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Construction of T-vector derived from pBluescript II SK with a positive selection marker, a rapid system for cloning

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ABSTRACT

A rapid DNA cloning system is a research interest of many scientists. TA cloning is one of the methods used for the cloning of PCR-amplified DNA molecules. The TA cloning method is a convenient and labor-saving replacement to traditional, restriction enzyme-mediated cloning strategies. A T-vector called pBlueskript II SK-1 with the lethal gene ccdB was designed to construct a positive selection vector. This lethal gene was inserted in multiple cloning sites of pBlueskript II SK. Then the vector digested with the endonuclease SmaI producing the blunt end. To directly clone the PCR product, a single 3'-A was added to a double-stranded DNA fragment by Taq polymerase and a T-vector with 3'-T overhang at each end using ddTTP and terminal transferase enzyme. The recombinant vector was transferred to the competent cells of host *Escherichia coli*. After DNA fragment entry, the activity of the ccdB gene eliminated, and the survival probability and host colony formation increased after transformation with the recombinant vector. The proliferation of the host of the T-vector was highly specific, and only hosts with the ccdA gene were able to receive this vector, to replicate the vector and survive. Therefore, after the insertion of the target gene, the lethal gene becomes inactivated, so there was no need to use a specific host and other selective markers, such as antibiotics. The TA cloning with a positive selection marker strategy is both simple and much more efficient than blunt-ended ligation and cohesive-end cloning.

Keywords: ccdB; Cloning; pBluescript II SK-1; Positive selection marker; TA vector

Introduction

Cloning of a gene is one of the common methods in recombinant DNA technology. Several methods for cloning of DNA fragments are used, including cohesive-end and blunt-end. The former method is based on the presence of a restriction site sequence at 5' ends of both gene and plasmid creating sticky ends. In the blunt-end method, two smooth ends of strands are involved. Both mentioned methods are ligation-dependent and need multi-steps purification after enzyme treatment that is time consuming and expensive

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(1). TA cloning method is based on the presence of adenine and thymine nucleotides at the 3' ends of DNA fragment and plasmid. In this method, nucleotide A is added to the 3' end of the PCR product and nucleotide T is added to the 3' end of blunt-ended plasmid, which can also be paired together. Most of the PCR products are amplified by Taq polymerase, thus possess a single 3'-A overhang at both ends. Linearized T vector with a 3'-T overhang at each end is used to direct cloning of the PCR products with 3'-A overhang (2-4). If cloning by any of the methods mentioned is not accomplished under optimal conditions, the success of screening of the inserted DNA fragment is reduced, so many transformers must be screened to obtain the desired positive clone.

To dissolve the problem with backgrounds, some strategies have been developed including insertional inactivation, directional cloning, phosphate treatment, and positive selection (5). The best way to separate correct clones without background from the selective medium plate was the use of positive selection vectors or direct selection vectors. Directional cloning requires double digestion of a gene and vector and could be difficult for successful digestion as well as purifying of digested DNA fragments from agarose gel electrophoresis. Phosphate treatment method had some disadvantages due to the variable efficiency of dephosphorylation (5).

A positive selection vector is a vector that can survive in host cells under specific conditions when foreign DNA fragments are inserted into the vector. The mechanism to perform positive selection includes the inactivation of functional genes of the vectors during insertion to foreign DNA fragments. The functional genes are generally controlled by their promotors (5). There are five groups of positive selection vectors: 1- compound dependence and compound sensitivity, 2- derepression of antibiotic resistance, 3- lethal genes, 4- palindrome, and 5- restriction/modification system. Out of five groups, compound sensitivity and lethal genes are the most common methods to obtain positive selection vectors. For positive selection, the expression of lethality genes toxic to host cells is used. These genes can originate from phages, bacteria, and eukaryotes. Among these genes, *ccdB* is a lethal gene encoding a protein that injures topoisomerase II (DNA gyrase) resulting in unrepairable DNA damage. This gene is located in F plasmid in some strains of *Escherichia coli* and is part of the toxin-antitoxin system encoded by the operon of *ccdB* that is responsible for the survival of plasmid in the cell. Another gene, called *ccdA*, is an antitoxin existing also in this operon and neutralizes the effect of this toxin in the cell and protects the cell. Molecular biologists have indicated the ability of the *ccdB* gene to increase cloning efficiency about 20 years ago. Meanwhile, a cloning vector containing this gene in the molecular cloning site is designed (6-7). Thus, any cell without F plasmid will die after transformation with a vector containing the *ccdB* gene when this gene is expressed.

