# Short Technical Reports

## 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*$^r$) to obtain a user-friendly plasmid in which the two desired restriction sites are separated by the *neo*$^r$ stuffer fragment (Figure 1). The *neo*$^r$ 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*$^r$ 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 *Hin*dIII-*Pst*I DNA fragment carrying the *neo*$^r$ gene was subcloned from the plasmid pMAM*neo* (CLONTECH Laboratories, Palo Alto, CA, USA) into *Hin*dIII-*Pst*I sites of pBluescript®

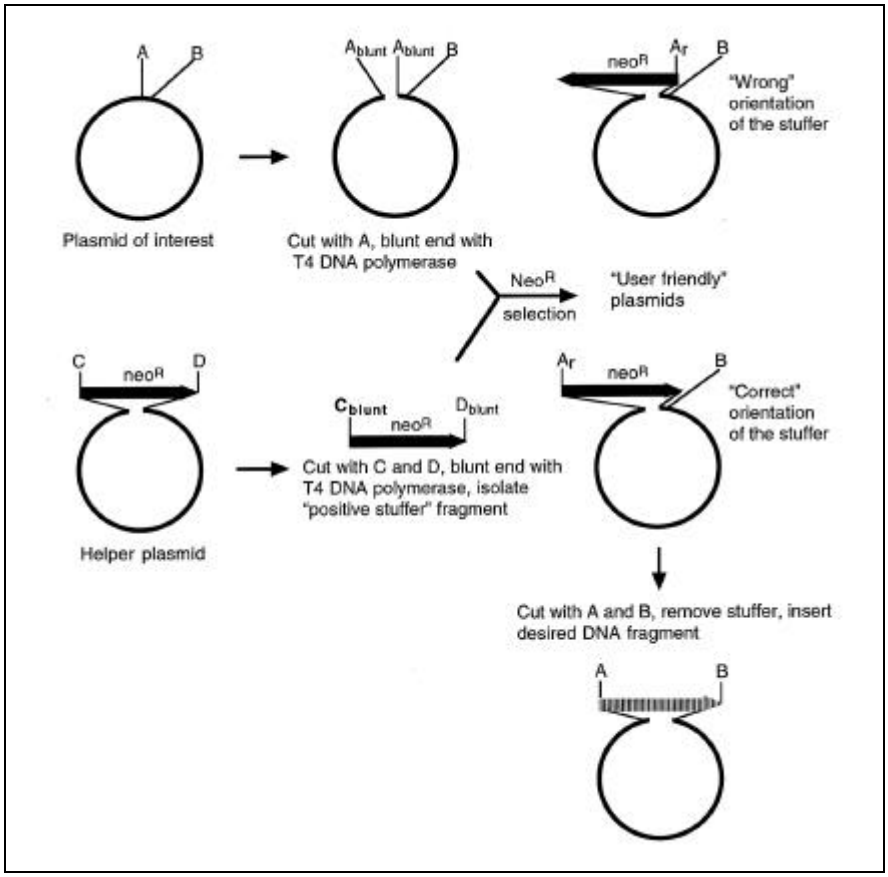


**Figure 1. Schematic representation of the "positive stuffer" cloning strategy.** A and B: desired restriction sites in the plasmid of interest; C and D: restriction sites used to cleave helper plasmid; $A_{blunt}$, $C_{blunt}$ and $D_{blunt}$: A, C and D sites blunt-ended with T4 DNA polymerase; $A_r$: reconstituted site A. Restriction enzyme C is chosen so that ligation of $A_{blunt}$ with $C_{blunt}$ reconstitutes site A. Insertion of *neo*$^r$ fragment in correct orientation generates an intermediate plasmid where sites A and B are separated by the stuffer. This user-friendly plasmid can be cut with A and B completely and then ligated with the desired fragment.

