One-step DNA Fragment Assembly and Circularization for Gene Cloning

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Abstract

This article describes a one-step procedure based on *Taq* polymerase for the precise assembly of DNA fragments into circular constructs as long as 6 kb. The only prior step needed was the amplification of the gene to be cloned and the linear vector backbone, and the whole process up to assembly and circularization lasted only 2 days, compared with the conventional method's 2 weeks. Furthermore, the final DNA construct was used to transform *Escherichia coli* directly without any further treatment. By circumventing the need for DNA ligase, our "Quick Assemble" method offers an improvement over the combination of long PCR and overlap extension PCR, and is expected to facilitate various kinds of complex genetic engineering projects that require precise in-frame assembly of multiple fragments, such as multiple site-directed mutagenesis and whole-DNA library gene shuffling, as well as the construction of new plasmids with any promoter, resistance gene marker, restriction site, or any DNA tag.

Introduction

Recombinant DNA projects generally involve the ligation of two or more long fragments of DNA, but this process is sometimes not possible with conventional cloning techniques, or unwanted sequences need to be inserted first (Shevchuk *et al.* 2004). Several alternative approaches have been developed, but they usually require the use of a single-stranded DNA template (Kunkel 1985), which must be pre-prepared from double-stranded DNA. One method not requiring single-stranded DNA is DNA shuffling, in which pools of selected mutant genes are randomly fragmented and reassembled by in vitro homologous recombination, followed by the polymerase chain reaction (PCR) (Stemmer 1994). Some other ligase-free cloning methods need to use either topoisomerase I (Heyman *et al.* 1999) or uracil DNA glycosylase (Rashtchian *et al.* 1992; Buchman *et al.* 1993). Both of them need special vectors. Another method is overlap extension PCR. For example, Shuldiner *et al* developed a PCR-induced (ligase-free) subcloning method (Shuldiner *et al.* 1990; Shuldiner *et al.* 1991). However, the PCR protocols are still limited by existing techniques of DNA purification to remove excess primers through an Utrafree-MC unit. The exact steps contain 1) PCR amplification from genomic DNA or cDNA, 2) Filtration to remove excess primers, 3) Two sets of PCR reactions with 2 sets primers, "ac" and "bd", 4) Combine 2 sets of PCR products and cyclization. 5) Concentrating the mixture. It is labor-intensive.

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In this article, we describe a modified PCR method that simultaneously and precisely anneals and ligates DNA fragments, thereby allowing recombinant products to be created in fewer steps than conventional restriction enzyme cloning. We have demonstrated this one-step method of DNA fragment assembly and circularization by successfully inserting the mouse alpha-synuclein gene into a vector based on the plasmid pcDNA3.1/V5-His-TOPO. This method contains 1) PCR amplification from genomic DNA or cDNA, 2) circularization of PCR product and linear plasmid. The PCR product can be used directly in the circularization without any purification. The circularization step does not need to add any primers.

Material and Methods

Cell Culture
Mouse ATDC5 cells, from an embryonal carcinoma-derived chondrogenic cell line, were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Flow Laboratories, Irvine, UK) containing 5% fetal bovine serum (GIBCO BRL, Gaithersburg, MD), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biofluids Inc., Rockville, MD, USA), and incubated at 37ºC in a humidified atmosphere containing 5% carbon dioxide. An inoculum of cells (3×10^5 cells in 30 mL of medium) was transferred to each of 7 Petri dishes. Lead nitrate was added to reach a final concentration of 200 µmol/L per plate to stimulate alpha-synuclein transcription, and glutamic acid was added to reach a concentration of 200 µmol/L to increase the solubility of the lead nitrate. The cells were harvested at 48 hours.

Generation of cDNA Pool
Total RNA was isolated from cells with an RNeasy Mini Kit (Qiagen Sciences, Germantown, MD). Reverse transcription of the mRNA to generate the cDNA pool was performed with oligo-(dT) primers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA).