In a previous study, the *ccdB* lethal gene was used for constructing positive selection vectors (5). When foreign DNA fragment is inserted into any one of multiple cloning sites, the toxic function of the *ccdB* gene is inhibited. Since the use of TA-based vector cloning is more cost-effective than conventional vector cloning, designing these vectors could be a new step in making effective vectors. In the present study, we designed a TA vector that had a positive selection marker to select the correct recombinant vectors from the background. In the first step, the *ccdB* gene was cloned into multiple cloning sites of the pBlueskript II SK cloning vector as a positive selection marker. In the second step, the vector was digested with blunt-end digestion enzyme after preparing as a T-vector with 3'-T overhang at each end using ddTTP and terminal transferase enzyme. Finally, a target gene was cloned into T-vector.

Materials and methods

The ccdB gene synthesis

In order to select the appropriate sequence of the gene, the corresponding sequences in GenBank (https://www.ncbi.nlm.nih.gov) were extracted. Multiple alignment was done at Clustal Omega to determine the identity of the sequences (www.ebi.ac.uk/service/clustal). Then the desired sequence with accession number EU496090.1 was selected for synthesis by ShineGene Molecular Biotech, Inc. (China). The selected sequence was 306 bp long from start to stop codons and contained two restriction sites, BamHI and HindIII, at the two ends. The synthesized sequence was incorporated in pUC57 plasmid.

Cloning of ccdB gene into vector

The amplified *ccdB* gene and pBlueskript II SK cloning vector were double digested with *Bam*HI and *Hind*III. The reaction was incubated at 37°C for 2 hrs. The ligation reaction including 50 ng digested vector, 150 ng digested *ccdB* gene, 5 units T4 DNA ligase with the final volume of 20 µl was stored at 22°C for 4 hrs.

Construction of T-vector

The T vector was generated based on the back bone of the pBlueskript II SK cloning vector. In order to construct a linear vector, the recombinant pBlueskript II SK was digested by *Sma*I to produce blunt-ended plasmid. Then *Taq* polymerase and ddTTP were used to add a 3'-T to the blunt-ended plasmid. For this reaction, 10 nM of ddTTP, 4 units *Taq* polymerase were added to digested vector. The reaction was performed in PCR for 30 mins at 70°C.

Adding a single A to the 3' end of the fragments

The polymerase chain reaction (PCR) to produce a single A at the 3' end of the amplified target gene was performed by *Taq* DNA polymerase, specific forward and reverse primers, template DNA, and dNTPs. By prolonging the final extension time, a single A nucleotide was added to both ends of the target gene.

Cloning of the target gene in T-vector and confirmation of cloning

For preparation of cloning reaction, 100 ng of amplified target gene with single A at the 3' end, 20 ng of linearized T-vector, and 2 units of T4 DNA ligase with a final volume of 15 µl were mixed. The reaction was done at 16°C overnight. Five µl of the ligation reaction was transferred to competent *E. coli* with heat shock at 42°C for 2 min. The transformed bacteria were transferred into LB agar and incubated at 37°C after 1 hr growth in the LB medium. After the formation of bacterial colonies on the plate, a few colonies were picked up and the colony PCR was performed with specific primers of the target gene for confirmation of transformation.

