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Full Length Research Paper

Cloning the *Pfu* DNA polymerase from DNA contaminants in preparations of commercial *Pfu* DNA polymerase

Ji Hye Heo and Suhng Wook Kim*

Department of Biomedical Science, College of Health Sciences, Korea University, Seoul 136-703, Korea.

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The *Pfu* DNA polymerase gene was cloned from commercial *Pfu* DNA polymerase by semi-nested polymerase chain reaction (PCR) using a contaminant template in the commercial *Pfu* DNA polymerase and the activity of the *Pfu* DNA polymerase itself. We confirmed the occurrence of residual bacterial DNA in standard *Pfu* DNA polymerase preparations and demonstrated that the commercial enzyme can be used directly as a PCR template for cloning, thus eliminating the laborious and time-consuming steps of cell recovery and DNA extraction. A simple method was developed for the purification of *Pfu* DNA polymerase by the heat-mediated (100°C, 10 min) lysis of *Escherichia coli* and denaturation of *E. coli* proteins, exploiting using the extremely thermostable properties of *Pfu* DNA polymerase, followed by chromatography on heparin columns. The whole purification process could be achieved within 2 h using one type of buffer, with no need for sonication or the use of toxic reagents. This advantageous alternative method is convenient and faster than previously reported methods.

Key words: *Pfu* DNA polymerase, DNA contamination, cloning, purification.

INTRODUCTION

Pfu DNA polymerase is an archaeal family B or human α-like DNA polymerase isolated from *Pyrococcus furiosus*, a hyperthermophilic archaeon that grows optimally at 100°C (Uemori et al., 1993). The thermostable *Pfu* DNA polymerase is commonly used in the polymerase chain reaction (PCR), which is one of the most powerful techniques in molecular biology used for *in vitro* amplification of DNA. *Pfu* DNA polymerase has a strong 3′ to 5′ exonucleolytic activity, in addition to its 5′ to 3′ DNA polymerizing activity, and is able to correct errors introduced during substrate polymerization (Cline et al. 1996). Based on the 3' to 5' proofreading exonuclease activity, *Pfu* DNA polymerase has superior fidelity compared to thermostable *Taq* DNA polymerase, which lacks the 3′ to 5′ proofreading exonuclease activity (Lundberg et al., 1991). The error rate for *Pfu* DNA polymerase is 7 to 10-folds lower than that of non-proofreading *Taq* DNA polymerases (Barnes, 1994; Flaman et al., 1994).

Since the frequent use of *Pfu* DNA polymerase for higher fidelity PCR often results in the need for large amounts of the enzyme, it is of some interest to develop a simple and rapid method by which an individual laboratory can easily purify large quantities of *Pfu* DNA polymerase for routine use. Therefore, the development of a rapid and effective cloning and purification protocol for *Pfu* DNA polymerase is of practical significance and commercial interest.

The presence of contaminating DNA in commercial preparations of *Taq* DNA polymerase has been convin-cingly established (Bottger, 1990; Corless et al., 2000; Hughes et al., 1994; Newsome et al., 2004; Rand and Houck, 1990; Schmidt et al., 1991). Most thermostable DNA polymerases are expressed as recombinant proteins in *Escherichia coli* harboring expression vectors. However,

*Corresponding author: E-mail: swkimkorea@gmail.com. Tel: 82-2-940-2811. Fax: 82-2-917-2388.

expression vectors are generally present in multiple copies and the products are usually contaminated with residual DNA from *E. coli* as a result of incomplete purification (Chiang et al., 2005; Perron et al., 2006; Tondeur et al., 2004). It is likely that during thermostable DNA polymerase purification, the DNA containing the polymerase gene is not completely removed, and it should be feasible to amplify the polymerase gene by PCR.

In this study, to develop a rapid and easy cloning and purification protocol, the *Pfu* DNA polymerase gene was amplified from a commercial *Pfu* DNA polymerase as a template by semi-nested PCR. Subsequently, recombinant *Pfu* DNA polymerase was purified by heat-mediated lysis of *E. coli* and its activity was analyzed.

