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Notes & Tips

Simplified one-tube "megaprimer" polymerase chain reaction mutagenesis

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Site-directed mutagenesis has become an essential tool in the investigation of gene structure and function as well as the products of gene expression, namely RNA and protein. Selected positions in a DNA sequence are changed, and the effects of the mutations are studied in vitro and/or in vivo. Although many different mutagenic strategies are available [1], the simplicity, cost effectiveness, and accuracy of "megaprimer" mutagenesis have made it a widely used and popular approach [2–14]. A number of modifications have been introduced to the original protocol, but the core idea remains the same, based on two rounds of PCR using two flanking primers and one internal primer that carries the desired mutation.

Most strategies require intermediate gel purification to remove leftover primers from the first round of PCR amplification. Gel purification introduces its own problems given that it is time-consuming, costly, and often a source of product loss. In response to this limitation, a number of efforts to skip the gel purification step and to develop one-tube procedures have been made [7,9,10,14]. Unfortunately some shortfalls remain. One of the modified procedures [9] is based on the use of a restriction enzyme to remove competing template that would result in unmutagenized products and decreased efficiency. The requirement for specific restriction sites, however, can be complicated and can significantly limit the flexibility of this approach. In an alternate strategy [10], the melting temperature of the flanking primers plays a critical role in which a low-melting temperature primer, used in the first PCR amplification step, is temperature inactivated during the second step. Although relatively simple, this procedure is limited by the need for careful primer design and an 80% average mutant frequency. The last two approaches are based on the use of alternate primer combinations [7] or carefully reduced primer concentrations [14]. These methods, however, have even lower mutagenesis frequencies (~50%) or require gel purification after PCR amplification.

In gene studies, PCR amplification is commonly used to prepare DNA template for sequence analyses. In such studies, the DNA products often are purified by gel electrophoresis or spin column to remove leftover primers and nucleotides that interfere with the sequencing reactions. A simple and effective alternative to such purification methods is the use of an exonuclease/phosphatase treatment that permits DNA sequencing without gel or column purification [15]. Here we describe a similar strategy that permits the application of PCR-based megaprimer mutagenesis as a "single-tube" method without a need for intermediate gel purification or even a precipitation step. After the standard first round of PCR amplification, the reaction mixture containing the megaprimer product is treated directly with exonuclease I, which is then heat inactivated. Additional nucleotides and the forward primer are next added directly to the mixture, and the second round of PCR amplification follows to produce the final mutant DNA fragment. As illustrated in Fig. 1, with this treatment of a megaprimer reaction for a U3 snRNA gene sequence the final product was essentially a single clear band (lane 3), whereas in the absence of the exonuclease treatment the final reaction mixture contained countless numbers of unwanted by-products (lane 2).

To further illustrate the reliability of this strategy, six different mutations were introduced into the upstream

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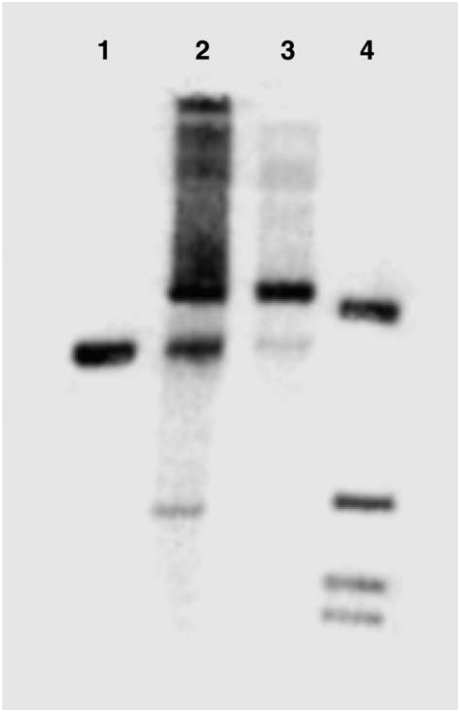

Fig. 1. Effect of exonuclease I treatment on a megaprimer-based PCR amplification. The megaprimer product of a first-step PCR amplification (lane 1) was used directly (lane 2) or treated with exonuclease I (lane 3) and used in a subsequent PCR step. Products of these reactions were fractionated on a 1.5% agarose gel together with size markers (lane 4) and were visualized using ethidium bromide stain.

