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An Efficient Method to Prepare PCR Cloning Vectors

Soon Gyu Hong[1,2]*, Ji Young Choi[1], Barry M. Pryor[2] and Hong Kum Lee[1]

[1]*Polar BioCenter, Korea Polar Research Institute, KORDI, Songdo Techno Park, Songdo-dong 7-50, Yeonsu-gu, Incheon 406-840, Korea*
[2]*Division of Plant Pathology and Microbiology, Department of Plant Sciences, College of Agriculture and Life Sciences, University of Arizona, Tucson, Arizona 85721, USA*

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An improved procedure for preparing PCR cloning vectors was developed. This procedure includes the incorporation of adapters to create XcmI restriction enzyme sites in pBluescript II SK(+) vectors, digestion with XcmI followed by further digestion of the small fragment produced by XcmI digestion with additional enzymes, and purification with PCR purification kits. Using this procedure, PCR cloning vectors with high ligation efficiencies and low blue or false-positive colonies were obtained.

KEYWORDS : PCR cloning vector, XcmI

Efficient cloning of PCR products is very important in molecular biology and various methods have been developed for this purpose. Preparation of PCR cloning vectors has been conducted by digesting a vector with restriction enzymes, such as XcmI, HphI, and MboII, that generate 3'-terminal unpaired deoxythymidine residues (Kovalic *et al*., 1991; Mead *et al*., 1991; Chuang *et al*., 1995; Borovkov and Rivkin, 1997) or by using terminal transferase or Taq DNA polymerase to add a single protruding T residue to the 3' termini of linearized vectors (Holton and Graham, 1991; Marchuk *et al*., 1991; Sambrook and Russell, 2001). Efficiency, small proportion of self-ligation or false positive colonies, and low cost are important criteria in choosing the optimal method for preparing PCR cloning vectors.

In this study, a restriction enzyme digestion method was employed to develop an improved method for PCR cloning vector preparation. To select the most appropriate restriction enzyme for vector preparation, comprehensive information on restriction enzymes from the REBASE database (http://rebase.neb.com) was examined with the following criteria in mind: 1) the enzyme produces a T overhang at the 3' end, 2) the enzyme does not digest commonly used vectors in molecular biology, such as pBluescript II and pUC vectors, and 3) the enzyme is available at a low price. Using these criteria, XcmI was selected as the best restriction enzyme for the preparation of PCR cloning vectors. pBluescript II SK(+) (Stratagene) was selected as a backbone vector because of the availability of various restriction sites in multiple cloning sites, blue/white selection, and high copy number. However, the strategy presented in this study can be applied to any vector system frequently used in molecular biology.

To introduce XcmI sites (CCANNNNN^NNNNTGG) into the multiple cloning sites of the pBluescript II SK(+) vector, two adapters (Fig. 1A and 1B) were incorporated into the vector. Adapter 1 was ligated into pBluescript II SK(+) vector digested with XbaI and BamHI to produce pBL-XcmT1. Adapter 2 was ligated into pBL-XcmT1 digested with ClaI and SalI to produce pBL-XcmTT. The adapters created an in-frame lacZ' gene when they were incorporated into the pBluescript II SK(+) vector for white/blue selection (Fig. 1C). The vector maintains in-frame lacZ' gene when XcmI sites are digested, T-overhangs are deleted and the vector is self-ligated to reduce false-positive colonies produced by self-ligation of damaged T vectors (Fig. 1D). BamHI restriction sites were introduced in both ends of the multiple cloning sites for easy examination of insert size by digesting clones with a single enzyme. The vector pBL-XcmTT was maintained and amplified in *E. coli* XL-1 blue cells.

To prepare the PCR cloning vector with T overhangs at both ends of the vector, the pBL-XcmTT vector was prepared by QIAGEN Plasmid Mini Kit (Qiagen) and digested with XcmI restriction enzyme (NEB). When 50 ng of T vector (prepared without purification) was ligated with 50 ng of PCR products of the 16S rRNA gene of *E. coli*, approximately 2,000 white colonies were obtained (Table 1). All of the white colonies we tested contained an insert of the expected size (Fig. 2A). However, there were more blue colonies than white colonies. When the digested vector was purified with QIAquick Gel Extraction Kit (Qiagen) to remove digested small fragments, blue colonies were not produced, but colony number was dramatically lower (Table 1). All of the white colonies we tested contained an insert of the expected size