Production of DNA Fragments
The linear vector backbone of pcDNA3.1/V5-His-TOPO was produced by high-fidelity PCR amplification of the circular plasmid pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, USA) using the forward primer of 5'-aag ggc aat tct gca gat atc cag c-3' (melting temperature, Tm=60.6ºC) and reverse primer of 5'-AAG GGC AAT TCC ACC ACA CTG GAC-3' (Tm=61.9ºC). Each 50-µL sample in the PCR thermocycler (Takara Bio, Shiga, Japan) contained 5 µL 10 × AccuPrime™ PCR Buffer I (Invitrogen), 1 µL of each primer at 10 µM, 40 ng of circular pcDNA3.1/V5-His-TOPO, and 0.2 µL *Taq* DNA polymerase (AccuPrime™ *Taq* High Fidelity; Invitrogen). The mouse alpha-synuclein gene was amplified from the cDNA pool from ATDC5 cells by high-fidelity PCR using the forward primer 5'-**gtc cag tgt ggt gga att gcc ctt** <u>acc atg</u> gat gtg ttc atg aaa gga ctt tca aag-3' (the Kozak consensus

sequence is underlined and the overlapping sequence with the vector is bolded) and reverse primer 5'-**GCT GGA TAT CTG CAG AAT TGC CCT T**TT AGG CTT CAG GCT CAT AGT CTT GGT AGC-3'. Each 50-µL sample in the PCR thermocycler contained 5 µL 10 × AccuPrime™ PCR Buffer I, 1 µL of each primer at 10 µM, 40 ng of cDNA derived from ATDC5 cells, and 0.2 µL AccuPrime™ *Taq* High Fidelity. The PCR cycling profile for all reactions was as follows: 94ºC for 2 minutes, 94ºC for 30 seconds, 52ºC for 30 seconds, and 68ºC for 3 minutes (for 35 cycles); the holding temperature was 4°C.

Assembly of DNA Fragments

Single-step DNA assembly and circularization was performed on non-purified PCR amplification products by high-fidelity PCR, simply by adding more *Taq* DNA polymerase. Each 50-µL sample contained 5 µL 10 × AccuPrime™ PCR Buffer I, 0.5 µL of raw reaction product mix containing the linear vector backbone, 2 µL of raw reaction product mix including the alpha-synuclein gene, and 0.2 µL AccuPrime™ *Taq* High Fidelity. The ratios of insert to vector were 4: 1. This ratio should change based on the size of the fragments. But for the insert range of 500 bp to 2000 bp, the ratios of insert to vector kept in 4:1 were worked well. The PCR cycling profile was as follows: 94ºC for 2 minutes, 94ºC for 30 seconds, 52ºC for 30 seconds, and 68ºC for 3 minutes (for 35 cycles); the holding temperature was 4°C. Because the primers included complementary directional overhangs, the correct head and tail sequences of the vector and gene were expected to anneal and assemble into plasmid pcDNA3.1/V5-His-TOPO-alpha-synuclein (Figure 1).

Transformation Procedure

One tube of 50 µL DH5α *Escherichia coli* cells was thawed on ice and 1 µL of raw reaction product mix from the previous step, which contained the assembled DNA construct, was immediately added and mixed gently. The tube was then incubated on ice for 30 minutes, heated for 60 seconds in a 42°C water bath, and placed on ice for 2 minutes. The contents were added to 950 µL of pre-warmed SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM glucose) in a 1.5-mL tube and shaken at a speed of 225 rpm at 37°C for 1 hour. Finally, 200 µL was spread onto a pre-warmed LB agar plate containing 100 µg/mL ampicillin, and the plate was incubated overnight at 37°C.

Plasmid DNA Extraction

A colony of transformed *E. coli* cells was picked and grown in 5 mL LB medium at 37°C overnight. Plasmid DNA was extracted with the Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO).

