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Communication:

Improvement of PCR reaction conditions for site-directed mutagenesis of big plasmids

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QuickChange mutagenesis is the method of choice for site-directed mutagenesis (SDM) of target sequences in a plasmid. It can be applied successfully to small plasmids (up to 10 kb). However, this method cannot efficiently mutate bigger plasmids. Using KOD Hot Start polymerase in combination with high performance liquid chromatography (HPLC) purified primers, we were able to achieve SDM in big plasmids (up to 16 kb) involving not only a single base change but also multiple base changes. Moreover, only six polymerase chain reaction (PCR) cycles and 0.5 μl of polymerase (instead of 18 PCR cycles and 1.0 μl of enzyme in the standard protocol) were sufficient for the reaction.

Key words: Site-directed mutagenesis (SDM), Mutant, Plasmid

QuickChange mutagenesis is a method for achieving site-directed mutagenesis (SDM) in a plasmid, by substitution, deletion, or insertion of nucleotides in the target plasmid. The method is fast, straightforward, and essential in functional studies, genetic engineering, and biochemistry. QuickChange mutagenesis employs polymerase chain reaction (PCR) using oligonucleotide primer pairs that carry the desired mutation, deletion, or insertion. There are many approaches for mutagenesis using PCR (Kunkel, 1985; Cormack, 1994; Ishii *et al*., 1998; Chapnik *et al*., 2008; Kumar and Rajagopal, 2008; Li *et al*., 2008; Liu and Naismith, 2008; Tseng *et al*., 2008; Edelheit *et al*., 2009; Fushan and Drayna, 2009). QuickChange is widely used for SDM of a plasmid which uses complementary primer pairs in the same PCR reaction. The parental methylated DNA can be eliminated from the newly synthesized unmethylated mutant DNA by digesting with *Dpn*I restriction enzyme. The mutant DNA product has a strand break (nick) which can be ligated by host repair enzymes. Although this method is simple, it has some limitations (Zheng *et al*., 2004). One limitation is the size of the target plasmid. Although there are some commercial kits for mutating big plasmids, they are costly. Home-made SDM (Laible and Boonrod, 2009) offers a low cost alternative. This method is, however, efficient only for plasmids smaller than 10 kb. The factors which may affect the efficiency of the method may be the quality and efficacy of the polymerases and primers used. In this study, we analyzed the efficacy of two polymerases in combination with two different purified primers and also identified the most efficient polymerase and the purity of primers for achieving SDM in a big plasmid using the QuickChange method. Moreover, we also investigated the minimal requirement of PCR cycles and the amount of enzyme used for the mutagenesis reaction.

The high-fidelity *Pfu* DNA polymerase commonly used in SDM can amplify a DNA template up to 20 kb (recommended by Fermentas, Germany). Indeed, using *Pfu* DNA polymerase for SDM of a plasmid less than 10 kb can be very efficient (Laible and Boonrod, 2009). However, our attempts to achieve SDM in a plasmid bigger than 10 kb were unfortunately not successful. This prompted us to investigate

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alternative polymerases and conditions for use in SDM of a big plasmid. KOD Hot Start DNA polymerase is a premixed complex of high-fidelity KOD DNA polymerase and two monoclonal antibodies that inhibit the DNA polymerase and 3′-5′ exonuclease activities at ambient temperature (Mizuguchi *et al*., 1999). The enzyme can amplify plasmids up to 21 kb and it exhibits a higher fidelity than *PfuTurbo* (Fujii *et al*., 1999; Kitabayashi *et al*., 2002). To validate the efficacies of the two polymerases for SDM of a big plasmid, an *Nco*I restriction site was deleted from the target sequence of binary vectors (pKB-DRD1-GFP-DRM-Red, 16 kb) kindly provided by Dr. Michael WASSENEGGER using the QuickChange method and was used as a marker for analyzing the successful mutation. The two complementary primers (forward pKB-DR-*Nco*I-delete, 5′-CACCATTTACGAACGATACC*TAC*GGTTTTGTTTACATTG-3′ and reverse pKB-DR-*Nco*I-delete, 5′-CAATGTAAACAAAACC*GTA*GGTATCGTTCGTAAATGGTG-3′, where italic and underlined alphabets indicate the mutated bases) were designed as described by Laible and Boonrod (2009) and purified by either desalting or high performance liquid chromatography (HPLC). The reaction was performed and the mixtures were incubated in a thermocycler according to the conditions shown in Tables 1 and 2.

The PCR products were electrophoresed in a 1% Tris-acetate-ethylenediaminetetraacetic acid (TAE) gel and visualized under ultraviolet (UV) light. In addition, the PCR products were digested with *Dpn*I restriction enzyme and then were transformed into *Escherichia coli* competent cells. The results revealed that the reaction using desalted primers in combination with either *Pfu* or KOD Hot Start polymerase did not yield any PCR products (Fig. 1). PCR products and transformants were obtained only when using HPLC-purified primers and KOD Hot Start polymerase (Fig. 1).

