Chapter 15

Simultaneous Removal of Multiple DNA Segments by Polymerase Chain Reactions

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Abstract

Precise DNA manipulation is a key enabling technology for synthetic biology. Approaches based on restriction digestion are often limited by the presence of certain restriction enzyme recognition sites. Recent development of restriction-free cloning approaches has greatly enhanced the flexibility and speed of molecular cloning. Most restriction-free cloning methods focus on DNA assembly. Much less work has been dedicated towards DNA removal. Here we introduce a protocol that allows simultaneous removal of multiple DNA segments from a plasmid using polymerase chain reactions (PCR). Our approach will be beneficial to applications in multiple sites mutagenesis, DNA library construction, genetic and protein engineering, and synthetic biology.

Key words Restriction-free cloning, Polymerase chain reaction, Synthetic DNA assembly and manipulation, Multiplex gene removal, Synthetic single-stranded bridging oligos

1 Introduction

Synthetic biology is an emerging interdisciplinary branch of biology that spans biotechnology, biophysics, biochemistry, and engineering [1]. It aims to design and construct biological modules, devices, systems, networks, and machines to rewire and reprogram biological organisms [2]. A key enabling technology in synthetic biology is precise manipulation and assembly of synthetic DNA constructs. Conventional approaches of DNA manipulation depend on restriction enzyme digestion, followed by ligation. Such approaches are limited by the presence of unique, specific restriction enzyme recognition sites, which are not necessarily available in a plasmid. Consequently, site-directed mutagenesis of genes is often needed to insert appropriate digestion sites, the use of which can affect endogenous structure and function of the encoded protein.

Restriction-free DNA cloning technologies have successfully removed limitations in restriction enzyme-based methods [3]. DNA assembly can be achieved by end-homology recombination

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methods including Gateway, overlap extension PCR [4], transfer PCR [5], DNA fragment assembly [6], restriction-free (RF) cloning [7], circular polymerase extension cloning (CPEC) [8], seamless ligation cloning extract (SliCE) [9], and prolonged overlap extension PCR (POE-PCR) [10]. Alternative methods based on DNA annealing include ligation-independent cloning (LIC) [11], In-Fusion [12], quick and clean (QC) cloning [13], sequence and ligation-independent cloning (SLIC) [14], Gibson [15], ligase cycling reaction (LCR) [16, 17], and PaperClip [18]. Approaches for mutation include single primer mutagenesis (SOMA) [19], simultaneous noncontiguous deletion [20], and multiplexing clonality [21]. Most of these restriction-free cloning approaches primarily target DNA assembly or mutation. Much less work has been devoted to DNA removal. The ability to remove specific DNA sequences is critical for structure-function analysis of proteins, DNA library construction, as well as genetic and protein engineering.

Here we introduce a PCR-based restriction-free cloning technique that allows simultaneous removal of multiple DNA segments from a plasmid (Fig. 1) [22]. We demonstrate simultaneous three-gene removal from a plasmid pCRII-U85 in a one-pot reaction. By properly designing gene-specific primers, one would be able to apply the same protocol to remove any DNA segments from a plasmid. The pCRII-U85 plasmid has a *u85* gene inserted into the *lacZ* sequence within the pCRII vector, which has both kanamycin