MATERIALS AND METHODS

Purification by heat-mediated lysis

E. coli strain BL21(DE3) was transformed with pET-*Pfu*NC. Large-scale cultures of *E. coli* containing the pET-*Pfu*NC plasmid were initiated by adding 2 mL of an overnight culture to 400 mL of Luria-Bertani (LB) medium with ampicillin (50 µg/mL). These cultures were grown at 37°C for 2 h, and then isopropyl-β-D-thiogalactoside was added to a concentration of 0.5 mM. After 5 h of induction, the bacteria were harvested by centrifugation for 5 min at 4000 *g*, and the cell pellet was stored at -70°C until use. For protein purification, the cell pellet was resuspended in 50 mM Tris-HCl (pH 8.0) buffer. The suspension was incubated in a water bath at temperatures of 75 or 100°C for different periods (10 to 40 min). After heat-mediated cell lysis, the cell debris and denatured proteins were removed from the lysate by centrifugation at 16000 *g* for 10 min at 4°C. The supernatant was applied to a HiTrap Heparin HP column (Amersham Biosciences, Piscataway, NJ) and washed with 50 mM Tris-HCl (pH 8.0) buffer. The column was eluted by a 0-500 mM KCl gradient in 50 mM Tris-HCl (pH 8.0). Fractions containing *Pfu* polymerase, detected by activity assay using PCR, were pooled and mixed in a 1:500 volume ratio with buffer containing 1 M Tris-HCl (pH 8.0), 1 M dithiothreitol, 0.1 M ethylenediaminetetraacetic acid (EDTA), and Tween-20. After the addition of equal amounts of glycerol, the sample was stored at −20°C. The protein concentration was determined by a Bio-Rad *DC* protein assay kit (Bio-Rad, Hercules, CA).

Activity assay

The activity of the purified *Pfu* polymerase was determined using a multiplex PCR amplification assay by titration against the commercial enzyme preparation (Stratagene, Santa Clara, CA). For the enzyme activity assay, the commercial and purified *Pfu* polymerase was diluted appropriately with 1× *Pfu* storage buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% Tween-20 and 50% glycerol) before adding it to the reaction mixtures. Titration of the enzyme was performed in 20 µL reaction mixtures with 1 µL of diluted enzyme added. The reaction mixtures consisted of 1 × commercial PCR buffer, 0.25 µM each of the two primer pairs (5'-GATGAGTTCGTGTCCGTACAACTGG-3' and 5'-GGTTATCGAAATCAGCCACAGCGCC-3'; 5´-TTCAGGCGGCGCATTTTTATT-3´ and 5´-ACGTCGATGACATTTGCCGTA-3´) that amplify 500 and 300 bp fragments corresponding to nucleotides 7131 to 7630 and 30,537 to 30,836 of the bacteriophage Lambda genome, respectively, 0.2 mM each dNTP, and 10 ng of lambda genomic DNA. Amplification involved initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 25 s, primer annealing at 60°C for 25 s, and extension at 72°C for 30 s. After PCR cycling was completed, 10 µL aliquots of the PCR products were analyzed by electrophoresis in 2% agarose gels.

RESULTS

Cloning of *Pfu* DNA polymerase from commercial *Pfu* DNA polymerase

We first attempted to clone *Pfu* DNA polymerase gene from commercial *Pfu* DNA polymerase sources. Our method included two steps. First, two primers covering the entire *Pfu* DNA polymerase gene were hybridized to a contaminant template DNA in the commercial *Pfu* DNA polymerase, and then amplified using the activity of the commercial *Pfu* DNA polymerase itself. Next, the first-round PCR products were amplified by a second-round semi-nested PCR step.