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 *Xho*I site (site A) is reconstituted when ligated with blunt-ended *Bam*HI (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"

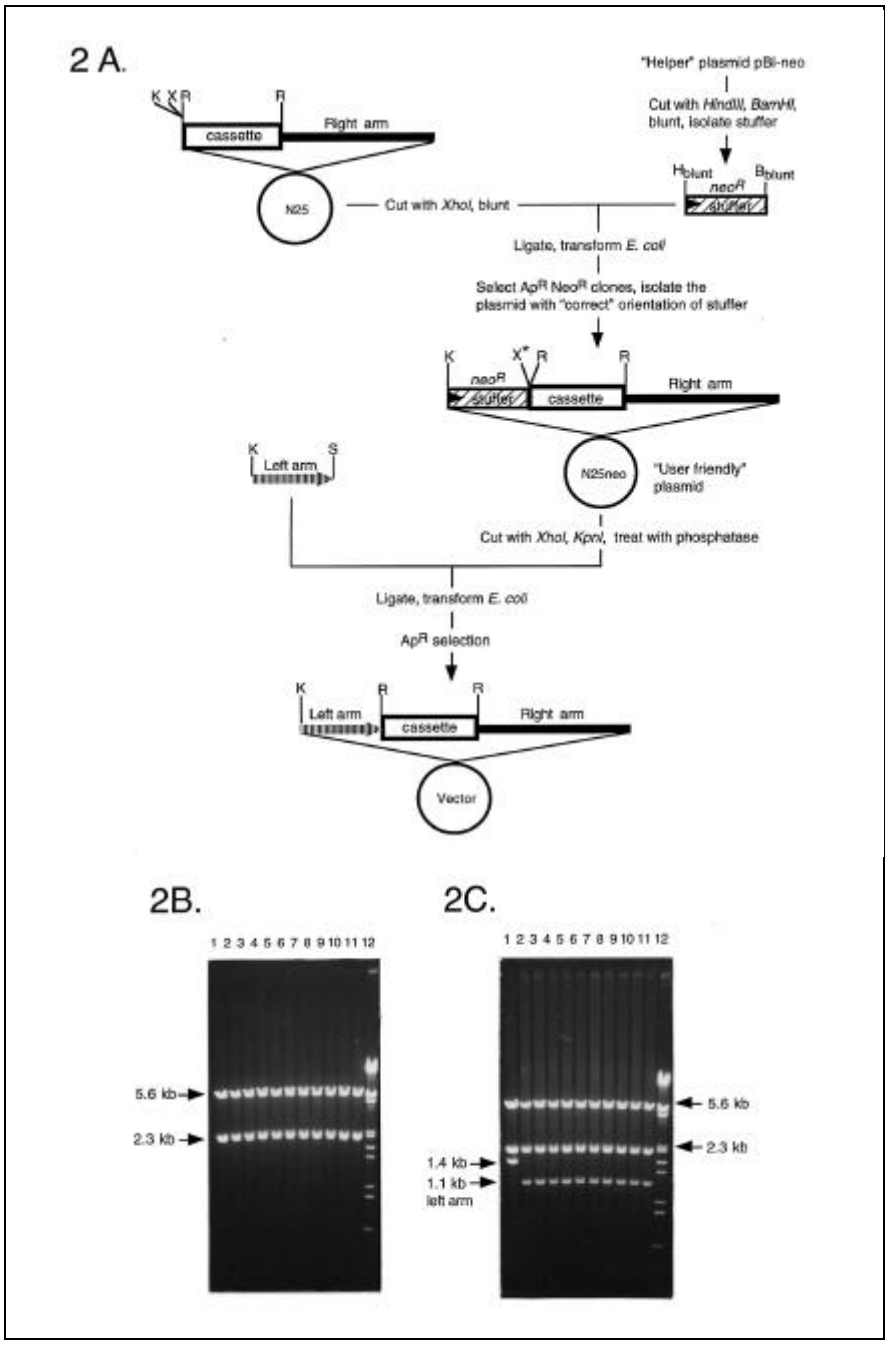


**Figure 2. Application of the "positive stuffer" strategy to clone the left arm into the tagging vector for smooth-muscle myosin heavy-chain gene mutagenesis.** (A) Schema shows cloning of the left arm through a user-friendly plasmid carrying the positive stuffer. K: *Kpn*I; X: *Xho*I; R: *Eco*RI; S: *Sal*I; $H_{blunt}$: blunt-ended *Hin*dIII; $B_{blunt}$: blunt-ended *Bam*HI; X*: *Xho*I site reconstituted after ligation with blunt-ended *Bam*HI. 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 *Kpn*I+*Eco*RI and run on 1% agarose gel. Lane 1: *Kpn*I+*Eco*RI digest of N25 DNA (control); lanes 2–11: plasmids from randomly picked ampicillin-resistant clones; lane 12: molecular weight markers (λ DNA digest with *Hin*dIII +*Eco*RI). 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 *Kpn*I+*Eco*RI (control); lanes 2–11: DNA isolated from ten independent clones cut with *Kpn*I+*Eco*RI; lane 12: molecular weight markers (λ DNA *Hin*dIII +*Eco*RI digest). 5.6-kb fragment containing the vector with the right arm, 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 *Kpn*I and *Xho*I. It was first cut with *Xho*I, then with *Kpn*I and treated with CIP. One hundred nanograms of this DNA were ligated with 200 ng of gel-purified, 1.1-kb *Kpn*I-*Sal*I "left arm" (*Sal*I has compatible ends with *Xho*I) and electroporated into *E. coli* XL-Blue. Plasmid DNA was isolated from ampicillin-resistant clones and analyzed by cleavage with *Kpn*I+ *Eco*RI. 