*Corresponding author <E-mail : polypore@kopri.re.kr>

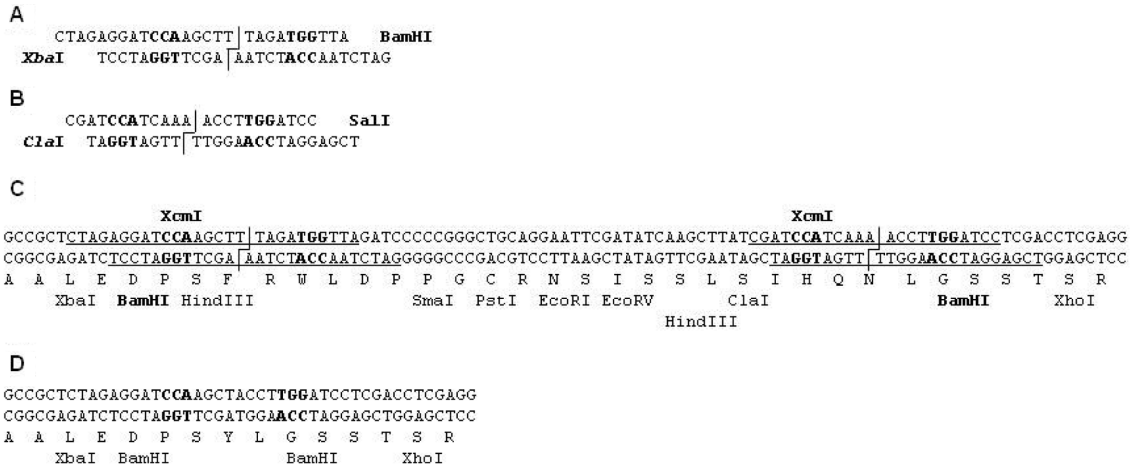

Fig. 1. Sequences of adapters and multiple cloning sites of constructed PCR cloning vectors. A, adapter 1; B, adapter 2; C, multiple cloning sites of pBL-XcmTT; D, multiple cloning sites after self-ligation of damaged T vector. The recognition site of XcmI is represented by bold letters.

Table 1. Cloning efficieny of PCR products by various treatments of the vector

Vector	pBL-XcmTT				Promega T easy vector
Enzyme digestion	XcmI		XcmI/ClaI/EcoRI/SmaI		
Purification	no	gel elution	PCR prep kit	gel elution	
White colonies	1984	106	4450	74	2432
Blue colonies	2251	0	257	0	23

*Cloning of PCR products was conducted by ligation of 50 ng of vector and 50 ng of 16S rRNA gene PCR products at 16°C for 16 hours and transformation into *E. coli* XL-1 blue.

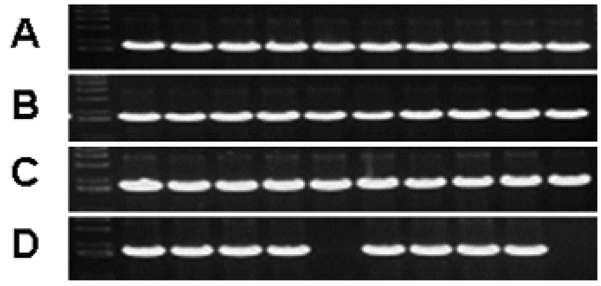

Fig. 2. Inserts of 10 clones from clone libraries constructed with vectors. A, XcmI digestion with no purification; B, XcmI digestion and gel purification; C, XcmI/ClaI/EcoRI/SmaI digestion and purification with PCR purification kit; D, XcmI/ClaI/EcoRI/SmaI digestion and gel purification.

(Fig. 2B).

Because no-purification or purification by gel extraction of digested vector did not give satisfying results, purification of the vector with QIAquick PCR Purification Kit was evaluated. To remove 60 bp-fragments produced by XcmI digestion, pBL-XcmTT was digested further with ClaI, EcoRI and SmaI to generate fragments smaller than 20 bp. When the pBL-XcmTT vector was digested with ClaI/EcoRI/SmaI in addition to XcmI, and purified with QIAquick PCR Purificaiton Kit, the number of white colonies was relatively high compared to other methods, and only a relatively small number of blue colonies were produced (Table 1). All of the white colonies we tested contained an insert of the expected size. When the pBL-XcmTT vector was digested with XcmI/ClaI/EcoRI/SmaI and purified with QIAquick Gel Extraction Kit (Qiagen), the results were similar to those as by XcmI single enzyme digestion and gel purification (Table 1 and Fig. 2D). In this study, the small fragment produced by XcmI was further digested with three additional enzymes to guarantee removal of the fragment by PCR purification kit. However, this procedure may be replaced by EcoRI single enzyme digestion to produce two 30 bp-fragments because most of the commercially available PCR purification kits guarantee removal of DNA fragments less than 40 bp.

The PCR cloning vector prepared by digestion with four enzymes and purified with a PCR purification kit had better cloning efficiency than commercial PCR cloning vectors (Table 1), a smaller proportion of blue colonies, and no false positive colonies. Because spin columns for plasmid preparation and PCR purification are reusable, the

cost for the preparation of this PCR cloning vector is very low.

Acknowledgements

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