MCT cloning: a seamless cloning strategy for inserting DNA fragments

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ABSTRACT
This study presents a seamless, efficient and restriction/ligation-independent cloning strategy for inserting large fragments: MCT cloning (based on the generation of Manmade Cohesive Termini). This method requires only four steps: (i) the first parallel PCR for the amplification of complete and truncated insert fragments; (ii) the second parallel PCR for the exponential amplification of linear plasmids with the same sequences and different break sites mediated by the first products and the corresponding reverse primers; (iii) the formation of manmade cohesive termini after DpnI digestion, mixing, denaturation and annealing; and (iv) transformation. By employing this strategy, large fragments (1–4 kbp) can be easily inserted into the pGADT7 vector (~8 kbp).

Abbreviations: PIPE: incomplete primer extension; UDG: uracil DNA-glycosylase; LIC: ligation-independent cloning; RF: restriction-free; CPEC: circular polymerase extension cloning; EMP: exponential megapriming PCR; IFPC: inverse fusion PCR cloning

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Introduction
Molecular cloning is a fundamental and essential technique in biological research. Traditional cloning methods often rely on restriction enzymes and DNA ligase during the cloning process, which requires specific sites or sequences in both the insert and vector. With the development of high-fidelity DNA polymerases and PCR technology, many restriction/ligation-independent cloning techniques have been presented and widely used. By using numerous carefully designed primers, manmade cohesive sites can be introduced in some seamless cloning methods, such as enzyme-free cloning [1], hetero-stagger cloning [2] and polymerase incomplete primer extension (PIPE) cloning [3,4]. Consequently, DNA fragments and linear vectors can be paired using the manmade cohesive termini, and the incision sites can be repaired in vivo after direct transformation into Escherichia coli. In addition, a cohesive terminus with 12 bases can be introduced in uracil DNA-glycosylase cloning (UDG) [5,6] and ligation-independent cloning (LIC) [7]; these cloning strategies take advantage of the 3’-5’ exonuclease activities of uracil DNA-glycosylase and T4 DNA polymerase, respectively.

RF cloning [8,9], according to the principle of QuikChange™ site-directed mutagenesis [10,11], provides a convenient, economical and accurate approach for inserting a single fragment into a vector. This method does not introduce any unwanted sequences, such as restriction sites or recombinant sequences. As a derivative method of RF cloning, CPEC cloning [12,13], which is based on the polymerase extension mechanism, extends overlapping regions between the insert and vector fragments to form a complete circular plasmid. However, the amplification of the DNA product in RF cloning and CPEC cloning is linear, which leads to lower yields [14,15]. Using overlap extension PCR [16] and introducing a reverse primer, exponential amplification can be achieved in EMP cloning [14] and IFPC cloning [17]. However, the disadvantage of EMP cloning and IFPC cloning is the use of some indispensable and expensive enzymes (T4 PNK [T4 polynucleotide kinase] and T4 DNA ligase) in the reaction process [15].

Homologous recombination is another widely used cloning method. The use of commercial recombinases, such as Gateway™ (Invitrogen, Carlsbad/CA) [18] and In-Fusion™ (Clontech, Mountain View/CA) [19], can...
achieve in vitro recombination in homologous regions between the linear insert and the vector. Although this method of homologous recombination is more convenient to manipulate, expensive recombinases are required to complete the entire cloning process. Therefore, several recombinase-free seamless DNA cloning methods using the endogenous homologous recombination activity of laboratory E. coli strains have been reported [20–24]. The Seamless Ligation Cloning Extract (SLiCE) technique uses homologous recombination activities in vitro, in seamless DNA assembly [20–22]. Original SLiCE required a specific strain, E. coli PPY, which expresses lambda prophage Red/ET system in E. coli [20]. To extend the utility of this system, the SLiCE preparation method was improved and the cloning efficiency was increased [24]. Improved SLiCE can be prepared from laboratory E. coli strains containing JM109, DH5a and XL10-Gold [24] without any protein expression and has low-cost and versatility for seamless cloning [21]. The in vivo E. coli cloning (iVEC) is an alternative low-cost seamless cloning method [22,23]. The iVEC-method can directly transform and assemble DNA fragments without any treatment to E. coli competent cells, using in vivo E. coli endogenous recombination activities [22,23]. However, the iVEC-method requires high-efficiency E. coli competent cells for efficient seamless cloning [22].

