

## Versatile PCR-mediated insertion or deletion mutagenesis

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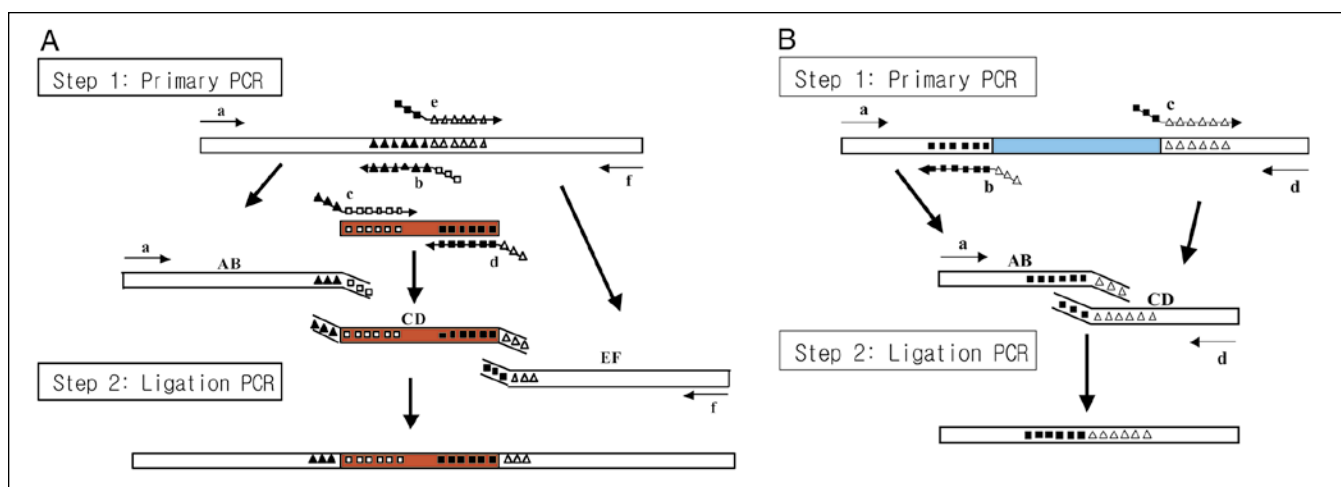
Site-directed mutagenesis is used widely to create desired changes in genes. Various site-directed mutagenesis protocols, both PCR and non-PCR based, have been described. Mutagenesis by overlap extension has been previously described as a method for site-directed mutagenesis to create base substitutions, insertions, or deletions (1,2), as well as for producing chimeric genes by combining two DNA fragments without the need for restriction sites (3). For insertion mutagenesis, mutagenic primers are typically designed to harbor the inserted sequence; thus, the insert size is limited by the length of the mutagenic primer (4). Therefore, insertions larger than 30 nucleotides have been difficult to create by traditional methods owing to the limitations of oligonucleotide synthesis. Here we describe a PCR-

based procedure that can be used to generate precisely located mutants with any length of insertion or deletion. The strategy for insertion mutagenesis employs an established overlap extension PCR protocol, but involves three templates including an insert fragment of any length.

The protocol for insertion requires four chimeric primers that are derived partly from the sequence to be inserted and partly from the template, in addition to two outermost flanking primers. Three PCR products are first prepared separately, including one insert fragment and two adjacent fragments using appropriate primer pairs. The insert fragment thus generated contains terminal complementarities at both ends to two adjacent fragments. In the second step, an overlap extension PCR is carried out, using the outermost primer

pair, to recombine the three fragments.

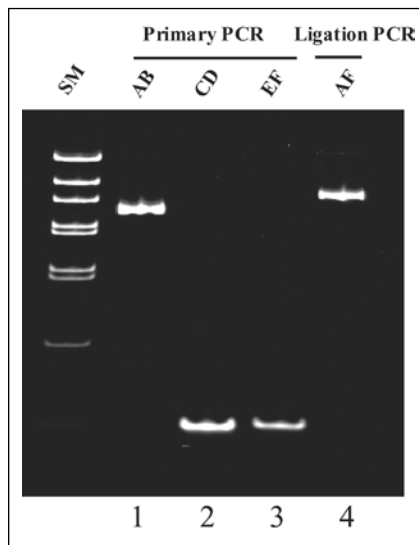
First, four chimeric primers are designed such that they all consist of an 18-nucleotide annealing segment and a 9-nucleotide anchor segment. An anchor segment is derived from sequences adjacent regions in a final desired product (Figure 1). The method employs a two-step PCR strategy. The first step involves three independent PCRs to generate three PCR products that are used as the template for a second ligation PCR. Two PCR products from the first step correspond to the flanking regions of an insertion site, whereas the third PCR product corresponds to the insertion cassette. An insertion cassette is prepared with primers that have terminal complementarities to two flanking fragments (Figure 1). Briefly, the flanking fragments are created by using one nonchimeric primer and one chimeric primer: primer a and b or primer e and f, respectively (Figure 1). Chimeric primers are designed to contain 18 nucleotides derived from the template sequences (triangles in Figure 1) and 9 nucleotides from the insert sequences (squares in Figure 1). The resulting PCR products AB and EF contain 9 nucleotides derived from an insert at one end (Figure 1). The three PCR fragments from the first step are gel-