Results

The *ccdB* gene sequence extracted from NCBI and pBluescript II SK map is shown in Figure 1. A schematic diagram of TA cloning is shown in Figure 2. In TA cloning, all DNA fragments were directly converted to double-stranded molecules having 3' A overhangs by treatment with Taq polymerase and dNTPs. These DNA fragments were successfully ligated to T-vectors. In this cloning, the efficiency up to 90% is achieved (1). The synthesized *ccdB* gene used as a positive selection marker was cloned successfully into pUC57. The amplified *ccdB* gene with 306 bp was visible on 1.5% agarose gel (Fig. 3). Following this step, the ligated vector with a 306 bp *ccdB* gene was transformed into *E. coli* O157H which is a specific host for the *ccdB* lethal gene that contains *ccdA* antitoxin gene. The colonies formed on a specific plate checked for ligation and insertion of the gene by extraction of the recombinant vector from a single colony isolated by ampicillin plate (Fig. 4). The recombinant T-vector digested with *Sma*I restriction enzyme for making a blunt-end and treatment the linearized vector with dTTP and *Taq* polymerase made a single 3' T overhang in the vector.

The complementarity between the vector 3' T overhangs and PCR product 3' A overhangs allowed direct cloning of the DNA fragment. After cloning of DNA fragment into constructed T-vector pBlueskript II SK-1, transformed into *E. coli* and then the colonies of transformants were formed on the plate. The recombinant TA-vector pBlueskript II SK-1 after insertion of DNA fragment inhibited the activity of lethal gene *ccdB*, thus the positive colonies were formed on the plate. In addition, T-vector pBlueskript II SK-1 without any new insert could express *ccdB*. Therefore, host bacteria could not grow on the plate due to the killing activity of the lethal gene (Fig. 5).

The inserted DNA fragment was introduced into T-vector pBlueskript II SK-1 and inhibited the positive marker (lethal gene). The mechanism of our positive selection was insertional inactivation. For confirmation of transformation, the colony PCR amplified the inserted gene. The band of an amplified inserted gene is shown in Figure 6.

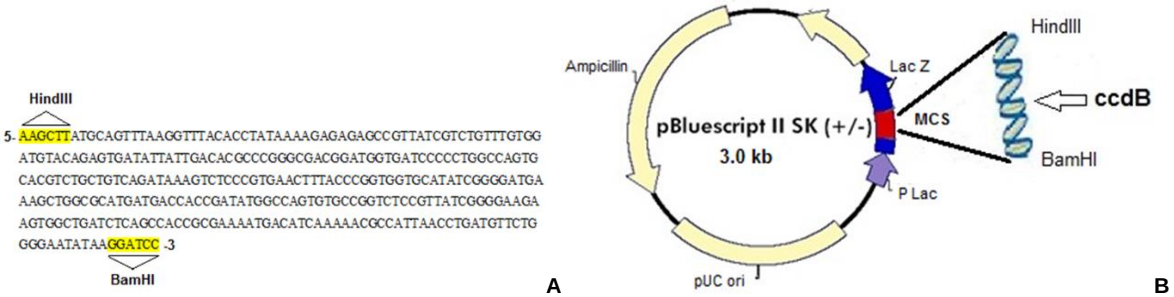


Figure 1. A. *ccdB* gene sequence. B. pBluescript II SK map and *ccdB* gene in molecular cloning site.

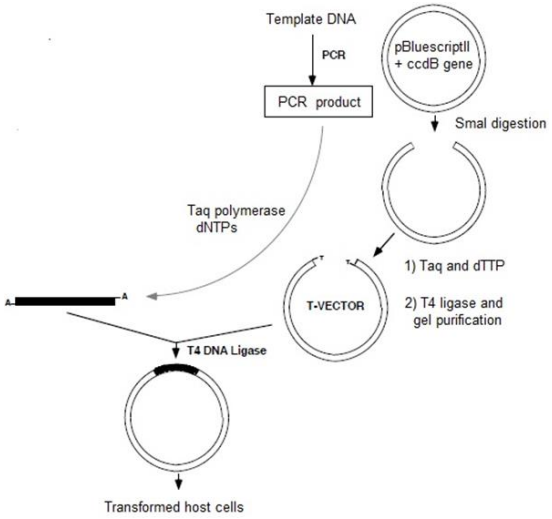


Figure 2. Schematic diagram outlining the cloning procedures using T-vector.

