Research Article

Directed-Mutagenesis and Deletion Generated through an Improved Overlapping-Extension PCR Based Procedure

Wirojne Kanoksilapatham[1*], Juan M. González[2] and Frank T. Robb[3]

[1]*Department of Microbiology, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand*
[2] *IRNAS-CSIC, P.O.Box 1052, 41080 Sevilla, Spain*
[3] *Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD 20274, USA*
[*]*Corresponding author. E-mail address: wirojne@su.ac.th*

Received May 1, 2007; Accepted September 20, 2007

Abstract

Generating either single or multiple point mutations and producing deletions and insertions into target DNA sequences are critical and widely used experimental procedures in molecular biological studies. This article presents a modified mutagenesis protocol based on an overlapping-extension PCR amplification method. The procedure benefits from the design of mutagenic primers to generate overlapping megaprimers without the need for intermediate purification to remove unused primers. Unused primers are diluted out during the successive amplification-extension reactions. A key success to this modified method is the use of two flanking primers after the overlapping extension reaction. The use of *Pfu* DNA polymerase increases, compared with *Taq* DNA polymerase, amplification accuracy. The proposed procedure represents a simple and efficient method that introduces many types of mutations into specific target DNA fragments and creates either hybrid DNA fragments or internal nucleotide deletions.

***Key Words*:** Directed-mutagenesis; Megaprimers; Overlapping primers; *Pfu* DNA polymerase

Introduction

Megaprimer and overlap PCR based methods (Wu et al., 2005; Angelaccio and Bonaccorsi di Patti, 2002; Kammann et al., 1989) are essential techniques for gene expression strategies. These techniques are commonly used to study the relationships between the function and structure of proteins. Methods using overlapping extension PCR (Ge and Rudolph, 1997) require complex combinations of primers to generate two or more overlapping DNA fragments. The standard procedure requires several purification steps of the intermediate DNA products. By generating complementary sequences at the overlapping regions, the DNA fragments can function as two megaprimers allowing an extension by DNA polymerases. Currently, a number of modified megaprimer PCR based methods have been described aiming to simplify the method and increase its efficiency (Nabavi and Nazar, 2005; Tyagi et al., 2004; Lai et al., 2003; Brons-Poulsen et al., 2002). The technique is based on two rounds of PCR amplifications using two different flanking regions and two internal primers designed to generate the desired mutations. During the first PCR round, the mutagenic primer and a reverse flanking

primer are used to generate double stranded portion of the target DNA sequence that is, then, purified from unused oligonucleotides. During the second PCR round, that double-stranded DNA fragment and a forward flanking primer are utilized to generate a mutated product corresponding to the total length of the target sequence. A subsequent modification introduced a digestion with a restriction enzyme (Wei et al., 2004), *Dpn*I, to remove a methylated DNA template, which was successfully developed to improve accuracy. However, purification of the intermediate product is still required in the above protocols resulting in a longer experimental time and a higher cost. Although a proposed megaprimer PCR based method using exonuclease I (Nabavi and Nazar, 2005) obviates the need for purification of the intermediate amplification products, the technique requires an expensive enzyme, and the protocol requires a several-hour digestion of leftover reverse primers.

Recently, a method using successive PCR amplification at differential melting temperatures for three primers and requiring no purification step was described (Wu et al., 2005). The success of this method was restricted to carefully designed primers with limited tolerance for mismatch mutagenesis. An overlapping method using two flanking primers and two mutagenic primers was proved to be useful as well as simple, but the protocol still requires intermediate purification steps (Angelaccio and Bonaccorsi di Patti, 2002). In the first PCR round, an internal megaprimer was produced using a mutagenic primer pair followed by purification to remove the mutagenic primers remaining unused after the reaction. In the second PCR round, the megaprimers and the appropriate flanking primer were employed to generate two overlapping DNA fragments in two separate tubes. The two overlapping DNA fragments and the flanking primers were then used to amplify full-length mutated DNA.

A generic PCR based method using an overlapping primer pair and two flanking primers is developed to both perform site directed mutagenesis and to generate hybrid DNA fragments and internal deletion mutations. The modified method does not require any intermediate purification step to remove unused primers. The method is simple, reliable, and highly accurate, representing a low-cost approach.

