Mol Biotechnol (2013) 55:10–16
DOI 10.1007/s12033-012-9597-5

RESEARCH

A Novel T-type Overhangs Improve the Enzyme-Free Cloning of PCR Products

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Published online: 4 June 2013

Abstract PCR product cloning is the foundational technology for almost all fields in the life sciences. Numerous innovative methods have been designed during the past few decades. Enzyme-free cloning is the only one that avoids post-amplification enzymatic treatments, making the technique reliable and cost effective. However, the complementary staggered overhangs used in enzyme-free cloning tend to result in self-ligation of the vector under some circumstances. Here, we describe a "T-type" enzyme-free cloning method: instead of designing the complementary staggered overhangs used in conventional enzyme-free cloning, we create "T-type" overhangs that reduce the possibility of self-ligation and are more convenient for multi-vector cloning. In this study, we systematically optimize "T-type" enzyme-free cloning, compare its cloning background with that in conventional enzyme-free cloning, and demonstrate a promising application of this technique in multi-vector cloning. Our method simplifies post-amplification procedures and greatly reduces cost, offering a competitive option for PCR product cloning.

Electronic supplementary material The online version of this article (doi:10.1007/s12033-012-9597-5) contains supplementary material, which is available to authorized users.

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Keywords Molecular cloning · PCR product cloning · Enzyme-free cloning · "T-type" enzyme-free cloning · Ligase-independent cloning

Introduction

Molecular cloning is a time-honored and ever-improving technology that is foundational to the life sciences. Since its invention in the 1970s [1–3], this traditional restriction and ligation based cloning method has been widely used. However, it is constrained by a requirement for suitable restriction sites in destination vectors and the use of specific endonucleases. Various innovations that overcome this limitation have been implemented and mainly involve the use of two kinds of enzymes: recombinase that promote recombination between genes and destination vectors, or exonucleases that create hetero-staggered overhangs which allow directional cloning of genes into vectors. Some of these technologies have already led to the production of efficient commercialized cloning kits, including the Gateway system from Invitrogen, the In Fusion system from Clontech, and the Cold Fusion cloning system from System Biosciences. The Gateway system depends on site-specific recombination between insert and destination vectors, while directional cloning is achieved in the latter two systems by the annealing of complementary single-stranded overhangs that flank linearized exonuclease-pretreated DNA fragments, similar to ligase-independent cloning (LIC) [4–6]. Sequence and ligation independent cloning (SLIC) is an improved version of LIC that allows assembly of multiple DNA fragments [7]. Other newly developed techniques, including restriction-free (RF) cloning [8, 9] and circular polymerase extension cloning (CEPC) [10],

are based on polymerase extension of the overlapping regions between inserts and vectors to amplify the whole plasmid backbone plus the insert.

Although each of the methods mentioned above have their own advantages and special applications, none of them avoid the use of expensive and easily degraded enzymes after PCR amplification of DNA. Tillett and Neilan [11] reported an enzyme-free cloning (EFC) method in 1999 with which they successfully cloned a short insert into plasmid pUC19 via a post-PCR denaturation-hybridization reaction. However, no systematic optimization of these procedures has been performed. Matsumoto and Itoh [12] modified the EFC method to allow the cloning of multiple fragments; however, their method, like LIC, uses complementary staggered overhangs [13] that sometimes result in plasmid self-ligations. Here we investigate the use of complementary "T-type" overhangs instead of the complementary staggered overhangs (we named them "Z-type" overhangs Fig. 3a) used in the conventional EFC methods mentioned above. We designed tailed primer sets to PCR amplify inserts or vectors to produce tailed DNA fragments. Tailed fragments were then mixed with their homologous non-tailed PCR products at a molar ratio of 1:1. A denaturation-hybridization reaction of the mixture produced "T-type" overhangs: a PCR product with a 5′ overhang and a 3′ overhang (Fig. 1). These overhangs were designed to be complementary between inserts and vectors. The remaining procedures were the same as those in the conventional EFC method. In this study, we optimized this "T-type" enzyme-free cloning (TEFC) method and demonstrated its advantages and one of its promising applications.

