Appl Biochem Biotechnol (2010) 160:1585–1599
DOI 10.1007/s12010-009-8658-0

Biochemical Properties and PCR Performance of a Family B DNA Polymerase from Hyperthermophilic Euryarchaeon *Thermococcus peptonophilus*

Jong Il Lee · Yun Jae Kim · Heejin Bae · Sung Suk Cho · Jung-Hyun Lee · Suk-Tae Kwon

Received: 30 January 2009 / Accepted: 23 April 2009 /
Published online: 8 May 2009

Abstract The *Thermococcus peptonophilus* (*Tpe*) DNA polymerase gene was expressed under the control of the T7*lac* promoter on pET-22b(+) in *Escherichia coli* BL21-CodonPlus(DE3)-RIL in order to fully elucidate its biochemical properties and evaluate its feasibility in polymerase chain reaction (PCR) application. The expressed enzyme was then purified by heat treatment followed by two steps of column chromatography after which optimum pH and temperature of the enzyme were evaluated to be 7.0 and 75 °C, respectively. The optimal buffer for PCR with *Tpe* DNA polymerase consisted of 50 mM Tris–HCl (pH8.0), 2 mM $MgCl_2$, 80 mM KCl, and 0.02% Triton X-100. *Tpe* DNA polymerase revealed a 3.6-fold higher fidelity (3.37×10^{-6}) than *Taq* DNA polymerase (12.13×10^{-6}) and performed significantly more efficiently in PCR amplification than both *Taq* and *Pfu* DNA polymerases. Ratios of 31:1 of *Taq* to *Tpe* DNA polymerases allowed PCR amplification of targets up to 15 kb in length with a 2.2-fold higher fidelity than *Taq* DNA polymerase. The results of the PCR experiments indicate that *Tpe* DNA polymerase may provide a higher fidelity DNA amplification in a shorter reaction time.

Keywords DNA polymerase · *Thermococcus peptonophilus* · Polymerase chain reaction · PCR efficiency · PCR fidelity · Archaea

Introduction

Archaea, a relatively new third domain of living organisms, is distinguishable from the Bacteria and Eukarya domains for all intensive purposes [1]. The archaea are divided into four phyla called Euryarchaeota, Crenarchaeota, Korarchaeota, and Nanoarchaeota [2, 3], with

J. I. Lee · H. Bae · S. S. Cho · S.-T. Kwon (✉)
Department of Biotechnology and Bioengineering, Sungkyunkwan University, 300 Cheoncheon-dong, Jangan-gu, Suwon 440-746, Republic of Korea
e-mail: stkwon@yurim.skku.ac.kr

Y. J. Kim · J.-H. Lee
Korea Ocean Research and Development Institute, P.O. Box 29, Ansan 425-600, Republic of Korea

Crenarchaeota and the Euryarchaeota containing the most culturable and well-investigated species. Hyperthermophilic species of Crenarchaeota and Euryarchaeota grow optimally at temperatures of 80 °C or higher, and therefore have raised considerable interest for both basic research and biotechnological applications. The exceptional thermostability of these hyperthermophilic enzymes has recently become very attractive for industrial processes, commercial uses, and new applications that can advance molecular biology and related areas [4]. Among the various applications, the most outstanding is the polymerase chain reaction (PCR) [5], based upon the use of thermostable *Taq* and *Pfu* DNA polymerases. Thus, various DNA polymerases from hyperthermophilic archaea have previously isolated and characterized [4, 6–19]. Archaeal DNA polymerases have been mostly identified as members of family B, similar to eukaryotic replicative DNA polymerases [20]. Common traits of the family B polymerases include remarkable accuracy during replication and strong $3' \rightarrow 5'$ exonuclease activity [6, 10–15]. The contribution of proofreading activity to DNA polymerase fidelity is also evident when the error rates of proofreading and non-proofreading enzymes are compared [21]. Two hyperthermophilic archaea from the genera *Pyrococcus* and *Thermococcus* contain family B DNA polymerases that have recently become popular for potential use in PCR because they offer high fidelity and considerable amplification [22].

