Simultaneous Introduction of Multiple Mutations Using Overlap Extension PCR


Introduction of multiple mutations can be accomplished through phage M13-based site-directed mutagenesis using several oligonucleotides (6). The major drawback of this method is the low efficiency of generating all the intended mutations simultaneously (7,9). The alternative methods for introducing multisite mutations are based on polymerase chain reaction (PCR) (1) and ligation chain reaction (LCR) (8), but they require complicated modifications to the current mutagenesis and PCR protocols. Overlap extension PCR has been used to introduce single mutations embedded in the oligonucleotide primers (2) and to fuse genes or gene segments (3). We have combined both applications of the overlap extension PCR to introduce multiple mutations within genes or gene segments simultaneously. In contrast to other methods of introducing multiple mutations, the current method is fast, simple and highly efficient. In addition, this method is very flexible.

To illustrate the usefulness of this method, we have applied this method to remove several restriction sites in both a promoter-modified cat gene (chloramphenicol acetyltransferase) (5) and the lacI gene. The method of Vallejo et al. (10) has been modified, and the Taq DNA polymerase has been replaced with a thermal stable polymerase with proofreading activity (Pfu). The method consists of four steps, described below.

(i) The PCR primers were designed where the mutations were placed 12–13 bases upstream from the 3' end of the cognate primers (Figure 1A). Pairs of primers annealing to the overlap regions of the mutagenic primers were used to amplify the segments of the genes that were not mutagenized. For lacI gene PCR primers (Figure 1B), the overlap regions of the mutagenic primers (B3 and B4) were shortened, and the primers, which were used to generate the unaltered segments of the gene (B2 and B5), were shifted to include some of the mutations. Primer B7 was added in the final assembly PCR to add the 3' end.

(ii) The fragments for overlap extension (Figure 1, A and B) were produced using Pfu DNA Polymerase (Stratagene, La Jolla, CA, USA) in a Perkin-Elmer DNA Thermal Cycler (Norwalk, CT, USA). Briefly, the plasmid carrying the modified cat gene (5) (ca. 100 ng) was mixed with 10× buffer (supplied with the enzyme), the primer pairs (25 pmol each, synthesized by GenSet, Paris, France) and dNTP (10 nmol) in a total volume of 50 µL. The mixtures were heated at 92°C for 3 min while Pfu DNA polymerase was added and cooled down to 50°C. The mixtures were incubated at 72°C for 1 min, followed by 14 cycles of PCR (92°C for 1 min; 50°C for 1 min and 72°C for 1 min) with a final extension at 72°C for 5 min. The PCR products were gel-purified (Qiagen, Hilden, Germany) and eluted in TE buffer (10 mM Tris-
HCl, pH 8.0, 1 mM EDTA).

(iii) The fragments were PCR-assembled using approximately equal amounts of the three fragments. The mixtures were heated at 94°C for 3 min while the Pfu DNA polymerase (2.5 U) was added. Two cycles (94°C for 1 min, 52°C for 2 min and 72°C for 1 min) were carried out. Then the 5′-end and 3′-end primers were added, and another 13 rounds of PCR under the same conditions were performed. For the lacI gene, the same procedure was applied except that the annealing temperature was raised to 60°C and 28 instead of 13 cycles of overlap extension PCR were performed. The full-length PCR products were gel-purified (Qiagen) and analyzed on 1% agarose gels (Figure 2 and data not shown).

(iv) The purified full-length PCR products were ligated directly into either pCR-Script™ (cat gene; Stratagene) or pZErO™-1 vector (lacI gene; Invitrogen BV, Leek, The Netherlands), which was used to transform purchased competent XL-1 Blue (cat gene) or TOP10F™ cells (lacI gene) prepared as described (4). Plasmids were isolated from 2-mL overnight cultures of single colonies using a QIAprep™ Spin Plasmid Kit (Qiagen) and subjected to restriction analysis. Selected clones containing the inserts were also subjected to sequence analysis (SequiServe, Vaterstetten, Germany).

Except for one clone with one restriction site unchanged, all the clones assayed (4 from cat and 10 from lacI) contain all the introduced mutations. The sequence analysis indicated that there was only a one-base mutation besides the introduced mutations in 4-kb sequences analyzed. The simplicity of the method is also accompanied by the speed with which the mutations can be introduced: from the start of PCR to generate the fragments to screening the correct clones required less than one week. We have applied this technique extensively in our laboratory, and the results have been quite satisfactory. In conclusion, this technique offers a rapid and reliable method to introduce multiple mutations simultaneously.

REFERENCES


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