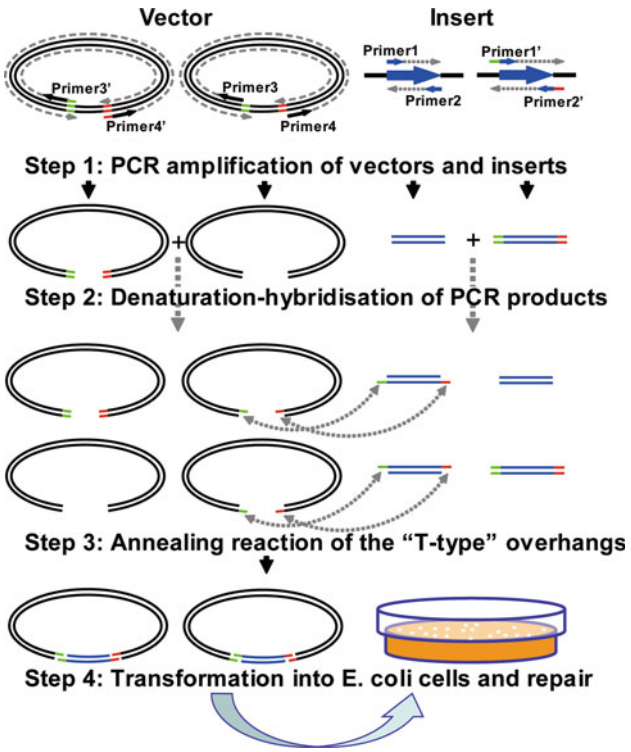

Fig. 1 Schematic illustration of TEFC. Four PCR reactions are conducted using four primer sets, two for vectors and two for inserts. The first pair of primers for vector PCR contain tailed sequences (*colored in green or red*) at their 5′ ends while the second pair does not. As a result, the first vector PCR product contains tailed sequences at both of its ends and the second vector product does not. Equimolar volumes of the two products are mixed and incubated in a denaturation-hybridization reaction. In theory, 50 % of the four resulting annealing products should contain a 5′ overhang and a 3′ overhang, named "T-type" overhangs. Primers for the insert are designed in a similar manner: one pair with a tailed sequence and the other pair without. The only difference is that the tailed sequence designed for the insert is reverse-complementary to the tailed sequence (*same color*) designed for the vector. As a result, the "T-type" overhangs from the vector will anneal with the "T-type" overhangs from the insert, allowing the assembly of two kinds of circular recombinant DNA with four nicks. These nicks will be repaired after transformation into *E. coli* cells (Color figure online)

Materials and Methods

Materials

PAGE-purified DNA primers were obtained from Sangon Biotech (Shanghai, China). Tris-base and EDTA were purchased from AMRESCO LLC. (Solon, OH, USA.); NaCl, DMSO, and other chemicals were obtained from Beijing Chemical Works (Beijing, China). The annealing buffer contained 10 mM Tris–HCl, pH 7.5, 100 mM NaCl and 1 mM EDTA.

T-type EFC Method

We amplified inserts, such as MSMEG_6199 (0.4 kb), in parallel using two sets of primers with a high-fidelity DNA polymerase (KOD plus; TOYOBO BIOTECH CO., LTD, Shanghai). One set of primers were non-tailed primers: Primer1, MSMEG_6199-F (5′-GTGTCAGCTACACACA CTGTCGCACGTAAG-3′) and Primer2, MSMEG_6199-R (5′-TCAGACTGCGCTGCGGTGCTTG-3′); and the other set were tailed primers: Primer1′, MSMEG_6199-20bL-F (5′-**TGGATATCGGA**GTGTCAGCTACACACACTGTC GCACGTAAG-3′) and Primer2′, MSMEG_6199L-20b-R (5′-**AAGCTTGTCGAC**GACTGCGCTGCGGTGCTTG-3′). Linear complementary vectors, such as pET20b (3.7 kb), were also produced in two parallel PCRs using either Primer3, pET20bS-F (5′-TGGCCATCGCCGGCTGGG CA-3′) and Primer4, pET20bS-R (5′-GCGGCCGCACT CGAGCACCAC-3′); or Primer3′, pET20bL-F (5′-**TCCGA TATCCA**TGGCCATCGCCGGCTG-3′) and Primer4′, pET20bL-R (5′-**GTCGACAAGCTT**GCGGCCGCACTC GAG-3′). The vector template was first linearized by PCR

or restricted endonuclease treatment. Sequences indicated in bold in the tailed primers were designed to be reverse-complementary between the insert and the vector. Detailed PCR conditions for both the inserts and the vectors are listed in table S1. The two MSMEG_6199 or two pET20b PCR products were mixed at an equal molar ratio and co-purified separately with a wizard gel purification kit (Promega, Madison, WI).

