Benchmarks

ever, all of the modifications that we have considered are significantly more complicated and would most likely result in a lower cloning efficiency.

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General Method for Site-Directed Mutagenesis

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Site-directed mutagenesis is used to introduce mutations into DNA fragments in a highly defined manner (5). Sequences with known mutations can thereby be generated for molecular biological studies such as the expression of altered proteins or for use as standard DNA sequences. Such standard DNA is often used in competitive PCR and for quality control of mutation detection assays. During the last decade, several strategies have been developed to simplify this approach and to increase its efficiency. The yield of mutated clones usually ranges from 20% to 60%. A general strategy is based on the generation of a "megaprimer", which is then used for amplification of a mutated sequence (1,4,7), or on the extension of two overlapping mutated sequences (3,8), which are then extended (often referred to as "overlap extension"). The latter approach has the disadvantage that, in addition to the external primers, two mutagenic oligonucleotide primers are necessary to amplify the whole fragment. The megaprimer approach is simpler because only three primers are needed, one of which is mutagenic. In our experience this is the more robust of the methods. The efficiency, on the other hand, is rather low because usually only a maximum of 60% of the clones carry the mutation (2). This is due to trace amounts of template that are always present and are co-amplified during the cycles with the outer primers that generate the whole sequence of interest. Recently, a more efficient method was described that is based on enzymatic cleavage of methylated maternal DNA, but this requires an additional passage through a particular *E. coli* strain (6). We have developed a strategy for site-directed mutagenesis based on the megaprimer approach using dUTP-containing templates. After subsequent enzymatic digestion of the dUTP sequences with uracil glycosylase, the efficiency, as defined by the ratio of clones with the mutation to all clones, is increased to about 95%. This approach does not require any additional subcloning or special

bacterial strains. It is completely based on enzymatic in vitro DNA amplification and can be accomplished within one working day.

The steps to generate a defined mutation using this technique are as follows:

Step 1. Isolated dsDNA (1 µg) was amplified with dUTP instead of dTTP for 30 cycles of enzymatic amplification using a proofreading polymerase system, Expand™ High Fidelity consisting of a mixture of *Taq* and *Pwo* DNA polymerases (Roche Molecular Biochemicals, Mannheim, Germany). This polymerase mixture was used because *Pwo* is an enzyme that avoids random amplification errors. PCR reaction buffer (50 µL) containing 2.5 mM MgCl₂, 10 nmol dNTPs each, and 25 pmol primers (*a-for* and *a-rev*) were used for this amplification. Primers were removed after this step using a PCR purification kit (Qiagen, Hilden, Germany), and the template DNA was then dissolved in 30 µL sterile water.

Step 2. The resulting dsDNA fragment was then used as template for the megaprimer generation in which only the primer *rev-mut* (25 pmol) was used in a first round of 20 cycles of linear enzymatic amplification using *Taq* DNA polymerase and dTTP, instead of dUTP, under the same reaction conditions as above (30-s elongation). After this step, the whole mixture was subjected to enzymatic digestion of the template using 2 U uracil DNA glycosylase at 37°C for 90 min, followed by inactivation of the enzyme at 95°C for 2 min.

Step 3. After this step, 10 pmol of the primer *a-for* and 1 U Expand High Fidelity DNA polymerase were added to 20 µL of the solution, and 20 PCR cycles were performed to generate the megaprimer. The Expand High Fidelity DNA polymerase was used to avoid adenosine overhangs that are produced by *Taq* DNA polymerase. Such 3' adenosine residues may lead to an adenosine insertion into the target DNA. Primers were again removed after this step, as described above, and the generated megaprimer was dissolved in 30 µL water.

Step 4. The megaprimer solution (2.5 µL) was then added to 1 µL of the template DNA solution (Step 1) for linear amplification (20 cycles, 45-s elongation) using the conditions described for Step 2. This enzymatic amplifica-

Benchmarks

tion was followed by digestion with uracil DNA glycosylase, as described in Step 2.

Step 5. One microliter of the resulting mixture was used for amplification with the primers *a-for* and *a-rev*. Twenty PCR cycles were performed as described in Step 2, using *Taq* DNA polymerase to generate the necessary 3' adenosine overhangs for cloning. The reaction mixture was used directly for the cloning reaction.

Step 6. The TOPO-TA PCR2.1® vector (Invitrogen, Groningen, The Netherlands) was used. Briefly, 2 µL PCR product were mixed with 1 µL vector reaction mixture and 2 µL water

and incubated for 5 min at room temperature. After the incubation period, the vectors were cloned into *E. coli* (Top 10 F'; Invitrogen).

Figure 1 gives a schematic overview of this strategy for site-directed mutagenesis. The final mutation efficiency is usually about 95%, as assessed by the ratio of clones containing the mutation versus all positive colonies.

We describe a simple and rapid method for site-directed mutagenesis that is capable of achieving almost complete effectiveness. The whole process up to the cloning step can be finished within one working day. This protocol was developed strictly to avoid unwanted

ed wild-type amplification. This was warranted by two template digestions and two primer removals at critical steps during the procedure. We have used this approach to generate megaprimers up to a length of 500 bp. If increased over that length, the overall efficiency tends to be lower. Therefore, the use of dUTP-containing templates with consecutive digestion with the specific enzyme uracil DNA glycosylase can easily be adapted to similar strategies, provides a substantial increase in efficiency, and avoids a time- and money-consuming search for positive clones.

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Figure 1. Schematic overview of site-directed-mutagenesis using dUTP-containing templates (open lines); dTTP-containing sequences are displayed in closed lines.