Based on the DNA sequence of the *Pfu* DNA polymerase (accession no. D12983), two primers - *Pfu*-N (5′-ATGATTTTAGATGTGGATTACATAACTG-3′) and *Pfu*-C (5′-CTAGGATTTTTTAATGTTAAGCCAGG-3′) - were synthesized and used for the first-round PCR reaction. The primer pairs were designed to anneal to upstream and downstream sequences just inside the polymerase gene and to amplify the full reading frame of *Pfu* DNA polymerase.

First-round PCR reaction was performed in a DNA thermal cycler (Corbett Research, Sydney, Australia) with a 50 µL reaction mixture containing 0.2 mM of each dNTP, 0.5 µM of each primer (*Pfu*-N and *Pfu*-C), and 2.5 units *Pfu* in 1 × *Pfu* buffer supplied with enzymes. The cycling conditions consisted of an initial denaturation step for 5 min at 95°C, followed by 60 cycles of 40 s at 95°C, 40 s at 55°C, 5 min at 72°C, and an additional 10 min for final elongation at 72°C.

In the second-round PCR reaction, two primer pairs were used: *Pfu*-Nde (5′-GGGAGCCATATGATTTTAGATGTGGATTACATA-3′, the *Nde*I recognition site is underlined) and *Pfu*-1001R (5′-CCATAAAGGTTGTCCAACTAATCTTG-3′), and *Pfu*-911F (5′-CCTTGAGAGAGTTGCCAAATACTCG-3′) and *Pfu*-Sal (5′-TCTATCGGTCGACTAGGATTTTTTAATGTTAAGCCA-3′, the *Sal*I recognition site is underlined). The primer pairs *Pfu*-Nde and *Pfu*-1001R were designed to amplify a 1035 bp fragment of the 5' end of the coding region of the *Pfu* DNA polymerase gene, and the primer pairs *Pfu*-911F and *Pfu*-Sal were designed to amplify a 1429 bp fragment of the 3' end of the coding region of the *Pfu* DNA polymerase gene. Following the first-round PCR reaction, two PCR fragments were amplified by semi-nested PCR using the first-round product as the template and the two primer pairs *Pfu*-Nde and *Pfu*-1001R, and *Pfu*-911F and *Pfu*-Sal. Semi-nested PCR reactions were performed

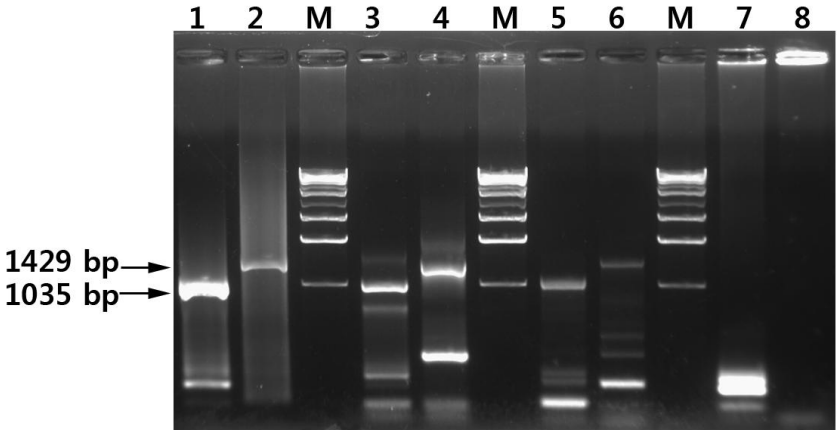

Figure 1. PCR amplification of *Pfu* DNA polymerase gene fragments from commercial *Pfu* DNA polymerase. *Pfu* DNA polymerase gene fragments were amplified by PCR from commercial *Pfu* DNA polymerase from company A (lanes 1 and 2), company B (lanes 3 and 4), and company C (lanes 5 and 6). PCR products were run on a 1% agarose gel with 0.5x Tris-acetate-EDTA buffer. Lane M, DNA size marker (1 kb ladder); lanes 1, 3, 5 and 7 contain PCR products using primer pair *Pfu*-Nde and *Pfu*-1001R, corresponding to the 5' half of the *Pfu* polymerase gene (1035 bp); lanes 2, 4, 6 and 8, PCR products using primer pair *Pfu*-911F and *Pfu*-Sal, corresponding to the 3' half of the *Pfu* DNA polymerase gene (1429 bp); lanes 7 and 8, PCR products after DNase I digestion of the commercial *Pfu* DNA polymerase before use as PCR template.