Purified MSMEG_6199 or pET20b mixtures were then added in a 20 µl denaturation and hybridization buffer system (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) at the DNA concentrations indicated. The mixture was heat-denatured for 5 min at 95 °C, then slowly cooled to room temperature. Theoretically, 50 % of the pooled DNA fragments should produce single-stranded "T-type" overhangs after the denaturation and hybridization reaction. The resulting MSMEG_6199 and pET20b PCR products were mixed together in equal volumes in a 10 µl annealing reaction system. The annealing reaction was held at 65 °C for 5 min, then slowly cooled to room temperature. Three microliters of the annealed mixtures were used to transform 30 µl of chemically competent Top10 *Escherichia coli* cells (Transgene, Beijing). The resulting colonies were verified by colony PCR and double digestion with restriction endonucleases.

Results and Discussion

Optimization of the TEFC Method

The key step in this method is the denaturation-hybridization reaction. Since the initial concentration of DNA influences the efficiency of renaturation [14], we first optimized this step by comparing cloning efficiency at different input DNA concentrations. Tailed and non-tailed MSMEG_6199 PCR products were copurified at a final concentration greater than 150 ng/µl. The mixture was then serially diluted from 1 to 100 ng/µl with annealing buffer and added to the denaturation-hybridization reaction. Tailed and non-tailed PCR products of vector pET20b were also copurified and serially diluted in the same way. Subsequent steps were the same as those described in the Methods section. Cloning efficiency was estimated as the number of recombinants produced per nanogram of input vector. As shown in Fig. 2a, maximum efficiency was reached at an initial DNA concentration of 2 ng/µl. Efficiency then decreased dramatically as input DNA concentration increased from 5 to 25 ng/µl. Cloning efficiency further reduced to a relatively low stable level when DNA concentrations were higher than 50 ng/µl. The accuracy of our cloning method was verified by PCR colony amplification and restriction enzyme digestion of randomly chosen recombinants