Recent studies have shown that various mixtures of non-proofreading and proofreading DNA polymerases synthesize higher yields of PCR product and allow amplification of templates longer than previously possible with single enzyme formulations [21]. This potential for discovering effective DNA polymerase mixtures, along with the increasing number of applications that employ PCR amplification, has intensified the demand for various thermostable DNA polymerases [23]. The hyperthermophilic Euryarchaeon *Thermococcus peptonophilus*, which was isolated from deep-sea hydrothermal vent areas in the western Pacific Ocean, is able to grow on tryptone and yeast extract as sole carbon sources [24]. *T. peptonophilus* showed exceptionally fast growth rate when compared to other species within the genus *Thermococcus* [24]. In this study, the gene encoding a DNA polymerase from *T. peptonophilus* was cloned and expressed in *E. coli*. We report the purification protocol and biochemical properties of the enzyme as well as the results of various PCR experiments. It was found a mixture of *Tpe* DNA polymerase and *Taq* DNA polymerase improves the performance of *Tpe* DNA polymerase for long and accurate PCR.

Materials and Methods

Bacterial Strains and Growth Conditions

T. peptonophilus (DSM 10343) was obtained from the German Collection of Microorganism and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ). *T. peptonophilus* cells were grown anaerobically in DSMZ medium 760, as recommended in DMSZ's protocol. A medium containing 37.4 g/l of Marine Broth 2216 (Difco, USA) and 0.5 mg/l of resazurin was prepared by adjusting the pH of the medium to 7.0 at 25 °C and incubation for 6 h at 95 °C. The heated medium was filtered through normal filter paper to remove sediments and was dispensed into 120 ml serum bottles (Wheaton Science Products, USA) containing finely divided sulfur (0.5% *w*/*v*) under 100% N_2 gas. After sealing each serum bottle, 0.3 ml of 5% $Na_2S{\cdot}9H_2O$ was added to the medium

to eliminate any traces of oxygen prior to sterilization at 100 °C. The cells were then inoculated with a syringe and incubated anaerobically at 85 °C for 20 h.

Escherichia coli DH5α and *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, USA) cells were used for plasmid propagation and gene expression, respectively. *E. coli* strains were cultured in Luria–Bertani (LB) medium at 37 °C with appropriate antibiotics.

Cloning and Expression of the *Tpe* DNA Polymerase Gene

Standard procedure [25] was used to isolate genomic DNA from *T. peptonophilus*. Restriction enzymes and other modifying enzymes were purchased from Takara (Takara, Japan). Small-scale isolation of plasmid DNA from *E. coli* was performed using a plasmid mini-prep kit (Qiagen, Germany).

The full-length gene encoding the *T. peptonophilus* DNA polymerase (GenBank accession no. E13952) flanked by *Nde*I and *Sal*I sites was amplified by PCR using genomic DNA and two primers (sense [5′-TAGGTGTCCCATATGATCCTCGACACTGACTAC-3′] and antisense [5′-CAAATGGAAGTCGACAGTTCCCTTCGGCTTCAGC-3′]; the underlined sequences indicate the *Nde*I site in the sense primer and the *Sal*I site in the antisense primer).

The amplified DNA fragment was digested with *Nde*I and *Sal*I, extracted with the Qiaquick® gel extraction kit (Qiagen, Germany), and finally ligated with the corresponding sites in the pET-22b(+) plasmid (Novagen, USA). *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, USA) were transformed with the expression construct by electroporation and correctly transformed clones were selected by restriction enzyme analysis using plasmid minipreps. The sequence of the entire *Tpe* DNA polymerase gene was confirmed by DNA sequencing (Macrogen, Korea). The resulting expression plasmid was named pEPEPOL.