Restriction Enzyme Digestion

Double digestion was performed on a sample of extracted plasmid DNA with *Hin*dIII (20,000 U/mL, New England Biolabs, Ipswich, MA) and *Xho*I (10,000 U/mL, New England Biolabs). The two restriction sites were just beyond the gene either side (Figure 1). Digestion products were subjected to agarose gel electrophoresis to determine the size of the inserted fragment.
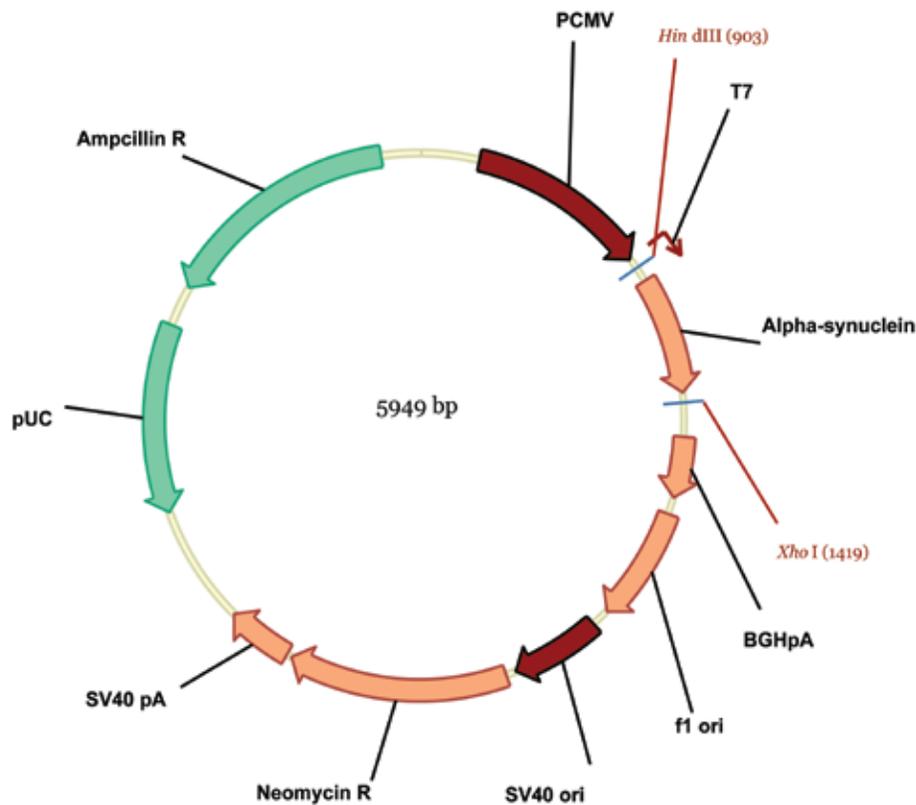

Figure 1. Diagram of plasmid pcDNA3.1/V5-His-TOPO-alpha-synuclein. Plasmid pcDNA3.1/V5-His-TOPO and mouse gene alpha-synuclein were amplified separately, and complementary head and tail sequences allowed directional self-assembly.
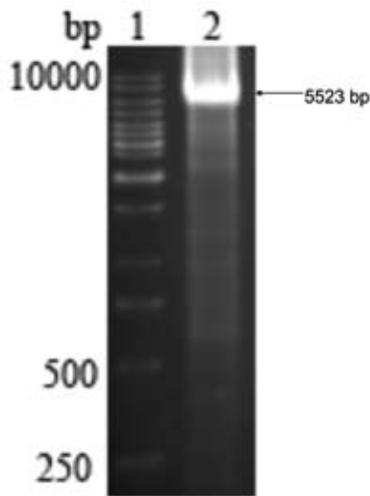
Figure 2. Amplification of linear backbone pcDNA3.1/V5-His-TOPO. Plasmid pcDNA3.1/V5-His-TOPO was amplified by the polymerase chain reaction using AccuPrime™ *Taq* High Fidelity polymerase. Lane 1: DNA molecular weight markers. Lane 2: amplified linear pcDNA3.1/V5-His-TOPO (5523 bp).

Western Blot
Complexes between pcDNA3.1/V5-His-TOPO-alpha-synuclein Lipofectamine™ 2000 Transfection Reagent (Invitrogen) were prepared according to the particular specifications of the manufacturer. Generally, after pcDNA3.1/V5-His-TOPO-alpha-synuclein-reagent complexes had formed, the mixture was exposed to cultures and incubated for 6 h before replacing the Dulbecco's modified Eagle's medium and Ham's F-12 medium. Lipofectamine™ 2000

Transfection Reagent was used as per guidelines for 6-well plates with a volume of 10 µl per well. The cells were immediately scraped off the plate after 48 h incubated at 37ºC in a humidified atmosphere containing 5% carbon dioxide. The sample was sonicated for 10-15 seconds to shear DNA and reduce sample viscosity. An aliquot of cell extract (15 µg of cytosolic soluble proteins) was used for determining alpha-synuclein protein content by purified mouse anti-alpha-synuclein antibody (BD Transduction Laboratories).