using 2.5 units of *Pfu* DNA polymerase in a 50 µL reaction volume of PCR buffer, 0.5 µM of each primer, 0.2 µM of each dNTP and 1 µL of the first-round PCR reaction product. The cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 40 s, 60°C for 40 s, 72°C for 3 min, and a final extension period of 72°C for 10 min.

After amplification, the PCR products were visualized in 1% Tris-acetate-EDTA (TAE) agarose gels and bands of the correct size (1035 and 1429 bp) were purified. The two gel-purified fragments were stepwise introduced into the expression vector pET21(b) (Novagen, San Diego, CA). The purified 1035 bp PCR fragment containing the 5' end of the coding region of the *Pfu* polymerase gene was digested with *Nde*I and *Eco*RI, and cloned into the *Nde*I/*Eco*RI site of the expression vector pET21(b) (Novagen), resulting in pET-*Pfu*N. Then, the purified 1429 bp fragment containing the 3' end of the coding region of the *Pfu* polymerase gene was digested with *Eco*RI and *Sal*I, and cloned into pET-*Pfu*N digested with the same enzymes to make pET-*Pfu*NC. The sequence and the site of insertion were verified by restriction analysis and DNA sequencing.

The developed method was tested for the cloning of *Pfu* DNA polymerase from commercial *Pfu* DNA polymerase from three different companies. When *Pfu* DNA polymerases from three different companies were tested, the desired two PCR products corresponding to the 5' half of the gene (1035 bp) and 3' half of the gene (1429 bp) were both observed (Figure 1). Although the desired fragments were accompanied by several DNA bands from non-specific amplifications, the amplification products could be easily purified from the agarose gel.

To show that the PCR products are amplified from the contaminating DNA template in the commercial *Pfu* DNA polymerase, and not from contaminating DNA at our laboratory, we treated the commercial *Pfu* DNA polymerase with DNase I before use as the PCR template. We could not detect the PCR products (Figure 1, lanes 7 and 8).

The purified amplification products were inserted into the pET21(b) vector to obtain the recombinant pET-*Pfu*NC plasmid containing the full-size *Pfu* DNA polymerase gene. When the nucleotide sequence of this construct was confirmed by sequencing analysis to ensure that the reading frame was correct, there were no discrepancies, except for two silent mutations.

Rapid purification by heat-mediated lysis

Two heating temperatures (75 and 100°C) for different periods (10, 20, 30 and 40 min) were used for a comparison of the effects of heat-mediated lysis for the purification of the *Pfu* DNA polymerase. The results from denaturing polyacrylamide gel electrophoresis (PAGE) (Figure 2) revealed *Pfu* DNA polymerase of the expected size (90 kDa) in the lysis buffer following the lysis of *E. coli* at both temperatures and at all heating times. A period of 10 min at 100°C seemed to be the most appropriate because it resulted in a high yield of *Pfu* DNA polymerase with nearly no contamination with other proteins (Figure 2B, lane 1). Longer times (20 to 40 min)

