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SHORT REPORTS

A strategy for in-house production of a positive selection cloning vector from the commercial pJET1.2/blunt cloning vector at minimal cost

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Abstract

Key message **In-house production of a positive selection cloning vector could be simple, efficient and low cost.**

Abstract DNA cloning technology requires a vector to harbour a gene of interest for multiplication of the gene in bacterial cells. Positive selection vector has become a popular type of cloning vector due to the simplicity and efficiency of the positive selection system. Due to the presence of a toxic gene, propagation of a commercial positive selection vector in common laboratory *E. coli* strains is infeasible. This study demonstrated a strategy for propagation and in-house production of a commercial positive selection vector, i.e., pJET1.2/blunt cloning vector, at low cost. This was done by insertion of a specially designed DNA fragment (MCS fragment), which can be easily removed later by *Eco*RV digestion, into the pJET1.2/blunt cloning vector to allow the propagation of the modified plasmid (termed pJET1.2M) in common *E. coli* strains. Removal of the MCS fragment from the pJET1.2M plasmid produces the pJET1.2/blunt cloning vector ready for gene cloning. The self-made pJET1.2/blunt cloning vector exhibited a cloning efficiency of 100%.

Keywords DNA cloning · Positive selection · Low cost · pJET1.2/blunt cloning vector

With the advancement in sequencing technology, tremendous genetic information is now available to the public. Cloning of a genetic element is a prerequisite for in-depth study of the genetic element. Therefore, DNA cloning technology has made astonishing leaps of progress in understanding gene function in this post-genomic era. As the DNA cloning technology has become an essential part of all biological research involving DNA manipulation, it turns out to be a routine procedure in many laboratories. This technology involves combining a cloning vector with an insert(s) using specific enzymes and followed by propagation of the recombinant plasmid in a bacterial host cell to produce identical copies of the DNA fragment in abundance (Green and Sambrook 2020). The cloned DNA fragment can be used for various analyses such as DNA sequencing, protein expression, and sequence alteration through site-directed mutagenesis (Seetaraman Amritha et al. 2020; Zhang et al. 2021). DNA cloning allows one to multiply and keep the transcripts of low abundance genes, facilitating the transfer of the DNA material from one laboratory to another when necessary.

Identifying a recombinant clone among countless bacterial colonies on the culture medium is a critical step in DNA cloning. Blue-white selection is a common screening strategy used to identify recombinant clones. It makes use of the *lacZ* gene present in a cloning vector. The product of the *lacZ* gene, namely β-galactosidase, cleaves X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) and eventually results in the formation of a blue pigment called 5,5′-dibromo-4,4′-dichloro-indigo which gives rise to blue colonies (Julin 2018). The incorporation of a DNA insert inactivates the *lacZ* gene and renders the recombinant clones to appear as white colonies. Although the strategy sounds faultless, it is common to obtain false positive and false negative results when applying blue-white selection (Padmanabhan et al. 2011; Zhang 2016). For instance, the formation of

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light blue colonies on a culture medium is common. Besides, this strategy also requires the supplementation of IPTG and X-gal in culture media alongside the antibiotic (Green and Sambrook 2019).

Positive selection is a popular strategy used in the selection of recombinant clones. This strategy makes use of a cloning vector that carries a lethal gene (or suicide gene) encoding a toxic or harmful product. One of the popular lethal genes used in the construction of positive selection vector is the *ccdB* gene (Ramos et al. 2017). As the cloning site is located within the lethal gene, incorporation of an insert alters the reading frame of the lethal gene and disrupts the production of the toxic protein in bacterial cells carrying the recombinant DNA molecule. On the other hand, uptake of the non-recombinant plasmid results in the production of a toxic protein that inhibits the growth or kills the host cells upon plating (Reece-Hoyes and Walhout 2018). Hence, only bacterial cells carrying the recombinant plasmid will form colonies on a culture medium, and the recombinant clones can be obtained effortlessly. The positive selection strategy offers several advantages, including extremely high cloning efficiency (99–100%), reduced time for identifying recombinant clones and exclusion of the costly IPTG and X-gal required for blue-white selection. Owing to the advantages of the positive selection strategy, many positive selection vectors have been developed (Choi et al. 2002; Motohashi 2019). Nowadays, some of the positive selection cloning vectors are commercially available, for instance, the *pEASY*®-Blunt Zero cloning vector (TransGen, China) and pJET1.2/blunt cloning vector (Thermo Scientific, USA).

