Construction of Recombinant DNA Plasmid for Production of 100 Base Pair DNA Ladder Based on PCR Technique

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ABSTRACT

The standard 100 base pairs (bp) DNA ladder is an essential molecular marker in the molecular biology laboratory. This standard DNA marker is normally used to determine the size of DNA fragments, plasmids and PCR products during gel electrophoresis. Since the standard DNA ladder is one of the major costs in molecular biological research and teaching; therefore, the unlimited in-house production of this DNA ladder could be an effective cost reduction strategy for laboratories. Thus, the purpose of this study is to prepare a DNA ladder template for laboratory use by constructing a plasmid DNA inserted with a synthetic 1,500-bp polynucleotide fragment. Using the PCR technique, the results showed that PCR was able to amplify 11 different fragments ranging from 100-1,000 bp, and a 1,500 bp fragment with high qualification and size accuracy. Finally, the purification of the PCR product fragments showed high quality and quantity for each individual band of the ladder. Our in-house procedure for the production of the 100 bp DNA ladder points to the possibility of a simple, inexpensive, time saving and an unlimited production method for laboratory-grade DNA markers.

Keywords: DNA ladder; synthetic DNA template; polymerase chain reaction

INTRODUCTION

DNA ladder is a standard molecular marker commonly used as a reference marker or a scale to determine the size of unknown DNA fragments or PCR products, which have been separated by the gel electrophoresis technique (Applichem, 2010). Most DNA ladders used in the laboratory are the typical 100 bp ladders, which generally range from 100-1,500 bp. Although DNA ladders are commercially available, the purchase of these ladders for use in a routine molecular biology laboratory is considered as a significant cost to the laboratories. Thus, the development of a reliable and cost effective 100 bp DNA ladder, with the possibility of endless usage will reduce the cumulative cost of the routine laboratory work. According to literature, several approaches have been employed successfully to produce 100 bp DNA ladders. One of the methods was developed by digesting the genomic DNA of a lambda bacteriophage or an *E. coli* plasmid with the restriction endonuclease (Cooney *et al.*, 1989; Polyarush *et al.*, 2003; Thomas *et al.*, 1975). The limitation of this approach was the inability of the technique to produce an equal interval of the 100 bp DNA bands, while also needing more practical processes for the propagation of the bacteriophage or the plasmid within a suitable host. The solution to the approach used in generating 100 bp DNA ladder fragments of equal intervals was the PCR method. For instance, DNA ladders can be produced using the single or multiplex PCR amplification method where the backbone region of commercially available vectors can be amplified to produce the target fragments (Abbasian *et al.*, 2015; Natarajan *et al.*, 2008; Riyajan. *et al.*, 2011). It is, however, difficult to design specific primers to amplify regions of regular sizes on the plasmid to generate 100 bp DNA ladders of exact intervals. Additionally, it is also difficult to control the intensity of the specific DNA bands using the multiplex PCR method. In the past, the production of DNA molecular weight markers through the PCR of target chromosomal regions of bacteria have been reported (Sekhavati *et al.*, 2015). The challenge with this approach is the high possibility of contamination of the DNA ladder amplicon with bacterial DNA that have spread about or lingered within the laboratory environment, which could cause a carryover effect in the PCR reaction when working with closely related bacterial species. Therefore, the present study attempts to overcome these problems by describing a new approach for the construction of a recombinant plasmid for use as PCR template by inserting a synthetic double stranded DNA of 1,500 bp that has a unique sequence and has not been identified to be associated with any living species.

MATERIALS AND METHODS

Designing of DNA template and primers

Approximately of 1,500 bp dsDNA template was manually designed by randomizing the nucleotide sequence that has no homology with any species. To make it easy for amplification and size precision of 100 bp DNA ladder, the position of every 100 bp intervals (100-1,500) of the synthetic dsDNA template was, therefore, specifically designed to be the binding site of each primer (Table 1). To ensure that the designed 1,500 bp dsDNA template is unique and not homologue to any living things DNA (except primer binding site), the designed template was analyzed by Blast search and Oligoanalyzer (https://sg.idtdna.com/calc/ analyzer) prior sending to synthesis (IDT: Integrated DNA Technologies Inc, USA).