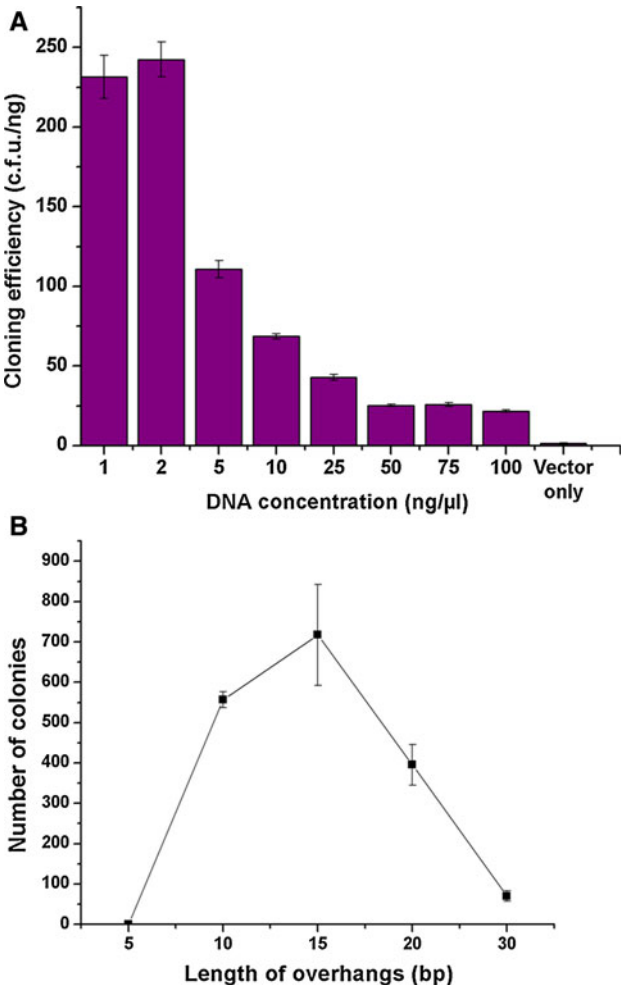

Fig. 2 Optimization of the TEFC method. **a** Optimization of DNA concentration. Two PCR fragments at various initial DNA concentrations. The 0.4 kb insert fragment is from MSMEG_6199 and the 3.7 kb vector fragment is from pET20b. Vector only indicates cloning without the insert. The number of colonies obtained per 1 ng pET20b PCR fragment is shown as colony-forming units (cfu). *Error bars* represent the SD ($n = 3$). **b** Optimization of the length of overhangs. Two PCR fragments with various lengths of "T-type" complementary overhangs. The 0.2 kb insert fragment was from MSMEG_0559 and the 3.7 kb vector fragment was from pET20b. The initial DNA concentration of both vector and insert was 5 ng/µl. The number of colonies obtained is shown as colony-forming units (cfu). *Error bars* represent the SD ($n = 3$)

(Supplementary Fig S1). Nine out of nine recombinants tested had correctly inserted fragments.

Another important factor affecting TEFC is the length of the tailed overhangs. Matsumoto and Itoh showed that cloning efficiency is positively correlated with overhang length when it is less than 12 nt in length [13]. Here, we gradually increased the length of overhangs from 5 to 30 nt at intervals of five. Unexpectedly, after reaching a maximum at an overhang length of 15 nt, the number of recombinant colonies decreased sharply (Fig. 2b). Primers were designed with tails of different lengths from 5 to 30 nt

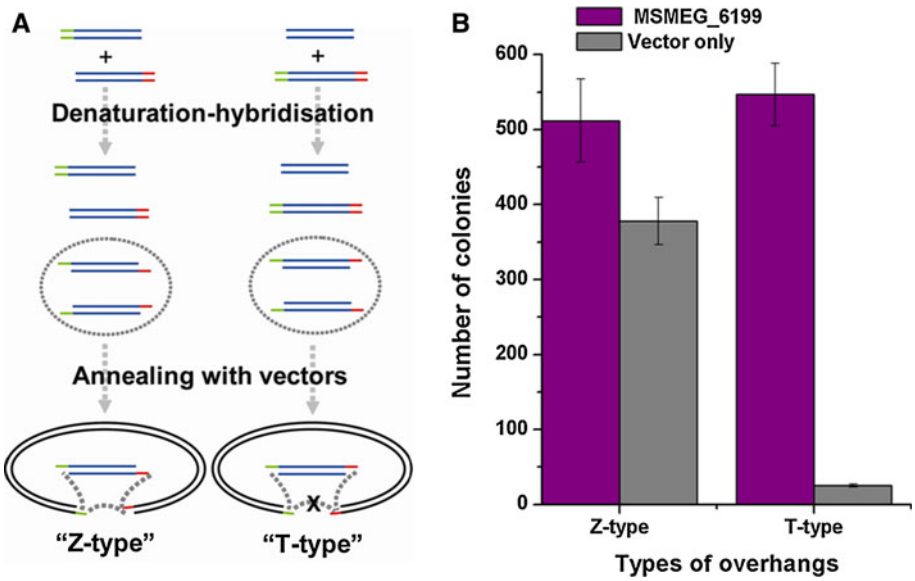

Fig. 3 Comparison of the two types of overhangs. **a** Schematic illustration of the creation of "Z-type" and "T-type" overhangs via a denaturation-hybridization reaction. Vectors with "Z-type" overhangs are more prone to be self-ligated. **b** Comparison of the cloning background interference between "Z-type" and "T-type" overhangs. Two PCR fragments were assembled using the two types of overhangs. The 0.4 kb insert fragment was from MSMEG_6199 and the 3.7 kb vector fragment was from pET20b. Overhangs flanking the vectors were designed to be complementary to each other. The initial DNA concentration of both the vector and the insert was 5 ng/μl. Vector only indicates cloning without the insert. MSMEG_6199 indicates cloning with the insert MSMEG_6199. "Z-type" and "T-type" indicates the two types of overhangs. The number of colonies obtained is illustrated as colony-forming units (cfu). *Error bars* represent the SD ($n = 3$)

(Supplementary Table S2). As indicated in Supplementary Table S3, the number of recombinant colonies yielded varied only slightly between different initial DNA concentrations from 2 to 10 ng/μl. We thus used a DNA concentration of 5 ng/μl in all following experiments to reduce the influence of differences in DNA concentration. Our experimental results suggest that the optimal length of overhangs for TEFC is around 10–20 nt; reducing the length of overhangs to less than 10 or increasing it to more than 20 decreases the efficiency of TEFC.

