Analytical Biochemistry 481 (2015) 7–9

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

Multiplex gene removal by two-step polymerase chain reactions

Vishnu Vardhan Krishnamurthy, John S. Khamo, Ellen Cho, Cara Schornak, Kai Zhang *

Department of Biochemistry, University of Illinois at Urbana–Champaign, Urbana, IL 61801, USA

ARTICLE INFO

Article history:
Received 22 February 2015
Received in revised form 18 March 2015
Accepted 19 March 2015
Available online 23 April 2015

Keywords:
Restriction-free cloning
PCR
Multiplex gene removal
Plasmids

ABSTRACT

Precise DNA manipulation is critical for molecular biotechnology. Restriction enzyme-based approaches are limited by their requirement of specific enzyme sites. Restriction-free cloning has greatly improved the flexibility and speed of precise DNA assembly. Most of these approaches focus on DNA assembly rather than gene removal. Here we present a polymerase chain reaction (PCR)-based cloning method that allows removal of multiple gene segments from plasmids without using restriction enzymes and thermostable ligase. We demonstrate simultaneous removal of three gene segments from a plasmid. This approach could be beneficial to DNA library construction, genetic and protein engineering, and synthetic biology.

Precise and simple manipulation of genetic materials is essential for genetics, biochemistry, molecular and cellular biology, and synthetic biology. Classical cloning and mutagenesis strategies are limited by their dependence on restriction enzyme sites. Restriction-free cloning has significantly improved the flexibility and speed of genetic manipulation. Current DNA assembly methods can be categorized into several groups: methods based on end–homology recombination, including Gateway, overlap extension polymerase chain reaction (PCR)[1] [1], transfer PCR [2], DNA fragment assembly [3], restriction-free (RF) cloning [4], circular polymerase extension cloning (CPEC) [5], seamless ligation cloning extract (SLiCE) [6], and prolonged overlap extension PCR (POE–PCR) [7]; methods based on DNA annealing, including ligation-independent cloning (LIC), In-Fusion, quick and clean (QC) cloning, sequence and ligation-independent cloning (SLIC) [8], Gibson [9], and PaperClip [10]; and specific methods for mutation, including single primer mutagenesis (SOMA) [11], simultaneous noncontiguous deletion [12], multiplexing clonality, and genetic barcoding [13]. Most recent updates on DNA assembly were summarized in a recent collection [14]. Nearly all of the above-mentioned approaches focus on DNA assembly or insertion. Much less work has been developed for DNA removal. The ability to remove specific DNA sequences is critical for structure–function analysis of proteins, DNA library constructions, and genetic and protein engineering. Here we describe a restriction-free method that allows removal of multiple gene segments from plasmids within several hours.

Removal of single gene segment from a plasmid can be accomplished by inverted PCR followed by blunt-end ligation. We tested this strategy on plasmids with sizes ranging from 4.0 kb to 9.6 kb (see Fig. 1 in Ref. [15]). It remains challenging, however, to apply this strategy for multiplex gene removal, where nonspecific blunt-end ligation would generate undesirable products. To address this issue, we have developed a PCR-based cloning method to simultaneously remove multiple segments from a plasmid. The strategy uses inverted PCR to exclude targeted gene segments. The resultant fragments are recircularized by a second-round PCR with single-stranded oligos (ss-oligos), generating nicked plasmids that are ready for use in transformation (Fig. 1A). Each ss-oligo (“FA”, “BC”, and “DE” in Fig. 1A) is 40 bases in length, sharing a 20-nt complementarity with the two fragments to be connected. Annealing and extension of ss-oligos generate progressively longer double-stranded (ds) fragments, which can ultimately yield nicked plasmids. In the second round of PCR, multiple annealing events can result in various intermediates that are eventually amplified into nicked circular forms. Figure 2 in Ref. [15] shows one possible scenario based on a specific ss-oligo, FA. Initial annealing and extension generate three possible linear products: ds-FB, ds-BD, and ds-DF (top left panel). The sense strand of ds-FB may anneal to the antisense of ds-BD (referred to as B*D*) and extend to form ds-FD (bottom left panel). Subsequently, the sense strand of ds-FD may anneal to the antisense strand of ds-DF (referred to as D*F*, bottom right panel) and extend to form the desired nicked circular product (top right panel). When transformed into competent cells, these nicked DNA should be repaired.

