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Modification of overlap extension PCR: A mutagenic approach

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In vitro site-directed repair or creation of a mutation is an invaluable technique in genetic and protein engineering. Several methods have appeared in literature but still require many modifications. We describe a rapid and efficient modified overlap extension PCR method for multiple uses in mutagenesis studies. The protocol is based on two rounds of PCR with the help of two sets of primers, two flanking and two internal mutagenic primers. Two fragments of DNA prepared in first round of PCR are then allowed themselves to anneal in the second stage of PCR using gradient annealing temperature without using flanking primers. This protocol has been used for correcting a mutation caused in exoglucanase (CBHII) gene of *Trichoderma* spp. We successfully synthesized the full length of gene from two fragments in the second round of PCR in lesser time.

Keywords: DNA polymerase, e*x taq*, overlap extension PCR, site directed mutagenesis, one step overlap extension-PCR

Introduction

Mutation repair and site-directed mutageneseis (SDM) are important tools in genetic and protein engineering. Desired mutation can simply be achieved by polymerase chain reaction (PCR) which amplifies specific segments of DNA using DNA polymerase. However, thermo stable DNA polymerase enzyme often creates unwanted mutation in the gene as it lacks in 3′→5′ exonuclease (proof reading) activity.

Many protocols have been developed to repair or create a desired mutation in DNA. All the techniques are based on synthetic oligonucleotide primers containing the desired mutation/s with complementary base sequence of intended gene[1]. Overlap extension PCR (OEP) and megaprimer methods are widely utilized techniques and have been modified regularly over a period of time.

Overlap extension PCR (OEP), which comprises two rounds of PCR, generates two DNA fragments having overlapping ends. These fragments are combined in a subsequent fusion reaction in which the overlapping 3′ overlap of each strand serves as a primer for 3′ extension of complimentary strand. The resulting fusion product is amplified further by PCR. Specific alterations in the nucleotide sequence could be introduced by incorporating nucleotide changes into the overlapping oligoprimers[2]. OEP was further modified to one step overlap extension PCR (OOE-PCR), which describes two stage PCR strategy in one PCR reaction[3].

To obtain high mutant yields, it is necessary to fractionate and purify the PCR products. After purifying, the fragments of DNA were allowed to anneal for two rounds of PCR and then further amplification was carried out with polymerase enzyme for few more PCR cycles[4]**.** Megaprimer also proved to be efficient and economical to use. Reaction can be carried out in single tube either by changing annealing temperature or by gradually adding primers[5-7].

PCR is more stringent to annealing temperature. Starting from lower annealing temperature the temperature is increased after each cycle until a predetermined annealing temperature is reached. This allows for more stringent annealing of common fragments and increases the specificity of the PCR products. As specific products accumulate in the first round, they can be made available as templates in the succeeding rounds, which have increased annealing temperatures leading to higher yield of products.

We used the above modified overlap extension PCR to repair mutation in a gene. While isolating the gene of exoglucanase (CBHII) from *Trichoderma* spp, a mutation was generated at one point in structural gene of 1515 base pairs. Two DNA fragments were generated using four primers (two flanking and two

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mutagenic) and were allowed to anneal at gradient temperatures for two cycles at each temperature. We introduced the gradient temperature in the annealing of common base pairs and extension process was carried out without using any primers. The common region of both fragments acted as a primer to amplify the whole fragment with the point mutation getting corrected.

Materials and Methods

The method consists of two rounds of PCR (Fig. 1) with the help of four primers forward, reverse, F-1 reverse and F-2 forward (Table 1) and *Ex Taq* polymerase (Takara, Japan). Primer F-1 reverse and F-2 forward share same sequence, which corresponds to 567-577 base pairs number of original sequence of exoglucanase gene (mutated nucleotide in bold) primers. Forward and reverse primers have unique *EcoR1*/*Xba1* enzyme sites.

First Phase of PCR

Two different sets of PCR were performed on Biometra thermocycler. In tube one, 10 p mole of primers, forward and F-1 reverse, were used to amplify fragment 1 while in tube two, 10 p mole of primers, F-2 forward and reverse, were used to amplify the second fragment. For each PCR reaction, 100 ng of template, 2.5 unit of *Ex Taq* 1X PCR buffer (Takara, Japan) and 25 m*M* of dNTP (Takara, Japan) were mixed together and final volume made to 100 µL with distilled water.

