International Pharmacy Acta. Volum2019;2(1):e6
Original Research Article
OPEN ACCESS
ISSN: 2645-3266

Use of Green Fluorescent Protein (GFP) Vector in Classical Restriction Enzyme-based Cloning Methods of Gateway Cloning System

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Article Info:
Received: May 2019
Accepted: July 2019
Published online:
August 2019

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ABSTRACT:

Introduction: Generation of an expression clone that can produce the pharmaceutical proteins in an efficient and soluble form at high levels is considered as an important step in pharmaceutical industry. Recombination-based cloning could be a quick and efficient way for generating expression vectors. Thus, both efficient and robust subcloning is vital for the construction of gene expression vectors. In this study, we used the traditional restriction enzyme-based cloning methods for generation of expression-ready clones by the most well-known commercial cloning technologies, Gateway.

Keywords: GFP; Gateway cloning technology; pEGFP-N1; pTracer-SV40; plasmid; PCR

Please Cite this article as: Naddafi F, Tabarzad M, H. Shirazi F. Use of Green Fluorescent Protein (GFP) Vector in Classical Restriction Enzyme-based Cloning Methods of Gateway Cloning System. Int. Pharm. Acta. 2019;2(1):e6
DOI: https://doi.org/10.22037/ipa.v2i1.22719

1. Introduction

Gateway cloning, the site-specific recombination system, is based on the robustly specific integration and excision reactions of bacteriophage λ into and out of the E. coli chromosome [1]. The Gateway cloning technology consists of two LR and BP recombination reactions. The BP reaction used to create an Entry clone by transferring DNA elements of interest in the Expression clone or the attB-flanked PCR product into a Donor vector. The LR reaction used to generate an Expression clone by recombination between attL and attR sites contained in the Entry vector (which is contained the gene for kanamycin resistance) and the Destination vector (which is contained all the necessary sequences for expression), respectively (Figure 1) [2]. It has been indicated that the Gateway system can provide an advance over classical restriction enzyme cloning by increasing the efficiency and reliability [3]. The Gateway cloning system is considered as a novel trend in molecular biology that eliminates the need for traditional cloning methods for transferring genes between vectors [4, 5]. In one study, adenovirus vectors containing the lumican gene had been constructed by Gateway cloning technology [6]. In another study, RNAi vectors, for SmNAC1 transcription factors of Salvia miltiorrhiza, had been constructed by Gateway technology. It has been proved that this technology could provide a rapid and highly efficient cloning method [7]. We created a PCR product, attB1- pSV40 - GFP- BGH polyA- attB2, to easily be cloned into pDONR™ 221 Gateway vector in a single recombination reaction.

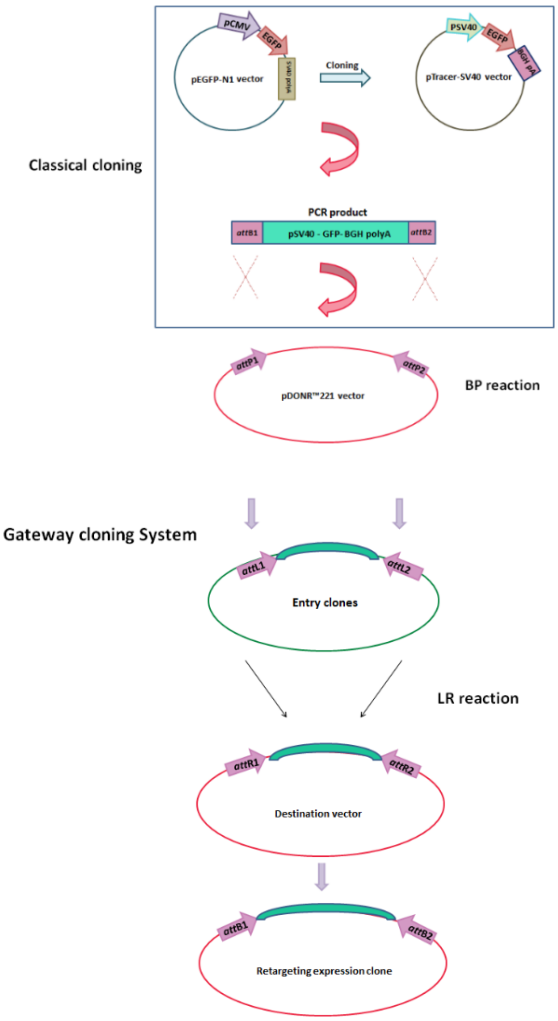

Figure 1. Schematic model of Gateway cloning system as well as the role of the classical restriction enzyme-based cloning methods in Gateway cloning system.

