An efficient method for integration of PCR fragments into adjacent or overlapping restriction sites during gene cloning

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Received: 7 November 2017 / Accepted: 13 March 2018 / Published online: 24 March 2018
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Abstract

In the present work, a simple and straightforward method was developed to clone any PCR-amplified products into restriction sites that are very close, adjacent or overlapping in the expression vector. The novelty of the methodology involves a crucial primer-designing step by adding appropriate overhangs to the 5' ends of primers based on the multiple cloning sites (MCS) (polylinker) region of expression vector. After PCR amplification, actual cloning is performed not in adjacent RE sites, but in sites that are little distant in the MCS. However, the sites lost during this cloning step are maintained intact since they are provided by the cloned PCR product (through the primer overhangs). Gene for green fluorescent protein (GFP) was cloned and expressed employing this strategy to demonstrate its simplicity. This method is highly useful for vector modification without losing the restriction sites present in the MCS.

Keywords Cloning · Restriction · Ligation · Multiple cloning sites · Green fluorescent protein

Abbreviations

RE Restriction enzyme
MCS Multiple cloning sites
GFP Green fluorescent protein
Pfu *Pyrococcus furiosus*

Introduction

Recombinant proteins are commonly produced in a heterologous host system for structural and functional studies. Before producing a recombinant protein, a necessary cloning of gene of interest in correct orientation into an expression vector is essential (Bellini et al. 2011). Although a number of cloning methodologies have been developed for creating recombinant vectors, directional cloning by restriction and ligation method is the most popular strategy owing to its low cost and unlimited choice of expression vectors available (Hartley 2006). However, there are minor drawbacks associated with this method when the desired sites are close, adjacent or overlapping in the expression vector or when the

desired restriction enzyme (RE) sites may be present in the DNA or gene to be cloned. In the first instance, when the two restriction sites are present adjacent or overlapping to each other in the expression vector, double digestion is highly problematic. This results in partial or incomplete double digestion of vector molecules, which recircularizes leading to high background colonies.

There are instances we encounter at times where a cloning event is needed to be performed in the close or adjacent restriction sites, although a choice of other RE sites are available in the vector. These cases are often encountered by molecular biologists working on creating custom-designed vectors (include protein tags for purification, quantification) by various DNA alterations into the existing vectors. In such cases, they may have to alter the vector by incorporating new DNA sequences into adjacent RE sites such that the remaining restriction sites are retained for other purposes. Various methodologies have been proposed for the successful cloning of DNA fragments into close or adjacent restriction sites such as creating an intermediate plasmid in which the adjacent RE sites are separated by cloning a positively selectable stuffer DNA with resistance to neomycin into one of the two restriction sites (Loukianov et al. 1997), treating simultaneously both insert and linearized vector with T4 DNA polymerase or exonuclease III enzymes leading to long cohesive ends (Li and Evans 1997), enzyme-free cloning method of creating complimentary staggered overhangs on

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both insert and vector using tailed PCR primer sets (Tillett and Neilan 1999) or by introducing homologous sites in the DNA which are complementary to the sites where the integration is desired based on adaptation of QuickChange Site-Directed Mutagenesis protocol (Geiser et al. 2001) or overlap extension cloning (Bryksin and Matsumura 2010). All of the above methods involve complex steps of either creating intermediate selectable plasmids or performing PCRs for amplifying lengthy plasmid and insert employing robust DNA polymerases followed by enzymatic treatments.

In the present work, our goal is to develop and demonstrate a simple, universal and straightforward method for directional cloning of any DNA fragment into adjacent or overlapping restriction sites of vector employing classical restriction and ligation strategy. We demonstrated the above strategy by cloning the DNA encoding for green fluorescent protein (GFP) into adjacent restriction sites of the pET22b vector and studying its functional properties. The GFP target was chosen because when fused to the C terminus of protein targets serves as a sensitive reporter that has enormously facilitated in gene expression studies and it is also useful as the fluorescent tag in experiment assays such as immunofluorescence and fluorescence microscopy. The methodology applied in this study can also be utilized in instances when the desired RE sequences of vector where the gene needs to be cloned are present within the gene.

