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*Full Length Research Paper*

# Construction of a high-efficiency multi-site-directed mutagenesis

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**Although site-directed mutagenesis has been used in many fields, it still has low rate of success and high cost because of low-yield target products. A modified method for multi-site-directed mutagenesis was developed with shifted primer design and cold-start polymerase chain reaction (PCR). The developed method was successfully applied to hexapeptide gene synthesis and recombinant enterokinase gene modification in the plasmids pET41a and pET24b-EK. The efficiency was pronounced at a 1:10 molar ratio of 7-base mutant products to 705-bp fragment products as control. Even in a 10-base substitution mutagenic PCR, a 1:50 molar ratio of mutant products to 705-bp fragment products was reached. Meanwhile, the quality of mutants was proved through the transformation efficiency and sequencing. This method was beneficial to prepare high-quality multibase mutagenesis and also implied that large-scale multibase mutagenesis was feasible, efficient, economical, and productive.**

**Key words:** Site-directed multibase mutagenesis, shift primer, hexapeptide gene, enterokinase gene.

## INTRODUCTION

Multi-site-directed mutagenesis (MSD) is a powerful tool to modify DNA sequences in molecular biological studies (Mullaney et al., 2010; Shaya et al., 2010; Zhao et al., 2010). The simplest and most broadly applicable protocol is the quick-change site-directed mutagenesis system developed by Stratagene Company. With this approach, the mutation is introduced in a single polymerase chain reaction (PCR) with one pair of complementary primer containing the interesting mutation. In this method, primer dimer will be easily formed rather than the primer-template annealing. Thus, mutational studies will become more difficult because it often fail to introduce any mutation. Later, the problem was overcome by Zheng et al. (2004). However, we still confronted many difficulties even when using the optimal method. This led us to explore more methods to improve the PCR and make MSD available.

Recombinant hexapeptide (Falla and Zhang, 2010) and enterokinase (EK) were two products developed in our laboratory. The gene for the hexapeptide could be synthesized easily in the plasmid pET41a through MSD since it is coded by an 18-base gene. The latter was expressed as inclusion body in BL21 (DE3) with no bioactivity after refolding, which was caused by its wrong sequences; therefore MSD could be used to amplify the gene or correct wrong sequences directly. Meanwhile, the rules for PCR-based MSD were exploited. The work was directed toward better definition of basic processes and the development of analytical procedures that could be

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**Abbreviations: PCR,** Polymerase chain reaction; **MSD,** multi-site-directed mutagenesis; **EK,** enterokinase; **LB,** lysogenic broth; **ccc,** circular covalently closed.

**Table 1.** The primer pair comparison between QuickChange-site-directed mutagenesis system and our study.

| Primer sequence | Length/ mutation (bases) | Successive complementarity of primer pair (bases) | Self-annealing Tm (°C) |
|---|---|---|---|
| HP_f** 5'-CGACGACAAGgaagagatgcaacgtcgctAGTCCCATGGGA-3' | 41/19 | 39 | 70.8 |
| HP_r** 5'-CCATGGGACTagcgacgttgcatctcttcCTTGTCGTCGTCA-3' | 42/19 | | |
| HP_f* 5'-ACAAGgaagagatgcaacgtcgctAGTCCCATGGGA-3' | 36/19 | 29 | 62.9 |
| HP_r* 5'-GGACTagcgacgttgcatctcttcCTTGTCGTCGTCA-3' | 37/19 | | |
| HP_f 5'-gaagagatgcaacgtcgctAGTCCCATGGGATATC-3' | 35/19 | 19 | 51.1 |
| HP_r 5'-agcgacgttgcatctcttcCTTGTCGTCGTCATCAC-3' | 36/19 | | |
| HP1_f** 5'-CGACGACAAGgaagagATGGGATATCGGGGATC-3' | 33/6 | 26 | 59.5 |
| HP1_r** 5'-GATATCCCATctcttcCTTGTCGTCGTCATCACC-3' | 34/6 | | |
| HP1_f* 5'-ACAAGgaagagATGGGATATCGGGGATC-3' | 28/6 | 21 | 43.4 |
| HP1_r* 5'-CCCATctcttcCTTGTCGTCGTCATCACC-3' | 29/6 | | |
| HP1_f 5'-gaagagATGGGATATCGGGGATC-3' | 23/6 | 6 | 18 |
| HP1_r 5'-ctcttcCTTGTCGTCGTCATCACC-3' | 24/6 | | |
| HP2_f** 5'-GGAAGAGATGcaacgtcgctGATCCGAATTCTGTACAG-3' | 38/10 | 31 | 64.4 |
| HP2_r** 5'-GAATTCGGATCagcgacgttgCATCTCTTCCTTGTCGTC-3' | 39/10 | | |
| HP2_f* 5'-AGATGcaacgtcgctGATCCGAATTCTGTACAG-3' | 33/10 | 26 | 53.8 |
| HP2_r* 5'-GGATCagcgacgttgCATCTCTTCCTTGTCGTC-3' | 33/10 | | |
| HP2_f 5'-caacgtcgctAGTCCCATGGGATATC-3' | 27/10 | 10 | 32 |
| HP2_r 5'-agcgacgttgCTTGTCGTCGTCATC-3' | 27/10 | | |
| EK1_f** 5'-GATATACATATGgTTGTCGGAGGAAGTGACTC-3' | 32/7 | 26 | 56.4 |
| EK1_r** 5'-CTTCCTCCGACAAcCATATGTATATCTCCTTC-3 | 32/7 | | |
| EK1_f* 5'-ATATGgTTGTCGGAGGAAGTGACTC-3' | 23/7 | 5 | 30 |
| EK1_r* 5'-GACAAcCATATGTATATCTCCTTC-3' | 21/7 | | |

