

“In-house” production of DNA size marker from a vaccinal *Bacillus anthracis* strain

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ABSTRACT

Background and Objectives: DNA molecular weight marker is widely used in molecular biology experiments incurring considerable costs on low-budget settings.

Materials and Methods: Here a PCR-supported procedure is described that uses 10 primer pairs targeting chromosomal DNA from the harmless vaccinal *Bacillus anthracis* Sterne 34F2 strain as template. A single PCR protocol is used to reproduce all the 10 fragments of a 100 bp DNA size marker.

Results and Conclusion: The unpurified amalgam of 10 PCR products can be directly loaded to agarose gels. This work was intended to develop a reasonably cost-effective DNA ladder that is useful for researchers in laboratories with limited funding.

Keywords: DNA size marker, PCR, sequencing, *Bacillus anthracis* Sterne 34F2

INTRODUCTION

DNA size markers are among the common daily-used consumables in molecular biology laboratories. Commercially, a broad range of DNA ladders from numerous suppliers are available in the market with relatively high prices (1). Essentially, a typical DNA size marker comprises from discrete DNA fragments of specific size ranging from tens to thousands of base pairs (bp) where application of these to agarose gel provides a visual reference to help to estimate size of unknown DNA molecules (2). DNA ladder or marker, nucleic acid ladder or marker, DNA standard, DNA size marker and molecular weight size marker are all similar jargons interchangeably used in the

current literature (2-5). While a number of dissimilar techniques have been proposed to prepare DNA size markers, the original method of restriction digestion of bacteriophage lambda DNA, plasmid DNA molecules and Simian virus genome, still seems to be one of the favorite and approved methods in the market (4, 6). Technically, the restriction enzyme used along with the nucleotide contents of DNA molecule employed and finally the digestion conditions experienced are the leading variables to determine length of produced fragments in this method (2, 5, 7, 8). A commonly used DNA molecular weight marker is the genome of the Lambda phage treated with the restriction enzyme *Hind* III (8). This is however not ideal as tedious cloning work, substantial laboratory skills as well as equipments are required (7, 8).

More recently, some workers have come up with the idea of replacing enzymatic digestion with enzymatic amplification (PCR) to reproduce DNA fragments of favorite sizes (6, 8, 9). Depend on the PCR system used, two versions of this preparation system have been introduced, in the earlier method developed by Chang *et al.* (6) an amalgam of specific DNA

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segments are inserted into an appropriate cloning vector such as pGEM-T Easy. These inserts are subsequently multiplied by application of only one specific primer pair. Later in 2010 Wang (9) claimed by using individual primers, discrete fragments can be amplified with no need for extensive cloning work required by Chang initiative. Therefore, PCR products with sizes of interest are propagated using purpose-designed primers when they are subsequently purified and mixed together at appropriate proportions to make up the DNA size marker (5). In 2012, Vo Thi Thuong Lan innovated a combined system of cloning, PCR and enzymatic digestion where self-ligation was used to produce tandem repeat units of 100 bp length (2). In the work presented here, a more simplified protocol for preparation of a 100 bp DNA ladder based on Wang work is described that is cost efficient and perfectly suitable for small laboratory settings with limited running budget.

MATERIALS AND METHODS

Bacterial strain and Culture. The required DNA template for PCR came from *Bacillus anthracis* Sterne 34F2, an avirulent strain safe to laboratory work that could be easily obtained from central and regional veterinary services. Using a sterile syringe 0.5 ml of a bottle content of Anthrax vaccine, manufactured by Razi Vaccine & Serum Research Institute, Iran, was aspirated and used to inoculate a trypticase soy agar (TSA) plate (10 cm). Incubation of the plate at 37°C continued for 16 hours when a test tube of trypticase soy broth was subsequently inoculated by a loopful of the fresh culture for a further overnight incubation at 37°C (10).

