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A Do-it-Yourself Protocol for Making Desired Mutant Proteins

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Abstract

The generation of a mutant protein is often crucial to the elucidation of the pathogenesis of a genetic disease. This requires site-directed mutagenesis of an expression vector containing the gene of interest. Current site-directed mutagenesis procedures are derivatives of either a restriction site elimination method or a PCR-based mutagenesis method, which involve a commercial kit manufactured by any one of a number of biotechnology companies. A new efficient economical site-directed mutagenesis procedure is described here to provide researchers an additional choice that does not to rely on a commercial kit. This novel method has been validated, and requires only basic molecular biological techniques and reagents available in most laboratories even in developing countries. The mutation efficiency of this new method is usually greater than 80%. In case several different mutants of a protein are required for elucidation of the protein structure-function relationships, this method has distinct advantages since multiple mutations can be derived from the initial single strand DNA template.

Keywords Site-directed mutagenesis; Protein structure-function relationship; Fatty acid oxidation enzymes; HSD10

Introduction

The etiology of most genetic diseases has been found to be a mutation in a gene's coding region. For example, various mutations have been identified on the *HSD17B10* gene, resulting in 17β-hydroxysteroid dehydrogenase type 10 (HSD10) deficiency and mental retardation, choreoathetosis, abnormal behavior (MRXS10) [1].
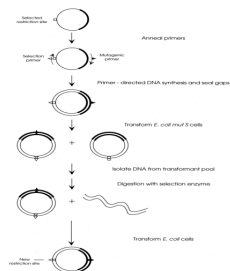

Figure 1: Strategy for site-directed mutagenesis with a high efficiency. The selection and mutagenic primers were prepared and annealed to ss DNA template for in vitro synthesis of the mutated second strand DNA. Restriction digestion with a selection enzyme at a unique site to get rid of the non-mutated recombinant DNA isolated from the *E. coli mutS* transformants could greatly increase the mutation frequency.

To elucidate the pathogenesis of many genetic diseases, it is necessary to synthesize mutants of their protein gene products. Site-directed mutagenesis is not only essential for this goal but is also critical to advances in biological science, especially for protein engineering and improved understanding of protein structure-function relationships.

Site specific mutagenesis was first achieved in 1970s [2]. Thereafter, the polymerase chain reaction (PCR) technique simplified the specific alteration of DNA sequences, and the unique restriction digestion raised the mutagenesis efficiency. Site-directed mutagenesis kits on the market are relatively expensive because of the proprietary DNA polymerases and competent cells they contain.

Here we report a do-it-yourself protocol for making desired mutant proteins. This efficient economical method could make a desired mutation at virtually any position in a target gene (Figure 1).

Materials and Methods

Anneal primer(s)

The single strand (ss) DNA template containing the target gene *fadB* [3] cloned into an M13 vector was prepared according to the published procedure [4,5]. It was then primed with one or more 5'phosphorylated mutagenic primer(s) and a 5'phosphorylated selection primer (5'-CAGGCATGCACGCGTGGCGTAATC-3') with a *Mlu* I restriction site instead of a *Hind* III site. The mismatched nucleotide(s) were underlined, and had to be flanked by at least 10 matched nucleotides on each side. The selection primer was designed to change a unique restriction site (Figure 1). The nucleotide sequence of mutagenic primer used in the current study was 5'-TGCGCTGGGCTTTGGCTGCGAA-3'. 0.1 μg of ssDNA template was mixed with appropriate primers (0.1 μg each), 2 μl of 10x annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl$_2$, and 500 mM NaCl) and water to a total volume 20 μl. After incubation at 100°C for 1 min

the annealing mixture was transferred into a 40°C water bath for 3 min, and then put on ice for 5 min.

Synthesis of second strand DNA

In order to extend the primers and to seal the gaps in the newly synthesized second strand, 2 µl of 10x synthesis buffer (100 mM Tris-HCl, pH 7.5, 5 mM each dNTP, 10 mM ATP and 20 mM dithiothreitol), 1 µl T4 DNA polymerase (3units). 1 µl T4 DNA ligase (6 units) and 5 µl H$_2$O were added into the annealing mixture, and then incubated at 37°C for 2 h. The *in vitro* synthesis reaction was stopped by heating the mixture at 70°C for 5 min. Controls were treated with the same procedures except that no primer was added (template control) or only one primer was included (primer control). Thereafter 5 µl of the reaction mixture and 5 µl of each control were analyzed by electrophoresis on a 1% agarose gel (Figure 2).
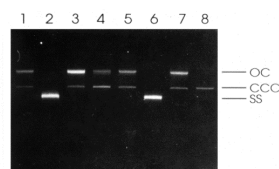

Figure 2: Analysis of DNA products from the second strand DNA synthesis with T4 DNA polymerase and gap(s) sealed with T4 ligase. DNA products were separated on 1% agarose gel and stained with ethidium bromide. Symbols: OC, open circles or nicked circular DNA; CCC, covalently closed circular DNA; SS, single strand DNA. Lane 2 showed the "template" (no primer) control while lanes 1 and 3 were "primer" controls (without the selection primer) in two parallel experiments. Lane 4 was the other "primer" (no mutagenic primer) control. Lanes 5 and 7 showed the outcomes of two parallel mutagenesis experiments. Lanes 6 and 8 were loaded with ss DNA and RF DNA, respectively, to serve as corresponding indicators.