Materials and Methods

Primer Sequences

The nucleotide sequences of the forward (Ff) and reverse (Rf) flanking primers are 5' ATG AAG GCT GAG AGC ATT CTA TAC TC and 5' GGA TAA CTG GGG CAT CAC, respectively. The nucleotide sequences of the two mutagenic primer pairs that are complementary to each other are 5' GGT TCA GGA CTC TGA AGG AAA GAA (Mf) and 5' GCT TTG TTC TTT CCT TCA GAG TCC (Mr); and 5' CTT TAA AAA GAA AGA ACA AAG CG (If) and 5' CTT TCT TTT TAA AGT CCT GAA CCA GCG (Ir) (bold font indicates mutated nucleotides). Each pair of these primers was designed to produce two mutated DNA fragments on a *transposase* gene sequence from *Pyrococcus furiosus* (GeneBank Accession No. AF443788). Mutated DNA fragments obtained were TA cloned into a pBAD TOPO® vector (Invitrogen, Carlsbad, CA) as described by the manufacturer.

PCR Conditions for Megaprimer Synthesis

PCRs were performed using cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA) for 30 cycles. A 50 µl reaction mixture consisted of 1-10 ng of plasmid DNA carrying the target DNA template, 1.3 mmoles of $MgCl_2$, 10 nmoles of dNTP, 20 pmoles forward primer, 20 pmoles reverse primer, and 2.5 units of DNA polymerase. Thermal cycling conditions were: 95°C for 3 minutes followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 40 seconds, and 72 °C for 60 seconds, and a final step at 72 °C for 8 minutes.

Overlapping Extension

Five microliters from a pair of the first PCR reactions were added into a 15 µl of a PCR reaction mixture that have no addition of franking primers. The reactions were carried out for ten cycles to generate

mutated template. Then, an equal volume (25 µl) of a PCR reaction mixture containing 20 pmoles of Ff and Fr primers and 2.5 units of *Taq* DNA polymerase was added into the reaction to obtain a 50 µl PRC reaction mixture. Amplification of the mutated product was performed for 30 cycles as described above. The mutated DNA product obtained was gel purified. The purified mutated product (50-100 ng) was cloned into the pBAD TOPO® vector. The vector was transformed into *E. coli* Top10. Ten white colonies growing on a LB containing ampicillin (100 µg) and X-gal (30 µg) agar plate were randomly picked for sequencing. Nucleotide sequences were analyzed using AssemblyLIGN™ (Oxford Molecular Group PLC, Oxford Science Park Medawar Centre Oxford, UK).

Western Blotting Analysis

Western blotting analysis was performed using semi-dry system (Pharmacia, Uppsala, Sweden). Recombinant transposase on the blotted Nitrocellulose membrane was detected using anti-6xHis tag (Life Technologies, Inc., Gaithersburg, MD) and chemiluminescent (Pierce Biotechnology, Rockford, IL).

Results

A generic protocol for site directed mutagenesis was developed with minor modifications of an overlapping extension PCR technique (Figures 1 and 2). Figure 1 describes the experimental design to obtain internal mutations in the original target sequence. In the first PCR step, each mutagenic primer pair, together with appropriate flanking primers, generated two mutated DNA fragments or megaprimers. These were named "Mega1" and "Mega2". The presence of the desired single bands with the expected sizes was corroborated by agarose gel electrophoresis. These first step PCR products were immediately transferred to the overlapping extension reaction. The mixture of Mega1 and Mega2 resulted in an extension by DNA polymerase generating full-length mutated templates. It is important to state here that the addition of the flanking primers (Ff and Rf) usually inhibits the full-length mutated template