Materials and methods

Materials

Dehydrated media and antibiotics were purchased from HiMedia labs, Mumbai, India. Deoxyribonucleotide triphosphates (dNTPs), *Pfu* DNA polymerase, FastDigest restriction enzymes and IPTG were purchased from Thermo Scientific (Fermentas), India. pET22b vector and *E. coli* BL21DE3 were from Novagen, USA. The pRSET-EmGFP vector and *E. coli* DH5 α host strain were from Invitrogen, USA. Primers were synthesized at primer synthesis facility at Sigma-Aldrich Ltd. Bangalore, India. Sequencing of cloned product was carried out at Xcelris genomics facility, Ahmedabad, India.

Primer designing

The strategy developed in this study was evaluated by cloning DNA sequence coding for the GFP of *Aequorea victoria* after amplifying by PCR using primers listed in Table 1 from the pRSET-EmGFP vector and is introduced into *NotI* and *XhoI* restriction sites which appear at the end of the multiple cloning sites (MCS) of pET22b vector and are adjacent to each other (Fig. 1A). The forward primer

Table 1 Primers used in the present study for cloning of the *gfp* gene into the adjacent RE sites

Primer	Sequence (5'–3')
GFP-For	CCGGAATTCGAGCTCCGTCGACAAGCT TGCGGCCGCAAGCAAGGGCGAGGAGC ^a
GFP-Rev	CCGCTCGAGCTTGTACAGCTCGTCCATGC ^b

^aBold and underlined letters stand for *EcoR* I (5' side- GAATTC) and *Not* I (3' side-GCGGCCG) restriction recognition sites

^bBold and underlined letters stand for the *Xho* I restriction site

(GFP-F) is introduced with a series of restriction sites (*EcoR* I, *SacI*, *SalI*, *HindIII*, *Not* I) (Fig. 1B) at the 5' end in the same order and reading frame as that of the pET22b (Novagen, USA) vector starting with the site in which the insert is actually cloned (*EcoR* I) and ending with the site (*Not* I) that is exactly adjacent to the restriction site provided by the reverse primer (*Xho* I). Thus, using this strategy any DNA insert can be cloned into adjacent restriction sites without altering MCS, but the actual cloning takes place in the restriction sites that are distant enough in the vector for efficient digestion to occur. Methodology employed for introducing *gfp* gene and the primer design strategy is represented diagrammatically in Fig. 1.

PCR amplification and cloning

PCR was performed using the GFP-F and GFP-R primers using the pRSET-EmGFP plasmid as template on Eppendorf Mastercycler pro gradient thermal cycler with 1 $\times$ *Pfu* buffer, 1.5 mM MgSO₄, 200 μ M of dNTPs, 5 pmol each of forward and reverse primers, 1.0 ng of DNA template and 1 U of *Pfu* DNA polymerase. The final volume was made up to 20 μ l with nuclease-free water. The following PCR conditions were used: 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension of 2 min at 72 °C. An initial denaturation of 4 min at 94 °C and a final extension of 72 °C for 8 min were also observed during PCR. The amplified PCR product and pET22b vector were digested with *EcoR* I and *Xho* I FastDigest restriction enzymes. Digested vector (50 ng) and *gfp* insert (25 ng) were ligated using T4 DNA ligase and transformed into chemically competent *E. coli* DH5 α host cells. Transformants were screened by colony PCR using T7 promoter and terminator primers with proper controls. One representative positive colony was sequenced at Xcelris genomics Pvt. Ltd., Ahmedabad, India.