**Figure 1. Overlap extension PCR protocol for insertion or deletion mutagenesis.** (A) A protocol for insertion mutagenesis by overlap extension PCR. The features of insertion sequences are denoted by squares (open or closed), whereas those of flanking regions are denoted by triangles (open or closed). Three PCR products are prepared first by separate reactions with appropriate primer pairs. In the second step, a ligation PCR is performed with the outermost primer pair (a and f) using a mixture of the three PCR fragments: AB, CD, and EF. A new 18-nucleotide annealing segment between two adjacent fragments (between AB and CD or between CD and EF) facilitates the extension of the second PCR. Finally, the recombined PCR product can be recloned by making use of appropriate restriction sites in two flanking regions. (B) A protocol for deletion mutagenesis by overlap extension PCR. Symbols are the same as in panel A. Two PCR products representing the flanking regions of the sequence to be deleted are prepared by using one nonchimeric and one chimeric primer. In the second step, two PCR products are used as the template for a ligation PCR that contains the outermost primer pair.

purified and are used as the template for the second PCR step. In the second step, the three products from the first step, owing to terminal complementarity, form an extended DNA product by forming duplexes during the second PCR. During this so-called overlap extension, the recombined product is amplified with the two outermost primers: primers a and f (see Figure 1).

In this study, we have successfully used the novel two-step overlap PCR method described above to insert an 87-nucleotide sequence encoding a hepatitis delta virus (HDV) antigenomic ribozyme at various positions within a hepatitis B virus (HBV) genomic construct, pCMV (cytomegalovirus)-HBV/30 (5). First, to create the HDV ribozyme insertion cassette fragment, two chimeric primers (27-mer) were designed to contain 9-nucleotide anchor sequences that are derived from each side of the insertion



**Figure 2. Application of overlap extension PCR to the insertion of an 87-nucleotide HDV ribozyme at specific position.** The first PCR yields three products: AB and EF products were generated by using pCMV-HBV/30 as a template, whereas CD product was generated by using a dimeric HDV genomic clone as a template. The PCR products AB and EF each contain the 9-nucleotide sequence derived from the HDV ribozyme sequence at one end. The PCR product CD contains a 9-nucleotide anchor sequence at both ends to the overlap AB and EF products. In a second step, ligation PCR generated the AF product bearing the ribozyme-encoding insertion. Samples were analyzed by 5% polyacrylamide gel electrophoresis. AB, 676 bp; CD, 85 bp; EF, 93 bp; AF, 820 bp; HDV, hepatitis delta virus; HBV, hepatitis B virus; CMV, cytomegalovirus.

site of HBV: forward primer (primer c in Figure 1), 5'-CACCTCTGC-GGGTCGGCATGGCATCTC-3' and reverse primer (primer d in Figure 1), 5'-GATGATTAGCTCTCCCTTAGC-CATCCG-3', in which the HDV ribozyme sequences are underlined. The first PCR was performed to produce an HDV ribozyme insertion cassette using a dimeric HDV genomic clone (a gift of John Taylor, Fox Chase Cancer Center) (6) (Figure 2, lane 2). Second, to prepare the two flanking HBV fragments, two primer pairs were designed in which one member is a chimeric primer containing a 9-nucleotide anchor sequence derived from the HDV ribozyme: (i) outermost forward primer (primer a in Figure 1), 5'-CATCAGCGCATGCGT-GGAAC-3' (the *Sph*I site is underlined) and reverse primer (primer b in Figure 1), 5'-TGCCGACCCGCAGAGGTGA-AAAAGTTG-3', where the HDV ribozyme sequences are underlined; and (ii) forward primer (primer e in Figure 1), 5'-AAGGGAGAGCTA-ATCATCTCTTGTTC-3', where the HDV ribozyme sequences are underlined and outermost reverse primer (primer f in Figure 1), 5'-TAGAATAGGGCCCTCTAGAA-3' (*Apa*I site underlined). Two separate PCRs were performed to produce two flanking HBV fragments using a wild-type HBV template (Figure 2, lanes 1 and 3). A mixture of three purified PCR products, which overlapped by 18 bp (9 nucleotides from each fragment), served as templates for a final ligation PCR performed with the outermost primers (Figure 2, lane 4). Finally, the resulting extended fragment [nucleotides 1238–1914 of HBV, nucleotides 900–815 of HDV ribozyme (6), and nucleotides 1915–1992 of HBV] was digested by *Sph*I and *Apa*I and then inserted between the *Sph*I (nucleotide 1238 of HBV) and *Apa*I (nucleotide 3069 of pDNA1/amp) sites of pCMV-HBV/30 (5). DNA sequencing analysis confirmed the precise insertion of the 87-nucleotide HDV ribozyme cassette at the insertion site with no change in flanking HBV sequences. Moreover, we found that the inserted HDV ribozyme carried out the specific cleavage of the viral pregenomic RNA at the insertion site, indicating that the inserted ribozyme is catalytically active (H.-J.

Lee, J. Lee, and W.-S. Ryu, unpublished data).

We have used a similar strategy to successfully generate deletion mutations (Figure 1B; data not shown). For deletion mutagenesis, two chimeric primers are used, which contain 18-nucleotide sequences derived from the flanking region and 9 nucleotides from the flanking region on the other side of the deletion region. Subsequent recombination of the two flanking regions was performed by an overlap PCR with the outermost primer pair (data not shown).

In summary, the overlap extension PCR strategy we describe here is a rapid and efficient method for creating insertions or deletions of any length at any position in a DNA molecule. This method is generally applicable and, therefore, represents a significant improvement to the now widely used overlap extension PCR method.

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