Purification of *Tpe* DNA Polymerase

Overexpression of the *Tpe* DNA polymerase protein was induced to a final concentration of 0.4 mM by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase (OD_{600} of 0.5), followed by an additional 6 h of incubation at 37 °C. The cells were then harvested by centrifugation (5,590×*g*, 4 °C, 20 min) and resuspended in 20 mM Tris–HCl buffer (pH7.8) containing 0.1 M KCl and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication and centrifuged (18,700×*g*, 4 °C, 15 min). The resulting supernatant was heat-treated for 30 min at 80 °C then centrifuged just as described. The sample was then applied to a HiTrap™ Heparin HP column (GE Healthcare, UK) equilibrated with buffer A (20 mM Tris–HCl, pH7.8, and 0.1 mM KCl). The column was washed with buffer A and the bound proteins were eluted with a 0.1–1.0 M NaCl gradient in buffer A. The peak fractions containing *Tpe* DNA polymerase were pooled and dialyzed against buffer B (20 mM Tris–HCl, pH7.8, and 0.5 M NaCl) then loaded onto a HisTrap™ HP column (GE Healthcare, UK) equilibrated with buffer B and washed in the same buffer. The combined proteins were eluted in a step-wise fashion using 0.1–0.5 M imidazole in buffer B. The purified enzyme was dialyzed against 20 mM Tris–HCl (pH7.4), 0.1 mM EDTA, 0.1% Tween 20, 0.5% Nonidet P40, 0.1 M KCl, and 50% glycerol, and was stored at −20 °C. Protein concentration was determined as previously described [26] using bovine serum albumin (BSA) as a standard. Protein purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedure.

DNA Polymerase Activity Assay

The DNA polymerase activity of the purified enzyme was measured as previously described [18]. The basic reaction mixture (50 µl) contained 50 mM Tris–HCl (pH7.5); 5 mM $MgCl_2$; 50 mM KCl; 1 mM 2-mercaptoethanol; 100 µM each of dATP, dCTP, and dGTP; 10 µM dTTP; 0.5 µCi of [methyl-^{3}H]thymidine 5′-triphosphate (30 Ci/mmol, GE Healthcare, UK); 1.25 µg of activated calf thymus DNA; and enzyme solution. One unit of *Tpe* DNA polymerase was defined as the amount of polymerase that incorporated 10 pmol of [^{3}H]TTP into an acid-insoluble product at 75 °C for 10 min.

Exonuclease Activity Assay

Measurement of exonuclease activity was performed as previously described [19]. The 3′-end-labeled DNA substrate was prepared by filling pBluescript SK-DNA linearized by *Not*I with a Klenow fragment in the presence of [α-^{32}P]dCTP. After labeling, DNA substrate was purified by gel filtration, followed by ethanol precipitation. For evaluation of exonuclease activity, end-labeled DNA substrate was placed in 50 mM Tris–HCl (pH7.5), 14 mM $MgCl_2$, 80 mM KCl, 0.01% BSA, and enzyme solution. The reaction mixture was incubated at 75 °C in the absence or presence of 0.4 mM dNTPs (final concentration), as described in our previous study [19].

Optimization of PCR Amplification Using *Tpe* DNA Polymerase

Oligonucleotide primers that anneal to λ DNA [27] were synthesized for PCR analysis. The primer sequences are presented in Table 1. PCR was performed with 1 U of purified *Tpe* DNA polymerase in a 50-µl reaction mixture containing 23 ng of λ DNA, 10 pmol each of the primers anchor-λ F and λ-2 R, 250 µM of dNTPs and PCR reaction buffer. The reaction buffer conditions are indicated in the corresponding figure legend. PCR was conducted as follows: 1 min at 94 °C; 30 cycles of 20 s at 94 °C, and 30 s at 72 °C; and 10 min at 72 °C.

Table 1 Primers used for PCR extension efficiency assays.