Transformation of *E. coli* mutator strain

When it was determined that in vitro synthesis was satisfactory as shown in Figure 2, only those reaction mixtures containing the desired mutant DNAs were used to transform the *E. coli mutS* competent cells prepared with a one-step method [6]. 100 µl competent BMH 71-18 *mutS* cells were gently mixed with 10 µl of the synthesis reaction mixture, and incubated on ice for 20 min. After a heat shock at 42°C for 1 min, the cells were gently mixed with 0.5 ml LB medium and incubated at 37°C for 45 min as a recovery period. The transformants were amplified by adding 5 ml LB medium, and incubated at 37°C overnight with shaking at 200 rpm.

Digestion with selection enzyme

The replicative form (RF) DNA was isolated from the transformant pool using a standard miniprep procedure [7], and then digested with the selection enzyme as follows: 0.1 µg RF DNA was mixed with 1 µl of the appropriate restriction enzyme, 10 units of *Hind* III (Promega, WI) in the current study, 2 µl of 10x digestion buffer, and water to a total volume of 20 µl, and incubated at 37°C for 2 h. Then, an additional 1 µl of the same restriction enzyme was added and incubated at 37°C for 1

h. The total enzyme used should not be more than 1/10 volume of the digestion system to avoid any inhibition caused by glycerol. The linearization of parental DNA decreased its transformation efficiency more than a hundred-fold.

Amplification of mutant DNA

5 µl of the restriction enzyme digest was used to transform 100 µl of competent *E. coli* MV1190 cells, and the transformants were plated to generate plaques. Several plaques were picked randomly, and each was incubated with 100 µl of overnight culture of MV1190 in 10 ml LB medium supplemented with 5.0 mM MgSO$_4$ at 37°C for 3 h with shaking at 300 rpm. The RF DNA was isolated from the infected *E. coli* cells by a standard miniprep procedure [7].

Verification of desired mutation(s)

Since the mutation frequency by this method is usually very high, a preliminary screening of mutants by restriction digestion was omitted. The desired mutation(s) was readily identified by DNA sequencing of the coding region of *fadB* gene.

Overexpression and purification of mutant protein

The mutant expression plasmids were transformed into *E. coli* BL21(DE3)pLysS according to one-step method [6]. The transformants were grown in 2YT medium to an absorbance of about 1.0 at 600 nm and then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h. Cells were harvested by centrifugation for 10 min at 3000x g, 4°C, and washed twice with ice-cold 200 mM KP$_i$, pH 8.0. The preparation of cell extract, purification of mutant protein FAOMP (fatty acid oxidation multifunctional protein) G116F, and the characterization of the mutant protein were performed as described previously [8].

Results and Discussion

Efficient *in vitro* synthesis of the mutated second strand DNA is essential for achieving a high frequency of mutation. As shown in Figure 2, almost all the single DNA template has been converted to double strand DNA either nicked or covalently closed circular DNA in this study (Figure 2). In contrast, the synthesis of the mutated second strand DNA was very poor in the unique site elimination (USE) method [9] because it was severely inhibited by the annealing of the two complementary parental strands. This provides an explanation for the failure of the improved USE method to mutate DNA cloned into some expression plasmid and phagemid vectors [9,10]. The synthesis buffer reported here contains ATP and dithiothreitol, indispensable for activities of T4 ligase and T4 polymerase, respectively. Thus, this economical site-directed mutagenesis method was successfully validated. In contrast to the improved USE method, this new efficient site-directed mutagenesis method has only one selection step rather than two, because (a) the residual ssDNA, if any, has very low transformation efficiency; (b) ssDNA is extremely refractory to restriction digestion; (c) T4 DNA polymerase does not have strand displacement activity; (d) the nicked or covalently closed circular, heteroduplex DNA product, containing only the mutagenic primer but not the selection primer, is a very minor component which is destroyed with the homoduplex parental DNA.