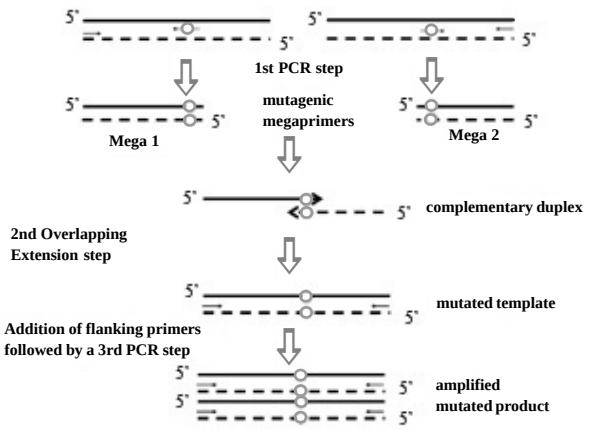

Figure 1 Site directed mutagenesis using double stranded megaprimers. A standard PCR using *Pfu* DNA polymerase was performed in two separate tubes to produce double stranded megaprimers (Mega1 and Mega2). The megaprimers were immediately transferred to the overlapping extension reaction. In this reaction, only one self-priming duplex (formed by the Mega1 and Mega2) is extended from the 3' hydroxyl group to generate a full-length mutated template. Then, the flanking primers were added, and the mutated products were amplified using a standard PCR amplification reaction. Solid and dotted lines represent top and bottom strands of DNA, respectively. Short arrows represent primers. Arrow head indicates 3' hydroxyl group. White circles indicate mutated nucleotides. Block arrows indicate steps.

(Figure 3). In the overlapping extension reactions, only one pair of the Mega1 and Mega2 can form self-priming duplexes that will be extended from the 3' hydroxyl groups toward their 5'-ending during the overlapping extension reaction. The removal of unused primers from the first PCR step is not necessary because the intensity of bands corresponding to the megaprimers and the accuracy of the final mutated DNA products were not affected (Figure not shown). This is because the concentration of the unused primers and the template DNA that may be carried over from the first PCR step reactions were diluted out in the overlapping extension reaction mixture and final PCR amplification (Tyagi et al.,

2004). After the overlapping extension reaction, the flanking primers were added to the reaction mixture following the final PCR step (Figure 1) carried out to amplify the mutated, full-length, products (Figure 3). Cloning and sequencing of the inserts revealed that all of the analyzed clones (ten) carried the desired mutations. The modified method described above is simple, versatile, and can be applied to generate single and multiple mutations (Figure 1) and to create either hybrid DNA fragments or internal nucleotide deletions (Figure 2) with slight modifications.

Figure 2 shows experimental design aiming to generate an internal deletion construct. The primers were designed to contain overhanging nucleotide sequences that are complementary to each other except the nucleotide sequence of the deleted DNA fragment that was skipped. All PCR and overlapping extension steps are described in Figure 1. This technique were employed successfully to generate several promoter-gene hybrid constructs where no appropriate sites for restriction enzyme digestion were present (unpublished data) and to construct an internal deletion on a recombinant gene encoding a heat shock protein from *Pyrococcus furiosus* (Laksanalamai et al., 2003).

In order to demonstrate the accuracy of the method, a cloned DNA fragment corresponding to a transposase gene (*tnp*) from *Pyrococcus furiosus* was used as a DNA template. This *tnp* gene (size of 702 nucleotide long) contains the sequence 522-T TTA AAA-528 (where nucleotide 1 corresponds to the A of the initiation codon) (Kanoksilapatham et al., 2004) that is similar to a translational frameshifting heptamer, a programmed translational frameshifting signal. The heptanucleotide slippery motif of X XXY YYZ (X, Y and Z represent nucleotides) is frequently observed in various *transposase* and *integrase* genes of transposons and retroviruses (Farabaugh and Bjork, 1999). Mf and Mr, and If and Ir, (see Primer Sequences in Materials and Methods) are two mutagenic primer pairs containing an overlapping common sequence. They were used to successfully generate two side

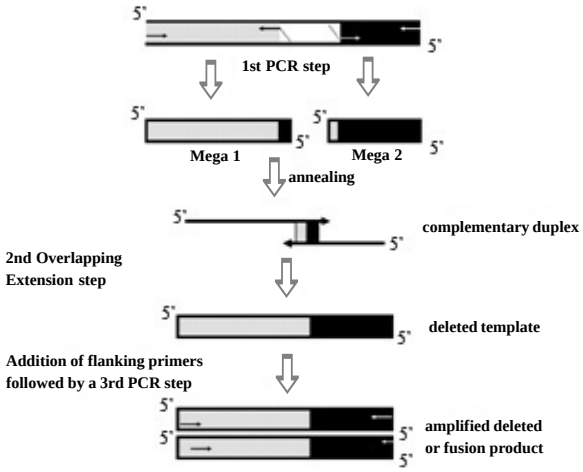

Figure 2 Internal deletion of a gene of interest using 5'-end overhanging primers. The primers were designed to contain overhanging nucleotide sequences that are complementary to each other. All PCR and overlap extension steps are described in Figure 1. Gray and black boxes indicate target nucleotide sequences of a gene of interest. The empty box represents an internal nucleotide sequence that is deleted in the final product. Arrow heads indicate 3' hydroxyl group. Short and bent arrows represent primers containing 5'-end overhanging nucleotides.

