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SHORT COMMUNICATION

Construction of DNA ladder based on 16S rRNA gene of *Bacillus subtilis* using touchdown PCR technique

Tran Gia Huy*, Nguyen Thi Kieu Anh and Nguyen Thi Lien

Biotechnology Research and Development Institute, Can Tho University, Vietnam.
Email: tghuy0603@gmail.com

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ABSTRACT

Aim: DNA molecular size markers or DNA ladders play a vital role in molecular biology laboratories where DNA electrophoresis experiments are usually conducted. This study aimed to produce a 100 bp DNA ladder at laboratory scale, which could be applied to determine the size of DNA fragments in molecular biology experiments.
Methodology and results: In this study, 14 primers including 4 forwards and 10 reverses were designed based on the 16S rRNA gene sequence of *Bacillus subtilis*. These primers were able to amplify 10 DNA fragments with accurate sizes from 100 to 1000 bp. Furthermore, touchdown PCR was involved to maximize the specificity and yield of PCR products. Ten DNA fragments with the sizes including 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 bp were synthesized, and such bands were equivalent with commercial DNA ladders. Moreover, the quantity and quality of PCR products were measured using a nanodrop spectrophotometer. The optimal concentration ratios between such fragments (100-1000 bp) were 800, 300, 150, 150, 500, 50, 50, 50, 50 and 50 (ng/µL), respectively. These ratios showed the clear and high resolution on 1.5% agarose gel.
Conclusion, significance and impact of the study: The results indicated that 16S rRNA gene of *B. subtilis* was a potential material for DNA ladder preparation due to the multiple copies number of this gene. Furthermore, in combination with touchdown PCR, the nonspecific bands were reduced, and the products could be used directly without the need of purification step.

Keywords: Bacillus subtilis, 16S rRNA gene, 100 bp DNA ladder, touchdown PCR

INTRODUCTION

In recent years, it is apparent that molecular biology plays a central role in biotechnology and brings a variety of applications in many fields such as agriculture, food technology, medicine. Moreover, DNA electrophoresis is an essential tool to study about DNA and genes. DNA ladder is a mixture of DNA fragments with standard sizes, during the migration process through agarose or polyacrylamide, the lengths of PCR or restriction enzyme products are compared with ladder, so their size are determined. Both commercial and homemade sources of DNA molecular size markers carry advantages and disadvantages. The commercial DNA markers provide reference fragments in standard increments and are usually pre-packaged in quality-controlled concentrations, allowing quantitation of experimental samples by visual comparison. However, this convenience comes at expensive cost that cannot always be satisfied for developing nations. There are two popular methods for generating DNA ladder. The first one is digestion by restriction enzyme (Vemuri and Shama, 2015), this method is suitable to create DNA fragments with larger size; however, the dependence of restriction site leads to the inaccurate band size. Another approach is amplification of DNA fragment by using PCR (Abbasian *et al.*, 2015), multiplex PCR (Wang *et al.*, 2010). By this method, the sizes of DNA fragments are defined, such technique is an appropriate solution for preparation of DNA ladders, which have small range under 1000 bp. So far, DNA from plasmid of *Escherichia coli* (Henrici *et al.*, 2017) and bacteriophage genome (Gopalakrishnan *et al.*, 2010) were used as main materials for ladder preparation.

In this study, the 16S rRNA gene was achieved as an ubiquitous and inexpensive material for generating a 100 bp DNA ladder. The aim of this study was to synthesize a DNA molecular size marker including 10 bands (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 bp) from 16S rRNA gene of *Bacillus subtilis*.

*Corresponding author

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MATERIALS AND METHODS

Primer design

On the basis of the known 16S rRNA gene of *B. subtilis*, 4 forward and 10 reverse primers were designed by using Primer-BLAST program from NCBI in order to amplify 10 DNA fragments ranging from 100 to 1000 bp in length. The software programs used to analyze primer characteristics and specificity were NetPrimer (Premier Biosoft, USA) and Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast/) respectively.

DNA extraction

In this study, *B. subtilis* was isolated and identified by Hien *et al.* (2017). Bacteria were cultured in lysogeny broth medium until the cell density reached 10^7 CFU/mL. The procedure for DNA extraction was modified from Cetyltrimethylammonium bromide (CTAB)-based extraction method. In the initial stage of the process, bacterial biomass was resuspended in a solution containing TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), 10% SDS, and 20 mg/mL proteinase K. The mixture was incubated at 65 °C for 20 min. This was followed by the addition of CTAB solution. Afterwards, a mixture of chloroform and isoamyl alcohol (volume ratio, 24:1) was added. By centrifugation at 13,000 rpm for 10 min, the supernatant was collected and precipitated by the addition of isopropanol. A round of centrifugation at 13,000 rpm was carried out to keep the pellet, which was then washed with 70% ethanol twice. With the completion of this step, the DNA pellet was re-dissolved in 30 µL of Tris-EDTA solution and kept at -20 °C for further experiments. The purity, integrity and concentration of extracted DNA were verified using 1% agarose gel electrophoresis and nanodrop spectrophotometer.