Primer name (target size)	Primer sequence	Lambda DNA sequence (bp)
Anchor-λ F	5′-CCTGCTC TGCCGCTTCACGC-3′	30,352–30,371
λ-500 R (0.5 kb)	5′-CGTCGATGACATTTGCCGTAGCG-3′	30,815–30,837
λ-1 R (1 kb)	5′-GGAGCAATGGCGATGACGCATCC-3′	31,343–31,365
λ-2 R (2 kb)	5′-CCATGATTCAGTGTGCCCGTCTGG-3′	32,326–32,349
λ-4 R (4 kb)	5′-CGAAGAGCATCCTCAGGATGTGATGG-3′	34,316–34,341
λ-6 R (6 kb)	5′-GAGATGGCATATTGCTACGCAAGA-3′	36,339–36,362
λ-8 R (8 kb)	5′-GCCTCGTTGCGTTTGTTTGCACG-3′	38,373–38,395
λ-10 R (10 kb)	5′-GCACAGAAGCTATTATGCGTCCCCAGG-3′	40,316–40,342
λ-12 R (12 kb)	5′-TCTTCCTCGTGCATCGAGCTATTCGG-3′	42,409–42,434
λ-15 R (15 kb)	5′-CTTGTTCCTTTGCCGCGAGAATGG-3′	45,228–45,251

Oligonucleotide primers are in antisense orientation (reverse primer) except for the anchor-λ F primer

DNA Polymerase Mixtures for Long-Range PCR

The amplification efficiency of a 15-kb DNA fragment with lambda DNA as a template was compared by varying the ratio of *Taq* to *Tpe* DNA polymerase from 1:1 to 255:1. The mixture of *Taq* and *Tpe* DNA polymerases was used for PCR with the *Taq* buffer (PCR buffer; Takara, Japan). PCR amplification for the 15-kb targets was carried out in 30 cycles of 94 °C for 20 s and 72 °C for 15 min. In comparing extension efficiencies between DNA polymerases, a set of primers (Table 1) was used to amplify DNA fragments of 2, 4, 6, 8, 10, 12, and 15 kb in size from the lambda DNA in a 50-μl reaction. The DNA polymerases *Tpe*, *Taq*, and *Tpe* plus (*Taq*/*Tpe* DNA polymerase, 31:1 ratio) were evaluated. PCR was performed at 94 °C for 1 min; 30 cycles of 94 °C for 20 s, 62 °C for 20 s, and 72 °C for 60 s/kb (target length).

PCR Amplification Efficiency Assay

PCR was performed with *Tpe*, *Taq* (Takara, Japan), and *Pfu* (Promega, USA) DNA polymerases in each enzyme's recommended buffer, with λ phage DNA (23 ng) [27] as a template. Comparison of amplification efficiency used primers (Table 1) in order to amplify amplicons of 0.5, 1, 2, and 4 kb in size from the template in a 50-μl reaction. The cycling protocol used is indicated in the corresponding figure legends. Upon completion of the PCR, 5 μl of the PCR was mixed with loading dye and loaded onto a 0.8% agarose gel.

