Original Article

Easy method for production of a home-made DNA ladder in every laboratory

Mahdi Abbasian, Hadieh Alsadat Eslampanah Seyedi, Zahra Khalili Boroujeni, Mohammad Reza Mofid

Bioinformatics Research Center, Department of Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Molecular DNA markers are one of the essential tools in molecular biology labs with varied applications. In the present study, we suggest an efficient and available strategy to produce molecular size marker in routine laboratories.

Materials and Methods: To achieve the desired sizes of DNA fragments, we recruited PCR and bioinformatics techniques to synthesize 14 DNA fragments ranging from 100 to 3000 bp.

Results: Holistic analysis of different parameters in primers design resulted in amplification of fragments in just one PCR program without any by-product and purification step. Our applied method enables researchers to modify amplified DNA fragments by wide range of chemical modifications toward varied applications.

Conclusion: Method of home-made DNA ladder production by available ingredients and routine techniques reported in this study can be used in common laboratories for different applications.

Key Words: Click chemistry, DNA ladder, home-made, PCR, primer

Address for correspondence:
Prof. Mohammad Reza Mofid, Department of Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Bioinformatics Research Center, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: mofid@pharm.mui.ac.ir
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INTRODUCTION

Determination of the molecular weight (mw) or the base pair (bp) length of nucleic acids is an essential urge in the molecular biology. This need comprises a wide range of nucleic acids weights or lengths in size ranging from mega bp down to very short oligonucleotides of only a few bp. Nucleic acid mw standards are useful tools for estimating the quality, size, and quantity of the nucleic acid sample. A standard is typically fractionated in parallel with the sample, and following detection a comparison is made between the sample band(s) and the bands of the standard. Knowing the standard size allows the size of the unknown fragment(s) to be estimated.[1] Typically, standard DNA markers are classified in two types I: Molecular weight markers and II: Molecular size markers or DNA ladders. Following reported methods of marker production are reviewed.

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Generally, the mw markers have been produced by enzyme digestion of *E. coli* plasmids[2] or genomic DNA of bacteriophages.[1,3-5] However, Barvish *et al*. described a method for preparation of a mw standard from a completely different DNA source, *Tenebrio molitor* genomic DNA, using *Eco* RI digestion.[6] Banding pattern of such DNA markers

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in electrophoresis is non-uniform while the size (ranging from one hundred to several thousand of nucleotides) and number of bands depend on the frequency of recognition site(s) for the enzyme(s) used in digestion.[7]

In contrast to type I DNA markers, molecular size markers (DNA ladders) have numerous bands that regularly increase in size, even intervals. Also, the bands of DNA ladder show an equivalent intensity upon staining.[7] Commercially produced DNA ladders are routinely generated through three basic strategies:

1. Ligation of uncloned subunits into concatemers;
2. Partial restriction digestion of a vector and insert, where the insert is composed of concatemerized subunits; and
3. Partial restriction digestion of an excised insert, composed of concatemerized subunits, without the plasmid DNA.[8]

Recently, some strategies have been reported in the literature based on different PCR techniques.[9-14] Currently, DNA ladders are commercially available from different vendors including Sigma, Pharmacia, Life Technologies, Promega, Boerhinger-Mannheim, Amersham, New England Biolabs, Stratagene, fermentas and Invitrogen with varied characters.

In spite of different methods that have been introduced in production of DNA ladder to date, there is not an easy and flexible technique that enables researchers in common laboratory to produce a DNA ladder with desired migration patterns and labeling strategies. In previous studies, some drawbacks such as tedious plasmid construction, limitation in length and number of amplified fragments have still remained. Additionally, in some studies expensive and time-consuming purification steps have been used for production of DNA ladder.

In this report, we introduced a method based on combination of PCR and bioinformatics techniques while all PCR ingredients (but not DNTPs) were produced in our laboratory. We first generated a *Taq* DNA polymerase and all essential buffers for PCR reaction in our own laboratory, and then synthesis of 14 DNA fragments ranging from 100 to 3000 bp were conducted using limited number of primers. PCR fragments were amplified with high qualification without any by-product and finally merged together to make DNA ladder without any purification step. The produced DNA ladder is flexible to be modified through either 5'-modified primer sets, or labeled nucleotides based on click chemistry technology toward variety of molecular biology applications.

MATERIALS AND METHODS

Vectors and biochemicals

The vectors used as a DNA template were pUC57 (2710 bp circular DNA) and pET-21 (5443 bp circular DNA) from Genscript and Novagen, respectively. The biochemicals ($MgCl_2$, and DNTPs) were purchased from Fermentas.

