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Improved T-Vector for the Cloning of PCR DNA Using Green Fluorescent Protein

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Abstract A new GFP-based T-vector for cloning of PCR products was developed by using a green fluorescent protein (GFP) as a marker. In order to facilitate the DNA inserts, multiple restriction sites, SP6 and T7 RNA polymerase promoter sites, were introduced close to the PCR DNA insertion site of a pCRGv vector. The *Xcm*I-digested pHNT plasmid can be used to clone a 3' A-overhanged PCR DNA amplified by *Taq* DNA polymerase. A potential method of easing some difficulties from its use along with its cost savings provided by this vector are likely to lead to the replacement of other T-vectors for PCR DNA cloning.

Key words: Green fluorescence, T-vector, cloning, PCR

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 [10]. Because light-stimulated fluorescence does not require any cofactors or substrates, GFP has been known to be effective in its use as a marker for gene expression [1]. To facilitate the cloning of PCR products generated by the polymerase chain reaction (PCR), a variety of methods are currently available [4]. In particular, the T-vector, known as a linearized plasmid containing a single 3' T-overhang, allows for the direct cloning of PCR products to take place by taking advantage of the terminal transferase activity of *Taq* DNA polymerase, and adds an A residue to the 3' end of its reaction product [3]. T-vectors containing 3' T-overhangs have been created by enzymatic tailing [8] or adding two adjacent nonidentical restriction sites for *Xcm*I [6]. Digestion of the vector with *Xcm*I generates a linear DNA molecule with a single T-overhang at both 3' ends.

We have previously described the construction of the GFP-based T-vector (pCRGv-T) for cloning of PCR products into T-overhang sites generated by *Xcm*I digestion [7]. The cloning site is placed between the *lacZ* and *gfp* genes in such a way that insertion of PCR DNA gives rise to a disrupt GFP reading frame. Recombinants usually do not produce fluorescence and can be easily identified by illuminating with UV light. However, the difficulty in the use of this vector is the fact that there is no simple way to release the DNA insert from the T-vector due to the lack of suitable restriction sites. To resolve this problem of the pCRGv-T vector, we have introduced multiple restriction sites, SP6 and T7 RNA polymerase promoter sites, close to the PCR product insertion site.

The PCRGv vector was modified by inserting a 39-nucleotide linker that contains the SP6 promoter and *Not*I sites into the *Xba*I site, and a 45-nucleotide linker that consists of T7 promoter, and *Bgl*II and *Not*I sites into the *Kpn*I site of pCRGv. The oligonucleotides (39-mer A: 5'-CTAGACGATTTAGGTGACACTATAGGATATCGCGG-CCGC-3' and 39-mer B: 5-CTAGGCGGCCGCGATATC-CTATAGTGTCACCTAAATCGT-3') were synthesized by Bioneer (Cheongwon, Korea). Fifty picomoles of 39-mer A oligonucleotide were annealed with a same amount of 39-mer B, providing a double-stranded linker with 5'-CTAG *Xba*I protruding ends. The pCRGv plasmid was digested with *Xba*I, followed by dephosphorylation with calf intestinal phosphatase. The ligation reaction was performed using approximately 100 ng of *Xba*I-cut pCRGv vector, 10 pmol of annealed 39-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* DH5α. After the transformation, the cells were spread on LB plates containing 50 μg/ml of ampicillin and incubated overnight at 37°C. The plates were then placed on a UV transilluminator (365 nm) and five nonfluorescent colonies were picked for colony PCR. Clones that contained an insert linker in the correct orientation were tested by colony PCR with SP6 primer (5'-CGATTTA-GGTGACACTATAG-3') and GFP specific primer (5'-CAC-CATCTAATTCAACAAG-3'). The resulting plasmid, pCRGv-M1, was digested *Kpn*I and dephosphrylated with CIP. The