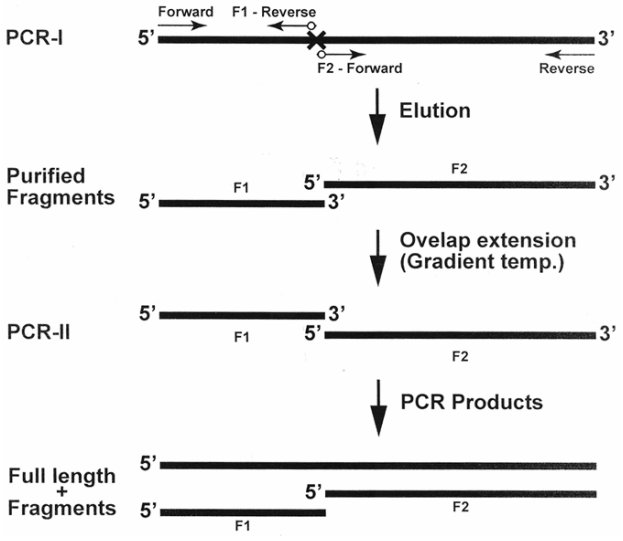

Fig. 1—Schematic diagram of mutagenesis protocol: In PCR 1, 2 fragments fractionated and purified using 2 different sets of primers and mutated gene as a template. Primers, F1- Reverse and F2- Forward share common 10 base pairs with desired mutation in single nucleotide base. In PCR II, fragments were simply allowed to anneal themselves at different gradient temperatures starting from low to high temperatures. By running two rounds of PCR desired full length DNA obtained.

Twenty cycles of amplification (94°C; 1 min, 48°C; 1 min, 72°C; 1 min) were performed followed by 5 min at 72°C and holding at 4°C. After the first round PCR, both the fragments were excised from gel and purified using silica column and dissolved in TE (tris-EDTA) buffer (*p*H 8.0). Purified bands were checked on 0.7% agarose gel for band intensity using EtBr and visualizing it under UV light and photographed on UV transilluminator (Core Biosystems).

Second Phase of PCR

The equimolar concentration of both fragments was mixed together in a tube containing 1X PCR buffer, 25 m*M* dNTPs and 5 units of *Ex Taq* and final volume made up to 100 µL with distilled water. The reaction was performed at gradient annealing temperature in a range from 44-54°C at 2°C intervals. At each annealing temperature, 2 cycles of PCR were carried out with 1 min of denaturation (94°C) and 1 min extension (72°C). Annealing time was kept 1.5 min at each temperature. PCR products were run on 0.7% agarose containing EtBr and checked the DNA under UV transilluminator.

After second round of PCR the newly synthesized full length PCR product was eluted and cloned in pBluscriptKS vector using *EcoR1*/*Xba1* enzyme site. The clone was used to transform DH5α competent cells. Plasmids were isolated from 2 mL of overnight cultures as described in Sambrook *et al*[8] and subjected to restriction analysis using *EcoR1* and *Xba1* (NEB, England). Selected clones containing insert of gene were sent for sequencing (Genotech, Korea).

Table 1—Primer sequences used to amplify fragments during first phase of PCR

Primers	Sequence
Forward	5′GAA TTC ATG TAT CAG AAA TTG GCC G 3′
Reverse	5′TCT AGA CAG GCA CTG AGA GTA GAA TGG 3′
F-1 Reverse	5′CAG GTC ACG A**A**G GCA CTG GCT 3′
F-2 Forward	5′GCC AGT GCC **T**TC GTG ACC TGA 3′

Forward and F-1 reverse primers were used to get fragment 1 while reverse and F-2 forward primers were used to multiply fragment 2. Mutated nucleotide showed in bold letters in primers F-1 reverse and F-2 forward.

Results and Discussion

The four PCR primers, forward, reverse, F-1 reverse and F-2 forward, were used in first round of PCR (Fig. 2A) to amplify two fragments of 576 and 939 base pairs, respectively. The primers were designed such that it would correct the mutation, while both flanking primers, forward and reverse, have unique restriction sites *EcoR1* and *Xba1*, respectively.

In order to avoid unwanted incorporations of fresh mutations (Taq polymerase induced) only 20 cycles of PCR were performed using previously cloned exoglucanase (CBHII) gene as a template. Both the PCR products were of the expected size.

In the second round of PCR, both the fragments, 1 and 2, were allowed to join to generate full length of fragment by gradient annealing temperatures using taq polymerase. Three clear bands, two fragments of 576 and 939 base pairs and one full length of 1515 base pairs of exoglucanase (CBHII) in agarose gel were observed (Fig. 2B). To authenticate the longer fragment, another round of PCR was carried out using the forward and reverse primers and amplifying the full length of gene (data not shown). The full length product was cloned into pBluescriptKS (-/+) and sequenced. Sequence data confirmed the authenticity of the clone. With this protocol, joining of fragments could be achieved within few hours.

