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A rapid and efficient one-tube PCR-based mutagenesis technique using *Pfu* DNA polymerase

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ABSTRACT

A rapid method for efficiently generating site-directed mutations on a clean sequence background is described. This modification of the megaprimer PCR mutagenesis approach can be performed in one tube in less than 4.5 hours, and does not require purification of intermediate products. High fidelity of DNA sequence replication is obtained by employing *Pfu* DNA polymerase and limiting the total number of amplification cycles to 30. The mutagenesis efficiency of the procedure is high enough to allow rapid, direct identification of mutants by restriction digest or sequencing techniques.

INTRODUCTION

Oligonucleotide-directed mutagenesis techniques are extensively used to study gene regulation and DNA and protein structure/function relationships. A number of PCR-based mutagenesis methods have been developed recently (1–6). In general, PCR-based approaches are simpler and faster than classical mutagenesis methods employing *in vivo* DNA synthesis, and a variety of them are now widely practiced. This communication reports an efficient, cost effective, high-fidelity, one-tube modification of the 'megaprimer' PCR mutagenesis procedure (3,4). In order to increase the utility of the method, we have extensively investigated a number of parameters that affect mutagenesis efficiency, DNA replication fidelity and DNA yield. This has resulted in the development of a protocol which is simple to perform, fast and efficient, and for which the rate of site-directed mutation has been optimized and the rate of second site mutation minimized. The procedure has been successfully used in our lab to produce numerous antithrombin III variants for structure/function studies.

MATERIALS AND METHODS

Materials

Polymerase chain reactions were performed on a Perkin-Elmer Cetus Thermal Cycler. *Pfu* DNA polymerase was purchased from Stratagene and dNTPs from Pharmacia. Oligonucleotides were synthesized on an Applied Biosystem 380B DNA synthesizer, or purchased from IDT (Coralville, Iowa).

The procedure requires three oligonucleotide primers: two flanking primers, which are upstream (U) and downstream (D) of the mutation site, and one mutagenic primer (M). The same set of flanking primers can be employed for many different mutagenesis projects, and it is often convenient to use 'universal' primers corresponding to plasmid sequences flanking a vector's cloning site. Principles for designing mutagenic primers include standard considerations, as well as the following specific points. First, due to the 3'→5' (proofreading) exonuclease activity of *Pfu* polymerase (7), at least ten perfectly matched bases are recommended on the 5' side of the mutagenic primer. We also recommend ten perfectly matched bases at the 3' end. Secondly, mixed primer pools can be employed to obtain numerous related mutants from single oligonucleotide synthesis and mutagenesis reactions in a cost effective manner. Third, we have been able to include translationally silent restriction site changes in greater than 90% of our mutagenic primers, and recommend this strategy whenever possible to facilitate initial analysis of the PCR product and rapid screening of subclones.

Circular double stranded plasmid DNA templates were prepared by Qiagen (Chatsworth, CA) purification and their concentrations determined by A260 measurement. 4.2–16.5 kb plasmids consisting of the 1.5 kb human antithrombin cDNA subcloned into 2.7–15 kb vectors were used as the templates for work described in this report.

Mutagenesis reaction

Mutagenesis is achieved by the three-step PCR procedure described below and illustrated in Fig. 1.

Step 1. 95 μl mutagenesis reactions contain 3.6 fmol template DNA (10 ng of 4.2 kb plasmid), 10 pmol (100 nM) each of the mutagenic (M) and downstream (D) primers, 2.5 U *Pfu* DNA polymerase, dNTPs at 0.2 mM, 20 mM Tris–Cl, pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100 and 0.1 g/l BSA. The enzyme is added last, after thoroughly mixing the other components, and the reaction is overlaid with mineral oil. Ten cycles of amplification (94°C, 1

