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.

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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-

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1F</td>
<td>AGATCTGAAGCTTGGGAG</td>
<td>17</td>
</tr>
<tr>
<td>F1RT</td>
<td>GGTTCCGTTATCTCAAGTTACAGTGTTTCCCAGTAG</td>
<td>40</td>
</tr>
<tr>
<td>F2FT</td>
<td>AACTTGGGAATAAGCCAGTGTACGTTTCAGTACACG</td>
<td>40</td>
</tr>
<tr>
<td>F2RT</td>
<td>GCCAAATCTCAATCAGGCCATTTCCACACTGAAAG</td>
<td>41</td>
</tr>
<tr>
<td>F3FT</td>
<td>GCCGGCGATTGAGAGATGCGAGGGACCCCTGAAAG</td>
<td>40</td>
</tr>
<tr>
<td>M13</td>
<td>CTGGCCGCTGTCTTAC</td>
<td>16</td>
</tr>
</tbody>
</table>

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


