Construction of a synthetic vector for preparation of a 100 base pair DNA ladder

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Abstract
DNA size markers are widely used to estimate the size of DNA samples on agarose or polyacrylamide gel electrophoresis (PAGE). DNA markers can be prepared by mixing PCR products with definite sizes. Alternatively, they are prepared by restriction enzyme digestion of the genomic DNA of bacteriophages or natural and synthetic DNA plasmids. The present study describes engineering of a synthetic plasmid which produces a 100 bp DNA ladder, a popular DNA size marker, upon digestion with a single restriction enzyme. Our strategy consisted on sequential cloning of ten PCR products of 100 to 1000 bp in plasmid pTZ57R, using the *Bam*HI and *Bgl*II restriction enzymes and releasing the fragments from the recombinant plasmid by enzyme *Eco*RV. This strategy could be applied to construct various complex synthetic vectors to produce different DNA ladders.
Keywords**:** Cloning; DNA marker; plasmid; 100 bp ladder

INTRODUCTION

Gel electrophoresis is a common technique in molecular biology for estimating the size of PCR products or DNA fragments digested by restriction enzymes. This technique has found widespread use in research as well as in practical applications. To determine the size of nucleic acid fragments on agarose or polyacrylamide gels, standard nucleic acid markers or DNA ladders are very useful tools. They are simultaneously used in electrophoresis with DNA samples in the neighboring lanes to serve as references. Following electerophoresis and gel staining, a comparison is made between the sample band(s) and the bands of the standard DNA Marker. Knowing the size (in base pairs) of the standard allows the size of the unknown fragment(s) of the sample to be estimated.

Generally, the DNA markers have been produced by enzyme digestion of *E. coli* plasmids (Polyarush *et al*., 2003) or genomic DNA of bacteriophages (Cooney, 1994). Banding pattern of such DNA markers in electrophoresis is non-uniform and the size and number of bands depend on the frequency of recognition site(s) for the enzyme(s) used in digestion. In contrast to traditional DNA markers, DNA ladders have numerous bands that increase in size in regular, even intervals. The bands of DNA ladder show also an equivalent intensity upon staining. DNA ladders can be produced by multiplex PCR (Wang *et al*., 2010) or single PCR amplification of desired DNA bands and then mixing the purified PCR products at certain concentrations (Microsugar *et al*., 2008; Amills *et al*., 1996). Alternatively, DNA ladders are produced by enzyme digestion of specially engineered plasmids which contain recognition sites for one or more specific restriction enzymes at particular intervals (Chen *et al*., 2009; Dongyi *et al*., 2008; Hartley, 2006; Hyman, 1998). The advantage of DNA marker production based on artificial plasmids is that DNA markers can be generated on an industrial scale through *E. coli* fermentation with a variety of simple processing techniques (Chen *et al*., 2009; Dongyi *et al*., 2008). Efforts

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to improve marker-producing plasmids have mainly focused on the incorporation of several DNA fragments into a single vector at the limited restriction sites.

The present study describes a method for making a recombinant plasmid which provides a 100 bp DNA ladder after digesting with a single restriction enzyme.

MATERIALS AND METHODS

PCR amplification of 100 to 1000 bp fragments: All DNA fragments to be cloned in the recombinant plasmid were amplified by PCR from pMAL-c2X plasmid (New England Biolabs, Canada), as the template. For this purpose, ten pairs of primers (Table 1) were designed for PCR amplification of DNA fragments of 100 to 1000 base pairs, the main DNA bands of commercial 100 bp ladders (Fermentas, Litvany).

The selected segments to be amplified did not contain any restriction sites for *Bam*HI, *Bgl*II and *Eco*RV enzymes. The forward primers harbored a recognition site for the enzyme *Bgl*II and the reverse primers contained the sites of *Bam*HI and *Eco*RV at their 5'ends. The forward primer of 100 bp DNA fragment had also an additional *Eco*RV site after the site of *Bgl*II. PCR reactions were carried out in a final volume of 50 µl containing 2.5 U of *Taq* DNA polymerase, 20 ng of pMAL-c2X DNA template, 50 pmol of each forward and reverse primer, 200 µM of dNTPs mixture, 1.5 mM of $MgCl_2$ and 5 µl of 10×PCR reaction buffer. The PCR amplifications were performed using a program of initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Construction of recombinant plasmid: PCR products were electrophoresed on 1% agarose gel and bands of the appropriate sizes were excised from the gel and purified by a DNA gel extraction kit (Qiagen, Germany). The purified DNA fragments were double digested with *Bam*HI and *Bgl*II enzymes and purified again from agarose gel, before cloning into pTZ57R plasmid (Fermentas, Litvany). The cloning process was commenced by 100 bp fragment. Therefore, pTZ57R plasmid was digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase (Fermentas, Litvany) based on the manufacturer's instruction. The 100 bp fragment was ligated into the plasmid and the construct transformed into chemically prepared DH5α competent cells (Chang *et al*., 1989). Transformants were screened using restriction digestion analysis of their plasmids. Those plasmids which

