Short Technical Reports

Restriction Site-Free Insertion of PCR Products Directionally into Vectors

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ABSTRACT

A restriction site-free cloning method has been developed for inserting a PCR product into a vector flexibly and precisely at any desired location with high efficiency. The method uses a pair of DNA integration primers with two portions. The 3' portion isolates the inserts by PCR, and the 5' portion integrates the PCR products into the homologous region of the vector. For mutagenesis, a third portion of mutation-generating sequences can be placed in between the 3' and 5' portions. This method has been used to clone the *E. coli* gene that codes for peptidyl-tRNA hydrolase, expressing it as a native protein and as a glutathione S-transferase fusion protein. It was also applied to convert a construct of the *E. coli* fatty acid biosynthesis protein with an N-terminal hexa-histidine tag into a construct with a C-terminal hexa-histidine tag.

INTRODUCTION

The procedure for cloning a gene into a vector generally needs several steps, which include amplifying the target gene using PCR, generating blunt or sticky ends for ligation and inserting the PCR products into a suitable vector. There are many ways to insert PCR products into a vector, such as introduction of restriction sites on both ends of the PCR products (5,10), blunt-end insertion (2,8), T vectors (9) or ligation-independent cloning (1,3). The cloning sites are often difficult to find because of the limited availability of suitable restriction sites in the vector or in the gene. The cloning process for inserting PCR products into a defined location of the vector can be complicated.

PCR and related techniques have developed rapidly in recent years. They have made it possible to add, delete, change and link nucleotides at any po-

Table 1. DNA Integration Primers Used for Construction of pQCH1 and pQCH2

Primer	5' Portion Sequence	3' Portion Sequence
1	TCACACAGAATTCATTAAAGAGGAGAAATTAAC	TATGACGATTAAATGATTGTCGGCC
2	CCCCAGGCGTTTAAGGGCACCAATAACTGCC	TTATTGCGCTTTAAAGGCGTGCAAT
3	ACCATCCTCCAAAATCGGATCTGGTTCCGCGTGGATCC	ATTAAATGATTGTCGGCCTGGCG
4	TTTTCACCGTCATCACCGAACGCGCGAGGCAGATCG	TTATTGCGCTTTAAAGGCGTGCAAT

sition on the DNA (4,7). Here, we describe a restriction site-free cloning method developed from these techniques that can flexibly and precisely insert a DNA fragment directionally into a vector at any desired location with high efficiency. Our method is not limited by the availability of appropriate restriction sites for insertion.

MATERIALS AND METHODS

Plasmids and Cells

Plasmid pDS56/RBSII (12) was used to construct expression plasmid pQCH1, which produces a native pro-

tein of the *E. coli* peptidyl-tRNA hydrolase (Pth). Plasmid pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used for construction of the pQCH2 expression plasmid, which produces a glutathione S-transferase (GST) fusion protein. Plasmid pQZ1, a derivative of pDS56/RBSII that produces an *E. coli* fatty acid biosynthesis (FabZ) protein with an N-terminal hexa-histidine (6×His) tag, was used for construction of expression plasmid pQCH3, which produces the *E. coli* FabZ protein with a C-terminal 6×His tag. *E. coli* SURE[®] 2 strain (Stratagene, La Jolla, CA, USA) was used for transformation and expression. All vectors used for this experiment were isolated from a *dam*⁺ *E. coli* strain.

Enzymes and Reagents

DNA polymerases were purchased from Roche Molecular Biochemicals (Expand[™] high-fidelity PCR kit) and Stratagene (*PfuTurbo*[™] DNA polymerase). The DNA sequencing kit, plasmid miniprep kit, dNTPs and restriction enzymes are from PE Biosystems (Foster City, CA, USA), Qiagen (Valencia, CA, USA), Roche Molecular Biochemicals (Indianapolis, IN, USA) and Stratagene, respectively. SOC medium (containing 2% tryptone, 0.5% yeast extract, 0.05% NaCl and 20 mM glucose) was used for recovery of the cells after electroporation.