Comparison of the Two Types of Overhangs

It is self-evident that, in theory, there should be no difference in the cloning efficiency of "T-type" and conventional "Z-type" overhangs under ideal conditions (assuming that no self-ligations occur). However, one drawback of "Z-type" overhangs is that they tend to produce self-ligated plasmids when the two-tailed overhangs are partially or totally complementary to each other, thus greatly increasing cloning background interference. Our "T-type" overhang effectively eliminates the possibility of self-ligation, thus reducing the background interference (Fig. 3). pET20b and MSMEG_6199 were used as vectors and inserts in validation experiments. We designed primer sets with or without tailed sequences (Supplementary Table S3). The tailed sequences were designed to be complementary to each other to permit self-ligation. These primers were used in different combinations to produce "T-type" or "Z-type" overhangs. The initial DNA concentration was 5 ng/μl. As shown in Fig. 3b, although the cloning efficiency of "Z-type" overhangs is almost the same with that of the "T-type" overhangs, the latter greatly reduced the number of the colonies of the negative control (Vector alone). As the number of the colonies produced by "Z-type" overhangs is close to its background, most of the colonies produced with "Z-type" overhangs would be self-ligations. On the contrary, "T-type" overhangs produced a more than 20-fold increase in the number of colonies compared to its negative control. Most of the colonies produced by "T-type" overhangs are, therefore, recombinants with inserts. Further colony PCR experiments confirmed that 10 out of 10 randomly chosen recombinants produced by "T-type" overhangs had inserted fragments, while only one out of 10 produced by "Z-type" overhangs contained the inserted fragments (Supplementary Fig. S2).

Application of TEFC in Multi-Vector Cloning

The advantages of "T-type" over "Z-type" overhangs is best illustrated when TEFC is applied to multi-vector cloning (Fig. 4). If one gene is cloned into three different vectors by conventional EFC (Fig. 4a), three pairs of primer sets are required to PCR amplify the gene. Each pair of

