Site-Directed Mutagenesis: A Two-Step Method Using PCR and DpnI

BioTechniques 23:588-590 (October 1997)

In recent years, a two-step mutagenesis protocol has been developed that requires the polymerase chain reaction (PCR) (2,3). In the first step, a specific mutation is introduced by using a mutagenic primer and flanking primer (Figure 1). The PCR product is then used as a primer along with an opposite end-flanking primer for a second PCR step. The final PCR product is cut at unique restriction sites and subcloned. The advantages of this two-step method are: (i) it permits mutagenesis at virtually any site; (ii) it is inexpensive because only one specific primer is required for a given mutagenesis reaction; and (iii) it allows the mutated PCR product to be easily introduced into different vectors without further subcloning steps.

We found that a significant problem with this two-step method was the generation of a high frequency of unmutagenized recombinant clones as a result of contamination with the parental plasmid used as a template for PCR. Gel purification was insufficient to correct this problem because we found that contaminating nonmutagenized supercoiled plasmid can migrate similar to PCR products. Reducing template concentration was also an inadequate solution because low-input template concentrations typically led to low or no detectable PCR product.

We provide an easy modification that simplifies the procedure and vastly improves the frequency of mutagenized products available for subcloning. The DpnI restriction enzyme is used to cleave contaminating parental plasmid before gel purification and final subcloning. DpnI recognizes a methylated A residue in its target sequence GA\(\rightarrow\)TC, and thus it will cleave the bacterially generated parental plasmid but not the PCR products. Although DpnI is used in some commercially available mutagenesis kits (e.g., from Stratagene, La Jolla, CA, USA), it should be stressed that other than the use of DpnI, the method described in this report differs fundamentally from these methods. In addition, our method is considerably less expensive than commercial kits and involves the use of only a single nonmodified specific oligonucleotide. Our method works efficiently in most laboratory bacterial strains. In situations in which Dam+ bacteria are required, a restriction enzyme that cuts at a unique site in the parental plasmid must be substituted for DpnI.

To test our modified method, we mutagenized a mouse T-cell receptor \(\beta\), 1.8-kb Sall/BamHI genomic fragment [containing the \(V_{\beta 8.1}\) leader exon, a rearranged \(V_{\beta 8.1}D_{\beta 2.1}J_{\beta 2.3}\) exon and a third exon composed of the \(C_{\beta 2.1}\) and \(C_{\beta 2.4}\) exons fused together (1)] inserted in the pBluescript® KS(+) vector (Stratagene). As shown in Figure 1, the
first PCR step was performed with the mutagenic primer (5′-ACTATCGATCCAAACTTACCTGGAACTCAACGTCT-3′, which is complementary to the V\_β\_8.1 sequence; the mutant nucleotides are underlined) and the T3 primer. The PCR mixture consisted of 100 ng DNA template, 150 \( \mu \)M of each dNTP, 200 ng of each primer, Pfu DNA Polymerase (Stratagene) and the buffer supplied with the enzyme in a total volume of 50 \( \mu \)L. It was performed for 25 cycles at 94°C, 55°C and 72°C for each segment of 45 s (the DNA was denatured at 94°C for 2 min before the first cycle). This PCR product was then gel-purified and used as a megaprimer (528 nucleotides in length) along with the T7 primer for the second PCR step. The constituents and reaction conditions of the second PCR were the same as for the first except for the substitution of the megaprimer (500 ng) and T7 primer (200 ng), and amplification was performed for 25 cycles with the following conditions: 45 s at 94°C, 15 min at 55°C and 2 min at 72°C. The product of this reaction was first cut with \( DpnI \) (10 U for 1 h at 37°C), then with \( SalI \) and \( BamHI \), gel-purified and inserted into the \( SalI \) and \( BamHI \) sites of the pH\( \beta \)-apr-1-neo expression vector. Sequence analysis revealed that DNA from 12 of 12 independent transformed colonies contained the mutation in the mutagenic primer. In contrast, when the two-step protocol was performed in an identical manner except for omission of the \( DpnI \) cleavage step, none of 12 independent colonies tested contained DNA that possessed the mutation.

Three primers corresponding to other regions of the V\_β\_8.1 exon were also used for the two-step protocol. All three primers yielded 6 of 6 independent colonies that displayed the appropriate mutation. With some primers, we found that lower amounts of input parental DNA (<10 ng) for PCR permitted the generation of a reasonable frequency of mutagenized clones (50%–80%) when the \( DpnI \) step was omitted. However, this required careful titration of the DNA and was only successful if the PCR product could be separated from nonmutagenized, supercoiled plasmid DNA by gel purification. We recommend inclusion of the \( DpnI \) cleavage step because it provides a reliable way of generating a large number and high frequency of mutagenized DNA clones.

**REFERENCES**


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Received 13 December 1996; accepted 27 March 1997.

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