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Fast and Almost 100% Efficiency Site-directed Mutagenesis by The Megaprimer PCR Method*

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Abstract A novel PCR-based mutagenesis method was reported, in which there is no need to purify megaprimers or design a special flanking primer. This method used one mutagenic primer and two sequencing primers ($T_m \leqslant 58$℃) as flanking primers. After first round PCR, 12.5 μl first PCR production was directly added into 50 μl second PCR system as template and megaprimer, and 10 rounds of asymmetrical PCR at high temperature of annealing (68℃) was to add in initiation of second PCR. This additional step greatly has increased the efficiency of mutagenesis *via* 600 bp or 800 bp long megaprimer. The results demonstrated that this method can achieve high fidelity, 97%～98% efficiency, high yield.

Key words PCR, mutagenesis, megaprimer
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Site-directed mutagenesis is a powerful technique to explore the relationship between the structure and function of DNA or protein[1]. In contrary to non-PCR methods, PCR-based site-directed mutagenesis methods have been widely used because of their simple and low-cost [2]. Among them, megaprimer approach is the particularly attractive and simplest, because it requires only one mutagenic primer, two flanking primers and two rounds of PCRs [3]. Derived from one flanking primer and the mutagenic primer, the first-round PCR product with the desired mutation is used as the "megaprimer" with the second flanking primer for the second-round PCR to produce the final product that contains the desired mutation in a particular DNA sequence[4].

The efficiency of site-directed mutagenesis of this approach proposed originally by Kammann is 10% ～ 30%[3]. After gel purification of the first PCR reaction product, the purified product can be used as megaprimer to promote the efficiency of mutation from 10% ～ 30% to 100% [5]. However, the gel purification step is time-consuming and laborious and a source of product loss. To eliminate the purification step and to ensure the efficiency of mutation, several modifications of megaprimer approach have been developed, such as enzyme treatment of PCR product [6～8], use of templates derived from two different vectors[9], design of special primers [10] and optimization of PCR parameters[11～14]. The former two modifications are not easy to use, and the third modification only reaches 82% efficiency of mutation. The last modification is most appealing because of its simplicity and low cost. However, the four existing protocols have their limitations,such as low efficiency (50%～60%)[11, 12], low yield derived from 0.05 pmole of the first flanking primer [13] and need to determine template reannealing temperature (T_{ra}) [14]. So it is still worthwhile to explore novel megaprimer protocol without purification step of the first PCR reaction product through the optimization of PCR parameters.

According to the Lai′s protocol that requires the very long primers and needs 4 mmol/L $MgCl_2$ in the second round of PCR [15] , we have developed simple, rapid and high efficient site-directed mutagenesis protocol without the very long primers and a higher

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$MgCl_2$ concentration in the second round of PCR. This protocol had successfully reached 100% efficiency of site-directed mutagenesis on some cDNA sequences of some vectors in our laboratory. For more accurate measurement of efficiency of site-directed mutagenesis of this protocol, we have designed two special mutagenic primers that can introduce their respective new restriction enzyme sites into target sequences. Therefore, efficiency of mutagenesis of this protocol can be simply evaluated by rapid, direct restriction enzyme digestion.

1 Materials and methods

1.1 Materials

Polymerase chain reactions were performed on a MJ Research Thermal Cycler. Pfu DNA polymerase was purchased from Tiangen, All restriction enzymes and dNTP were purchased from TaKaRa. The plasmid pQE30-APEX was derived from pQE30 (Qiagen) into which a APE/ref-1 cDNA(accession number: AF309114) of ~980 bp from PC12 cell was inserted between *Bam*HⅠ and *Pst*Ⅰ sites [16]. The derived vector was propagated in *E. coli* JM109. Primers used for PCR amplifications were purchased from Invitrogen and were as follows: (1) the forward sequencing primer P (S1): 5′ CGGATAACAATTTCACACAG 3′; (2) the reverse sequencing primer P (S2) : 5′ GTTCTGAGG-TCATTACTGG 3′; (3) the mutagenic primer P(M1): 5′TAGAGATGCCGGAAGCTTTC 3′; (4) the mutagenic primer P(M2): 5′ AGACCCCTTCCTGCAGTCGG 3′. The mutagenic primers were designed for introducing *Hin*dⅢ or *Pst*Ⅰ sites in PCR products for identification of the mutation by restriction enzyme digestion, and all base substitutions of them are underlined. According to the instruction manual provided by Qiagen Company, these sequencing primers are corresponding to both sides of multiple cloning site of pQE30 vector.