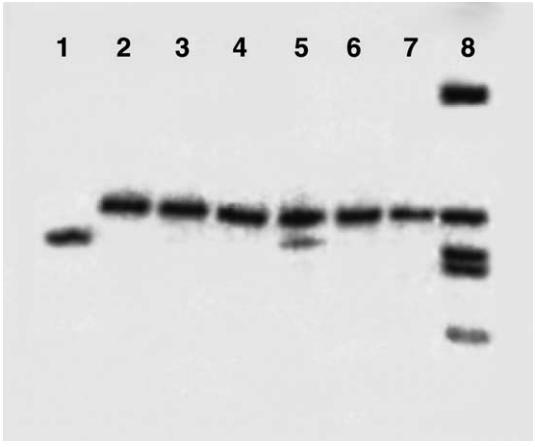

Fig. 2. PCR-amplified mutant *S. pombe* U3 snRNA sequences using six different exonuclease I-treated megaprimers (lanes 2–7). Products of the second amplification step were fractionated on a 1.5% agarose gel together with an example megaprimer (lane 1) and size markers (lane 8).

region of the U3 snoRNA gene of *Schizosaccharomyces pombe* cells. The six reactions were performed simultaneously starting with 50-μl reaction mixtures containing 0.2 mM dNTPs, 3 ng plasmid template, 30 pmol of each primer, and 1 unit of Taq1 polymerase. After an initial denaturation step at 95 °C for 5 min, PCR was performed for 30 cycles with 1 min at 94 °C, 1 min at 40 °C,

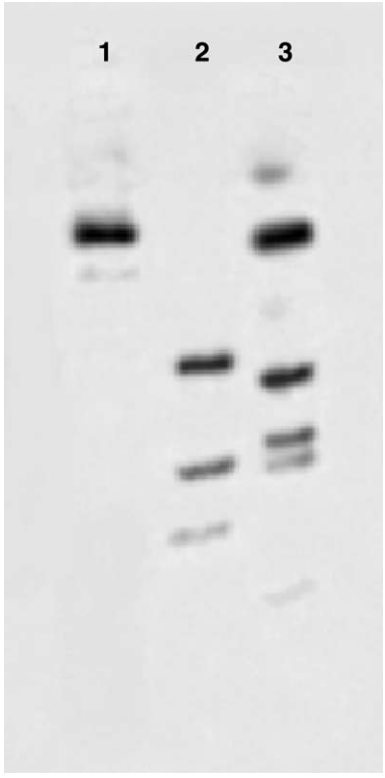

Fig. 3. *Hin*fI restriction digest of a mutant U3 snRNA sequence. A second *Hin*fI cleavage site was introduced into an *S. pombe* U3 snRNA sequence, and the final product was digested with *Hin*fI endonuclease (lane 2) prior to fractionation on a 1.5% agarose gel. An undigested control and size markers also were applied in lanes 1 and 3, respectively.

and 30 s at 72 °C. The product of each PCR reaction was a single band of 380 bp with a yield of approximately 1 μg. The product of the first PCR reaction was sequentially digested with two 5-unit aliquots of exonuclase I (Fermentas, Hanover, MD, USA), each for 2 h at 37 °C, followed by enzyme inactivation for 10 min at 75 °C. For the second round of PCR amplification, the reaction mixture was diluted further with 50 μl of buffer containing 3 ng template, 0.2 mM dNTPs, 30 pmol flanking primer, and 2 units *Taq* polymerase. After denaturation for 5 min at 95 °C, amplification again was performed for 30 cycles with the annealing time extended to 15 min as described previously [6]. As shown in Fig. 2, in all but one case (lanes 2–7) there was essentially a full conversion of megaprimer to the final product. A small amount of megaprimer remained in one case (lane 5).

For further analyses, all six reaction mixtures were treated with buffer-saturated phenol solution and precipitated with ethanol containing 2% potassium acetate. After digestion with *Bam*HI restriction endonuclease, all were cloned into the pFL20 plasmid vector [16] and the individual mutant sequences were confirmed by DNA sequencing. Only mutant sequences were observed (results not shown). To further confirm this high mutation yield, a mutant PCR product containing an

additional *Hin*fI site also was examined after enzyme digestion (Fig. 3). Again, only mutant fragments were present (lane 2), consistent with a yield that was essentially 100%.

In summary, the method described in this report provides for a simplified one-tube megaprimer approach to PCR-based mutagenesis with an efficiency that approximates 100%. The need for megaprimer purification is circumvented with a low-cost exonuclease treatment. The method provides mutant DNA in high yield without the primer loss that often is experienced with gel purification.

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