Table 1 Sequence of primer and annealing temperature

No	Primers	Sequence (5'-3')	Annealing Temp.
1	F-1500	ATG GCG ATT ACT GGA TAG ATG G	51
	R-1500	AGT CGG GGT CTT GGT TGT GAG	51
2	R-1000	CGC TGT TTA TTC TGT GTC TTGTC	51
3	R-900	TTG AAG TAC CTC ATC CCA CCA	51
4	R-800	TTT ACG ACT CAC TTG CCT AAC G	51
5	R-700	TAC CTT CTG GCG TAC CCA CTT T	51
6	R-600	CGA TCT TGT TAG CYG TTG TAG TG	51
7	R-500	GAG TTG TCC ATC TCT TTG TTC TG	51
8	R-400	GAC AGC CTC TCT TTC TCC ACA	51
9	R-300	TGG AAC GAA GGC TAC GTA	51
10	R-200	CCT ATA ACC AGA CCG TTC AG	51
11	R-100	TCA CAG ACG GCA TGA TGA AC	51

Construction of recombinant plasmid

The synthetic 1,500 bp ds-DNA template was firstly amplified with forward and reverse 1,500 primer to generate 1,500 bp PCR product using *Taq* DNA Polymerase (Thermo Scientific: USA). The PCR product was purified by the PCR purification kit (company, country). Next, the purified PCR product was cloned in the pGEM T-Easy vector system (Promega; USA) and recombinant plasmid were transformed into *E. coli* DH-5α using electroporation system (Bio-Rad). The *E. coli* that ability to grow in Tryptic Soy Agar (TSA) supplemented with 100 μg/mL of ampicillin was randomly selected and tested for present of the inserted 1,500 bp DNA by direct colony PCR (Figure 1). Positive colonies were subcultured onto TSA agar supplemented with 100 μg/mL of ampicillin prior preserved in the glycerol broth (Tryptic Soy Broth with 20% glycerol) and stored in the deep freezer (-80 °C). Another part of the positive colony was collected for plasmid purification using a plasmid extraction kit.

PCR amplification

Polymerization of target DNA was carried out by *Taq* DNA polymerase (Thermo Fisher Scientific, MA, USA). The reaction mixture (50 mL) composts of 1X PCR reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM each primers, 1.25 Unit of DNA polymerase and 10 ng of plasmid DNA template. Amplification condition was performed as described, initially with denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 50 min, annealing for 1 min at the temperature depending on the Tm of each primer (Table 1), primer extension at 72 °C for 1 min, and one cycle with a final extension step at 72 °C for 5 min. The amplification product was analyzed and visualized by 1.5% gel electrophoresis stained with GelRed^{Tm} (Thermo Fisher Scientific, MA, USA) and visualized under LED transilluminator (UltraBright LED: Maestrogen; Korea). For long term storage and quality preservation of the DNA markers, PCR products can be purified with PCR purification kit (The E.Z.N.A.®Cycle-Pure Kit; USA).

RESULTS

The outcome of the use of the dsDNA template and primers for the generation of the DNA ladder showed successful amplification. As shown in Figure 2A, the amplified PCR product of the DNA ladder presented bands of 100 to 1,000 bp, and 1,500 bp, which were clearly observable on 1.5% agarose gel. The size of all amplified DNA bands, in comparison to the commercially available DNA markers, were closely related. However, we found that non-specific DNA bands could occur during the amplification process, but does not have to interfere with the productive DNA ladder as small unwanted bands can be removed via a DNA purification process and some unwanted bands have the same or closely related size to 100 bp ladder.

For long term storage and quality preservation of the DNA markers, PCR products were purified. Before the purification process, the PCR products were

divided into 4 pools, which were 100-400, 500, 600-900 and 1,000-1,500 bp, respectively. The results showed that purification can enhance the quality of the 100 bp DNA ladder. Moreover, the results also indicated that it is easier to regulate the density of the DNA bands at 500, 1,000 and 1,500 bp (Figure 2B).

