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Enhancing allele-specific PCR for specifically detecting short deletion and insertion DNA mutations

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ABSTRACT

Allele-specific PCR (AS-PCR) has been widely used for the detection of single nucleotide polymorphism. But there are some challenges in using AS-PCR for specifically detecting DNA variations with short deletions or insertions. The challenges are associated with designing selective allele-specific primers as well as the specificity of AS-PCR in distinguishing some types of single base-pair mismatches. In order to address such problems and enhance the applicability of AS-PCR, a general primer design method was developed to create a multiple base-pair mismatch between the primer 3′-terminus and the template DNA. This approach can destabilize the primer–template complex more efficiently than does a single base-pair mismatch, and can dramatically increase the specificity of AS-PCR. As a proof-of-principle demonstration, the method of primer design was applied in colony PCR for identifying plasmid DNA deletion or insertion mutants after site-directed mutagenesis. As anticipated, multiple base-pair mismatches achieved much more specific PCR amplification than single base-pair mismatches. Therefore, with the proposed primer design method, the detection of short nucleotide deletion and insertion mutations becomes simple, accurate and more reliable.

1. Introduction

AS-PCR is a rapid, low-cost, and sequence specific method for genotyping Single Nucleotide Polymorphisms [1]. In AS-PCR, *Taq* DNA polymerase is used, which lacks 3′ to 5′ exonuclease activity. Thus the enzyme does not correct primer 3′-terminal nucleotides that form mismatched base-pairs with the template DNA [2,3]. Therefore, this DNA polymerase amplifies the target DNA efficiently only when the primer is fully complementary to the target DNA sequence. Conversely, PCR amplification is refractory to mismatches at the primer 3′-terminus.

AS-PCR has been largely applied in the characterization of single nucleotide substitution [1,3–7]. Many algorithms have been developed to optimize the specificity of AS-PCR [8–10]. Also, AS-PCR has shown some success in detecting short deletion or insertion, but it requires different primer design strategies for forming primer–template mismatches, which vary from one to several base-pairs [2,11–14]. To simplify the design, forming a multiple base-pair mismatch in AS-PCR is often desired because it enables greater specificity than does a single base-pair mismatch [2,7]. This type of specificity-enhanced AS-PCR would be valuable for a rapid and reliable diagnosis of short deletion- or insertion-associated diseases [15–25] and also for other sequence screening purposes. Therefore, we were motivated to develop a general primer design method for efficient detection of such mutations.

In vitro site-directed mutagenesis plays an important role in modifying DNA sequences in molecular biological studies and genetic engineering as well as for studying protein structure–function relationships [26–30]. QuikChange™ Site-Directed Mutagenesis (Stratagene, La Jolla, CA, USA) is one of the most popularly used mutagenesis methods. Yet in some cases, the mutagenesis efficiencies are still very low; sometimes no mutations are achieved even after intensive efforts [31,32]. Primer–dimers, the nature of annealing sequences, and the efficiency of the denaturation of the template DNA are the major reasons for the low mutagenesis efficiency [32–34]. Although various mutagenesis methods have been developed for high mutagenesis efficiencies [32,34–39], in practice PCR amplification conditions have to be optimized case by case. Optimization is somewhat time-consuming in that successful mutants have to be verified by other methods, e.g. DNA sequencing. Since most mutant targets do not have an easily accessible phenotype, rapid and reliable ways to screen desired DNA constructions prior to routine DNA sequencing are helpful. Yet most available screening methods are still not time- and cost-effective [32,40].

We are investigating a rapid method that can conveniently identify short deletion and insertion mutations directly using DNA polymerase, independent of a complex analytical instrument. A method of primer design for the enhancement of AS-PCR in detecting

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such types of DNA variations was developed. The method was validated through colony PCR screening for the target plasmid DNA with short deletion and insertion. It features high specificity for detecting deletion and insertion mutations.

2. Materials and methods

2.1. Materials

NEB Turbo competent *Escherichia coli* cells, restriction endonuclease *Dpn* I, *Taq* DNA polymerase with standard *Taq* buffer, and dNTPs were purchased from New England Biolabs (Ipswich, MA, USA). *Pfx50* DNA polymerase was purchased from Invitrogen Co. (Carlsbad, CA, USA). Oligo DNA (sequence is shown in Table 1) was synthesized by Integrated DNA Technologies Inc (Coralville, IA, USA). DNA sequencing service was provided by the Virginia Bioinformatics Institute at Virginia Tech (Blacksburg, VA, USA). pET26b-xr that contains wild-type xylose reductase from *Neurospora crassa*, as described elsewhere [41], was from Professor Huimin Zhao. Other chemicals were purchased from Sigma–Aldrich, Inc (Saint Louis, MO, USA).

2.2. Site-directed mutagenesis

Site-directed mutagenesis was carried out according to the QuikChange™ protocol. Using plasmid pET26b-xr as template DNA, primers X-D-F/X-D-R and X-I-F/X-I-R were used to create nucleotide deletion and insertion mutants, respectively. Amplification conditions were 94 °C for 30 s, 18 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 3 min, and a final extension step at 68 °C for 5 min. After PCR amplification, 5 μL of the PCR reaction product were used to transform NEB Turbo competent *E. coli* cells after digestion by the restriction endonuclease *Dpn* I.

