Table 1. Microprep Procedure

1. Transformed *Escherichia coli* are spread on an LB agar dish with the appropriate antibiotic and, after incubating overnight, individual colonies are picked and used to inoculate 5 mL of LB plus antibiotic in numbered tubes, which are then incubated at 37°C overnight with shaking at 250 rpm.
2. A 500-µL aliquot of overnight culture is transferred to a 1.5-mL microcentrifuge tube, and the bacteria are pelleted by spinning 1 min in a microcentrifuge at 14 000× *g*.[a]
3. The LB is completely removed and discarded and the bacterial pellet resuspended in 19 µL H_2O plus 1 µL 10 mg/mL RNase A by vigorous vortex mixing.
4. The plasmid DNA is liberated from the bacteria by boiling the tubes 1 min.
5. Bacterial debris is pelleted by spinning the tubes for 1 min in a microcentrifuge.
6. Enough supernatant is then removed to a fresh 1.5-mL microcentrifuge tube containing premixed restriction enzyme buffer and restriction enzymes (≤1 U per reaction) to obtain a final total volume of 20 µL.
7. After a 1-h incubation, 5–10 µL of the restriction digests can be analyzed using agarose gel electrophoresis.
8. Once recombinant plasmids containing the largest cDNA inserts are identified from the agarose gels, the corresponding positive overnight LB cultures are then retrieved and used for purifying plasmid DNAs using a standard miniprep procedure or kit.

[a]This amount of bacterial suspension was determined to be optimal for efficient subsequent restriction enzyme digestion and visualization on an agarose gel.

LB cultures of individual colonies for use in restriction-enzyme analysis. This procedure is cost-effective because it saves the use of expensive miniprep kits for use with only positively identified recombinant clones and limits typically time-consuming miniprep steps. Our method can be accomplished in a few minutes in two microcentrifuge tubes with minimal enzyme, expense, no phenol/chloroform extractions and no ethanol precipitations. We routinely prepare overnight cultures on day one, perform the microprep isolation and restriction enzyme analysis the following morning and then perform a miniprep isolation (e.g., using S.N.A.P.™ Nucleic Acid Isolation Kit; Invitrogen, Carlsbad, CA, USA) of positive recombinants in the afternooon yielding DNA that is ready for sequencing, transfection, in vitro transcription/translation, PCR, restriction mapping, ligation, or transformation.

REFERENCES

1. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
2. **Shepard, A.R. and J.L. Rae.** 1997. Magnetic bead capture of cDNAs from double-stranded plasmid cDNA libraries. Nucleic Acids Res. *25*:3183-3185.
3. **Shepard, A.R. and J.L. Rae.** 1998. Ion transporters and receptors in cDNA libraries from lens and cornea epithelia. Curr. Eye Res. *17*:708-719.

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Simple Version of "Megaprimer" PCR for Site-Directed Mutagenesis

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Site-directed mutagenesis (SDM) is used to introduce a defined mutation into a target DNA of known sequence to study gene expression and protein structure/function relationship. In the past few years, numerous methods have been developed for achieving SDM, including several based on polymerase chain reaction (PCR) amplification (10). Among the PCR-based SDM methods, two of them are particularly appealing because they are simple and efficient: (*i*) "overlap-extension" PCR (5) and (*ii*) "megaprimer" PCR (6,13).

The overlap-extension PCR method requires three PCR amplifications and

four oligonucleotide primers, two of which are mutagenic primers. A modified version of this method was developed and successfully implemented in one previous study investigating allele-specific oligonucleotides for cystic fibrosis gene therapy (3). Although a one-step version of overlap-extension PCR that reduced the entire procedure to only one PCR amplification was recently reported (14), four oligonucleotide primers were still necessary.