Sequencing
Sequencing of the final plasmid construct was performed by the Genome Research Centre, The University of Hong Kong, with the ABI BigDye sequencing method, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Results
Linear backbone of pcDNA3.1/V5-His-TOPO
As revealed by agarose gel electrophoresis, linear pcDNA3.1/V5-His-TOPO was produced by high-fidelity PCR from circular pcDNA3.1/V5-His-TOPO in sufficient quantity and was the expected size of 5523 bp (Figure 2).

Mouse alpha-synuclein
The mouse alpha-synuclein gene that was amplified from mouse cDNA was the expected size of 423 bp (Figure 3).

The assembly product
For assembly both vector and insert, the quantities of vector and insert were 234.6 ng and 938.4 ng, respectively. The concentration of vector and insert were determined by spectrophotometer at λ_{260}. The idle ratios of the insert to vector were expected that both the vector and insert are completely assembly together. However, the ratios were not significant from 10:1 to 4:1. We used the ratios as 4:1. Both the linear vector pcDNA3.1/V5-His-TOPO and amplified
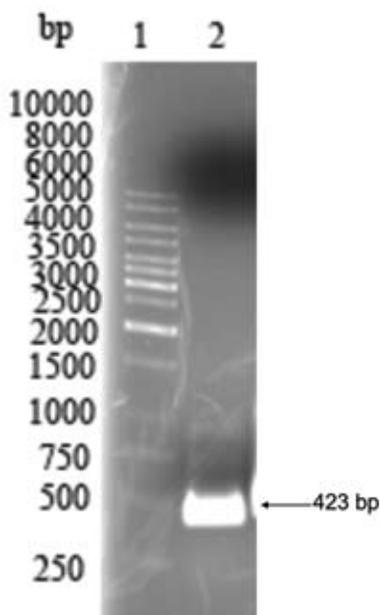

Figure 3. Amplification of the mouse alpha-synuclein gene. Alpha-synuclein was amplified by the polymerase chain reaction using AccuPrime™ *Taq* High Fidelity polymerase. Lane 1: DNA molecular weight markers. Lane 2: amplified linear alpha-synuclein gene (423 bp).
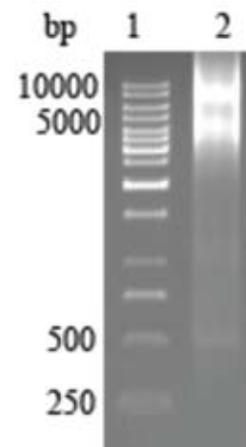

Figure 4. Circularization of fragments. Amplified linear pcDNA3.1/V5-His-TOPO and alpha-synuclein were joined and circularized by the polymerase chain reaction using AccuPrime™ *Taq* High Fidelity polymerase. Lane 1: DNA molecular weight markers. Lane 2: circularization products of pcDNA3.1/V5-His-TOPO and alpha-synuclein.
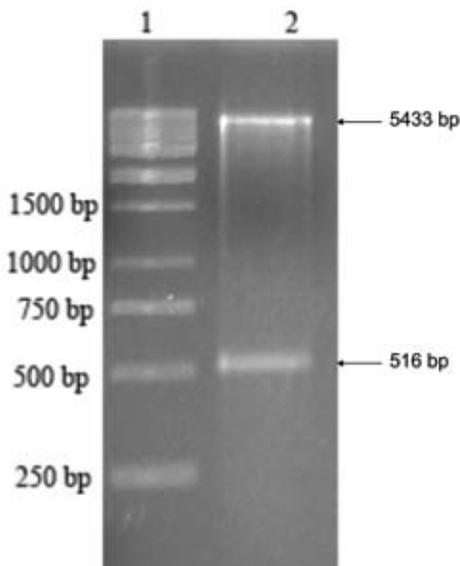
Figure 5. Confirmation of assembled plasmid pcDNA3.1/V5-His-TOPO-alpha-synuclein. The pcDNA3.1/V5-His-TOPO-alpha-synuclein plasmid was cleaved by *Hind*III and *Xho*I. Lane 1: DNA molecular weight markers. Lane 2: Two digestion fragments of 5433 bp and 516 bp.

alpha-synuclein gene annealed and ligated together during PCR using DNA polymerase. The raw assembly product was a mixture of circular DNA molecules that ranged in size from 500 bp to more than 10,000 bp (Figure 4). Because we added ACC before the start site in the alpha-synuclein gene to generate the Kozak consensus sequence (to assist in the initiation of translation), the exact size of the desired plasmid containing the vector and gene was expected to be 5949 bp.