PCR Fidelity Assay

PCR fidelity assays were performed by first constructing the plasmid pJR2-*lacZ* [19] using pJR [28] and pLEX/*lacZ* (Invitrogen, USA) as follows (Fig. 1). pJR contains *Bam*HI sites in the front of the 5′ region *tac* promoter and the 5′ region of the multiple cloning site, of which the former must be removed in order to form plasmid pJR2. In order to eliminate the *Bam*HI site near the front of the pJR *tac* promoter, pJR was first partially cleaved with *Bam*HI and the *Bam*HI ends were filled-in, ligated, and pJR2 then selected. On the other hand, the 3-kb *Bam*HI–*Pst*I fragment containing the entire gene for β-gactosidase from pLEX/*lac*Z DNA was obtained and then joined to the multiple cloning *Bam*HI–*Pst*I site of pJR2. At this point, pJR2-*lacZ* (5.7 kb), an expression plasmid containing the entire *lacZ* gene, was constructed and ready to be used as a template in PCR fidelity evaluations. An 832-bp fragment containing the 5′ region of the *lacZ* gene was amplified using the primers Lac-B (5′-NNNNGGATCCAATGATAGATCCCGTCGTTTTAC-3′) and Lac-C (5′-NNNNATCGATAATTTCACCGCCGAAAGGCGC-3′), in which the *Bam*HI and *Cla*I sites, respectively, are underlined [19, 29]. PCR was performed with the *Tpe*, *Tpe* plus, and *Taq* DNA polymerase in the optimized buffer or the buffer supplied by the manufacturer at 94 °C for 1 min; 30 cycles of 94 °C for 20 s, and 62 °C for 1 min; and 72 °C for 3 min. PCR with *Pfu* DNA polymerase was also performed in the supplied buffer at 94 °C for 1 min; 30 cycles of 94 °C for 20 s, and 62 °C for 1 min; and 72 °C for 3 min. The PCR products were digested with *Bam*HI and *Cla*I endonuclease, purified, and ligated with the 4.9 kb *Bam*HI/*Cla*I fragment from pJR2-*lacZ*. *E. coli* DH5α cells were transformed with each ligate by electroporation and then plated on LB agar plates containing 50 μg/ml ampicillin, 0.3 mM IPTG, and 20 μg X-gal. Pale blue and white colonies were formed by cells containing mutant plasmids, while blue colonies were formed by cells containing intact plasmids. Error rates (ER) were calculated using the equation $ER = mf/(bp \times d)$, where mf is the mutation frequency, bp is the number of

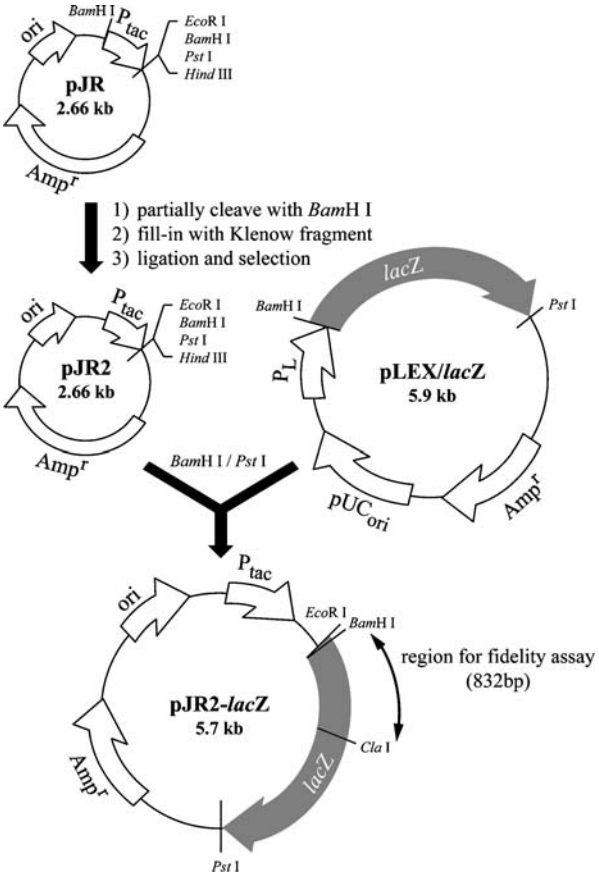

Fig. 1 Construction of the plasmid pJR2-*lacZ* for PCR fidelity assay. Plasmid pJR2 was constructed from pJR. The *Bam*HI–*Pst*I fragment containing the entire gene for β-gactosidase from pLEX/*lacZ* DNA was obtained and then joined to the multiple cloning *Bam*HI–*Pst*I site of pJR2. An 832-bp fragment containing the 5′ region of the *lacZ* gene is used for fidelity assay. Amp^r, ampicillin resistance gene; Ori, replication origin; P_{tac}, *tac* promoter; P_L, λP_L promoter

detectable sites in the region of *lacZ* (832 bp), and *d* is the number of template doublings [12, 21].