For all primers, mutagenized positions are denoted in lowercase. The single asterisk stands for the primer with the mutation site at the fifth base from the 5'-terminus. The primers without asterisk have the mutation site at the 5'-terminus. The primers, which are designed according to the instructions given by Strategene Company, are marked with double asterisk. In all the primers of EK, there is a 6-base deletion compared with partial template: 5'-CATATG~~GCTAGC~~ATTGTCGGAGGAAG-3'.

applied to successful mutagenesis.

## MATERIALS AND METHODS

### Plasmid construction

Plasmids pET41a and pET24b-EK were used as mutagenic templates. All primers designed to introduce multibase mutations were synthesized and purified by Takara and all restriction enzymes were purchased from Takara (Dalian, China). *Pfu* DNA polymerase and competent cells were purchased from Dingguo Biotech (Beijing, China), while *Dpn* I was purchased from Biolabs (Beijing, China). The self-annealing temperature (Tm) of the primer was calculated with the formula given by Stratagene (http://www.stratagene.com/manuals/200519.pdf). The characteristics of the primers are listed in Table 1. The gene fragment 5'-GAAGAGATGCAA CGTCGC-3' encoding hexapeptide EEMQRR was inserted into the downstream of the EK cleaving site. EK gene (AY682203) was cloned into pET-24b at the *Nde* I/*Xho* I sites.

### PCR mutagenesis

One hundred ng (1 µl) plasmid pET41a or pET24b-EK was used as mutagenesis template. Five µl *Pfu* DNA polymerase buffer 10× reaction buffer, 2 µl primers (each at a concentration of 20 µM), 2 µl (4 U) *Pfu* DNA polymerase, 2 µl dNTP mix (10 mM each) and 36 µl water were combined and placed in the block of a Eppendorf temperature cycler, at 95°C for 2 min, followed by 18 cycles of 95°C for 1 min, 55°C for 1.5 min, and 68°C for 14 min. Finally, one cycle of 68°C was carried out for 14 min.

Another PCR was performed with cold start. 100 ng (1 µl) plasmid pET41a or pET24b-EK was used as mutagenesis template. Five µl *Pfu* DNA polymerase buffer 10 × reaction buffer, 2 µl primers (each at a concentration of 20 µM), and 36 µl water were combined and placed in the block of an Eppendorf temperature cycler, at 95°C for 2 min. The thermal cycler was turned off to allow the samples to cool slowly to room temperature. The reaction mixture was placed on ice while adding 2 µl (4 U) *Pfu* DNA polymerase, 2 µl dNTP mix (10 mM each). The reaction mixture was then placed in the block of the cycler at room temperature and the unit was turned on so that the block could slowly heat to 68°C. The reaction

**Table 2.** PCR and transformation efficiency comparison between QuickChange site-directed mutagenesis system and our study.

| Primer | Molar ratio of mutant products to enterokinase gene products | Molar ratio of mutant products to enterokinase gene products (with cold start) | Transformation efficiency (colonies) |
|---|---|---|---|
| HP_f** /HP_r** | No mutant products could be seen | No mutant products could be seen | 0 |
| HP_f* /HP_r* | <1:200 | <1:200 | 3 |
| HP_f /HP_r | <1:200 | <1:200 | 0 |
| HP1_f** /HP1_r** | 1: 100 | 1:100 | 2 |
| HP1_f* /HP1_r* | 1: 30 | 1:10 | 200 |
| HP1_f /HP1_r | 1: 15 | 1: 8 | 30 |
| HP2_f** /HP2_r** | <1:200 | <1:200 | 2 |
| HP2_f* /HP2_r* | 1: 100 | 1: 50 | 20 |
| HP2_f /HP2_r | 1: 50 | 1: 40 | 5 |
| EK1_f** /EK1_r** | 1: 100 | 1:100 | 3 |
| EK1_f* /EK1_r* | 1: 20 | 1:10 | 300 |

