Modular construction of plasmids through ligation-free assembly of vector components with oligonucleotide linkers

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We have developed a modular method of plasmid construction that can join multiple DNA components in a single reaction. A nicking enzyme is used to create 5′ and 3′ overhangs on PCR-generated DNA components. Without the use of ligase or restriction enzymes, components are joined using oligonucleotide linkers that recognize the overhangs. By specifying the sequences of the linkers, desired components can be assembled in any combination and order to generate different plasmid vectors.

Traditionally, to construct a plasmid, DNA fragments are cut by restriction enzymes and then joined using ligase. Unfortunately, ligase-based methods can be relatively inefficient and generally only two fragments are joined at once in order to minimize undesired products. The method also requires that the targeted restriction sequences be present only at the ends and not the middle of the fragments—a requirement more difficult to satisfy as constructs become larger and more complex. More recently, ligation-independent cloning (LIC) methods have been developed. These methods have used uracil DNA glycosylase (UDG) (1), the 3′ to 5′ exonuclease activity of T4 (2) or poxvirus DNA polymerase (3), PCR primers containing ribonucleotides (4), or a nicking enzyme (5) to create long, 10 to 20 bp overhangs on PCR-generated DNA or plasmid vectors. Without ligase treatment, these PCR-generated DNA fragments can be annealed with the plasmid vector, or with other PCR-generated fragments, to generate plasmids that can be immediately amplified in *Escherichia coli*. Use of the long overhangs and removal of the ligase step greatly increases the efficiency of plasmid construction. Additionally, because overhangs are generated without restriction enzymes, DNA can be assembled independent of its sequence.

Here we have developed an LIC method to assemble multiple DNA fragments in a modular fashion into plasmids. We generated PCR products with primers containing the recognition site for the nicking enzyme *Nt.Bbv*CI. Digestion of the PCR fragments with *Nt.Bbv*CI produced long single-stranded overhangs—a 3′ overhang on the 5′ end of the DNA and a 5′ overhang on the 3′ end (Figure 1A). Without using ligase, we then stitched them together with oligonucleotide linkers to construct a plasmid (Figure 1B). By specifying the sequence of the linkers to be complementary to selected overhangs, any combination of nicked DNA fragments can be joined in any order.

In this case, we assembled plasmids with up to four PCR fragments. Using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) and standard reaction conditions, we amplified four genes: pUC origin (*ori*), kanamycin resistance (Kan^R), apramycin resistance (Ap^R), and *lacZα* (primers and templates described in Table 1). The PCR primers (Integrated DNA Technologies [IDT], San Diego, CA, USA) included a 15–20 bp tail containing the 7 bp *Nt.Bbv*CI nicking enzyme site (CC′TCAGC) and additional nucleotides that will form a 13–15 bp overhang sequence after nicking (Figure 1A). Additionally, the primer on the antisense strand of the Ap^R gene contained a 34-bp loxP recombinase site, a feature we wanted our plasmid to contain. The PCR products were separated by electrophoresis on a 0.5% agarose gel and recovered using silica gel columns (QIAquick Gel Extraction kit, Qiagen, Valencia, CA, USA). Forty-five microliters of each PCR product were digested with 1 µl *Nt.Bbv*CI (10 units/µl) (New England Biolabs, Ipswich, MA, USA) overnight at 37°C and

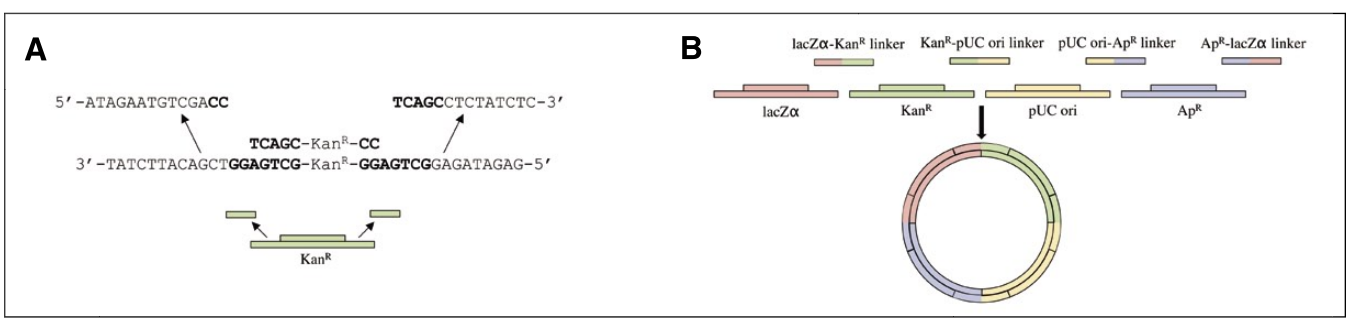

Figure 1. Modular construction of plasmids without ligase. (A) Module preparation using the Kan^R module as an example. Sequences at the ends of the module are shown with the *Nt.Bbv*CI recognition site in bold. (B) Schematic representation of the annealing and assembly of the pUC-Ap^R-lacZα-Kan^R plasmid from four modules and four linkers.