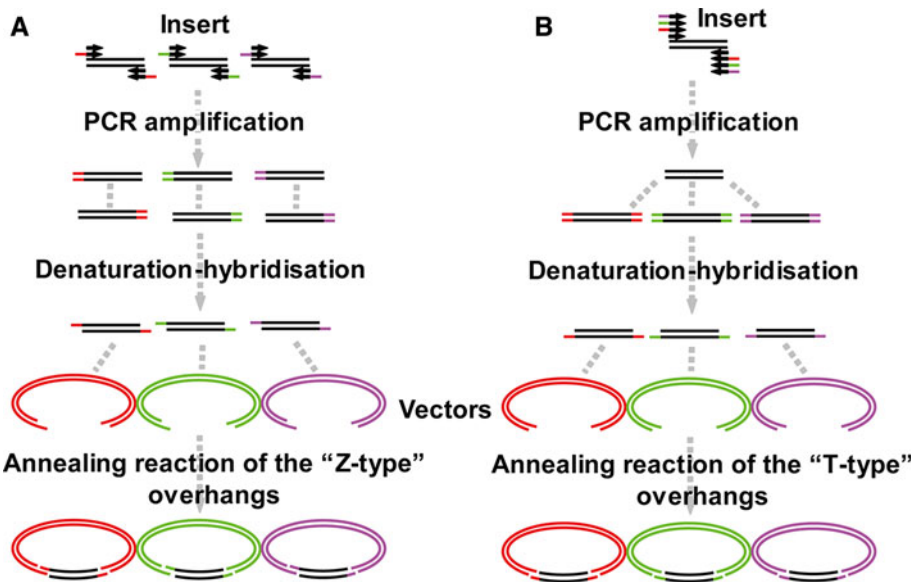

Fig. 4 Schematic illustration of conventional EFC and TEFC in multi-vector cloning. **a** Multi-vector cloning by conventional EFC. One insert MSMEG_1622 is separately cloned into three different vectors, pET20b, pQE30, and pGEX6p-1. Six primer sets are used to PCR amplify the insert. The 5′-tailed insert is paired with its corresponding 3′-tailed PCR product. Denaturation and hybridization of the mixture produces inserts with three different kinds of "Z-type" overhang, which are then annealed with their corresponding pretreated "Z-type" vectors. **b** Multi-vector cloning by TEFC. One insert MSMEG_1622 is cloned separately into three different vectors, pET20b, pQE30, and pGEX6p-1. Four primer sets are used to PCR amplify the insert. The non-tailed insert is paired separately with the other three tailed PCR products. Denaturation and hybridization of the mixture will produce inserts with three different kinds of "T-type" overhangs which anneal with their corresponding pretreated "T-type" vectors

PCR products is then mixed in the denaturation-hybridization reaction to produce "Z-type" inserts, which are finally annealed with corresponding pretreated "Z-type" vectors. If the number of destination vectors is n, then the number of PCR products of the gene required to perform conventional EFC would be 2n. In contrast, TEFC using "T-type" overhangs (Fig. 4b) reduces the number of PCR products required to $n + 1$. The one non-tailed PCR product pairs with the tailed PCR products separately to generate n kinds of "T-type" inserts, which are then annealed with the corresponding pretreated "T-type" vectors. Compared with conventional EFC, our TEFC method simplifies the procedures of multi-vector cloning. We demonstrated this TEFC application by cloning 1.6 kb MSMEG_1622 into vector pET20b (3.7 kb), pQE30 (3.4 kb), and pGEX6p-1 (4.9 kb). The primers used in this TEFC application are listed in Supplementary Table S5. The resulting recombinant colonies were identified by colony PCR and restriction enzyme digestion (Fig. 5); all of them had correctlyinserted fragments.

Discussion

Enzyme-free cloning avoids the use of easily degraded enzymes while achieving a seamless transfer of genetic elements in vitro without a requirement for specific vector sequences. Moreover, throughout EFC cloning procedures, DNA is constantly incubated in annealing buffer which helps to stabilize the denaturation-hybridization products. "T-type" and "Z-type" vectors can, therefore, be prepared in large quantities as stocks and can be preserved for lengthy periods. It should, however, be noted that, EFC still has at least one weakness in that primer design is more complicated than for other conventional cloning methods. The advantages of EFC still outweigh this drawback in primer design. Considering the relatively low cost of reagents and the shorter time required for this method, EFC is a very competitive alternative for routine or high throughput cloning.

Conventional EFC, however, similar to the LIC and SLIC procedures [4–7], utilizes "Z-type" overhangs for the self-assembly reaction, giving rise to a tendency to produce self-ligated plasmids under some circumstances. Here, we have improved the conventional EFC method by introducing "T-type" overhangs to replace "Z-type" overhangs, reducing the incidence of plasmid self-ligations. This is probably because "T-type" overhangs reduce the stability of self-ligated products and tend to produce the twisted self-ligated plasmids which is impossible to replicate in vivo. Further investigations are needed to discover the mechanisms behind. Nevertheless, it is the first time that the use of a specially designed "T-type" overhang has been reported in molecular cloning.

