A variety of methods are currently available to clone the DNA products generated by polymerase chain reaction (PCR) (3). For Taq DNA polymerase, adding an A residue to the 3′ end of its reaction product (2) can be used to ligate a 3′ A-overhanged PCR product directly to a T vector containing compatible 3′ T overhangs produced by enzymatic tailing (6) or XcmI digestion (5). In the selection system for the recombinants, all of these kinds of vectors contain a multiple cloning site (MCS) within the α-peptide coding region of β-galactosidase, making an insertional disruption of the lacZ reading frame to allow recombinant clones to be directly identified by blue/white screening on a plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

The gfp gene of the bioluminescent jellyfish Aequorea victoria encodes the green fluorescent protein (GFP), which absorbs light at 395 nm and fluoresces at 508 nm (7). Because light-stimulated GFP fluorescence does not require any cofactors or substrates, GFP has been...
Benchmarks

reported to be useful as a marker for gene expression (1).

We describe the construction of a new T vector for the cloning of PCR products into a T overhang-linearized plasmid. The cloning site is between the lacZ promoter and the gfp gene of the pGFPuv plasmid (CLONTECH Laboratories, Palo Alto, CA, USA) such that insertion disrupts gfp expression (Figure 1). Recombinants usually do not fluoresce and can be rapidly identified by illumination with UV light.

The new T vector was constructed by inserting a 35-nucleotide linker that contains two XcmI sites into the XbaI and XmaI sites of pGFPuv. The oligonucleotides (35-mer A: 5′-CTAGACC-ATGGATATCATGGCCAACAAAAC-CATGG-3′ and 35-mer B: 5′-CCGG-CCATGGTTTTGTTGGCA TGATA-TCCATGG-3′) were synthesized by Biosynthesis (Lewisville, TX, USA). Fifty picomoles of 35-mer A oligonucleotide were annealed with the same amount of 35-mer B, providing a double-stranded linker with a 5′-CTAG-XbaI protruding end on the left and a 5′-CCGG-XmaI overhang at the right end. The cloning site is between the lacZ promoter and the gfp gene of the pGFPuv plasmid. The pGFPuv plasmid was digested with XbaI and XcmI, followed by ligation of the vector with GENE-CLEAN III Kit (Bio 101, Vista, CA, USA). The ligation reaction was initiated using approximately 100 ng of XbaI- and XmaI-cut pGFPuv vector, 10 pmol of annealed 35-mer linker and 1 U of T4 DNA ligase in a 10-µL reaction volume. The ligation was incubated at 12°C for 4 h. Two microliters were used to transform E. coli strain DH5α (Life Technologies, Gaithersburg, MD, USA). After transformation, the cells were spread on LB plates containing 50 µg/mL of ampicillin and incubated overnight at 37°C. The plates were placed on a UV transilluminator (365 nm), and five green fluorescent colonies were randomly picked for further analysis.

The resulting plasmid, pCRGv, was digested with XcmI to generate a 3′-T overhang at both ends. To prepare the PCR DNA for this study, a 1.0-kb amplified DNA fragment was made as follows. The PCR mixture (50-µL final volume) contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 200 µM each dNTP, 10 µM each primer [forward 5′-CTAGC CACTCAATTTACAT-3′], reverse 5′-TACTGCAGAGTTAAGGAG-3′, derived from the Candida CIP1 gene (4)], 100 ng of Candida chromosomal DNA and 2.5 U of Taq DNA polymerase. PCR was routinely performed for 30 cycles in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) with denaturation for 1 min at 94°C and annealing for 1 min at 54°C, followed by a 2-min extension at 72°C.

Approximately 100 ng of the PCR product, 50 ng of XcmI-cut pCRGv vector and 2 U of T4 DNA ligase were mixed and incubated at 4°C in a 20-µL reaction volume. Five microliters of ligation mixture were used to transform E. coli DH5α.

