Universal Restriction Site-Free Cloning Method Using Chimeric Primers

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ABSTRACT

A universal restriction site-free cloning method has been developed to precisely insert a DNA fragment into a vector at any desired location without altering any nucleotide(s) in either the DNA fragment or the vector. The technique employs two pairs of chimeric primers, each containing a ribonucleotide. One pair of primers is used to amplify a target DNA fragment and another is used to prepare a linear vector. The ribonucleotide is used as a specific site for cleavage promoted by rare-earth metal ions such as La^{3+} or Lu^{3+}. Therefore, blunt-ended PCR products can be converted into a dsDNA with single-stranded 3′ overhangs for efficient ligation. The primers are designed so that both the target DNA fragment and vector PCR products create defined 3′ overhangs to permit the formation of a seamless plasmid during the subsequent ligation. This method has been used successfully to clone the E. coli *gene coding for peptidyl-tRNA hydrolase.*

INTRODUCTION

PCR has largely been used to rapidly amplify specific DNA fragments for cloning. Constructing native proteins, creating chimeric genes, and studying interactions between proteins and DNA all require a cloning method that allows one to insert a DNA fragment into a defined position of the vector without changing the DNA sequence. Although there are several methods that can achieve this objective, such as blunt-end ligation (2,9), sticky end ligation (created by using class IIS restriction endonuclease digestion) (8,11), and restriction site-free cloning (3), some drawbacks still exist. The ligation of blunt-ended DNA fragments is always associated with low efficiency. The efficient ligation with sticky ends, which uses class IIS restriction endonuclease digestion, is very dependent on the recognition sites in the DNA fragment or vector. For example, when *Eam*1104I is used, 5-methyl-deoxycytosine is needed in the PCR process to protect the internal recognition site of the products from enzyme attack (11). The recognition sequence of most class IIS enzymes is five or six nucleotides long, and the cleavage site from the recognition sequence varies from 1 to 20 nucleotides in length (14). Therefore, at least 10 nucleotides that are not related to the sequence of the plasmid or the insert must be added to the 5′ end of each primer for efficient cleavage. The site-free cloning method also requires long primers, but the preparation of long primers often introduces errors. To overcome these obstacles, we have developed an efficient cloning method that can precisely insert DNA fragments into a defined position of a vector without altering any nucleotide(s).

MATERIALS AND METHODS

Plasmids and Cells

The pDS56/RBSII plasmid (13) was used for the construction of the expression plasmid that produced a native protein of the *E. coli pth*. Sure® 2 strain (Stratagene, La Jolla, CA, USA) that was used for transformation.

Enzymes and Reagents

We used the Expand High Fidelity PCR System, T4 DNA ligase (both from Roche Molecular Biochemicals, Indianapolis, IN, USA), T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA), dNTPs (Roche Molecular Biochemicals), lanthanum chloride (Sigma, St. Louis, MO, USA), and lutetium chloride (Aldrich Chemical, Milwaukee, WI, USA). Chimeric primers were synthesized by Microsynth GmbH (Balgach, Switzerland).

Primer Design

The primers in this cloning method were used to link two unrelated DNA fragments together at defined positions to form a new plasmid. This was

achieved by designing primers so that blunt-ended PCR products of both vector and target DNA would have identical overlapping nucleotide sequences at their ends. A ribonucleotide was introduced within the primer as a specific cleavage site for rare-earth metal ions (6,10). Therefore, the rare-earth metal ions would selectively cleave the phosphodiester bond between the ribonucleotide and deoxyribonucleotide in the blunt-ended PCR products to form defined ssDNA 3′ overhangs that were complementary between the vector and target DNA fragment. A desired plasmid could then be obtained during the following ligation (Figure 1). Table 1 lists the primers used to prepare the linear vector and to amplify the *pth*-encoding region.

Table 1. Primers for Preparation of Vector and *pth*-Encoding Region

Preparation of a linear vector	
Forward primer	CGTCATAGT[**U**]AATTTCTCCTCTTTAATGAA
Reverse primer	GCAATAAGG[**C**]AGTTATTGGTGCCCTTAAAC
Preparation of a DNA fragment for *pth*-encoding region	
Forward primer	AACTATGAC[**G**]ATTAAATTGATTGTCGGCCT
Reverse primer	GCCTTATTG[**C**]GCTTTAAAGGCGTGCAAT

The underlined nucleotide in brackets is the ribonucleotide. Primers are in the 5′→3′ direction.