2. Materials & Methods

Strains, plasmids and culture media

The expression cassette for GFP was prepared in pTracer-SV40 plasmid (Thermo Fisher Scientific). The sequence was sub-cloned from the pEGFP-N1 plasmid (Clonetech), into the pTracer-SV40 plasmid by PstI / NotI restriction enzymes. E. coli strain Top10 F' (Novagen) was used as the host to propagate plasmids. E. coli was cultured in LB agar and Broth (Bio Basic). Zeocin selection reagent was purchased from Thermo Fisher Scientific. PstI, NotI, and BpiI restriction enzymes were obtained from Fermentas.

GFP cloning in the pTracer-SV40 expression plasmid

The GFP gene was cloned into the expression plasmid pTracer-SV40 with PstI and NotI restriction enzymes, respectively. The pEGFP-N1 plasmid and the pTracer-SV40 plasmid were double-digested with the PstI and NotI restriction endonucleases for 16h at 37 °C. Then digested fragments were electrophoresed on the 1 % agarose gel stained with ethidium bromide. Subsequently, the fragments were purified using the NucleoSpin® Gel and PCR Clean-up kit (MN) according to manufacturer's instructions. Ligation was performed by using T4 DNA ligase enzyme (Fermentas). 100 ng of purified double-digested of cloned pTracer-SV40 and 3 - 5 fold molar excess of insert (GFP) were incubated with T4 DNA ligase enzyme and 10X T4 DNA ligase buffer at 22 °C for 4 hr. The recombinant plasmid was transformed into the competent Top10 F' by standard calcium chloride method. Transformants were selected on low salt LB-agar medium containing Zeocin (25-50 µg /mL). A single colony of E. coli cells carrying the ligated plasmid was grown in 5 ml low salt LB medium containing Zeocin (25-50 µg /mL). Plasmid extraction was performed using the FavorPrep Plasmid Extraction Mini Kit (FAPDE050- Favorgen Biotec Corp). Subsequently clones containing ligated plasmid were screened with BpiI restriction enzyme .

Amplification of attB1- pSV40 - GFP- BGH polyA- attB2 gene with PCR

attB1- pSV40 - GFP- BGH polyA- attB2 gene was amplified by PCR using the following primers: attB1- pSV40 - GFP- BGH polyA - Fwd 5′- GGGACAAGTTTGTACAAAAAAGCAGGCT CGGCTGTGGAATGTG- 3′ and attB2- pSV40 - GFP- BGH polyA -Rev 5′ GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA AGAGCCCACCGCATC- 3′. The underlined bases show attB1 and attB2 sites, respectively and the PCR products were flanked by these sites. The PCR mixture consisted of 5 µL of 10 × PCR buffer, 2.5 mM MgCl2, 0.2 mM for each dNTP, 250 nM for each primer, 1 µL of template DNA, and 5 units of Pfu DNA polymerase (Fermentas) in the final volume of 50 µL. The amplification consisted of 35 cycles on a thermocycler (Eppendorf) as follows: preliminary denaturation for 5 min at 95 °C followed by

25 cycles including denaturation for 1 min at 95 °C, annealing for 1 min at 53 °C and extension for 1 min at 72 °C, and final extension for 10 min at 72 °C. PCR product fragment was electrophoresed on the 1% agarose gel and stained with ethidium bromide. After the PCR process, the amplified DNA fragments are size-separated by agarose gel electrophoresis and purified using the NucleoSpin® Gel and PCR Clean-up kit (MN). Then, the DNA fragment should be combined with a pDONR™ 221, attP1 and attP2 sequences containing vector, with BP Clonase II enzyme supplied by Jump-In™ TI™ Gateway® Vector Kit (A10896, Life Technologies). Moreover, pDONR™ 221 could not be used to generate multi-fragment entry clones.

3. Results

In this study, we used classical cloning system to generate a suitable PCR product for Gateway® BP reaction with a donor vector (flanked by attP1 and attP2 sites). Thus, the PCR product should be flanked by attB1 and attB2 sites.Generation of the attB1- pSV40 - GFP- BGH polyA- attB2 PCR product.

Due to lack of promoter and polyadenylation sequences in Gateway cloning vectors, we designed the reporter gene, GFP, with SV40 promoter and BGH polyadenylation sequences. The GFP gene was sub-cloned from the pEGFP-N1 vector, into the pTracer-SV40 plasmid with PstI and NotI restriction enzymes (Figure 2, 3). In electrophoresis of cloned pTracer-SV40 plasmid, two different bonds could be detected (2100 and 2700 bp). pSV40 - GFP- BGH polyA gene was amplified by attB1- pSV40 - GFP- BGH polyA - Fwd and attB2- pSV40 - GFP- BGH polyA -Rev primers by addition of attB1 and attB2 sites. Successful amplification of 1300 bp of attB1- pSV40 - GFP- BGH polyA- attB2 gene was visualized on 1% agarose (Figure 4). The suitable PCR product was generated to be cloned into the pDONR™ 221 plasmid.