Results and Discussion

Expression and Purification of *Tpe* DNA Polymerase

The *Tpe* DNA polymerase gene was amplified by PCR and inserted into the *Nde*I and *Sal*I sites of pET-22b(+) to facilitate overexpression and purification of pEPEPOL. *E. coli* BL21-CodonPlus(DE3)-RIL cultures harboring pEPEPOL were generated, cells harvested, and then initially sonicated. Purification of the *Tpe* DNA polymerase was performed through a combination of heat, a HiTrap™ Heparin HP column (GE Healthcare, UK), and a HisTrap™ HP Ni^{2+} affinity column (GE Healthcare, UK). The specific activity of the

purified enzyme was determined to be 692.9 U/mg, and based on the total activity of the sonicated extract, the recovery was determined to be approximately 18.8%. The purification of the enzyme was monitored by SDS-PAGE (Fig. 2), which revealed a 90-kDa major protein band consistent with the 89,707-Da molecular mass calculated based on the 774 amino acid sequence.

Characterization of *Tpe* DNA Polymerase

Tpe DNA polymerase activity exhibited pH dependence in the pH range of 6.5–10.0. The buffers used were 50 mM MOPS–NaOH (pH6.0–7.0), 50 mM Tris–HCl (pH7.0–9.0), and 50 mM glycine–NaOH (pH9.0–10.0). The enzyme activity was highest at pH7.0 (Fig. 3a). The dependence of the *Tpe* DNA polymerase activity on temperature was found in the range of 50–90 °C. From these experiments, the optimal temperature for the *Tpe* DNA polymerase was determined to be approximately 75 °C (Fig. 3b). The DNA polymerase was highly dependent on $MgCl_2$, with maximal activity at 14 mM $MgCl_2$ and no detectable activity in the absence of $MgCl_2$ (Fig. 3c). KCl also affected the activity of *Tpe* DNA polymerase, with an optimal concentration of 80 mM (Fig. 3d). Thermostability of *Tpe* DNA polymerase (0.1 μg/μl concentration) was tested by measuring the decrease in activity after preincubation at 90 and 95 °C. Thermostability was revealed to be remarkably high, as was expected from a thermostable protein. The half-life of the enzyme was 4 h at 90 °C (Fig. 4).

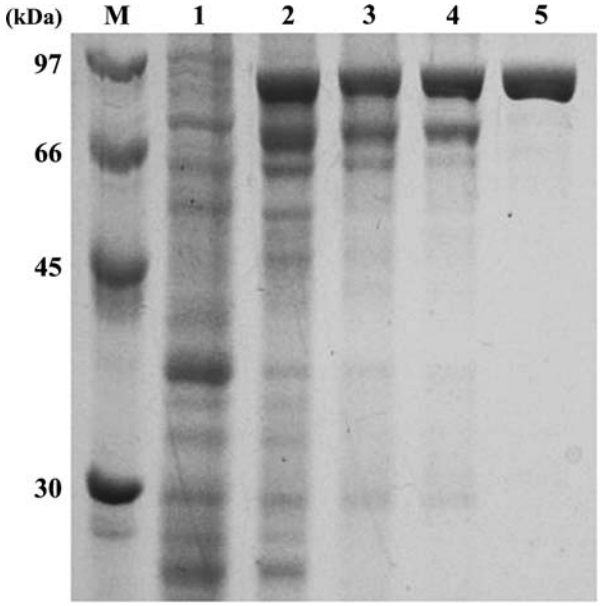

Fig. 2 SDS-PAGE analysis of *Tpe* DNA polymerase. Electrophoresis was performed on a vertical, 10% polyacrylamide gel, stained with Coomassie Brilliant Blue R-250. Lane assignments: *M*, low molecular mass markers; *lane 1*, sonicated extract of uninduced cells; *lane 2*, sonicated extract of induced cells; *lane 3*, heat treatment; *lane 4*, HiTrap™ Heparin HP column chromatography; *lane 5*, HisTrap™ HP column chromatography

