Note

Minimum Length of Homology Arms Required for Effective Red/ET Recombination

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The original protocol of Red/ET recombination requires 50-bp sequence homology with target vector on both sides of the DNA fragment. To make it more cost effective, we investigated to determine the minimal length of homology required for the system to work. We found that a homology of 9-bp was sufficient to achieve homologous recombination with more than 50% efficiency.

Key words: Red/ET recombination; glutaredoxin5 (Glrx5); mitochondria; short homology arms

There are several methods for the introduction of foreign DNA fragments into plasmid vectors. The most common involves digestion of both vector and foreign DNA fragment with the same combination of restriction enzymes and joining of them by DNA ligase. Cloning with ligation is often hampered as the total size of insert and vector increases, since a large molecule tends to be concatametered and is difficult to circularize under ordinary concentrations of DNA. Furthermore, the limited number of appropriate restriction sites in the insert and vector DNA render the construction of the recombinant molecule less flexible. To overcome these constrains, several methods have been reported, based on either site-specific recombination or homologous recombination.

Site-specific recombinations cause rearrangements of DNA segments by taking advantage of recombinases that recognize short specific DNA sequences (sites), at that they cleave the DNA backbone, exchange the two DNA helices involved, and rejoin the DNA strands. Several methods based on site-specific recombination are available, including the Gateway™ system, the Cre-loxP system and the FLP/FRT system. The Gateway™ system is derived from lambda phage system, requires 22 or 25 bp specific sequences called attB flanking each side of the insert for the reaction.1,2 For Cre-loxP or FLP/FRT recombination, 34-bp specific sequences, called loxP and FRT respectively, are also required.3,4 Since these specific sequences always remain after recombination at the site of recombination, this method is not suitable for in-frame insertion of foreign DNA into an open reading frame.

In contrast to these methods, cloning systems based on homologous recombination including In-Fusion™ cloning and Red/ET recombination, have wider potential for use.3,5 Since homologous recombination does not require specific sequences for recombination, the seamless insertion of any DNA fragment at any desired position can be achieved in vitro or in vivo. The In-Fusion™ enzyme reaction can join any two pieces of DNA that have 15-bp of identity at their ends.3,5 The 15-bp overlap can be achieved through its inclusion in the primers used to PCR amplify a segment of DNA. Thus the average length of primer is around 35 nucleotides, containing an average length of 20 nucleotides corresponding to the PCR template. Although the cost of primer synthesis may not be high, the expensive enzyme used in the In-Fusion™ makes this system unsuitable for routine molecular cloning. Similarly, Red/ET recombination, a powerful homologous recombination system based on the Red operon of lambda phage or RecE/RecT from Rac phage, requires 50-bp of homology to join two pieces of DNA.3,5 Thus a total length of about 75 nucleotides is needed in each primer for PCR amplification despite the negligible cost of the recombinase encoding plasmid.

To reduce the cost of Red/ET homologous recombination employing primers of shorter length, we investigated to ascertain the minimal length of homology required for Red/ET recombination. We found that 9-bp homology was sufficient to achieve effective homologous recombination. We further applied this method in the construction of EGFP fusion protein by inserting the human Glrx5 gene, that product is localized in the mitochondria, in-frame.3,5 When we transfected this plasmid into COS7 cells, the mitochondrial localization of the GLRX5-EGFP fusion protein was observed, indicating the usefulness of our protocol.

pUC119-SacB was constructed by inserting the PCR-amplified SacB gene from pDN1 (Invitrogen, Carlsbad, C/) into XbaI-digested, Klenow DNA polymerase-

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Abbreviations: EGFP, enhanced green fluorescent protein; Glrx5, glutaredoxin5; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; FBS, fatal bovine serum
treated pUC119 vector. Linearized pUC119 vector was prepared from pUC119-SacB by treating it with the indicated restriction enzymes to remove the SacB gene, and then was treated with the Klenow DNA fragment to fill the 5′-protruding ends. The SacB gene acts as a negative selection marker that it possible to select against the bacteria still containing a nonlinearized vector, and thus makes to reduce the background growth of non-recombinant colonies. To quantify the cloning efficiency and the recombination efficiency of Red/ET recombination in model experiments, the Renilla luciferase gene and the Firefly luciferase gene were used as target DNA fragments. pUC119-Renilla Luci was constructed by inserting the 935-bp NheI/XbaI Renilla luciferase gene from pRL-TK (Promega, Madison, WI) into the XbaI site of pUC119. pUC119-Firefly Luci was constructed by inserting the 1656-bp NheI/XbaI Firefly luciferase gene from Pikagene Basic vector2 (Toyo Ink Group, Tokyo) into the XbaI site of pUC119. The PCR-amplified Renilla and Firefly luciferase genes used for Red/ET recombination were prepared using primers (Figs. 1A and 2A) and pUC119-Renilla Luci or pUC119-Firefly Luci as template. The Glrx5 gene was amplified by RT-PCR using primers cagatctcga accatgacgggtccctcgg and gtacctcgactggactttctttct (underlined sequences indicate short homology arms).