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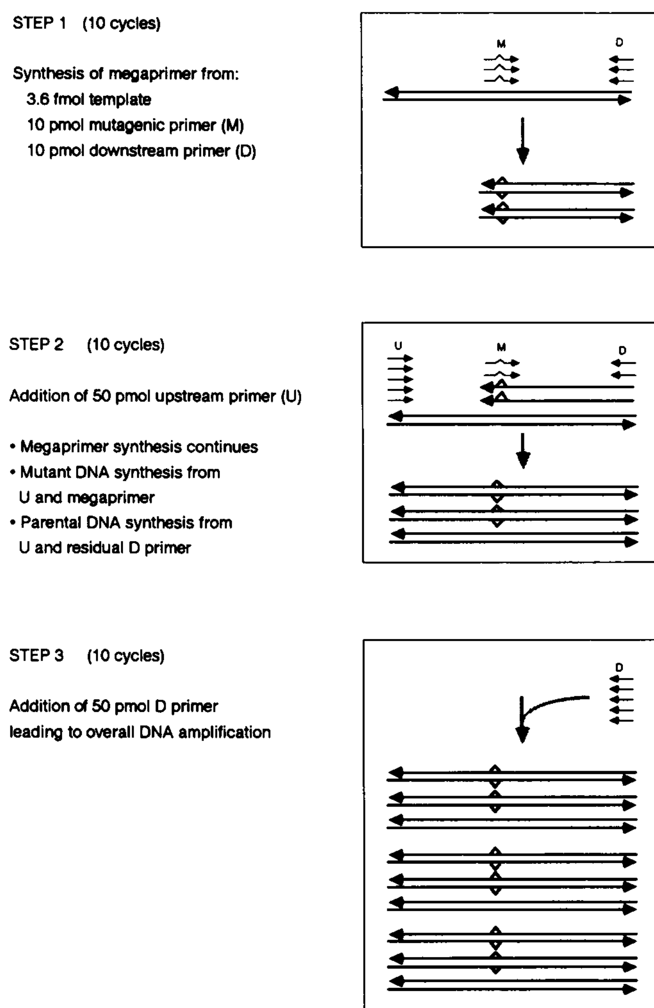


Figure 1. Principle of the mutagenesis method.

min; 48°°C, 1 min; 72°C, 2 min) are performed, followed by 5 min at 72°C, and holding at 4°C.

Step 2. Step 2 is initiated by adding 50 pmol upstream (U) primer (2.5 μl of a 20 μM solution) to the aqueous phase and submitting the tube to the amplification program described in Step 1.

Step 3. Step 3 is initiated by adding 50 pmol downstream (D) primer (2.5 μl of a 20 μM solution) and again submitting the tube to the amplification program described in Step 1.

Set up time for 1–10 mutagenesis reactions is less than 30 minutes, and the 10 cycle amplification program for each Step of the method requires 80 minutes on our Perkin-Elmer Thermal Cycler. Thus, the entire mutagenesis procedure can be completed in less than 4.5 hours if primer additions are made promptly at the conclusions of Steps 1 and 2.

Analysis of mutagenesis reaction products and subcloning

Following PCR, a 5 μl aliquot of the 100 μl reaction is analyzed by 5% polyacrylamide gel electrophoresis. If the mutagenic primer encoded the introduction or destruction of a restriction site sequence, the sample can also be checked for the presence of mutant molecules by digesting an aliquot with the enzyme. After the electrophoretic verification of full length DNA (and mutant molecules) in the reaction mixture, 50 μl of the remaining sample is used to prepare insert for subcloning. This may be by restriction enzyme digestion to generate cohesive ends, or by blunt end ligation. Restriction sites for subcloning can be conveniently generated by using 'universal primers' as flanking primers for mutagenesis of sequences inserted into common vectors, or by designing them into the flanking primers.

The favorable mutagenesis efficiency and low error rate of the method make it unnecessary to analyze a large number of subclones. We routinely analyze six subclones for each mutagenesis reaction. In most cases mutagenic oligonucleotides have been designed to include restriction site changes, and restriction digests immediately reveal one or more mutants among the six miniprepped subclones. One mutant subclone is sequence verified over the extended region that will be subsequently subcloned for expression (or other studies). In the rare event that misincorporation has occurred and there is an unplanned sequence change in addition to that introduced by the mutagenic oligonucleotide, one of the other mutant subclones can then be sequenced. If the mutagenic oligonucleotide does not carry a restriction site marker, mutants can be identified by limited dideoxysequencing (i.e., use only one ddNTP, as in 'G-tracking') of the six subclones and a control sample of the template DNA (8). Full sequence verification of one of the mutants then proceeds as above.

RESULTS

Mutagenesis reaction parameters

Mutagenesis is performed as a one-tube PCR with three consecutive steps of 10 amplification cycles each. Fig. 2B shows the DNA products of the PCR reactions after Steps 1, 2, and 3. Trace amounts of megaprimer (mp) are visible after Step 1. During Step 2, this megaprimer is further amplified and 'full length' (fl) molecules begin to accumulate. The full length molecules are amplified during Step 3, at the expense of the megaprimer. The full length molecules produced during Steps 2 and 3 include mutant DNAs, as indicated by the presence of 551 bp bands (B′) in aliquots digested with *EagI* (the mutagenic oligonucleotide contained an *EagI* site, see Fig. 2A).