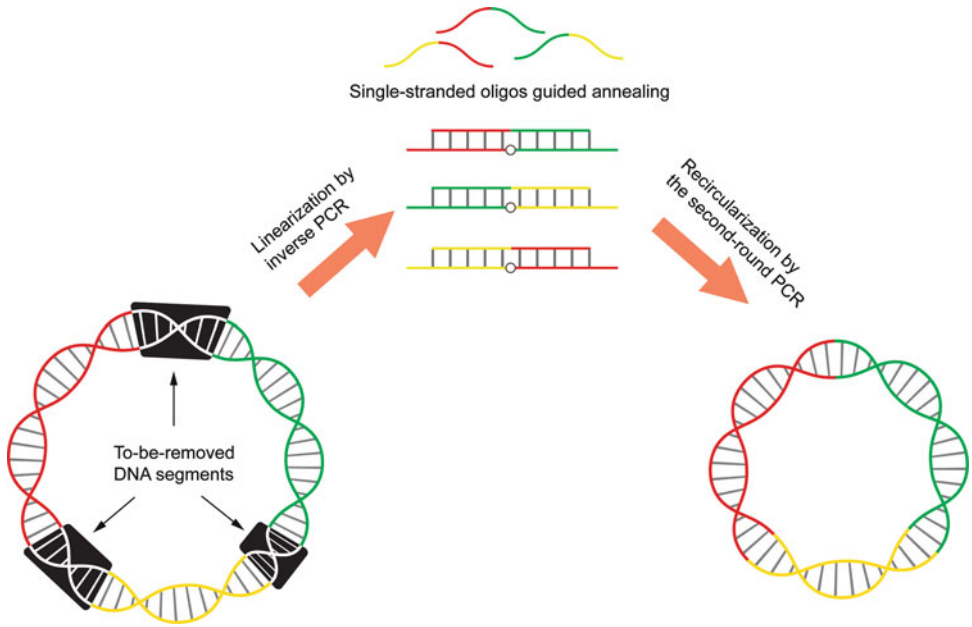

Fig. 1 Overall scheme of multiple gene removal based on two-step polymerase chain reactions. The first round of PCR generates linear products, which are recombined by the second round of PCR guided by complementary single-stranded oligos

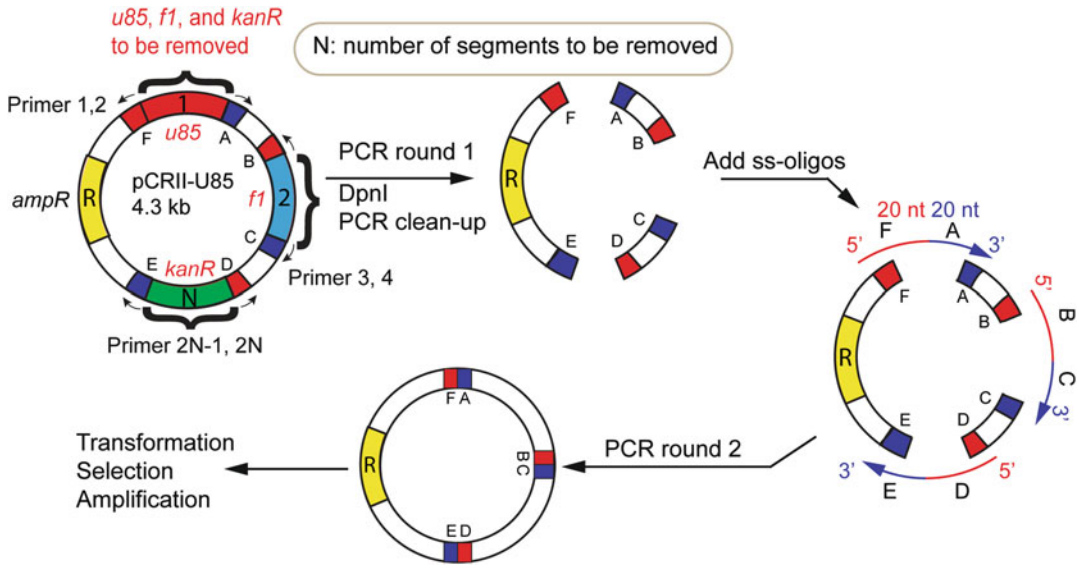

Fig. 2 pCRII-U85 plasmid with gene segments *u85*, *f1*, and *kanR* to be removed. To remove N segments, 2 N primers are used to generate N linear fragments in the first round of PCR. The product mixture is then treated with DpnI to remove the original template, followed by PCR clean-up. To set up the second round of PCR, fresh master mix and N ss-oligos are added to the purified PCR products. Each oligo shares a 20-nt complementarity to two neighboring fragments to be connected. Final PCR products containing nicked plasmids are used in transformation. Reproduced from [22] with permission from Elsevier

and ampicillin resistance genes (*kanR* and *ampR*). This construct allows quick readout of successful gene removal. We will simultaneously remove *u85*, *kanR*, and part of *f1-ori* (abbreviated as *f1*) segments from pCRII-U85 (Fig. 2). Removal of *u85* should produce blue colonies in a blue/white colony screening assay; removal of *kanR* while retaining *ampR* should allow colonies to grow selectively only on ampicillin plates. The final sequences are confirmed by DNA sequencing. By using synthetic single-stranded oligos as bridging sequences, our approach does not require alteration of template DNA to create overlap between neighboring segments. Consequently, this approach does not produce residual sequence between neighboring segments.