To confirm that the plasmids were mutated, plasmids were prepared from 10 different colonies and analyzed by *Nco*I restriction digestion as described by Zhang *et al*. (2009). The results showed that all tested plasmids were mutated. In addition, the mutants were confirmed by sequence analysis. The sequence analysis of the whole insert gene from three different positive clones revealed that no additional mutants were found (data not shown). However, we could not exclude additional mutants among the mutated clones.

We conclude that KOD Hot Start DNA polymerase in combination with HPLC-purified primers is the key factor for achieving SDM in a big plasmid. To confirm that this combination can be applied to SDM with more than single base changes, we performed other sets of mutagenesis trials in which 5 or 8 bases were substituted. The results revealed that all mutants were successful and 99% of the tested transformants were correctly mutated (data not shown).

To reduce time and costs of the reaction, we investigated the reaction conditions by reducing the number of cycles and the amount of the polymerase.

Table 1 PCR reaction components

Polymerase	Template plasmid DNA (ng)	Volume (μl)						
		Forward primer 10 pmol	Reverse primer 10 pmol	Buffer	$MgCl_2$ 50 mmol/L	dNTPs 10 mmol/L	DNA polymerase	Final volume by adding H_2O
Pfu	70	1	1	5	0	1	1	50
KOD Hot Start	70	1	1	5	3	1	1/0.5	50

Pfu polymerase (2.5 U/μl) purchased from Fermentas, Germany; KOD Hot Start polymerase (1.0 U/μl) purchased from Novagen, Germany

Table 2 PCR reaction conditions

Polymerase	Denaturated temperature/time		Annealing temperature/time	Extension temperature/time	Holding temperature (°C)
	Template	Activated enzyme			
Pfu	95 °C/1 min	95 °C/30 s	55 °C/1 min	72 °C/12 min	4
KOD Hot Start	94 °C/2 min	98 °C/10 s	57 °C/30 s	68 °C/6 min	4

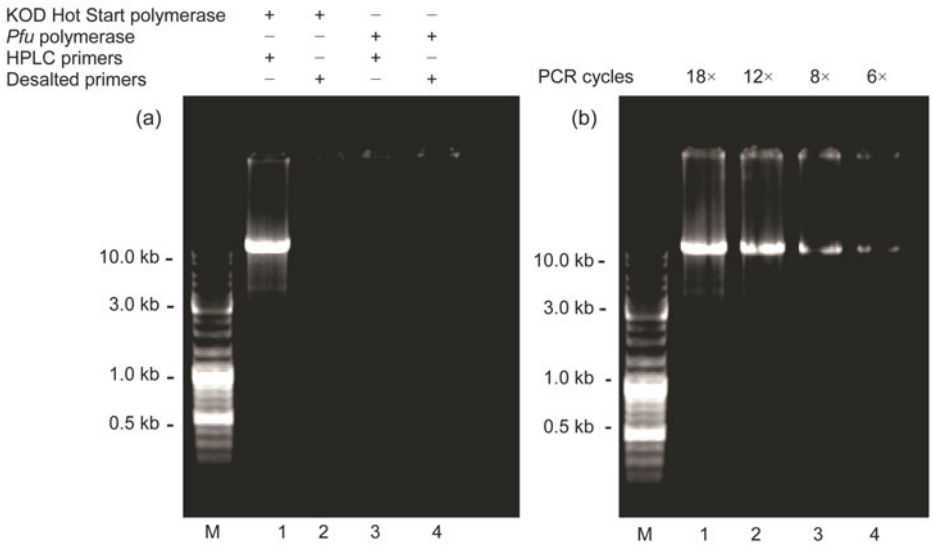

Fig. 1 Gel electrophoresis of PCR products

(a) Comparison of the efficiencies of two DNA polymerases using desalted and HPLC-purified primers. The plasmid pKB-DRD1-GFP-DRM-Red (16 kb) was mutated using 1 μl of either *Pfu* or KOD Hot Start DNA polymerase in combination with either desalted or HPLC-purified primers. The reactions were performed as described in Tables 1 and 2 using 18 cycles. (b) Comparison of the different PCR cycles required for the mutagenesis reaction. KOD Hot Start DNA polymerase (0.5 μl) and HPLC-purified primers were used for SDM of the pKB-DRD1-GFP-DRM-Red (16 kb) plasmid in varying PCR cycles (from 18 to 6). The PCR reactions were performed as described in Tables 1 and 2. After PCR, 5 μl of each reaction was electrophoresed in 1% TAE agarose gel

The pKB-DRD1-GFP-DRM-Red plasmid was used as a template and the reactions were performed as described in Tables 1 and 2, except that the PCR reactions were performed by varying the number of PCR cycles (from 18 to 6) and only 0.5 μl of the KOD Hot Start DNA polymerase was used. The result (Fig. 1) showed that the plasmid was amplified and transformants were obtained under all conditions tested. The restriction digestion and sequence analysis indicated that the plasmids obtained from all reactions were 100% mutated (data not shown). These results indicated that only six reaction cycles and 0.5 μl of polymerase were sufficient to generate a mutant.

In conclusion, KOD-polymerase in combination with HPLC-purified primers is efficient for achieving SDM in big plasmids (up to 16 kb). Only 0.5 μl of the enzyme and six cycles of reaction are sufficient for the PCR reaction.

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