A Simple Method to Construct T-Vectors Using XcmI Cassettes Amplified by Nonspecific PCR

Chulman Jo and Sangmee Ahn Jo

Division of Degenerative Diseases, Department of Biomedical Sciences and Biomedical Brain Research Center, National Institute of Health, Seoul, Korea

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Polymerase chain reaction (PCR) is one of the most powerful tools in cloning genes. For the direct cloning of PCR products, T-vectors, which contain complementary 3'-thymidine overhangs, are widely used. In the present study, we developed a plasmid, pNB-T, which was constructed by cloning an XcmI cassette with a sufficient length of DNA (over 500 bp long) between two XcmI restriction sites into pBluescript SK(+). An XcmI cassette was made by nonspecific PCR using a primer containing recognition sequences of XcmI so that pNB-T can easily be converted into a T-vector by restriction of the plasmid with XcmI. In addition, the recognition sequences for BamHI and NcoI were added at 5'-end of the primer in order to facilitate subcloning of the gene cloned in the T-vector. The cloning efficiency of a PCR product, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, was approximately 90%. Digestion of the recombinant plasmid containing the GAPDH gene with BamHI or NcoI liberated the DNA fragments with the expected size, demonstrating the usefulness of extra restriction sites. The method described in this report is quite simple and enables us to construct a variety of useful T-vectors.

Key Words: T-vector; XcmI cassette; nonspecific PCR.

The rapid amplification and isolation of specific DNA fragments by polymerase chain reaction (PCR) have become routine for a variety of molecular biological studies and applications. It is known that most PCR products have adenosine residues at the 3' end due to the terminal transferase activity of Taq polymerase (Clark, 1993). Therefore, for the direct cloning of PCR products, DNA vectors that contain complementary 3' thymidine overhangs (T-vector) were developed and have been widely used.

T-vectors could be prepared by tailing a single thymidine residue at both 3' ends of a linearized, blunt-ended plasmid with terminal deoxynucleotidyl transferase (Holton and Graham, 1991). Alternatively, they could be prepared by constructing a vector designed to contain restriction sites of the enzymes such as XcmI (Arashi et al., 1999; De Vries et al., 1998; Borovkov and Rivkin, 1997; Cha et al., 1993; Harrison et al., 1994; Kovalic et al., 1991; Mead et al., 1991), AspEI (Ichihara and Kurosawa, 1993; Mead et al., 1991), HphI (Mead et al., 1991), or MboII and to produce a single thymidine overhang when digested with the corresponding enzyme. In the latter case, insertion of oligodeoxyribonucleotides which contain two tandemly arrayed recognition sites for the restriction enzyme into the cloning vector is followed by digestion with the corresponding restriction enzyme. Among restriction enzymes mentioned above, XcmI has been widely used because the cloning vectors commonly used such as pUC-based plasmids do not contain the sequences recognized by XcmI.

Usage of XcmI to construct T-vectors, however, is limited since the vectors incubated with XcmI are often partially digested, leading to a high background of nonrecombinant transformants (Harrison et al., 1994; Mead et al., 1991). In order to overcome the problem, several approaches such as dephosphorylation of T-vector (Cha et al., 1993) or inclusion of XcmI enzyme in the ligation reaction mixture (Harrison et al., 1991).
1994) have been employed, but these methods did not improve the cloning efficiency. Alternatively, purification of the completely digested vectors from the partially digested ones is thought to be the best way to remove the non-recombinant transformants. However, it is not easy to separate the fully cut T-vectors from the uncut or singly cut T-vectors on the agarose gel because the size of the DNA between the two XcmI sites is too small. Therefore, insertion of a spacer DNA between XcmI sites which is long enough to make an observable difference in migration on the gel has been proposed before (Testori and Sollitti, 1996), but their method requires another step for inserting a spacer DNA fragment between the XcmI sites, which is time consuming.

Therefore, in the present study, we introduce a simple, one-step procedure to obtain XcmI cassettes with sufficient length of DNA (over 500 bp long) between two XcmI restriction sites. Our strategy is based on fishing a long XcmI cassette produced by nonspecific PCR using a primer which contains a recognition sequence of XcmI as well as BamHI and NcoI (Fig. 1A; 5’-CGGGATCCCATGGTTGTGATGGG-3’) at the annealing temperature lower (48°C) than that calculated (75°C; Thein and Wallace, 1986) to increase annealing efficiency. Here, inclusion of the restriction sites of BamHI and NcoI at the 5’ end of the primer was intended to facilitate subcloning of the gene cloned in the T-vector. The restriction site of BamHI can be substituted for that of other restriction enzymes and several other restriction sites can be additionally attached.

