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Notes & Tips

Rapid gene splicing and multi-sited mutagenesis by one-step overlap extension polymerase chain reaction

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ABSTRACT

Gene splicing and site-directed mutagenesis (SDM) are important to introduce desired sequences in target DNA. However, introducing mutations at multiple sites requires multiple steps of DNA manipulation, which is time-consuming and labor-intensive. Here, we present a rapid efficient gene splicing and multi-sited mutagenesis method that introduces mutations at two distant sites via sequential connection of DNA fragments by one-step overlap extension polymerase chain reaction (OE–PCR). This bottom-up approach for DNA engineering can be broadly used to study protein structure–function, to optimize codon use for protein expression, and to assemble genes of interest.

Gene splicing and site-directed mutagenesis (SDM)[1] provide essential engineering tools to modify genes and have been widely used to elucidate protein structure–function, to optimize codon use for gene expression, and to assemble genes of interest. The polymerase chain reaction-based overlap extension (OE–PCR) approach has proven to be useful to generate chimeric DNA sequences [1,2]. This approach enables researchers to introduce desired mutations into full-length DNA template prior to cloning the gene into plasmids, which offers an advantage over existing strategies using time-consuming and labor-intensive plasmid-based SDM [3,4]. Despite simplicity, the procedure of OE-PCR is till length when mutations at multiple sites are needed [5]. Here, we report one-step OE–PCR that rapidly and efficiently introduced multi-sited mutagenesis and gene splicing by sequential connection. As demonstrated in Fig. 1, the presented method enabled site-directed mutagenesis at two sites via hinge-pair primers, which were designed to primarily match existing template sequences and to introduce mutation sequences at the far end. The adjacent hinge-pair primer was designed to reverse complement the mutated sequences. Once two adjacent DNA templates were combined, the mutated sequences would appear only once. Depending on the positions, one or two hinge-pair primers were used to amplify each DNA fragment.

The amplification protocol consisted of two rounds of PCR to introduce mutations at two distant sites. In the first round of PCR, three DNA fragments (F1, F2, and F3) were independently amplified from a recombinant plasmid using mutagenic primers (F1F and F1RT, F2FT and F2RT, and F3FT and F3R; see Table 1). The first 20 bp of F1RT and F2FT were reverse complements of each other; the same was true for F2RT and F3FT. Thus, the sequences at the 5′ end of F2 were identical to those at the 3′ end of F1; similarly, the sequences at the 3′ end of F2 were identical to those at the 5′ end of F3. The PCR was conducted in a standard mixture (a volume of 100 μl) containing 2.5 mM $MgCl_2$, 100 μM deoxyribonucleotide phosphates (dNTPs), 0.2 μM of each primer, 0.4 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 10 ng of recombinant plasmid. The reaction was carried out using the following program: 94 °C (5 min); 35 cycles of 95 °C (30 s), 52 °C (30 s), and 72 °C (30 s); and a final elongation at 72 °C (5 min) prior to storage at 4 °C. Agarose gel analysis (Fig. 2A) showed successful amplification of PCR fragments with the expected sizes for F1 (69 bp), F2 (125 bp), and F3 (170 bp).

In the second round of PCR, the overlapping sequences served as primers to allow for extension of the intermediate chimeric products (i.e., F1–F2 and F1F2–F3). Briefly, F1, F2, and F3 were sequentially added into a PCR tube to generate a full-length chimeric DNA fragment, which was then amplified by two flanking primers (F1F and F3R). To create the chimeric products, a presumable PCR

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[1] *Abbreviations used:* SDM, site-directed mutagenesis; OE–PCR, polymerase chain reaction-based overlap extension.