Table 1. Oligonucelotide primers designed based on the plasmid pMAL-c2X sequence data for PCR amplification of 100 to 1000 bp DNA fragments.

Primer name	Primer sequences
100 Forward	5' GCGAGATCT(*Bgl*II) GATATC(*Eco*RV)TGCTCCCGGCATCC 3'
100 Reverse	5'GCGGGATCC (*Bam*HI) GATATC(*Eco*RV)TGCCTCGCGCGTTT 3'
200 Forward	5' GCGAGATCT(*Bgl*II) AACCGCGTGGCACA 3'
200 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)TGCACCGCCGCTTT 3'
300 Forward	5' GCGAGATCT(*Bgl*II)CCGCAGCCGAACGA 3'
300 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)TCCCGGAGACGGTCA 3'
400 Forward	5' GCGAGATCT(*Bgl*II)GCGCCCGGAAGAGA 3'
400 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)GCGTTGCGCGAGAA 3'
500 Forward	5' GCGAGATCT(*Bgl*II)GGGGGCGGAGCCTA 3'
500 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)TGCCTCGCGCGTTT 3'
600 Forward	5' GCGAGATCT(*Bgl*II)GCGGGCAAACAGTCG 3'
600 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)GCCCAGCGCCATCTG 3'
700 Forward	5' GCGAGATCT(*Bgl*II)GACGCGCCCTGACG 3'
700 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)GGCGGGGTTGCCTTA 3'
800 Forward	5' GCGAGATCT(BglII)CTGCGCCCCGACAC 3'
800 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)TTCGGGCAGCGTTGG 3'
900 Forward	5' GCGAGATCT(*Bgl*II)ACCCGCCGCGCTTA 3'
900 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)TGCGCTCGGTCGTTC 3'
1000 Forward	5' GCGAGATCT(BglII)AAGCCGGCGAACGTG 3'
1000 Reverse	5' GCGGGATCC(*Bam*HI)GATATC(*Eco*RV)GCTGCGCTCGGTCGT 3'

released a 100 bp fragment after digestion with *Eco*RV, were analyzed for insert orientation by PCR using M13F and 100 Reverse primers. A recombinant plasmid with the 100 bp fragment in the correct orientation was digested with *Bam*HI, dephosphorylated and used for cloning the 200 bp fragment, as above. This process was continued up to cloning all of ten fragments. From the cloning of 200 bp fragment onward, the screening of transformants was carried out only by electrophoresis in agarose gel and *EcoR*V digestion. During screenings, recombinant plasmids with apparently more than one copy of each insert, were identified and excluded based on comparative electrophoretic mobility and the fact that such plasmids produced a more intensively stained insert after digestion with *EcoR*V. However, to increase the intensity of 100 bp fragment in the final product, an additional 100 bp insert was deliberately cloned after cloning the 1000 bp fragment. The final recombinant plasmid was eventually digested with *Eco*RV and its banding pattern was compared with a commercial ladder.

RESULTS

PCR carried out with the designed primers and pMAL-c2X plasmid as DNA template, successfully resulted in amplification of the expected (100 to 1000 bp) DNA fragments (Fig. 1).

After purification of PCR products and double digestion with *Bgl*II and *Bam*HI enzymes, the fragments were successively cloned into plasmid pTZ57R, digested with *Bam*HI. The recombinant plasmids produced after ligation of each fragment and digested with enzyme *Eco*RV are shown in Fig 2A. The results indicate that the recombinant plasmids release the cloned fragments after digestion with *Eco*RV in a regular pattern. However, intensity of 100 bp fragment was found to be insufficient following cloning of 400 bp fragment on ward. Therefore, an additional copy of the 100 bp fragment was introduced in the recombinant plasmid after cloning of the 1000 bp fragment. Banding pattern of the final recombinant plasmid digested with *Eco*RV, was comparable to a commercial and standard 100 bp ladder (Fig. 2B).