2 Materials

2.1 Components for Two-Step PCR Reaction

1. Oligonucleotides as primer pairs: 10 μM in Molecular grade water.
2. Guiding single-stranded oligonucleotides (ss-oligos): 20 μM in Molecular grade water.
3. Plasmid template: 10 pg/μL in Molecular grade water.

4. 2× Phusion DNA polymerase master mix.
5. 2× DreamTaq PCR master mix.
6. 1× TAE buffer: 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.
7. DNA ladder.
8. DpnI: 10 U/μL.
9. Agarose gel apparatus.
10. PCR clean-up kit.
11. Thermocycler.
12. Nanodrop.
13. Blue transilluminator.

2.2 Components for Confirmation of Successful Removal of DNA Segments

1. Competent cells (e.g., DH5α).
2. Luria-Bertani (LB) medium.
3. Luria-Bertani (LB) agar plate with appropriate antibiotics (e.g., ampicillin or kanamycin-resistant).
4. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) stock solution: 20 mg/mL in dimethylformamide (DMF).
5. 14 mL sterile cell culture tubes.
6. Agarose gel premixed with SYBR green.
7. Agarose gel apparatus.
8. Minicentrifuge.
9. Shaker incubator.

3 Methods

3.1 Primer Design

1. For each gene segment to be removed, three nonphosphorylated primers are designed: two for the first round of PCR and one for the second round of PCR.
2. For the first round of PCR, design primers so that the sense primer is the vector sequence downstream of the gene to be excised and the antisense primer is the reverse complement of the vector sequence upstream of the gene to be excised (Fig. 2).
3. Vary the length of both primers between 18 and 22 bases so that their annealing temperatures are within 4 °C of each other.
4. For the second round of PCR, design a 40-base single-stranded oligo with a 20-nt complementarity to each of the two fragments to be connected (Fig. 2).

3.2 Linearization by the First-Step PCR

1. To linearize the vector in the first round of PCR, mix 10 μL Phusion polymerase 2× master mix with 1 μL each of the sense and antisense primers (10 μM), 1 μL template (10 pg/μL), and 7 μL of water (*see* **Note 1**).
2. In each reaction cycle, the reaction mixture is denatured at 98 °C for 15 s, annealed for 15 s (*see* **Note 2**), and extended at 72 °C (*see* **Note 3**). Mixing multiple pairs of primers does not degrade the quality of linear products compared to separate primer pairs (Fig. 3).
3. Repeat the cycle 25 times before a final extension of 10 min at 72 °C.
4. Hold the tubes at 4 °C if not used immediately.
5. The first step of PCR product was mixed with 2 μL DpnI (10 U/μL) and incubated at 37 °C for 1 h (*see* **Note 4**).

3.3 Clean-Up of the PCR Product

1. Clean up PCR product following manufacturer's instructions (*see* **Note 5**).
2. The final concentration of the fragments is measured by NanoDrop (*see* **Note 6**).