The frequency of the altering of the restriction site in this method is about 90%. For example, conversion of the *Hind* III site into *Mlu* I site

of an expression vector containing *fadB* gene was found to be 9/10, 13/16 and 12/12 in three independent experiments. Since the 5'phosphorylated mutagenic primer was annealed with the ssDNA template simultaneously in the same reaction mixture, it would be incorporated into the mutated second strand DNA as readily as the selection primer (Figure 1). The mutagenesis frequency of this method is, therefore, not only much higher than the improved USE method [9] but also comparable to those announced for commercial PCR-based mutagenesis kits in the market, i.e., >80% mutation efficiency for single site mutagenesis. A potentially misleading statement that it is possible "....to introduce a variety of mutations in any vector in a single day" was also found in an announcement of the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent), in which the *Dpn* I restriction digestion serves as the selection step to eliminate the methylated DNA template. However, if bacterial transformation is necessary for converting the mutated PCR product to a useful preparation of mutated expression vector, any PCR-based site-directed mutagenesis method cannot be achieved in a single day. With regard to introducing a mutation into a sequence, the first stage of this method (Figure 2) could be, indeed, completed within a few hours.

When the restriction enzyme digests of RF DNA isolated from *E. coli mutS* transformants were analyzed, the supercoiled and relaxed circular DNA resistant to the digestion due to the mutation accounted for about half of the total DNA (see lanes 2 and 5 of Figure 3). In contrast, the wild type MV1190 competent cells cannot serve for this purpose (see lanes 3 and 6 of Figure 3). As shown in Figure 2, the template (no primer) and one primer controls of this method serve to monitor the success in the synthesis of the mutated second strand DNA. This efficient and economical site-directed mutagenesis method is especially suitable for protein engineering and studies on structure-function relationships of an important protein such as the fatty acid oxidation and related enzymes: hydratase, HADH (3-hydroxyacyl-CoA dehydrogenase), and HADHII (17β-hydroxysteroid dehydrogenase type 10) [11-13]. The mutant protein FAOMPG116F was found to lose both enoyl-CoA hydratase and 3-hydroxyacyl-CoA epimerase activities while retaining 3-hydroxyacyl-CoA dehydrogenase and some of its Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activities due to the substitution of phenylalanine for glycine at residue 116. If several different mutants need to be made and characterized, the ssDNA template could be repeatedly utilized (Table 1).

Mutagenic primer Nucleotide sequence	Mutant protein	Ratios of specific activities of mutant vs WT (%)				Ref
		Hydratase	Dehydrogenase	Isomerase	Epimerase	
5'-CCGGATCTGCA AATCGGTCTGC-3'	FAOMPR134Q	62	106	155	122	14
5'-CGGTCTGCCGC AAACCAAACT-3'	FAOMPE139Q	0	115	33	0	14
5'-CTGGGTGCAGC GATTATGGG-3'	FAOMPG322A	170	46	103	ND[a]	15
5'-CTGCGGGATGG CCTTCTTTAACC-3'	FAOMPH450A	63	0	64	ND[a]	15
5'-CGGGATGCAAT TCTTTAACC-3'	FAOMPH450Q	45	0	33	ND[a]	15
5'GGTGGCTGCCAATGCGTGCT-3'	FAOMPE119Q	0.9	62	5	ND[a]	16
5'GTCGGTGCTAATATCACCGAA-3'	FAOMPD69N	9	95	76	ND[a]	16
5'-GCAAGATCGCC AAAGTGATG-3'	FAOMPD524A	48	11	69	ND[a]	17
5'ATCTGCTGGCCGTTGTGGG-3'	FAOMPD542A	93	6	32	ND[a]	17
5'-GCCGTTGGTAC AAATTATTCGCG-3'	FAOMPE462Q	62	0.4	37	ND[a]	17
5'-GCCGTTGGTAG CAATTATTCGCG-3'	FAOMPE462A	83	1.5	47	ND[a]	17

*A selection primer (5'-CAGGCATGCACGCGTGGCGTAATC-3') was used for site-directed mutagenesis experiments. In each of these 11 experiments 5 clones were chosen at random for sequence analysis. All clones contained the desired mutations.

[a]ND, not done.

Table 1: Utilization of a ss DNA template for making various mutant proteins*

Moreover, the production of M13 phage and ssDNA has become much easier [5]. Last but not least, this method requires only basic molecular biological techniques and reagents that are readily available in most laboratories in economically developed as well as in developing countries.
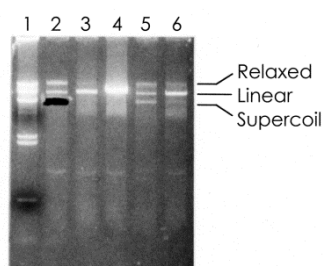
Figure 3: Comparison of the selection enzyme digests of RF DNA isolated from *E. coli mutS* and MV1190 transformants. DNA was separated by electrophoresis on a 1% agarose gel. Lane 1 was molecular weight markers (1 μg of λDNA/*Hind* III fragments). Lanes 5 and 6 were the digests of RF DNA of the no mutagenic primer control isolated from *E. coli mutS* and MV1190, respectively. Lanes 2 and 3 were corresponding to lanes 5 and 6 of a parallel experiment, respectively. The supercoiled DNA in lane 2 was cut from the gel [18] to confirm that it did carry the desired mutation. Lane 4 was the digest of RF DNA of the no selection primer control from *E. coli mutS* transformants.

Acknowledgement

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