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Robust multi-type plasmid modifications based on isothermal in vitro recombination

Qin-Long Zhu *, Zhong-Fang Yang, Qun-Yu Zhang, Le-Tian Chen, Yao-Guang Liu *

State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, College of Life Sciences, South China Agricultural University, Guangzhou 510642, China

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ABSTRACT

A robust strategy for multi-type plasmid modifications is developed based on the isothermal in vitro recombination technology, by which any combination of the sequence modifications can be efficiently achieved in plasmids at any desired position in a seamless manner. As an example, we showed that a plasmid modification with insertion of a *GFP* gene, deletion of a 623-bp fragment, and substitution of an ampicillin resistance gene by a kanamycin resistance gene was accomplished simultaneously by this method. Therefore, the isothermal in vitro recombination-based multi-type plasmid modification strategy is a useful approach for broad application prospects in molecular biology studies.

1. Introduction

Plasmid construction and modification are essential processes of molecular biology studies, including sequence insertion, substitution, deletion, and site-mutagenesis. However, it is time-consuming and labor-intensive task using restriction digestion and ligation. Beside the traditional methods a variety of ligation-independent methods for plasmid construction and modification have been reported, including Gateway (Sasaki et al., 2004), uracil-specific excision reagent (USER) (Nour-Eldin et al., 2006), sequence- and ligase-independent cloning (SLIC) (Li and Elledge, 2007), overlapping PCR (Cha-aim et al., 2009), circular polymerase extension cloning (CPEC) (Quan and Tian, 2009), nicking DNA endonuclease (NiDE) (Yang et al., 2010) and Ω-PCR (Chen et al., 2013). However, these methods still have some limitations and are not able to achieve simultaneously multiple types of sequence modifications in single reactions. Although the Golden Gate cloning-based method based on the use of the type II restriction enzymes, such as *Bsa* I, enables ligation of multiple fragments simultaneously in some cases (Yan et al., 2012; Zhou et al., 2013), the possible presence of the enzyme site(s) within the target fragments or parental plasmids limits its applications.

Among the reported DNA assembly methods, the isothermal in vitro recombination reaction, also called "Gibson Assembly", has powerful capabilities to assemble multiple overlapping DNA molecules up to several hundred kilobases (Gibson, 2011; Gibson et al., 2009, 2010). This method takes advantage of the collaborative actions of an enzyme mixture containing the 5′-T5 exonuclease, the Phusion polymerase and the *Taq* DNA ligase in a single reaction at 50 °C. The T5 exonuclease chews back the overlapping 5′ ends of DNA fragments (PCR products or restriction enzyme-cut plasmids) to generate single-stranded 3′ overhangs. By annealing of complementary fragments at the overlapping regions, Phusion polymerase fills in the gaps and then *Taq* DNA ligase seals the nicks. Based on this technique, Jiang et al. have developed a method to construct hairpin RNA interference vectors in one-step manner (Jiang et al., 2013).

In this study, we demonstrated that the isothermal in vitro recombination technique can be efficiently applicable to create plasmids with multiple types of sequence modification in single reactions. As an example, we constructed a green fluorescent protein (GFP) expression vector by introducing three types of sequence modification on the basis of a plasmid pGEX-4T-2: an insertion of the GFP gene; a substitution of an ampicillin (Amp) resistance gene by a kanamycin (Kan) gene; and a deletion of a 623-bp fragment.

2. Materials and methods

2.1. Primer design

The primers for assembling fragments contain an extra 5′-end overlapping homologous region and a fragment specific sequence at 3′-end. Based on the sequences of the target and plasmid backbone fragments, the 5′-overlapping homologous regions were designed into the primers (Fig. 1A). The desired lengths of overlapping regions for isothermal recombination reaction are about 20 bp or more (Jiang et al., 2013). All primers used in this paper are listed in Table S1, and the locations of them in the plasmid construct are shown in Fig. 1B.