1.2 Mutagenesis reaction system

Mutagenesis is achieved by the two-step PCR procedure described below and illustrated in Figure 1. In first PCR reaction, 50 μl reactions contained 0.5 ng template plasmid pQE30-APEX plasmid, 20 pmol (0.40 μmol/L) each of the mutagenic primer (P(M1) or P (M2)) and the forward sequencing primer (P (S1)), 1.25 U Pfu DNA polymerase, 5 nmol of dNTPs, 20 mmol/L Tris-HCl, pH 8.75, 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgCl_2$, 0.1% Triton X-100 and 0.1 g/L BSA. After denaturation at 94℃ for 100 s, 22 cycles of amplification (94℃, 20s; 50℃, 20 s; 72℃, 140 s) were performed, followed by 5 min at 72℃, and holding at 5℃. In second PCR reaction, in addition to 12.5 μl first PCR production as template and megaprimer, 37.5 μl reactions contained 20 pmol (0.40 μmol/L) the reverse sequencing primer (P(S2)), 15 pmol(0.30 μmol/L)the forward sequencing primer (P(S1)), 1.25 U Pfu DNA polymerase, 7.5 nmol of dNTPs, 20 mmol/L Tris-HCl, pH 8.75, 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgCl_2$, 0.1% Triton X-100 and 0.1 g/L BSA. 50 μl total reactions were mixed. After denaturation at 94℃ for 100 s, 10 cycles of amplification (94℃, 20 s; 68℃, 20 s; 72℃, 80 s) and 22 cycles of amplification (94℃, 20 s; 50℃, 20 s; 72℃, 140 s) were performed, followed by 5 min at 72℃, and holding at 5℃.

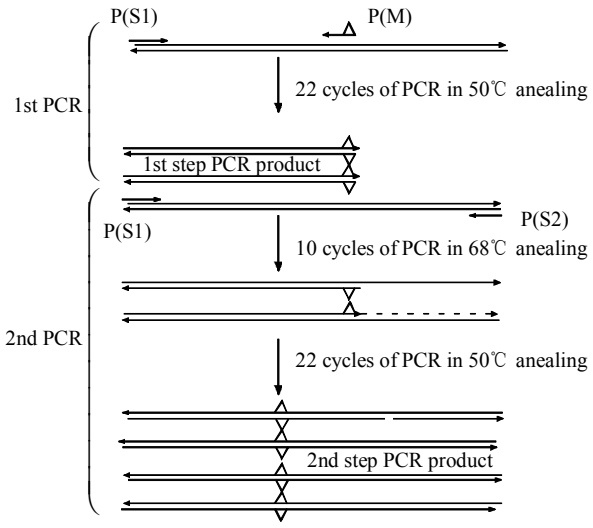


Fig. 1 A schematic outline of the PCR mutagenesis strategy in this study

In first PCR, the megaprimer is synthesized using the mutagenic, the reverse sequencing primers and the wild type template. In second PCR, the addition of 10 cycles at a high annealing temperature of 68℃ can improve mutagenesis efficiency without gel purification. Then full-length products can be synthesized by performing another 22 cycles of PCR using the sequencing primers at a low annealing temperature.

