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Short Communication

Blunt-end vectors generated by polymerase chain reaction (PCR) for direct cloning of blunt-end DNA fragments

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Blunt-end cloning is a convenient way to clone polymerase chain reaction (PCR) products generated by proof-reading DNA polymerase. However, it is a time consuming procedure to prepare the linearized blunt-end vector, which usually involves plasmid extraction and restriction enzyme digestion. Moreover, 5' dephosporylation of the vector is usually required to avoid vector self-ligation. Here, we reported a method for generating linearized blunt-end vector pBSK-blunt by PCR. Vector generated in this way has no 5'-phosphate groups, hence completely avoiding vector self-ligation and yielding almost 100% positive clones.

Key words: Blunt-end cloning, phosphorylated DNA fragment, dephosphorylated blunt-end vector.

INTRODUCTION

The rapid progress of genome sequencing projects in the post genomic era has yielded large amounts of open reading frames (ORFs) whose functions need to be elucidated. It is necessary to clone these ORFs into a plasmid vector for subsequent molecular biological experiments, including sequencing, and expression. A strategy for rapid cloning of polymerase chain reaction (PCR) amplified DNA fragments is thus critically needed. PCR products can be cloned into the vectors by any of these three ways: i) restriction enzyme based DNA recombinant method; ii) TA cloning (Ito et al., 2000; Kwon et al., 1998; Mead et al., 1991); iii) blunt-end DNA cloning (Koesters et al., 2002). Generally, amplification of gene of interest by PCR using proof reading DNA polymerase, such as *Pfu*, *KOD* and Primerstar, is preferred since the PCR products with a higher degree of fidelity are required in many investigations. However, traditional blunt-end cloning method for direct cloning of blunt-end PCR products is not efficient since non-recombinant clones will be produced by self-ligation of the vectors yielded by restriction enzymes (e.g., *EcoR*V) digestion. More efforts are needed to screen the clones with inserts from the "empty" clones. To circumvent this problem, Liu and Schwartz, (1992) have developed a *Sma*I cloning method and Tachibana et al., (2007) have further improved this method. This method was simple without pretreatment of the vector, including restriction digestion and dephosphorylation and required only the addition of *Sma*I restriction enzyme to the ligation mixture before transformation (Liu and Schwartz, 1992). However, incompletely digestion of vector by *Sma*I also results in high ratio of non-recombinant clones.

Here, we developed a PCR-based method to generate blunt-end vectors for blunt-end DNA fragment cloning. With this method, a lot of steps are saved, which includes restriction endonuclease digestion, 5' dephosphorylation and positive clone screening.

METHODS AND MATERIALS

Construction of pBSK-blunt vector

Two oligonucleotides with *EcoR*I site at 5' ends (underlined), R7: 5'-GAATTCTCCCTTTAGTGAGGGTTAAT-3' and R3: 5'-GAATTCCCCTATAGTGAGTCGTATTA-3' were synthesized. PCR was performed

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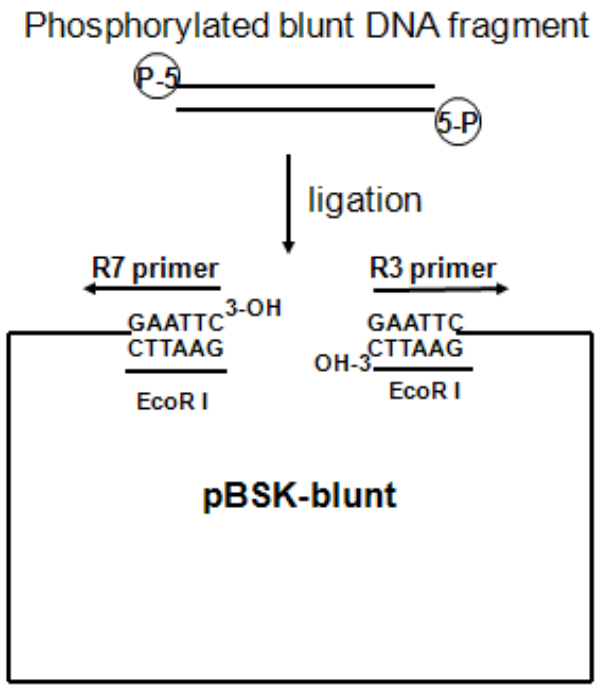

Figure 1. Principle of cloning blunt-end DNA fragment with pBSK-blunt. The pBSK-blunt was generated by PCR with primer R7 and R3 and did not have 5'-phosphate groups, while the PCR products to be cloned were phosphorylated to contain 5'-phosphate groups, which facilitated the ligation with pBSK-blunt vector.