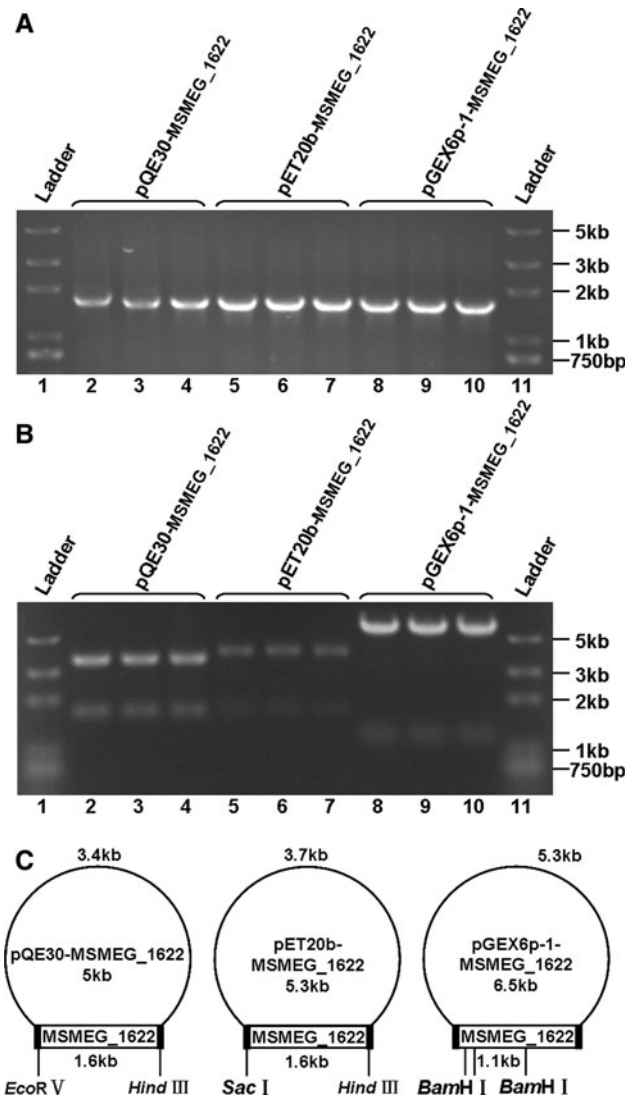

Fig. 5 Analysis of TEFC application for multi-vector cloning by PCR and restriction enzyme digestion. **a** Identification of the recombinants inserted with MSMEG_1622 by PCR. *Lanes 1 and 11* DNA ladder, *lanes 2–4* recombinants of pQE30-MSMEG_1622, *lanes 5–7* recombinants of pET20b-MSMEG_1622, *lanes 8–10* recombinants of pGEX6p-1-MSMEG_1622. **b** Identification of the recombinants by restriction enzyme digestion. *Lanes 1 and 11* DNA ladder, *lanes 2–4* a Sac I–Hind III restriction analysis of pQE30-MSMEG_1622, *lanes 5–7* an EcoR V–Hind III restriction analysis of pET20b-MSMEG_1622, *lanes 8–10* a BamH I restriction analysis of pGEX6p-1-MSMEG_1622. **c** Restriction maps of the above recombinant plasmids. Our TEFC-derived pGEX6p-1-MSMEG_1622 contained three BamH I restriction sites inside the ORF of MSMEG_1622. BamH I restriction analysis of pGEX6p-1-MSMEG_1622 will produce three fragments: 0.1, 1.1, and 5.3 kb, the 0.1 kb fragment is hard to be detected after Goldview staining

We systematically optimized our improved EFC method. TEFC can be performed at initial DNA concentrations from 1 to 100 ng/μl, with the most efficient initial DNA concentration being around 2 ng/μl. The most suitable length of overhangs for TEFC was found to be from 10 to 20 nucleotides. Moreover, results from EFC using self-complementary overhangs demonstrated that "T-type" overhangs greatly reduce the cloning background interference compared with "Z-type" overhangs. Most importantly, we illustrated the benefits of TEFC in multi-vector cloning by separately cloning MSMEG_1622 into vectors pET20b, pQE30, and pGEX6p-1. TEFC reduced the number of PCR products and reactions needed in conventional EFC, simplifying the procedures for multi-vector cloning. In conclusion, our TEFC method benefits from the strengths of EFC without having its drawbacks, and is thus a better choice for PCR product cloning.

Acknowledgments The authors thank Dr. Zhongdao Li, Shiqiang Lin, Liwei Wang, and Jinjun Wu for their comments and suggestions; Dr. J.E. Fleming for careful proofreading and revision of the manuscript. The authors thank Dr. Mingzhang Yang and Shanghua Fan for providing some of the experimental materials. This study was financially supported by the National Natural Science Foundation of China (Grant No: 31170132), National Basic Research Program of China (Grant No.: 2012CB518700).

Conflict of interests None.

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