1.3 Construction and identification of mutated recombinants

The full-length products (~1 100 bp) of second PCR were purified and digested with *Bam*HⅠ and *Pst*Ⅰ (for P(M1) primer yielding product) or *Bam*HⅠ and *Hin*dⅢ (for P (M2) primer yielding product). The resulting fragments were purified and cloned into pQE30 vector after digestion of vector with *Bam*HⅠ and *Pst*Ⅰ or *Bam*HⅠ and *Hin*dⅢ. The ligation products

were used to transform *E. coli* strain JM109 competent cells and the transformed culture was cultured on Luria-Bertani agar plates supplemented with 60 mg/L ampicillin. The plasmids isolated from selected colonies were identified by digesting with *Bam*HⅠ and *Hin*dⅢ (for P(M1) primer yielding plasmids) or *Bam*HⅠ and *Pst*Ⅰ (for P(M2) primer yielding plasmids).

2 Results

2.1 The identification of PCR products

After the first round and the second round of PCR reactions, PCR products were analyzed by agarose gel electrophoresis (Figure 2). According to the result, the first round of reaction has specifically amplified an 800 bp or 600 bp megaprimers, respectively, according to the expected length as designed with the primers P (M1) or P(M2), and the second round of reaction has successfully amplified the 1 100 bp full-length product with its corresponding megaprimer. The bright 1 100 bp bands indicate high yield of desired PCR products.

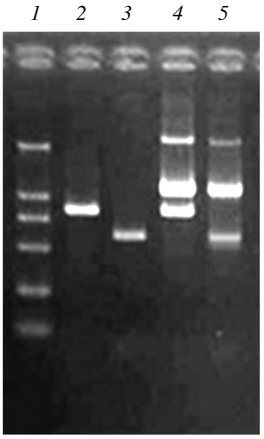


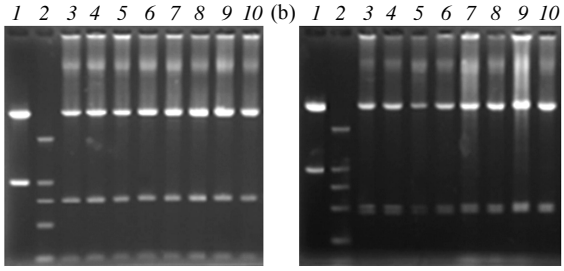
Fig. 2 Electrophoretic analysis of the PCR products of this protocol

Samples analyzed on a 1% agarose gel. *1*: Marker DL2000; *2*: P(M1) primer yielding megaprimer (800 bp); *3*: P (M2) primer yielding megaprimer (600 bp); *4*: P (M1) primer yielding final product, full-length product (1 100 bp) with its megaprimer(800 bp); *5*: P(M2) primer yielding final product, full-length product (1 100 bp) with its megaprimer (600 bp).

2.2 Mutagenesis efficiency (restriction enzyme assay)

For more accurate qualitative determination of efficiency of site-directed mutagenesis of this protocol, the recombinant plasmids were identified by digestion with *Bam*HⅠ and *Hin*dⅢ(for P(M1) primer yielding plasmids) or *Bam*HⅠ and *Pst*Ⅰ(for P(M2) primer yielding plasmids). The products of digestion were then analyzed by agarose gel electrophoresis(Figure 3). Except for about 4 200 bp pQE30 itself, the pQE30-APE plasmid, the wild type plasmid, generated only 980 bp bands, while the expected mutated plasmids generated about 750 bp and 230 bp bands(Figure 3a) or 520 bp and 460 bp bands(Figure 3b). Total 129 recombinant plasmids yielded by P(M1) primer and 118 recombinant plasmids yielded by P (M2) primer were identified by restriction digestion. Among them, 127 and 115 recombinant plasmids were respectively confirm in mutation indeed(Figure 3c). So efficiency of mutagenesis of this protocol is 98.4% or 97.5% .