Inspired by the aforementioned cloning methods, a new and effective cloning method was introduced. In this method, based on two parallel overlap extension PCRs, two linear plasmids with the same sequences and different break sites were amplified. After mixing, denaturing and annealing of the two parallel linear plasmids, a manmade cohesive terminus was generated and paired, and the two nicks in the circular plasmid were repaired in vivo in E. coli. Since the new cloning method can generate Manmade Cohesive Termini, we named it MCT cloning. With MCT cloning, the assembly of large DNA fragments (1–4 kbp) can be simply performed in one day. Compared to other seamless cloning methods, MCT cloning is much more efficient and economical for longer fragment insertions.

Materials and methods

Materials

E. coli TOP10F’ (Invitrogen, RecA- strain) was used for cloning. Chemically competent TOP10F’ cells were homemade (approximately 10⁶ cfu/μg). High-fidelity DNA polymerase (KOD-FX) was purchased from TOYOBO (Osaka, Japan). All insert fragments generated from hNa1.5 were inserted into the plasmid pGADT7 (7988 bp, Clontech). All primers were purchased from Sangon (Shanghai, China). The sequences of insert fragments, insertion sites and primers are provided in the Supplementary Tables S1 and S2.

Overview of MCT cloning

The mechanism of MCT cloning for the insertion of single fragments is shown in Figure 1. MCT cloning requires four steps. The first step is the exponential amplification of the interested fragments (Figure 1A). This amplification process involves two completely independent PCRs, requiring a total of two pairs of primers (F1/R1 and F2/R2 in Figure 1A). The primer pair F1/R1 and the primer pair F2/R2 were used to amplify the target gene in the first round of PCR, and produce the PCR products M1 and M2, respectively. The reverse primers R1 and R2 contain 3’ regions (the black part in Figure 1A) homologous to the insert gene and 5’ regions homologous to the vector (the sky blue part in Figure 1A). The forward primer F1 was homologous to the target genes (the orange part and black part in Figure 1A), while the forward primer F2 was truncated. Compared with the F1 primer, the forward primer F2 deliberately deleted a partial sequence of the 5’ part (the orange part in Figure 1A). Therefore, after the first PCR, compared to M1, M2 lacked some of the base pairs (the orange part in Figure 1A).

In the second step, the PCR products M1 and M2 were used together with the short reverse primers R3 and R4 to exponentially amplify the vector pGADT7 to get the PCR products M3 and M4, respectively. The reverse primer R3 was homologous to the vector (the reddish-purple part in Figure 1B), while the 5’ end of the reverse primer R4 added a complementary part of the orange part (the orange part in Figure 1B), which was deleted in primer F2. Finally, the orange parts were in the 5’ and 3’ ends of M3 and M4, respectively.

In the third step, the methylated templates of the second step were digested with DpnI. After the mixing, denaturing and annealing of M3 and M4, we obtained the final products M5 and M6, in which the orange part sequences were turned into cohesive terminus (Figure 1C).

In the fourth step, the final products M5 and M6 were transformed into chemically competent E. coli Top 10F’ cells, in which the incision sites in the M5 and M6 can be repaired in vivo (Figure 1D).

The first PCR step: amplification of insert fragments

DNA fragments of different sizes (1–4 kbp) were amplified from hNa1.5 using 1 U of KOD-FX enzyme (TOYOBO, Osaka, Japan) supplemented with 0.2 μmol/L of each primer, 1 × reaction buffer, 0.4 mmol/L dNTPs and 10 ng template DNA in 50 μL of the reaction mixture. Mixtures
were first denatured (2720 Thermal Cycler, Applied Biosystems, USA) at 94 °C for 2 min, subjected to 25 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s and elongation at 68 °C for 25 cycles (1 kbp/min), and followed by a final 10-min extension step at 68 °C. PCR products were analyzed by agarose gel electrophoresis, and target fragments were purified using PCR purification kits (BioSci, Hangzhou, China).

**The second PCR step: insertion of fragments**

Fragments purified from the first PCR step and the corresponding reverse primer (R3, R4) were used together to exponentially amplify the plasmid pGADT7 (as shown in Figure 1B). The 50 μL of PCR mixture contained 0.2 μmol/L primer, 10 ng of recipient plasmid, 1 × PCR Buffer, 0.4 mmol/L dNTPs and 1 U KOD-FX polymerase. Mixtures were pre-degenerated at 94 °C for 2 min, subjected to 25 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s and elongation at 68 °C (1 kbp/min), and followed by a final 10-min extension step at 68 °C. PCR products were analyzed by agarose gel electrophoresis, and the second PCR products were purified using PCR purification kits or gel extraction kits (BioSci, Hangzhou, China).