* Corresponding author at: Department of Biochemistry, School of Molecular and Cellular Biology, University of Illinois at Urbana–Champaign, 600 South Mathews Avenue, 314B Roger Adams Laboratory, Urbana, IL 61801, USA. Fax: +1 217 244 5858.

E-mail address: kaizkaiz@illinois.edu (K. Zhang).

[1] *Abbreviations used:* PCR, polymerase chain reaction; ss-oligo, single-stranded oligo; ds, double-stranded; T_a, annealing temperature.

http://dx.doi.org/10.1016/j.ab.2015.03.033
0003-2697/

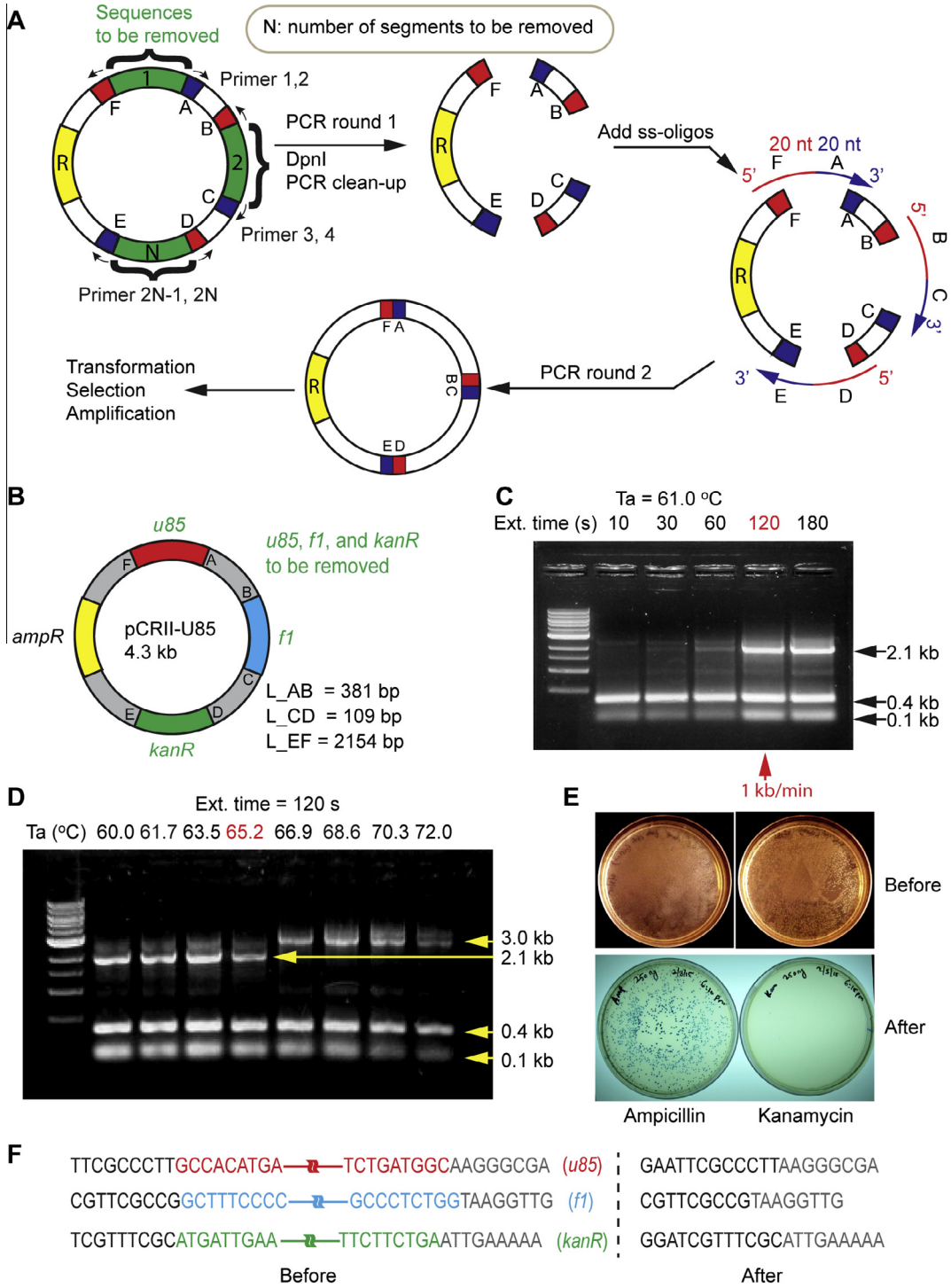

Fig.1. Scheme for multiplex gene segment removal via two-step PCR. (A) To remove *N* segments, 2*N* primers are used to generate *N* linear fragments in the first round of PCR. The product mixture is then treated with DpnI to remove the original template, followed by PCR cleanup. To set up the second round of PCR, fresh master mix and *N* ss-oligos are added to the purified PCR products. Each oligo shares a 20-nt complementarity with two neighboring fragments to be connected. Final PCR products containing nicked plasmids are used in transformation. (B) pCRII–U85 plasmid with gene segments *u85*, *f1*, and *kanR* to be removed. Expected lengths of linear fragments after the first round of PCR are 381, 109, and 2154 bp. (C) Yield of the largest segment of the first-round PCR product increased with the extension time. The optimal result occurred with a 120-s extension time (~1 kb/min). (D) Dependence of the first-round PCR product on the annealing temperature. High annealing temperature generated nonspecific products around 3.0 kb. (E) Images of agar plates with plasmids before and after gene removal. Before gene removal, white colonies on ampicillin and kanamycin plates indicated that *u85*, *ampR*, and *kanR* genes were intact. After gene removal, blue colonies on the ampicillin plate indicated that *u85* was removed and *ampR* was intact; no colonies on the kanamycin plate indicated that *kanR* was removed. (F) DNA sequencing results confirmed that *u85*, *f1*, and *kanR* gene segments were cleanly removed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We demonstrate three-gene removal ($N = 3$) from a plasmid, pCRII–U85 (Fig. 1B). This plasmid has a *u85* gene inserted into a *lacZ* sequence within the pCRII vector, which has both kanamycin and ampicillin resistance genes (*kanR* and *ampR*). This construct allows quick readout for successful gene removal. We aimed to remove *u85*, *kanR*, and part of *f1-ori* (abbreviated as *f1*) segments

