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Improved method for PCR-mediated site-directed mutagenesis

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Site-directed mutagenesis using two subsequent PCR amplifications has become over the past years a method of choice (1, 2). These procedures require just one mutagenic primer and two flanking universal primers. In the first PCR amplification, the mutagenic primer is used together with the corresponding antiparallel universal primer to yield a fragment that is subsequently used as a megaprimer in the second PCR reaction in conjunction with the second universal primer. The resulting amplified fragment contains the mutation and can be cloned after digestion with the appropriate enzymes (2). In practice, a frequent drawback of this method is the very low yields of the full-length fragment, due to inefficient priming by the megaprimer fragment in the second amplification. Long megaprimers can adopt secondary structure that may affect negatively extension by Taq polymerase. In addition, when used at concentrations required for efficient priming in conventional PCR reactions, reannealing of the double-stranded megaprimer itself will be favoured against priming of the template DNA.

Here we describe a modification of the second PCR reaction of the reported protocol (2), that takes advantage of exponential amplification to increase the yield of the desired fragment. The general strategy is outlined in Figure 1. The target for mutagenesis must be present in two different vectors. Vector 1 (black bars) is the template in the first PCR reaction, using primer A (universal primer specific for vector 1) and the mutagenic primer M. The product is the fragment M/A which should be isolated from a gel to be used in the subsequent reaction (Fig. 1A). Vector 2 (hatched bars) does not contain primer A sequence, and is used as the template in the second PCR reaction, with primers A and B (universal primer for vector 2) and the fragment M/A resulting from the first PCR reaction as megaprimer. As shown in Fig. 1B, the megaprimer M/A forms a heteroduplex with the template sequence in vector 2, which is extended by Taq polymerase. This results in a hybrid molecule that contains the mutated sequence flanked by the sequences of the two different vectors. Even if produced with low efficiency during the first cycles of PCR, this hybrid molecule can be amplified exponentially with primers A and B in the subsequent cycles. However, the wild type sequences of the template can be only linearly amplified with primer B, resulting in a very low background of false positive mutants. The resulting fragment containing the mutation can be cloned in a suitable vector after digestion with restriction enzymes *X* and *Y*.

The requirement of two vectors in which the target sequence must be available may appear as a restriction for the general application of this protocol. Actually, the only limitation of this protocol is that the sequence of the primer used for the first PCR reaction must not be present in the vector used as template in the second PCR reaction. Nevertheless, the presence of a given sequence in different vectors (having different sequencing primers) is not an infrequent circumstance. In case that several mutants will be prepared in a certain region of a cDNA or promoter, the effort employed for an additional subcloning step will be compensated by the high yield of the final product and the very low frequency of wild-type false positive clones. The improved protocol has been successfully used in our laboratory to produce point mutants of the chicken $T_3R\alpha$ (3), as well as mutants in the human $RAR\beta_2$ promoter. In all cases the percentage of wild-type sequences resulting from the cloning was below 5%. The results from three different cloning experiments using the improved procedure are shown in Table 1. Figure 2 shows an example of the different products of the first and second PCR reactions. In this example, the goal was to introduce a mutation in the TATA box of the RA-responsive reporter construct RARE O1-TK_{109} Luciferase (4). Conditions for both PCR reactions (100 µl) were: 10 mM Tris–HCl (pH 8.3 at 20°C), 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg/ml Gelatine, 50 ng template DNA, 200 µM dNTPs, 2 µM primers, 2.5 units Taq polymerase (Boehringer-Mannheim), 0.5 units PerfectMatch (Stratagene). The reactions were overlaid with 75 µl mineral oil and 30 amplification cycles of 1 min. at 94°C, 1.5 min. at 45°C, and 1 min. at 72°C were performed. In addition, the second PCR reaction included different amounts of the megaprimer obtained in the first PCR reaction. The mutagenic primer TK_{spm3} (CGAGGCCACACGCGTCACCTTTTTACACGAAGTGG-ACCTGGG) was designed according to Kuipers *et al.* (5), to avoid additional mutations by untemplated addition of a nucleotide by Taq polymerase. TK_{spm3} primer was used together with the upper strand oligonucleotide of the RARE O1 RAR/RXR binding site (TCGAGCGAGTGAACTTTCGGTGAACCCTACCCG) as primer and RARE O1-TK Luciferase as template, to generate the 201 bp megaprimer O1u/TK_{spm3} (Lane 2). The fragment was isolated from an agarose gel, purified using QiaEx resin (Quiagen) following the instructions of the manufacturer, and resuspended in 50 µl of distilled water (Lane 3). A series of second PCR reactions were set up, which included 0.5, 1, 2, and 5 µl of the isolated megaprimer (between 5 and 50 ng, as estimated from gel), together with the parental construct TK_{109} Luciferase (4), that lacks the RARE O1 RAR/RXR binding site, and primers RARE 01 u and Luciferase (GGATA-GAATGGCGCCGGGCC) (Lanes 4–7). In general, the appearance of the 331 bp final product is favoured at low

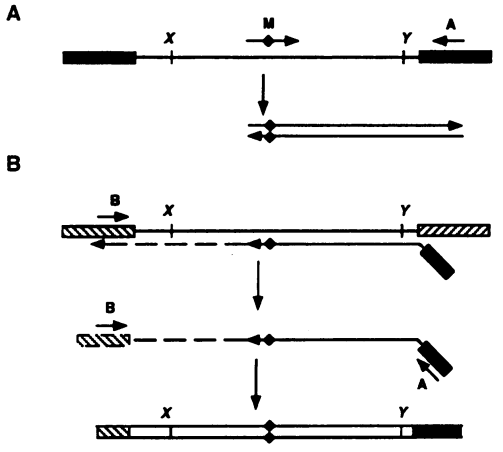

Figure 1. Scheme of the mutagenesis procedure. **A.** First PCR reaction. **B.** Second PCR reaction.

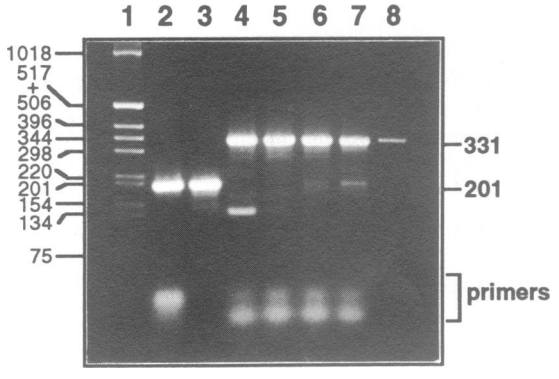

Figure 2. Ethidium bromide stained 2% agarose gel. Lane 1: Molecular weight standards (1 kb ladder). Lane 2: product of PCR I (1/10 of the reaction). Lane 3: Isolated 201 bp O1u/TK_{spm3} fragment (5 μl). Lanes 4 to 7: Product of the different second PCR reactions, including 0.5, 1, 2, and 5 μl of the purified megaprimer shown in Lane 3 (1/10 of the reaction). Lane 8: Isolated 331 bp final product. The sizes of the standards (left) and those of the expected PCR products (right) are shown on the sides of the picture.

Table 1.

Experiment number	clones tested	clones positive
1	17	16
2	10	10
3	6	6

megaprimer concentrations. The 331 bp fragment was isolated from an agarose gel and purified as described above (Lane 8). After digestion with BglII and SalI, the resulting fragment was cloned into BglII/SalI digested RARE O1-TK_{109} Luciferase DNA.

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