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Research Paper

Quick inactivation of *Bgl*I sites in pUC18 using in-fusion® mediated site-directed mutagenesis

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Hongmin Li*, Jihong Cui, Shasha Yang, Sili Wei, Xin Xie and Fulin Chen

College of Life Sciences, Northwest University, Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of China, Taibai North Rd 229, 710069, Xi'an, Shaanxi Province, China.

*Corresponding author. E-mail: lihm2006@nwu.edu.cn, lihm2006@126.com. Tel: 86-029-88302634; Fax: 86-029-88303572.

ABSTRACT

In-Fusion® strategy is a highly regarded strategy in gene cloning manipulation. Here, we reported a successful case of site-directed mutagenesis mediated by In-Fusion® strategy. The 1118 bp fragment between the two sites of *Bgl*I in pUC18 was amplified using the primers containing mutation site and recombined with the 1568 bp fragments of pUC18 derived from the digestion of *Bgl*I in virtue of the 15 bp homologous sequence added to the 5'-end of each primer that precisely match the ends of the 1568 bp fragment. Restriction digestion and DNA sequencing showed that the plasmids isolated from the 6 single colonies selected randomly were successfully mutated with the *Bgl*I$_{436}$ replaced by *Xho*I$_{436}$ and inactive *Bgl*I$_{1554}$, indicating mutation efficiency in this work was very high and the manipulation was simpler than other means as the fragment containing mutation site was not required to be treated by restriction endonuclease before recombination. Furthermore, the cost of this technique was lower than gene synthesis when designed mutation site was just only a base, a site of restriction endonuclease or other short elements. These results proved that In-Fusion® cloning technique can be used as an effective approach for site-specific mutagenesis.

Keywords: Quick-mutation, gene cloning, recombinant, elastin-like protein.

INTRODUCTION

In-Fusion® mediated site-directed mutagenesis

Plasmid pUC18, with high copy number and a length of 2686 bp was created by Yanisch-Perron. The "p" prefix denotes "plasmid" while the abbreviation "UC" stands for "University of California". This plasmid is the most widely used cloning vector as a standard *Escherichia coli* vector for the convenient recombinant selection based on ampicillin resistance or blue-white spot screening (Messing et al., 1977; Yanisch-Perron et al., 1985; Vieira and Messing, 1982).

However, some uses of cloning vectors requires special recognition site of restriction endonuclease, such as *Bgl*I matched *Pfl*MI often used in the elongation of the gene coding for elastin-like peptides (ELPs) through recursive directional ligation (RDL) (Meyer and Chilkoti, 1999; Kim and Chilkoti, 2008), but *Bgl*I site is not included in the multiple cloning sites (MCS) and on the contrary, there are two sites of *Bgl*I locating 436 bp (*Bgl*I$_{436}$) and 1554 bp (*Bgl*I$_{1554}$) of pUC18 (GenBank: L08752.1). In order to make pUC18 invisible for the elongation of ELPs-encoding gene through RDL, it is essential to mutate the two sites of *Bgl*I. Here, we report the inactivation of *Bgl*I$_{436}$ and *Bgl*I$_{1554}$ in pUC18 through In-Fusion® cloning mediated site-directed mutagenesis based on polymerase chain reaction (PCR).

MATERIALS AND METHODS

Reagents

All restriction endonucleases, including *Bgl*I, *Nde*I, *Xho*I and *Pfl*MI (MBI Fermentas, Ontario, Canada) were purchased from Xi`an Zhou Dingguo Biotechnology Company, Limited,