Primer Design

The key role of the DNA integration primers used in this cloning method is to link two DNAs at defined positions to form a new plasmid. This is achieved by designing a pair of primers so that their nucleotide sequences at the 3' portion are homologous to the DNA fragment to be inserted for amplification during PCR. The nucleotide sequences in the 5' portion are related to the insertion region sequence of the vector and are

Short Technical Reports

used to generate the 3' ends of the PCR products for extension along the vector in the thermal cycling elongation step (Figure 1). This results in a fusion between the DNA of the fragment and the vector to form a recombinant plasmid.

Table 1 lists the primers for the construction of pQCH1 (primers 1 and 2) and pQCH2 (primers 3 and 4). The pQCH1, which expresses the native *E. coli* Pth, was designed so that the first nucleotide of the initiation codon ATG and the last nucleotide of the stop codon TAA were inserted at position 115 and at position 959, respectively, in pDS56/RBSII. In pQCH2, which expresses the GST fusion protein, the first amino acid (methionine) codon ATG of *E. coli* Pth was omitted, and the first nucleotide of the second amino acid codon ATT was designed to be inserted at position 936, following a *Bam*HI site. The last nucleotide of the stop codon TAA was at position 980 in pGEX-4T-3.

For the mutagenesis, the DNA integration primers can be functionally divided into three parts: a 3' portion, a mutation-generating portion and a 5' portion. The 3' portion is for amplification

Table 2. DNA Integration Primers Used for Mutagenesis in Construction of pQCH3

DNA Integration Primer	5' Portion Sequence	Mutation Generating Portion	3' Portion Sequence
Forward	TCACACAGAATTCAT TAAAGAGGAGAAATT AACTATG		ACTACTAACACTCAT ACTCTGC
Reverse	CCCCAGGCGTTTAAAG GGCACCAATAACTGC CTTA	GTGATGGTGATG GTGATG	GGCCTCCCGGCTACG AGCAC

and the 5' portion is for integration. The mutation-generating portion contains nucleotides to be changed or inserted; the removal of nucleotides in this portion will generate a deletion.

Table 2 lists the DNA integration primers used for construction of pQCH3. The forward DNA integration primer used to delete the N-terminal 6×His tag was designed so that 33 nucleotides from plasmid pQZ1 are deleted. The reverse DNA integration primer was designed to add the 6×His tag to the C-terminus of the FabZ protein

and, concomitantly, a DNA fragment of about 850 bp that contains the *CAT* gene was deleted.

Amplification of *E. coli* pth Gene-Encoding Region and an *E. coli* fabZ Gene

All PCR experiments were performed on a Biometra® TRIO-Thermoblock. A typical 100 µL PCR consisted of 0.5 µg *E. coli* BL21 strain chromosomal DNA or 5 ng pQZ1 plasmid DNA as template, 0.5 µM primers each, 200 µM dNTPs each and 2.5 U Expand high-fidelity DNA polymerase in its 1× PCR buffer with Mg²⁺. A 25 cycle, two-step PCR program was applied, with denaturing at 95°C for 30 s and annealing/extension at 68°C for 1 min with a final step of extension at 72°C for 10 min. The PCR products were purified on a 1% agarose gel in TAE buffer followed by a DNA gel extraction step.

Cloning of PCR Products

The thermal cycling elongation was also performed on the TRIO-Thermoblock. A 25 µL thermal cycling elongation reaction consisted of 5–10 ng pDS56/RBSII, pGEX-4T-3 or pQZ1 plasmid, 50–100 ng purified PCR products as described above, 200 µM dNTPs each and 1 U *Pfu*Turbo DNA polymerase in its 1× PCR buffer. The thermal cycle program was denaturation at 95°C for 2 min and annealing/elongation at 68°C for 10 min with 20 cycles. After the reaction, 5 U of restriction enzyme *Dpn*I was added to digest for 1–2 h at 37°C. The enzyme will selectively cut the parental plasmid and hybrid plasmid because it recognizes