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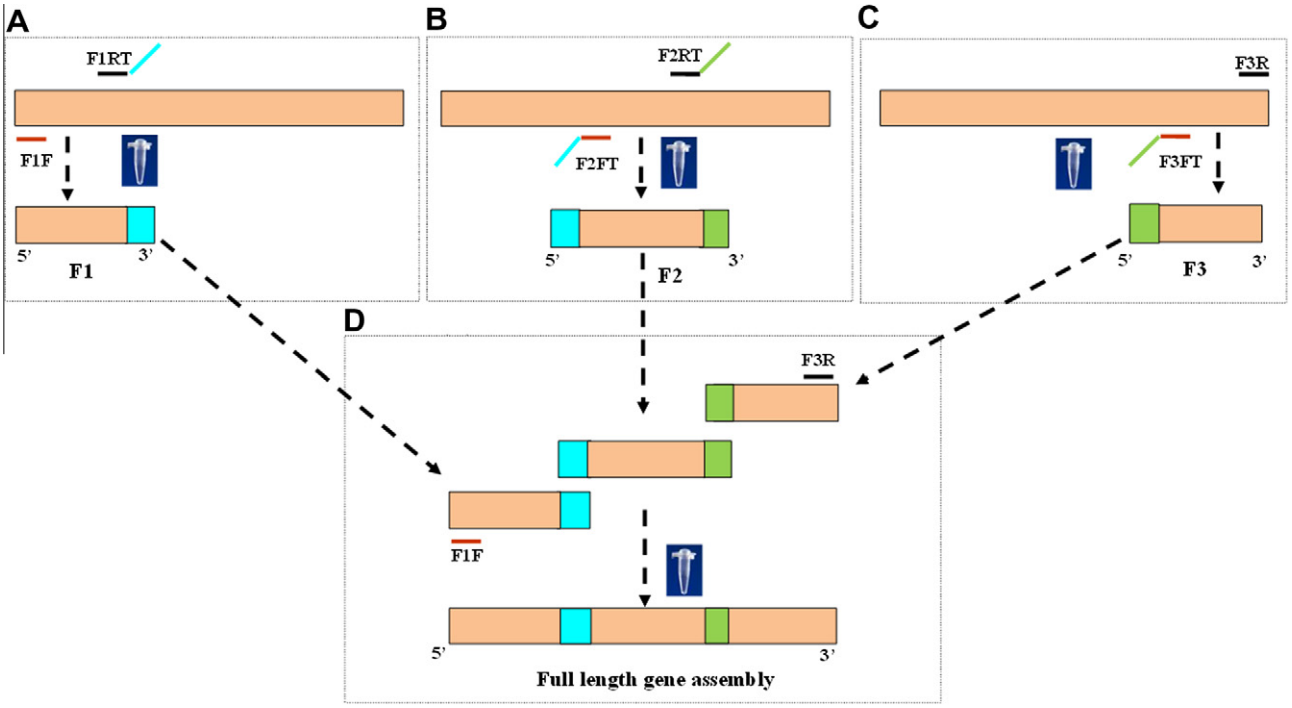

Fig.1. Gene splicing and SDM by modified OE–PCR. Solid boxes indicate double-stranded DNA. The bottom line indicates the antisense strand, and the top line indicates the sense strand. In the first round of PCR (A–C), DNA fragments F1, F2, and F3 were individually amplified using paired primers F1F and F1RT, F2FT and F2RT, and F3FT and F3R, respectively. F1 had addition of tail sequences at the 3′ end (cyan), F2 had addition of tail sequences at both the 5′ (cyan) and 3′ (green) ends, and F3 had addition of tail sequences at the 5′ end (green). In the second round of PCR (D), sequential addition of F1 and F2 in a PCR mixture without primers, and then F3, and then primers (F1F/F3R) allowed for the amplification of a full-length DNA in one reaction tube. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Primers used for site-directed mutagenesis by OE–PCR.

Name	Sequence (5′–3′)	Length (nt)
F1F	AGATCTGAGCCTGGGAG	17
F1RT	GGTTCCGTTATTCCCAAGTTTTAAGCAGTGGGTTCCCTAG	40
F2FT	AACTTGGGAATAACGGAACCGAGTGCTTCACTTGATACAG	40
F2RT	GCCAATCTCTCAATCGCGGCATTTTCCACACTGACTAAAAG	41
F3FT	GCCGCGATTGAGAGATTGGCCAGGGACCTGAAAGCGAAAG	40
M13	CTGGCCGTCGTTTTAC	16

Note: Underlined sequences correspond to overlapping bases. nt, nucleotides.

program consisted of 5 cycles of 95 °C (30 s), 52 °C (30 s), and 72 °C (30 s). Initially, F1 and F2 were added to the same PCR mixture except for primers to generate the F1–F2 intermediate. Then, F3 was added to create the F1F2–F3 product. Finally, with the addition of primers F1F and F3R (0.2 μM), large amounts of the full-length chimeric fragment were generated by 35 cycles of the same PCR program. To ensure that all products were fully extended, the reaction was incubated at 72 °C for 5 min prior to storage at 4 °C. As a control, F1, F2, and F3 were added to the same PCR mixture containing primers F1F and F3R and amplified in parallel for only 35 cycles (without 10 cycles of preamplification). As shown in Fig. 2B, sequential addition of DNA fragments (F1, F2, and F3) and flanking primers in the OE–PCR obtained a clear DNA band of 324 bp; by contrast, the control PCR showed only very faint DNA bands at the 324-bp position. These results indicate that sequential connection of DNA fragments, as opposed to randomized connection, is of importance to achieve high fusion efficiency. The obtained full-length DNA fragment was cloned in a pCRII T vector (Invitrogen). The colony PCR using primer M13/F3R results showed a clear DNA band of 435 bp, indicative of insertion of target DNA (Fig. 2C).