The single asterisk stands for the primer with the mutation site at the fifth base from the 5'-terminus. The primers without asterisk have the mutation site at the 5'-terminus. The primers which were designed according to the instructions given by Strategene Company were marked with double asterisk.

incubated at this temperature for 5 min followed by 18 cycles of 95°C for 1 min, 55°C for 1.5 min, and 68°C for 14 min. Finally, one cycle of 68°C was carried out for 14 min in order to evaluate the PCR efficiency. Meanwhile, EK gene (708 bp) was amplified as a control.

At the end of temperature cycling, the PCR products were evaluated by agarose gel electrophoresis. Meanwhile, 10 U (0.5 µl) of *Dpn*I restriction enzymes were added to 8.5 µl PCR reaction mixture and incubated at 37°C for 1 h. Following *Dpn* I digestion of parental DNA, 5 µl reaction mixture were used to transform XL1-Blue competent cells. The transformed cells were spread onto lysogenic broth (LB) medium plates containing 50 µg/ml kanamycin. A total of 18 colonies (two colonies for each sample, Table 2) were selected and their plasmids were isolated by mini-prep. The positive mutants were verified by sequencing.

#### Other work preparation

A prerequisite for the success of PCR-based multibase mutagenesis was the development of cost-effective and a generic process for plasmids DNA production. However, to satisfy the strict rule, the material must be available and highly purified with homogeneous super coiled circular covalently closed (ccc) pDNA. Alkaline lyses of the bacteria damages the pDNA, resulting in a reduced recovery of ccc pDNA and an increase in partially open circular forms. Shear stress in these processes needs to be tightly controlled; buffer composition and pH also need to be optimized.

## RESULTS AND DISCUSSION

For hexapeptide gene synthesis, we encountered difficulties with the Quick-Change protocol in the course mutational studies, where we were completely unable to introduce any mutation following the recommended protocol (Table 2). These difficulties might be caused by the high self-annealing temperature of primer pair (Table 1), which led to an enormous amount of primer dimers formation and a colossal amount of PCR products reduction.

We developed a simple modification of the primer design, which not only overcame the limitation of self-annealing temperature of primer design, but also made multiple mutations available. The mutant products could be visualized on agarose gel to confirm that the plasmids were successfully amplified. During a 6-base-substitution and a 6-base-deletion-and-1-base-substituion mutagenic PCR, the efficiency was pronounced at a 1:10 molar ratio of mutant products to 705-bp fragment products as control (Table 2) when the mutation was placed as close as five bases away from the 5’-terminus. Typically, around 2.5 µg of asymmetry double-nicked circular DNA was generated from each 50 µl of the PCR reactions. Meanwhile, the transformation efficiency was high (Table 2). The results suggested that the primers in the pair should complement each other at the 5’-terminus instead of the both sides to avoid primer self-extension. Thus, all the self-annealing temperature of inter-primers was not too high.

Comparatively, the amounts of PCR products were more than that earlier described when the mutation was placed at the 5’-terminus but the transformation efficiency is extremely low (Tables 1 and 2). When the mutation was placed at the 5’-terminus, the circularization of PCR products hardly occurred after transformation. Therefore, the transformation efficiency can be used to evaluate the

quality of mutant products.

All the 18 multibase mutations were proved correctly through sequencing. In the modification of pET24b-EK, we changed the first five residue of EK, so the N-terminus of the expressed mature recombinant EK would be MVGGS- DSRE. The residue could be cleaved by methionine aminopeptidase, thereby the modified EK had the same bioactivity as the native EK (Tan et al., 2007). In comparison, all attempts failed if mutant sites started the 5'-terminus in primers or complete overlapping primers were used, even when no products appeared on agarose gel (Table 1 and 2).

A few rules can be deduced from our experiments: (1) The mutation can be placed no less than five bases close to the 5'-terminus and at least 10 to 15 bases from the 3'-terminus; (2) more mutations can be introduced in one primer, with a mutation content up to almost 30% (HP2, 10 out of 38 bases) and (3) cold start PCR appeared to increase the efficiency of PCR (Table 2).

In summary, the methods introduced here was beneficial to broaden the usefulness and facilitate the preparation of high-quality and high-yield MSD, since most reagents were made dead cheaply and no kits were used in the whole process and also documented that, large scale MSD was feasible, efficient, economical, and productive when combined with other methods (Spiess et al., 2010; Zhang et al., 2008).

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