(a) *1 2 3 4 5 6 7 8 9 10* (b) *1 2 3 4 5 6 7 8 9 10*



(c)

Mutagenesis reaction	Subclones analyzed	Mutant subclones	Mutagenesis efficiency
P(M1) primer	129	127	98.4%
P(M2) primer	118	115	97.5%

Fig. 3 Determination of mutagenesis efficiency

(a) Restriction endonuclease analysis for mutant screening of P(M1) primer yielding product. *1*: *Bam*HⅠ and *Hin*dⅢ enzyme digestion of the wild type plasmid; *2*: Marker DL2000; *3*～*10*: *Bam*HⅠ and *Hin*dⅢ enzyme digestion of recombinant plasmids. (b) Restriction endonuclease analysis for mutant screening of P(M2) primer yielding product. *1*: *Bam*HⅠ and *Pst*Ⅰ enzyme digestion of the wild type plasmid; *2*: Marker DL2000; *3*～*10*: *Bam*HⅠ and *Pst*Ⅰ enzyme digestion of recombinant plasmids. (c) Analysis of mutagenesis efficiency in two consecutively performed reactions.

3 Discussion

Our aim in developing new megaprimer method was to obtain a fast, simple and efficient PCR mutagenesis protocol that is economical in terms of oligonucleotide synthesis and labor expenditures. At 68℃, the sequencing primers ($T_m \leqslant 58$℃) cannot be annealed to template DNA. In contrast, megaprimer can efficiently anneal to templates and extends itself. Noting this fact, we designed this two-step PCR protocol that avoids purification of PCR intermediates. In order to promote high overall fidelity of DNA

sequence replication, the total number of amplification cycles was limited to 54 cycles (*vs*. 80 cycles for the method described in reference [17], and 60 cycles for the methods presented in reference [18]). This PCR amplifications were carried out with Pfu DNA polymerase that has minimized the rate of extra mutations because of its high-fidelity. The sequencing primers corresponding to both sides of multiple cloning site of pQE30 vector were used as flanking primers. That can make this protocol more economical.

In the first round PCR, for optimization of the PCR reaction, 0.5 ng pQE30-APE was added as template and the concentration of primers was 0.4 μmol/L. After 22 cycles of the first round PCR, 12.5 μl first PCR production was directly added into 50 μl second round PCR system as megaprimer and template from the residual wild-type template of the first round of PCR amplification. When 10 rounds of asymmetrical PCR was performed at high temperature of annealing (68℃) in second PCR, only megaprimer can anneal to templates and extends itself and the sequencing primers as flanking primers had no intervention in the PCR reaction because of their lower T_m(≤58℃). Therefore the extended megaprimers with desireable mutation accumulated arithmetically through this asymmetrical PCR and the level of them can finally reach many times more than that of wild type templates. At last, the extended megaprimers and wild type templates were subsequently amplified and the high yield of the desired mutagenic products can be reached by performing another 22 cycles of PCR using the two sequencing primers at a low annealing temperature. Finally, the results of analysis of agarose gel electrophoresis and restriction enzyme assay had demonstrated that this simple, rapid protocol of site-directed mutagenesis can achieve high yield and 97%～98% efficiency in generating the desired mutation of PCR products when incorporating a megaprimer as large as 600 or 800 bases. After all, the 97%～98% efficiency of mutagenesis eliminates the need of a time-and money-consuming screening for positive clones.

This protocol will be of particular utility for producing the mutations in long target template with long megaprimer, and there was no need to adjust particularly critical parameters, such as template reannealing temperature (T_m) [14] and higher $MgCl_2$ concentration in the second PCR [15]. The rapidity, simplicity, high yield and high efficiency of this protocol demonstate the versatility of our strategy that could be applied to most constructs for site-directed mutagenesis.

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快捷的定点突变效率近100%的大引物PCR方法*

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摘要 报道了一种新的PCR突变方法，它不需要纯化大引物或设计特别的旁侧引物．利用一个诱变引物和两个测序引物(T_m≤58℃)作为旁侧引物．第一轮PCR产物12.5 μl直接加入到50 μl的第二轮PCR反应体系作为模板和大引物，在开始第二轮PCR反应时，增加在68℃退火温度下进行10个循环的不对称PCR，这一步骤大大提高了通过600 bp或800 bp大引物所导致的突变效率．结果表明，该方法的产物能够达到高保真、97%～98%的突变效率和高产率．

关键词 聚合酶链反应，定点突变，大引物
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