Touchdown PCR amplification of ten DNA fragments

Ten DNA fragments were amplified by Touchdown PCR using 4 forward and 10 reverse primers. This method represents a useful technique to reduce non-specific PCR products and increase the specificity as well as the yield of specific ones. The amplification was brought about in a total volume of 25 µL. The final concentration of each components consisted of 10 ng/µL of DNA template, 5 µL of 5× Mytaq buffer (Bioline, England), 1 µL of each forward and reverse primers (10 pmol/µL), 0.25 µL of *Taq* DNA polymerase (MyTaq 2,500 U, Bioline, England), and an appropriate amount of deionized distilled water up to 25 µL. The PCR conditions were shown in Table 1 (Bio-rad C1000, USA). The amplification products were detected by 1.5% agarose gel at 150 V for 45 min, then quantified by using Nanodrop spectrophotometer (Thermo scientific 2000C, USA). The gel images and DNA band migration were analyzed by Quantity One software (Bio-rad, USA) version 4.6.3.

Combination and analysis of PCR products

The ten PCR products subsequently used to prepare the 100 bp ladder with the volume of 100 µL. It was involved to determine their optimal concentrations for sharper and more intense bands. The 500 bp band, which considered as a reference band, should be had higher concentration compared to the others. The detailed DNA concentrations between each combination were described in Table 2.

Table 1: Thermal cycle of Touchdown PCR procedure.

Phase 1	Stage	Temperature (°C)	Duration
1	Initial denaturation	95	3 mins
2	Denaturation	95	30 sec
3	Annealing	70 (decrement 1 °C/cycle)	30 sec
4	Extension	72	1 min
Repeat 10 cycles from 2 to 4			
Phase 2	**Stage**	**Temperature (°C)**	**Duration**
5	Denaturation	95	30 sec
6	Annealing	60	30 sec
7	Extension	72	1 min
Repeat 20 cycles from 5 to 7			
Phase 3	**Stage**	**Temperature (°C)**	**Duration**
8	Extension	72	5 min
9	Storage	10	30 min

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Table 2: DNA concentrations in 5 combinations to prepare DNA ladder.

Size (bp)	Combination 1 (ng/µL)	Combination 2 (ng/µL)	Combination 3 (ng/µL)	Combination 4 (ng/µL)	Combination 5 (ng/µL)
100	400	500	600	700	800
200	100	150	200	250	300
300	150	150	150	150	150
400	150	150	150	150	150
500	100	200	300	400	500
600	50	50	50	50	50
700	50	50	50	50	50
800	50	50	50	50	50
900	50	50	50	50	50
1000	50	50	50	50	50

Table 3: Combination of forward and reverse primers.

Combination	Product length (bp)	Number of pairing positions on *B. subtilis* genome*	Number of heterodimer**
F4 x R1	100	10	2
F4 x R2	200	10	3
F10x R3	300	10	0
F4 x R4	400	10	4
F9 x R5	500	10	2
F4 x R6	600	10	4
F8 x R7	700	10	4
F4 x R8	800	10	3
F9 x R9	900	10	3
F10 x R10	1000	10	0

*From the Ribosomal RNA Database, rrndb.umms.med.umich.edu
**Information were accessed from NetPrimer (Premier Biosoft, USA).

RESULTS AND DISCUSSION

Primer design

The remarkable result to emerge from the data is that only 4 forward and 10 reverse primers were used to amplify 10 desirable DNA fragments in the PCR reaction. This can be explained that some PCR products uses common primers, resulting in the 30% reduction of primer cost. Besides F7, which was only the forward primer for one band (700 bp), other forward primers had multiple roles. For example, F4 worked as a primer for the amplification of 5 bands (100, 200, 400, 600 and 800 bp) whereas F9 and F10 primers were responsible for amplifying 2 bands (500 and 900 bp for F9; 300 and 1000 bp for F10). As expected, these primers paired at 10 locations on the *B. subtilis* genome, which corresponds to 10 copies of the 16S rRNA gene (Tables 3 and 4).