DISCUSSION

The challenging step in DNA marker production from artificial vectors is the design and construction of complex synthetic vectors. For this purpose, appropriate strategies should be used to allow cloning of multiple fragments of DNA into a plasmid in tandem in a manner that they can be released after digestion.

In our strategy, initially, ten DNA fragments of 100 to 1000 bp were produced by PCR. Then, fragments were successfully cloned in plasmid pTZ57R. Sequential cloning of multiple PCR fragments in pTZ57R was achieved through the *Bgl*II and *Bam*HI restriction sites introduced in fragments by forward and reverse primers, respectively. During the cloning process, the selected plasmid was digested only with *Bam*HI, and PCR products were double digested by *Bgl*II and *Bam*HI. Enzymes *Bgl*II and *Bam*HI are Isocaudomer for each other and both produce a similar protruded sequence (5' GATC) at termini of the digested DNA. Therefore, the ends of DNA fragments digested with both enzymes are compatible and can be ligated to each other. Moreover, after ligation of *Bgl*II- and *Bam*HI-digested DNAs, none of these enzymes are capable to digest the new sequence. This feature let us to linearize the plasmid construct after digestion with BamHI. By using this strategy, a successive

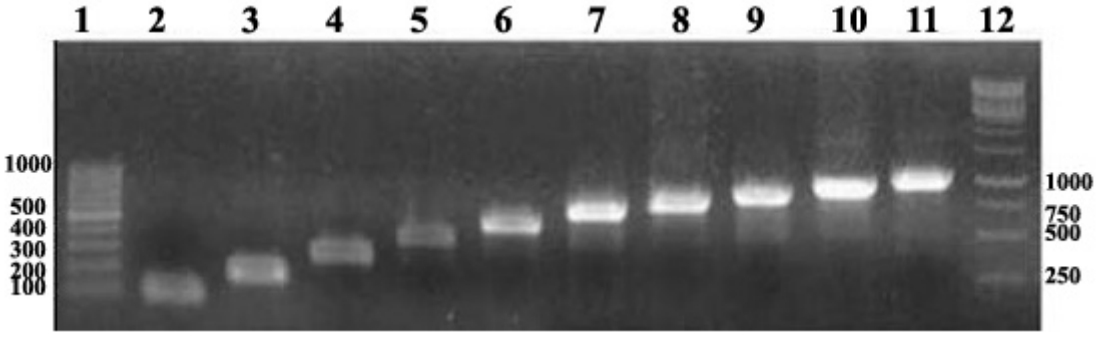

Figure 1. Electrophoresis of 100 to 1000 bp PCR products in agarose gel. lane 1: 100 bp DNA ladder (Fermentas, Litvany), Lane 2-11: 100-1000 bp DNA fragments amplified by PCR using 10 primer pairs described in Table 1, lane 12: 1 Kb DNA ladder (Fermentas, Litvany).