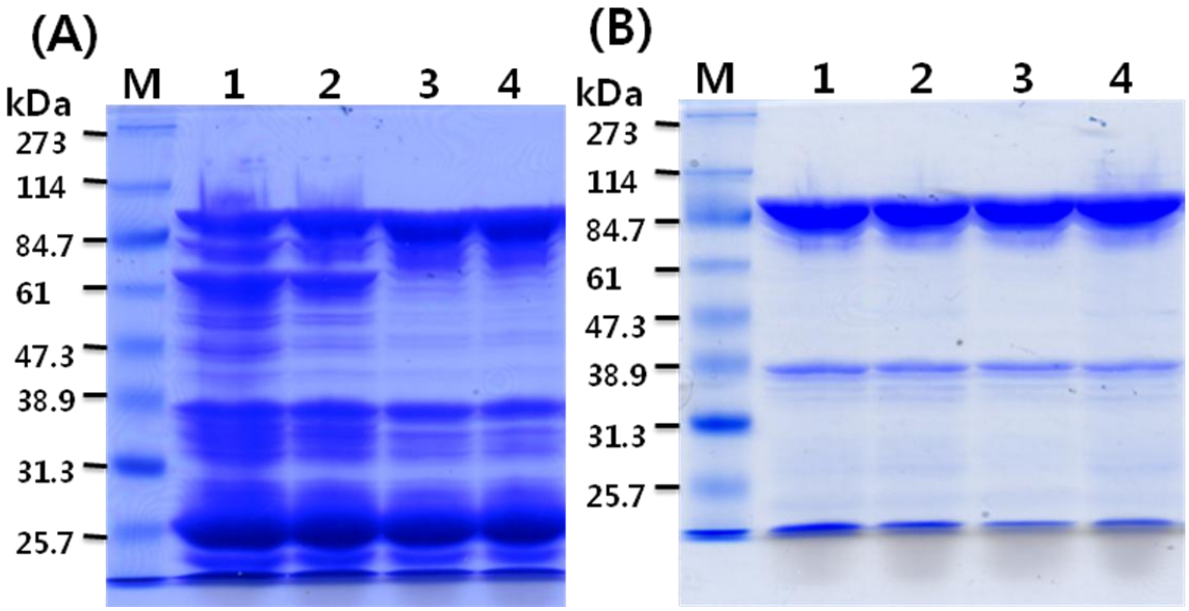

Figure 2. SDS-PAGE analysis of the heating-lysed *Pfu* DNA polymerase. Two heating temperatures of 75°C (A) and 100°C (B) were set for a comparison of the effects using heat-mediated lysis for the purification of the *Pfu* DNA polymerase. Lanes 1 to 4, the *Pfu* DNA polymerase remaining in the supernatant after heating for 10, 20, 30 and 40 min, respectively. M, Molecular weight markers.

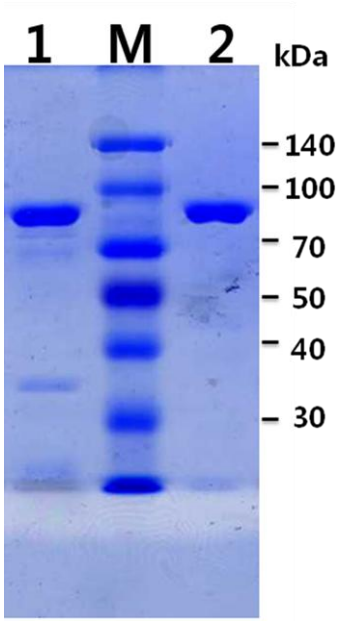

Figure 3. SDS-PAGE analysis of the fractions obtained by purification of *Pfu* DNA polymerase. SDS-PAGE was performed under reducing conditions in 10% gels and the proteins were visualized by Coomassie Brilliant Blue R-250 staining. M, Molecular weight markers; 1, cleared lysate after heat treatment; 2, proteins eluted from heparin column.

did not appreciably increase the yield (Figure 2). Furthermore, longer incubation at 100°C could destroy the stability of *Pfu* DNA polymerase. *Pfu* DNA polymerase was further purified by chromatography on heparin columns. The purified *Pfu* DNA polymerase was homogeneous as judged by 10% sodium dodecyl sulfate-PAGE (SDS-PAGE), where a single band with an apparent molecular mass of 90 kDa was observed (Figure 3).