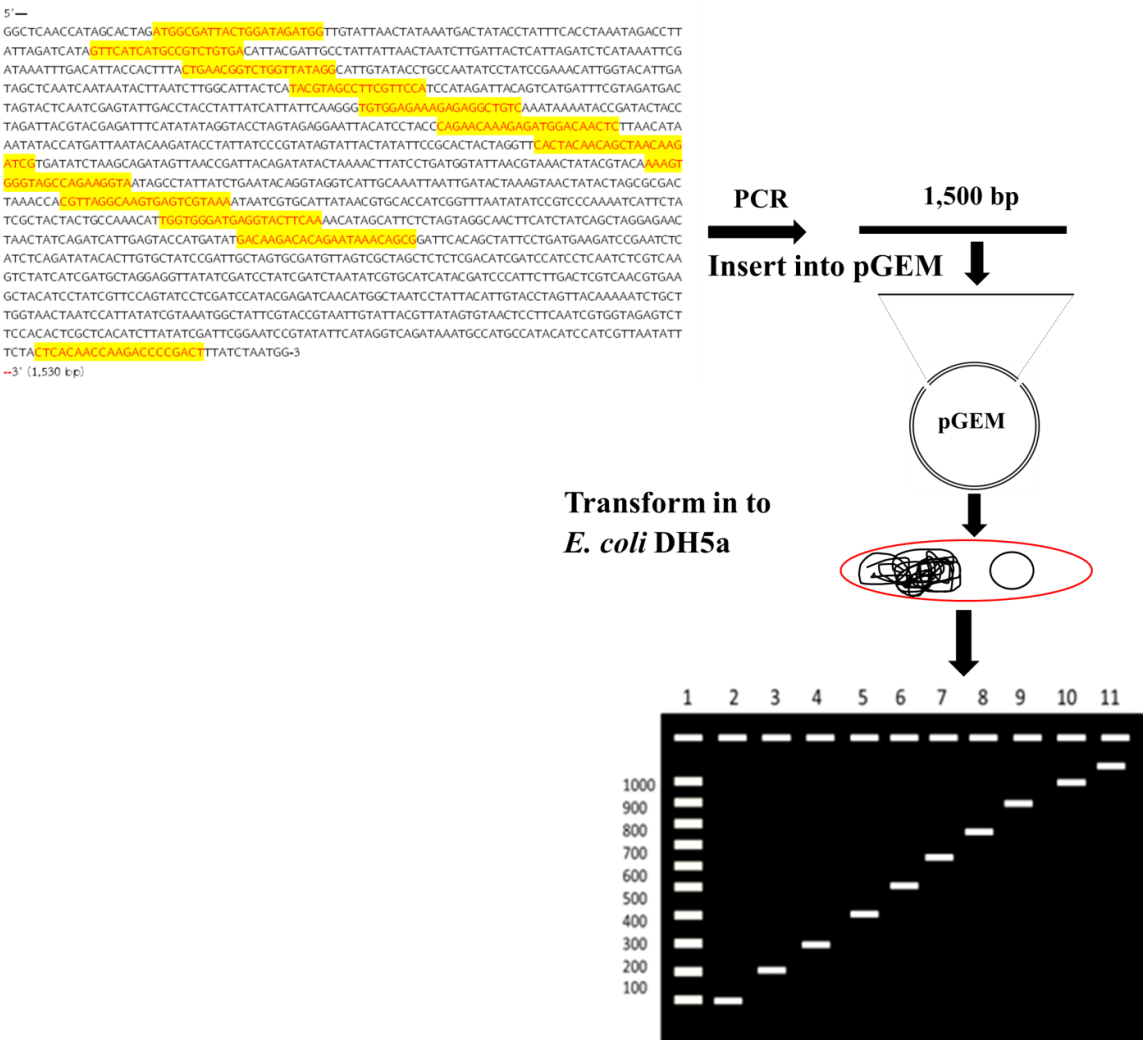

Figure 1 Schematic representation of construction and generation of the 100 bp DNA ladder.

