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Improved site-directed mutagenesis method using PCR

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Several methods for site-directed mutagenesis using PCR have been described in the last few years. One of the most rapid, universal and economical methods was described by Landt et al. (1). This procedure requires just one mutagenic primer and two universal primers, which may contain convenient restriction sites for cloning. Essentially, it uses two of subsequent amplification rounds, the first with the mutagenic oligonucleotide and the antiparallel universal primer and the second one using the purified first fragment as a primer, together with the second universal primer, and subsequent digestion and cloning of the fragment. A possible problem described by these authors is the untemplated addition of one nucleotide at the 3′ site-specifically altered end of the first amplified fragment by Taq-polymerase, which can give rise to unwanted mutations in the second generated fragment. The authors advise to use lower concentrations of dNTPs to avoid untemplated addition of a nucleotide. A drawback of this procedure is a lower yield and no guarantee for the absence of a 3′ additional residue. A second solution for the problem is to remove the additional 3′ residue by the action of e.g. T4-polymerase prior to performance of the second PCR. This means that an additional enzymatic modification step has to be performed, which might not be fool-proof. As has been observed by several authors the 3′ additional nucleotide appears almost invariably to be an A-residue when using Taq polymerase (2, 3). Making use of this observation we have successfully applied a modification in the method which can generally be used to exclude the described difficulties. A mutagenic oligonucleotide is used for the first PCR reaction which is designed in such a way that the first 5′ nucleotide of the primer follows a T-residue in the same strand of template sequence. Thus, whether or not the amplified primer fragment from the first PCR contains an additional 3′ A-residue, in both cases the amplified product will have the correct sequence, without need for further modifications. Since in almost every case it should be possible to find a T-residue at a reasonable distance from the site of mutation, this adjusted method is generally applicable. We performed several different site-directed mutagenesis experiments by this method on the nisA gene (4). The following experimental conditions were used. Approximately 10 ng of plasmid DNA harbouring the nisA gene was used as template for PCR in a total volume of 50 µl, containing 1 U of Taq-polymerase (BRL), 50 mM NaCl, 10 mM Tris-HCl pH 8.8, 2 mM MgCl₂, 10 µg gelatine, 200 µM of dNTPs, 10 pmol of each primer, 2.5 µl of stabilizer (1% W-1, BRL) and covered with 100 µl of light mineral oil. PCR was performed in 30 cycles, each cycle consisting of a denaturing step at 93°C for 1 min., a primer annealing step at 54°C for 1.5 min. and an extension step at 72°C for 2.5 min. using a Biomed Thermocycler 60. The DNA-fragments were purified by TAE-agarose gel electrophoresis and recovered using the GeneClean procedure (Bio 101, La Jolla, California). Fig. 1. shows the sequence of the nisA gene and that of one of the oligonucleotides we used for site-directed mutagenesis. In each case we obtained the designed mutated fragment without undesired substitutions as was shown by dideoxy sequencing of six independent clones from each of several different mutagenesis experiments. This shows that no other nucleotide than an A-residue, or no nucleotide at all, had been applied to the 3′ end of the amplified primer fragment although we used up to 200 µM of dNTPs in all PCR reactions. In two other mutagenesis experiments using the same PCR conditions as described above, primers were used which followed another nucleotide than a T-residue at the 5′ end (in our case a C-residue). Following subcloning of the digested fragments, six clones obtained from each mutagenesis experiment were sequenced. In ten out of twelve cases a T for C substitution was encountered on the left side of the 5′ end of the first mutagenic primer, simultaneously with the desired mutation. In one case the wild-type sequence was observed, probably originating from a cloned template fragment and in one case the desired mutation and the correct sequence at the 5′ end of the primer were found. Thus, when using this mutagenesis method, a well considered choice of primer sequences can considerably increase the frequency of correctly mutated sequences.

REFERENCES


Figure 1. Sequence of nisA and primers used for PCR. Primers are shown in bold, the template T-residue 5′ to the mutagenic primer (Ser5 → Ala) is indicated by an asterisk and sites of mutation are underlined. The non-coding sequence of the nisA gene is shown in italics.