In order to obtain XcmI cassettes, PCR was performed in the PCR buffer (Promega, Madison, WI) using 50 ng of chromosomal DNA prepared from rat liver as a template, 20 pmol of a primer (Fig. 1A), and 2.5 U of Taq DNA polymerase in a total volume of 50 μl. Amplification was performed using a GeneAmp 2400 thermocycler (Perkin–Elmer, Norwalk, CT) by 30 cycles as follows: a denaturing step at 95°C for 45 s, a primer annealing step at 48°C for 45 s, and an extension step at 72°C for 1 min. The 550-bp DNA fragment amplified as a major product was purified using a gel-extraction kit (Qiagen, Chatsworth, CA) and cloned into the BamHI site of pBluescript SK(+) (Stratagene, La Jolla, CA). Thus, the plasmid, named pNB-T (Fig. 1B), was constructed and T-vectors were generated by cutting the plasmid with XcmI (Fig. 2) and purified.

To examine the cloning efficiency of PCR products using the T-vector prepared by our method, 970-bp of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Genbank Accession No. M17701) was amplified from cDNA prepared from rat liver using a forward primer (5’-CATTGTGCCATCAACGACC-3’) and a reverse primer (5’-CTCTCTTGCTCTCAGTATCC-3’). PCR was performed using the same amount of components and volume as described for the amplification to obtain XcmI cassettes. Each PCR cycle consisted of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, and polymerization at 72°C for 1 min. Amplified GAPDH gene was purified with a purification kit (Qiagen). Ligation reaction was performed at 4°C for 12 h in the recommended buffer (Promega) containing 150 ng of the amplified product, 50 ng of the T-vector, and 3 U of T4 DNA ligase in a total volume of 10 μl. The ligate was transformed into Escherichia coli (XL1-blue) with an efficiency

![FIG. 1. DNA sequence of the primer used for PCR and map of pNB-T plasmid. A single-strand oligonucleotide used to amplify an XcmI cassette is shown in A. The recognition site for XcmI is indicated as bold, and those for BamHI and NcoI are underlined. The structure of pNB-T plasmid that is constructed by our method is illustrated in B. The restriction sites present in the multicloning site of pBluescript SK(+) are omitted.](image-url)
of approximately $6 \times 10^5$ cfu/mg DNA. To identify the correct recombinant clones, each colony was picked with a sterile toothpick, resuspended in 10 mM NaOH, and boiled at 95°C for 10 min. Then, the same volume of PCI (phenol/chloroform/isoamylalcohol, 25:24:1) was added into the solution, and the tube was vortexed and centrifugated at 10,000g for 10 min. PCR was performed using 1 μl of supernatant as template as previously described for the amplification of GAPDH cDNA and a PCR product (970 bp) was separated on 1% agarose gel, stained with ethidium bromide, and visualized with UV light. When the cloning efficiency was calculated by examining the presence of a 970-bp PCR product, the ratio of recombinant versus nonrecombinant clones was 90% (27 of 30 colonies randomly chosen). Figure 2 shows that the digestion of a recombinant plasmid with BamHI (lane 5) or NcoI (lane 4) liberates the DNA fragments with expected size. This result suggests that extra restriction sites are useful for subcloning.

In summary, the method described here to construct T-vectors using XcmI cassettes amplified by nonspecific PCR is simple and has several advantages compared to the methods previously reported (De Vries et al., 1998; Borovkov and Rivkin, 1997; Cha et al., 1993; Harrison et al., 1994; Kovalic et al., 1991; Testori and Sollitti, 1996). First, this method enables us to produce a variety of plasmids that contain different length and DNA sequences between the two XcmI sites either by changing the template DNA or by setting PCR at the different annealing temperature. Second, by inserting recognition sequences for extra restriction enzymes at the 5′ end of the primer in addition to XcmI, we could simply construct plasmids containing a variety of restriction enzyme sites which can be utilized in subcloning. Third, the plasmid we constructed contains restriction sites arrayed symmetrically since one primer is used to obtain XcmI cassettes, and therefore an insert in the vector can be cut off by single digestion with a corresponding restriction enzyme. Finally, by subcloning the XcmI cassette into other prokaryotic or mammalian vectors, we could easily convert them into T-vectors.

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