PBRU SCIENCE JOURNAL
2022.19(1) : 64-71

การใช้พีซีอาร์ในการทำดีเอ็นเอมาตรฐานต้นทุนต่ำ

Low Cost PCR Based DNA Ladder

ประดิพันธ์ ทองแถม ณ อยุธยา* และ จุฑามาส กาญจนวราภรณ์
Pradipunt Thongtam na Ayudhaya* and Jutamas Karnjanawaraporn
สาขาวิชาชีววิทยา คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏเพชรบุรี
Department of Biology, Faculty of Science and Technology, Phetchaburi Rajabhat University
*Corresponding author; E-mail: pradipunt.t@gmail.com
Received: 05 July 2021 /Revised: 16 August 2021 /Accepted: 03 November 2021

Abstract

DNA ladder has been widely used to determine the size of DNA fragments by electrophoresis in routine molecular biology laboratories. Commercial DNA ladders are typically used for comparing the size and estimating the DNA concentration of unknown DNA sample. However, it is quite expensive. Here, we developed a simple strategy for the preparation of 100 bp DNA Ladder which comprises the 12 designed primers to amplify 100 bp to 1,000 bp and 1,500 bp DNA fragments from the vector (pRGEB32) as a template for polymerase chain reaction (PCR). All primers could be used for amplification of all individual fragments in the same PCR profile. Therefore, the total running time of PCR was reduced. In addition, there were no undesired PCR products and the requirement of the purification step. Our procedure for production of the DNA ladder was simple and time saving. The total cost was also inexpensive compared with those of the commercial ones. This result indicates that it can be used for molecular studies.

Keywords: DNA Ladder, PCR, vector, base pair, primer

Introduction

DNA molecular weight markers, also called DNA ladders, are the essential molecular chemicals in molecular laboratory. They permit important determination of molecular weight or the base pair (bp) length of nucleic acid fragment sizes in a wide variety of experiments including restriction enzyme digestions, polymerase chain reaction (PCR) amplifications, Northern and Southern blotting, and every application that operates on basic gel electrophoresis. The standard nucleic acid, especially DNA marker, is helpful to

approximate the size, quality, and quantity of the nucleic acid sample. A set of standard DNA ladder with known molecular weight is used to estimate the size of the unknown DNA by comparing the distance or relative mobility (Rf) of the sample bands and those of ladder bands. The fixed standard band size can be estimated the size of the sample fragment. Typical 100 bp ladders afford fragment increments from 100 bp to 1.5 kilobase (kb), and 1 kb ladders spread from 250 bp to several thousand base pairs.

The DNA ladder has been fractionated by restriction enzyme digestion of plasmids [1,2] or genomic DNA of bacteriophages [3,4] or small size plasmid [5] Generally, the commercial DNA ladders are generated within three simple approaches, Firstly, a partial restriction digestion of a vector and insert, where the insert is made of fractionated subunits. Secondly, a ligation of uncloned subunits into the concatenated fragment. Finally, a partial restriction enzyme digestion of excised insert, composed of fractionated subunits, without plasmid [5] Presently, there are numerous DNA ladders that are commercially existing from various sellers. However, the commercial DNA ladder is rather expensive in Thailand.

In this research, we described a method based on combination of PCR, an uncomplicated, effective and appropriate method used to generate numerous amounts of amplicon, and simple primer designation to produce 100 bp DNA ladder with a discoverable vector in laboratory and it is the potential and useful protocol to produce the low-cost DNA ladder marker for laboratory use nowadays.

Materials and Methods

Primer design

Primer pairs in this experiment were designed conferring to CRISPR-Cas9 vector (pRGEB32) which was kindly gifted from Yinong Yang (Department of Plant Pathology and Environmental Microbiology and the Huck Institutes of the Life Sciences, Pennsylvania State University [6]) and further applied in our laboratory. A forward primer sequence was acquired from M13 promoter sequence, a universal forward primer with the name of M13 promotor from pUC vector on the reverse strand. Eleven reverse primers were manually designed subsequently from 100 bp to 1.5 kb.Then all melting temperature of reverse primers were determined by OligoAnalyzer (www.idtdna.com/pages/tools/ oligoanalyzer) and synthesized from Bionics (Korea). Moreover, the landing locations of reverse primers were selected from base positions of 9,186 to 10,687 which were referred from the universal forward primer. The primer position and sequence information are shown in (Figure 1) and (Table 1), respectively.

