Cloning DNA Fragments Between Two Adjacent/Overlapping Restriction Sites Using a “Positive Stuffer”

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ABSTRACT

Here we describe a solution to a common problem encountered in recombinant DNA cloning when directional cloning of a DNA fragment into a predetermined plasmid requires the use of restriction enzymes with adjacent or overlapping recognition sites. In preparing the double-digested plasmid, only one enzyme will often cut, whereas the second will not because of the lack of a sufficiently long stretch of double-stranded DNA at its recognition site. The problem can be solved by construction of a “user-friendly” intermediary plasmid in which the desired restriction sites are separated by a positively selectable stuffer with resistance to neomycin. This approach is particularly useful in cases where the choices of restriction sites are severely limited, for example, when it is necessary to clone an additional piece of DNA into a complex vector already containing multiple gene cassettes.

INTRODUCTION

Directional cloning of a DNA fragment into a plasmid vector cleaved with two different restriction enzymes is the most efficient cloning method (8). This procedure yields recombinant molecules in which the insert is oriented in the desired direction and minimizes plasmid self-ligation.

When making complex recombinant DNA constructs such as gene-targeting vectors or cDNA expression vectors, investigators are usually limited in their choice of restriction sites. Often the desired sites may be adjacent or overlapping. In this case, the major difficulty is to completely digest the DNA with both enzymes (3,4). The vector molecules cleaved by only one enzyme self-ligate and generate high background. This leads to laborious screening for the insertion, including isolation of numerous minipreps or colony hybridization. In this report, we describe a “positive stuffer” strategy to solve this problem.

MATERIALS AND METHODS

DNA manipulations were done using standard procedures (6). All restriction enzymes, calf intestinal phosphatase (CIP), T4 DNA Polymerase and T4 DNA Ligase were purchased from New England Biolabs (Beverly, MA, USA). Competent bacterial cells E. coli XL1-Blue were transformed by electroporation according to standard protocol (7).

RESULTS AND DISCUSSION

The “positive stuffer” strategy is based on the use of kanamycin/neomycin resistance gene (neo) to obtain a user-friendly plasmid in which the two desired restriction sites are separated by the neo stuffer fragment (Figure 1). The neo positive stuffer serves as a positive selection marker for its own insertion. This user-friendly plasmid can be easily cut with both restriction enzymes and, if necessary, the vector DNA can be purified from the stuffer with conventional gel purification methods. Subsequent ligation of the vector with the desired fragment is of high efficiency.

The neo gene was originally derived from transposon Tn5 (1,2) and confers resistance to 100–200 mg/mL of neomycin on E. coli (5). A 1.4-kb HindIII-PstI DNA fragment carrying the neo gene was subcloned from the plasmid pMAMneo (CLONTECH Laboratories, Palo Alto, CA, USA) into HindIII-PstI sites of pBluescript®.

![Figure 1. Schematic representation of the “positive stuffer” cloning strategy.](image)
SK(+) (Stratagene, La Jolla, CA, USA) to generate a helper plasmid pBl-neo. In this plasmid, neo\(^r\) fragment is flanked by multiple restriction sites, providing multiple choices for stuffer excision.

The “positive stuffer” strategy is illustrated in Figure 1 and described below. This strategy is used to clone a DNA fragment between the two adjacent/overlapping restriction sites A and B as follows: (i) the plasmid of interest is linearized with the restriction enzyme A and blunt-ended with T4 DNA polymerase; and (ii) 1.4-kb neo\(^r\) positive stuffer is excised from the pBl-neo helper plasmid with the restriction enzymes C and D, blunt-ended with T4 DNA polymerase, gel-isolated and ligated with the linearized plasmid of interest to obtain the user-friendly construct. Enzyme C, to cut pBl-neo, is chosen so that ligation with blunt-ended site C provides for reconstitution of site A when the stuffer is inserted. For example, the XhoI site (site A) is reconstituted when ligated with blunt-ended BamHI (site C). DNA is transformed into E. coli, and clones resistant to two antibiotics are selected. Neomycin resistance is provided by the stuffer, and the other antibiotic resistance (ampicillin, tetracycline, etc.) is provided by the plasmid of interest. The wrong orientation of stuffer in the plasmid of interest results in proximity of sites A and B. Stuffer in correct orientation separates A and B from each other. The two orientations of the stuffer can be easily distinguished since only the stuffer in correct orientation can be excised with A and B. User-friendly plasmid with correct orientation of stuffer is cleaved with A and B and ligated with the desired fragment.

We found the use of the 1.4-kb neo\(^r\) fragment as a positive stuffer very convenient because of the following: (i) the size of the stuffer fragment (ca. 1.4 kb) allows for easy purification for further manipulations; (ii) it provides for very efficient selection for the insertion of the stuffer with very low background—usually all the ampicillin/neomycin-resistant clones carry the plasmid with stuffer, half in correct orientation; and (iii) it can be applied to most of the commonly used vectors that have ampicillin or tetracycline resistance markers.