Fig. 2C shows the effect of DNA template input on the mutagenesis procedure. Full length mutant DNAs were obtained overa 25-fold range of template DNA concentrations (2–50 ng of 4.2 kb plasmid DNA in a standard 100 μl reaction), and the yield increased with increasing template input. However, we recommend using 3.6 fmol template per reaction (10 ng of 4.2 kb plasmid) in order to get a satisfactory yield of full length DNA molecules with minimal PCR 'background' products.

Fig. 2D shows the effect of megaprimer length on the mutagenesis procedure. Good yields of full length DNA product were obtained using 303 bp (center, '–' lane) and 133 bp (right, '–' lane) megaprimers, but not with the 568 bp megaprimer (left, '–' lane). We have used megaprimer sizes ranging from 114 to 413 bp to synthesize mutated DNAs up to 910 bp with satisfying results, and recommend megaprimer lengths of less than about 400 bp for best results with this procedure.

Mutagenesis efficiency

The described procedure produced mutation rates of 25–100% for the nine consecutively performed mutagenesis reactions shown

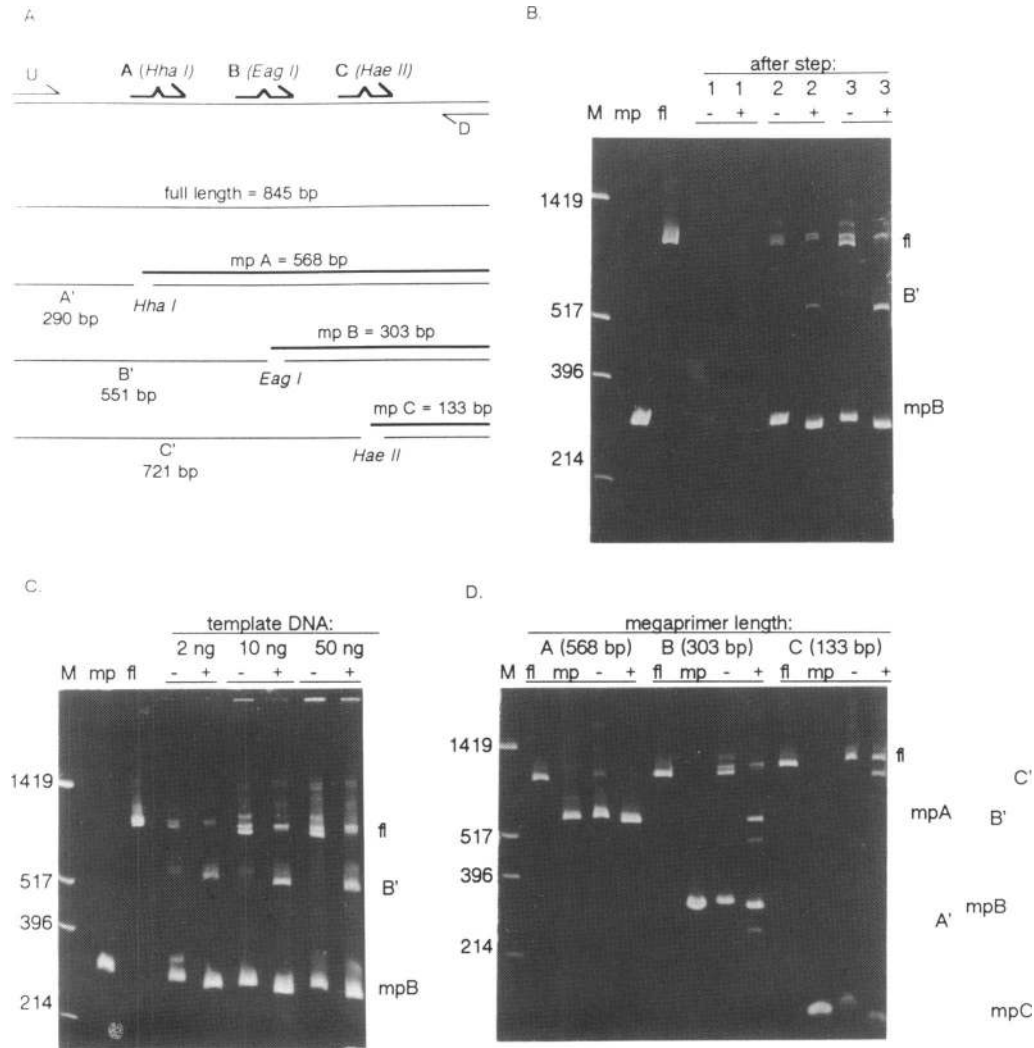


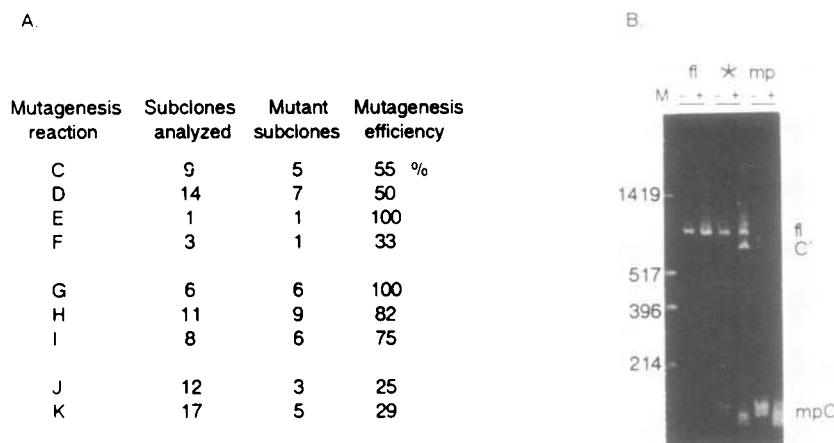
Figure 2. Characterization of the mutagenesis method. **A.** Schematic diagram showing primers used in the 2B, 2C and 2D reactions, and their relationships to megaprimer and full length PCR products. U and D are upstream and downstream flanking primers. A, B, and C are mutagenic primers which each specify an amino acid substitution (not shown) and introduce a restriction site as indicated. In the bottom part of the diagram, the thick lines depict the megaprimer generated in a mutagenesis reaction with the indicated mutagenic primer. The thin lines show the products of a restriction digest of the full length product with the indicated enzyme. **B.** Progress of a mutagenesis reaction with primer B and flanking primers U and D. After each 10-cycle step of the procedure (see Fig. 1), 10 μl of the mutagenesis reaction were diluted into restriction enzyme buffer and divided into aliquots that were not digested (−) or digested (+) with *EagI*. The samples were analyzed on a 5% polyacrylamide gel. Markers are: M, pUC18-*HinfI* digest; mp, megaprimer length DNA from PCR using B and D primers; fl, full length DNA from PCR using U and D primers. The fl, B′ and mpB labels on the right side of the gel indicate the positions of the full length, 5′ *EagI* fragment and megaprimer B bands, respectively. **C.