Construction of a New T-Vector: Nickase (Nt.*BspQI*)-Generated T-Vector Bearing a Reddish-Orange Indicator Gene

Ji-Young Choi^{1†}, Chulman Jo^{2†}, Sangmee Ahn Jo^{1,3*}

¹Department of Nanobiomedical Science & BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, Cheonan, Korea

²Division of Brain Research, Center for Biomedical Sciences, National Institute of Health, Osong, Korea

³Department of Pharmacy, College of Pharmacy, Dankook University, Cheonan, Korea

T-vectors are widely used for cloning the polymerase chain reaction (PCR) products. However, the low conversion efficiency of a plasmid into the linear T-vector usually results in non-recombinants. Here, we designed a new plasmid pNBQ-T to easily select the recombinant colonies harboring PCR products. pNBQ-T plasmid, which contains a DsRed indicator gene between two Nt.*BspQI* restriction cassettes, each of which contains palindromic sequences susceptible to Nt.*BspQI* nickase (5'-GCTCTTCT[^]GAAGAGC-3') at each end. Thus, this plasmid can be easily converted into T-vectors by a nickase (quadruple nicking), which results in two double strand breaks with 3'-thymidine overhangs. DsRed indicator gene, which is inserted between the restriction sites, helps identifying the PCR recombinants. Using this pNBQ-T plasmid the insertion efficiency of a PCR product was examined. We successfully identified white colony of the recombinants with the inserted myostatin promoter gene: the cloning efficiency was 93%. Therefore, this simple method utilizing pNBQ-T plasmid will serve as a useful and efficient technique for preparation of home-made T-vectors.

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INTRODUCTION

Since the polymerase chain reaction (PCR) products acquire adenosine residues at their 3'-ends by the terminal transferase activity of *Taq* polymerase, T-vectors containing thymidine overhangs at their 3'-ends have become widely used in gene cloning [1]. T-vectors can be prepared by using terminal deoxynucleotidyl transferase, which adds a single thymidine residue at each 3'-end of a linearized, blunt-ended plasmid [2]. Alternatively, they can be prepared by constructing a plasmid that contains the restriction sites of enzymes such as *XcmI* [3-5], *AspEI* [6,7] or *HphI* [7], which produces a single thymidine overhang upon digestion with the restriction endonuclease. Among these restriction enzymes, *XcmI* has been widely recommended because the common cloning vectors such as the pUC-based plasmids do not contain sequences recognized by

XcmI. However, the use of *XcmI* for T-vector construction is limited because the vectors incubated with *XcmI* are often only partially digested, and this failure leads to a high background of non-recombinant transformants [3,7]. Therefore, T-vectors with *lacZ* or green fluorescent protein (GFP) coding sequences as indicators were designed to overcome this difficulty. In these vectors, the insertion of PCR products disrupts the expression of the indicator gene, rendering the colonies that harbor the recombinant plasmids devoid of β -galactosidase activity or non-fluorescent [8-10]. As an alternative, *Discosma* sp. red fluorescent (DsRed) gene can be used as the indicator. One of advantages of DsRed gene is that its fluorescence is visible by naked eyes without UV or blue light while GFP-mediated fluorescence is only observed under UV.

Typically, restriction endonucleases hydrolyze both strands of the DNA double strands at the same time when they bind to their DNA recognition sites. Recently, engineered nicking endonucleases that targets one DNA strand and produce a nick in the DNA have become commercially available from NEB (Ipswich, MA, USA). The nicks created by these enzymes can serve as initiation points for a variety of other enzymatic reactions, including replacement, DNA synthesis, strand-displace-

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***Corresponding author:** Sangmee Ahn Jo, Department of Nanobiomedical Science & BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, 119 Dandae-ro, Dongnam-gu, Cheonan 31116, Korea. Tel: 82-41-550-1433, Fax: 82-41-559-7899, E-mail: smahn@dankook.ac.kr

[†]These two authors contributed equally.

ment amplification, and creation of small gaps.

Nt.*BspQI*, one of the available nicking endonucleases (nickase), recognizes a seven-base sequence plus one undefined base at the 3'-end (5'-GCTCTTCN-3') and makes a nick at the 3'-end of the recognition site. That being said, a commercially available pGEM-T Easy vector was deployed as a template and DsRed gene-containing two tandem Nt.*BspQI* nickase cassettes was inserted into this vector. Then the digestion of 5'-GCTCTTCTGAAGAGC-3' with palindromic recognition sites of Nt.*BspQI* would produce a T-overhang at the 3'-end. Thus we expected that cloning two tandem sequences into an appropriate plasmid and digesting it with Nt.*BspQI* could transform it into a T-vector. Here, we present this approach for preparing a T-vector using Nt.*BspQI* nickase.

