘bubble-linker’ structure. There are three forms of ‘bubbles’ when the poly-linker primer binds to the template, called ‘small’, ‘middle’, and ‘large’ bubbles. When the 3 nt natural bases at targets complements with 3 nt dI poly-linker (3 vs. 3), a ‘small’ bubble
is formed. The ‘3 vs. 1 or 3 vs. 2’ forms middle bubbles, and ‘3 vs. 0’ forms ‘large’ bubbles (Fig. 1A). We hypothesize that poly-linker primer carried ‘large’ bubble-linker has more excellent single-base mismatch distinguishing ability than others.

To demonstrate the sensitivity and specificity of different types of poly-linker primer, HBV genotype C was used as an example. In China, the most prevalent HBV genotypes are Genotypes B and C. By comparison of genotype A–H complete genome, HBV could be genotyped by detection of single-base mismatch (Supplementary Fig. S1). Poly-linker primers 1–4 with 3 nt dI insertion forming ‘small’, ‘middle’, and ‘large’ bubble-linker were designed to detect HBV genotype C. Primers were used to detect 1 × 10² IU/ml–1 × 10⁶ IU/ml full-length plasmids of HBV genotype C. All primers (p1–p4) could detect 1 × 10⁵ IU/ml HBV genotype C plasmid. For the same concentration of the plasmids, Cₜ value was almost the same, with <0.5 cycle variation.

To evaluate the specificity of the four primers, experiments were carried out to detect 1 × 10⁵ and 1 × 10⁷ IU/ml Genotype B plasmids, respectively. The results proved our hypothesis that Primer 1 (forms large bubble) was more specific than the others. The Cₜ values were 26.45 ± 0.34 and 27.94 ± 0.30, respectively, for the detection of 1 × 10⁵ and 1 × 10⁷ IU/ml Genotype B plasmid. It was about two cycles delay compared with detection of 2 × 10⁵ IU/ml Genotype C plasmids (Supplementary Fig. S2A). When Primers 2–4 (carrying ‘small’ and ‘middle’ bubble-linker) were used to detect 1 × 10⁵ IU/ml Genotype B plasmids, the Cₜ values were 19.9 ± 1.01, 22 ± 0.49, and 20.75 ± 0.99, respectively. When Primers 2–4 were used to detect 1 × 10⁵ IU/ml Genotype B plasmid, the Cₜ value were almost the same as that in the detection of 1 × 10⁵ IU/ml Genotype C plasmids (Supplementary Fig. S2B–D). So the poly-linker primer that formed ‘large’ bubble-linker has better single-base mismatch detection ability.

To further evaluate the number of dI inserted, Primers 5 and 6 contained 4 and 5 nt dI forming ‘large’ bubble-linker were applied to detect serially diluted plasmids (1 × 10⁵–1 × 10⁶ IU/ml) of Genotype C plasmids and 1 × 10⁸ ∼ 1 × 10⁷ IU/ml Genotype B plasmids, respectively. It was found that the more dI inserted, the higher the specificity was acquired, but some sensitivity was lost. Only 1 × 10⁵ IU/ml HBV Genotype C could be stably amplified by Primers 5 and 6 (Supplementary Fig. S3A,B). So the poly-linker primer with 3 nt dI inserted and formed ‘large (3 vs 0)’ bubble-linker had excellent capability of distinguishing single-base mismatch.

To evaluate the distinguishing ability of HBV genotypes B and C mixed population, 1 × 10⁷ IU/ml mixed population T1 and T2 were