Alternatively, the megaprimer PCR method is more cost-effective because it entails the use of three oligonucleotide primers for two rounds of PCR amplification. In addition, only a single mutagenic primer is required for a given SDM reaction. Two different approaches have been used in the development of the numerous versions of the megaprimer PCR method. In earlier studies, a specific mutation is introduced in the first PCR amplification, using the forward external primer and the internal mutagenic primer. The resultant mutant PCR product then becomes a primer (megaprimer), which in conjunction with the reverse external primer, primes a second round of PCR to amplify a longer region of the same template (2,6,13). The purification of megaprimer from the residual primers of the first-round PCR amplification seemed to increase the efficiency of the technique from 10%–30% (6) to 100% (2) and avoided amplification of wild-type sequences. In the second megaprimer approach, the megaprimer is used for an initial extension step in the second-round PCR amplification, rather than as a primer. The extended megaprimer is subsequently amplified with the two flanking external primers (1,4). The work of Herlitze and Koenen (4) showed that an excess of purified extended megaprimer, compared to the template DNA, is a critical factor for the efficiency of SDM. Using a 10-fold to 20-fold excess of the purified megaprimer for an initial elongation step, they showed an overall efficiency of mutagenesis of at least 50%. Although several protocols have been developed

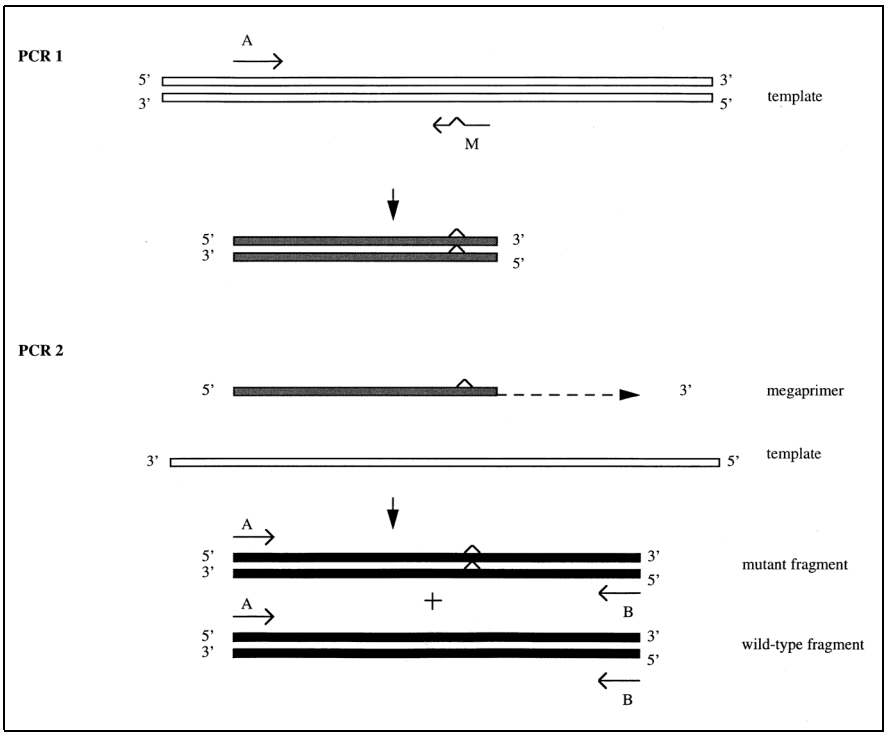

Figure 1. Schematic illustration of the mutagenesis method. The first PCR (PCR 1) is performed using external forward (A) and mutagenic reverse (M) primers to amplify a mutagenic fragment. The second PCR (PCR 2) is performed using external forward (A) and external reverse (B) primers. During the first cycle(s) of PCR 2, the megaprimer product formed in the first PCR is extended with DNA polymerase. In the subsequent cycles of PCR 2, due to the large excess of mutant amplification products, the mutant fragment is preferentially amplified with respect to the original wild-type sequence.

to circumvent the purification step of the first PCR product from residual primers, some of the PCR parameters still require optimization (9,11,12).

This report describes an additional simplification of the megaprimer approach based on a dilution of the products from the first round of PCR and its use as a template for the second-round amplification. Figure 1 depicts our method, which did not require purification of the intermediate PCR product. In the first PCR amplification, a wild-type sequence was mutated with a nonmutating forward primer (A) and a mutagenic internal primer (M). The desired mutation(s) give rise to a new restriction enzyme cleavage site and/or a functional mutation in the amplified product. Then a diluted aliquot of this first amplification was used in a second round of PCR with the external forward and reverse primers (A and B, respectively). During the first cycle, the mutated fragment is extended with DNA polymerase using the wild-type sequence as the template. In the subsequent cycles, due to the large excess of mutant fragment with respect to the original wild-type sequence, the mutagenic sequences will be preferentially amplified with both primers A and B. Ultimately, this results in a relatively high frequency of mutated fragment compared to the original wild-type sequence (>60%).

