

Full Length Research Paper

Amplification of deoxyribonucleic acid (DNA) fragment using two-step polymerase chain reaction (PCR)

Chang-Qin Jing^{1,2}, Li Wang¹, Tian-Yun Wang^{1*}, Jun-He Zhang¹, Wei-Hua Dong¹ and Xiu-Hua Zhang²

¹Department of Biochemistry and Molecular Biology, Xinxiang Medical University, Henan 453003, China.

²Department of Life Sciences and Technology, Xinxiang Medical University, Henan 453003, China.

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Polymerase chain reaction (PCR), an essential tool in many fields such as molecular biology, normally comprises three steps: denaturation at a high temperature, annealing at a low temperature and elongation at a moderate temperature. Here, we report a two-step PCR method which incorporates annealing and elongation step for significant time-saving and reduction in reagent use. To investigate whether the two-step method is as useful as the common three-step method, different lengths of DNA fragments were amplified by two-step PCR and three-step PCR and the influence of incorporated temperature and the amount of Taq polymerases were performed in the present study. The results showed that, all the DNA fragments of 300 to 1000 bp could be amplified by two-step PCR method and the temperature from 50 to 60.8°C could provide a viable range for annealing/extension steps and the fidelity of the PCR has not been affected by the two step method presented. Taken together, the two-step PCR could be used to amplify DNA fragment, which is time-saving, reliable and inexpensive.

Key words: Polymerase chain reaction (PCR), two-step polymerase chain reaction, three-step polymerase chain reaction, time-saving.

INTRODUCTION

Polymerase chain reaction (PCR) has been widely used in many fields such as molecular biology, illness diagnosis, food detection, etc. (Saiki et al., 1985; Xiong et al., 2004; Umeda et al., 2010; Kawasaki et al., 2009; Yabuki et al., 2007; Sambrook and Russell, 2001). Generally, each PCR cycle comprises three steps, a 95°C step to denature the duplex DNA, an annealing step of around 55°C to allow the primers to bind and a 72°C elongation step. In practice, about 30 to 35 cycles are commonly used. So a complete PCR often takes more than two hours by this normal procedure (Sambrook and Russell, 2001). The annealing step and elongation step could be incorporated into one step which, along with the

denaturation step is named the two-step PCR method in many labs. The two-step protocol is designed for significant time-savings and a reduction in reagent use, relative to the standard PCR protocol. However, the knowledge of this method is limited now. In the present study, we investigated the influence of temperature and Taq polymerases on this method, whether different lengths of DNA fragments could be amplified by two-step PCR and the difference of DNA product quality produced by the two methods.

MATERIALS AND METHODS

PCR template and reagents

Enterobacteria phage lambda DNA (GenBank no: V00636) served as template and PCR reagents were all purchased from TaKaRa Biotechnology Company Limited (Dalian, China). Primers were designed according to the template gene sequence and synthesized by Shanghai Sangon Biological Engineering Technology and Co., Ltd (Shanghai, China) and their oligonucleotides sequences are shown in Table 1.

*Corresponding author. E-mail: wtianyuncn@126.com. Tel: 86-373-3029354.

Abbreviations: PCR, Polymerase chain reaction; **BLAST**, Basic Local Alignment Search Tool; **NCBI**, National Center for Biotechnology Information.

Table 1. Primers used in the experiment.

Length (bp)	Primer	Position
1000	5'-GCGGCACGGAGTGGAGCAAG-3'	6631
900	5'-GTTTCGATCCGAAAGGCTGGGCGCT-3'	6731
750	5'-CATCGTCGTGTATTCCGGACAGTA -3'	6881
600	5'-ACGCCTCTGCCCGTTACCCGAA-3'	7031
500	5'-GATGAGTTCGTGTCCGTACAACCTG-3'	7131
400	5'-CCGCTCGCTGGGTGAACAA-3'	7231
300	5'-ACGGATGAAACTGCCGGTCAGGACA-3'	7331
0	5'-GTTATCGAAATCAGCCACAGGGC-3'	7630