** Effect of template concentration on the mutagenesis reaction. Mutagenesis reactions used primers B, U and D, and 2, 10 or 50 ng of 4.2 kb template DNA. 10 μl aliquots of the completed reactions were diluted into restriction enzyme buffer and divided into aliquots that were not digested (−) or digested (+) with *EagI*. Electrophoresis, markers and labelling are as in 2B. **D.** Effect of megaprimer length on the mutagenesis reaction. Mutagenesis reactions were performed with primers U, A and D (left); U, B and D (center); and U, C and D (right). 10 μl aliquots of the completed reactions were diluted into restriction enzyme buffer and divided into aliquots that were not digested (−) or digested (+) with *HhaI* (A), *EagI* (B) or *HaeII* (C). The megaprimer lanes (mp) are from PCRs of the indicated mutagenic primer and downstream flanking primer D. The full length DNA lane (fl) is from a PCR with U and D primers. Labelling on the right side of the gel shows mobilities of full length DNAs (fl), the megaprimers (mpA, mpB, mpC) and the 5′ fragments of the digests (A′, B′ and C′).

in Fig. 3A. Mutagenesis efficiencies were always ≥ 25%, and in six of the nine reactions, the mutation rate was ≥ 50%. Thus, in practice it is virutally always possible to obtain one or more mutants by miniprepping six subclones of a given mutagenesis reaction.

Initial estimates of mutation efficiency can be obtained by restriction analysis of an aliquot of the completed PCR reaction, if the mutagenic oligonucleotide included a restriction site change. Fig. 3B shows the analysis for the C reaction from Fig. 3A, which produced 55% mutants. The mutant derived band (C′) and the full length parental-derived band (fl) in the digested (+) aliquot of the mutagenesis reaction (*) are of approximately equal intensities, in agreement with the 55% mutation rate determined by restriction (and sequence) analysis of nine subclones.

Error rate

Since DNAs modified by site-directed mutagenesis are often used in functional studies, it is important to insure that sequence



A.