Design of primers

To amplify exact DNA fragment lengths, a set of 17 oligonucleotide including 6 forward and 11 reverse primers were designed using Oligo v.5 software, on the basis of the known pUC57 and pET21 sequences. Thermodynamic details of primer dimers were considered by the Oligo v.5 software. The sequence of the primers with their characters and the corresponding expected amplified products have been displayed in Tables 1 and 2, respectively.

5x PCR master mix and reagents

To perform PCR reactions we prepared a home-made 5x master mix containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% Glycerol, 0.08 % NP-40, 0.05% Tween-20, 67 units/ml *Taq* Polymerase pH 8.6 at 25°C in 1x reaction.

Taq DNA polymerase expression in *Escherichia coli* and purification

The amplified *Taq* DNA polymerase encoding fragment was cloned into pET-15b expression vector in our laboratory (unpublished data). rpET-15b containing *Taq* polymerase encoding DNA was transformed into expression host BL21 (DE3) and subsequently induced with IPTG to produce recombinant *Taq* DNA polymerase.[15] Optimum production occurred with the addition of 0.75 mM IPTG for 3 h. Recombinant enzyme was harvested from bacterial[16] and supplied for PCR amplification in storage buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20 and 50% glycerol. The electrophoresis (SDS-PAGE) of *Taq* polymerase enzyme was performed using 12% polyacrylamide gel with Coomassie blue R-250 for gel staining.[15]

PCR condition

PCR amplification was done in a total volume of 30 µl. Each reaction consisted of 1X master mixed with 10 pM of each primer per reactions and 10 ng of template plasmid. PCR was performed under the following conditions: Initial denaturation at 95°C for 4 min followed by 35 cycles of at 95°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 80 sec, and final extension at 72°C for 10 min.

Table 1: Some important thermodynamic parameters analyzed by the oligo v.5 software to evaluate optimum primers. Primer length (mer), annealing temperature (T_m), 3' stability (DG), upper-lower, upper-upper, lower-lower duplex formation at 3' and 5'

Number of primer	Primer sequence 5'→3'	Primer characters
F1	TGGCGGGTGTCG	12 mer/T_m: 60.9/3' DG= -8.4/Duplex: -3.6 at 5'/No false priming site
F2	AAATACCGCATCAGG	15 mer/T_m: 55.9/3' DG= -8.2/Duplex: -3.6 at 5'/No strong false priming site
F3	GATGTGCTGCAAGG	14 mer/T_m: 54.7/3' DG= -8.5/Duplex: -6.9 at 3'/No false priming site
F4	TATGTAGGCGGTGCT	15 mer/T_m: 56.7/3' DG= -7.9/Duplex: -3.6 at 3'/No false priming site
F5	CTATTTCGTTCATCCA	16 mer/T_m: 52.9/3' DG= -8.1/Duplex: -3.6 at 5'/No strong false priming site
F6	GCACAATCTTCTCG	14 mer/T_m: 50/3' DG= -8.4/Duplex:-3.6 at 5/'No strong false priming site
R1	TGACCATGATTACGC	15 mer/T_m: 54.3/3'DG:-9.0/Duplex: -5.3 at 5'/No strong false priming site
R2	AACCGCCTCTCC	12 mer/T_m: 52.1/3' DG= -7.9/Duplex:-3.6 at 5'/No strong false priming site
R3	ACAGAAAAGCATCTTACG	18 mer/T_m: 57.0/3' DG= -7.8/Duplex: -3.6 at 3'/No strong false priming site
R4	ATCCCGTATTGACGC	15 mer/T_m: 58.8/3' DG=-9.6/Duplex: -4.9 at 3'/No strong false priming site
R5	CCAGGGGGAAACG	13 mer/T_m: 60/3' DG= -8.7/Duplex: -5 at 5'/No strong false priming site
R6	AGATCGCTGAGATAGGTG	18 mer/T_m: 59.3/3' DG= -7.9/Duplex: -4.7 at 5'/No strong false priming site
R7	AAACGCCAGCAAC	13 mer/T_m: 54.3/3' DG= -8.2/Duplex: -3.2 at 5' No strong false priming site
R8	TTAAAAGGATCTAGGTGA	18 mer/T_m: 53.6/3' DG= -7.9/Duplex: -4.8 at 5'/No strong false priming site
R9	CGGTCGGGCTGA	18 mer/T_m: 59.7/3' DG= -8.2/Duplex: -3.6 at 5'/No strong false priming site
R10	TCCGAAGGTAACTGG	15 mer/T_m: 55.8/3' DG= -7.9/Duplex: -3.6 at 5'/No strong false priming site
R11	AGGCAACTATGGAT	14 mer/T_m: 47.7/3' DG=-8.1/Duplex: -3.1 at 5'/No strong false priming site

