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Simple Cloning: direct transformation of PCR product (DNA multimer) to *Escherichia coli* and *Bacillus subtilis*

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ABSTRACT

We developed a general restriction enzyme-free and ligase-free method for subcloning up to three DNA fragments into any location of a plasmid. The DNA multimer generated by prolonged overlap extension PCR was directly transformed in *Escherichia coli* [e.g., TOP10, DH5α, JM109, BL21(DE3)] and *Bacillus subtilis* for obtaining chimeric plasmids.

Keywords: enzyme-free cloning, DNA multimer, *Escherichia coli*, Phusion polymerase, overlap extension PCR

The limited choices of restriction enzymes, relatively low efficiencies in digestion and ligation, and possible self-ligation of the digested plasmid may result in difficulties in constructing chimeric plasmids. Recently, several companies have developed recombinase-based technologies, such as the Invitrogen Gateway cloning technology, Clonetech In-Fusion, BioCat Cold-Fusion, and Red/ET Recombination, but these rely heavily on specialized kits containing vectors, enzymes, or hosts (4, 7, 10, 11, 14, 15). Several overlap extension PCR-based methods were developed for subcloning. However, RF-cloning (9) and overlap extension PCR cloning (2) require DpnI to digest the vector template. Additionally, the maximum inserted DNA length is ~6.7 kb (2). Another technology called "Quick Assemble" has low positive cloning efficiencies of ~33% (16).

We developed a sequence-independent 'Simple Cloning' method without the need of restriction and ligation enzymes. The protocol includes three steps (Fig. 1): (i) linear

DNA fragments (i.e., inserted DNA fragment and vector backbone), both of which contained a 3' and 5' 40-50 bp overlapping termini, generated by high-fidelity PCR with the New England Biolabs (NEB) Phusion polymerase (Ipswich, MA), (ii) the DNA multimer was generated based on these DNA templates by prolonged overlap extension PCR (POE-PCR) with Phusion polymerase, and (iii) the POE-PCR products (DNA multimer) was transformed in competent *E. coli* or *B. subtilis* strains directly, yielding the desired chimeric plasmid.

A 1.3-kb insertion fragment (Cherry-cbm17) encoding a cherry fluorescent protein and a family 17 carbohydrate-binding module from *Clostridium cellulovorans* cellulase 5A (1) was subcloned into a 3.6-kb pET20b vector backbone, yielding a 4.9-kb plasmid pET20b-cherry-cbm17, where the fusion protein was controlled by a T7 promoter. A linear vector backbone was amplified by using the forward primer VF (5'TAGCCTGGACAATATCAAATTTACC**CTCGAGCACCACCACCACCACCAC T**3') and the reverse primer VR (5'**TATCCTCCTCGCCCTTGCTCACCATA** TGTATATCTCCTTCTTAAAGTTAA3'). VF and VR contain the last 25 bp of the 3' terminus of the insertion sequence (underlined) and the first 25 bp of the 5' terminus of the vector sequence (highlighted). Similarly, the insertion fragment was amplified by a pair of primers IF (5'**TTAACTTTAAGAAGGAGATATACATA** TGGTGAGCAAGGGCGAGGAGGAT3') and IR (5'AGTGGTGGTGGTGGTGGTGCTCGAG**GGTAAATTTGATATTGTCCAGGCT A**3'). IF and IR have reverse complementary sequences of VR and VF, respectively. The standard extension time (SET) in PCR was calculated based on the amplified fragment

length divided by 3 kb/min for Phusion polymerase at 72^{o}C. Two linearized DNA fragments were purified with Zymo DNA Clean & Concentration Kit (Irvine, CA) (Fig. 2A&C).

In POE-PCR, each tube contained 0.2 mM dNTP, 2 ng µl^{-1} purified insertion DNA fragment, equimolar purified vector backbone, and 0.04 U µl^{-1} Phusion polymerase without the addition of primers, where the insertion and vector fragments were concomitantly used as primers and templates (Fig. 1). The POE-PCR reaction was conducted at 98 ^{o}C denaturation for 30 s; 25-30 cycles at 98 ^{o}C denaturation for 10 s, 60 ^{o}C annealing for 10 s, and extension at 72 ^{o}C at a rate of 2 kb/min based on the length of desired chimeric vector. When the extension time was shortened (e.g., 30% of the SET), the PCR products smeared in 0.8% agarose gel (Fig. 2A). Extending the SET (0.9 fold or longer), large-sized DNA multimers of repeated insertions and vector backbones in tandem was formed, which cannot move in the gel (Fig. 2A). Although the highest transformation efficiency was obtained at a 0.9 SET (Fig. 2B), it was recommended that the extension time for POE-PCR was 1.3 SET (i.e., 2 kb/min) because of (i) a distinct formation of DNA multimers and (ii) an acceptable transformation efficiency.