MATERIALS AND METHODS

Construction of pNBQ-T plasmid

A variant of DsRed gene [11] was amplified using an RNAi-Ready pSIREN-RetroQ-DsRed-Express plasmid (Clontech, Mountain View, CA, USA) as the template, 20 pmole of a primer set with the palindromic recognition sequences (bold) of Nt.*BspQI* (sense, 5'-GCTCTTCTGAAGAGCTGGCCTCC TCCGAGGACGT-3'; anti-sense 5'-GCTCTTCTGAAGAGC TACAGGAACAGGTGGTGGC-3'), and 2.5 units of EF-*Taq* DNA polymerase (SolGent, Daejeon, Korea) in a total volume of 50 μ L. Amplification was performed using a GeneAmp 9600 thermocycler (Perkin Elmer, Norwalk, CT, USA) for 30 cycles of denaturation at 97°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The 706-bp DNA fragment amplified as a major PCR product was purified using a gel-extraction kit (Qiagen, Chatsworth, CA, USA) and was ligated into pGEM-T Easy vector (Promega, Madison, WI, USA). Thus we named this new plasmid pNBQ-T.

Preparation of T-vectors

In order to generate T-vector from pNBQ-T plasmid, pNBQ-T plasmid was incubated with 10 U of Nt.*BspQI* endonuclease at 50°C for 30 min and the digested vector was purified with a gel-extraction kit (Qiagen).

Cloning of myostatin promoter gene using pNBQ-T

To verify the easiness and efficiency of pNBQ-T usage cloning with a PCR product was tested. In this case a promoter region of the mouse myostatin gene was amplified from mouse genomic DNA using forward (5'-ACCCGGGATCCTTGCA GAGGCACTT-3') and reverse primers (5'-AGAGCTCTTT TTCCCTTGTTCTTATTTCTTC-3'). The PCR reaction was performed as described above for the amplification of the

DsRed gene. Each PCR cycle consisted of denaturation at 97°C for 45 s, annealing at 57°C for 45 s, and polymerization at 72°C for 1 min. The resulting PCR products, without further amplification, were ligated into the pNBQ-T (50 ng) using T4 DNA ligase (Promega, Madison, WI, USA) at room temperature for 2 h. The ligate was transformed into *E. coli* (DH5 α) with the heat-shock method and the transformant was spread on LB agar plates containing ampicillin (50 μ g/mL).

RESULTS

In order to develop a new T-vector, pNBQ-T, we first produced a T-vector-convertible plasmid (Fig. 1A) by inserting the DsRed gene (706-bp DNA fragment) into a pGEM-T Easy vector using a primer set containing the palindromic Nt.*BspQI* recognition sequences as described in Materials and Methods (Fig. 1B). In this case we designed the primers in order for the inserted DsRed gene to fit into the reading frame of the *lacZ* gene in pGEM-T Easy vector, which allowed red colonies to form on LB agar plates after transformation into *Escherichia coli* (DH5 α). As a result, we observed many surviving colonies presenting reddish-orange colonies without use of UV or blue light and the plasmid from each colony was purified with the QiaprepTMSpin Mini-prep kit (Qiagen, Chatsworth, CA, USA). This plasmid was thus named pNBQ-T plasmid. The DNA sequence of the plasmid was confirmed by sequencing (data not shown).

This pNBQ-T plasmid was then converted into T-vector by incubation with Nt.*BspQI* nickase. As shown in Figure 2, incu-

Figure 1. Map of the pNBQ-T plasmid. The three Nt.*BspQI* restriction sites in the pNBQ-T plasmid are shown in pink (A). Other *Eco*RI and *Pvu*II restriction sites are indicated by their positions in base pairs (bps). Of these, the sequences of the two tandem Nt.*BspQI* cassettes, designed for the conversion to a T-vector, are shown in (B). Arrows indicate the nick sites to be made by Nt.*BspQI*.

bation of the plasmid purified from a colony illuminating strong and bright color at 50°C for 30 min generated a T-vector of 3032-bp DNA fragment (top band) and a 691-bp DNA fragment (low band) cut out from the pNBQ-T plasmid.

Next, the cloning efficiency of pNBQ-T vector prepared by our method was tested. For this purpose we amplified the promoter region of mouse myostatin (MSTN) gene by PCR and the PCR product was ligated into pNBQ-T vector as described

Figure 2. Conversion of the pNBQ-T plasmid to T-vector by digestion with *Nt.BspQI*. The pNBQ-T plasmid was incubated with *Nt.BspQI* at 50°C for 30 min to convert a T-vector and the mixture were analyzed by 1% agarose gel. A 3032-bp DNA fragment (T-vector; top band) and a 691-bp DNA fragment (low band) cut out from the pNBQ-T plasmid were detected.