Abbreviations: PCR, polymerase chain reaction; GST, glutathione S-transferases; GFP, green fluorescent protein; ISO buffer, isothermal reaction buffer.

* Corresponding authors.
E-mail addresses: zhuql@scau.edu.cn (Q.-L. Zhu), ygliu@scau.edu.cn (Y.-G. Liu).

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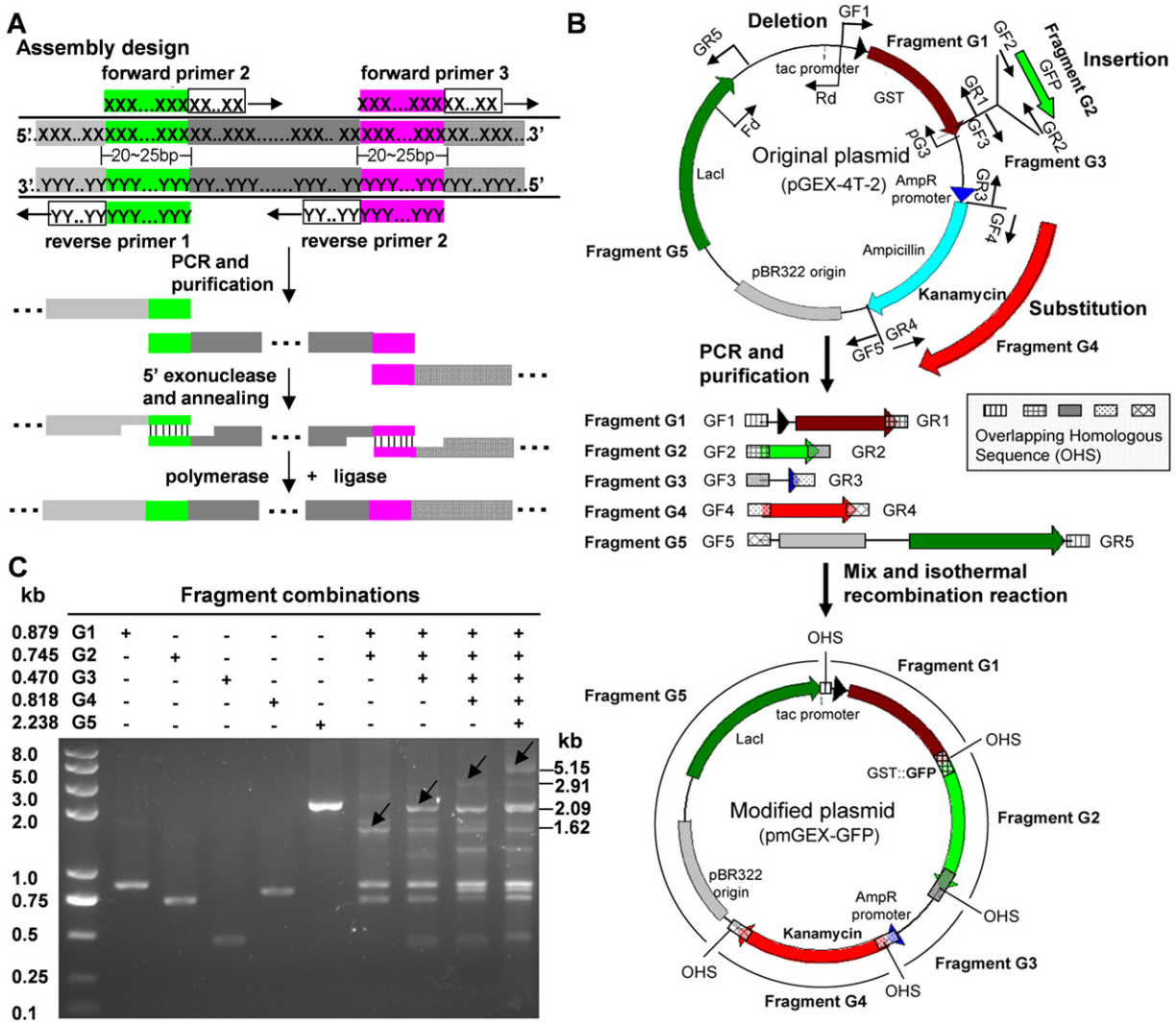