After transformation of *E. coli*, only cells hosting intact pcDNA3.1/V5-His-TOPO-alpha-synuclein plasmids could grow on LB plates containing ampicillin. The others still kept in liner form were not grown at all. Using this method, we got 7 colonies per transformation (transformation efficiency was 1.864×10^2 colonies per microgram of PCR insert). We picked up 3 colonies. One of them contained the 423 bp insert. The other 2 contained inserts of different sizes (250

Figure 6. Western blot of assembled plasmid pcDNA3.1/V5-His-TOPO-alpha-synuclein. The pcDNA3.1/V5-His-TOPO-alpha-synuclein plasmid was transfected to mouse ATDC5 cells. Lane 1: pcDNA3.1/V5-His-TOPO-alpha-synuclein plasmid was transfected to mouse ATDC5 cells. Lane 2: pcDNA3.1/V5-His-TOPO empty vector was transfected to mouse ATDC5 cells.

bp and 1000 bp). Double digestion of extracted plasmid DNA with *Hind*III and *Xho*I yielded two fragments on a 1% agarose gel, of sizes 5433 bp and 516 bp, representing linear vector pcDNA3.1/V5-His-TOPO and alpha-synuclein gene, respectively (Figure 5). We also performed a western blot proving that the gene was functional (Figure 6). The pcDNA3.1/V5-His-TOPO-alpha-synuclein expressed more alpha-synuclein protein in ATDC5 cells than the control empty pcDNA3.1/V5-His-TOPO. These findings indicated that mouse alpha-synuclein had been successfully inserted into pcDNA3.1/V5-His-TOPO and cloned.

Sequencing
The DNA sequence of the complete pcDNA3.1/V5-His-TOPO-alpha-synuclein construct was 100% in agreement with the sequences of the two constituent DNA fragments (data not shown).

Discussion
In conventional gene cloning, suitable restriction enzymes need to be identified before genes can be inserted into a vector plasmid. Both vector plasmid DNA and gene insert are typically cut with the same restriction enzyme(s) and then ligated together with DNA ligase (Russell and Sambrook 2001). However, the gene may sometimes contain internal restriction sites that are also recognized by the chosen enzyme, and these require prior deletion by site-directed mutagenesis. Many approaches to do this are available, because site-directed mutagenesis is widely used to study protein function. An oligonucleotide-based method was developed by Michael Smith, who was awarded a Nobel Prize in 1993 for this contribution (Zoller and Smith 1987). There are also methods based on PCR. Stemmer et al. used DNA shuffling to synthesize both strands of the promoter and structural regions of the *bla* gene from 56 individual oligonucleotides. Those researchers introduced five point mutations to create five new restriction sites to differentiate the synthetic gene from the naturally occurring *bla* gene (Stemmer *et al.* 1995). However, a shortcoming of current PCR protocols, including long PCR and overlap extension PCR, is the time and resources needed for DNA purification, digestion, and ligation.

Our "Quick Assemble" method requires only 2 days to construct the final plasmid, compared with the conventional method's 2 weeks. Shuldiner *et al.* reported their work to subclone polymerase chain reaction products in a single day (Shuldiner *et al.* 1990; Shuldiner *et al.* 1991). Their method is more complicated than ours. It contains 5 steps. 1) PCR amplification from genomic DNA or cDNA, 2) Filtration to remove excess primers, 3) Two sets of PCR reactions with 2 sets primers, "ac" and "bd", 4) Combine 2 sets of PCR products and cyclization. 5) Concentrating the mixture. It is labor-intensive.