Figure 3. Cloning of the myostatin (MSTN) gene promoter using the pNBQ-T vector. (A) In order to examine the cloning efficiency, the MSTN promoter gene amplified by polymerase chain reaction (PCR) was ligated into the pNBQ-T vector and the vector was transformed into *E. coli* (DH5 α). On the next day, colonies with non-recombinants showed reddish-orange colonies on the LB agar plate while those with recombinants white color. (B) In order to confirm insertion of the myostatin gene promoter the pNBQ-T and pNBQ-T/MSTN promoter gene plasmids were incubated with *EcoRI* (lane E) or *PvuII* (lane P) at 37°C for 2 h. The incubation of the pNBQ-T and pNBQ-T/MSTN promoter gene plasmids with *EcoRI* produced the DNA fragments corresponding to DsRed (727-bp) and MSTN promoter (1224-bp) genes from the plasmids, respectively. *PvuII* digestion of the pNBQ-T produced 2,564, 860 and 299-bp DNA fragments while that of the pNBQ-T/MSTN promoter gene 2564-bp and 1656-bp DNA fragments. M: 1-kbp DNA ladder.

in Materials and Methods. The ligated vector was transformed into *E. coli*. As a result, white or reddish-orange colonies were formed (Fig. 3A) with an efficiency of approximately 2.4×10^5 cfu/ μ g DNA. Since DsRed gene in pNBQ-T vector worked as a negative selector, the recombinant transformants containing the target gene appeared white colonies whereas the non-recombinants reddish-orange colonies. To calculate the cloning efficiency of pNBQ-T vector, thirty white and ten reddish-orange colonies were randomly selected for PCR analysis. The result showed 93% cloning efficiency as twenty eight out of thirty white colonies had a 1185-bp PCR product corresponding to the MSTN promoter gene. In addition, the cloning of MSTN promoter sequence was verified by both restriction of *EcoRI* and *PvuII* enzymes and by sequencing of the plasmid (data not shown). *EcoRI* digestion of the pNBQ-T/MSTN promoter gene plasmid produced the 2996-bp and 1224-bp DNA fragments, while *PvuII* digestion generated 2564-bp and 1656-bp fragments (Fig. 3B). In contrast, none of the 10 reddish-orange colonies showed no expression of the MSTN promoter gene. These findings suggest that the pNBQ-T vector is a very useful tool for direct cloning of non-purified PCR products.

DISCUSSION

An essential step in preparation of T-vectors is to reduce non-recombinant transformants which originate from partially digested or undigested vector [4]. Thus, techniques to exclude non-recombinant transformants from recombinant ones containing the PCR product were developed [8,10] and such examples are T-vectors containing *lacZ* or GFP coding sequences as non-recombinant markers.

Our approach described in this paper using the DsRed gene as an indicator has an advantage compared to the case using the GFP gene. It was reported that a variant of DsRed protein expressed in mammalian cells produces a red fluorescent color under fluorescent microscope [11]. Interestingly, colonies containing the DsRed gene were seen with naked eye and it was not difficult to differentiate the reddish-orange colonies from white ones. Thus, this aspect provides a huge advantage to prevent mutation of the DNA that could be induced by UV light.

Moreover, there is an additional advantage for using the *Nt.BspQI* enzyme. The generation of T-vector using restriction enzymes such as *XcmI*, *AspEI* and *HphI* is applicable to only plasmids which have no such recognition sequences. In contrast, the pNBQ-T plasmid was not cut by the enzyme although the plasmid has one *Nt.BspQI* recognition site at 1109, because the enzyme makes only a nick on one strand not digesting the plasmid. Overall, the cloning efficiency using the new vector was 93%, which is useful in practical use.

Although this method is very simple and efficient several other aspects should be considered to improve this vector. First, Ds-Red is reported to be toxic in *E. coli* if sufficiently expressed and thus the T-vectors containing strong promoters should be cautious. Second, mutation of Nt.*BspQI* recognition site present in the pNBQ-T plasmid (at 1109) is desirable albeit there was no problem in converting to T-vectors in the present experiment. Last, although the cloning of MSTN promoter gene PCR products (1185 bp) was highly successful DNA amplicons with various size should be tested to further enhance the reliability.

In summary, this approach using the Nt.*BspQI* nickase improves significantly the previously published methods of preparing T-vectors and allows easy selection of the recombinants.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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