from pCRII–U85 in a one-pot reaction. Removal of *u85* should give 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. In the first round of PCR, three primer pairs were used to linearize pCRII–U85. The amount of template could be varied from 5 ng to 1 pg without decreasing the yield of the PCR products (Fig. 3 in Ref. [15]). As expected, the first-round PCR generated three linear products with sizes of 381 bp (*u85–f1*), 109 bp (*f1–kanR*), and 2154 bp (*kanR–u85*). Optimal yield of the products, however, occurred with an extension time longer than 120 s, indicating an optimal extension time of 1 kb/min (Fig. 1C). Under a constant extension time of 120 s, we further optimized the annealing temperature using a gradient from 60.0 to 72.0 °C. We observed the expected products for annealing temperatures up to 65.2 °C, consistent with the manufacturer's recommendation for Phusion PCR polymerase (annealing temperature [T_a] = 61.0 °C). Higher annealing temperatures generated a larger product around 3 kb (Fig. 1D). We reasoned that primers cannot anneal well at such high temperatures. Instead, the longer PCR products may anneal to each other and be amplified. At optimal T_a and extension time, a single reaction containing all three primer pairs produced results comparable to those generated by three separate reactions containing individual primer pairs (Fig. 4 in Ref. [15]). We then treated the PCR products with DpnI, followed by PCR cleanup to remove the parent template and primers. DpnI treatment ensures that a minimal amount of full-length parent template propagates into the next round of PCR. Otherwise, undesired full-length template can be amplified because its antisense strand contains complementary sequences to ss-oligos. We determined the digesting performance of DpnI in various buffers and found that complete digestion can be achieved in both the Pfusion master mix and the vendor-recommended fast-digest buffer at 37 °C for 30 min (see Fig. 5 in Ref. [15]). DpnI did not work in pure water. Therefore, no buffer exchange was needed before DpnI treatment. The second round of PCR contained fresh master mix (2×), three purified linear fragments (250 ng total), and three ss-oligos (final concentration 1 μM) (see Ref. [15] for detailed PCR conditions). The transformation-ready product can be achieved within 8 h (PCR round 1: 2 h; DpnI treatment: 0.5 h; PCR cleanup: 0.5 h; PCR round 2: 3.5–4.5 h). After the second round of PCR, 5 μl of product was used to transform 30 μl of DH5α competent cells, which were then plated on both kanamycin and ampicillin agar plates pre-plated with X-gal. As expected, we observed blue colonies on the ampicillin plate and no colonies on the kanamycin plate, indicating that both *u85* and *kanR* genes were successfully removed (Fig. 1E). To confirm successful removal of the *f1* segment, we used colony PCR to examine the sizes before and after gene removal. Colony PCR showed that eight of eight randomly selected colonies produced plasmids with the correct size (Fig. 6 in Ref. [15]). DNA sequencing confirmed that *u85*, *f1*, and *kanR* segments were cleanly removed (Fig. 1F). We did observe some white colonies in the blue/white screening assay, indicating that full-length parent template may have been amplified. By varying the amount of template in the first round of PCR, we were able to generate a sufficient amount of PCR products using 1 pg of template (see Fig. 3 in Ref. [15]), which significantly reduced the percentage of white colonies in the final screening.

Our scheme used two-step PCR and ss-oligos to remove specific genes from a plasmid. A similar scheme has been used for DNA assembly using ligase cycling reaction (LCR) [16,17], where a thermostable ligase was used to ligate two single-stranded parent templates during PCR amplification. We reasoned that nicked plasmids can be repaired in cells, and therefore the ligation step can be skipped. By using synthetic ss-oligos as bridging sequences, our approach does not require generation of overlap between neighboring segments by PCR. Consequently, this approach does not generate extra sequence between neighboring segments. This approach does, however, require gene-specific bridging oligos because sequences outside the to-be-removed gene may vary in different vectors. It is worth noting, however, that a set of universal primers can be designed for partial gene truncation. In such a case, one can design primers using sequences within the gene so that the same set of primers can be used to partially truncate a gene from different plasmids.

In summary, we have presented a restriction-free cloning method for quick removal of multiple DNA segments from a plasmid via two-step PCR. The first round of PCR generated linear products, which were recircularized by the second round of PCR using ss-oligos. No thermostable ligase was used in the procedure. We achieved three-gene removal in a one-pot reaction. Our approach will be beneficial to applications in multiple-site mutagenesis, DNA library construction, genetic and protein engineering, and synthetic biology.

Acknowledgments

K.Z. is thankful for funding support from the University of Illinois at Urbana–Champaign (UIUC), United States. We thank Joseph Gall (Carnegie Institution for Science) for providing the pCRII plasmid and thank X. L. Nan (Oregon Health and Science University) for providing the ERK–GFP plasmid. We thank Sandra McMasters from the cell media facility of UIUC for providing DH5α competent cells and for her insightful comments on our experiments.