directed DNA fragments in which the mutated sequences are 522-T CTG AAG-528 and 522-T TTA AAA A-529 (bold font indicates mutated nucleotides), respectively (Figure 3). The reading frame of the latter mutated sequence thus was interrupted. They were cloned into the pBAD TOPO® vector such that their 3'-ends were tagged with the six histidine codons. Transformants, named "GAAG" and "AAAA", were obtained, respectively. The mutated nucleotide sequences were confirmed by nucleotide sequencing in all clones. The results indicate high accuracy of the technique. Expressed recombinant transposases are shown in Figure 4. As expected, two histidine tagged proteins were observed in the strain carrying wild type sequence (522-T TTA AAA-528) (lanes 1-2) and the strain GAAG (lanes 3-4). On the other hand, the strain AAAA (lanes 5 and 6)

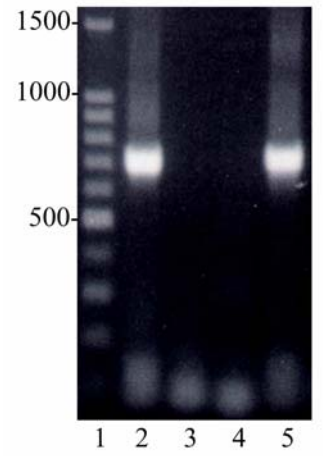

Figure 3 PCR products generated from the 3rd step of the directed mutagenesis. Lane 1 represents 100 bp DNA ladder. Lanes 2 and 5 represent samples from overlapping extension reactions performed without the addition of the corresponding flanking primers. Lanes 3 and 4 represent samples from overlapping extension reactions performed with the corresponding franking primers. The expected 702 bp long mutagenic DNA fragments were obtained in lanes 2 and 5. On the other hand, no PCR product was observed in lanes 3 and 4. The trace amount of the corresponding Mega1 and Mega2 pairs (sizes of 536 and 188; and 529 and 190, respectively) carried over from the preceding reactions were not observed (lanes 3 and 4) because they were diluted.

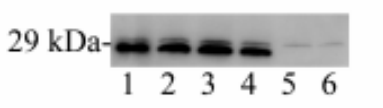

Figure 4 Western blotting analysis shows his-tagged transposases. Lanes 1-2 represent samples from *E. coli* Top 10 carrying the wild type sequence (522-T TTA AAA-528). Lanes 3 - 4, and 5 -6 represent samples from strains GAAG (522-T CTG AAG-528) and AAAA (522-T TTA AAA A-529) carrying the mutated sequences, respectively. Bold font indicates mutated nucleotides. Two identical 10% slap gels were performed using SDS-PAGE technique. They were blotted onto two nitrocellulose membranes and stained using Ponceau S dye, respectively. Molecular size markers were located, and a membrane was stained using anti-6xHis tag and chemiluminescent techniques. As expected, the clones carrying the wild type sequence (lanes 1 - 2) and the strain GAAG (lanes 3 - 4) expressed a 29 kDa his-tagged protein. On the other hand, the strain AAAA produces truncated protein, lacking the six histidine residuals (lanes 5 - 6). The band with the size of slightly smaller than 29 kDa is an unknown host protein bound by the anti-histidine monoclonal antibody.

carrying an A insert at nucleotide number 529 produces truncated protein; and, thus, 6xHis tagged recombinant protein was not observed. The band with the size of slightly smaller than 29 kDa is an unknown host protein bound by the anti-histidine monoclonal antibody. The expression results confirm the sequencing results.