Figure 1. Scheme for the restriction site-free cloning method. A pair of DNA integration primers is designed to contain the nucleotide sequences from both the insertion region of the vector (black) and the gene (gray). First, it is used to amplify the DNA fragment to be inserted into the vector. The 3' portion of the DNA integration primers will anneal to the target DNA sequence and form a PCR product that contains insertion region sequences at both ends. Then, in the thermal cycling elongation step, both ends of each strand of the PCR products will anneal to their complementary strands of the vector, and their 3' ends will be extended by DNA polymerase using the vector as a template in 3' to 5' direction along the plasmid till to its 5' end. The designed plasmid is selected after *Dpn*I digestion as *Dpn*I cuts the parental and hybrid plasmids (11,13).

Short Technical Reports

Table 3. Efficiency of Restriction Site-Free Insertion of PCR Products for pQCH1, pQCH2 and pQCH3

Experiment ^a	Number of Colonies Picked	Number of Designed Plasmid	Number of Parental Plasmid	Efficiency
1	20	17	3	85%
2	12	11	1	92%
3	5	5	0	100%
4	9	7	2	78%
5	8	5	1	63%

^aExperiments 1, 2 and 3 are for construction of pQCH1. Experiment 4 is for pQCH2, and experiment 5 is for pQCH3.

fully methylated double-stranded DNA (dsDNA) and will also cut hemimethylated dsDNA with much less efficiency, but not unmethylated dsDNA (11,13). The transformation was performed on a Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, USA) with the supplied electroporation protocol. Fifty microliters of *E. coli* SURE 2 electroporation-competent cells and 1 µL *DpnI*-treated mixture were used for transformation. After electroporation, the cells were immediately mixed with 450 µL SOC medium and incubated at 37°C for 30 min, and 100 µL of the transformation culture were plated onto a LB agar plate containing 100 µg/mL ampicillin. The plate was incubated at 37°C overnight. Plasmids were purified from single-colony overnight cultures using a miniprep kit from Qiagen and analyzed on a 1% agarose gel after restriction enzyme digestion. The insertion was verified by DNA sequencing.

RESULTS

Amplification of the *E. coli pth* and the *E. coli fabZ* Gene-Encoding Regions

The *E. coli pth* gene-encoding region was isolated from *E. coli* BL 21 chromosomal DNA by using PCR with the DNA integration primers listed in Table 1. The PCR resulted in two bands. The lower-density band had the expected molecular weight, and the material was purified using a gel extraction kit from Qiagen. The *E. coli fabZ* gene was isolated from pQZ1 by using the pair of

DNA integration primers listed in Table 2. The N-terminal 6×His tag and some unrelated nucleotides were deleted from *fabZ*, and nucleotides coding for a 6×His tag were added to the *fabZ* at the protein C-terminal end.

Cloning of the *E. coli pth* PCR Products into pDS56/RBSII and pGEX-4T-3 and the Construction of pQCH3

The insertion of the *E. coli pth* PCR products and the *E. coli fabZ* PCR products into the appropriate location of the vectors was achieved using a thermal cycling elongation reaction. One microliter of *DpnI*-treated thermal cycling elongation mixture resulted in 20–300 colonies per 100 µL transformation solution. About 80% of randomly picked colonies contained plasmids that had the expected molecular weight after the restriction enzyme analysis. The results were repeatable, and the average efficiency was about 80% (Table 3). DNA sequencing confirmed the presence of the expected inserts at the designed locations in pQCH1, pQCH2 and pQCH3 expression plasmids. Simultaneously, an approximate 850 bp DNA fragment containing the *CAT* gene was deleted from pDS56/RBSII and pQZ1.

In experiment 5 in Table 3, two colonies contained plasmid pQCH3 with additional mutations. One had a nucleotide change from C to G at position 585 in pQCH3, and the other had nucleotide T deleted at position 555. This deletion created an *XhoI* site de-

tected after the restriction enzyme analysis. Both additional mutations were located in the region of the reverse DNA integration primer. Therefore, these mutations are probably caused by impurities in the primers.