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 *Xho*I and blunt-ended with T4 DNA polymerase. The 1.4-kb *neo*$^r$ stuffer was excized from pBl-neo helper with *Hin*dIII and *Bam*-HI, blunt-ended with T4 DNA polymerase and gel-purified. The *Bam*HI site, when blunted and ligated to the blunted *Xho*I site, reconstitutes the *Xho*I 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 *Xho*I and *Kpn*I 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 *Kpn*I-*Sal*I left-arm DNA fragment (*Sal*I and *Xho*I 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 *Kpn*I+*Eco*RI (Figure 2C). All the clones carried the desired 1.1-kb left arm, and none had the 1.4-kb *neo*$^r$.

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


1. **Beck, E., G. Ludwig, E.A. Auerswald, B. Reiss and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferasegene from transposon Tn5. Gene *19*:327-336.
2. **Berg, D.E., J. Daevis, B. Allet and J.D. Rochaix.** 1975. Transposition of R-factor genes to bacteriophage. Proc. Natl. Acad. Sci. USA *72*:3628-3632.
3. **Kaufman, D.L. and G.A. Evans.** 1990. Restriction endonuclease cleavage at the termini of PCR products. BioTechniques 9:304-306.
4. **Moreira, R.F. and C.J. Noren.** 1995. Minimum duplex requirements for restriction enzyme cleavage near the termini of linear DNA fragments. BioTechniques *19*:56-59.
5. **Rao, R.N. and S.G. Rogers.** 1979. Plasmid pKC7: a vector containing ten restriction sites suitable for cloning DNA segments. Gene *7*:79-82.
6. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. **Seidman, C.E., K. Struhl and J. Sheen.** 1994. Introduction of plasmid DNA into cells, p. 1.8.1.–1.8.8. *In* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, K. Struhl (Eds.), Current Protocols in Molecular Biology. John Wiley & Sons, New York.
8. **Struhl, K.** 1994. Construction of hybrid DNA molecules, p. 3.16.1.–3.16.11. *In* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman, K. Struhl (Eds.), Current Protocols in Molecular Biology. John Wiley & Sons, New York.



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