Benchmarks

PCR Mutagenesis: Treatment of the Megaprimer with Mung Bean Nuclease Improves Yield

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The megaprimer method of site-directed mutagenesis is an important technique for creating mutations at specific sites using the polymerase chain reaction (PCR). The original method was developed by Sarkar and Sommer (9) and Landt et al. (6) and was later modified by Barik and Galinski (2). The method requires two successive PCRs using three oligonucleotide primers, one of which contains the desired mutation. The other two are the terminal primers. The first reaction uses one terminal primer and the internal primer containing the mutation to synthesize a megaprimer. This megaprimer is used in the next reaction with the other terminal primer to synthesize the full mutated product. The use of the megaprimer in the second step represents a challenge because of the nature of the DNA products generated by commonly used heat-stable DNA polymerases. Such enzymes add almost exclusively a single deoxyadenosine to the 3′ terminus of most of the amplification products in a template-independent manner (3). This type of product can not act as a primer in the next PCR because the additional deoxyadenosine does not hybridize with the template DNA. Hence, in spite of using a higher concentration of the megaprimer, the yield of the product remains very low in the second reaction. Several procedures have been suggested and used in the past to circumvent the problem of nontemplated addition of adenosine (1,5,7).

We present a simple technique with which we have produced a significant improvement in the PCR amplification of the full-length product by incubating the megaprimer with mung bean nuclease for a brief period. This mung bean nuclease-treated megaprimer was then used in the next PCR. We have illustrated the method using as template the *P* gene of Chandipura virus (PC), cloned in pET3a vector. We used *Chp*I, a 40-mer primer that maps to the 5′ terminus of the *P* gene and *Chp*III, an internal 27-mer primer containing the desired base changes around the 190th base of the gene in the first reaction to synthesize a 200-bp megaprimer. pET3a-PC (100 ng) was used as the template in a standard 100-µL reaction mixture containing the buffer recommended by the manufacturer (Perkin-Elmer, Norwalk, CT, USA), 2.5 mM of dNTPs, 300 nmol of each primer and 2.5 U of AmpliTaq® DNA Polymerase (Perkin-Elmer). PCR was carried out for 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and elon-

gation for 1 min at 72°C. The 200-bp megaprimer was purified from a 1.5% agarose gel (Life Technologies, Gaithersburg, MD, USA) by extraction with QIAEX® II (Qiagen, Chatsworth, CA, USA) (Figure 1, lane 2). In the next step, one set of reactions was carried out with 4 µg of purified megaprimer, 2 µg of pET3a-PC template and 300 nmol of *Chp*II, 3′-terminal 40-mer primer, keeping other conditions the same. The total reaction product (100 µL) was then analyzed on a 1.5% agarose gel (Figure 1, lane 3). In other reaction set, 4 µg of purified megaprimer were incubated with 0.1 U of mung bean nuclease (Life Technologies) at 30°C for 10 min. The DNA was extracted with phenol-chloroform, precipitated with ethanol and used in the PCR with 2 µg of the same template and 300 nmol of *Chp*II. Reaction product (20 µL) was analyzed in the same gel as above (Figure 1, lane 4). Improvement in the yield of full-length mutated product after the mung bean nuclease treatment of the megaprimer is shown in lane 4 of Figure 1, where only one fifth of the reaction product was loaded instead of 100% as in lane 3. The reason for the lower yield in the untreated reaction can be understood by the presence of a large amount of unused megaprimer and smaller products.

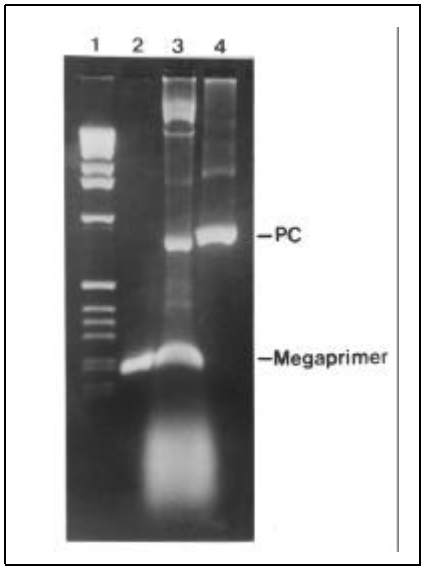

Figure 1. PCR mutagenesis with mung bean nuclease-treated megaprimer. Ethidium bromide-stained 1.5% agarose gel showing 1-kb DNA ladder (Life Technologies) as the standard molecular size marker (lane 1). QIAEX II-purified megaprimer is shown in lane 2. Lane 3 shows the analysis of total reaction product (100 µL) in the second-step PCR with untreated megaprimer. Full-length product and unused megaprimer are shown. One fifth of the reaction product (20 µL) of second-step PCR with mung bean nuclease-treated megaprimer was analyzed (lane 4).

Mung bean nuclease removes single-stranded overhangs in the double-stranded DNA leaving the duplex region intact (8). The possible explanation for the higher yield with the mung bean nuclease-treated megaprimer is that the enzyme removed the additional deoxyadenosine incorporated by AmpliTaq DNA Polymerase at the 3′ terminus of the megaprimer, enabling the total megaprimer to be used in the second step. The advantage of using mung bean nuclease instead of S1 nuclease is that mung bean nuclease does not cleave opposite nicks in double-stranded DNA (4) that may have been generated during purification of the megapimer from the gel. It is possible that mung bean nuclease may remove one or more nucleotides from the 3′ terminus of the double-stranded product because of local melting of some molecules. However, considering the brief incubation period and the fact that the mutation is located well inside the megaprimer, the probability of the altered bases being removed is very low. Standardization of the incubation period and the amount of mung bean nuclease would further reduce this possibility.

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