Direct cloning of DNA fragments generated by Taq DNA polymerase is based on the digestibility of the cloning vector with XcmI to yield a linear molecule with 3′ unpaired T bases at both ends. XcmI recognizes and cleaves the DNA sequence 5′-CCANNNNN-NNNTGG-3′ (N represents A, T, C or G), resulting in 5′-CCANNNNN and
NNTTGG-3'. Two partially complementary 35-mer oligonucleotides containing the 5' XbaI CTAG- and 3' XmaI CCGG-protruding sequences were synthesized. Two XcmI digestion sites were also included in these oligonucleotides. The XhoI and XmaI sequences within the MCS of pGFPuv were replaced with these 35-mer linkers (Figure 1). Upon digestion with XcmI, the resulting plasmid, pCRGv, yields a vector with the desired unpaired 3' T residues and central stuffer fragment, which can be removed by elution. The polylinker region of this vector was sequenced (8) from both directions to verify that the 35-mer linker was correctly inserted and the GFP reading frame was maintained.

The XcmI-digested pCRGv vector (pCRGv-T) has been used to directly clone a 1.0-kb-long CIP1 gene (4) from Candida sp., amplified by PCR. Approximately one fifth of the E. coli transformants from the ligation were not fluorescent, while the other four fifths were green fluorescent under the 365-nm longwave UV light (Figure 2). This value is similar to that obtained when the pDK101 vector, which has lacZ reporter gene instead of gfp gene, was used as a PCR cloning vector (5).

For the insert analysis, DNA preparations from 10 randomly chosen fluorescing and nonfluorescing colonies were digested with NcoI, which has its target sites at both ends of the 35-mer linker. Non-recombinant fluorescing clones produced two fragments; all the nonfluorescing recombinants appeared to have an additional 1-kb insert (data not shown). When five white recombinants among them were checked for the vector-insert junction sequence by DNA sequencing, we observed the correct nucleotide sequences. In contrast, by DNA sequencing we also noticed that plasmids from most of the green fluorescent colonies had a single XcmI site without the insert PCR DNA. These plasmids might be attributed to incomplete digestion by the XcmI used to make a cloning T vector of pCRGv.

When GFP is used as a reporter for the cloning of PCR product, it has a number of advantages compared to that of lacZ. First, there is no need to use relatively expensive chemicals such as X-gal or isopropyl-β-d-thiogalactopyranoside (IPTG). Even if the gfp gene is activated by the lacZ promoter, its basal level of expression is enough to allow for the detection of fluorescence in the absence of IPTG. Second, there are no ambiguities in identifying nonfluorescing colonies. Cells harboring non-recombinant vectors are fluorescent, whereas those containing inserts are not. However, β-galactosidase sometimes shows ambiguous and variable activities such as pale blue, making it difficult to identify true recombinant. Furthermore, the fluorescence of GFP works in a wide range of host strains, but α-complementation of lacZ on lacZ-based systems requires the use of specific strains carrying an F' episome encoding the omega segment of lacZ. Finally, when the GFP is used as a reporter, the initial identification for the true recombinant is immediate and easy by briefly exposing the plate to 305–365-nm UV light.

We have demonstrated that XcmI-digested pCRGv vector can be used to directly clone the PCR product that has been generated by Taq DNA polymerase and that recombinants can be easily detected with a UV hand monitor.

REFERENCES

This work was supported by the Academic Research Fund (Grant No. GE 97-278) of the Ministry of Education, Republic of Korea. Address correspondence to Dr. Soon-Yong Choi, HanNam University, Department of Microbiology, 133 Ojung-Dong, Taeduk-Ku, Taejon 306-791, Republic of Korea. Internet: sychoi@eve.hannam.ac.kr

Received 12 November 1997; accepted 8 April 1998.

Jung Kwon, Kill Soon Park, Seong Weon Park and Soon-Yong Choi
HanNam University and Korea Ginseng and Tobacco Research Institute Taejon, Korea