Fig. 1. Multi-type sequence modification strategy based on isothermal in vitro recombination reaction. (A) Primer design for assembly of DNA fragments. X and Y are any pair of complementary nucleotides in desired assembly products. For each primer, it contains an extra 5′-overlapping region marking on green or pink background and 3′-specific sequence in boxes. DNA fragments are linked by base paring between the overlapping ends, extending by polymerase, and ligated by ligase. (B) Plasmid construction strategy by one-step assembling with three types of sequence modification (insertion, substitution and deletion). An insertion of the *GFP* gene, a substitution of the kanamycin gene, and three fragments of the plasmid pGEX-4T-2 backbone with a sequence deletion are simultaneously linked to generate a novel plasmid pmGEX-GFP in a single isothermal in vitro recombination reaction. (C) The assembly reactions with different numbers of the PCR fragments. The linked product (G1 + G2 + G3 + G4 + G5, 5.15 kb) and other intermediate combinations (e.g. G1 + G2, G1 + G2 + G3, G1 + G2 + G3 + G4) were displayed on agarose gel electrophoresis. Arrows indicate the predicted target products of the fragment combinations.

2.2. PCR amplification and purification of DNA fragments

The primer pairs GF2/GR2 and GF4/GR4 were used to amplify the *GFP* gene (insert fragment G2) and kanamycin gene (substitution fragment G4), respectively. Other primer pairs amplify the fragments G1, G3, and G5 from the pGEX-4T-2 plasmid, by which a 623-bp fragment was deleted (Fig. 1B). The PCRs were performed using a high fidelity KOD FX Polymerase (KFX-101, TOYOBO, Japan) under the recommended reaction conditions. The PCR products were purified using a gel purification kit, or were treated with *Dpn* I to digest the original plasmid followed by purification with a PCR product purification kit.

2.3. One-step isothermal in vitro recombination reaction

The agent of isothermal in vitro recombination reaction was homemade and modified according to Gibson et al. (2009). Briefly, the 2× master mixture was prepared by combining 160 μL 5× ISO buffer (25% PEG-8000, 500 mmol/L Tris-HCl pH 7.5, 50 mmol/L $MgCl_2$, 50 mmol/L DTT, 1 mmol/L each of dNTPs, 5 mmol/L NAD, stored at −20 °C), 1.5 units of T5 exonuclease (Epicentre), 20 units of Phusion polymerase (NEB), 2000 units of *Taq* ligase (NEB), and deionized water to a final volume of 0.4 mL. The concentrations of PCR fragments were determined by agarose gel electrophoresis and NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). All PCR products were mixed in equimolar amounts (about 0.1 pmol for each fragment, with about 3.5 ng for 100 bp sequence) in 10 μL, and then 10 μL of 2× master mixture was added and mixed. The reaction was carried out at 50 °C for 30 min. Then, 2 μL of the reaction product was transformed into 50 μL of *Escherichia coli* DH5α competent cells, plated on LB-Kan plates, incubated overnight at 37 °C and then screened for the desired plasmid construct (pmGEX-GFP).

3. Results

In this example of assembly reaction, five fragments of the target sequences and plasmid backbone were involved. The whole process could be summarized as three steps: primer design, PCR amplification of DNA fragments, and one-step isothermal in vitro recombination reaction (Fig. 1A and B). To evaluate the efficiency of the method, 10 μL of reaction products for combinations of the five fragments (G1–G5) was detected by agarose gel electrophoresis (Fig. 1C). The transformed DH5α