Characterization of pET22-GFP plasmid

Recombinant clones were induced and characterized using methods such as SDS-PAGE, Western blotting using anti-histidine antibodies described elsewhere (Reddy et al. 2012). Additionally, periplasmic extracts were recovered from

Fig. 1 Schematic representation of the strategy used for cloning PCR products into adjacent restriction enzyme sites. **A** Map of pET22b vector with *peB* signal sequence, polylinker region and $\times 6$ His tag. **B** Diagrammatic representation of the forward and reverse primers; the *Xho* I site was added as overhang to the GFP-R primer and *Not* I was added as overhang to the GFP-F primer along with four other restriction sites (*Eco*R I, *Sac* I, *Sal* I, and *Hind* III). **C** PCR was carried out with the GFP-F and GFP-R primers. **D** The restriction sites

now present at the termini of the PCR product are *Eco*R I and *Xho* I which are quite distant in the pET22b vector and thus can be double digested easily and cloned, but the cloning has actually taken place in *Not* I and *Xho* I sites which are adjacent in vector. **E** Representation of special overhang primer used in the study where the RE site in which cloning is desired (*Not* I) is placed close to the primer, followed by other RE sites (*Eco*R I, *Sac* I, *Sal* I and *Hind* III) in the same order as that of the vector

induced and uninduced clones by osmotic shock and checked for fluorescence by observing under UV light as well as with multimode microplate reader (Tecan, Austria).

Results

Cloning and plasmid sequencing

DNA fragment coding for GFP protein was amplified after performing PCR with primers mentioned in Table 1 by using the pRSET-EmGFP plasmid as template. pET22b vector and PCR product were subjected to restriction and ligation followed by transformation. The transformation efficiency was $\sim 4.1 \times 10^3$ CFU/ μ g of ligated recombinant plasmid. Transformed colonies were screened by colony PCR using T7 sequencing primers to check the integration of DNA fragment (Fig. 2a). A single clone was sent for sequencing, the result was analyzed using Gene Runner software ver 3.05 (Hastings Inc, USA) and observed results were

exactly coinciding with expected result such that the *gfp* gene was cloned into *Eco*R I and *Xho* I sites and the rest of the polylinker region (*Sac* I, *Sal* I, *Hind* III, *Not* I) lost during double digestion was revived by the forward primer and was cloned in correct order and open reading frame.

Expression studies of GFP protein in *E. coli*

Clones harboring pET22b-*gfp* gene were induced using IPTG along with proper controls at 37 °C. The induced and uninduced clones were analyzed by SDS-PAGE and Western blotting. Recombinant GFP was found to be expressing at ~ 28 kDa after staining of SDS-PAGE gel with Coomassie brilliant blue R250 (Fig. 2b) and on Western blots probed with anti-histidine antibodies.

Functional characterization of pET22-*gfp* plasmid

The functional properties of *gfp* gene cloned in pET22b-*gfp* vector was characterized by inducing GFP expression in

Fig. 2 Structural and functional characterization of the pET22b-GFP vector **a** Colony PCR of clones transformed with the pET22b vector (control 309 bp) and pET22b-GFP vector (1020 bp) using T7 primers. Lane M, 1 kb ladder; lanes 1–3, control; lanes 1–3, GFP-positive clones. **b** SDS-PAGE analysis of induced and uninduced clones har-

boring the pET22b-GFP vector for expression of recombinant GFP (r-GFP) protein at the 30 kDa region. **c** Graph showing the fluorescence readings of periplasmic extracts of induced and uninduced clones. **d** Tube showing green fluorescence along with negative control when exposed to long UV radiations

recombinant clones with IPTG and studied for fluorescence properties. Periplasmic extracts were prepared from induced and uninduced clones. Only extracts from induced clone was positive for fluorescence after excitation at 487 nm and recording the emission at 509 nm and also by visual inspection by exposing to UV light on UV transilluminator (Fig. 2c, d).