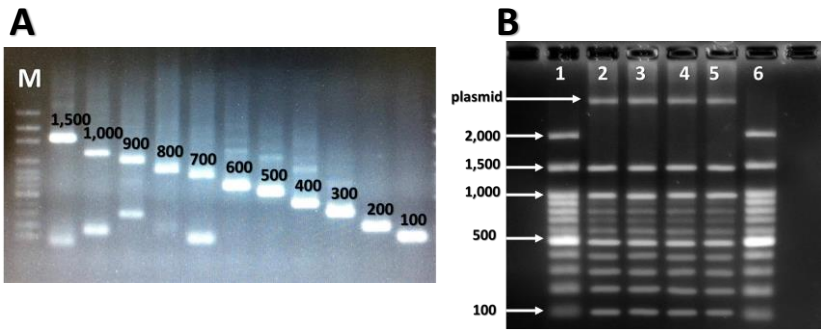

Figure 2 Agarose gel (1.5%) electrophoresis of amplified PCR products, A: Amplified PCR products of 100-1,500 bp DNA ladders. Lane M = DNA ladder. B: Combination of PCR products of 100-1,500 bp DNA ladder in a-1.5% agarose gel electrophoresis. Ten µL of purified products were loaded in each lane. Lanes 1 and 6 = commercially available 100 bp DNA ladder, lanes 2-5 mixed 100 bp DNA ladder produced in this study.

DISCUSSION

The 100 bp DNA ladder is one of the important costs of molecular biology research laboratory because of the volume of use on a regular basis. In order to limit the external costs, the development of an in-house DNA ladder could prove to be highly useful, cost-effective and beneficial, both for molecular research and teaching. In this study, we developed a new approach in generating 100 bp DNA ladder. By using a synthetic 1,500 bp DNA template inserted into a cloning vector, we found that an endless amount of DNA template can be generated. Our system is very simple and cost effective when compared to several previously described methods. One of the former DNA ladder production methods made use of bacteriophage and plasmids digested with restriction enzymes to generate DNA fragments; however, the drawback was the inability to generate regular sizes of the fragments (Polyarush *et al.*, 2003). Although the construction of synthetic plasmids containing restriction sites for the generation of 100 bp DNA ladder have been described before (Rashno *et al.*, 2012), it is however a sophisticated, laborious technique with a limitation in terms of large-scale production. To resolve the limitation of this restriction enzyme based method, several PCR based methods have been applied in conjunction. The 100 bp DNA ladder can be directly amplified from the backbone of a pGEM-T Easy plasmid vector (Riyajan. *et al.*, 2011). Although the PCR approach is simple and cost effective, the designing of the primers specific to distinct regions of the plasmid to generate 100 bp ladder is nonetheless difficult and requires several primer pairs. Moreover, direct amplification from commercially available plasmids may lead to the infringement of certain patents, which could prove problematic. In this study, through the synthesis of a 1,500 bp template where every 100 bp interval bands comprise specific nucleotides at the primer binding sites, shows that we were able to resolve this general issue that was persistent in the past. Our 100 bp DNA ladder presented with regular sizes where only 1 universal forward primer was used with 11 reverse primers. Through the application of this model, we can also construct plasmids with larger inserts of DNA template segments in order to generate DNA ladder larger than 1,500 bp. Because the template is a randomly synthesized DNA sequence, it is highly likely to be a unique DNA sequence unlike any sequence from other living species. Our designed DNA ladder, therefore, takes advantage of this uniqueness to form DNA markers that are different from the commercial markers that are directly amplified from bacterial DNA. Even though primers used in this study have already been used in our laboratory, the amplicons do not affect or cause contamination to our routine work because the forward and reverse primers used in the generation of the DNA ladders do not match with any of those used in our routine work.

ACKNOWLEDGEMENTS

We would like to thank the Department of Biology, Faculty of Science, Thaksin University for financial support and research activities throughout this study.

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