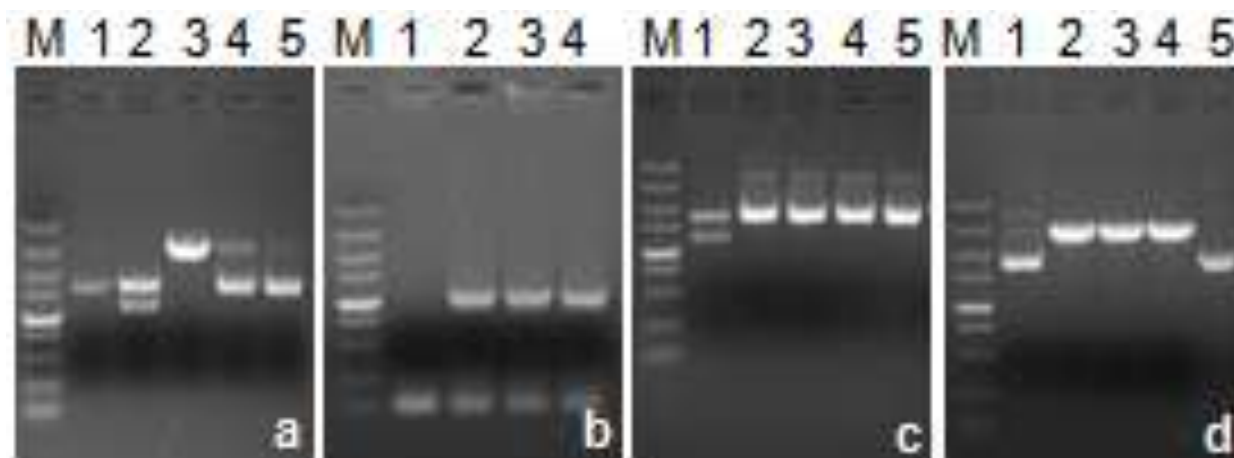

Figure 1: Restriction endonucleases analysis of pUC18-mut m: DL5000; a1: plasmid DNA of pUC18; a2, c1: pUC18 digested by *Bgl*I; a3: pUC18 digested by *Nde*I; a4: pUC18 digested by *Xho*I; a5: pUC18 digested by *Pfl*MI; b1: negative control with H_2O as template; b2-4: PCR products for In-Fusion® cloning; c2-4: pUC18-mut digested by *Bgl*I; c5: plasmid DNA of pUC18-mut; d1: pUC18 digested by *Xho*I; d2-4: pUC18-mut digested by *Xho*I; d5: plasmid DNA of pUC18-mut.

China. The AxyPre™ DNA Gel Extraction Kit for DNA purification and The AxyPre™ plasmid MiniPrep Kit for plasmid DNA extraction was obtained from Axygen Biosciences (Union city, CA). In-Fusion® HD cloning Kit, dNTP (2.5 mM for each) and DNA marker DL5000 are the products of TaKaRa Biotechnology (Dalian) Company, Limited. The PCR primer used for In-Fusion® cloning was also synthesized by TaKaRa Biotechnology. Both strains of *E. coli* Top 10 were used as cloning host cells and the plasmids pUC18 retained by the Laboratory of Tissue Engineering, College of Life Sciences, Northwest University, China.

PCR manipulation and In-Fusion® mediated mutagenesis of BglI sites

The forward primer is 5'-cccaacagttgcgcactcgagatggcgaatggcgcc-3' and reverse primer is 5'- cagcaataaaccagccaaccggaagtgccgagcgc -3'. Within the primers, the first 6 bases of *Bgl*I$_{436}$ site was replaced by that of *Xho*I (CTCGAG) in the forward primer, while the fourth and the last base of *Bgl*I$_{1554}$ site was mutated into A and T respectively. The PCR system was composed of 4.7 µl sterile deionized H_2O, 0.3 µl PrimeStar® HS DNA polymerase, 2 µl 5 × PrimeStar® HS DNA polymerase reaction buffer, 0.8 µl dNTP, 1 µl forward primer (10 pM), 1 µl reverse primer (10 pM) and 0.2 µl pUC18 plasmids DNA (ng/ml) as template. The parameters of PCR are as follows: 94°C for 3 min; 94°C for 30 s; 65°C for 30 s; 72°C for 30 s; 39 cycles and 72°C for 10 min. The PCR was carried out in a BioRad C1000 thermal cycler (Bio-Rad Laboratories Company, Limited, California, USA). The 1149 bp PCR products and the 1568 bp fragments excised from pUC18 using *Bgl*I was isolated on 1% agarose gel and purified using AxyPre™ DNA Gel Extraction Kit. The purified 1118 bp fragments and 1568 bp fragments were mixed and recombined according to the manual of In-Fusion® cloning Kit. The combined mixture was arranged to transform the competent cells of *E. coli* Top 10. Single transformed colony was inoculated into liquid LB media with 100 µg/ml ampicilin and cultured overnight at 37°C on a rotary shaker with 200 rpm speed. After centrifugation, plasmids as mutant candidate were extracted according to the manufacturer protocol of AxyPre™ plasmid MiniPrep Kit and confirmed by restriction endonucleases digestion and DNA sequencing. The actual mutant was termed pUC18-mut.