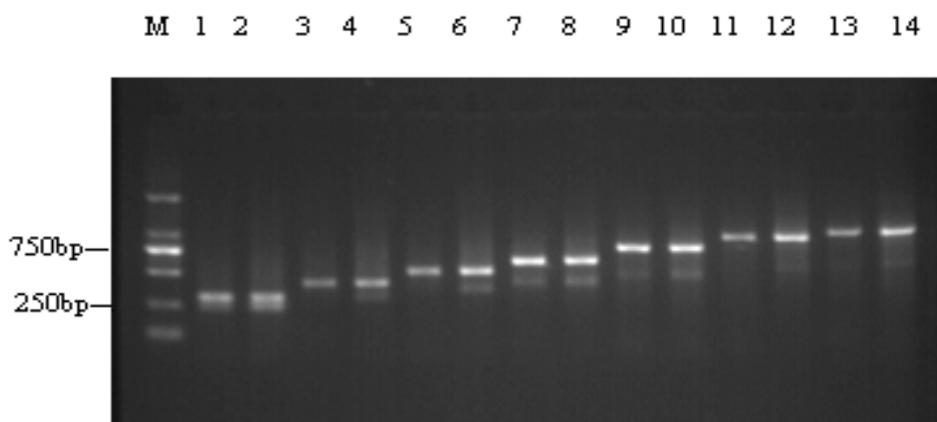


Figure 1. DNA fragments of different length amplified by two-step PCR and three-step PCR. M: DL2000 DNA Ladder; Lane 1 and 2:300 bp DNA fragment; Lane 3 and 4:400 bp DNA fragment; Lane 5 and 6: 500 bp DNA fragment; Lane 7 and 8:600 bp DNA fragment; Lane 9 and 10:750 bp DNA fragment; Lane 11 and 12:900 bp DNA fragment; Lane 13 and 14: 1000 bp DNA fragment. Lane 1,3,5,7,9,11,13: DNA fragments amplified by two-step PCR; Lane 2,4,6,8,10,12,14: DNA fragments amplified by three-step PCR.

DNA fragments amplified by PCR

Seven different lengths of DNA fragments were amplified by two-step PCR in comparison with that by three-step PCR. Reaction mixture (100 μ l) for each quantitative PCR contained 100 ng template DNA, 0.2 mM dNTP, 0.1 mM of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl (pH 8.8), 2.5 U Taq polymerase. The two-step PCR method contained the following procedure: Predenaturation for 3 min at 95°C and subjected to 30 cycles of 30 s denaturation at 94°C, 50 s step at 65°C, and 1 min extension at 65°C. The three-step PCR procedure was denaturation for 3 min at 95°C and subjected to 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C, 30 s elongation at 72°C and 1 min extension at 72°C.

Gradient denaturation temperature and Taq polymerases

The DNA fragment of 600 bp was amplified at a gradient of temperatures by two-step PCR method. The PCR system was prepared as previously described and the PCR procedure included denaturation for 3 min at 95°C and subjected to 30 cycles of: 30 s denaturation at 94°C, 50 s incorporated step at the gradient of 25 to 75°C. The DNA fragment of 600 bp was amplified at a gradient of amount of Taq polymerases by two-step PCR method. The PCR

procedure was as same as two-step PCR method described previously, while the PCR system included a gradient of Taq polymerases of 0.5, 0.4, 0.3, 0.2, 0.1, 0.08, 0.072, 0.064, 0.056 and 0.048 units, respectively.

Purified and sequence of amplified DNA fragments

After PCR, the results were detected by 2.0% agarose gel electrophoresis, then they were purified and sequenced using T7/SP6 primers synthesized by Shanghai Sangon Biological Engineering Technology and Co., Ltd (Shanghai, China). Basic Local Alignment Search Tool (BLAST) was carried out at the National Center for Biotechnology Information (NCBI) server ([http://www.ncbi.nlm.nih.gov/blast/ Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/)).

RESULTS AND DISCUSSION

DNA fragments amplified by two-step PCR procedure

As shown in Figure 1, the seven different DNA fragments from 300 to 1000 bp in length could be effectively amplified, although, the quantity of DNA fragments was less in

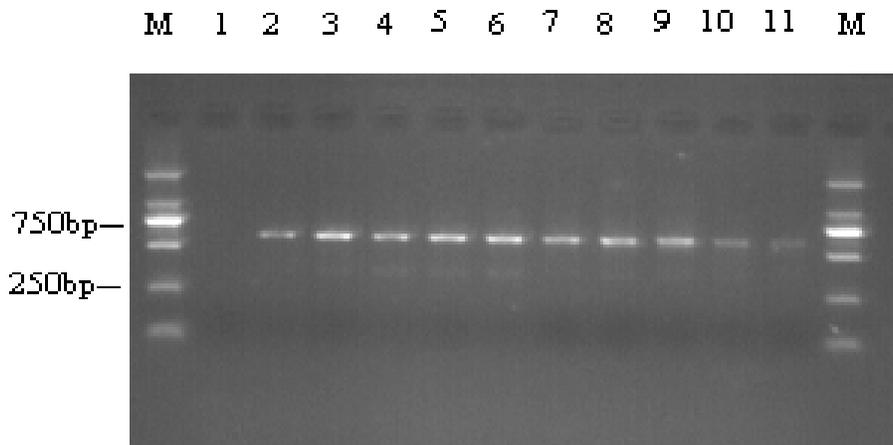


Figure 2. DNA fragments of 600bp amplified in a two-step gradient experiment with a temperature range of 25 to 75°C. M: DL2000 DNA Ladder; Lane 1: 75°C; Lane 2:70°C; Lane 3:65°C; Lane 4:60°C; Lane 5:55°C; Lane 6:50°C; Lane 7:45°C; Lane 8: 40°C; Lane 9:35°C; Lane 10:30°C; Lane 11:25°C.