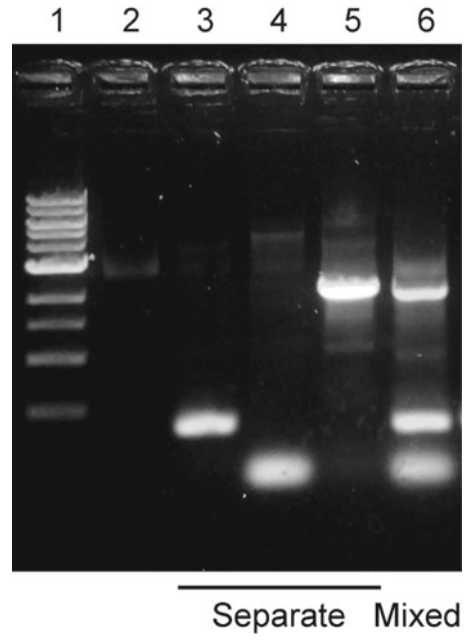

Fig. 3 Mixing three pairs of primers does not degrade the quality of linear products compared to separate primer pairs. *Lane 1*: DNA ladder, *Lane 2*: DNA template alone, *Lane 3–5*: PCR products with primer pair 1, 2, and 3 in three separate reactions, *Lane 6*: PCR products with all three pairs of primers mixed in one reaction. Reproduced from [23] with permission from Elsevier

3.4 Recircularization by the Second-Step PCR

1. Mix 250 ng of the PCR products (*see* **Note 7**) from the first round with 10 μL 2× Phusion polymerase master mix, 1 μL of ss-oligos (20 μM each, final concentration 1 μM), and water to make a 20 μL reaction mix.
2. In each reaction cycle, the reaction mixture is denatured at 98 °C for 15 s (*see* **Note 8**), annealed at 55 °C for 15 s, and extended at 72 °C (*see* **Note 9**). The reaction is repeated for 20 cycles.
3. The product is then ready for use in transformation. The transformation-ready product can be achieved within 8 h (PCR round 1: 2 h, DpnI treatment: 0.5 h, PCR clean-up 0.5 h, and PCR round 2: 3.5–4.5 h) (*see* **Note 10**).

3.5 Transformation and DNA Amplification

1. Thaw competent cells on ice for 10 min.
2. For each transformation reaction, transfer 30 μL competent cells to a sterile 14-mL culture tube. Incubate with 5 μL products of the second-round PCR on ice for 30 min (*see* **Note 11**).
3. Incubate the culture tube in a 42 °C water bath for exactly 45 s.
4. Transfer the culture tube back to ice and incubate for another 2 min.
5. Add 1 mL LB medium without antibiotics to the culture tube. Incubate the tube at 37 °C with vigorous shaking (225–250 rpm) for 1 h.
6. Spread 250 μL of cell culture evenly onto agar plates and incubate at 37 °C overnight.

3.6 Confirmation of Successful Removal of DNA Segments by Blue/White Colony Screen Assay

1. Spread 120 μL X-gal stock solution (20 mg/mL) to pre-made LB agar plates using a glass spreader at room temperature (*see* **Note 12**).
2. Incubate the plates at 37 °C for at least 30 min to dry.
3. Spread recovered competent cells onto the plates and incubate the plate at 37 °C overnight.
4. Colonies containing plasmids with successful DNA removal should appear blue (Fig. 4).

3.7 Confirmation of Successful Removal of DNA Segments by Colony PCR Reactions

1. For each colony to be tested, add 4 mL LB medium into a 14-mL sterile cell culture tube.
2. Use a sterile toothpick or pipette tip, pick a single colony from the agar plate.
3. Drop the tip or toothpick into the LB medium with appropriate antibiotics.
4. Repeat **steps 2–3** and randomly pick a total of 6–8 colonies from the same agar plate.
5. Incubate the cells at 37 °C for 4 h with vigorous shaking (*see* **Note 13**).