Amplification of Vector and DNA Fragment of *E. coli pth*-Encoding Region

All the PCR experiments were performed on a TRIO-Thermoblock (Biometra GmbH, Göttingen, Germany). A typical 100-µL reaction consisted of 0.5 µg *E. coli* BL21 strain chromosomal DNA or 5 ng pDS56/RBSII plasmid DNA as template, 0.5 µM primers each, 200 µM each dNTPs, and 2.5 U Expand High Fidelity DNA polymerase in 1× reaction buffer with Mg^{2+} (supplied with kit). A 35-cycle, three-step PCR program was applied. The denaturation was at 94°C for 45 s, annealing at 50°C for 1 min, and extension at 68°C for 1 min for the amplification of the DNA fragment or 3 min for the vector. The final extension step was at 68°C for 15 min. The PCR products were then purified by electrophoresis on a 1% agarose gel in TAE buffer, followed by a DNA gel extraction step.

Creating 3′ Overhangs for Ligation

The purified PCR products of the vector and DNA fragment were incubated at 50°C in a 10-mM $LaCl_3$-NaOH (or $LuCl_3$-NaOH) solution, pH 9.5, for 120 min to selectively hydrolyze the phosphodiester bond between the deoxyribonucleotide and the ribonucleotide. Both samples were then precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 vol ethanol and were quantified on an agarose gel before phosphorylation. The phosphorylation reaction was carried out at 37°C in 10 µL 1× T4 DNA ligation buffer using 10 U T4 DNA polynucleotide kinase for 30–60 min. T4 DNA polynucleotide kinase-treated vector (25–50 ng) and DNA fragment (10–20 ng) were combined in an Eppendorf® tube, and 1× T4 DNA ligation

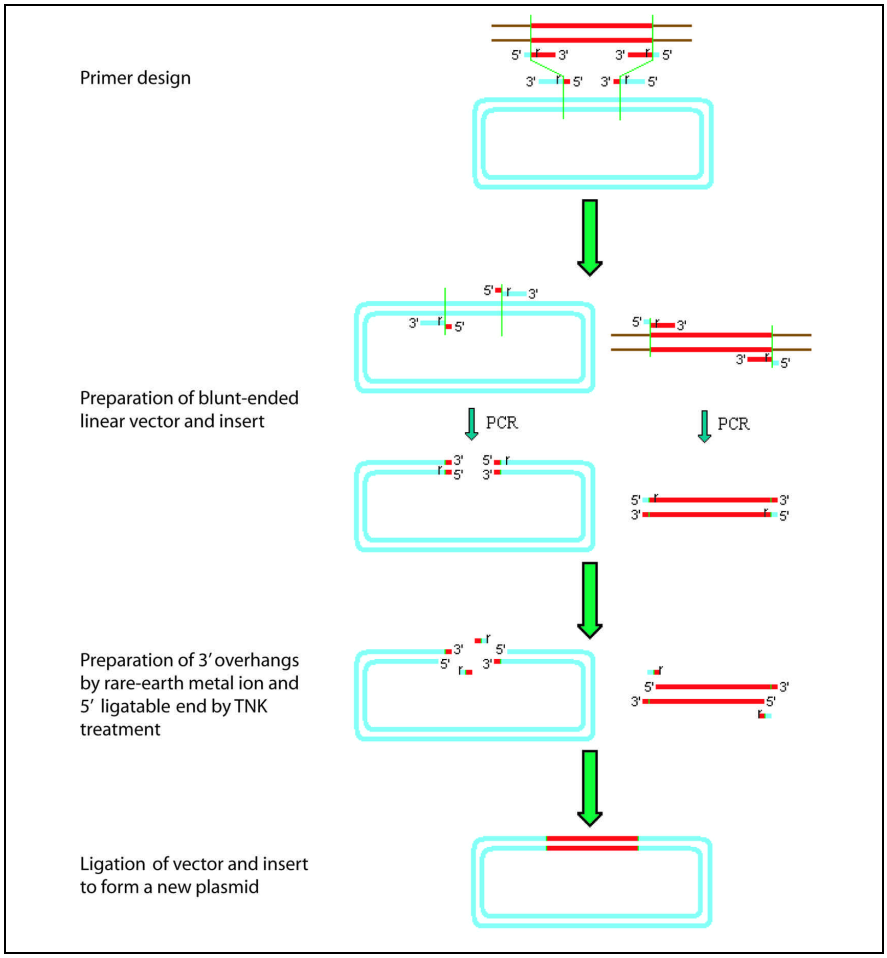

Figure 1. Outline of the universal restriction site-free cloning method using chimeric primers. Each primer is designed to contain a ribonucleotide (r) and nucleotide sequences (at the 5′ end) that are complementary between the pair of primers for the vector and the pair of primers for the insert. Blunt-ended PCR products are produced by using a blend of polymerases such as *Taq* DNA polymerase, which can use ribonucleotides as templates, and *Pwo*, which produces blunt end. The defined 3′ overhangs are generated by the incubation of PCR products with rare-earth metal ion solution. After phosphorylation to add a phosphate to the 5′ end, the two PCR products are ligated directionally to form a new plasmid.

