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# Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method

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## ABSTRACT

**We describe a rapid and efficient megaprimer PCR procedure for site-directed mutagenesis that does not require any intermediate purification of DNA between the two rounds of PCR. This protocol is based on the design of forward and reverse flanking primers with significantly different melting temperatures ($T_m$). A megaprimer is synthesized in the first PCR reaction using a mutagenic primer, the low $T_m$ flanking primer and a low annealing temperature. The second PCR reaction is performed in the same tube as the first PCR and utilizes the high $T_m$ flanking primer, the megaprimer product of the first PCR and a high annealing temperature, which prevents priming by the low $T_m$ primer from the first PCR reaction. We have used this protocol with two different plasmids to produce cDNAs encoding seven distinct mutated proteins. We have observed an average mutagenesis efficiency of 82% in these experiments.**

Oligonucleotide-directed site-specific mutagenesis is used routinely to introduce desired mutations into target DNA sequences. A variety of protocols have been established to achieve efficient mutagenesis, including several that use the polymerase chain reaction (PCR) (1). Among the PCR-based protocols, the 'megaprimer' method introduced by Kammann *et al.* (2), and later modified by Sarker and Sommer (3,4) and Landt *et al.* (5), appears to be particularly simple and cost-effective. This method involves two rounds of PCR that utilize two 'flanking' primers and one internal mutagenic primer containing the desired base substitution(s). The first PCR is performed using the mutagenic internal primer and the first flanking primer. The product of this first PCR, the 'megaprimer', is purified and used, along with the second flanking primer, as a primer for a second PCR. The final PCR product contains the desired mutation in a particular DNA sequence.

Most mutagenesis strategies based on this two-step PCR scheme require an intermediate purification step of the first PCR reaction products to prevent left-over primers from the first PCR from interfering with the second PCR step. This purification step, usually accomplished by agarose gel electrophoresis and subsequent elution of the DNA of interest, is time-consuming and labor intensive. One approach to megaprimer PCR mutagenesis which eliminated the agarose gel electrophoresis step has been reported recently (6); however, this protocol employs several additional manipulations and enzyme treatments of PCR products. In this study, we describe a convenient two-step PCR protocol which provides rapid and highly efficient site-directed mutagenesis in a single tube without any intermediate purification steps or additional manipulation or treatment of PCR products.

The new mutagenesis strategy utilizes primers with significantly different melting temperatures ($T_m$) to initiate the two PCR steps. This experimental design allows the use of an elevated annealing temperature in the second reaction to ensure selective synthesis utilizing only two of the primers present in the reaction mixture. As summarized in Figure 1A, the two flanking primers are different in length. The short, 'reverse' flanking primer is usually 15–16 bases long and typically has a calculated $T_m$ of 42–46°C. The long, 'forward' flanking primer is designed to be 25–30 base long and has a calculated $T_m$ between 72 and 85°C.

The first PCR reaction is carried out using the short flanking primer, the internal mutagenic primer and a low annealing temperature. Without a purification step, the second, high $T_m$ flanking primer is added directly to the reaction tube. The second step PCR is performed using a high annealing temperature, usually 72°C. The high annealing temperature assures that the final PCR product will be generated through selective priming by the megaprimer product of the first PCR and the high $T_m$ flanking primer. The short flanking primer that remains from the first PCR does not interfere with the second PCR synthesis because of its poor efficiency of annealing to the template at 72°C. For the same reason, the two flanking primers are also unable to generate copies of the wild type DNA template during the second PCR (data not shown). This simple modification of primer design to incorporate primers with substantially different $T_m$s eliminates the cumbersome purification step between the first and the second PCR and allows rapid, efficient site-directed mutagenesis with a megaprimer approach.

To test the new mutagenesis strategy, two different DNA templates were used in seven mutagenesis experiments (Table 1). A mutant of tissue plasminogen activator (t-PA), t-PA/R70E, was generated using the expression vector pSVT7/t-PA (7) as a template. The forward flanking primer sequence used in this experiment, 5′-AGCCTCACCGAGTCGGGTGCCTCC-3′, had an estimated $T_m$ of 82°C. The reverse flanking primer sequence, 5′-ATTGTCGTAAGTGTC-3′, had a calculated $T_m$ of 42°C. The mutagenic primer used to create t-PA/R70E was 5′-TGATCTTGGGCGAGACATACCGGGTGG-3′ (nucleotides which represent mutations are underlined). Six mutants of plasminogen activator inhibitor type I (PAI-1) were also produced using as a template the

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expression vector pPAI.HIS, which contains a full length cDNA encoding human PAI-1 (8).

**Table 1.** Results of mutagenesis experiments using the single tube, megaprimer protocol

| Mutant name | Template plasmid | Original sequences | Mutant sequences | Mutagenesis efficiency[a] (%) |
|---|---|---|---|---|
| tPA/R70E | pSVT7/tPA | CGC | GAG | 13/16 = 81.3 |
| pAI/P3R | pPAI,His | TCA | CGC | 21/24 = 87.5 |
| PAI/P4Q | pPAI,His | GTC | CAG | 5/6 = 83.3 |
| PAI/P4Q3R | pPAI,His | GTCTCA | CAGCGC | 12/16 = 75.0 |
| PAI/69P2I | pPAI,His | GG | AT | 4/6 = 66.7 |
| PAI/69P2F | pPAI,HIS | GG | TT | 5/6 = 83.3 |
| PAI/69P2D | pPAI,His | GC | AT | 5/6 = 83.3 |

[a]Mutagenesis efficiency was calculated as the number of confirmed mutants/total number of clones analyzed.