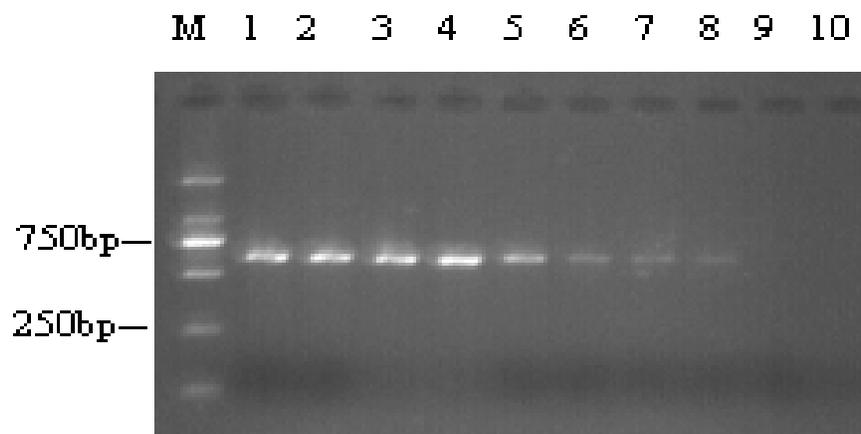


Figure 3. DNA fragments of 600 bp amplified at a gradient of amount of Taq polymerases by two-step PCR method. M: DL2000 DNA Ladder; Lane 1: 0.5U, Lane 2:0.4 U, Lane 3:0.3U, Lane 4:0.2 U, Lane 5:0.1 U, Lane 6:0.08U, Lane 7:0.072 U, Lane 8:0.064U, Lane 9:0.056U, Lane 10: 0.048 U.

two-step PCR compared with three-step PCR. In addition, time could be saved, two-step PCR took 62 min while three-step PCR only needed 87 min.

Gradient denaturation temperature and Taq polymerases

We amplified the 600 bp DNA fragment at gradient denaturation temperature and Taq polymerases. The results demonstrated that, 600 bp DNA fragment could be obtained by two-step PCR at temperatures as high as 70°C and as low as 35°C (Figure 2). The PCR was effective when the temperatures were from 50 to 65°C (Lane 3 to 6, Figure 2). Although, DNA fragments could be amplified at 30 and 25°C, the PCR amount diminished

greatly. In two-step PCR, annealing temperature and elongation temperature were quite adjacent, and the lower temperature was in favor of annealing, whereas, the higher temperature was fit for elongation, so the proper temperature will benefit both processes. In the present study, the large quantity of DNA could be produced at temperature from 65 to 35°C. The amount of DNA fragments will be less greatly and were not amplified when the temperature was beyond this range.

Optimum usage of Taq polymerase is necessary for PCR, too less could lead to the failure of PCR, but too much also could not benefit the results (Giovannelli et al., 2004; Al-Shanti et al., 2009; Abe et al., 2008; Wang et al., 2007). DNA fragments of 600 bp were amplified at a gradient of the amount of Taq polymerases by two-step PCR method (Figure 3). In 20 µl PCR system, the amount of

Enterobacteria phage lambda, complete genome
 Length=48502

Score = 1664 bits (901), Expect = 0.0
 Identities = 901/901 (100%), Gaps = 0/901 (0%)
 Strand=Plus/Minus

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Query  37  GGTTATCGAAATCAGCCACAGCGCTCCCGTTATTGCATTCTGCCAGGCCCGCTCTGT  96
      |||
Sbj ct 7630 GGTTATCGAAATCAGCCACAGCGCTCCCGTTATTGCATTCTGCCAGGCCCGCTCTGT  7571

Query  97  CATTTCCGCTGCCACACCGGCAGAGACAGAAACGCCGTTCCCGCCAGCACAAATGCCAC  156
      |||
Sbj ct 7570 CATTTCCGCTGCCACACCGGCAGAGACAGAAACGCCGTTCCCGCCAGCACAAATGCCAC  7511

Query  157  AGGTTTCATCCCGCGTGGCGTGAAGTGCATCAGTATGCAGCTTACCAGTGCCACGACCGT  216
      |||
Sbj ct 7510 AGGTTTCATCCCGCGTGGCGTGAAGTGCATCAGTATGCAGCTTACCAGTGCCACGACCGT  7451

