A Comparison of Three Site-Directed Mutagenesis Kits

Paxton Loke and Tiow-Suan Sim*

Department of Microbiology, Faculty of Medicine, National University of Singapore, MD4A, 5 Science Drive 2, Singapore 117597. Fax: 65-7766872. E-mail: micsimts@nus.edu.sg

*Author for correspondence and reprint requests

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In this comparative study, three different mutagenesis kits, namely the MutaGene phagemid *in vitro* mutagenesis kit (Bio-Rad), the Transformerä Site-Directed mutagenesis kit (Clontech) and the Quik-change site-directed mutagenesis kit (Stratagene) were used for the mutagenesis of IPNS genes. However, a large difference in mutation efficiencies among these kits was encountered. Furthermore, these kits employ different strategies with its own individual strengths and weaknesses. Thus, a comparison among these three kits to evaluate their usefulness and improvements on the strategy adopted by the Quik-change site-directed mutagenesis kit, which was the kit of choice for our work, are presented for the benefit of research work.

Introduction

Studies on the structure and function of a particular protein are predominantly supported by the application of *in vitro* site-directed mutagenesis, in addition to the more arduous task of obtaining three-dimensional crystal structures. This procedure involves an extensive variety of techniques and strategies whereby a short mutant oligonucleotide is incorporated into a longer segment of DNA (Botstein and Shortle, 1985).

We have used three different commercial site-directed mutagenesis kits, namely the MutaGene phagemid *in vitro* mutagenesis kit (Bio-Rad), the Transformer™ Site-Directed mutagenesis kit (Clontech) and the QuikChange™ site-directed mutagenesis kit (Stratagene) for the mutational analysis of isopenicillin N synthase (IPNS), a key enzyme in the penicillin and cephalosporin antibiotic biosynthetic pathway. The IPNS genes used in our study are from the fungus *Cephalosporium acremonium* and the bacterium *Streptomyces clavuligerus*, with a G+C content of 63% and 66% respectively (Cohen *et al.*, 1990). We found the strategy employed by the QuikChange™ site-directed mutagenesis kit (Papworth *et al.*, 1996) to be the most efficient in achieving our intended mutations. As these kits differ in their mutagenesis strategy and their preparation set-up, a comparison among these three kits is appropriate (Table I). Furthermore, additional suggestions on the strategy adopted by the QuikChange™ site-directed mutagenesis kit are also presented (Table II).

Comparison of the three mutagenesis kits

The MutaGene phagemid *in vitro* mutagenesis kit is based on the selection method devised by Kunkel (1985) and requires the laborious and time-consuming task of acquiring single-stranded DNA and a subcloning step in order to transfer the gene of interest into the specialized phagemid vector. Besides, in the actual experimental environment, not all the single-stranded DNA generated are uracil-containing templates. Moreover, DNA mismatch repair to remove the mutation generated or the insufficient inactivation of the non-mutated parental strands can happen in the *E. coli* host provided, which is not a mismatch repair defective strain.

Nevertheless, this kit allows the formation of covalently closed circular DNA upon successful annealing of the mutagenic primer and *in vitro* DNA synthesis reaction to be observed via agarose gel electrophoresis. Hence, evidence of covalently closed circular DNA would most likely signal a successful mutagenesis reaction. The mutagenesis efficiencies encountered in the creation of mutants D218L and R281L of *C. acremonium* IPNS (cIPNS) were 10% and 33% respectively (Loke *et al.*, 1997a; Loke and Sim, 1998).

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Table I. Overall comparison of the three mutagenesis kits.

	MutaGene phagemid	Transformer	QuikChange
Preparation	1. Subcloning into a phagemid vector 2. Generation of U-ssDNA 3. One mutagenic primer	1. Subcloning into a vector that has a restriction enzyme site for the selection primer 2. One mutagenic and one selection primer respectively	1. Any double-stranded plasmid 2. Two complementary mutagenic primers
Experimental steps required	1. Two transformations 2. Subcloning	1. Two transformations 2. Subcloning	1. One transformation
Number of clones needed to be screened for one mutation	6–10	6–8	2–6
Strengths	1. Annealing and polymerization can be monitored		1. Polymerization can be monitored 2. Most rapid protocol
Possible weaknesses	1. Laborious in generating ssDNA 2. Rigorous optimization of annealing conditions required	1. Simultaneous annealing of both primers difficult to achieve	1. Optimization of cycling conditions
Time required for one mutation (from setup to sequencing)	Months	Weeks	Days

The Transformer™ Site-Directed mutagenesis kit which is based on the method of Deng and Nickoloff (1992) relies on the likelihood that two primers (selection primer and mutagenic primer) will anneal simultaneously to the same strand of a double-stranded plasmid. Furthermore, the *E. coli* host involved in the first transformation is deficient in mismatch repair (Zell and Fritz, 1987) and the linearized parental DNA after restriction enzyme digestion (the selection step) would be a hundred times less efficient in transforming bacterial cells. In addition, viral transduction to obtain single-stranded DNA is not required.