This SDM strategy was used with cDNAs encoding for both the normal and mutant cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. A 4.7-kb cDNA of wild-type (N) and mutant (ΔF508) *CFTR* was cloned into separate pREP eukaryotic episomal expression vectors (Invitrogen, Carlsbad, CA, USA) (pREP 4β-NCFTR and pREP 4β-ΔF508CFTR, respectively) in a fashion similar to that described previously (Reference 8 and K.K. Goncz, K. Kunzelmann and D.C. Gruenert, unpublished data). The external forward primer (A = CF9A) 5′-GGTGATGACAGCCTCTTCTTCAG-3′ starts at nucleotide (nt) 61 of exon 9, while the external reverse primer (B = CF11B) 5′-GTGATTCCACCTTCTCCAAGAAC-3′ starts at nt 57 of exon 11. The mutagenic primer (M = CF10XB) 5′-TACCCTCTGAAGGCTCGAGTTCTC-3′ used in this study starts at nt 49 of exon 10 and contains a silent mutation (G→C at nt 33 in exon 10) that gives rise to an *Xho*I restriction enzyme cut site. PCR amplifications were performed in a GeneAmp® PCR System 9600 (PE Biosystems, Foster City, CA, USA). The primary amplifications were carried out in a total volume of 20 µL containing 250 µM dNTPs, 1 ng of template plasmid (pREP 4β-NCFTR or pREP4β-ΔF508CFTR) DNA, 0.5 µM each of the external forward and the internal mutagenic primers, 1 U cloned *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA), in 1× *Pfu* reaction buffer [10 mM KCl, 10 mM $(NH4)_2SO_4$, 20 mM Tris-HCl, pH 8.75, 20 mM $MgSO_4$, 1% Triton® X-100, 1000 µg/mL bovine serum albumin (BSA)]. The PCR mixture was held at 94°C for 3 min and then cycled 25 times at 94°C for 20 s, 58°C for 20 s and 72°C for 1 min followed by 5 min at 72°C in the final cycle. After determining the size (172 bp) and purity of the intermediate PCR products from the primary amplification on a 2% agarose gel (Figure 2A), 2 µL of each PCR mixture were diluted (1:25 and 1:50) and used in a secondary PCR amplification. The amplification was performed as described above, except that the reverse external primer was substituted for the internal mutagenic primer, and no additional template DNA was added. The amplified products (371 bp) (Figure 2A) were ethanol-precipitated and cloned into pCR-Script™ Amp SK(+)