Table 2: Length of expected fragments based on primer pair used in this study

PCR product length (bp)	Primer pairs
100	F3 and R1
200	F2 and R1
300	F1 and R1
400	F2 and F5
500	F1 and F5
600	F2 and R7
700	F4 and R9
800	F1 and R5
900	F4 and R10
1000	F4 and R4
1200	F3 and R8
1500	F1 and R6
2000	F1 and R3
3000	F6 and R11

Visualizing and analysis of PCR products

The amplification products were mixed with 1x loading buffer (10mM Tris-HCl (pH:7.6), 10 mM EDTA, Glycerol 10%, 0.005% bromophenol blue) and separated using 1.5% agarose gel in 0.5X TBE buffer and stained with ethidium bromide visualized using a Fluor-S Multi Imager. The mw of each band was estimated by comparing with a co-migrating known size DNA ladder 100 bp (SMO321, Fermantas).

RESULTS

Design of primer

In the present study, a set of 16 primers were designed [Table 1] to amplify 14 fragments [Table 2] from the two universal vectors pUC57 and pET21a and subsequently amplicons used as a DNA ladder. In this report, appropriate primers were designed by the oligo v.5 software. In order to achieve the specific amplification, we tried to avoid formation of bothering structures: Mismatching, mispriming, hairpin formation and primer-primer dimmers. Primers were selected with average length of 12-18 bp with annealing temperature between 50ºC and 60ºC and the 3' ΔG above −7.8.

Production of *Taq* DNA polymerase

In our laboratory the encoding DNA of *Taq* polymerase enzyme was cloned and placed in the pET15b expression vector to obtain large amounts of *Taq* DNA polymerase.[17] Production of *Taq* DNA polymerase in *Escherichia coli* BL21 (DE3) cells was induced by incubation with isopropyl-b-D-thiogalactopyranoside (IPTG). SDS-PAGE analysis of cell extract showed a prominent polypeptide band of the expected molecular mass [Figure 1, lane 2] and the recombinant *Taq* DNA polymerase was purified according to Desi Method[18] [Figure 1, lane 3]. The activity of the obtained enzyme was measured by comparing the intensities of the produced DNA bands in PCR reactions. Results well showed an obtained active and stable enzyme which could be used in our laboratory for PCR reactions. Finally, produced recombinant enzyme was added to home-made master mix for the next step.

PCR amplification and DNA ladder

We were able to optimize the PCR amplification for all fragments at similar conditions using 6 forward primers and 11 reverse primers to generate fragments ranging from 100 bp to 3000 bp. Totally 14 fragments were amplified with their specific primer pairs in separated tubes under one PCR program. Each 100 and 2000 bp fragments were synthesized using pUC57

as a template, while 3000 bp fragment was made using pET21 as a template. The amplified PCR fragments were quantified by spectrophotometer and the bands size were estimated by gel electrophoresis along with a co-migrating commercial 100 bp ladder (Fermentas) in 1.5% TBE agarose gel [Figure 2]. The prepared DNA ladder bands were clear and could be seen under UV light. Finally, the PCR products were mixed together to create the DNA ladder via calculation of the precise concentration for every band [Figure 2].

DISCUSSION

It is well established in the art that ladders can be produced by partial restriction digestion of synthetic plasmids which contain tandem repeats of a DNA fragment.[1,8,9,18,19-24] The main advantage of DNA marker production based on artificial plasmids is that DNA markers can be generated in an industrial scale through *E. coli* fermentation with a variety of simple processing techniques.[23,24] But such ladders suffer three drawbacks: Only the tandem repeat portion of the plasmid generates the ladder, the largest DNA fragment is confined to the size of the tandem repeat, and the upper bands containing the vector region of the plasmid do not contribute as useful bands to the ladder.[20,21] To overcome this problem, Hyman *et al*. developed a method utilizing the entire plasmid to generate useful DNA fragments, having a maximum fragment size equal to the entire plasmid size, and not producing a by-product region of upper bands.[20,21] Some disadvantages of these methods are referring to the tedious cloning experiments which must be done to construct the vector with several tandem repeats besides the irreproducibility of restriction digestion resulting in variable yield each time. On the other hand, the production efficiency of small DNA fragments (<500 bp) by digestion of synthetic vector seems poor.[14]

Due to powerful capacity of polymerization and convenient manipulations of polymerase chain reaction, PCR is a alternatively common choice for the preparation of small DNA fragments in the biological companies and molecular labs.[14] Therefore, generation of DNA ladder using the PCR technique is one of noticeable strategies. Three methods based on PCR can be used for DNA ladder production:

1. Single PCR amplification of desired DNA bands and then mixing the purified PCR products at certain concentrations,[10-12,25]
2. Multiplex PCR,[13]
3. Combination of PCR and restriction digestion.[14]

Amills *et al*. introduced marker primer directed synthesis (MPDS) to allow the synthesis of DNA ladders.[10] This method involves the amplification of each DNA target using two sets of primers to produce a 6 bp ladder ranging from 90 to 204 bp. However, these approaches proved time consuming, requiring many primers, specific template and adjusting PCR condition to produce all the desired bands. Chang *et al*. described a PCR-synthesized marker (PSM) method for synthesizing 100 bp DNA markers. They attempted to amplify all designed fragments by only one pair of primer in a series of synthetic plasmid template harboring different length insert.[12] But for many routine laboratories, construction of desired synthetic plasmids to ladder production are not satisfying methods.

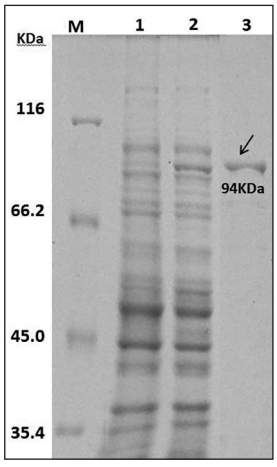

Figure 1: SDS-PAGE analysis for verification of expression and purification recombinant *Taq* DNA polymerase. M: Protein marker, lane 1: Total soluble protein extracted from *E. coli* before addition of IPTG, lane 2: Soluble extracted protein from *E. coli* 3 h after addition of IPTG, lane 3: purified *Taq* DNA polymerase

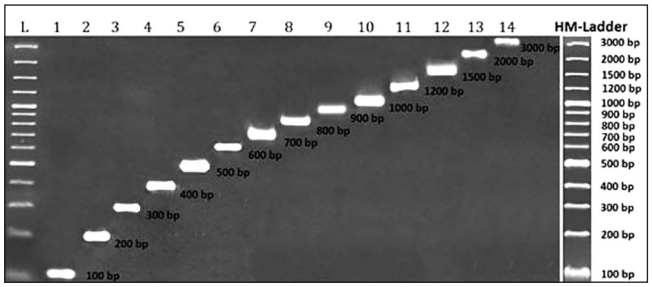

Figure 2: Agarose gel analysis for verification of PCR products ranging from 100 to 3000 bp along with mix of entire amplified fragments after precise estimation of each band concentration to make final ladder. The image show high quality and quantity of each fragment which resulted in so clear DNA ladder called as HM ladder. Produced ladder located on the right image in contrast to Fermentas ladder located on the left image displays high quality. L: 100 bp plus DNA Ladder, SMO321 Fermantas, lane 1-14: 100-3000 bp amplified fragments under the annealing temperature 54°C in similar PCR condition without nonspecific fragments, HM: Home-made DNA ladder synthesized by mixing amplified fragments based on appropriate concentrations

Also, demand for ladders with different capacities is one of the interesting aspects in production of ladder. In this report, we used simple PCR technique and homemade DNA polymerase to amplify a model migration pattern (100-3000 bp) from universal templates without further nucleic acid purification. To design every efficient primer sets, parameters such as 3' stability, upper-lower, upper-upper, lower-lower duplex formation (specifically strong duplexes formed at 3' duplexes avoided) and false priming was considered [Table 1].

Use of the PCR technique for ladder production enables us to apply any chemical modification and label amplified fragments. Recently, a wide variety of companies have offered a complete set of oligonucleotides chemical modification at 5' or 3' ends. Also, recent developments in DNA click chemistry have created powerful tools for attaching any favorite label to DNA fragments.[26-28] Ladder production based on our method is capable to be used with varied click reactions in order to different applications. For example, this strategy enables researchers in diagnostic medicine and plant pathologist to innovate rapid detection kits based on PCR technique. The mentioned method can be followed via attaching both ladder DNA fragment and marker related amplified fragments to a high-sensitive fluorescent dye in order to detect minimal amount of DNA. Moreover, attaching ladder DNA fragment to an eye visible dye enables researcher's online monitoring DNA migration on gel electrophoresis.

CONCLUSION

The purpose of this study is to introduce a rapid and cost-effective method in production of DNA ladder which is applicable for routine laboratories. Our produced ladder has potential to be developed toward other molecular applications by click chemistry. Moreover, most of the essential components for synthesizing DNA ladder were made in our own laboratory. Therefore, produced DNA ladder in this project is entitled as "Home-made DNA ladder with flexible application".

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