Figure 1. Schematic of primer location on pRGEB32. A part of the selected site of the circular vector begins from 9,186 to 10,687. The forward M13/pUC Rev serves as the universal forward primer and the reverse primers were designed subsequently.

Amplification of ladder amplicons and visualization

The PCR reagents were composed of 10 µl of 2X PCR master mix (Biotechrabbit, Germany), 0.2 µl of primer (10 µM), and 1 µl of pRGEB32 vector (0.5 ng/µl) in a final volume of 20 µl for 11 tubes of different reverse primer. PCR condition was performed under the following cycles: Initial denaturation at 95°C for 3 min followed by 20 cycles of denaturation at 95°C for 30 sec, annealing temperature, was an average among of all primer melting temperatures minus with five, at 59°C for 30 sec, extension at 72°C for 60 sec, and final extension at 72°C for 5 min. Three microliters of each PCR product were mixed with 1 µl of 10X redsafe dye (Intronbio, Korea) before subjecting to DNA electrophoresis with 1.5% agarose gel in 1X TAE buffer. The PCR products were visualized by using MaXiDoc (Daihan, Korea). The fragment size was estimated by comparing with the known size of the commercial 100 bp DNA ladder (Intronbio, Korea).

Table 1. The characteristic features of primers evaluated by OligoAnalyzer™

Primer name	Primer sequence 5' → 3'	Fragment size (base pair)	Melting temperature (°C)
M13/pUC Rev	AGCGGATAACAATTTCACACAGG	- (Forward primer)	65.8
100R	GATCTGTTCGTATGTTTAAAGATTCC	100 (M13/pUC Rev + 100R)	61.7
200R	CATCTGATTCCTCCAAGATCC	200 (M13/pUC Rev + 200R)	62.4
300R	TTGCTGGTAGCACCAGTAGAAGAC	300 (M13/pUC Rev + 300R)	65.4
400R	GGCTTCATGGCCCACTAC	400 (M13/pUC Rev + 400R)	63.2
500R	GCTTTGATCTATGTGGATAGCCG	500 (M13/pUC Rev + 500R)	65.5
600R	CTTGCTATTTCTAGCTCTAAAACCG	600 (M13/pUC Rev + 600R)	62.2
700R	ACCTGCAGGCATGCAC	700 (M13/pUC Rev + 700R)	62.4
800R	GTTGGTCGCCGTTAGGAC	800 (M13/pUC Rev + 800R)	63.2
900R	AAGATAGGTTTAAGGGTGATCCAAA	900 (M13/pUC Rev + 900R)	63.6
1000R	GCCGATCTCCTCTATATATGGTTCA	1,000 (M13/pUC Rev + 1000R)	64.9
1500R	CAAATCACAACCATCAGATCTACAAC	1,500 (M13/pUC Rev + 1500R)	64.3

Results

Ladder amplification

We optimized PCR amplification condition and successfully created 100 bp DNA ladder with 11 fragments ranging from 100 to 1,500 bp. All 11 fragments were generated by using only one forward primer and 11 reverse primers under the same PCR cycle and profile. The amplified PCR fragments were estimated by gel electrophoresis along with the commercial 100 bp ladder (Intronbio, Korea) in 1.5% agarose gel (Figure 2). The cocktail DNA ladder was pooled from all individual fragments of 100 bp to 400 bp for 2.5 µl each and 1 µl for the rest. The bands were sharp and visualized under UV light. By the way, pooling of the 11 PCR products was produced the clear-cut DNA ladder but they were still not suitable to use for DNA concentration measurement.