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second oligonucleotides (45-mer A: 5'-GCGGCCGCGAT-ATCCCCTATAGTGAGTCGTATTAAGATCTGGTAC-3' and 45-mer B: 5'-CAGCTCTTAATACGACTCACTATA-GGGGATATCGCGGCCGCGTAC-3') were annealed to produce a double-stranded linker with 3'-CATG *Kpn*I protruding ends. *Kpn*I-digested pCRGv-M1 plasmid and second linker were ligated according to the same procedure described above. The transformants were selected by visualizing the colonies with UV light. Five green fluorescent colonies were picked, followed by colony PCR with SP6 and T7 primers to choose the correct orientation. The nucleotide sequence of the multicloning site was sequenced from both directions to verify that two linkers were correctly inserted and that the GFP reading frame was maintained (Fig. 1).

Direct cloning of DNA fragments generated by *Taq* DNA polymerase is based on the digestibility of the new cloning vector, pHNT, with *Xcm*I to yield a linear molecule with 3' unpaired T-bases at both ends. *Xcm*I recognizes and cleaves the DNA sequence 5'-CCATGGATATCATGG-3'

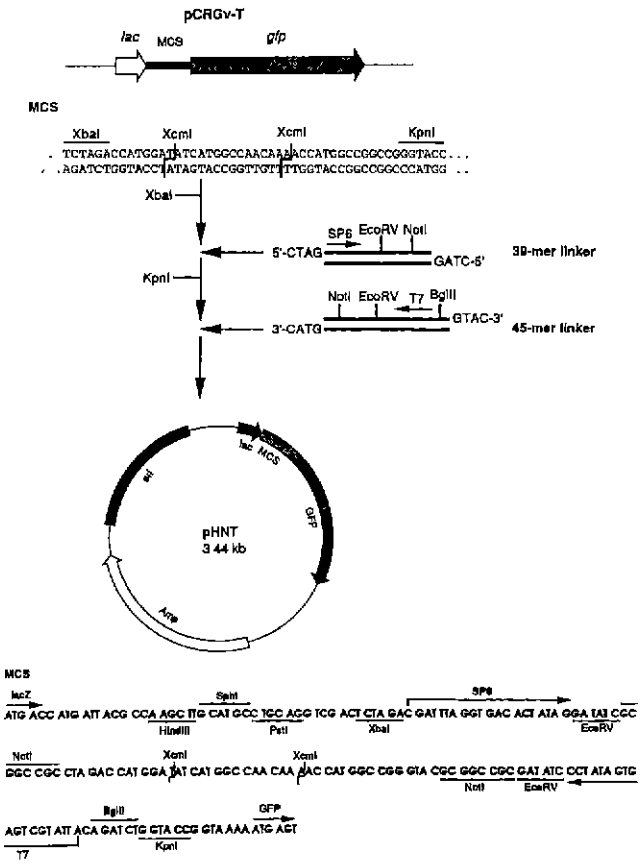

Fig. 1. Schematic representation of the construction of pHNT vector.

A 39-mer linker and a 45-mer linker were inserted into the *Xba*I and *Kpn*I sites of pCRGv, respectively. The nucleotide sequence of the multicloning site is shown with several restriction sites, SP6 and T7 RNA polymerase promoter sites. The digestion of pHNT with *Xcm*I gives rise to a single T-overhang at the 3' ends

or 5'-CCATGGTTTTGTTGG-3' of pHNT, resulting in 5'-CCATGGAT and complimentary 5'-CCATGGTT. The digested plasmid yields a vector with a desired unpaired 3' T-residue and a central stuffer fragment, which can be removed by elution. The SP6 and T7 promoter sites have been positioned in this vector to amplify the insert DNA, and to prepare strand-specific RNA probes. In addition, there are two *Not*I sites flanking the cloned PCR product for the isolation of insert DNA, two *Eco*RV sites for another blunt-end cloning, and a unique *Bgl*II site for directional cloning of DNA inserts.