The pJET1.2/blunt cloning vector is a component of the CloneJET PCR Cloning Kit (Catalogue number: K1231), and it is not sold individually. It contains an *eco47IR* gene that encodes for a restriction enzyme *Eco*47I that is harmful to the common laboratory *E. coli* strains used in molecular cloning. Therefore, the plasmid propagation requires a special host cell resistant to the toxic product, i.e., the *E. coli* strain RFL47, which produces *Eco*47I in nature (Janulaitis et al. 1983). However, the *E. coli* strain RFL47 is not widely available, rendering the propagation of the pJET1.2/blunt vector in common laboratories infeasible. As a result, users are forced to endlessly purchase the cloning kit from the manufacturer. In the present study, we developed a strategy to allow the propagation and in-house production of the pJET1.2/blunt cloning vector using the common laboratory *E. coli* strains such as DH5α and JM109. This study aims to produce the pJET1.2/blunt cloning vector for routine cloning activities at minimal cost.

In the strategy employed in this study, a short DNA fragment was inserted into the commercial pJET1.2/blunt cloning plasmid, generating a recombinant plasmid (an intermediate) termed pJET1.2M plasmid. The insertion disrupts the *eco47IR* gene and allows the propagation of pJET1.2M plasmid in common laboratory *E. coli* strains. Subsequently, the inserted DNA fragment was removed from the pJET1.2M plasmid to produce the pJET1.2/blunt vector ready for DNA cloning. As the commercial pJET1.2/blunt vector is prepared by restriction digestion of the pJET1.2 plasmid at the *Eco*RV site (5'-GATATC-3'), retention of the *Eco*RV site in the pJET1.2M plasmid would facilitate the production of the pJET1.2/blunt vector at a later stage. This is possible when the inserted DNA fragment contains the "GAT" and "ATC" sequences at the 3' and 5' ends, respectively. Upon ligation of this DNA fragment and the pJET1.2/blunt cloning vector, two *Eco*RV sites will be produced in the pJET1.2M plasmid (Fig. 1). The pJET1.2/blunt vector could easily be produced from the pJET1.2M plasmid by removing the insert through *Eco*RV digestion.

To prove the idea, a pair of oligonucleotides, namely MCS-F (5′-ATC GAA TTC ATA TGG TAC CGA T-3′) & MCS-R (5′-ATC GGT ACC ATA TGA ATT CGA T-3′) were synthesized. The oligonucleotides were designed to have a sequence complementary to each other, to allow the formation of a double-stranded DNA fragment when hybridized together. The lyophilized oligonucleotides were resuspended in an annealing buffer (10 mM Tris–HCL, pH 7.5, 50 mM NaCl, 1 mM EDTA) to a concentration of 100 µM. Then, 20 µL of each oligonucleotide was mixed in a 0.2 mL PCR tube and incubated in a thermocycler to produce a 22 bp double-stranded DNA fragment, designated as MCS fragment. The thermal cycling profile used for the annealing procedure was as follows: 3 min at 95 °C for 1 cycle, followed by 1 min at 95 °C (− 1 °C each cycle) for 70 cycles. The concentration of the MCS fragment was determined using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific, USA). Subsequently, 2.2 ng of the MCS fragment (1:6 molar ratio of vector:insert) was cloned into the commercial pJET1.2/blunt cloning vector (50 ng) according to the manufacturer's instructions. The ligation product was transformed into *E. coli* strain DH5α competent cells by the heat-shock method. The transformed bacterial cells were spread on LB agar supplemented with 100 µg/mL ampicillin and cultured overnight at 37 °C. A single colony was selected for plasmid preparation and sequencing.