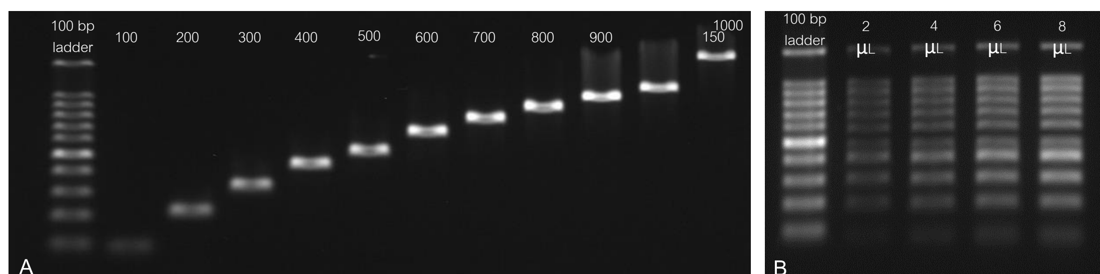

Figure 2. Agarose gel electrophoresis of amplified fragments; (A) individual fragment comparison with 5 µl of each PCR product, (B) comparison of the fragment cocktail quantity with 3 µl of commercial standard ladder on the left-handed well

Cost comparison

The budget of the created ladder in this study was composed of the ready to load 2X PCR master mix, which cost 720 THB per 50 reactions and was able to perform PCR reaction up to 2,500 µl from 1 vial (50 µl/reaction), the primer expensed of 2,500 µl PCR reactions was 15.12 THB, and the power consumption was 4.55 THB (Table 2). Consequently, the overall budget for the ladder in this study was only 728 THB and the price comparison among commercial ladders based on an available website is shown in (Table 3.)

Table 2. Cost calculation of ladder amplification

Ingredients	Cost	Overall reactions	Cost per one microliter
2X master mix	50 reactions = 720 THB	2,500 µl	0.288 THB
Primers	9 THB x 270 bases = 2,430 THB	15.12 THB/ 2,500 µl reaction	0.006 THB
Power consumption*	1.4 kW x 3.25 THB = 4.55 THB	4.55 THB/ 2,500 µl reaction	0.001 THB
		Total	0.295 THB

* Power consumption has been calculated from maximum power of thermal cycler (Biorad T100) for 2 hrs.

Table 3. Cost comparison between PCR-based ladder (this study) and the available commercial DNA ladders from different manufacturers (Exchange rate in Febrauary, 7th 2021)

Commercial brand	This study	Enzynomics*	Biotechrabbit*	Thermo*	Sigma-aldrich*	Promega*
Catalog no.	-	DM001	BR0800201	SM0243	D3687	G8291
Price (THB)	739.67/2,500 µl	1,200/500 µl	2,500/500 µl	2,560/500 µl	3,268/375 µl	2,903/300 µl
Price per µl	0.295/ µl	2.40/ µl	5.00/ µl	5.12/ µl	8.71/ µl	9.68/ µl

* 7% Vat. excluded

Discussion

As a result of effective ability of polymerization and convenient directions of polymerase chain reaction, PCR is an ordinary option for the generation of small DNA fragments in the biological companies and molecular laboratories [7] Hence, the production of DNA ladder using the PCR technique is one of perceptible strategies. Commonly, there are three methods based on PCR, which can be used for the production of DNA ladder. The first method is the authentic PCR amplification of preferred DNA bands and then blend together at accurate concentrations [8,9]. Second method is multiplex PCR, which is quite complicated since the numerous primers caused dimers and interfere results [10]. Third method is the combination of PCR and restriction digestion [7].

The former DNA ladder invention processes made use of bacteriophage and digestion of plasmids with restriction enzymes to produce DNA fragments; however, the