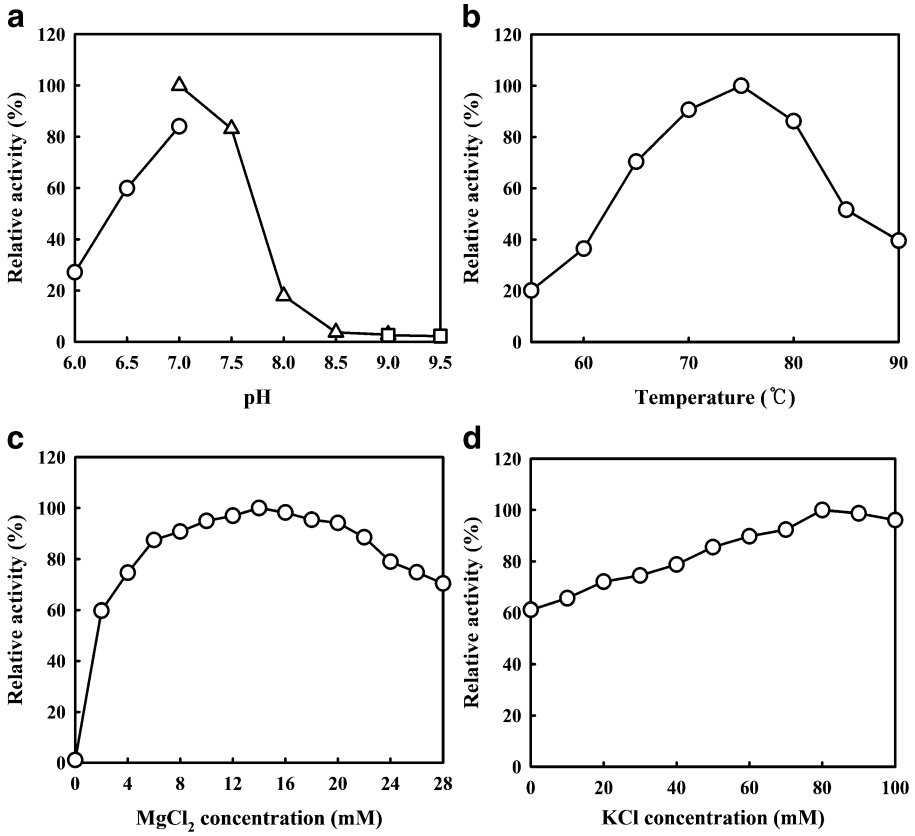

Fig. 3 Characterization of *Tpe* DNA polymerase. **a** Effect of pH on *Tpe* DNA polymerase activity: 50 mM MOPS–NaOH (*empty circle*), 50 mM Tris–HCl (*empty triangle*), and 50 mM glycine–NaOH (*empty square*). All buffer pH values were measured at 75 °C. **b** Effect of temperature on *Tpe* DNA polymerase activity. **c** Effect of $MgCl_2$ on *Tpe* DNA polymerase activity. **d** Effect of KCl on *Tpe* DNA polymerase activity

Exonuclease Activity of *Tpe* DNA Polymerase

Almost all archaeal family B DNA polymerases are known to have associated 3′ → 5′ proofreading exonuclease activity, which is responsible for the correction of mismatched dNTPs. Likewise, we wished to investigate the presence of proofreading activity in DNA polymerase from *T. peptonophilus*. Highly conserved amino acids in three domains (Exo I, Exo II, and Exo III) could be identified within the deduced amino acid sequence from *Tpe* DNA polymerase (data not shown). Incubation of *Tpe* DNA polymerase with linear DNA fragments in the absence of dNTPs led to degradation of the fragments indicating the existence of exonuclease activity in the preparation. This activity was checked by assaying the ^{32}P-labeled product released from an end-labeled DNA substrate, both with and without dNTPs. *Tpe* DNA polymerase could release about 80% ^{32}P from the 3′-end of the substrate DNA within 30 min in the absence of dNTPs (Fig. 5) and also about 10% ^{32}P from the 3′-end of the substrate DNA within 30 min in the presence of dNTPs. This result demonstrated that *Tpe* DNA polymerase possesses a 3′ → 5′ exonuclease activity, which is consistent with its deduced amino acid sequence as described above. This 3′ → 5′ exonuclease