to amplify the vector pBluscript II SK (Stratagene, CA, USA) with proof reading polymerase PrimeSTAR HS DNA (Takara, Dalian, China). The PCR reaction was performed for 30 cycles of denaturation at 98℃ for 10 s, primer annealing at 50℃ for 30 s, and extension at 72℃ for 3 min. The resulting PCR products were purified by PCR clean Kit (Axygen, CA, USA). To eliminate the template vector, the purified PCR product was treated by *Dpn*I (New England BioLabs, MA, USA) according to the manufacturer's instructions. The digested PCR product was then gel purified using the AxyPrep^{TM} DNA gel extraction kit (Axygen, CA, USA) and the desired 2900 bp band was recovered. This vector was named as pBSK-blunt.

Cloning of thioredoxin gene

The thioredoxin (TRX) gene amplified from pET32a (Novagen, Darmstadt, Germany) by PCR with proof reading polymerase Primerstar (TaKaRa) was used to test the cloning efficiency of the vector. Primers (TRX upper: 5'-ATGAGCGATAAAATTATTCA-3' and TRX lower: 5'-TGCACCCACTTTGGTTGCCGCCA-3') were synthesized. The primers were phosphorylated by T4 polynucleotide kinase (PKN, TaKaRa). The reaction in a volume of 20 µl, which contained 8.5 pM each of the pair primer, 1×T4 PKN buffer, 5 U PKN and 1 mM ATP (Fermentas, Vilnius, Lithuania) was carried out at 37℃ for 30 min. And then, aliquots of the resulting primers mixture were used for PCR. The PCR reaction was performed for 30 cycles of denaturation at 95℃ for 20 s, primer annealing at 50℃ for 30 s, and extension at 72℃ for 40 s. The resulting PCR product (about 260bp) was gel purified and ligated with the pBSK-blunt. Mixtures of 3 µl pBSK-blunt (~10 ng/µl), 1 µl purified TRX (~20 ng/µl), 1 µl T4 ligase (TaKaRa) and 5 µl 2X rapid ligation buffer (Promega, WI, USA) were incubated at room temperature for 10 min. The ligation mixture was transformed into competent DH5α *Escherichia coli* cell and plated on Luria–Bertani plates containing ampicillin.

Colony PCR using T7 (5'-TAATACGACTCACTATAGGG-3') and T3 (5'- ATTAACCCTCACTAAAGGGA-3') primers were performed to indentify the clones harboring inserts. The PCR reaction was performed for 30 cycles of denaturation at 95℃ for 20 s, primer annealing at 50℃ for 30 s and extension at 72℃ for 40 s. Plasmids from those trasformants were extracted using mini-prep kits (Axygen) and analyzed by *EcoR*I digestion according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The principle of PCR-prepared blunt-ended vector for cloning PCR product is illustrated in Figure 1. The pBSK-blunt vector was prepared by inverse PCR using primers R3 and R7 which were without phosphorylation modification. The PCR products to be cloned were phosphorylated before cloning into the pBSK-blunt vector. In comparison with blunt-end vectors prepared by restriction endonuclease enzyme digestion, blunt vectors prepared by PCR do not contain 5'-phosphate groups. For this reason, vector self-ligation is completely avoided and all clones that grew up will be positive clones. Although the blunt-end DNA fragments need to be phosphorylated before ligation, we saved the effort in clone screening, which is more time consuming.

To test the efficiency of PBSK-blunt for cloning, the TRX gene was chosen. The TRX fragment was phosphorylated before ligation with vector pBSK-blunt and the ligation products were transformed into DH5α cell. In our case, the transformation efficiency was approximately 8×10^5 cfu/mg DNA. Nine colonies were picked at random and they were all confirmed to be positive clones by colony PCR (use T7 and T3 as primer). Plasmids extracted from these colonies were further analyzed by *EcoRI* digestion analysis. The results show that all nine clones contained inserts of expected size (Figure 2A and B). Furthermore, lipase A from *Candida antarctica* (*CALA*, about 1300 bp) was also efficiently cloned using vector pBSK-blunt (data not shown). These results demonstrated that the pBSK-blunt as a valuable vector could be used for cloning blunt-end PCR products.

Although the cloning method is feasible, one limitation of this technology should be mentioned. The error rate of the proof-reading DNA polymerase Primerstar reported by Takara is 12 errors per 250,000 bp, meaning that few mutations would be present in the vector pBSK-blunt. To overcome this obstacle, proof-reading DNA polymerase with higher fidelity can be used to generate pBSK-blunt vector to decrease the mutation rate. Meanwhile, the plasmids with mutation would be depleted only when they can not replicate or resist antibiotics in the bacteria host.