Assessment of enzyme activity

The activity of the purified *Pfu* DNA polymerase was quantified by a PCR-amplification reaction titrated against commercial enzyme (Figure 4). The purified *Pfu* DNA polymerase was highly concentrated and had to be diluted before use, since *Pfu* DNA polymerase shows a decrease in activity at high concentrations. When fairly large amounts of purified *Pfu* DNA polymerase (1:2 diluted enzyme) were used, no PCR product was made (Figure 4, lane 4). It is possible that the high levels of *Pfu* DNA polymerase inhibited the reaction, as occurs for *Taq* DNA polymerase (Desai and Pfaffle, 1995; Engelke et al., 1990) or that the exonuclease activity of proofreading *Pfu* DNA polymerase degraded primers. The amplified products using 1:40-diluted purified *Pfu* DNA polymerase (Figure 4, lane 8) was nearly equivalent to amplified products using 2.5 units of commercial enzyme (Figure 4, lane 1). The activity of the typical concentration of the final purified *Pfu* DNA polymerase of undiluted stocks

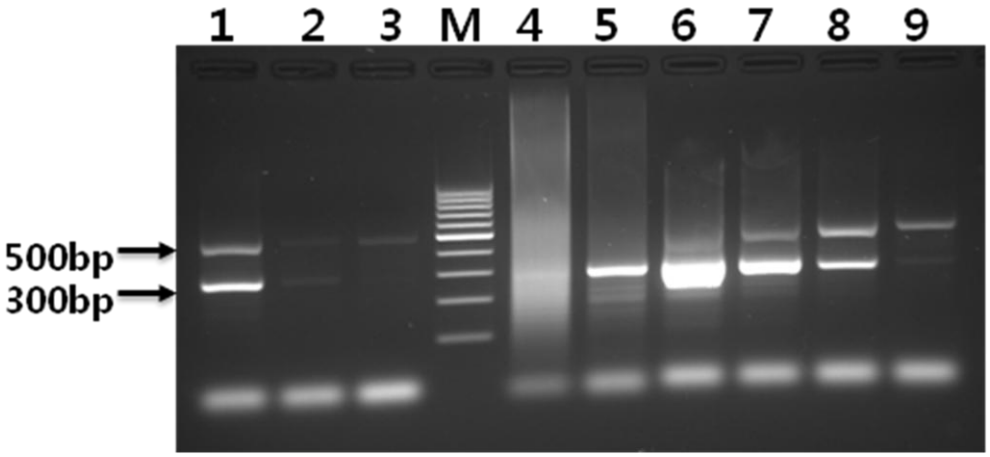

Figure 4. Activity assay of purified *Pfu* DNA polymerase in different dilutions. The activity of the purified *Pfu* DNA polymerase was quantified by PCR titrated against commercial enzyme. The expected amplified DNA fragments are marked with arrows. The commercial *Pfu* DNA polymerase (2.5 units/μL) was diluted 1:1 (lane 1), 1:2 (lane 2), and 1:4 (lane 3). The purified *Pfu* DNA polymerase was diluted 1:2 (lane 4), 1:5 (lane 5), 1:10 (lane 6), 1:20 (lane 7), 1:40 (lane 8), and 1:80 (lane 9) with storage buffer. M, DNA size marker (100 bp ladder).

reached a value of 100 units/μL.

DISCUSSION

Cloning of a particular gene in a target genome usually requires isolation of the genomic DNA from that organism. However, the presently-reported cloning method is a rapid and simple approach, in which the commercial recombinant enzyme is used directly as a PCR template, thus eliminating the laborious and time-consuming steps of cell recovery and DNA extraction. To our knowledge, this is the first report showing the efficacy of the use of a commercial recombinant enzyme as a DNA source in practical cloning procedures. The speed and efficiency of this method, along with the availability of commercial recombinant enzymes, suggest that this method could be adapted to cloning strategies for commercial recombinant enzymes from many sources.