Query  217  GACCAGTTCAGACGATCCAGAATCACGGTATCCGCTGCGCTGATCCACCTCATTTTC  276
      |||
Sbj ct 7450 GACCAGTTCAGACGATCCAGAATCACGGTATCCGCTGCGCTGATCCACCTCATTTTC  7391

Query  277  ATGTCGGTCAGACATTTTCCCGCTGAGAGGGGTGTCCTGACCGGCAGTTTCATCCGT  336
      |||
Sbj ct 7390 ATGTCGGTCAGACATTTTCCCGCTGAGAGGGGTGTCCTGACCGGCAGTTTCATCCGT  7331

Query  337  GTCATCAGCTCCTCTTTCAGCTCTGCCACACGGAGCGCCAGTCTTCTTTGTCGCCGT  396
      |||
Sbj ct 7330 GTCATCAGCTCCTCTTTCAGCTCTGCCACACGGAGCGCCAGTCTTCTTTGTCGCCGT  7271

Query  397  CAGGCTGACATCACGGTTCAGTTGTTCACCACGGAGCGGAGACCGGCAATCAGTTCATC  456
      |||
Sbj ct 7270 CAGGCTGACATCACGGTTCAGTTGTTCACCACGGAGCGGAGACCGGCAATCAGTTCATC  7211

Query  457  TTTGTCATGGACTCCTCCACAGAGAAACAATGCCCGGAGGCGCCATGATTACGCCAGT  516
      |||
Sbj ct 7210 TTTGTCATGGACTCCTCCACAGAGAAACAATGCCCGGAGGCGCCATGATTACGCCAGT  7151

Query  517  TGTAACGACACGAACTCATCAGGGTCAGCCAGCAGCATCAGGGTGCTGACTGAATCATG  576
      |||
Sbj ct 7150 TGTAACGACACGAACTCATCAGGGTCAGCCAGCAGCATCAGGGTGCTGACTGAATCATG  7091
    
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Figure 4B. Continued

0.2 units Taq polymerase was the most optimum usage.

Fidelity of the PCR

The fidelity of the PCR is very important in DNA fragment amplification (Wu et al., 2010). To investigate the fidelity of the two-step PCR method, we purified and sequenced the PCR-amplified 900 bp DNA fragment and analyzed

the sequence by BLAST on the web <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>. The results showed that, the PCR-amplified DNA was 100% homology with the template DNA (Figure 4), suggesting that two-step PCR method could amplify the DNA fragments reliably.

In conclusion, this experiment demonstrated that 300 to 100 bp DNA fragments could be amplified by two-step PCR and the temperature from 50 to 60.8°C, which provides a viable range for annealing/extension steps.

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Query 577 GTGAAGCTCAGCGCGCGGATCGCGGCGGTCACCCAGTTTTTGGGGTAAACGGGAGAGCGG 636
          |||
Sbj ct 7090 GTGAAGCTCAGCGCGCGGATCGCGGCGGTCACCCAGTTTTTGGGGTAAACGGGAGAGCGG 7031

Query 637 TTAATGCCCTTCGGCTGFGCGTCCGCATCCTGAATGCAGCCATAGGTGGCAGAACCGCGT 696
          |||
Sbj ct 7030 TTAATGCCCTTCGGCTGFGCGTCCGCATCCTGAATGCAGCCATAGGTGGCAGAACCGCGT 6971

Query 697 GCCTGAGTGTCCCGAGCAACCATCGTGTGTGCCGACGGAAGTCTTTTTGACCGCGT 756
          |||
Sbj ct 6970 GCCTGAGTGTCCCGAGCAACCATCGTGTGTGCCGACGGAAGTCTTTTTGACCGCGT 6911

Query 757 TCACGFACTGTCCGGAATACAGCAGATGCGCACATCGCCATACATCCCTTATAGGAC 816
          |||
Sbj ct 6910 TCACGFACTGTCCGGAATACAGCAGATGCGCACATCGCCATACATCCCTTATAGGAC 6851

Query 817 ACGCCTTTGCCAGGTCCTTACCGCTGCTCCAGCTCGGAATTAGAGCCAGCAACGGGTA 876
          |||
Sbj ct 6850 ACGCCTTTGCCAGGTCCTTACCGCTGCTCCAGCTCGGAATTAGAGCCAGCAACGGGTA 6791

Query 877 TCCAGCTTCTCCTTGACGGCTTTGAGGAACGGAAACAGCGCCACGCCITTCGGATCGAAC 936
          |||
Sbj ct 6790 TCCAGCTTCTCCTTGACGGCTTTGAGGAACGGAAACAGCGCCACGCCITTCGGATCGAAC 6731

Query 937 A 937
          |
Sbj ct 6730 A 6730
    
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B

Figure 4B. Continued

Two-step PCR, a thought provoking method, could reduce the time consume and improve the efficiency.

ACKNOWLEDGEMENTS

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