Our method contains only 2 steps. They are 1) PCR amplification from genomic DNA or cDNA and linear plasmid, 2) circularization of PCR product and linear plasmid. There is no reason our method need 1 day more than Shuldiner's method. The stop point for us is getting the *E. coli* colonies which hosted the recombinant plasmid. For sure, Shuldiner's method needs 2 days if they set the end point as getting the colonies. First, in our method, the individual parts of the desired construct are separately generated by PCR as linear DNA fragments. Then, the PCR products, which do
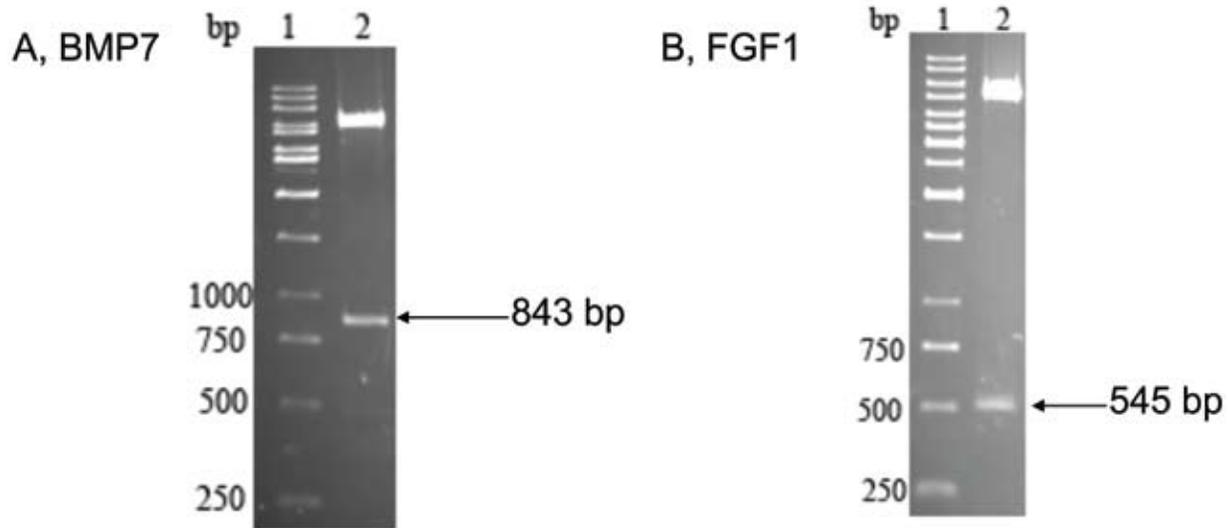
Figure 7. Confirmation of assembled plasmid pcDNA3.1/V5-His-TOPO-BMP7 and pcDNA3.1/V5-His-TOPO-FGF1.The pcDNA3.1/V5-His-TOPO-BMP7 and pcDNA3.1/V5-His-TOPO-FGF1 plasmids were cleaved by *KpnI* and *NotI*. A, Lane 1: DNA molecular weight markers. Lane 2: The size of BMP7 was 843bp. B, Lane 1: DNA molecular weight markers. Lane 2: The size of FGF1 was 545bp.

not need be gel extracted or purified, are directly assembled together in one PCR tube together with buffer and DNA polymerase without any primers. Owing to the directional complementary overhangs in the fragments and their large combined length (about 6 kb), the next PCR step allows annealing and intramolecular ligation of the plasmid without the need for DNA ligase. During single stranded melting, PCR created the complete second strand. Furthermore, we have shown that the circularized final plasmid can be cloned via *E. coli* transformation, again without any additional treatment, gel extraction, or purification.

For check the repeatability of this method, 2 other genes, bone morphogenetic protein 7 (BMP7), and human fibroblast growth factor 1 (FGF1) were cloned into vector pcDNA3.1/ V5-His-TOPO (Figure 7). Both the recombinant vectors were confirmed by the insert size generated by restriction enzyme *KpnI* and *NotI* digestion and the sequences.

Our new 2-day PCR-based method could be used to generate both circularized and linear final assembled products. Smith et al. have recently described techniques of synthesizing approximately 5-kb segments of DNA, followed by their assembly into longer sequences by conventional cloning methods (Smith *et al.* 2003). The size of the synthesized DNA can reach 31,656 bp (Kodumal *et al.* 2004). Our method could make the synthesis and assembly of these large molecules in a quick and efficient way, while circumventing the use of DNA ligases. Our ligase-free method also offers an additional improvement over the combination of long PCR and overlap extension PCR, and is expected to facilitate various kinds of complex genetic engineering projects that require precise in-frame assembly of multiple fragments, such as multiple site-direct mutagenesis and whole-DNA library gene shuffling. In addition, new restriction sites could be created. We have recently deleted 2 *Pvu*II restriction sites in the pUC19 plasmid with this technique (P. Zuo and J. Tian, 2007, unpublished data).