Five µl of the POE-PCR product (i.e., approximately one µg DNA multimer) was mixed with 100 µl of the competent *E. coli* BL21 cells or other cells. [Note: Commonly-used *E. coli* strains are capable of cleaving assimilated DNA multimers into the circular plasmid (3, 5)]. Through the standard chemical transformation protocol (6), nearly all colonies appeared red in the LB Petri dish (Fig. 2D), suggesting that more than 99% of the

transformants contained the plasmid expressing red fluorescent protein. The plasmid randomly isolated from the dish was digested by two restriction enzymes, exhibiting two fragments as expected (Fig. 2C, lane 6). The plasmid sequence was further verified by DNA sequencing.

The POE-PCR product (DNA multimer) can be transformed in other commonly-used chemical competent and electrocompetent *E. coli* hosts, such as JM109, DH5α, and Top10, as well as *B. subtilis* hosts. Transformation efficiencies of DNA multimerswere 3.3 X 10^4/μg in commercial DH5α competent cells, approximately five orders of magnitude lower than the intact circular plasmid (Table 1). By using home-made (low-transformation efficiency) *E. coli* strains BL21, JM109, and Top10, the transformation efficiencies were 43-420/μg of DNA multimer. However, such efficiencies were sufficient for subcloning. Much higher transformation efficiencies were obtained in *B. subtilis* (Table 1) because of its preference to assimilate DNA multimer to circular plasmid (12, 13).

We highly recommend using Phusion polymerase in both PCR amplifications due to its high fidelity and high speed. DNA multimer could be obtained even when the overlap length between two templates was shortened to 20 bp (data not shown), as regular overlap extension PCR (8). However, to ensure positive result, 40-50 bp overlap lengths are recommended. The concentration of each DNA template in POE-PCR is recommended to be *ca.* 2 ng/μl or higher, with a 1:1 molar ratio. The extension time of POE-PCR is calculated based on a rate of 2 kb/min for Phusion polymerase to ensure the

formation of DNA multimers rather than the use of shorter extension time described elsewhere (2, 9, 16).

By using this method, more than one hundred plasmids containing one DNA insertion fragment ranging in length from 0.2 to10 kb were constructed and the largest plasmid size tested was 11 kb (data not shown). For one DNA fragment insertion cloning, the success rate was 95%. Simple Cloning did not work when the DNA assembly by overlap PCR failed. Simple Cloning was enabled to assemble up to four fragments containing overlap regions at both 5' and 3' termini in tandem, yielding the desired plasmids (data not shown). In conclusion, Simple Cloning is rapid, efficient, inexpensive, and flexible.

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Figure legends

Fig. 1. The scheme of Simple Cloning. First, two 3' and 5' overlapped insertion and vector fragments are generated by regular PCR. Second, DNA multimer is formed by POE-PCR without primers and with a prolonged extension time. Third, *E. coli* or *B. subtilis* strains cleaved transformed DNA multimer to a circular plasmid, a desired chimeric plasmid.

Fig. 2. Optimization of extension time in POE-PCR. A, PCR products generated by the overlap extension PCR at different extension times from 0.3 to 2.5 SET; M, 1 kb DNA ladder from NEB; Lane V, vector backbone generated by PCR; Lane I, inserted DNA generated by PCR. B, transformation efficiency of DNA multimers as a function of extension time. C, analysis of 0.8% agarose gel for the case that a 1.3-kb DNA was subcloned into 3.6-kb plasmid. Lane 1, PCR linearized vector; Lane 2, PCR linearized insertion; Lane 3, DNA multimer generated by modified overlap extension PCR; Lane 4, PCR products digested with two restriction enzymes; Lane 5, resulting plasmid from a randomly selected *E. coli* colony; Lane 6, resulting plasmid digested with two restriction enzymes; and M, a 1-kb DNA ladder from NEB. D, the *E. coli* BL21(DE3) transformants containing pET20b-cherry-cbm17, where red fluorescent protein was expressed. (Note: *E. coli* TOP10, DH5α, and JM109 are preferred to BL21 for regular subcloning.)

198 **Table 1**. Transformation efficiency of DNA multimers to different hosts.

Stains	Source	Transformation efficiency (/μg)*		References
		Multimer	Plasmid	
E. coli				
DH5α	Commercial (Invitrogen)	3.3 X 10^4	2.7 X 10^9	
	Home-made	2.8 X 10^2	4.5 X 10^7	
BL21(DE3)	Home-made	4.2 X 10^2	6.7 X 10^6	Invitrogen, Carlsbad, CA
JM109	Home-made	4.3 X 10^1	5.1 X 10^5	
Top10	Home-made	3.5 X 10^2	2.0 X 10^7	Invitrogen, Carlsbad, CA
B. subtilis				
SCK6	Home-made	1.0 X 10^7	1.0 X 10^4	(12,13)

199 *All the *E. coli* cells are chemical competent.

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