An example of the “positive stuffer” strategy is shown in Figure 2. Application of the “positive stuffer” strategy to clone the left arm into the tagging vector for smooth-muscle myosin heavy-chain gene mutagenesis. (A) Schema shows cloning of the left arm through a user-friendly plasmid carrying the positive stuffer. K: KpnI; X: XhoI; R: EcoRI; S: Sall; H\(\text{blunt}\): blunt-ended HindIII; B\(\text{blunt}\): blunt-ended BamHI; X*: XhoI site reconstituted after ligation with blunt-ended BamHI. Cassette: markers for positive and negative selection in embryonic stem cells. (B) Results of cloning of the left arm (1.1 kb) into N25 by conventional method. Plasmid DNA isolated from ampicillin-resistant clones was cut with KpnI+EcoRI and run on 1% agarose gel. Lane 1: KpnI+EcoRI digest of N25 DNA (control); lanes 2–11: plasmids from randomly picked ampicillin-resistant clones; lane 12: molecular weight markers (λ DNA digest with HindIII+EcoRI). The 2.3-kb fragment containing the selection cassette and the 5.6-kb fragment containing the vector with the right arm are indicated. Note that none of the plasmids contain the 1.1-kb left arm. (C) Results of left arm cloning using the “positive stuffer” strategy. Lane 1: N25-neo DNA cut with KpnI+EcoRI (control); lanes 2–11: DNA isolated from ten independent clones cut with KpnI+EcoRI; lane 12: molecular weight markers (λ DNA HindIII+EcoRI digest). The 2.3-kb fragment containing the selection cassette, 1.4-kb stuffer fragment and 1.1-kb left arm fragment are indicated. Note that all the plasmids (lanes 2–11) contain the 1.1-kb left arm but not the 1.4-kb neo\(^r\) stuffer.
strategy is shown in Figure 2A. To target the smooth-muscle myosin heavy-chain gene, we needed to make a tagging vector. During vector construction, we had difficulties cloning the left arm into the plasmid N25, which carried the right arm together with the cassette of markers for positive and negative selection in the embryonic stem cells. Initially, we attempted the conventional cloning method. To insert the left arm, plasmid N25 had to be cleaved with the two adjacent sites KpnI and XhoI. It was first cut with XhoI, then with KpnI and treated with CIP. One hundred nanograms of this DNA were ligated with 200 ng of gel-purified, 1.1-kb KpnI-SalI “left arm” (SalI has compatible ends with XhoI) and electroporated into E. coli XL-Blue. Plasmid DNA was isolated from ampicillin-resistant clones and analyzed by cleavage with KpnI+ EcoRI. None of the clones carried the insert (Figure 2B).

Therefore we applied the “positive stuffer” strategy and made a user-friendly plasmid N25-neo. Briefly, N25 plasmid was cut with XhoI and blunted with T4 DNA polymerase. The 1.4-kb neo fragment was excised from pBl-neo helper with HindIII and BamHI, blunted with T4 DNA polymerase and gel-purified. The BamHI site, when blunted and ligated to the blunted XhoI site, reconstitutes the XhoI site. One hundred nanograms of the stuffer were ligated with 100 ng of linearized N25 DNA and electroporated into E. coli XL-Blue. Plasmids were isolated from ampicillin/neomycin-resistant clones and analyzed. All of the isolates contained the stuffer fragment; approximately one half were in correct orientation (data not shown). This user-friendly plasmid was cut with XhoI and KpnI to release the stuffer and treated with CIP to minimize religation with the stuffer fragment. One hundred nanograms of the vector DNA were then ligated without further purification with 200 ng of 1.1-kb KpnI-SalI left-arm DNA fragment (SalI and XhoI produce compatible cohesive ends) and transformed into E. coli XL-Blue. Plasmids were isolated from ampicillin-resistant clones by alkaline lysis and analyzed by cleavage with KpnI+EcoRI (Figure 2C). All the clones carried the desired 1.1-kb left arm, and none had the 1.4-kb neo.

The “positive stuffer” strategy has been successfully applied in our laboratory to solve the most difficult cloning problems arising from proximity of the restriction sites. Simple and highly reliable, this method will be helpful for many researchers faced with similar problems.

REFERENCES


Address correspondence to Muthu Periasamy, Director of Molecular Cardiology, Division of Cardiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, ML 0542, Cincinnati, OH 45267-0542, USA. Internet: periasm@ucbh.edu

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Evgeny Loukianov, Tanya Loukianova and Muthu Periasamy
University of Cincinnati College of Medicine
Cincinnati, OH, USA