We used semi-nested PCR because the level of contaminating DNA template in commercial *Pfu* DNA polymerase is very low. Although standard PCR is a remarkably sensitive technique, its sensitivity can be further increased by performing semi-nested PCR. Semi-nested PCR involves taking an aliquot of the product from the first-round PCR, and using it as a template for the second-round PCR. To avoid further amplification of nonspecific products generated in the first-round PCR, a different set of primers is employed in the second-round semi-nested PCR. For the semi-nested PCR, one of the primers used in the first round PCR is used again and the other primer is within the target sequence. Based on the fact that their sequences are different from the primary set of primers, they will not amplify artifacts or nonspecific products generated in the first-round PCR, enabling product specificity to be maintained over the high number of amplification cycles combined in the first-round and second-round PCRs.

We have also developed a rapid protocol for the purification of *Pfu* DNA polymerase by exploiting the extremely thermostable properties of *Pfu* DNA polymerase. *Pfu* DNA polymerase was easily purified by heating at 100°C for 10 min to lyse *E. coli* and denature the released proteins (except for the polymerase), followed by recovery of the polymerase by chromatography on heparin columns. To date, several procedures have been used for the purification of *Pfu* DNA polymerase to cope with commercial or experimental needs (Evans et al., 2000; Lu and Erickson, 1997). However, these methods are generally laborious and time-consuming, and require sonication and the use of reagents that can be toxic and/or expensive reagents (phenylmethanesulfonylfluoride and lysozyme). As well, the present method is rapid, requiring only 1.5-2 h to complete the integrated purification process using one kind of buffer. The protocol rapidly and easily isolated about 1.2 mg of *Pfu* DNA polymerase from 400 mL of crude bacterial cultures with a high activity of 3.2×10^5 units, as confirmed by PCR. The in-house purified *Pfu* DNA polymerase has been successfully used in routine applications including PCR screening, cloning, and mutagenesis (Figure 5).

In conclusion, we demonstrate that commercial preparations of *Pfu* DNA polymerase are contaminated with polymerase gene and detail a method to efficiently clone the polymerase gene from commercial *Pfu* DNA polymerase by semi-nested PCR. In addition, we have developed a more reliable, rapid, and simpler method for

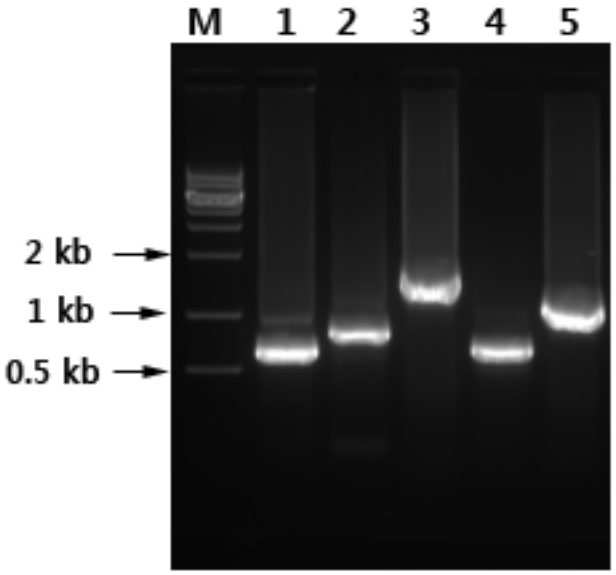

Figure 5. PCR amplification of different lengths of genes using the purified *Pfu* DNA polymerase. To confirm the effectiveness of the *Pfu* DNA polymerase purified by our protocol, the enzyme was applied to amplify different lengths of genes. Lane M, DNA size marker. Lane 1, Amplification product of the *Thermus thermophilus* single-stranded DNA binding protein gene (792 bp); lane 2, the product of the *Thermus thermophilus* RecA gene (1,144 bp); lane 3, the product of the *Thermotoga maritima* ATP-dependent DNA helicase gene (1,947 bp); lane 4, the product of the *Escherichia coli* otsB gene (801 bp); lane 5, the product of the *Escherichia coli* otsA gene (1,425 bp).

purification of *Pfu* DNA polymerase for routine use.

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