Initially, a mutation efficiency of 83% was obtained for the D214A mutants of *S. clavuligerus* IPNS (Loke *et al.*, 1997b) but subsequent attempts to generate mutants in cIPNS were unsuccessful. One disadvantage is that confirmation of the annealing of the mutagenic primer cannot be observed experimentally. Accordingly, this leads to the danger of the possibility that the mutagenic and selection primers do not anneal together at the same time and to the same strand. In fact, when putative mutant clones were screened by DNA sequencing and restriction enzyme digestion, the annealing of the selection primer was successful but the mutagenic primer was not, generating only wildtype clones. Thus, relatively low success rates for IPNS genes were observed.

The QuikChange™ site-directed mutagenesis system eliminates the need to generate single-stranded DNA and allows site-directed mutagenesis to be performed using any double-stranded plasmid. Thus, in our case, any recombinant vector carrying IPNS genes could be used directly for mutagenesis without the need for any subcloning step. Following that, expression analysis of the mutants could be carried out immediately without further subcloning or changing of expression vectors.

The QuikChange™ strategy allows for site-specific mutations via the incorporation of two complementary mutagenic primers into newly synthesized DNA during gene amplification. Successful amplification of the double-stranded mutants can be observed on agarose gel electrophoresis. How-

Table II. Additional suggestions for the QuikChange™ site-directed mutagenesis strategy.

Parameter	Suggestions/helpful hints
Primers	1. The shortest primer tried is 21 bp. Generally, 24–30 bp primers are adequate. 2. Around 50–100 pmol of each primer is used per reaction.
Cycle conditions: A. Annealing	1. Adjust annealing temperature 2–5 °C upwards when non-specific priming is seen. An annealing temperature of up to 65 °C has been attempted. 2. Lower the annealing temperature when expected product is not seen.
B. Polymerization	1. Polymerization cycles must be increased to 20–25 cycles.
C. Others	1. DMSO can be added up to 8%. 2. Template and amount of polymerase can be increased if no product is seen. 3. Scale-down of total reaction volume by half to 25 μl is possible.
Digestion step	1. *Dpn*I digestion time can be increased to 4 hours if necessary.
Transformation	1. Direct transformation into any *E. coli* host of choice for analysis.
Screening	1. At least, one successful mutant from four transformants. 2. At best, 100% of the transformants carry the mutagenized gene of interest.

ever, optimization of the thermal cycling conditions to obtain a successful mutant product is required. Modifications in the amplification conditions can be realized by adjusting the annealing temperature and/or the number of polymerization cycles.

A strong selection step is possible as the mutant product is subjected to a restriction endonuclease digestion with *Dpn*I. The *Dpn*I endonuclease has a target recognition site of 5′-G^{m6}ATC-3′ and is specific for methylated and semimethylated DNA. Therefore, only the parental (or wildtype) DNA will be susceptible to *Dpn*I digestion as plasmids isolated from almost all *E. coli* strains would be *dam* methylated. The mutation efficiencies achieved among the IPNS mutants generated by this strategy ranged from 25% to 100% and the maximum number of mutants needed to be isolated for screening is six although for most cases, four was sufficient. A review of our mutagenesis work in IPNS genes has been published (Sim and Loke, 2000).

Additional suggestions to the QuikChange™ site-directed mutagenesis strategy

For our mutagenesis work in IPNS genes, certain improvements were made to the QuikChange™ site-directed mutagenesis kit to obtain successful mutagenesis reactions (Table II). Primarily, this was done in the optimization of the cycling conditions, most critically the annealing temperature for the mutagenic primers. Variations to the annealing temperature range from the suggested value of 55 °C to 65 °C if non-specific priming is encountered. However, when the expected product is not seen, a decrease in the annealing temperature is necessary. A significant time-saving point is that direct transformation into any *E. coli* host of choice can be attempted instead of using the *E. coli* XL-1 Blue supplied by the kit. This is especially evident in our studies as the transformation of mutated plasmids into *E. coli* BL21(DE3) allows for immediate expression analysis without the need of going through an intermediate transformation step.

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