During the pairing phase of the PCR reaction, although there are many DNA sequences that were highly homologous to primers, the primer only prefers to attach to the site to form a highly durable structure. The stability of this cohesion is measured by free energy △G, as described by Qu and Zhang (2015), the smaller the free energy, the stronger the bond between primer and DNA template. This study also shows that the stability of 3' end played an important role, because only when 3' end was completely attached to the template, *Taq* polymerase catalyzed the synthesis of polynucleotide chains in the extended period. The greater of 3' stability of the primer, the smaller of the △G value. As a result, it is better for annealing stage and the PCR product would be more easily amplified. However, if △G is less than -12 kcal/mol, the probability of occurrence of non-specific amplicons was increased. In contrast, the higher endurance of 5' end strengthened primer-template binding. Of the 14 designed primers, the endurance of 3 'is from -6.5 to -12.8 kcal/mol, which is within the acceptable range so that primers can be paired correctly at the priming position as well as the term non-specific amplification products.

Amplification of DNA fragments by using Touchdown-PCR

By applying of Touchdown PCR, 14 primers were successfully amplify 10 DNA segments with increasing size from 100 to 1000 bp as shown in Figure 1. There is no band in the negative control sample showed no contaminated DNA into the sample during manipulation. Based on the gel electrophoresis image, DNA bands of

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Table 4: Structural characteristics of 14 primers.

Code	Sequence (5'-3')	Length (bp)	Position*
F4	AGTTGGTGAGGTAACGGCTC	20	256-275
F9	CGGGGAGGGTCATTGGAAAC	20	631-650
F10	ACCTAACCAGAAAGCCACGG	20	494-513
F8	CCGCAATGGACGAAAGTCTG	20	375-394
R1	TCCCGTAGGAGTCTGGGC	18	355-338
R2	TTCCCTAACAACAGAGCTTTACGA	24	456-432
R3	CCTGTTCGCTCCCCACG	17	793-777
R4	CCCCAGTTTCCAATGACCCT	20	655-636
R5	AAGATCAAGGGTTGCGCTCG	20	1130-1111
R6	GGCGGAAACCCCCTAACA	18	855-838
R7	GACGACAACCATGCACCAC	19	1074-1056
R8	CTGTCACTCTGCCCCCG	17	1055-1039
R9	ACCTTCCGATACGGCTACCT	20	1530-1511
R10	AATCATCTGTCCCACCTTCGG	21	1493-1473

*Information were accessed from Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast/).

size corresponding to the 100 bp standard commercial scale bands could be found. The DNA bands were clear and there was no extra bandage and no self-priming primer phenomenon, suggesting that the thermal cycle of PCR is suitable for primers to pair specifically with the desired position on the template. High annealing temperature (70 °C) at the first cycle created the extreme condition for primer binding, so it contributed significantly to limit the ability of paring in non-specific sequences.

Through the brightness and size of the amplified products compared to commercial ladder, the quantity and specificity of PCR products was indicated (Figure 1). A considerable feature is the brightness of band 100 and 200 bp were weaker than other bands. According to Yilmaz *et al.* (2012), during the DNA migration process, small pieces of DNA were easily diffused through gel holes, resulting in the fuzzy band. To reduce this diffusion, increase the agarose concentration to about 2 to 3% will see 100 and 200 bp bands clearly.

By measuring the optical absorbance at a wavelength of 260 nm, DNA concentration of PCR products was determined from 5000 ng/µL, so the yield of amplified reaction was confirmed. Large copies of amplified target DNA fragments expressed by such DNA concentrations were diluted and mixing these products together (Table 5). The ratio A260/A280 was much higher than 1.8 (an accepted value for purified DNA) because there was a large amount of amplicons produced, which resulted in the increased absorbance at 260 nm. In other words, this parameter reflected the high yield of PCR products and did not show the poor purity. Additionally, the high ratio of A260/A230 illustrated that small amount of salt was present.