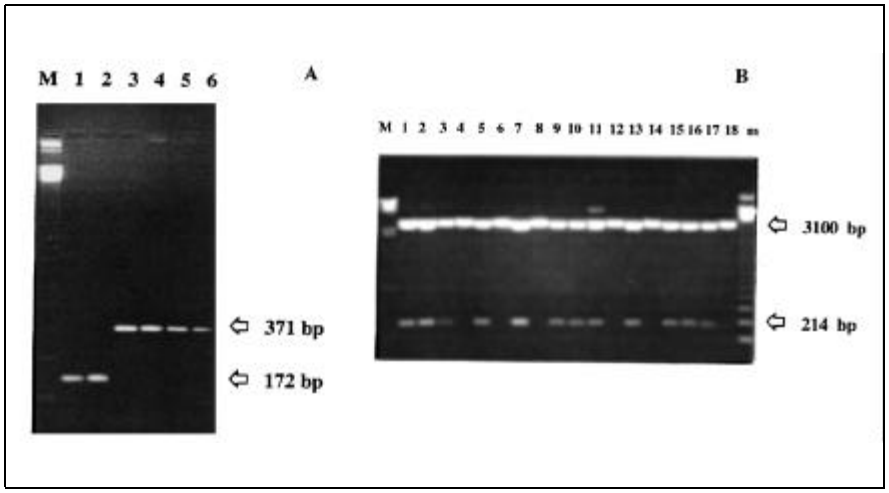

Figure 2. Application of the mutagenesis method. (A) Analysis of the PCR products: a 5-µL aliquot of each PCR product was analyzed on 1.8% agarose gel. Lane M, 123-bp DNA marker. Lanes 1 and 2, PCR 1 products (172 bp) from plasmid pREP 4β-NCFTR and pREP4β-ΔF508CFTR, respectively. Lanes 3 and 4, PCR 2 products (371 bp) using dilutions of 1:25 and 1:50 of the PCR 1 product (pREP 4β-NCFTR). Lanes 5 and 6, PCR 2 products (371 bp) with dilutions of 1:25 and 1:50 of the PCR 1 product (pREP4β-ΔF508CFTR). (B) *Xho*I digestion of 18 randomly chosen recombinant plasmids. Lane M, λ *Hin*dIII DNA marker. Lanes 1–9, recombinant plasmids from pREP 4β-NCFTR mutagenesis experiment. Lanes 10–18, recombinant plasmids from pREP 4β-ΔF508CFTR mutagenesis experiment. Lane m, 123-bp DNA marker. Mutant plasmids carry two *Xho*I sites and yield 214- and 3100-bp fragments (lanes 1–3,5,7,9–11,13 and 15–17). Nonmutant plasmids carry one *Xho*I site (lanes 4, 6, 8, 12, 14 and 18).

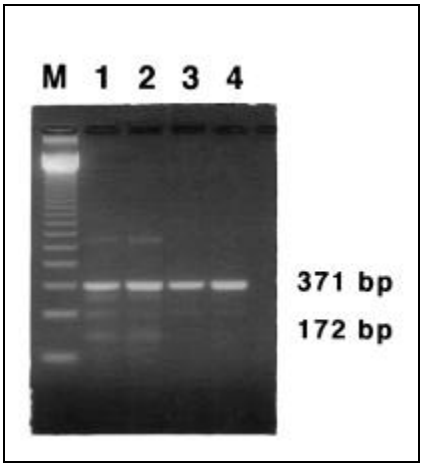

Figure 3. First-round PCR product dilution effect on the second round of PCR amplification. Lane M, 123-bp DNA marker. Lanes 1 and 2, second-round PCR products (371 bp) generated using 2 and 1 µL, respectively, of the undiluted first-round PCR product (pREP 4β-NCFTR). Lanes 3 and 4, second-round PCR products (371 bp) generated using 1:25 and 1:50 dilutions of the first-round PCR product (pREP 4β-NCFTR). The 172-bp band represents residual detectable megaprimer carried over from the first-round PCR.

vector (Stratagene), according to manufacturer's instructions. The ligation products were transformed into *Escherichia Coli* XL1-Blue MRF′ Kan Supercompetent cells (Stratagene), and 50 µL of transformation mixture were spread on LB plates supplemented with ampicillin (100 µg/mL), tetracycline (12.5 µg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG) (40 µg/mL) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg/mL). Nine white colonies were isolated from each transformation and selected for amplification in LB/ampicillin. Plasmids were extracted using the Wizard™ Plus SV Miniprep Kit (Promega, Madison, WI, USA), and 400 ng were subjected to enzymatic digestion using *Xho*I to confirm the proper insertion of the mutation. In both experiments, 6 of 9 clones contained the desired mutation (Figure 2B). Then, three recombinant clones from each experiment were sequenced with M13 universal primers. No additional mutations were detected (data not shown).

This two-step PCR method was simple, rapid and has a relatively high efficiency. Also, there were no particularly critical parameters required for this protocol. PCR amplifications were carried out with AmpliTaq® DNA Polymerase (PE Biosystems) and *Pfu* DNA polymerase and showed similar results (data not shown). The mutagenic primer was designed according to Kuipers et al. (7) such that its 5′ end immediately follows a T residue in the same strand. While the nontemplate addition of a 3′ dA residue facilitates the TA-based cloning of the amplified fragment, the addition of an out-of-sequence dA effectively introduces another mutation into the PCR product and undermines extension of the megaprimer in the initial cycle(s) of the secondary amplification, because of a mismatch between the 3′ end of the megaprimer and the template. Although the use of *Pfu* DNA polymerase is preferred because of its higher fidelity, this method is not limited to a particular DNA polymerase, unlike other strategies (11).