RESULTS

In-fusion® mediated mutagenesis of BglI sites in pUC18

MCS is essential for gene cloning or expression. Within the MCS of pUC18, multiple common recognition sites were involved which undoubtedly contributes to the most often application of it in bacterial gene-transformation and other routine molecular manipulation of many eukaryotic genes. Unfortunately, *Bgl*I cannot be directly used as cloning site. According to the gene information (GenBank: L08752.1), pUC18 sequence contains two *Bgl*I sites *Bgl*I$_{436}$ and *Bgl*I$_{1554}$, unique *Nde*I site at 498 bp and none of *Xho*I and *Pfl*MI. These recognition sites were confirmed by restriction endonucleases digestion in view of the application purpose of the prospective mutated pUC18. The covalently closed circular pUC18 DNA was cut into two fragments of 1118 bp and 1568 bp by *Bgl*I (Figure 1a2), or unique band of 2686 bp digested by *Nde*I (Figure 1a3) and could not be cleaved if the enzyme was *Xho*I (Figure 1a4) or *Pfl*MI (Figure 1a5), while the next three enzymes were deliberately involved for

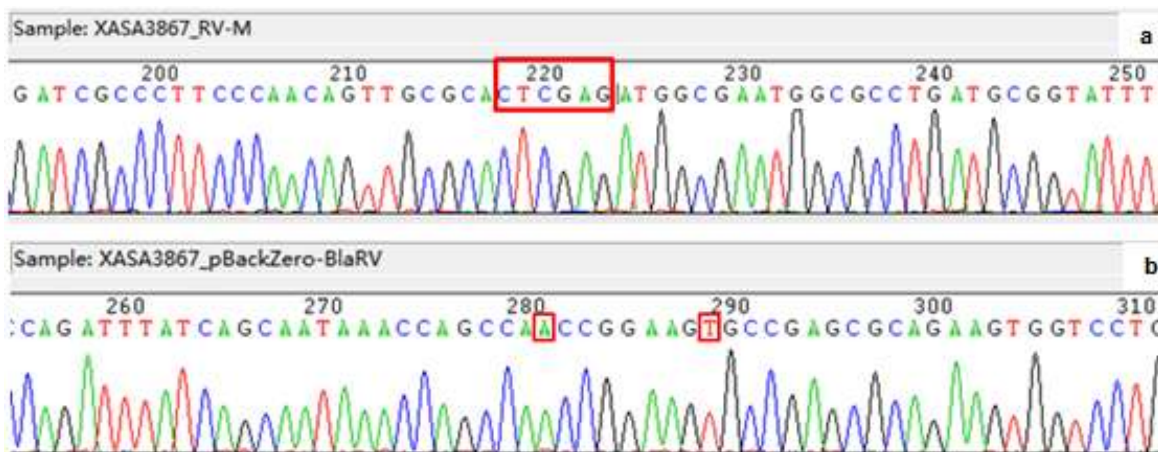

Figure 2: Sequencing analysis of the two mutated *Bgl*I sites in pUC18-mut a: Sequencing result of mutated *Bgl*I$_{436}$; b: Sequencing result of mutated *Bgl*I$_{1554}$.

the elongation and expression of ELPs encoding sequence.

Based on the results of restriction endonucleases digestion, the primers used for In-Fusion® cloning was designed as earlier described. The fragment between *Bgl*I$_{436}$ and *Bgl*I$_{1554}$ was successfully amplified and the size of PCR products approximately 1149 bp (Figure 1b2-4). The PCR products were extracted after agarose electrophoresis and inserted into the region between *Bgl*I$_{436}$ and *Bgl*I$_{1554}$ in strict accordance with the manufacturer's protocol of In-Fusion® cloning kit.

The results of restriction endonucleases digestion showed that the original fragment between the two *Bgl*I sites of pUC18 was successfully replaced by PCR product containing inactivated *Bgl*I$_{436}$ and *Bgl*I$_{1554}$. The putative mutated pUC18 could not cleaved by *Bgl*I (Figure 1c2-4), while pUC18 was cut into two fragments (Figure 1a2 and c1). At the same time, the recognition site of *Xho*I was introduced into the mutated pUC18 because it could be cleaved by *Xho*I to produce unique band on the verge of 2686 bp (Figure 1d2-4) while pUC18 could not (Figure 1 d1).