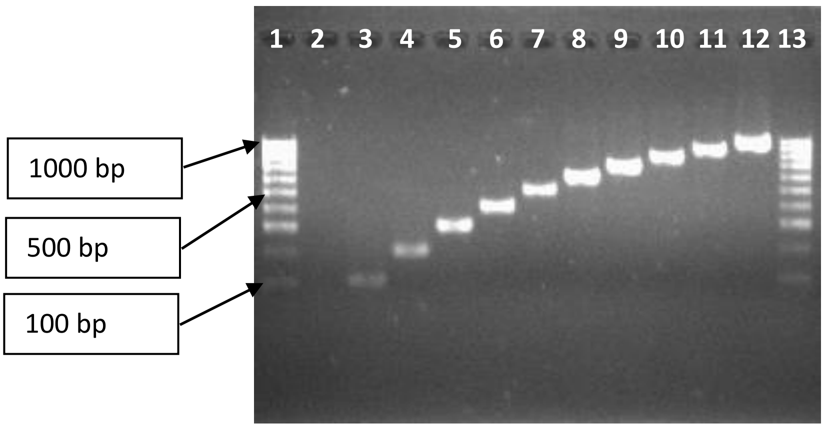

Figure 1: The gel pattern of ten PCR products with the size from 100 to 1000 bp on 1.5% agarose gel. Lane 1,13-Commercial 100 bp ladder (Bioline, England); Lane 2-Negative control; Lane 3-100 bp; Lane 4-200 bp; Lane 5-300 bp; Lane 6-400 bp; Lane 7-500 bp; Lane 8-600 bp; Lane 9-700 bp; Lane 10-800 bp; Lane 11-900 bp; Lane 12-1000 bp.

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Table 5: The DNA concentration and purity of PCR products.

PCR products	DNA concentration (ng/µL)	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{230}
100 bp	5722.7	114.454	1.948	58.77	11.09
200 bp	6233.4	124.667	2.110	59.10	11.22
300 bp	6263.7	125.273	2.040	61.41	11.50
400 bp	6242.8	124.855	2.058	60.68	11.35
500 bp	6417.4	128.348	2.045	62.75	11.63
600 bp	4082.8	81.656	1.448	56.40	11.38
700 bp	6138.2	122.764	1.916	64.06	11.76
800 bp	6032.8	120.657	2.105	57.31	10.90
900 bp	6465.2	129.305	2.077	62.24	11.57
1000 bp	6423.0	128.460	2.006	64.05	11.74

Combination of PCR products to form a complete DNA ladder

Ten amplified products with different concentrations were mixed for orientation to the increase the visualization of 500 bp band and 2 bands of small size with 100 and 200 bp. The results of electrophoresis (Figure 2) shows that Combination 5, which the concentrations of 100, 200, 500 bands are 800, 300 and 500 ng/µL, were optimal clearly displayed on the gel. In this combination, the band of 100 and 200 bp were visualized on the gel background. For the bands from 300 to 1000 bp in all combinations, such bands were bright visible and clearly separated. Based on the reference band (500 bp), the position of the other bands could be detected.

Thus, the molecular size of DNA is inversely proportional to the distance in the electric field but is directly proportional to the brightness of the band. This phenomenon can be explained by the interaction between the dye and DNA molecules, the larger the size can be associated with more dye molecules, so with the same DNA concentration but the larger size bands will be brighter than smaller bands. Another hypothesis should be considered is the DNA is negatively charged. When the electrophoresis moves in the electric field from negative to positive, the dye molecule has a positive charge, so it moves in the opposite direction. When electricity increases, the luminosity of the bands decreases due to partial loss of dye molecules, and the smaller dimmer bands will lose more (Lee and Bahaman, 2012).

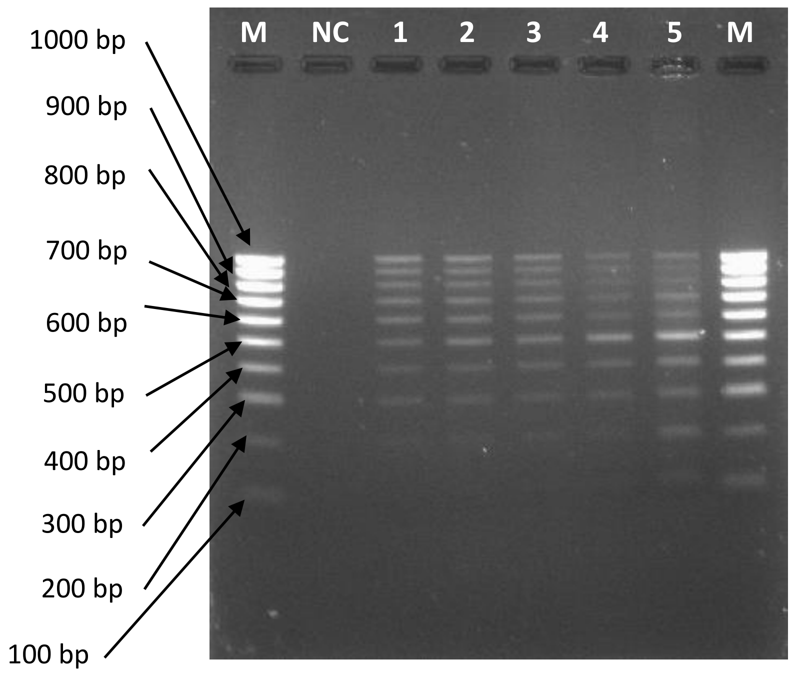

Figure 2: Band separation between five combinations on 1.5% agarose gel. M-Commercial 100 bp ladder (Bioline, England); NC-Negative control; 1-Combination 1; 2-Combination 2; 3-Combination 3; 4-Combination 4; 5-Combination 5.