As anticipated, the pJET1.2M plasmid produced contains an MCS fragment. The incorporation of the MCS fragment resulted in the formation of two *Eco*RV sites along with three additional unique restriction sites on the pJET1.2M plasmid (Fig. 1). Although the MCS fragment could be cloned in two different directions, the resulting recombinant plasmids only differ in the positions of the *Nde*I and *Kpn*I sites. Either one could be used in our study as the difference does not matter to the subsequent steps. Hypothetically, digestion of the pJET1.2M plasmid using *Eco*RV will produce two DNA fragments, i.e., the MCS fragment and the pJET1.2/blunt cloning vector. The resulting pJET1.2/blunt

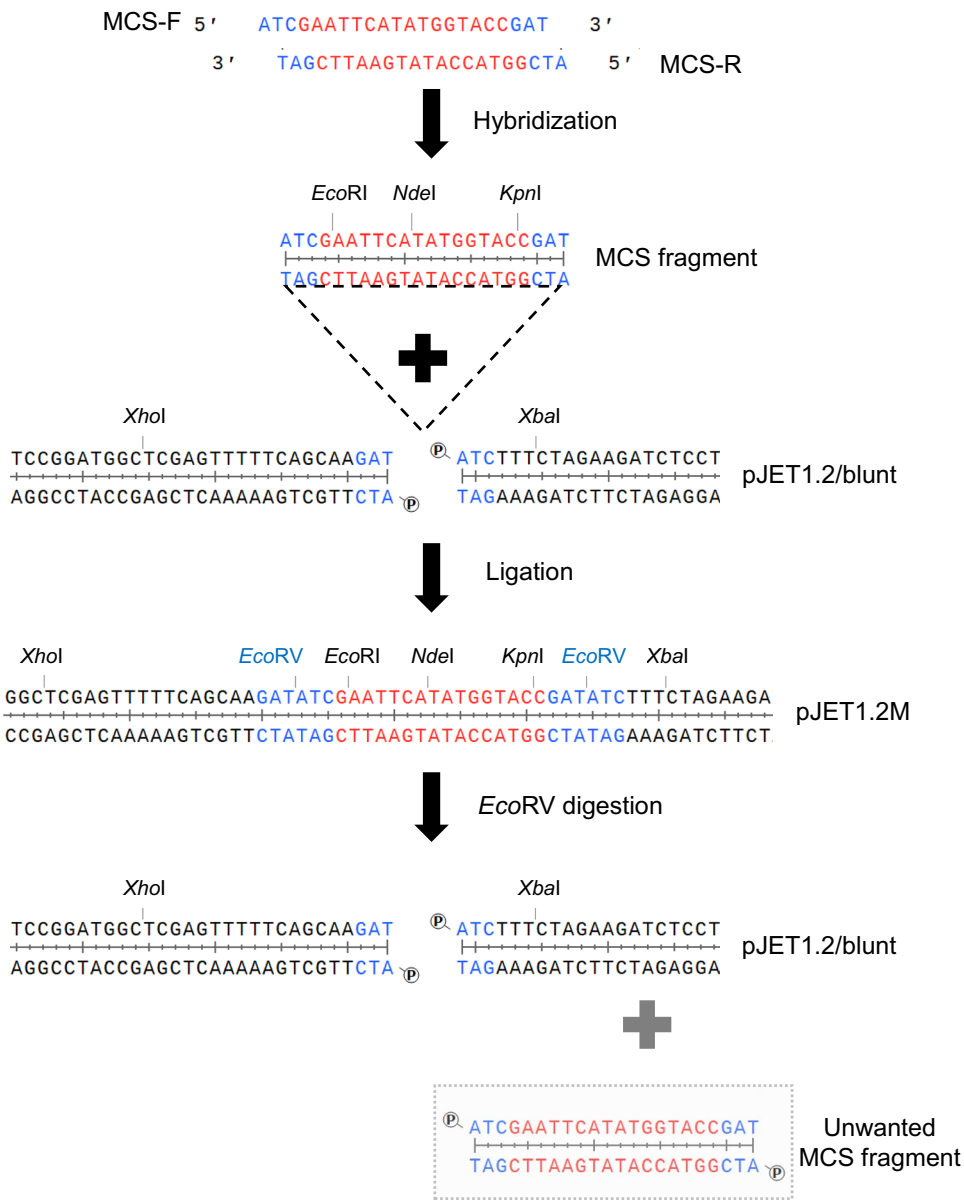

Fig. 1 Schematic representation of the strategy for in-house production of pJET1.2/blunt cloning vector

cloning vector can be purified from the digestion product and used as a cloning vector for other cloning works. To show the concept is feasible, 3 µg of pJET1.2M plasmid was digested overnight with 20 units of *Eco*RV (Promega, USA) and cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Since the MCS fragment is just 22 bp in length and most of the DNA and PCR clean-up kits in the market claim that they are only efficient in recovering DNA fragments of 100 bp to 10 kb, we expected that extraction of the target DNA fragment from agarose gel is not required.

To verify if the MCS fragment has been eliminated in the clean-up step, 50 ng of the purified DNA was used to set up a ligation reaction without an insert DNA, and the ligation product was transformed into *E. coli* strain DH5α competent cells. No colony was expected to form if the clean-up system had successfully eliminated the MCS fragment. Surprisingly, the transformed cells yielded 14, 20 and 15 colonies on three LB agar plates (Online Resource 1), indicating that some of the MCS fragments were recovered by the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and the MCS fragments carried over were re-ligated into the pJET1.2/blunt cloning vector. Hence, agarose gel electrophoresis was performed to separate the *Eco*RV-digested pJET1.2M plasmid and the DNA fragment corresponding to the pJET1.2/blunt cloning vector was recovered from gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). To evaluate the purity of the gel-purified pJET1.2/blunt cloning vector, a ligation reaction was set up in the absence of an insert DNA. Under a condition identical