2.3. Screening for the mutants

E. coli single colonies from an Agar plate were inoculated into 150 μL LB medium containing 50 μg/mL ampicillin in a 98-well plate and were grown at 37 °C with 200 rpm shaking. When A_{600} reached 0.1–0.2, 1 μL of culture was added into 9 μL of PCR mixture to get a final concentration of 10 mM Tris–HCl, 50 mM KCl, pH 8.3 (25 °C), 0.5 μM of primer pairs, 0.1 mM dNTPs, and 20 U/mL of *Taq* DNA polymerase. PCR analysis conditions were 98 °C for 2 min, 25 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 60 s, and a final step of 72 °C for 3 min. In both types of mutants, the generated 1.0 kb PCR products were analyzed by electrophoresis on an Agarose gel.

3. Results

3.1. Method of primer design for the specific characterization of deletion and insertion mutations

We take single nucleotide deletion and insertion as examples to illustrate the primer design for enhancing AS-PCR to detect nucleotide deletion and insertion mutants. Fig. 1A shows the scheme of a mutant-specific primer *r–m* binding to the parental DNA or its one-nucleotide deleted mutant. In the parental DNA, Nm is used to represent the homo-polynucleotide of *m* monomers ($m \geq 1$), where the mutation of the nucleotide deletion can happen. In fact, Nm includes both the simplest case where the deleted nucleotide is of a different type than either flanking nucleotide ($m = 1$) and the more complicated case where the deletion happens within homo-polynucleotide ($m > 1$). To build a multiple base-pair mismatch, the first aligned nucleotides for both parental and deletion mutant strands are to the right of the dashed box. Therefore, the mutant-specific primer *r–m* perfectly matches with the mutant DNA. However, the binding of the primer *r–m* with the parental DNA will result in a multiple base-pair mismatch, e.g. the three base-pair mismatch in Fig. 1A. The multiple base-pair mismatch is expected to cause a more restrained amplification than does single base-pair mismatch. Thus, it can dramatically increase the specificity of AS-PCR.

Table 1
Oligodeoxynucleotides used in this study.

Primer name[a]	Sequence[b]
X-D-F	5′-gaacctctggattttcggtagagatccgaattcgagctccgtcgac-3′
X-D-R	5′-gtcgacggagctcgaattcggatctctaccgaaaatccagaggttc-3′
X-I-F	5′-gaacctctggattttcggtgctagagatccgaattcgagc-3′
X-I-r	5′-gctcgaattcggatctctagcaccgaaaatccagaggttc-3′
XRF	5′-atggttcctgctatcaagctc-5′
XRD-m1	5′-gctcgaattcggatctctac-3′
XR-p1	5′-gctcgaattcggatctctaa-3′
XRD-m3	5′-gctcgaattcggatctctaccg-3′
XR-p3	5′-gctcgaattcggatctctacc-3′
XRI-m1	5′-gctcgaattcggatctctag-3′
XRI-m3	5′-gctcgaattcggatctctagca-3′

[a] Primer name with -m denoting a primer completely matched with mutant DNA, and -p indicating complement with parental DNA. The numbers 1 and 3 indicated potential to form a one base-pair and three base-pair mismatch, respectively.
[b] Underlined nucleotides were the variable 3′-terminal nucleotides.

Fig. 1B shows a diagram of the insertion mutant DNA-specific primer *r–m* binding to the parental DNA and its one-nucleotide inserted mutant. A random nucleotide (X) is inserted between the N^{n+1} and N^{n} positions. The dashed box region shows that insertion of merely one nucleotide will also cause a shift of DNA information within primer binding sites, similar to the aforementioned nucleotide deletion case (Fig. 1A). Therefore, the 3′-terminus of allele-specific primers can be designed to have from one to several deliberate mismatched nucleotides.

3.2. Screening for nucleotide deletion DNA mutants

In Fig. 2A, after deleting T^4 from the parental DNA in the shown region through site-directed mutagenesis, the sequences were changed from $C^8G^7G^6T^5T^4A^3G^2A^1$ in parental DNA to $C^{7'}G^{6'}G^{5'}T^{4'}A^3G^2A^1$ in mutant DNA. Because of the consecutive T^5T^4 in the displayed region, according to the primer design method described in Fig. 1A, the nucleotide G^6 (later becomes $G^{5'}$ in mutant DNA), which is immediately next to the consecutive homo-polynucleotide T^5T^4, was chosen as the discriminating nucleotide for single base-pair mismatch.

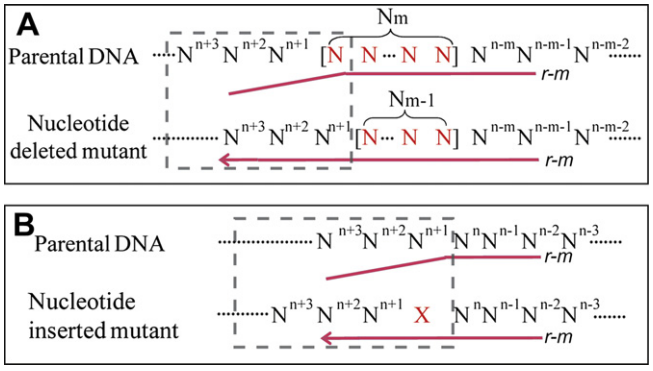

Fig. 1. Schemes of the primer design for the detection of short nucleotide deletion and insertion DNA mutants. (A) Binding of the deletion mutant-specific primer *r–m* to the parental DNA and its one-nucleotide deleted mutant. (B) Binding of the insertion mutant-specific primer *r–m* to the parental DNA and its one-nucleotide inserted mutant. The dashed box regions denoted the binding of the primer 3′-terminus with the template DNA. Arrows indicated *r–m* perfectly matched with mutant DNA allowing PCR amplification, while a bent line indicates a mismatch that could not proceed with PCR amplification.