DNA Extraction. Bacterial genomic material was prepared using the Fermentas genomic DNA purification kit (Product No: 0512, Fermentas, USA) according to the manufacturer's recommendations. In brief, 400 µl of lysis buffer was added to 200 µl of overnight bacterial TSB culture in a microfuge tube and the mixture was incubated at 65°C for 5 min. This was followed by addition of 600 µl of chloroform to the whole content. The tube was then inverted for 3-5 times and subjected to a round of centrifuge at 12,800 g for 2 min. The supernatant was transferred to a fresh microfuge tube and 800 µl of precipitation solution was added. The mixture was centrifuged again at 12,800 g for 2 min and supernatant was

removed. One hundred microlitres of NaCl solution plus 300 µl of cold ethanol (70%) were added and the mixture was kept at -20°C for 10 min. A final round of centrifugation at 12,800 g for 3-4 min was conducted on the mixture and the supernatant was removed. the end, the DNA pellet was air-dried.

To liquefy the extracted dried DNA, the pellet was redissolved in 100 µl of TE buffer and kept at refrigerator (4°C) or freezer (-20°C) for next short or long use respectively. Extracted DNA was quantified by spectrophotometer and also gel-electrophoresis.

PCR Primers. To enable PCR amplification of 10 fragments with precise lengths of 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 bp, Primer-BLAST software (11), available on <http://www.ncbi.nlm.nih.gov/tools/> was used to design 10 primer pairs. This was conducted in a way that a relatively short 10 Kb-long stretch of *Bacillus anthracis* Stern 34F2 genome carrying no repetitive elements such as VNTRs was used to include all the expected primers. On the grounds of commercial interests, nucleotide structure of the designed primers is not publicized here. Synthesis of the oligonucleotides was conducted at a collaborating laboratory (Macrogen, Korea).

PCR protocols designing and optimizations. For optimization, eight designated PCR protocols were used to assess impact of DNA content, MgCl₂ and primer concentrations in amplification specificity and yield. Besides, an identical gradient annealing phase ranging from 58 to 68 °C (58, 60, 62, 64, 66 and 68) was applied for all the 10 PCRs.

PCR reactions were performed with 1.5 or 3 µl of DNA template, 6 µl of PCR premix (2x Master Mix RED, Ampliquor, Denmark), 1.125 or 2.5 µl of each forward and reverse primers (5 pmol/µl), zero or 0.24 µl of MgCl₂ solution (50 mM) plus appropriate amount of double-distilled water to make up the final volume of 12 µl depends on the ingredient protocol used. The thermal conditions, provided by an Eppendorf thermocycler (Germany), were a single cycle of 95 °C for 5 min, 35 cycles of 95 °C (30 sec), 64 °C (30 sec), 72 °C (30 sec) plus a single cycle of 72 °C for 5 min.

Sequencing of PCR products. In order to authenticate accuracy of PCR amplifications, all the 10 amplicons were sequenced at the collaborating Macrogen laboratory. The returning data from

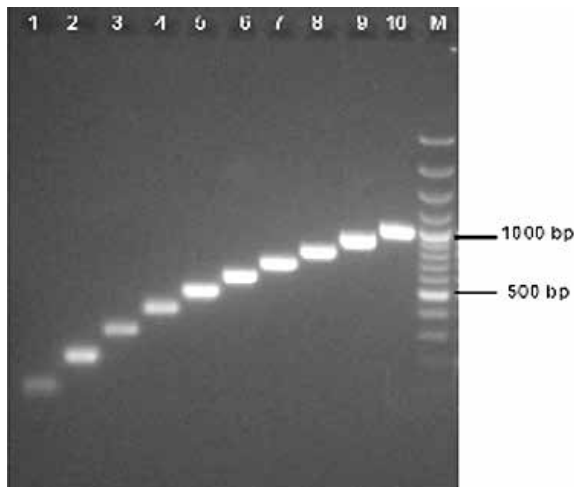


Fig. 1. Agarose gel (2%) electrophoresis representing PCR amplification of 10 constituent fragments of the 100-bp ladder. Lane 1 = 100 bp; lane 2 = 200 bp; lane 3 = 300 bp; lane 4 = 400 bp; lane 5 = 500 bp; lane 6 = 600 bp; lane 7 = 700 bp; lane 8 = 800 bp; lane 9 = 900 bp; lane 10 = 1,000 bp, Lane M = a commercial 100 bp DNA ladder (Product No. SM0323, Fermentas, USA). The 1,000 and 500 bp bands of the DNA ladder are specifically marked.