to the condition used in the previous ligation step, only a colony was observed on one of the LB agar plates used, and no colony was found on the other two LB agar plates (Online Resource 1). The result indicates that the MCS fragments have been successfully separated from the pJET1.2/blunt cloning vector. Most likely, the colony that grew on the plate harbours a pJET1.2M plasmid resulting from the circularization of a partially digested plasmid.

To assess the cloning efficiency of the self-made pJET1.2/blunt cloning vector, the gel-purified pJET1.2/blunt cloning vector was used to clone a 538 bp fragment of the *EgCAD2* promoter. After transformation, large quantities of bacterial colonies were observed on the LB agar plate (Online Resource 2). Out of the 30 colonies analysed by colony PCR, all contained an insert with the target size (Fig. 2a), yielding a cloning efficiency of 100%. The result evidenced that the strategy designed for in-house production of the pJET1.2/blunt cloning vector is feasible. Next, the cloning efficiency of the self-made pJET1.2/blunt cloning vector was further evaluated using another two larger DNA fragments, i.e., the promoter fragment of *EgPAL1* gene (≈2.3 kb) (Yusuf et al. 2018a) and the open reading frame (ORF) of *Eg4CL1* gene (≈1.6 kb) (Yusuf et al. 2018b). A considerable number of bacterial colonies were produced on the culture plates after overnight incubation (Online Resource 2). Colony PCR results revealed that all the analysed colonies harboured the desired insert (Fig. 2b). These results indicate that the self-made pJET1.2/blunt cloning vector is efficient for cloning of DNA fragments of varying sizes. In addition, the self-made pJET1.2/blunt cloning vector is comparable with the commercial product in terms of its cloning efficiency. Due to its high cloning efficiency and effective selection strategy, the self-made vector has been used for DNA cloning in our laboratory routinely. Recently, we have used our self-made pJET1.2/blunt cloning vector to clone the cDNAs and the promoter fragments of *EgCAD1* and *EgCAD2* genes from oil palm (Yusuf et al. 2022).