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According to Slater and Noolandi's study (1989), the travel distances of nucleic acid molecules in the electric field correlate with their molecular sizes. Specifically, the movement distance of DNA fragments is inversely proportional to the decimal logarithm of the molecular size. By applying this relationship, it is possible to build the correlation lines of these two quantities. The comparison of two linear correlation lines between commercial ladder from Bioline (Figure 3A) and the ladder constructed in this study (Figure 3B) was carried out. The R^2 value from the former ladder was 0.9900 and the latter was 0.9795. It is indicated that the migration of ten bands between these two ladders had high uniformity. Therefore, the accuracy of this homemade ladder was strengthened.

In particular, the use of PCR technique to synthesize DNA fragments is more favorable than restriction enzyme method due to the independence on specific sites on DNA template and enzymatic requirements. On the other hand, mixing of PCR products helps the researchers to be able to alternate the concentration of PCR products, thereby changing the brightness of the DNA bands to match the experimental conditions. Moreover, the material source is *B. subtilis* 16S rRNA gene, which is a beneficial bacteria group and easily isolated for many laboratories.

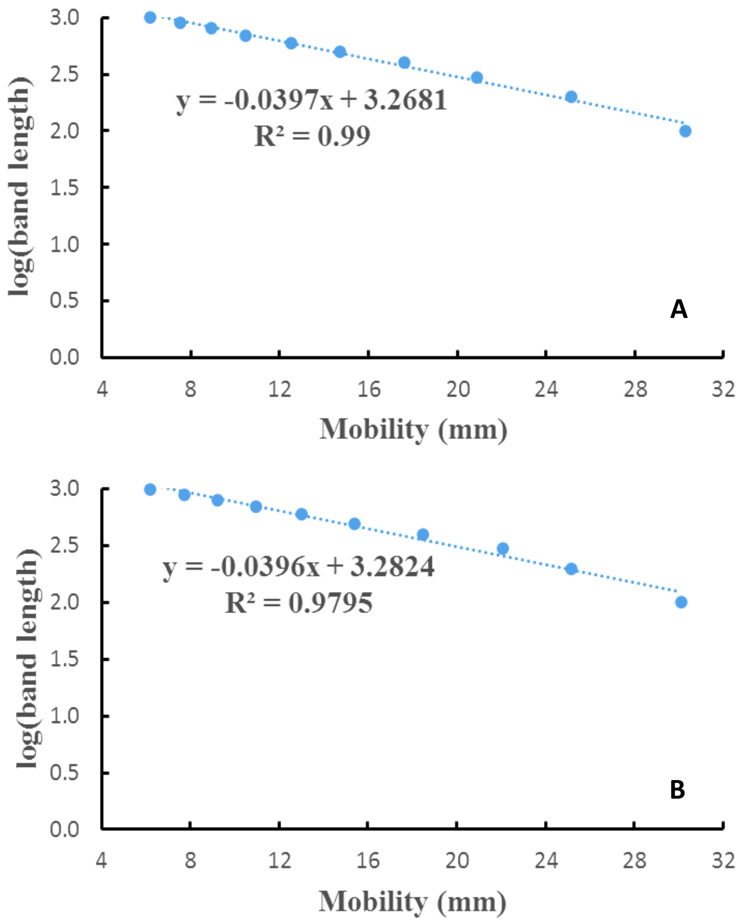

Figure 3: Mobilily plot of 100 bp DNA ladder from (A) commerical ladder from Bioline, England and (B) DNA ladder constructed in this study.

CONCLUSION

In this paper, based on 16S rRNA gene region of *B. subtilis*, 14 primers (4 forward and 10 reverse) were designed to synthesize 10 DNA amplicons ranging in size from 100 to 1000 bp. The success of this research originated from the use of Touchdown PCR technique to minimize non-specific amplification products as well as maintain the PCR yield, resulting in the formation of clearly separated bands. The present findings so far have been promising in commercial production due to the available material source, free of importing charge and reliability. Further experimental investigations are needed to increase the brightness of DNA bands and to determine their suitable storage conditions.

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