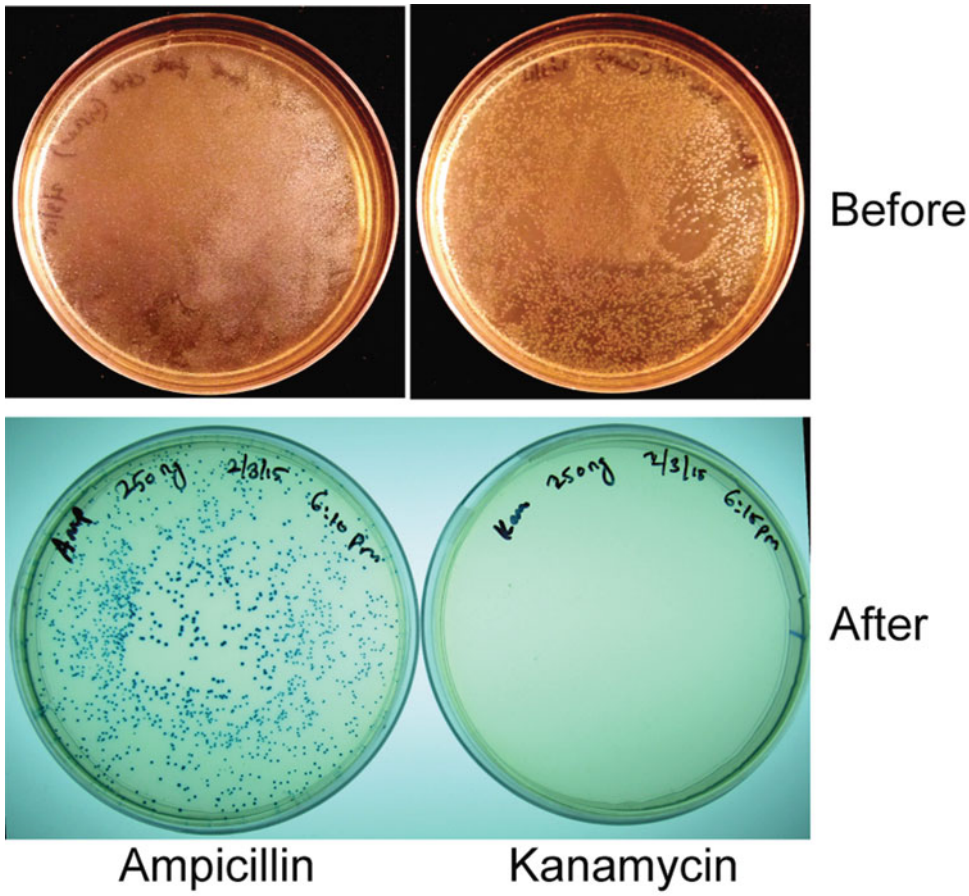

Fig. 4 Images of agar plates with plasmids before and after gene removal. Before gene removal, white colonies on ampicillin and kanamycin plates indicate that *u85*, *ampR*, and *kanR* genes were intact. After gene removal, *blue* colonies on the ampicillin plate indicate that *u85* was removed and *ampR* is intact; no colonies on the kanamycin plate indicate that *kanR* was removed. Reproduced from [22] with permission from Elsevier

6. Transfer 1 mL of cell culture to a 1.5 mL microcentrifuge tube (*see* **Note 14**).
7. Return the rest of the cell culture back to the shaker incubator (*see* **Note 15**).
8. Spin the cultures down at 13,000 rpm (15,700 rcf) for 1 min in a minicentrifuge (*see* **Note 16**).
9. Carefully remove the supernatant by decanting or with a pipette.
10. Resuspend each cell pellet with 50 μL sterile water with a pipette.
11. Place each tube into a 100 °C dry heat bath for 5 min to lyse the cells (*see* **Note 17**).
12. After cell lysis, transfer each tube to ice and incubate for 2 min.

13. Spin down the cell lysates at 13,000 rpm (15,700 rcf) for 1 min in a microcentrifuge.
14. Use 2 μL clear supernatants as the template for the following colony PCR reaction.
15. Use a full-length plasmid as a control.
16. For each reaction, mix 10 μL 2× DreamTaq master mix with 1 μL of sense and antisense primers (10 μM stock), 2 μL template, and 6 μL of water.
17. In each reaction cycle, the reaction mixture is denatured at 95 °C for 30 s, annealed for 30 s, and extended at 72 °C (1.5 min/kb) (*see* **Note 18**).
18. Repeat the cycle 20 times before a final extension of 10 min at 72 °C. Hold the temperature at 4 °C.
19. After the reaction, load 10 μL PCR product from each tube onto a 1% agarose gel premixed with SYBR green.
20. Run the agarose gel at 90 V for 30 min in TAE buffer (*see* **Note 19**).
21. Transfer the gel from the gel tray to a blue transilluminator and record gel images (*see* **Note 20**).
22. Compare the band from colony PCR with that from the control (no DNA removal). With DNA segments successfully removed, those bands should migrate faster than that from the control (Fig. 5).
23. For colonies that generate correct size of band, purify DNA using the rest of 3 mL culture 24 h after initial inoculation.
24. Confirm successful DNA removal by DNA sequencing.