heat-inactivated at 80°C for 20 min. Finally, each sample was heated to 65°C and purified using silica gel columns according to the QIAquick PCR Purification kit (Qiagen). The column binding step was performed at 65°C to denature the small single-stranded DNA left by the nicking reaction and to recover only the large PCR product.

Three annealing reactions were performed using various combinations of the four plasmid components and six oligonucleotide linkers (IDT) listed in Table 1. The first contained two modules and two linkers (pUC ori, Ap^R, pUC-Ap^R linker, Ap^R-pUC linker), the second three modules and three linkers (pUC ori, Ap^R, Kan^R, pUC-Ap^R linker, Ap^R-Kan^R linker, Kan^R-pUC linker), and the third four modules and four linkers (pUC ori, Ap^R, lacZα, Kan^R, pUC-Ap^R linker, Ap^R-lacZα linker, lacZα-Kan^R linker, Kan^R-pUC linker; see Figure 1B). We believe it is important to add equimolar concentrations of each fragment, since variations in the concentration of the individual components might favor assembly of incomplete, non-circularized constructs (6). In the annealing reactions, 0.075 pmol of each module and linker were combined in 33 mM Tris-acetate (pH 7.9), 66 mM sodium acetate, 10 mM magnesium acetate, and 1 mM DTT (same conditions as standard T4 DNA polymerase reaction buffer). The three annealing reactions were heated to 65°C for 1 min and allowed to cool to room temperature over 5 min before 3.3 µl of 25 mM EDTA were added. Ten microliters of each annealing mixture were used to transform 100 µl of chemically competent XL1-Blue *E. coli*. Cells were heat-shocked at 42°C for 45 s, chilled at 4°C for 2 min, after which 500 µl of LB medium were added. After incubation at 37°C for 1 h, 175 µl of each were plated onto LB-agar plates supplemented with 50 µg/mL apramycin.

The pUC-Ap^R annealing reaction yielded 261 colonies, the pUC-Ap^R-Kan^R reaction yielded 16, and the pUC-Ap^R-lacZα-Kan^R reaction yielded 3. Restriction enzyme mapping showed that all of the pUC-Ap^R and pUC-Ap^R-Kan^R

Table 1. Oligonucleotide Sequences

Gene-specific Primers	
pUC-F	5′-**AACTCGATAGTATCCTCAGC**CCCGTAGAAAAGAT-CAAAGGATCTTCT-3′
pUC-R	5′-**CATAACTACGCTGAGG**ATGTGAGCAAAAGGCCAGCAA-3′
Kan^R-F	5′-**ATAGAATGTCGACCTCAGC**CCGGAATTGCCAGCTGGG-3′
Kan^R-R	5′-**GAGATAGAGGCTGAGG**TCAGAAGAACTCGTCAAGAAGGCG-3′
Ap^R-F	5′-**TAGACCATTGATCCTCAGC**GTCTGACGCTCAGTGGAACGAA-3′
Ap^R-loxP-R	5′-**TATTGATGGCTGAGG**ataacttcgtataatgtatgctatacgaagttatTCAGC-CAATCGACTGGCG-3′
lacZα-F	5′-**GTCAGGCTTATCCTCAGC**GCGCAACGCAATTAATGTGAGTT-3′
lacZα-R	5′-**TGTATCTCGCTGAGG**tcaCCATTCGCCATTCAGGCTG-3′
Oligonucleotide Adapters	
pUC- Ap^R linker	5′-TCAGCGTAGTTATG-TAGACCATTGATCC-3′
Ap^R-lacZα linker	5′-TCAGCCATCAATA-GTCAGGCTTATCC-3′
lacZα-Kan^R linker	5′-TCAGCGAGATACA-ATAGAATGTCGACC-3′
Kan^R-pUC linker	5′-TCAGCCTCTATCTC-AACTCGATAGTATCC-3′
Ap^R-pUC linker	5′-TCAGCCATCAATA-AACTCGATAGTATCC-3′
Ap^R-Kan^R linker	5′-TCAGCCATCAATA-ATAGAATGTCGACC-3′

Overhang sequences and nicking sites shown in bold.
The pUC ori and kanamycin resistance gene and promoter were amplified from pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA).
Apramycin resistance gene (*aac(3)IV*) and promoter were amplified from pIJ6902 (John Innes Centre, Norwich, UK).
Additional loxP sequence on Ap^R-loxP-R shown in lowercase.
The *lacZα* gene and promoter were amplified from pUC18.