The prospective mutated pUC18 tested by restriction endonucleases digestion was hereupon arranged for complete DNA sequencing. The result showed that the first 6 bases of *Bgl*I$_{436}$ site in pUC18 was replaced by that of *Xho*I marked with box in the sequencing chromatogram (Figure 2a), while the fourth and last base of *Bgl*I$_{1554}$ recognition site in pUC18 was respectively mutated into A and T indicated with box (Figure 2b), although the sequence present in chromatogram was the reverse complementary sequence of pUC18. The final mutated pUC18 was named pUC18-mut in which the *Bgl*I sites were inactive and an *Xho*I site was introduced into the site of initial *Bgl*I$_{436}$.

DISCUSSION

Site-directed mutagenesis, also called site-specific or oligonucleotide-directed mutagenesis was firstly achieved by Charles Weissmann who induced a transition of GC to AT using a nucleotide analogue N^4-hydroxycytidine (Flavell et al., 1975; Muller et al., 1978). However, the specificity of this method was not high enough and was limited by the kind of mutation. Hutchison et al. (1978) developed a more flexible approach for site-directed mutagenesis using DNA polymerase extending oligonucleotides in a primer for which Michael Smith shared the Nobel Prize in Chemistry in 1993 with Kary B. Mullis who produced PCR technique. Later, a large number of methods were invented to increase the mutation rate and the specificity of site-directed mutagenesis. Unfortunately, most of them are now largely supplanted by newer techniques such as Kunkel's method (Kunkel, 1985), cassette mutagenesis (Wells and Estell, 1988) and PCR site-directed mutagenesis (Rabhi et al., 2004).

These techniques allow for simpler and easier ways to introduce expected mutation into genes with site specificity and high efficiency. However, the whole process of Kunkel's method must undergo twice of *E. coli* transformation and requires the host cells of *E. coli* strain deficient in two enzymes of dUTPase (*dut*$^-$) and uracil deglycosidase (*ung*$^-$) (Kunkel, 1985).

Cassette mutagenesis is inevitably limited by the availability of suitable restriction endonucleases sites flanking the site planning to mutate, although, this method is simpler and can generate mutants close to 100% efficiency. The inherent deficiency of cassette mutagenesis was the requirement of a suitable restriction site near the mutation site. In contrast, the Clontech In-Fusion® PCR Cloning System does not strictly require a proper restriction site near the desired mutation site. Here, the PCR primers was designed and the 3'/5' homologous regions are produced by adding 15 bp extensions to both PCR primers that precisely match the ends of the 1568 bp vector linearized by *Bgl*I digestion. When the 1149 bp PCR

fragments were combined with the linearized pUC18, the special In-Fusion® enzyme converted the double-stranded extensions of PCR product into single-stranded DNA and fuses these regions to the corresponding ends of the linearized pUC18 (http://www.clontech.com/CN/Products/Cloning_and_Competent_Cells/Cloning_Kits/Cloning_Kits-HD-Liquid?sitex=10022:22372:US).

After the transformation of *E. coli*, six single colonies were randomly selected and cultured for plasmids extraction. The analysis of restriction digestion and DNA sequencing showed that the plasmids isolated from the six single colonies were successfully mutated with the *Bgl*I_{436} replaced by *Xho*I_{436} and *Bgl*I_{1554} inactivation. These results showed that the mutation efficiency in our work was properly high and the recombination was directional and seamless. The manipulation was simpler than cassette mutagenesis or PCR site-directed mutagenesis as the fragment containing mutation was not required to be treated by endonuclease before recombination, such that the recombination could be finished in a short reaction time.

Furthermore, the cost of this technique was lower than gene synthesis when designed mutation site was just only a base, a site of restriction endonuclease. It was not observable that the deletion of the short sequence between *Nde*I_{498} and *Xho*I_{436} had obvious effects on the replication and stability of the recombinant plasmid pUC18-mut-ELPs when it was transformed into the host cell of *E. coli* Top10 upon continuously passaging 50 times (data was not showed), to which the sequence might contribute no clear and definite function involved in the specialties of pUC18 according to the gene information (GenBank: L08752.1). Duration of the DNA manipulation, detrimental effects on the replication and stability of the recombinant plasmid was not investigated when the original sequence between the *Bgl*I_{436} and the *Nde*I_{398} was deleted.

Taken together, In-Fusion strategy was successfully applied in our experiment to mutate the recognition sites of *Bgl*I_{436} and *Bgl*I_{1554} simultaneously and the *Bgl*I_{436} was replaced by *Xho*I_{436}. The mutated pUC18 with *Xho*I_{436} and inactive *Bgl*I_{1554} was achieved and named pUC18-mut which has been conveniently used for the seamless and directional oligomerization of the gene coding for ELPs (data was not showed).

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