References

[1] A.V. Bryksin, I. Matsumura, Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids, Biotechniques 48 (2010) 463–465.
[2] A. Erijman, A. Dantes, R. Bernheim, J.M. Shifman, Y. Peleg, Transfer-PCR (TPCR): a highway for DNA cloning and protein engineering, J. Struct. Biol. 175 (2011) 171–177.
[3] P.J. Zuo, A.B.M. Rabie, One-step DNA fragment assembly and circularization for gene cloning, Curr. Issues Mol. Biol. 12 (2010) 11–16.
[4] F. van den Ent, J. Lowe, RF cloning: a restriction-free method for inserting target genes into plasmids, J. Biochem. Biophys. Methods 67 (2006) 67–74.
[5] J.Y. Quan, J.D. Tian, Circular polymerase extension cloning of complex gene libraries and pathways, PLoS One 4 (2009) e6441.
[6] Y. Zhang, U. Werling, W. Edelmann, SLiCE: a novel bacterial cell extract-based DNA cloning method, Nucleic Acids Res. 40 (2012) e55.
[7] C. You, X.Z. Zhang, Y.H. Zhang, Simple cloning via direct transformation of PCR product (DNA multimer) to *Escherichia coli* and *Bacillus subtilis*, Appl. Environ. Microbiol. 78 (2012) 1593–1595.
[8] M.Z. Li, S.J. Elledge, Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC, Nat. Methods 4 (2007) 251–256.
[9] D.G. Gibson, L. Young, R.Y. Chuang, J.C. Venter, C.A. Hutchison, H.O. Smith, Enzymatic assembly of DNA molecules up to several hundred kilobases, Nat. Methods 6 (2009) 343–345.
[10] M. Trubitsyna, G. Michlewski, Y. Cai, A. Elfick, C.E. French, PaperClip: rapid multi-part DNA assembly from existing libraries, Nucleic Acids Res. 42 (2014) e154.
[11] T. Pfirrmann, A. Lokapally, C. Andreasson, P. Ljungdahl, T. Hollemann, SOMA: a single oligonucleotide mutagenesis and cloning approach, PLoS One 8 (2013) e64870.
[12] R. Krishnakumar, C. Grose, D.H. Haft, J. Zaveri, N. Alperovich, D.G. Gibson, C. Merryman, J.I. Glass, Simultaneous non-contiguous deletions using large synthetic DNA and site-specific recombinases, Nucleic Acids Res. 42 (2014) e111.
[13] K. Cornils, L. Thielecke, S. Huser, M. Forgber, M. Thomaschewski, N. Kleist, K. Hussein, K. Riecken, T. Volz, S. Gerdes, I. Glauche, A. Dahl, M. Dandri, I. Roeder, B. Fehse, Multiplexing clonality: combining RGB marking and genetic barcoding, Nucleic Acids Res. 42 (2014) e56.
[14] S. Valla, R. Lale (Eds.), DNA Cloning and Assembly Methods (Methods in Molecular Biology, vol. 1116), Humana, Totowa, NJ, 2014.
[15] V.V. Krishnamurthy, J.S. Khamo, E. Cho, C. Schornak, K. Zhang, Polymerase chain reaction-based gene removal from plasmids, Anal. Biochem. Data in Brief, accepted.
[16] S. de Kok, L.H. Stanton, T. Slaby, M. Durot, V.F. Holmes, K.G. Patel, D. Platt, E.B. Shapland, Z. Serber, J. Dean, J.D. Newman, S.S. Chandran, Rapid and reliable DNA assembly via ligase cycling reaction, ACS Synth. Biol. 3 (2014) 97–106.
[17] B. Paetzold, C. Carolis, T. Ferrar, L. Serrano, M. Lluch-Senar, In situ overlap and sequence synthesis during DNA assembly, ACS Synth. Biol. 2 (2013) 750–755.