The *Xcm*I-digested pHNT vector has been used to directly clone a 1.5 kb-long *CIP2* gene [9] or a 1 kb-long *CAD* gene [2] from yeast amplified by PCR. Approximately one third of the *E. coli* transformants gave rise to be non-fluorescent, while the others were shown to be green fluorescent under the 365-nm-long wave UV light (Fig. 2). High backgrounds of nonrecombinants appear to be the result of partial digestion of the plasmid DNA generating a low level of single-cut plasmid molecules that can be efficiently self-ligated [5]. For the insert DNA analysis, DNA preparations from 5 randomly chosen fluorescent and nonfluorescent colonies were digested with *Eco*RV as well as PCR amplification with SP6 and T7 primers. Nonrecombinant fluorescing clones produced no inserts, while all the nonfluorescing recombinants appeared to have 1.5 kb insert DNA (Fig. 3).

When *gfp* is used as a reporter for the cloning of the PCR product, it has a number of advantages compared to that of *lacZ*. First, the GFP-based pHNT vector eliminates the use of relatively expensive chemicals such as X-gal or isopropyl-β-D-thiogalactopyranoside (IPTG). Even if the expression of the *gfp* gene in pHNT can be driven by the *lac* promoter, its basal level of expression seems to be enough to allow for the detection of

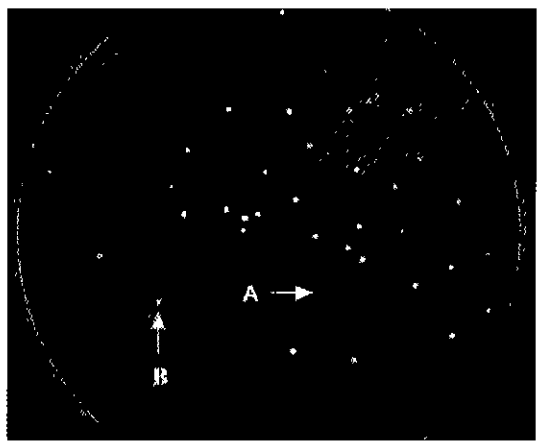

Fig. 2. UV illumination of *E. coli* transformants.

E. coli DH5α was transformed with pHNT::*CIP2* ligation and plated onto LB agar plate containing ampicillin. Colonies were irradiated with 365 nm UV source. (A) Nonfluorescent colony (B) Green fluorescent colony.

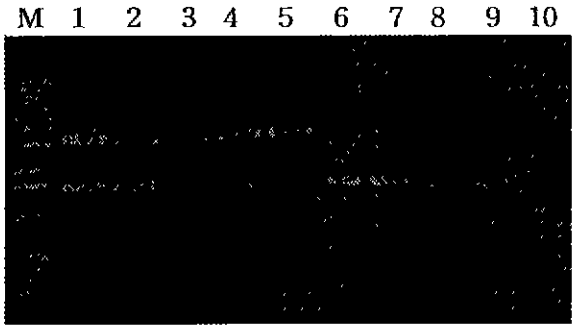

Fig. 3. *Eco*RV digestion and PCR amplification of transformants. Nonfluorescent (lanes 1-4, 6-9) and green fluorescent transformants (lanes 5 and 10) were digested with *Eco*RV (lanes 1-5) or PCR amplified with SP6 and T7 primers (lanes 6-10). M, molecular weight markers, 1 kb ladder.

fluorescence in the absence of IPTG. Second, there are no ambiguities in identifying nonfluorescing colonies. Cells harboring nonrecombinant 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 the true recombinant. Furthermore, the fluorescence of GFP can be applied in a broad 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*. Another advantage of the GFP vector is that the initial identification of true recombinant can be seen immediately and is simple to use by briefly exposing the covered plate to 305-365 nm UV light. Finally, similarly to the *lacZ*-based commercial T-vector, the multicloning site and PCR primer sites of the pHNT vector have facilitated the additional manipulation of DNA inserts. Therefore, the potential ease of use of this vector, in addition to cost savings provided by, are likely to influence the replacement of other markers such as β-galactosidase in the T-vector system.

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