Preparing the pJET1.2/blunt cloning vector from the pJET1.2M plasmid is straightforward and technically easy. When a DNA clean-up kit that is efficient in the elimination of small DNA fragments is used, separation of the digestion product on an agarose gel will be no longer required. This would further simplify the protocol and shorten the time required for the in-house production of the pJET1.2/blunt cloning vector.

Conclusion

The present study enables the in-house production of a positive selection vector at low cost. The pJET1.2M plasmid developed in this study can be propagated in common *E. coli* strains anytime needed. The pJET1.2/blunt cloning vector derived from the pJET1.2M plasmid is high quality and it allows the cloning of a blunt-ended DNA fragment at high efficiency. Furthermore, phosphorylation of the DNA fragments prior to ligation is not a prerequisite. Using this positive selection vector would hasten the process of recombinant clone screening and reduce the costs incurred in molecular cloning.

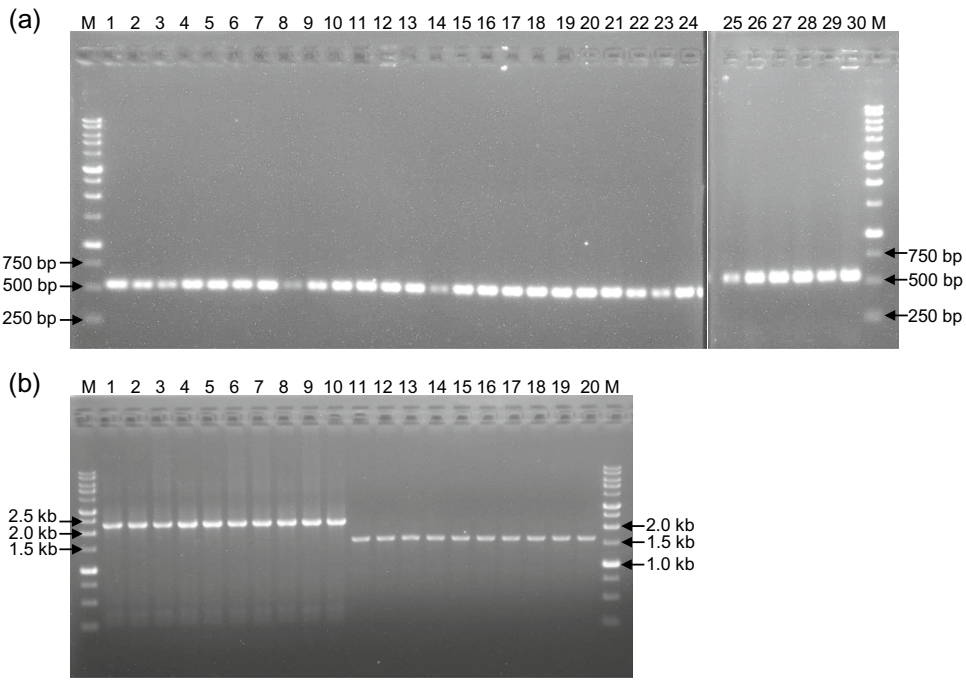

Fig. 2 Agarose gel electrophoresis of colony PCR products. **a** PCR products amplified from the recombinant clones harbouring a 538 bp promoter fragment of *EgCAD2* **b** PCR products corresponding to the promoter fragment of *EgPAL1* (≈2.3 kb, lanes 1–10) and the ORF of *Eg4CL1* (≈1.6 kb, lanes 11–20). The number above each lane represents the individual bacterial colony analysed. M, 1 kb DNA ladder (1st BASE, Malaysia). Refer to Online Resource 3 for the details of colony PCR

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Author contributions MPA and CYLY conceived and designed the research. ON performed the study. CYLY and MPA prepared the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Data availability Online Resource 1-3 are provided as electronic supplementary material.

Ethical approval This study does not involve any animal or human participants.

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