A method for multi-site-directed mutagenesis based on homologous recombination

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We have developed an efficient method for the simultaneous introduction of up to three mutations in a plasmid DNA via homologous recombination. The strategy is compatible with a variety of mutations, including degenerate codons in plasmids of different sizes. In contrast to other methodologies, this approach employs the same set of reagents for both single- and multi-site mutagenesis assays, minimizes the required protocol steps, and exhibits remarkably high mutagenesis efficiencies.

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The increasing need to understand the relationship between the structure and function of proteins and to modulate gene expression has led to the emergence of a variety of single- and multi-site-directed mutagenesis methods [1–8]. Due to the simplicity of their protocols, reduced hands-on time, and relatively high mutagenesis efficiency, the QuickChange mutagenesis kits (Agilent Technologies, La Jolla, CA, USA) have become standard. The single-site mutagenesis approach is based on the amplification of a double-stranded DNA (dsDNA) plasmid using a pair of complementary mutagenic oligonucleotides [9], whereas the multi-site strategy is based on primer extension from multiple mutagenic oligonucleotides annealed to the same strand of a circular episome using a proprietary enzyme mix of undisclosed formulation [10]. Shortcomings of these techniques include the following: (i) different sets of reagents are needed, depending on whether the pursued number of mutations is one or multiple ones; (ii) multi-site mutagenesis efficiencies lower than 50% are usually obtained with large-size plasmids; and (iii) numerous rounds of strand synthesis are required with multi-site mutagenesis due to nonexponential amplification. Protocols that intend to apply the single-site mutagenesis approach above to multiple sites or to provide alternative strategies require the use of either phosphorylated oligonucleotides [7] or multiple polymerase chain reactions (PCRs) [2,8] or are inefficient [5]. Additional disadvantages of these and other approaches have been discussed previously [10].

We recently showed that the inclusion of a homologous recombination step after the amplification of a circular episome with a pair of mutagenic primers significantly boosts the mutagenesis efficiency of a single site [11]. Here we show that the procedure can be adapted to accomplish multi-site-directed mutagenesis.

The strategy is summarized in Fig. 1A. Briefly, either a single multiplex or three independent PCRs are performed, where each pair of mutagenic primers is used. The mutation site in each oligonucleotide must be flanked with at least 10 unchanged nucleotides at both sides. Thus, the DNA fragments generated by PCR will share end-terminal homology, required for homologous recombination. After amplification, an aliquot of the reaction is subjected to a 15-min pulse of recombination activity, followed by transformation. Recombination is promoted by a dsDNA repair reaction [12,13]. Different template removal techniques, such as CpG methylation [14] and DpnI restriction [15], can be used. In our particular case, we employed CpG methylation (for further details, see Supplementary Material).

To introduce three mutated sites, typically three pairs of overlapping forward and reverse primers are used unless a pair of long primers is used to cover the mutation sites that are in very close proximity. The PCR mixture can be used as is or split into three PCR tubes. For a three-site mutagenesis reaction, we observed slightly higher mutagenesis efficiencies following the three-tube strategy compared with a single multiplexed PCR. For the three-tube strategy, the primer distribution should be as follows: (i) forward primer of site 3 and reverse primer of site 1, (ii) forward primer of site 1 and reverse primer of site 2, and (iii) forward primer...
The annealing temperature should be set to 5°C for 15 to 20 min (methylase reaction), and then the temperature is raised to 94°C for 15 to 20 min (methylation reaction), and then the temperature is lowered to 37°C for 15 to 18 cycles of 30 s at 94°C (denaturation), 30 s at 55°C (annealing; see below), and a variable time at 68°C (extension; see below). The annealing temperature should be set to 5°C below the oligonucleotide melting temperature. The extension time is primarily determined by the largest fragment to be amplified and the polymerase’s extension rate. For example, AccuPrime Pfx exhibits a polymerization rate of 30 s per kilobase. A variety of dsDNA repair enzyme sources may be used for the recombination step (see Supplementary Material and Refs. [12,13]). In our particular case, we followed the protocol listed in the Supplementary Material, which employs a 6-µl PCR sample and 15-min incubation at room temperature. After stopping the reaction with the addition of 1 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA), a 3-µl sample was used to transform DH5α competent cells. It is important to note that for the elimination of CpG methylated templates, only strains containing the McrBC restriction system must be used [14].

To evaluate the mutagenesis efficiency, we generated 5-, 10-, and 14-kb plasmids containing a 1.2-kb insert in which the spectinomycin gene was fused in-frame to the N terminus of the lacZα gene (for further details, see Supplementary Material). Different sets of three mutations, of 1 or 3 bp each, were introduced, where each individual mutation gave rise to white colonies (for the corresponding sequences, see Supplementary Material). The restoration of β-galactosidase activity occurred only when all three mutated sites changed back to the original wild-type sequence. The multi-site mutagenesis efficiency was determined by calculating the percentage of blue bacterial colonies that appeared on plates containing 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal).

Using a combination of the conditions above, we compared the mutagenesis efficiencies between this new protocol and the most widespread and efficient multi-site mutagenesis approach [10]. Our new method showed consistently higher mutagenesis efficiencies in a wide range of plasmid sizes (Fig. 1B). The approach was also evaluated using plasmids and sequences that would not confer any specific phenotype to the colonies. Sequencing of a sample of those clones revealed results consistent with the results above (not shown).

The approach is not severely affected by the distance between the sites (Fig. 1C). In any case, for very proximal sites (e.g., distances <70 bp), the mutation sites can be covered by a single pair of oligonucleotides, bringing the mutagenesis efficiency back up to 5 to 6 percentage points (Fig. 1C). The mutagenesis rate remains virtually constant between 90 and 100% for three sites placed more than 200 bp apart from each other, regardless of the plasmid size up to at least 14 kb (not shown).

Finally, by employing the lacZ platform above, we examined the viability of our approach for the generation of mutant libraries using six pairs of oligonucleotides harboring three degenerated nucleotides (NNN) in the middle of their sequences. More than 80% of the colonies exhibited a lacZ phenotype, which is an underestimation of the real mutagenesis efficiency, because those clones containing stop codons at one or more of the mutated sites are not represented in the positive population. Sequencing of a fraction of the positive clones did not reveal any apparent bias (not shown).

In the absence of an in vitro recombination step, plasmid reconstitution relies exclusively on the Escherichia coli endogenous homologous recombination, which was shown to be extremely low [16]. This level of recombination is not high enough to produce more than one simultaneous strand exchange (not shown).

In conclusion, we have developed a rapid and efficient multi-site-directed mutagenesis method that expands the circular polymerase mutagenesis approach to cover up to at least three sites in a single episome. The strategy uses the same set of reagents readily available from different sources, regardless of whether mutating one or multiple sites, and exhibits increased efficiency compared with the standard methodologies.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.05.002.

References