**The third step: DpnI digestion, mixing, denaturation and annealing of the second PCR products**

After purification, the second PCR products were digested using DpnI as follows: purified products...
(8 µL) supplemented with 1 µL of 10 × reaction buffer were digested with 1 µL of FastDigest™ DpnI (Thermo
Fermentas, Burlington, Canada) at 37 °C for 1 h. After
digestion, the two products were mixed (M3 and M4 in
Figure 1C) in equal amounts (Unless otherwise
stated, the dosage of DNA transformed to competent
cells in this study was 50 ng for each PCR product).
Mixtures were denatured at 98 °C for 30 min and
annealed at 37 °C for 20 min.

The fourth step: transformation, colony PCR and
DNA sequencing

The products of the third step were transformed di-
rectly into 50 µL of chemically competent TOP10F‘
cells. All cells were spread onto Luria–Bertani (LB)
plates containing 100 µg/mL of ampicillin sodium salt
and incubated overnight at 37 °C. Colony numbers per
plate were determined to estimate the cloning effi-
ciencies. Colony PCR was used to screen 24 colonies

Figure 2. Efficiency and fidelity of MCT cloning for different gap sequence sizes, purification methods and dosage of second PCR
products. (A) The first PCR of insert-1 (1 kbp, Supplementary Table S1). For lanes 1–6, insert-1 was amplified from hNav1.5 with
primers P1/P7, P2/P8, P3/P8, P4/P8, P5/P8 and P6/P8 (Supplementary Table S2), respectively, to produce complete gene and genes
lacking 10, 15, 20, 25 and 30 bp, respectively. The arrow indicates the target bands of the first PCR. (B) and (C) For lanes 1–6, the
pGADT7 vector was amplified by the first PCR products (lanes 1–6 in Figure 2A, respectively) with reverse primers P9-P14
(Supplementary Table S2), respectively. The fragments were purified by PCR purification kits or by gel extraction kits. The arrow
indicates the target band of the second PCR products. (D) and (E) Colony numbers and percentage of positive clones per plate
were counted to estimate the cloning efficiencies and fidelities for different sizes of gap sequences, respectively. (F) and (G)
Colony numbers and positive clones per plate were counted to estimate the cloning efficiencies and fidelities for different dosages
of the second PCR products, respectively. The results are mean values ± SEM of three independent experiments. M: DNA molecular
weight marker.
randomly picked from each group. The percentages of positive clones obtained via colony PCR were used to estimate cloning fidelity. Colony PCR was performed using DreamTaq DNA polymerase (Thermo Scientific, USA) according to the manufacturer’s instructions. Further DNA sequencing was performed by Sangon (Shanghai, China).

**Results and discussion**

*Efficiency and fidelity of MCT cloning*

To confirm the feasibility of the MCT cloning method, we first attempted to insert a fragment (1 kbp) into pGADT7 (7988 bp) with different gap sequence sizes. In the first PCR, we used six pairs of primers to amplify the interesting fragment and obtained two PCR products of A (Figure 2A, lane 1) and B1 to B5 (Figure 2A, lanes 1–6). The product A was the complete gene of the insert, while the 5’ ends of B1 to B5 were lacking 10, 15, 20, 25 and 30 bp, respectively. In the second PCR, the first PCR products A, B1, B2, B3, B4 and B5 were used as primers for pGADT7 amplification with the corresponding reverse primers. The second PCR products of A’, B1’, B2’, B3’, B4’ and B5’ were purified using PCR purification kits (Figure 2B, lanes 1–6) and gel extraction kits (Figure 2C, lanes 1–6). After the second PCR, we obtained two linear PCR products with different termini (such as M3 and M4 in Figure 1B), then we denatured the two products and mixed them in equal amounts to denature and anneal them further to get the two circular plasmids (such as M5 and M6 in Figure 1C) with a manmade cohesive termini, which could be complemented and repaired in vivo in E. coli. After transformation, more colonies (>400 cfu with 25 bp and 30 bp of cohesive terminus) were obtained with a longer cohesive terminus, while the positive efficiencies were all high (all approximately 100%) (Figures 2D and E). Interestingly, compared to the other cohesive terminus region length groups, the group with the 10 bp of cohesive terminus yielded enough colonies (>200) and a positive percentage (approximately 100%). Furthermore, for the clear bands of inserts after the second PCR, the procedure of gel extraction did not help to increase the number of colonies and positive percentages (Figures 2D and E).