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>Forward</td>
<td>5'-CCCGCAGTCCCAAATCTIICGCAGTC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AGATGAGGCATAGCAGGAGAT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>FAM-5'-AGGAAGATGATAAACAGCGCGGA-3'-TAMRA</td>
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<tr>
<td>Forward 1</td>
<td>5'-CCTTGCGCCGTTGCTCCTIIICACTTT-3'</td>
</tr>
<tr>
<td>Forward 2</td>
<td>5'-CCTTGGCTCCTGCCTGCCTIIICACTTT-3'</td>
</tr>
<tr>
<td>Forward 3</td>
<td>5'-CCTTGGCGCCGTTGCTCCTIIICACTTT-3'</td>
</tr>
<tr>
<td>Forward 4</td>
<td>5'-CCTTGGCGCCGTTGCTCCTIIICACTTT-3'</td>
</tr>
<tr>
<td>Forward 5</td>
<td>5'-CCAGATGATGGGATGGGAAT-3'</td>
</tr>
<tr>
<td>Forward 6</td>
<td>5'-ACAGCAACAAGAGGGGAAACATAGAGGT-3'-TAMRA</td>
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**Figure 1. Principle of poly-linker primer system**  (A) A draft of the bubble formed in the PLPS. (B) Examples of the small, middle, and the big bubbles. (C) The process of the PLPS amplification.
used. T1 mixtures contained: 50%, 10%, 1%, 0.1, 0.01%, and 0.001% Genotype C full-length genome DNA and 1 x 10^7 IU/ml Genotype B, respectively; T2 mixtures included 0.001% to 50% Genotype B genome DNA and 1 x 10^7 IU/ml Genotype C. RT-PLPS could detect 0.01% Genotype B or C contained in mixed populations. A total of 159 clinical samples got from HBV infected patients were detected by PLPS and sequencing. The results are summarized in Supplementary Table S1. Among the 159 samples, 48 were Genotype B, 108 were Genotype C, and 3 were B/C mixture. The results in 95.5% of the samples were completely consistent with the sequencing results.

In summary, we have developed a new, sensitivity and specificity SNP detection system, RT-PLPS, which is an improvement of the DPO system. First, the DPO system was originally developed for multiple PCR. To block non-specific amplification, 5 nt dl are inserted into natural base primers and there are five bases in target sequence complementary with 5 nt poly-linker, forming analogously a ‘small’ bubble-linker. As for PLPS, 3 nt dl insertion at n-6–7 position at 3’-segment formed a ‘large’ bubble-linker (3 vs. 0). Second, in PLPS, two bases are added to primer 5’-segment terminal to promote sensitivity and specificity of poly-linker primer (data not shown). Third, the distinguishing part of PLPS is composed of six to seven base sequence, and single-base mismatch or SNP detection position is located at the 3’-segment n-3 position. Due to these improvements, RT-PLPS exhibits excellent SNP detection ability. The RT-PLPS could detect 1 x 10^2 IU/ml HBV, and detect 0.01% mutants in 1 x 10^7 IU/ml wild-type templates. When RT-PLPS was used to genotype HBV, 159 clinical samples were genotyped, and the results are almost completely consistent with those by sequencing.

Many reports have shown that HBV could be genotyped by detecting single base or two bases disparity [8,9]. Accumulated data demonstrated that HBV genotypes influence the clinical profile, anti-virus drug-response, the long-term prognosis, and seroconversion [10]. In China, Genotypes B and C take great dominance compared with other genotypes, and Genotype C is more prevalent than Genotype B. In addition, HBV genotype C can cause more danger for liver cirrhosis and hepatocellular carcinoma [11], lower response rate to anti-virus drugs, and higher rate of A1762T/G1764A double mutation in the basal core promoter region [12]. So HBV genotyping is very important for the adjustment of the treatment program and long-term prognosis.

Several assays have been developed to genotype HBV by detection of single- or two-base disparity. However, those assays either need an additional support from melting-curve analyses [9], or are based on Taqman probe [8]. Although these methods are very sensitive, non-specific amplification is still an unsolved issue. The RT-PLPS improves DPO system. By using TaqMan probe as signal system, RT-PLPS effectively avoids non-specific amplification, and realizes real-time detection. In conclusion, the RT-PLPS is a novel SNP and single-base mismatch detection assay which can be widely used to develop molecular assays useful in clinical labs.

### Supplementary Data

Supplementary data are available at ABBS online.

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### References