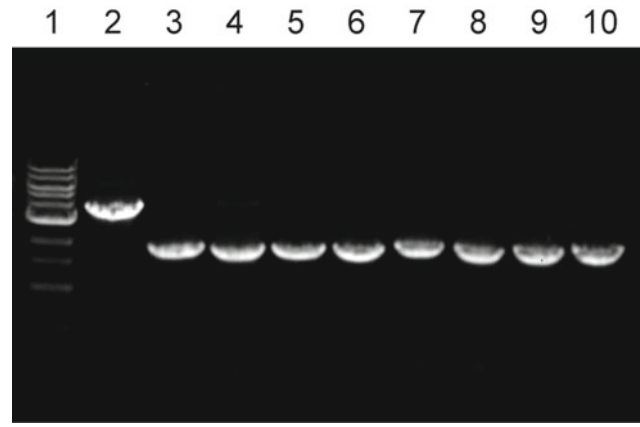

Fig. 5 Colony PCR showed that products from eight randomly selected colonies have all three gene segments (*u85*, *f1*, and *kanR*) removed from the plasmid pCRII-U85. *Lane 1*: DNA ladder, *Lane 2*: products of full-length pCRII-U85. *Lane 3–10*: products of eight randomly selected colonies. Reproduced from [23] with permission from Elsevier

4 Notes

1. Typically 1 pg to 5 ng template produces similar yield of PCR products. The amount of template should be adjusted so that residual template generates no false-positive colonies after transformation.
2. Use the Tm calculator for Phusion polymerase (e.g., from NEB website) to estimate the annealing temperature. When multiple primer pairs are used, use the lowest annealing temperature of all primers.
3. Given a processive rate of 1 kb/min for the polymerase, the extension time depends on the longest linear product (e.g., 2 min for a 2 kb linear product).
4. No buffer exchange is required for DpnI treatment.
5. PCR clean-up removes digested DNA template segments, primers, and enzymes. Use Molecular grade water to elute the PCR products.
6. A typical concentration of the products ranges from 30 to 50 ng/μL with 50 μL elution buffer**.**
7. The 250 ng products include a mixture of all three linear DNA segments from the PCR clean-up.
8. Longer denaturing time may degrade performance of Phusion polymerase.
9. The extension time depends on the longest DNA segments with 1 kb/min extension rate.
10. Fresh samples generate best results in transformation. Alternatively, intermediate products can be stored in a –20 °C freezer overnight to allow flexibility in schedule.
11. Extensive exposure of the competent cells to room temperature before heat shock can lead to loss of competency.
12. If the blue/white screen is preferred, select strains with *lacZ(del)M15* genotype that encodes a mutant form of β-galactosidase (also referred to as ω-peptide). Successful removal of the DNA segments from within the *LacZα* regenerates LacZα, which should rescue the activity of β-galactosidase via α-complementation. Other methods (e.g., colony PCR) can be used in more general cases of DNA removal.
13. Loosely cover the culture with a cap to ensure sufficient air flow.
14. A slightly turbid culture should appear if cells in the selected colony were successfully transformed.
15. These cell cultures will be used for DNA amplification for selected colonies 24 h after inoculation.

16. A cell pellet of about 1 mm^2 should appear at the bottom of the microcentrifuge tube.
17. Wear appropriate personal protective equipment (PPE) (safety goggle, mask etc.) as caps from the heated microcentrifuge tube may pop up. Alternatively, use microcentrifuge tubes with security lock.
18. An extension rate of 1.5 min/kb ensures complete extension reactions for DreamTaq polymerase.
19. A voltage setting of 5–8 V/cm is recommended.
20. Record the gel image immediately after gel electrophoresis. Leaving the gel in TAE buffer for extensive time may lead to broadening of DNA bands due to diffusion.

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