Discussion

Despite the development of numerous alternative cloning methodologies (Van Den Ent and Löwe 2006; Mathieu et al. 2014; Wang et al. 2014), classical restriction digestion and ligation mediated cloning is used more frequently (Zhang et al. 2017; Reddy et al. 2013; Babu et al. 2017; Idrees et al. 2016). In spite of its popularity, this method has certain setbacks when the desired restriction sites for directional cloning are very close to each other in the expression vector or when the desired sites are present in the DNA to be cloned. This problem has been the subject of discussion in many online molecular biology forums (<http://www.protocol-onlin>

[e.org/biology-forums/posts/38291.html](http://www.protocol-onlin.org/biology-forums/posts/38291.html), https://www.researchgate.net/post/Can_I_do_a_double_digest_when_my_restriction_sites_are_next_to_each_other, https://www.researchgate.net/post/Why_2_enzymes_having_very_close_restriction_sites_are_not_recommended_for_double_digestion_of_plasmid). Owing to these drawbacks and the limitations associated with the existing techniques, we developed a simple and universal strategy for cloning of DNA fragments into close or adjacent RE sites present anywhere in the polylinker region of any expression vector.

In vitro amplification of DNA fragments by polymerase chain reaction (PCR) is a routine technique and it has facilitated the incorporation of additional, non-complementary but experimentally necessary DNA sequences into the 5' ends of PCR primers. Generally, most of the expression vectors are designed with minimum of four or more RE sites in its polylinker region. Taking this fact as advantage in the present method, the actual cloning is not carried out between adjacent sites within the MCS but between non-adjacent sites, which permits efficient double cleavage of the vector DNA. In order to maintain the desired cloning junction sequence, we incorporated within one of the PCR

primers (used to prepare the insert DNA) the sequence that is removed from the vector during the double digestion by the non-adjacent enzymes. Although theoretically cloning was carried out in RE sites that are farther in the vector, the actual cloning occurs at the adjacent sites because the PCR product provides the remaining restriction sites lost during the double digestion step. In our work, we have used *gfp* gene as an example to demonstrate the above strategy because cloned gene products can be easily characterized by tagging with the GFP protein. A similar strategy of primer design can be applied when the desired RE sites are present in the gene of interest by cloning into a different set of RE sites, but the RE sites lost are restored by the RE sites provided by the primer overhangs. In the work presented here, GFP was cloned into sites that are present at the end of the polylinker region and hence forward primer is designed differentially with stretch of restriction sites. However, if the PCR product is wished to be cloned into sites at the beginning of polylinker region then the reverse primer has to be designed differentially employing the same principal. Plasmid sequencing showed that all the lost restriction enzyme sites were recovered after the cloning step which was provided by the forward primer. The cloned *gfp* gene expressed at high levels and the protein localized in the periplasmic extracts for easy downstream purification and fluorescence applications. Although the presented technique is conceptually a simple procedure, it is by far the most unique, unattempted and uncomplicated method. Due to the above reason, the presented technique can be a convenient alternative strategy to enzyme-free cloning and overlap extension methods for cloning of PCR amplified products into close restriction sites. This strategy can be applied during design and construction of new vectors such that additional DNA coding for various features can be added without losing the original restriction sites.

Despite the unique advantages of this method there are some limitations with the presented method. The first limitation is that the approach is feasible only when nearby alternative restriction sites are available close to (so that this sequence can be incorporated into the primer) one of the two intended RE sites where cloning is desired. The second limitation is this method requires the synthesis of a larger than normal primer with substantial superfluous sequence on its 5' end. These design parameters also result in some discrepancy in the melting temperature for the amplification primers, which sometimes may complicate the PCR amplification of the target sequence.

Acknowledgements The authors thank the administration and management of Vignan's Foundation for Science, Technology and Research for providing the necessary facilities for undertaking this work. Prakash Narayana Reddy is a DST-INSPIRE Faculty [DST/INSPIRE/04/2017/000565] sponsored by INSPIRE division, Department of Science and Technology, Govt. of India.

Author contributions SM participated in conceiving and performing the experiments. KS helped in technical discussion, writing the manuscript and proofreading. VRD helped in discussions, correcting the English language and proofreading the manuscript. PNR participated in conceiving, performing the experiments and writing the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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