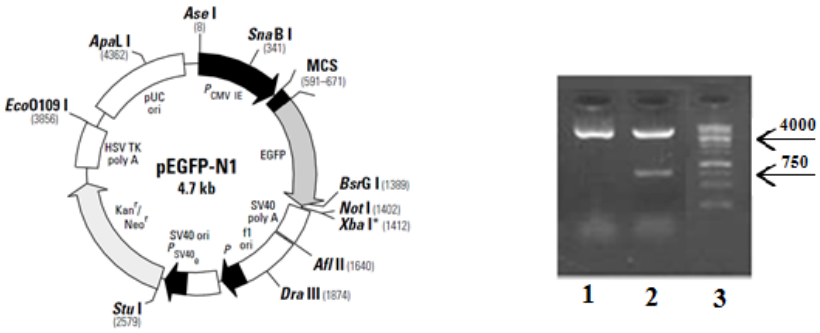

Figure 2. Agarose gel electrophoresis of digested pEGFP-N1 with PstI and NotI restriction enzymes. Lane 1 corresponds to undigested pEGFP-N1; Lanes 2 corresponds to digested pEGFP-N1 vector (700 bp GFP); Lane 3 shows 1kbp DNA Ladder (Fermentas).

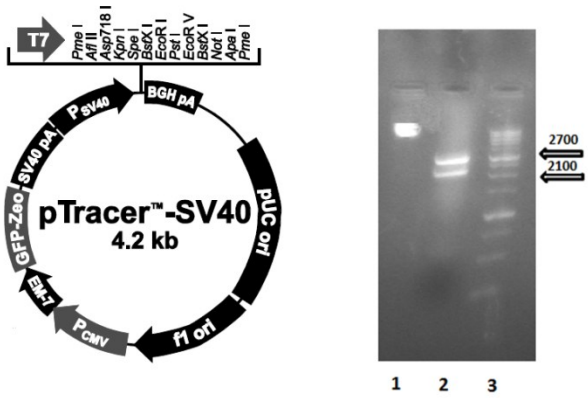

Figure 3. Agarose gel electrophoresis of digested pTracer-SV40 with BpiI restriction enzyme. Lane 1 corresponds to undigested the pTracer-SV40; Lanes 2 corresponds to digested pTracer-SV40 vector (2100 and 2700 bp); Lane 3 shows 1kbp DNA Ladder (Fermentas).

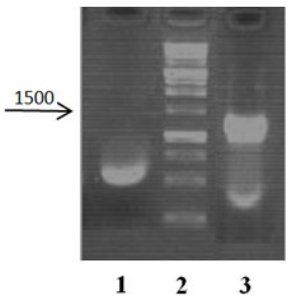

Figure 4. Agarose gel electrophoresis of amplified *att*B1- pSV40 - GFP- BGH polyA- *att*B2 (pTracer-SV40 plasmid). Lane 1 shows negative control; Lane 2 shows 1kbp DNA Ladder (Fermentas); Lane 3 Shows amplified *att*B1- pSV40 - GFP- BGH polyA- *att*B2 gene.

4. Discussion and Conclusion

Traditional cloning requires the use of restriction enzymes, T4 DNA ligase, or alkaline phosphatase that could be expensive and need long-term optimization, while Gateway cloning system represents a fast, efficient, and robust cloning method for the generation of an over-expressed vector. The Gateway cloning system uses site-specific recombination to transfer DNA elements between vector backbones [8]. In one study, a construct (pDONR-R4-R3) was generated that can convert any available Gateway destination vector to a MultiSite Gateway vector in a single recombination reaction [9]. In this studies, the reporter gene (GFP) was first cloned into Multiple Cloning Site (MCS) of pTracer-SV40 plasmid. Promoter sequences (pSV40) were located directly upstream of cloned GFP and BGH polyA was located downstream of the reporter gene as polyadenylation site. Since Gateway cloning vectors do not have promoter and cannot be expressed without necessary sequences, and pTracer-SV40 plasmid contains GFP-Zeo sequence, we had to construct a standard Gateway cassette (attB1- pSV40 - GFP- BGH polyA- attB2) by PCR. In conclusion, the creation of DNA constructs could be a rate-limiting step in biological experiments. Recombination cloning of single DNA segments by using the Gateway cloning system can provide an advance over classical restriction enzyme cloning due to its high efficiency and reliability.

Conflict of interest

There is no conflict of interest.

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