The SDM strategy presented here relies on the fact that a dilution of the first PCR amplification product (used as a template in the second round of PCR amplification) can improve the yield of the desired mutant clones relative to those carrying unaltered parental sequences. Using our experimental conditions, 3.2–1.6 ng of mutated product and 4–2 pg of wild-type plasmid DNA are present in the second PCR amplification mixture when a 2-µL aliquot of a 1:25 or a 1:50 dilution of the first-round PCR amplification were used, respectively. Under these conditions, the mutated amplification product is approximately 800-fold in excess of the wild-type plasmid DNA and, thus, preferred by the DNA polymerase. The dilution of the first-round PCR product was necessary to avoid spurious amplification products. When 1 or 2 µL of the undiluted first-round PCR product were used as the template, nonspecific amplification products were observed after the second-round PCR amplification (Figure 3).

The rapidity, simplicity and reproducibility of the method outweighs any potential inconvenience in the generation of a specific number of nonmutated PCR amplification products. An awareness of this modification should facilitate SDM strategies overall.

REFERENCES

1. **Barrettino, D., M. Feigenbutz, R. Valcarcel and H.G. Stunnenberg.** 1993. Improved method for PCR-mediated site-directed mutagenesis. Nucleic Acids Res. *22*:541-542.
2. **Giebel, L.B. and R.A. Spritz.** 1990. Site-directed mutagenesis using a double-stranded DNA fragment as a PCR primer. Nucleic Acids Res. *18*:4947.
3. **Goncz, K.K., K. Kunzelmann, Z. Xu and D.C. Gruenert.** 1998. Targeted replacement of normal and mutant CFTR sequences in human airway epithelial cells using DNA fragments. Hum. Mol. Genet. *7*:1913-1919.
4. **Herlitz, S. and M. Koenen.** 1990. A general and rapid mutagenesis method using polymerase chain reaction. Gene *91*:143-147.
5. **Higuchi, R., B. Krummel and R.K. Saiki.** 1988. A general method for in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. *16*:7351-7367.
6. **Kammann, M., J. Laufs, J. Schell and B. Gronenborn.** 1989. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). Nucleic Acids Res. *17*:5404.
7. **Kuipers, O.P., H.J. Boot and W.M. de Vos.** 1991. Improved site-directed mutagenesis method using PCR. Nucleic Acids Res. *19*:4558.
8. **Lei, D.C., K. Kunzelmann, T. Koslowsky, M.J. Yezzi, L.C. Escobar, Z. Xu, A.R. Ellison, J.M. Rommens, L.C. Tsui, M. Tykocinski and D.C. Gruenert.** 1996. Episomal expression of wild-type CFTR corrects cAMP-dependent chloride transport in respiratory epithelial cells. Gene Ther. *3*:427-436.
9. **Ling, M. and B.H. Robinson.** 1995. A one-step polymerase chain reaction site-directed mutagenesis method for large gene-cassettes with high efficiency, yield, and fidelity. Anal Biochem. *230*:167-172.
10. **Ling, M.M. and B.H. Robinson.** 1997. Approaches to DNA mutagenesis: an overview. Anal Biochem. *254*:157-178.
11. **Marini, F., A. Naeem and J.-N. Lapeyre.** 1993. An efficient 1-tube PCR method for internal site-directed mutagenesis of large amplified molecules. Nucleic Acids Res. *21*:2277-2278.
12. **Picard, V., E. Ersdal-Badju, A. Lu and S.C. Bock.** 1994. A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. Nucleic Acids Res. *22*:2587-2591.
13. **Sarkar, G. and S.S. Sommer.** 1990. The "megaprimer" method of site-directed mutagenesis. BioTechniques *8*:404-407.
14. **Urban, A., S. Neukirchen and K.E. Jaeger.** 1997. A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. Nucleic Acids Res. *25*:2227-2228.

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