sequencing laboratory were viewed by Chromas and aligned by Clustal X (ver 2.0.11) against the corresponding stretches of *Bacillus anthracis* Sterne 34 F2 genome.

Formulation and preparation of the 100 bp ladder. Quantification of unpurified PCR products was done by spectrophotometry and confirmed by gel-electrophoresis (Fig. 1). Adjustments were performed on all of the 10 PCR products to reach the unique concentration of 10 µg/µl. To make up the 100 bp ladder, an identical proportion of 100, 200, 300, 400 bp fragments (7% each) plus appropriate amount of 500, 600, 700, 800, 900 and 1,000 bp fragments (13.3, 11.6, 10.4, 9.1, 7.8 and 18.9 % respectively) were transferred to a fresh microfuge tube. The non-purified content of this ready-to-use cocktail was subsequently mixed thoroughly and submitted directly to gel electrophoresis (Fig. 2).

In order to assess stability of the produced DNA ladder, shortly after preparation, eight microlitres of the ready-to-use preparation along with a corresponding amount of Fermentas 100 bp ladder was loaded to a ethidium bromide-stained 1.5% agarose gel and electrophoresed at 5 v/cm for 15 m. The ready-to-use solution was kept at 4 and -20 °C for next 7, 30 and 60 days post-preparation when its integrity was examined again by gel electrophoresis.

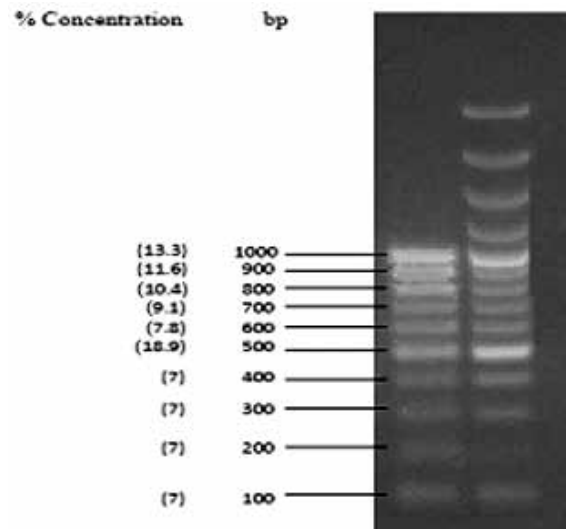


Fig. 2. Submission of 5 µl of the prepared DNA ladder to agarose gel (2%) electrophoresis. Figures on left depict the constituent fragments size in bp while numbers in brackets represent their proportional concentration in the DNA ladder preparation.

RESULTS

Altogether 480 individual PCR reactions representing 10 primer pairs targeting 10 DNA fragments were conducted. When results from gel-electrophoresis of amplification products from all reactions were cumulatively compared it was noticed, a combination of 1.125 picomol of each forward and reverse primers, a final concentration of 1 mM of MgCl₂ and as little as only 1 µl of DNA template produced the optimum mass of yield. Similarly, 63 °C proved as the optimal annealing temperature (Fig. 3). Nucleotide structure analysis of the 10 amplicons were identical to their matching stretches of *B. anthracis* 34F2 Sterne strain genome (data not shown). Storage of the ready-to-use DNA-marker solution with no kind of preservative material at either of 4 and -20 °C for 7, 30 and 60 days post-preparation resulted in no observable disintegrity of ladder preparation.