colonies analyzed and two of the three pUC-Ap^R-lacZα-Kan^R colonies were correct. As expected, the two correct pUC-Ap^R-lacZα-Kan^R clones produced blue colonies on plates supplemented with 50 µg/mL apramycin and 50 µg/mL kanamycin, and overlaid with 100 µl of 10 mM IPTG and 100 µl of 2% X-Gal. While these results show promise, further optimization may still be possible in the annealing (e.g., ramped cooling, different salt concentrations, and concentration of components), transformation (e.g., amount and concentration of constructed plasmid transformed), and module purification (e.g., better removal of the small single-stranded nicked fragments) steps.

In summary, we have demonstrated construction of a plasmid from multiple modules in a single assembly step using LIC. The method was efficient, annealing up to four modules and four linkers simultaneously. By annealing with higher concentrations of DNA and transforming larger numbers of *E. coli*, we estimate that five or more fragments can be assembled. To our knowledge, this is the first LIC method to use (*i*) a nicking enzyme to produce the complementary "sticky" ends of the PCR-generated DNA fragments and (*ii*) oligonucleotide linkers to specify the joining and order of these fragments. The nicking reaction produces DNA with both a 5′ and 3′ overhang, a feature essential for the joining of fragments with oligonucleotide linkers. Internal nicking sequences that result in single-stranded nicks in the DNA fragments should not interfere with the cloning reaction; the assembled plasmids will remain intact and the *E. coli* will repair the nicks later. While two nicking sites on opposite strands within close proximity (we estimate close to be ~15 nucleotides) may cause double-stranded cleavage, these occurrences should be more rare. We cannot say whether two internal nicking sites in close proximity on the same strand will be problematic, but this might also be repaired later by the *E. coli*. Although any nicking enzyme could probably be used with our method, we chose *Nt.Bbv*CI because (*i*) its seven-base recognition site will be relatively rare and (*ii*) its nicking site is in the middle of the recognition sequence, making for better linker-overhang recognition at both ends of the DNA.

Many plasmid vectors share common components, for example, promoters, enhancers, multi-cloning sites, viral long-terminal repeats, fluorescent protein fusion cassettes, and polyadenylation sites; it is the order and combination of these components that often distinguish plasmids. While other methods of modular plasmid construction using LIC require oligonucleotide PCR primers containing ribonucleotides (4), or use a proprietary polymerase with exonuclease activity (3), our method is arguably more convenient in that it uses oligonucleotides synthesized from standard deoxynucleotides, and a nicking enzyme, of which several are commercially available. In these other modular construction methods (3,4), because the specificity of end joining comes from the complementarity between fragment overhangs, the order and total number of fragments cannot be altered. For example, if fragments A, B, and C can be joined to create a circular plasmid A-B-C, these same fragments cannot be annealed to form other arrangements such as A-C, A-C-B, B-A, C-B; in contrast, with our method any of these arrangements can be made. Commonly used components only need to be fabricated once. These modules can then be assembled in any combination or order by adding oligonucleotide linkers that specify the plasmid design. Note though, that if reversing the orientation of a gene or module is desired, it is necessary to reamplify it with a different set of primers. Otherwise, additional oligonucleotides could be used to create double-stranded linkers with the appropriate single-stranded overhangs so that the orientation of the module is reversed.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Rashtchian, A., G.W. Buchman, D.M. Schuster, and M.S. Berninger.** 1992. Uracil DNA glycosylase-mediated cloning of polymerase chain reaction-amplified DNA: application to genomic and cDNA cloning. Anal. Biochem. *206*:91-97.
2. **Aslanidis, C. and P.J. de Jong.** 1990. Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. *18*:6069-6074.
3. **Zhu, B., G. Cai, E.O. Hall, and G.J. Freeman.** 2007. In-fusion assembly: seamless engineering of multidomain fusion proteins, modular vectors, and mutations. BioTechniques *43*:354-359.
4. **Donahue, W.F., B.M. Turczyk, and K.A. Jarrell.** 2002. Rapid gene cloning using terminator primers and modular vectors. Nucleic Acids Res. *30*:e95.
5. **Kodumal, S.J., K.G. Patel, R. Reid, H.G. Menzella, M. Welch, and D.V. Santi.** 2004. Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. Proc. Natl. Acad. Sci. USA *101*:15573-15578.
6. **Smith, H.O., C.A. Hutchison III, C. Pfannkoch, and J.C. Venter.** 2003. Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides. Proc. Natl. Acad. Sci. USA *100*:15440-15445.

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