Like the short flanking primer, the internal mutagenic primer can be designed to have a low estimated $T_m$. Longer mutagenic oligonucleotides can also be utilized successfully in this procedure. In cases where the estimated $T_m$ of the mutagenic primer is significantly higher than that of the short flanking primer, however, two modifications of the mutagenesis protocol significantly improve the yield and purity of the final PCR product. First, we use a 10-fold lower molar concentration of the internal, mutagenic primer than the short flanking primer. This modification may lead to a significant depletion of the high $T_m$ mutagenic primer during the first PCR and therefore decrease any potential interference by this primer during the second PCR. Second, it is sometimes beneficial to perform five rounds of linear amplification with the high $T_m$ mutagenic oligonucleotide, using a high annealing temperature, prior to the first PCR. By using these modifications, we consistently generated specific first PCR products using mutagenic primers with relatively high $T_m$s without observing heterogeneous bands in the agarose gel analysis (Fig. 1B). Similarly, to improve the efficiency of the megaprimer amplification step, we routinely adopted a strategy described by Datta (9), in which five cycles of linear amplification were performed with the megaprimer before addition of the high $T_m$ flanking primer and subsequent exponential amplification. Since there is no purification step between the first and the second rounds of PCR in our protocol, these five cycles of linear amplification can be included in the program for the first PCR. Adopting this experimental design also reduces manipulation of the reaction vessels by allowing the use of a single addition of high $T_m$ primer, DNA polymerase and dNTPs between the two PCR steps. The advantages of this new protocol will prove particularly important when many samples are being processed simultaneously and, in addition, should significantly diminish the effort required to accomplish automated PCR mutagenesis.

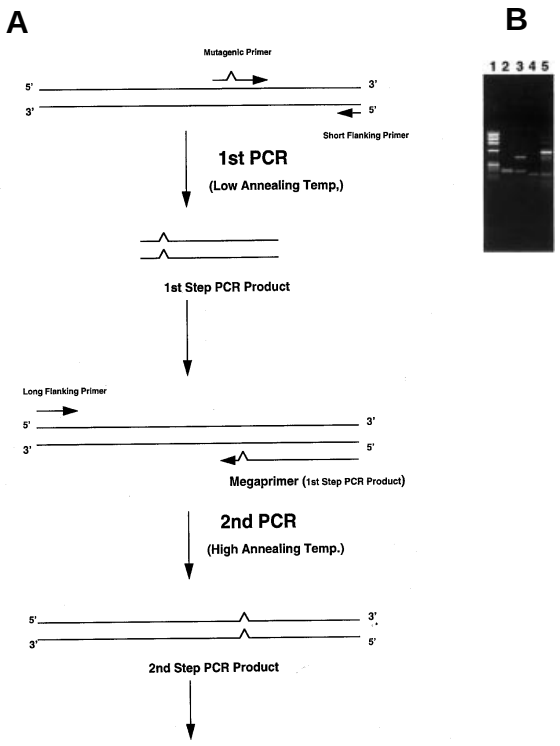


**Figure 1.** (**A**) Schematic outline of the mutagenesis protocol used in this study. PCR amplifications were performed in a Perkin-Elmer 9600 thermocycler. The 100 µl reaction mixtures contained 0.5 µl Hot Tub DNA polymerase (Amersham), 200 pg plasmid DNA template, 200 µM of dNTPs in the 1× DNA polymerase buffer supplied by the manufacturer. Ten pmol of mutagenic primer and 100 pmol of common, reverse flanking primer were employed for 25 cycles of amplification using the following reaction conditions: 94°C for 40 s (except for a 4 min first cycle), 42°C for 1 min and 72°C for 40 s with a final extension step for 5 min at 72°C. Production of a megaprimer of appropriate size and purity was verified by agarose gel electrophoresis (Fig. 1B). After completion of the first PCR, 0.5 µl DNA polymerase and 3 µl containing 7.5 nmol of each dNTP were added to the reaction tube, which was mixed gently and spun briefly. The second PCR was then initiated asymmetrically by subjecting the reaction mixture to five cycles of 94°C for 40 s and 72°C for 90 s. Following the five asymmetric cycles utilizing only the megaprimer, 100 pmol of forward flanking primer was added, and the reaction was continued for 25 cycles with the same two-step temperature profile. Efficient production of the desired final PCR product was verified by agarose gel electrophoresis (Fig. 1B). This DNA product was then digested with appropriate restriction enzymes, purified by agarose gel electrophoresis, and ligated into a suitable expression vector. The resulting ligation mixture was used to transform competent *Escherichia coli* (TG-1). Several colonies from each mutagenesis reaction were selected and screened for the presence of the desired mutation by Southern hybridization and DNA sequencing. In selected plasmids containing the appropriate mutation, DNA corresponding to the entire final PCR product was sequenced to confirm the absence of any additional mutations. During these studies, a total of 23 mutated cDNA fragments, either 299 or 472 bp in length, were completely sequenced, and no 'second site' mutations were observed. (**B**) Electrophoretic analysis of megaprimer PCR mutagenesis products on a 1% agarose gel. Lane 1, *Hae*III digest of ϕX174 DNA; lane 2, DNA products of first PCR during PAI/P4Q mutagenesis (175 bp); lane 3, DNA products of second PCR during PAI/P4Q mutagenesis (419 bp); lane 4, DNA products of first PCR during t-PA/R70E mutagenesis (107 bp); lane 5, DNA products of second PCR product during t-PA/R70E mutagenesis (534 bp).

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