DISCUSSION

The rapid development of PCR and related techniques has made it possible to link two unrelated DNA fragments and introduce deletion, insertion and substitution mutations in a target DNA (4,7). The insertion of PCR products into a vector can be achieved using different strategies (1–3,5,8–10). Normally, several steps are needed to generate ligation ends in vectors and/or PCR products. It can be complicated to insert a PCR product directionally into a desired location because suitable restriction enzyme sites are lacking from the vectors.

The restriction site-free cloning method is a development of existing strategies. In this method, vectors do not need to have a polylinker or a restriction site. The DNA fragment can be flexibly and precisely inserted into any desired location. Using this method, we have successfully inserted the *E. coli pth* gene into pDS56/RBSII and pGEX-4T-3 expressing it as a native protein and as a glutathione S-transferase fusion protein, respectively. The efficiency of this method in both cases was around 80%. In both cases, only two kinds of plasmid were obtained. One was the designed plasmid, and the other was the parental plasmid. The *DpnI* digestion of the thermal cycling elongation products is a critical step to reduce the background caused by parental plasmid and hybrid plasmid. This may also be achieved by using other restriction systems such as the Kunkel method (6) in which the *E. coli dut⁺ ung⁺* strain will eliminate in vivo the parental plasmid that contains deoxyuracil after transformation. In addition, PCR by-products (nonspecific bands) will also increase the background because their 3' ends (generated from the 5' portions of the DNA integration primers) will also anneal to the insertion region and generate unwanted plasmids. Therefore, purifying the PCR products is recommended.

Additional mutations can be introduced to the plasmid by impurities of the DNA integration primers—the longer the primer, the more impure it is. Some unwanted mutations can be avoided by using high-quality primers and by designing primers that are as short as possible. Both the PCR and the thermal cycling elongation steps can generate unwanted mutations, but the errors are different. Errors generated during early PCR steps can be amplified to a dramatic extent. They can be reduced by using more templates, less PCR cycles and high-fidelity DNA polymerases. However, errors generated in the thermal cycling elongation step will not be amplified because the original vectors are always used as the templates. Using high-fidelity DNA polymerases seems the only way to eliminate unwanted plasmids. The specificity of the insertion depends on the nucleotide sequences of the 5' portion of the DNA integration primers. The DNA sequence of the insertion region on the vector should be unique, and sequences that exist in other locations should be avoided. We failed to insert the *E. coli pth* into pGEX-4T-3 at the designed location because there is a region with an identical DNA sequence (more than 40 nucleotides) in another location on pGEX-4T-3. We did not get the designed plasmids, but all plasmids sequenced showed that the A(TG) starting codon insertion position was at position 4525 instead of at position 257 in the vector, whereas the stop codon was at the correct position. This resulted in the deletion of about 500 nucleotides.

This method is useful not only for cloning genes but also for mutagenesis. We have changed an expression plasmid (pQZ1), which produces *E. coli* FabZ protein with an N-terminal 6×His tag, to plasmid (pQCH3), which produces *E. coli* FabZ protein with a C-terminal 6×His tag. The deletion of N-terminal 6×His tag and the insertion of a C-terminal 6×His tag were carried out simultaneously on plasmid DNA. It can be applied further to site-directed mutagenesis for studying the interaction between amino acid residues. It is possible to simultaneously mutate bases coding for two amino acids that are far away from each other in a protein and not to replace each one sequentially.

The successful removal of about 850 bp of unwanted DNA fragment from vectors during thermal cycling elongation is also very useful. It could be applied to the blue-white color screening cloning method in which the *lacZ* gene will produce a nonfunctional LacZ' fusion protein when a DNA fragment is inserted. Readthrough of the inserted DNA fragment may still produce functional LacZ' protein leading to blue colonies in the presence of X-Gal. With this method, the partial or complete deletion of the *lacZ* gene during insertion of a DNA fragment avoids the isolation of false-negative colonies because of the readthrough. In addition, removal of unwanted DNA from vectors will keep plasmids small and easy to manipulate at steps such as thermal cycling elongation and transformation.

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