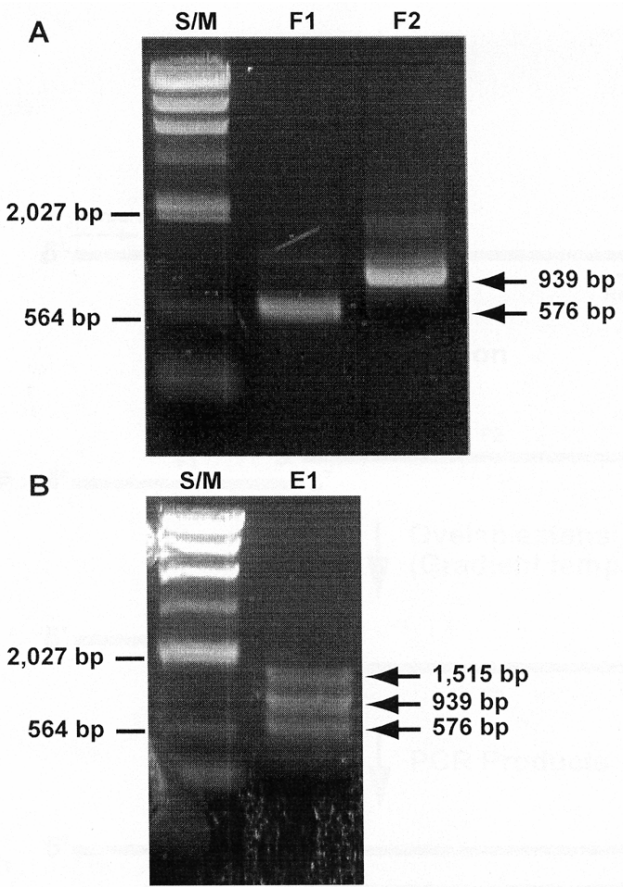

Fig. 2—Agarose gel electrophoresis: (A) An agarose gel showing eluted fragments. Fragment 1 (F1) has 576 base pairs while fragment 2 (F2) has 939 base pairs of nucleotides. (B) After gradient annealing temperature, PCR gel photo showing 3 clear bands. Both fragments and full length of 1515 base pairs amplified after modified overlap extension PCR.

To save time and avoiding laborious work, it is important to work with efficient mutagenesis method. This mutagenic PCR based method allows corrections to be carried out more efficiently in lesser time than traditional methods. Moreover, it is possible to achieve many types of mutations, for example, insertion, single point mutation and deletion depending upon the area of research.

The present investigation has advantage over classic OEP and megaprimer PCRs, which are routinely used techniques in mutagenic studies. In classic OEP, the fragments are simply allowed to anneal at fix *Tm* in order to amplify full length of the product, however, the product yield remains low. It requires one more PCR step to get sufficient quantity of the product[2-4]. On the other hand, in the present technique, high quantity of mutagenic products could be obtained without using any flanking primers. To maintain high fidelity of replicated DNA sequence, we have reduced the number of cycles during overlap extension to avoid polymerase dependent unwanted incorporation of nucleotides, and improved the final yield of product so as to continue further cloning process.

Megaprimer technique is effective in lesser time as the intermediate purification steps are not required; also the use of high *Tm* often leads to optimizing annealing time and screening for desired clone[5-6]. However, in this simplified method, the PCR fragments containing common nucleotide base pairs at 3′ and 5′ terminus of either of the fragments, are annealed at different temperatures during reaction.

In conclusion, this protocol provides as a facile method for the correction and deletion of nucleotides, single point mutations and could be accomplished with the above features by two round of PCR.

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References

1 Smith M, *In vitro* mutagenesis, *Annu Rev Genet*, 19 (1985) 423-462.

2 Steffan N H, Hunt H D, Horton R M, Pullen J K, Pease L R *et al*, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, *Gene*, 77 (1989) 51-59.

3 Andreas U, Neukirchen S & Jaeger K E, A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR, *Nucleic Acids Res*, 25 (1997) 227-228.

4 An Y, Ji J, Wu W, Lv A, Huang R, Wei Y *et al*, A rapid and efficient method for multiple-site mutagenesis with a modified overlap extension PCR, *Appl Microbiol Biotechnol*, 68 (2005) 774-778.

5 Picard V, Ersdal-Badju E & Lu ABock S C, A rapid and efficient one-tube PCR-based mutagenesis technique using *Pfu* DNA polymerase, *Nucleic Acids Res*, 22 (1994) 2587-2591.

6 Wu W, Jia Z, Liu P, Xie Z & Wei Q, A novel PCR strategy for high-efficiency, automated site-directed mutagenesis, *Nucleic Acids Res*, 33 (2005) 1-7.

7 Ke S H & Madison E L, Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method, *Nucleic Acids Res*, 25 (1997) 3371-3372.

8 Sambrook J & Russel D W, *Molecular cloning: A laboratory manual,* 3rd edn, vol 1, (Cold Spring Harbor Laboratory Press, New York) 2001, 32-37.