DISCUSSION

A major drawback of digestion-based protocols in production of DNA markers is their intrinsic impotence to produce stepwise fragments as size of digested DNA stretches is essentially pre-determined by restriction site position(s) of the selected enzymes. This justifies intentional selection of a PCR-based

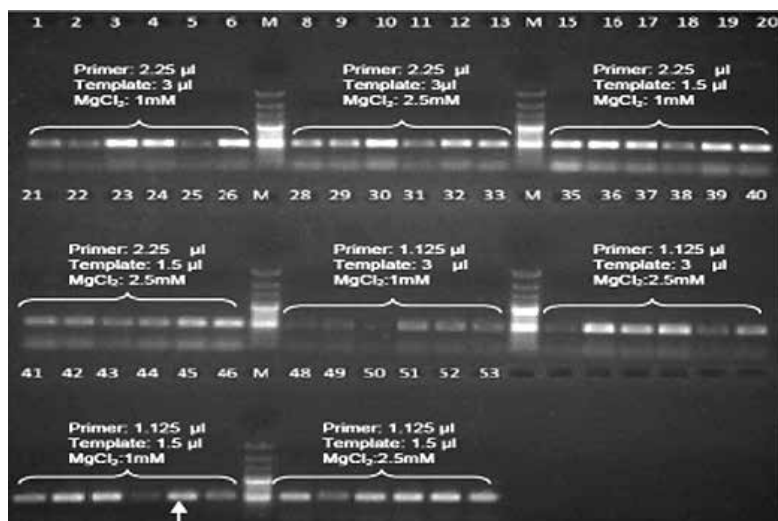


Fig. 3. Agarose gel electrophoresis of PCR products from 8 different amplification protocol sharing the same primer pair (500 bp DNA ladder fragment), template (genomic DNA from *Bacillus anthracis* 34F2 Sterne strain) and annealing temperature range (58-68 °C). Conditions applied to well No 45 (marked by arrow) were subsequently selected for optimum amplification yield. Numbers indicate individual PCR reactions with wells characterized by M representing DNA 100 bp ladder (Product No. SM0323, Fermentas, USA).

strategy for the present work.

In the original work by Wang, lambda DNA was used as template in amplification of DNA segments. Lambda phage is not a commonly available item in molecular biology laboratories therefore an alternative template, if available, will simplify the whole method. In this study, genomic DNA from the harmless vaccinal *B. anthracis* Sterne 34F2 strain has been used. Currently, a 100 ml anthrax vaccine bottle costs less than 0.25€ in the Iranian market and is readily available through central/regional veterinary offices and/or authorized vaccine dealers (12).

The observation that an eight-week long storage of the preservative-free, DNA marker solution at ambient temperature resulted in no observable disintegrity of bands was interesting as commercial DNA size markers are normally required to be kept at -20 °C for long-time storage. In contrast to many other bacteria such as *E. coli* and *Salmonella* spp. that their endonuclease content are typically involved in degradation of bacterial genomic material, this property in *B. anthracis* does not seem to be that active (13). Work on detrimental impacts of longer storage periods e.g 6 months or more on stability of the DNA size marker and suitability of application of an appropriate preservative, if necessary, is still ongoing at our laboratory.

The work presented here describes a simplified primer-directed PCR-based method to prepare a 100 bp DNA size marker using 10 discrete primer pairs

through a mutual amplification protocol. Unless intentionally required, there is no practical need for purification of PCR products as amplified fragments can be directly used in agarose gel electrophoresis. Economically, this domestically-developed DNA ladder is reasonably cost-effective as based on our rough estimation its cost-price is 7 to 15 times less than DNA ladders commercially available in the Iranian market.

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Contribution of authors

MS performed the laboratory work and prepared the first draft of this paper. RB, RG and GS participated in designing the primers and developing manuscript of the paper. NK helped with sequencing work. AJ and KT conceived the study plan and developed the paper. Authors have all read this paper and declared there is no conflict of interest between them.

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