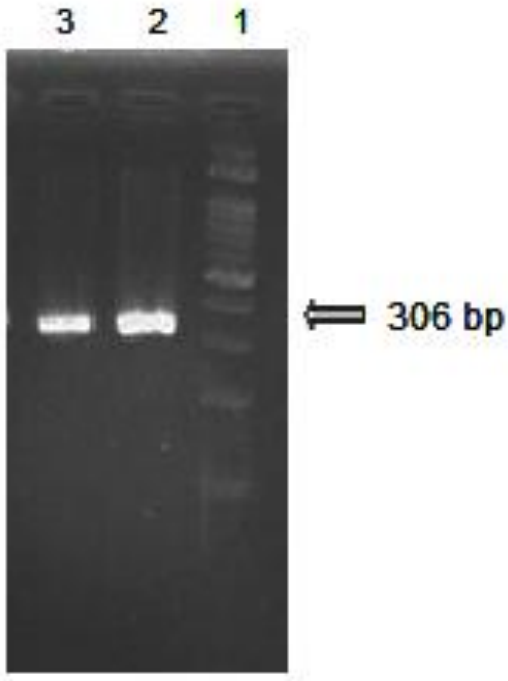


Figure 3. PCR product of amplified *ccdB* gene on 1.5% agarose gel. Lane 1, 100 bp DNA ladder; lane 2 and 3, 306 bp amplified gene.

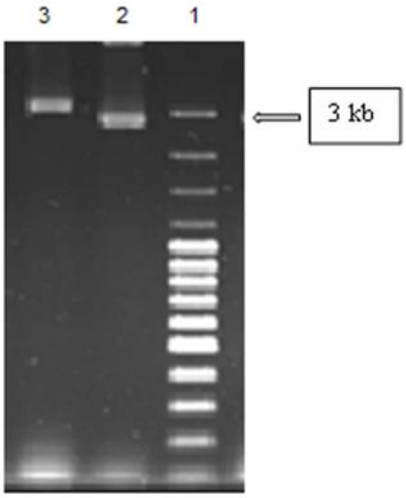
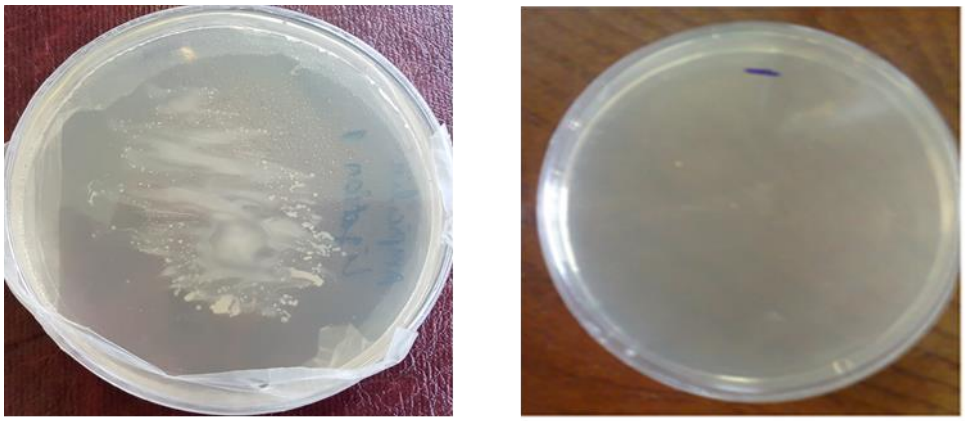


Figure 4. Agarose gel of recombinant vector. Lane 1: 100 bp plus DNA ladder; lane 2, pBluescript II SK vector (3 kb); line 3, recombinant vector containing *ccdB* gene (3.3 kb).