Discussions

The protocol described in this study, with minor modified features, is simple. The mutagenic primers were designed in such a way that their 5'-ends contain overhanging nucleotide sequences complementary to each other (Figures 1 and 2). Thus, the intermediate products of Mega1 and Mega2 contain complementary sequences at their ends corresponding to the mutagenic primers. Although, the use of *Pfu* DNA polymerase to replace *Taq* DNA polymerase is not novel, the amplification of the megaprimers using a blunt end generating DNA polymerase makes less primer design complicate, since any position on the template can be the candidate for the 3'-end nucleotide of the mutated primers. As well, *Pfu* DNA polymerase presents higher proofreading activity than *Taq* DNA polymerase (Wunschel and Smith, 2004; Bi and Stambrook, 1998) and, thus, minimizes the error rate during the amplification and extension steps. The lack of amplification errors is essential in mutagenesis/deletion experimental procedures to deter the production of DNA fragments with undesired sequences. The second PCR reaction mixture was modified by not adding the flanking

primers to the overlapping extension reaction mixture. This modification is crucial for success in a subsequent PCR step (Figure 3). In conclusions, the improved overlapping-extension PCR based method developed in this study is generic and can be employed to create various kinds of *in vitro* mutations, including point mutations, nucleotide substitutions, insertions, deletions, and hybrid promoter-gene fusion without the purification steps of the intermediate PCR products. The method is therefore simple, reliable, highly accurate, and inexpensive.

Acknowledgements

The followings supports are acknowledged: the Interagency Program in Metabolic Engineering (NIH, NSF, EPA, DOE) through the Grant BES0224717 awarded to Frank T. Robb, the Spanish Ministry of Education and Science under the Project REN2002-00041/GLO awarded to Juan M. Gonzalez, and the Research and Development Institute, Silpakorn University, Thailand awarded to W. Kanoksilapatham.

References

Angelaccio, S., and Bonaccorsi di Patti, M. C. (2002) Site-directed mutagenesis by the megaprimer PCR method: variations on a theme for simultaneous introduction of multiple mutations. *Analytical Biochemistry* 306: 346-349.

Bi, W., and Stambrook, P. J. (1998) Detection of known mutation by proof-reading PCR. *Nucleic Acids Research* 26: 3073-3075.

Brons-Poulsen, J., Nohr, J., and Larsen, L. K. (2002) Megaprimer method for polymerase chain reaction-mediated generation of specific mutations in DNA. *Methods in Molecular Biology* 182: 71-76.

Farabaugh, P. J. and Bjork, G. R. (1999) How translational accuracy influences reading frame maintenance. *The EMBO Journal* 18: 1427-1434.

Ge, L. and Rudolph, P. (1997) Simultaneous introduction of multiple mutations using overlap extension PCR. *Biotechniques* 22: 28-30.

Kammann, M., Laufs, J., Schell, J., and Gronenborn, B. (1989) Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). *Nucleic Acids Research* 17: 5404.

Kanoksilapatham,W., Gonz lez, J. M., Maeder, D. L., DiRuggiero, J., and Robb F.T. (2004) A proposal to rename the hyperthermophile *Pyrococcus woesei* as *Pyrococcus furiosus*, sub sp. *woesei*. *Archaea* 1:277-283.

Laksanalamai, P., Jiemjit, A., Bu, Z., Maeder, D. L., and Robb, F.T. (2003) Multi-subunit assembly of the *Pyrococcus furiosus* small heat shock protein is essential for cellular protection at high temperature. *Extremophiles* 7:79-83.

Lai, R., Bekessy, A., Chen, C. C., Walsh, T., and Barnard, R. (2003) Megaprimer mutagenesis using very long primers. *Biotechniques* 34: 52-56.

Nabavi, S. and Nazar, R. N. (2005) Simplified one-tube “megaprimer” polymerase chain reaction mutagenesis. *Analytical Biochemistry* 345: 346-348.

Tyagi, R., Lai, R., and Duggleby, R. G. (2004) A new approach to ‘megaprimer’ polymerase chain reaction mutagenesis without an intermediate gel purification step. *BMC Biotechnology* 4: 2

Wei, D., Li, M., Zhang, X., and Xing, L. (2004) An improvement of the site-directed mutagenesis method by combination of megaprimer, one-side PCR and *DpnI* treatment. *Analytical Biochemistry* 331: 401-403.

Wu, W., Jia, Z., Liu, P., Xie, Z., and Wei, Q. (2005) A novel PCR strategy for high-efficiency, automated site-directed mutagenesis. *Nucleic Acids Research* 33: e110.

Wunschel, D. S., Masselon, C., Feng, B., and Smith, R. D. (2004) Proofreading activity of P*fu* thermostable DNA polymerase on a 6-O-methylguanine-containing template monitored by ESI-FTICR mass spectrometry. *Chembiochem* 5: 1012-1015.