Mutagenesis reaction	Subclones analyzed	Mutant subclones	Mutagenesis efficiency
C	9	5	55 %
D	14	7	50
E	1	1	100
F	3	1	33
G	6	6	100
H	11	9	82
I	8	6	75
J	12	3	25
K	17	5	29

Figure 3. Determination of mutagenesis efficiency. **A.** Analysis of mutagenesis efficiency in nine consecutively performed reactions. Subcloned molecules from the PCR mutagenesis reactions were identified as mutant or parental by restriction digest. Tabulated data represents nine consecutive mutagenesis reactions with nine different mutagenic primers ranging in length from 25 to 35 nucleotides, and carrying 2 to 4 mismatches. These reactions used three different templates (circular plasmid DNAs ranging from 4.2 to 16.5 kbs), and three different pairs of flanking primers which generated full length molecules ranging from 572 to 910 bp in length. Megaprimer lengths ranged from 114 to 413 bp. **B.** 5% polyacrylamide gel electrophoresis of PCR mutagenesis reaction for mutagenesis C from 3A. Ten μl of the completed mutagenesis reaction was used on the gel shown here, and 50 μl was used to prepare insert fragments for subclones subsequently analyzed in 3A. The 10 μl aliquot (*) was diluted into restriction enzyme buffer and divided into equal portions that were either not digested (−) or digested (+) with *EagI*. Aliquots of the corresponding full length (fl) and megaprimer (mp) PCR reactions primed with U and D, and M and D, respectively, were also not digested (−) or digested (+) with *EagI*.

changes are limited to those introduced via the mutagenic oligonucleotide. We therefore developed the mutagenesis procedure with a minimal number of PCR cycles and chose to use *Pfu* polymerase for DNA amplification. The error rate for this PCR mutagenesis procedure is about 0.024% per base sequenced (1/4125). We sequenced 16,500 bp of DNA from 37 subcloned mutant molecules and observed four second site mutations.

DISCUSSION

Our intention in developing yet another PCR mutagenesis method was to obtain a fast and simple procedure in which the goals of minimizing background misincorporation rates and achieving reasonable mutagenesis efficiencies were balanced. Thus, we have worked out a high fidelity, one-tube protocol which utilizes *Pfu* DNA polymerase, involves a limited number of amplification cycles, and avoids steps requiring isolation of PCR intermediates.

Pfu DNA polymerase was selected for this method because it is reported to have 12-fold increased fidelity of DNA synthesis compared to *Taq* polymerase (1.6×10^{-6} *vs.* 2×10^{-5} per base incorporated), due to the presence of a 3′ to 5′ exonuclease activity (7). *Pfu* polymerase also lacks terminal transferase activity (9), which is present in *Taq* polymerase and further decreases *Taq* polymerase megaprimer mutagenesis fidelity and efficiency by adding an extra A to the 3′ ends of amplified double stranded DNAs (10). Thus, the choice of *Pfu* polymerase was critical for obtaining high overall fidelity and efficiency of the method reported here. An initial version of this procedure employed *Taq* polymerase and had to be abandoned because a high percentage of molecules contained 'extra' mutations, in addition to intended site-directed changes.

To minimize the labor involved in mutation projects, it is important to work with an efficient mutagenesis method. High mutagenesis efficiencies bypass the slower processes of oligonucleotide hybridization and mutant enrichment, and allow the use of rapid, direct restriction digest and sequencing approaches. Very high mutagenesis efficiencies can be achieved by starting with small amounts of template and amplifying for many cycles (3,5,6). However, repeated amplifications increase the level of polymerase-dependent misincorporation into 'background' sequences. In order to promote high overall fidelity of DNA sequence replication in this method, we limited the total number of amplification cycles to 30 (vs. 80 cycles for the method described in ref. 5, and 60 cycles for the methods presented in refs. 3 and 6). This favors the introduction of site-directed mutations on sequence backgrounds that are free of polymerase-associated misincorporation errors. Whereas others have used gel isolation of intermediate products (3,4) or templates derived from two different vectors (6) to increase mutagenesis efficiencies, we staggered the addition of flanking primers to increase the final ratio of mutant to parental sequences. To favor the incorporation of mutation-containing megaprimers into full length DNAs and the early production of mutant molecules, upstream (but not downstream) flanking primer is added at the beginning of Step two (see Fig. 1). Ten cycles later, at the beginning of Step 3, a final phase of overall amplification is initiated by the addition of downstream primer.

In conclusion, we have described an efficient PCR mutagenesis method which is economical in terms of oligonucleotide synthesis and labor expenditures. The rate of extra mutations has been minimized by employing *Pfu* DNA polymerase and limiting the number of amplification cycles to 30, and mutagenesis efficiency has been enhanced by staggering the addition of flanking primers to favor mutant DNA synthesis at an early stage.

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