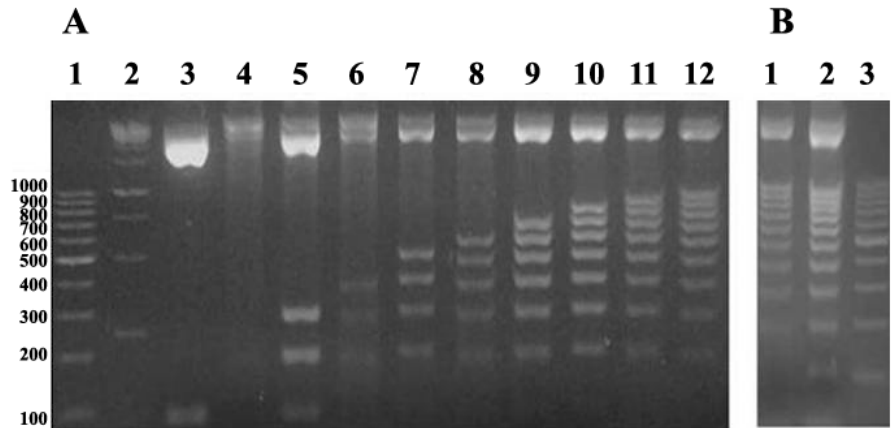

Figure 2. Agarose gel electrophoresis of recombinant plasmids digested with *Eco*RV. A: Lanes 1 and 2 represent 100 bp and 1 Kb DNA ladders, respectively (Fermentas, Litvany); Lanes 3-12: the electrophoretic pattern of *Eco*RV digested recombinant plasmids after sequential cloning of 100 to 1000 bp DNA fragments. B: Lane 1: a recombinant plasmid containing one copy of each DNA fragments of 100 to 1000 bp, lane 2 a recombinant plasmid as in lane 1 which contains 2 copies of the 100 bp DNA fragment, lane 3: 100 bp DNA ladder (Fermentas, Litvany).

cloning of *Bgl*II and *Bam*HI digested DNA fragments into the plasmid digested only with *Bam*HI has been done. In addition to *Bgl*II and *Bam*HI restriction sites, the site of *Eco*RV was also introduced into the fragments by the reverse primers. This sequence permitted releasing of the cloned fragments and a rapid screening of plasmids for correct orientation of the inserts, after digestion with *Eco*RV. Fragments inserted in a correct orientation had *Bam*HI-*Bgl*II ligation at the left side and could be released by *Eco*RV digestion. The final recombinant plasmid harbored all DNA fragments with definite sizes and could produce a 100 bp DNA size reference following digestion with *Eco*RV.

Articles describing the preparation of DNA markers are rare because the commercial companies need to protect their confidential protocols. However, there have been efforts to engineer recombinant plasmids which release the expected DNA bands after digestion with restriction enzyme(s).

To produce a plasmid based 100 bp DNA ladder, Hyman (1998) exploited the strategy of cloning multiple copies of a gene described by Hartley and George (1983). This method is based on asymmetrical digestion of the sequence CTCGGG by enzyme *Ava*I. Asymmetric digestion by *Ava*I makes it possible to blunt one end of the digested DNA while the other end remains sticky for a DNA digested with the same enzyme. This condition permits a polymerization of the fragments at the digested restriction site, because over several days of incubation in the presence of T4 DNA ligase, several copies of DNAs are ligated to each other (polymerization) at the place. To obtain a circular plasmid, eventually the end of last fragment will be blunt and ligated to the other end. By using this method, Hyman, (1998) cloned 20 copies of a 100 bp DNA fragment in pUC 18 and by partially digestion of the construct, a 100 bp DNA ladder was produced.

Hartley (2006) prepared 3 PCR products of different sizes which ligation of them to each other led to formation of a plasmid. Then, digestion of the plasmid with a restriction enzyme which recognition site was present in the PCR products led to formation of a DNA ladder. In that study, ligation of PCR products to each other was achieved through uracil DNA glycosylase treatment of PCR products amplified by specially designed primers or multiple compatible cohesive ends generated by restriction enzymes digestions.

Dongyi *et al*. (2008) and Chen *et al*. (2009) amplified several fragments of the lambda phage DNA by PCR and then ligated appropriate PCR products to each other by T/A mechanism or cohesive ends generated by restriction enzyme digestions. Thereafter, recombinant PCR products were purified either directly from agarose gel or after amplifying by PCR using suitable primers. The recombinant PCR products were finally cloned and the resulting plasmids were digested by the enzymes which recognition sites have been introduced by primers.

The main purpose of the related studies has mostly been to develop a cloning strategy of multiple DNA fragments which is rapid and eliminates the individual cloning process of each fragments. By applying our strategy, multiple DNA fragments of different sizes are independently cloned into a target vector. In the first

place, this process seems to be laborious. However, it is simplify because of the application of only two restriction enzymes for digestions of all fragments and screening of the cloned fragments with a single enzyme. Moreover, the main advantage of our protocol is its flexibility for ligation of multiple DNA segments in a more efficient way. Using this strategy, one can add more inserts into the plasmid in order to intensify some bands, based on specific applications. In conclusion, we described a simple and flexible method for construction of an artificial vector through a careful design of primers and use of routine techniques of molecular biology.

To prepare a commercial ladder based on a recombinant plasmid, it is very important to determine the most efficient conditions of bacterial propagation, plasmid purification and especially final digestion of the plasmid to release the inserts. In this regard, application of a restriction enzyme like *Eco*RI which is one of the most efficient and inexpensive enzyme, would be more suited than *Eco*RV.

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