A **B**

Figure 5. Transformation of recombinant TA vector and pBlueskript II SK into *E. coli*. A. colonies of recombinant TA vector; B. negative control, the bacteria transformed with pBlueskript II SK contains *ccdB* gene.

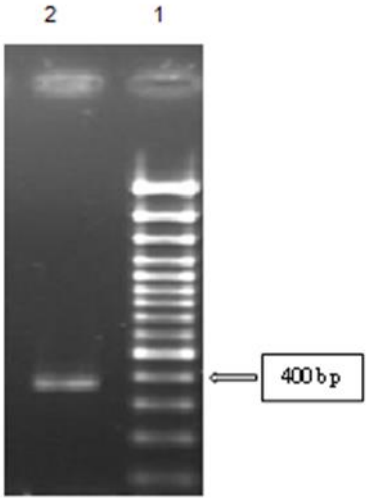


Figure 6. PCR colony of the gene inserted onto TA vector. Lane 1, 100 bp plus DNA ladder; lane 2, 400 bp amplified gene.

Discussion

Today, for easier gene cloning, different strategies are considered. One of these methods is the production of T-vector to directly clone the genes (5, 8). TA cloning method used in the present study to facilitate the cloning of PCR products. T-vector can have a positive selection marker to select the correct recombinant vectors and therefore they present the best way to separate clones from backgrounds (5). The positive selection vector can survive its host cell when foreign DNA is inserted to the vector, therefore transformants with uncut or self-ligated vectors cannot grow. In this study, we designed a T-vector with a *ccdB* gene as a positive selection marker. A *ccdB* gene encodes a lethal protein, which injures topoisomerase II (DNA gyrase), resulting in unrepairable DNA damage. To construct positive selection vectors, the *ccdB* gene was inserted into multiple cloning sites of pBluescript II SK. When DNA fragments are inserted into multiple cloning site, the toxic function of the *ccdB* gene is relieved.

Linearized T-vector produced with *Sma*I restriction enzyme digestion in our study generated a blunt end. There are two different strategies for producing T-vector, adding a T to both the 3' ends of a linearized vector and making single 3' T overhangs of a linearized vector using restriction endonuclease digestion (2-3, 9).

The DNA fragment was amplified here with Taq polymerase to produce 3' A overhangs (10). Taq DNA polymerase can add a single 3' A to the end of double-stranded DNA by terminal transferase like activity (10-12), which occurred also in our study. Most of the PCR products amplified by Taq polymerase would possess a single 3' A overhangs at both ends (2, 4).

The use of the *ccdB* gene as a positive selection marker is an important improvement over conventional TA cloning in that it eliminates the necessity of blue/white color screening (9). Using T-vectors prepared by this improved method, the entry clones for PCR products or restriction enzyme fragments could be created simply, efficiently, and inexpensive (9). A zero background TA cloning system that provides a simple method and high-efficiency direct cloning of DNA fragments with almost no self-ligation that also was provided by our method. The positive selection marker gene *ccdB* eliminated the self-ligation background after transformation. Thus, this cloning provides a general, cost-efficient, and high-throughput platform that complements a cloning system for gene and functional genomics studies (13).

Conclusion

TA cloning vectors are convenient and have a high cloning efficiency that is commercially available. In the present study, the formation of the recombinant colonies increased after transformation with the recombinant vector. Due to the presence of a positive selection marker, the need for antibiotics as a marker was eliminated. Also, the presence of non-recombinant colonies was removed by this method.

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Editorial Note

Volume 7, issue 2 of Progress in Biological Sciences was initialy scheduled to be published in December 31, 2017. However, some administrative changes leaded to a major delay in processing of the manuscripts. This issue is actually published in May 1, 2020. Editor-in-chief apologizes deeply for any inconvenience caused especially to the authors.