disadvantage was the inability to generate regular sizes of the fragments [1]. Although the construction of synthetic plasmids containing restriction sites for the generation of 100 bp DNA ladder has been described before [11], it is however a complex, laborious technique with a limitation for large-scale production. To solve the drawback of restriction enzyme based method, several PCR based methods have been operated in conjunction. In this study, all primers were designed on the basis of pRGEB32 sequences which are located at 9,186 bp to 10,687 bp and the amplicons can be directly amplified from the backbone of pRGEB32. Therefore, the size of 1,500 bp of PCR product also acts as the co-template with pRGEB32. The usage of only 1,500 bp is a good idea for performing each fragment but it needs to be purified and easy to degrade. The enormous amount of linear constructed DNA was quickly made by PCR reaction, providing enough materials for reproduction of DNA ladder. Actually, 1 ml of PCR product can produce 300 runs of DNA 100 bp ladder for electrophoresis on agarose gels or 600 runs on 12% acrylamide gels [12]. This means our ladder is able to produce up to 500 runs on agarose gels and up to 1,000 run on acrylamide gels. We recommend that loading volume of the mixture of 4-6 μl is enough to observe. In case of the procedure of PCR, we tried on the multiplex PCR; however, the result was unsatisfied since the non-specific amplicons from primer -dimer were appeared. As PCR products showed the difference in sizes and concentrations, so we have to mix them in an altered volume. From this trouble, the PCR performance in each size should be precise and easy to handle. Evidently, this scheme was quite simple, time saving, and especially inexpensive. For example, the price of 100 bp DNA ladder for 500 μl is listed 1,200 THB (Enzymomics), 2,500 THB (Biotechrabbit), 2,560 THB (Thermo Scientific), and lower volume from Sigma-aldrich and Promega are more expensive (3,268 THB and 2903 THB, respectively). We calculated that our procedure consumes only 0.295 THB per microliter while the commercial ladders are described in Table 3. 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 marker that is different from the commercial markers (directly amplified from bacterial DNA).

Conclusions

Our report in this paper is a simple and flexible procedure for the preparation of the 100 bp DNA ladder that contains 11 fragments ranged from 100 to 1,500 bp with the discoverable plasmid in laboratory and 12 primers. Not only pRGEB32, other known sequence plasmids could be used to design the complementary primers following our scheme to diminish cost certainly. Furthermore, our strategy could be applied to create the different varieties of DNA ladders, which will be useful for most molecular studies.

References

1. Polyarush SV, Egamberdiev SS, Mansurov DR, Azimova SS. Preparation of DNA markers based on *E. coli* plasmid DNA. Chemistry of Natural Compounds 2003;39:592-4.
2. Henrici RC, Pecen TJ, Johnston JL, Tan S. The pPSU Plasmids for Generating DNA Molecular Weight Markers. Scientific Reports 2017;1(2438). Doi:10.1038/s41598-017-02693-1.
3. Parker RC, Watson RM, Vinograd J. Mapping of closed circular DNAs by cleavage with restriction endonucleases and calibration by agarose gel electrophoresis. Proceedings of the National Academy of Sciences of the United States of America 1977;74(3):851-5.
4. Cooney CA. Techniques and high-resolution DNA size markers for pulsed field gel electrophoresis. Molecular Biotechnology 1994;2:119-27.
5. Abbasian M, Seyedi HA, Boroujeni ZK, Mofid MR. Easy method for production of a home-made DNA ladder in every laboratory. Advanced Biomedical Research 2015;4(70). Doi:10.4103/2277-9175.153894.
6. Xie K, Minkenberg B, Yang Y. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proceedings of the National Academy of Sciences of the United States of America 2015;112(11): 3570-5.
7. Wu J, Ye C. Tandem PCR: A novel and efficient unit amplification model for the preparation of small DNA fragments. Molecular Biology Reports 2011;38:2729-31.
8. Amills M, Francino O, Sánchez A. Primer-directed synthesis of a molecular weight marker. Genetic analysis 1996;13:147-9.
9. Chang M, Wang JH, Lee HJ. Laboratory production of 100 base pair DNA molecular

weight markers. Journal of Biochemical and Biophysical Methods 2008;70:1199-1202.

10. Wang TY, Guo L, Zhang JH. Preparation of DNA ladder based on multiplex PCR technique. Journal of Nucleic Acids 2010;421803. Doi:10.4061/2010/421803.
11. Rashno M, Shapouri MRSA, Jolodar A. Construction of a synthetic vector for preparation of a 100 base pair DNA ladder. Iran Journal of Biotechnology 2012;10:106-10.
12. Lan VTT, Loan PTT, Duong PAT, Thanh LT, Ha NT, Thuan TB. Straightforward Procedure for Laboratory Production of DNA Ladder. Journal of Nucleic Acids 2012; Doi:10.1155/2012/254630.