Figure 3. Insertion of 1–3 kbp of fragments by MCT cloning. (A) For lanes 1–6, insert-1, insert-2 and insert-3 (Supplementary Table S1, 1 kbp, 2 kbp and 3 kbp, respectively) was amplified by the primers P1/P7 and P2/P8, P1/P15 and P2/P16, and P1/P17 and P2/P18 (Supplementary Table S2), respectively. The arrow indicates the target band of the first PCR products. (B) The pGADT7 vector was amplified by the first PCR products (lanes 1–6 in Figure 3A, respectively) with respective reverse primers (P9 for lanes 1, 3 and 5, and P10 for lanes 2, 4 and 6, Supplementary Table S2). The arrow indicates the target band of the first PCR products. (C) and (D) Colony numbers and percentage of positive clones per plate were counted to estimate the cloning efficiencies and fidelities for various lengths of large fragments (1 kbp, 2 kbp and 3 kbp) with 10 bp of gap sequences. The results are mean values ± SEM of two independent experiments. M: DNA molecular weight marker.
We then examined the effect of the dosage of the second PCR products for cloning efficiency and fidelity. A series tests were run using equal amounts of M5 and M6 (10, 25, 50 and 100 ng each) of the second PCR products as shown in Figures 2F and G. With the increase in the second PCR products, the number of recombinant colonies was normally distributed in the bell-shaped curve (Figure 2F). When the amounts were less than 50 ng for each product, M5 and M6, the number of recombinant colonies increased (Figure 2F). However, the number of recombinant colonies decreased when the amounts were more than 50 ng for each product, M5 and M6 (Figure 2F). Despite this, the positive percentage may have changed very little (>90%) (Figure 2G).

**Insertion of long fragments of different sizes**

To verify whether the cloning method has a wider applicability, a series of fragments (1–4 kbp) were tested by inserting them into the pGADT7 vector. According to the previous experimental results, we used a 10 bp gap sequence length in this group of experiments (Figure 3). The 1–3 kbp of insert fragments M1 and M2 were clearly amplified in the first PCR (Figure 3A) and produced the products M3 (Figure 3B, lanes 1, 3 and 5) and M4 (Figure 3B, lanes 2, 4 and 6) as primers in the second PCR. For the 1-kb and 2-kb inserts, 10 bp of cohesive sequence produced enough recombinant colonies (>100) and high positive percentages (close to 100%). In contrast, for the inserts longer than 2 kbp (3 kbp, in Figure 3C), recombinant colonies of the groups with 10 bp of cohesive terminus dropped (below 100 cfu), and the positive percentage decreased to only approximately 20%, although this was partly due to the poor amplification in the second PCR (Figure 3B, lanes 5 and 6).

Therefore, longer cohesive sequences (30 bp) were tested to overcome the limitation, as shown in Figure 4. The insert fragments M1 and M2 of 3 kbp and 4 kbp were clearly amplified in the first PCR.
amplification (EMP cloning and IFPC cloning), we developed a novel cloning method that provides an alternative approach to inserting DNA fragments into plasmids. This method accomplished large fragment (1–4 kbp) assembly with high efficiency and high fidelity. MCT cloning, as a derivative of RF cloning, and its derivatives (CPEC cloning, EMP cloning and IFPC cloning) offer the advantage of both low cost (RF cloning and CPEC cloning) and exponential amplification (EMP cloning and IFPC cloning). We expect MCT cloning to play a significant role in functional genomic studies, protein engineering and other related processes.

Conclusions

Here, we developed a novel cloning method that provides an alternative approach to inserting DNA fragments into plasmids. This method accomplished large fragment (1–4 kbp) assembly with high efficiency and high fidelity. MCT cloning, as a derivative of RF cloning, and its derivatives (CPEC cloning, EMP cloning and IFPC cloning) offer the advantage of both low cost (RF cloning and CPEC cloning) and exponential amplification (EMP cloning and IFPC cloning). We expect MCT cloning to play a significant role in functional genomic studies, protein engineering and other related processes.

Disclosure statement

The authors declare they have no competing interests.

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