Red/ET recombination was carried out by following the instruction manual from Gene bridges, with some
modifications. The linearized vectors (100 ng) and the PCR-amplified target gene (50 ng) were mixed with Top10 competent cells (Invitrogen) pre-transformed with pRedET (Gene Bridges, Heidelberg) for the expression of Red/ET recombinase. After recombination, colonies grown on agarose plates containing 1% sucrose and ampicillin or kanamycin were inoculated in LB medium, and plasmids were extracted from them.

COS7 cells were maintained in DMEM medium containing 10% FBS. Cells were transfected with plasmid using FuGENE HD transfection reagent (Roche, Basel) and cultured for 48 h.

To identify the minimum length of homology arms required for Red/ET recombination between the PCR-amplified target gene and the linearized plasmid, the Renilla luciferase gene was amplified by PCR using a series of primers flanked by 5–50 bp of sequence nucleotide with multi cloning sites of pUC119 (Fig. 1A). Red/ET recombination was carried out with EcoRI/HindIII-digested/blunt-ended pUC119 prepared from pUC119-SacB and the Renilla luciferase gene containing various lengths of homology arms, and plasmids were extracted from the transformed E. coli. As shown in Fig. 1B, the Renilla luciferase gene containing 50-bp homology arms on both sides was successfully introduced into pUC119 with a recombination efficiency of 92.5% and high cloning efficiency. Similarly, the Renilla luciferase gene with 9-bp homology on both sides was also successfully introduced into pUC119 with a recombination efficiency of 64.2%, although the cloning efficiency decreased to about 50% as compared to that of the 50-bp homology arms. Recombination efficiency, however, dropped sharply, below 37.5%, when the length of the homology arms flanking Renilla luciferase gene was less than 9-bp on both sides, as shown in Fig. 1C, D.

Next we tested whether the DNA sequence within and between homology arms would influence recombination efficiency. The Firefly luciferase gene flanked by 5–30 bp of homology arms on both sides was amplified by PCR (Fig. 2A). Red/ET homologous recombination was carried out between BamHI/SalI-digested/blunt-ended pUC119-SacB and the Firefly luciferase gene with various lengths of homology arms. As shown in Fig. 2B and C, the Firefly luciferase gene flanking the 30-bp homology arms on both sides was successfully introduced into pUC119 with a recombination efficiency of 100% and high cloning efficiency. While a recombination efficiency of 83.3% was found in the case of 9-bp homology, the cloning efficiency was less than 20% as compared to that of observed with the 30-bp flanking sequence. These results indicate that homology arms longer than 9-bp are sufficient in most cases of Red/ET recombination to introduce the PCR product into the linearized vector.
Although the observed recombination efficiency was more than 60%, the cloning efficiency was influenced by the sequence of DNA fragments and that of its flanking homology arms.

The $\text{Glrx5}$ gene encodes a 16.6-kDa mitochondrial protein that is required for the mitochondrial biogenesis of Fe–S clusters.\(^5\) To test whether the Red/ET recombination with short homology arms can be used in routine subcloning, we amplified the $\text{Glrx5}$ gene and tried to insert it in-frame into mammalian EGFP expression vector, pEGFP-N1, to express the GLRX5-EGFP fusion protein in the mitochondria. As shown in Fig. 3A, the PCR-amplified $\text{Glrx5}$ gene carrying 10-bp homology arms with multi cloning site of pEGFP-N1 were successfully introduced into pEGFP-N1 (Fig. 3B). DNA sequencing of the plasmids containing the 474-bp insert revealed that the $\text{Glrx5}$ gene was in-frame fused with the EGFP gene in all the clones examined. To confirm the function of the resulting plasmids, COS7 cells were transiently transfected with the PCR-amplified the $\text{Glrx5}$ gene and the linearized pEGFP-N1 and then plated on kanamycin agar plates. Homology arms are outlined with solid boxes. B. Agarose gel electrophoresis of the $\text{BglII/SalI}$-digested plasmid. Ten colonies were isolated, and plasmids were extracted from them. Agarose gel electrophoresis showed that 9 of the 10 plasmids contained the target $\text{Glrx5}$ gene (0.5Kb). C. Localization of the GLRX5-EGFP fusion protein in the mitochondria. COS7 cells transfected with the Glrx5-EGFP expression plasmid was observed under a fluorescent microscope, and the localization of the GLRX5-EGFP fusion protein was determined.

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Wang et al. have reported that the minimum length of identity required for Red/ET recombination with two single-strand phagemids is somewhere between 11 and 30bp at both arms, with less than 5% recombination frequency.\(^7\) In contrast, our tests using two linearized double-strand DNA molecules of an insert and a vector showed that a homology of 9-bp is sufficient to achieve homologous recombination with more than 50% recombination efficiency. Thus, our results clearly showed the usefulness and reliability of Red/ET recombination with short homology arms for the routine cloning of the PCR product into the linearized vector.

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