"TA cloning" is a popular method of cloning without the use of restriction enzymes; instead, PCR products are amplified with only *Taq* DNA polymerase and other polymerases. These polymerases lack 5'-3' proofreading activity and add an adenosine triphosphate residue to the 3' ends of the double-stranded PCR products. Such PCR amplified products can thus be cloned in a linearized vector that has complementary 3' thymidine triphosphate overhangs. The major problem is that the gene has a 50% chance of getting cloned in the reverse direction. The method also needs DNA ligase. The vector pcDNA3.1/V5-His-TOPO is a plasmid used in TA cloning (Tekin *et al.* 2006), and we used it as the template for the linear vector backbone in our protocol, implying that our "Quick Assemble" technique could also be applied to improve TA cloning.

The number of vectors in molecular cloning is continually growing and the correct vector must be carefully chosen to match the purpose of the research. However, for some projects no vector will be suitable. With our protocol, any kind of vector, or even any part(s) of a vector, could be assembled together and used as a template to generate any vector backbone for gene insertion without the need for ligase. Our new method would hence allow the construction of new, tailor-made plasmids to fulfill a variety of needs. The following reporter genes could conceivably be constructed:

1) Green fluorescent protein that fluoresces green when exposed to blue light (Prendergast and Mann 1978) or ultraviolet light (Tsien 1998);
2) Red fluorescent protein (Campbell *et al.* 2002);
3) β-Galactosidase that reacts to substrates of different β-galactosidases, including ganglioside GM1, lactosylceramides, lactose, and various glycoproteins (Matthews 2005);
4) Luciferase (from lightening bugs) (Greer and Szalay 2002);

5) Chloramphenicol acetyltransferase (from bacteria) (Leslie *et al.* 1988);
6) β-Glucuronidase commonly used in plants (Wynn *et al.* 1981).

Alternatively, our new method could attach reporter or other tags to a targeted gene. For example, polyhistidine tags are often added to allow the affinity purification of polyhistidine-tagged recombinant proteins after expression in *E. coli* (Hengen 1995). Human immunoglobulin Fc fragment, maltose-binding protein, glutathione S-transferase, and thioredoxin could also function as tags when fused with target genes (Xu *et al.* 2008). Furthermore, our method would enable researchers to replace promoters or modify resistance to antibiotics by using different or multiple resistance gene markers such as ampicillin, kanamycin, neomycin, chloramphenicol, gentamycin, tetracycline, erythromycin, streptomycin, vancomycin, and spectinomycin (Wang *et al.* 2009).

In summary, we describe a new one-step "Quick Assemble" method of precisely and simultaneously joining multiple DNA fragments followed by intramolecular ligation. This technique of plasmid assembly and circularization could be used to easily construct new plasmids with any promoter, resistance gene marker, restriction site, or tag. In the future, DNA shuffling might become easier and quicker with this method to offer new opportunities in genetic engineering.

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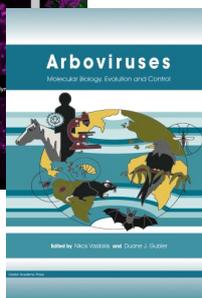
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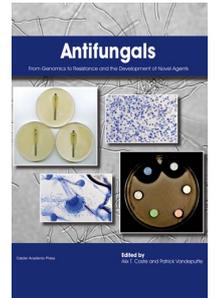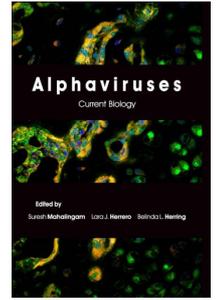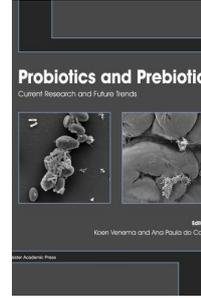
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