In summary, we report a simple method to generate linearized blunt-end vector by PCR for efficient cloning of blunt-end phosphorylated DNA fragments. A major advantage of this cloning strategy is that screening for

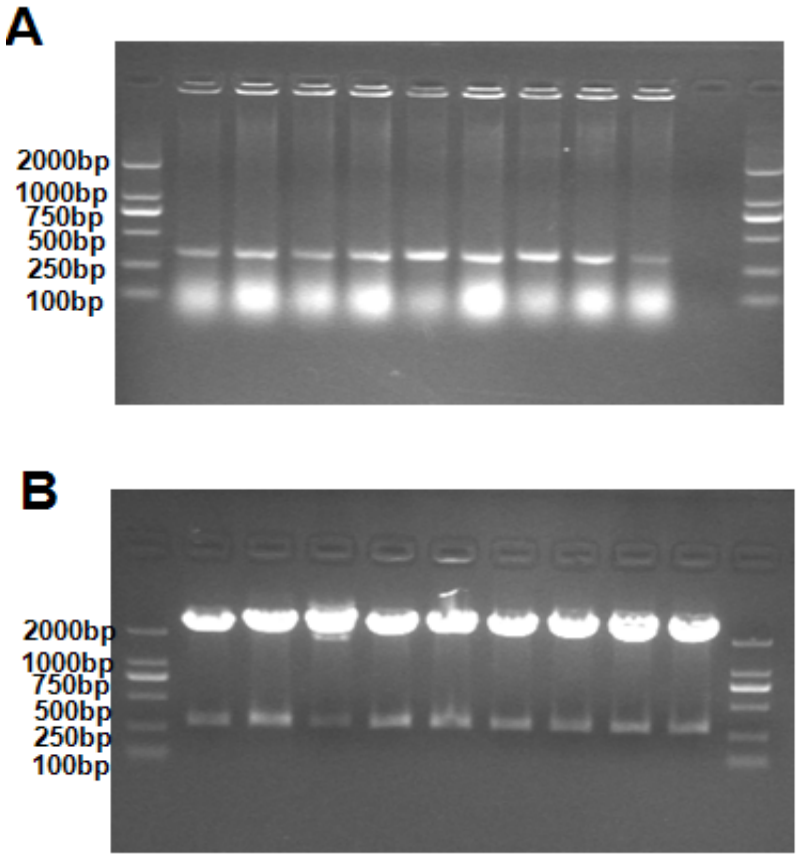

Figure 2. Analysis of the cloning efficiency using pBSK-blunt. A: Agarose gel electrophoresis of PCR products obtained from colony PCR. B: Restriction analysis of isolated plasmid DNA. The isolated plasmid DNA was digested by *EcoR* I. The digestion product was analyzed by agarose gel electrophoresis.

clones with inserts is unnecessary, thus saving time and labor. It is also economical to prepare linearized blunt-end cloning vectors by this PCR method, as it avoids bacterial culture and plasmid extraction. The principle of this method can also be used in other applications such as generation of prokaryotic and eukaryotic expression vectors for analysis of gene expression or genomic library construction (Kawata et al., 1998).

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REFERENCES

Ito Y, Suzuki M, Husimi Y (2000). A T-extended vector using a green fluorescent protein as an indicator. Gene. 245(1): 59-63.

Kawata Y, Yano S, Kojima H (1998). Efficient library construction with a TA vector and its application to cloning of the phytoene synthase gene from the cyanobacterium Spirulina platensis. Curr. Microbiol. 37(4): 289-291.

Koesters R, von Knebel Doeberitz M (2002). Improved blunt-end cloning by replacing EcoRV with Eco32I. Biotechniques, 32(6): 1244-1246.

Kwon J, Park KS, Park SW, Choi SY (1998). T vector for direct selection using green fluorescent protein. Biotechniques, 25(2): 192-196.

Liu ZG, Schwartz LM (1992). An Efficient Method for Blunt-End Ligation of PCR Products. Biotechniques, 12(1): 28-30.

Mead DA, Pey NK, Herrnstadt C, Marcil RA, Smith LM (1991). A universal method for the direct cloning of PCR amplified nucleic acid. Biotechnol. N. Y. 9(7): 657-663.

Tachibana A, Tanabe T, Yamauchi K (2007). SmaI cloning with regeneration of the SmaI site for sequential PCR product cloning. Anal. Biochem. 361(1): 143-145.