Table 2. Efficiency of the Universal Restriction Site-Free Cloning Method

Experiment	Total colonies	Plasmids analyzed	Expected plasmids
1 (La^{3+} treatment)	33	10	5
2 (La^{3+} treatment)	96	10	6
3 (Lu^{3+} treatment)	67	10	5

buffer was added up to a final volume of 20 µL. The mixture was then heated at 60°C for 2 min and was slowly cooled to room temperature. T4 DNA ligase (5 U) was used for ligation at 4°C overnight. Afterwards, the ligated DNA sample was cleaned up by phenol extraction and precipitated by ethanol.

The transformation was performed on a Gene Pulser™ electroporation system (Bio-Rad Laboratories, Hercules, CA, USA) with a protocol supplied by the manufacturer. *E. coli* Sure 2 electroporation-competent cells (50 µL) and the above DNA sample were used for transformation. After electroporation, the transformants were immediately mixed with 450 µL SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, and 20 mM glucose) and incubated at 37°C for 30 min, and the culture was plated onto LB agar plates containing 100 µg/mL ampicillin. The plates were incubated at 37°C overnight. Plasmids were purified from single-colony overnight cultures using a QIAprep® Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and analyzed by restriction enzyme-digestion mapping and DNA sequencing.

RESULTS AND DISCUSSION

Between 30 and 100 colonies were obtained using the above procedure (Table 2). Restriction mapping of plasmids from randomly selected colonies showed that over 50% of the colonies contained the expected plasmid, while the rest of the colonies contained a background vector. Sequencing of the 12 plasmids with the expected digestion map from several individual experiments confirmed that the plasmids all had the correct sequences at the ligation junctions as planned. No mutations were observed in the *E. coli pth*-encoding region. The cloning efficiency would be improved by using highly purified chimeric primers and/or by applying more blunt-end polishing enzymes. In our experiments, the impurity of the chimeric primers (50%–70% pure) and the use of the Expand High Fidelity PCR System, in which the ma-

jority PCR products would still be non-blunt-ended (12), might produce non-ligatable DNA fragments.

The successful insertion of the *E. coli pth*-encoding region into the defined position of pDS56/RBSII demonstrated the usefulness of this universal restriction site-free cloning method. It provided a flexible and efficient alternative for cloning DNA fragments without restriction sites. The universal restriction site-free cloning approach has a number of advantages over other methods. The method enables one to create a defined ssDNA 3′ overhang with the desired length and sequence for efficient and directional ligation. The DNA fragments can be cloned into the vector independent of a restriction enzyme site. This is particularly useful for ensuring that the DNA fragment is cloned into the correct reading frame for native or fusion protein expression. This approach may also enable directional ligation of several DNA fragments simultaneously to facilitate the construction of chimeric genes within a single cloning step. It may also be used for the ligation-independent cloning (1) but without introducing additional nucleotide sequences.

The primers used in this method contain a ribonuleotide. To make a full-length, blunt-ended PCR product, the Expand High Fidelity PCR System was used in the PCR amplification step. The Expand High Fidelity PCR System meets the above requirement because it contains both *Taq* and *Pwo* DNA polymerases. *Taq* DNA polymerase can use ribonucleotides as a template, while *Pwo* DNA polymerase is able to create blunt ends.

Only one ribonucleotide inside the primer is required to create an ssDNA 3′ overhang. Although the primer could contain additional ribonucleotides, this would increase the frequency of error because the DNA polymerase was more error-prone when using ribonucleic acid as a template (4). The primer we used has some advantages over those used in earlier report (4). First, the ribonucleotide is located inside the primer and is flanked by dNTPs. Therefore, the primer may be more stable than the primer with ribonucleotide(s) at the 5′-terminal and will resist an attack by most ribonucleases. Second, the primer reduces the frequency of error, and, third, it decreases the cost of the primer synthesis.

Alkaline treatment (0.2 M NaOH solution) may also be used to break the linkage between the ribonucleotide and deoxyribonucleotide to create the 3′ overhangs. However, DNA molecules were also denatured in the alkaline solution because we did not obtain any colonies without renaturing the DNA before ligation (data not shown). The rare-earth metal ion treatment was performed at a pH at which the DNA molecule could be in its native conformation. This avoids renaturing DNA for ligation.

Because primers contain a ribonucleotide, it is possible to make a PCR product with 5′ overhangs by using DNA polymerases (such as *Pfu* or *Vent*) that are unable to use ribonucleotides as a template. Theoretically, these DNA fragments could also be ligated to form a desired plasmid containing ribonucleotides. We did not obtain the expected results after directly transforming the ligated DNA. This may due to the low efficiency of the DNA-to-DNA ligation by the T4 DNA ligase at the junction of the DNA and the RNA template because it was reported that T4 DNA ligase catalyzed the joining of DNA fragments hybridizing to RNA strands extremely slowly (5,7). With further development and new findings, the use of chimeric primers to produce defined 3′ and 5′ overhangs will have a big impact on the development of cloning techniques and molecular biology.

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