In this study, we improved OE–PCR for introducing mutations at multiple sites with high efficiency. This OE–PCR method is distinct from previous gene assembly approaches [5–10] in that we sequentially connected DNA fragments in a wanted order, thereby improving the efficiency of assembling the full-length DNA fragment as we recently published [11]. This claim was supported by a comparison of our modified OE–PCR method and the standard OE–PCR, in which multiple DNA fragments were added simultaneously (Fig. 2B). The low efficiency observed in standard OE–PCR may be due to a considerable amount of incomplete intermediates, which were created by the presence of flanking primers during the initial rounds of PCR. In this case, the presence of incomplete intermediates creates null events, such as reannealing of the original fragments and incomplete intermediates, and drives down the number of effective interactions between the appropriate DNA fragment ends, compared with sequential assembly in the absence of flanking primers. Accordingly, a poorer assembly efficiency may be expected when more than three DNA fragments are simultaneously added to achieve multi-sited mutagenesis. To assemble multiple DNA fragments, several rounds of OE–PCR can be performed to improve the efficiency. However, this strategy requires much longer time to obtain the full-length DNA with intended mutations than our approach (6.5 h: 2.5 h for the first round of PCR +1.5 h for gel electrophoresis and gel extraction +2.5 h for the second round of PCR). Furthermore, this approach enabled us to introduce continuous point mutations up to 20 bp at two sites in one reaction.

In conclusion, the presented OE–PCR with a modified protocol significantly improves the efficiency of gene splicing and SDM at multiple sites. The modified OE–PCR protocol would be particu-

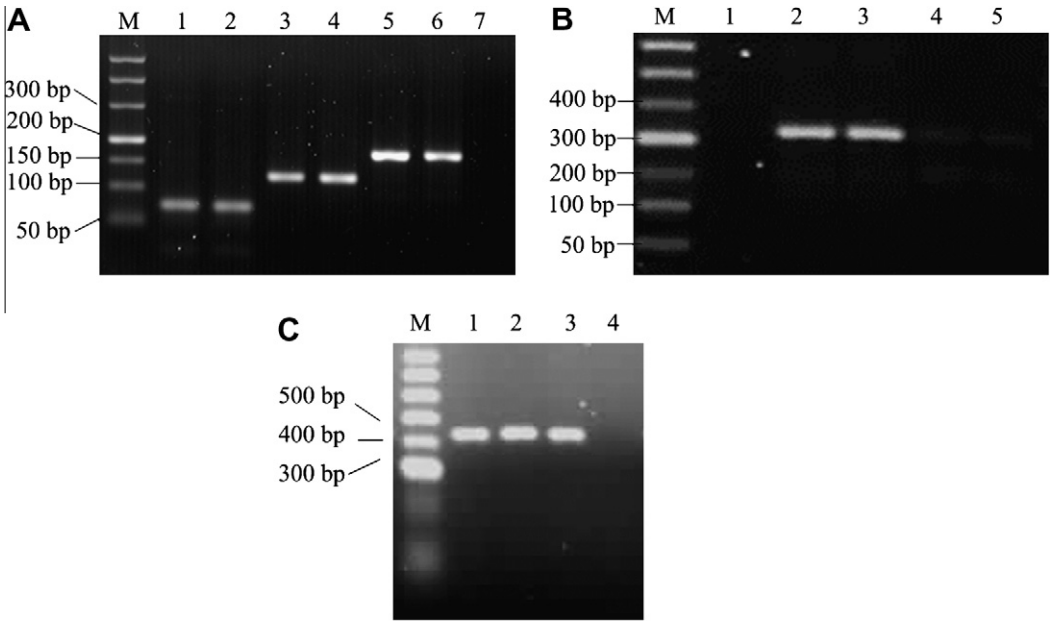

Fig.2. Electrophoresis of PCR products on agarose gels (1.5%). (A) F1, F2, and F3 of a DNA template were individually amplified from a recombinant plasmid by standard OE–PCR. M, DL500 DNA marker (Takara); lanes 1 and 2: F1 products; lanes 3 and 4: F2 products; lanes 5 and 6: F3 products; lane 7: negative control. (B) Full length of fused DNA fragment was amplified by a modified OE–PCR. M, 100-bp DNA standard; lane 1: negative control; lanes 2 and 3: full length of fused DNA fragment, which was amplified by sequentially adding DNA fragments F1, F2, and F3, and primers F1F/F3R; lanes 4 and 5: full length of fused DNA fragment, which was amplified by simultaneously adding DNA fragments F1, F2, and F3 together with primers F1F/F3R. (C) Three white colonies (lanes 1–3) and one blue colony (lane 4) were analyzed using colony PCR.

larly useful to assemble genes of desired sequences when multiple DNA fragments are involved. In addition, this protocol is amenable to insertions and deletions at intended sites assuming that mutagenic primers are appropriately designed. Hence, the modified OE–PCR offers great convenience for DNA manipulations that can facilitate a variety of gene-related research.

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