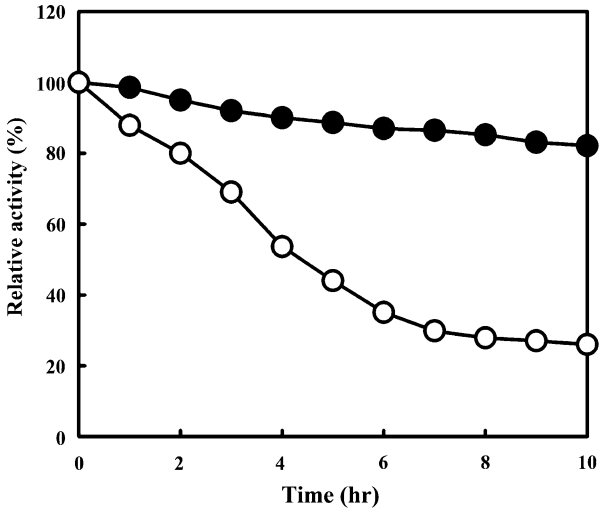

Fig. 4 Thermostability of *Tpe* DNA polymerase. Purified *Tpe* DNA polymerase was incubated separately at 90 °C (*filled circle*) and 95 °C (*empty circle*). Aliquots of the mixture were removed at intervals up to 8 h later and quenched on ice. The residual activity of the quenched samples was measured in the basic reaction mixture

activity, which can remove mispaired bases (proofreading activity) up to five nucleotides after the misincorporation, is ultimately responsible for the fidelity of DNA polymerases [30]. The importance of proofreading activity has been demonstrated for *Thermococcus litoralis* (Vent) and *Pfu* DNA polymerases, which exhibit 5-fold and 7–40-fold increase in error rate, respectively, when 3′ → 5′ exonuclease activity is inactivated [13, 21]. The

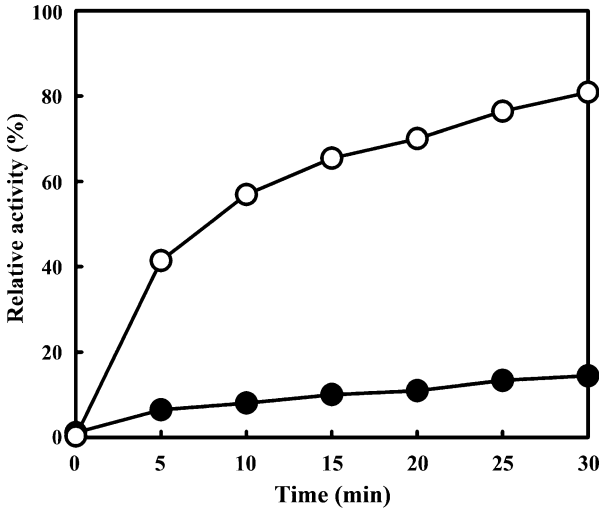

Fig. 5 Exonuclease activities of *Tpe* DNA polymerase. The 3′ → 5′ exonuclease activity was measured at 75 °C in the absence (*empty circle*) or presence (*filled circle*) of 0.4 mM dNTPs (final concentration). Activity was calculated as the amount of supernatant radioactivity/total radioactivity

significant increase in error frequency is also observed when 3′ → 5′ exonuclease activity is inactivated in *Thermococcus zilligii* (*Tzi*) DNA polymerase [10].

Optimization of PCR Amplification Using *Tpe* DNA Polymerase

PCR technology is widely used in molecular biology and molecular genetics research, including genome mapping, site-directed mutagenesis, and sequencing studies. However, more precise reaction buffer optimizations can surely improve yield and specificity in PCR. The optimal buffer for PCR with *Tpe* DNA polymerase was therefore determined. To determine the optimum pH, PCR was performed in the pH range of 7.0–9.0 using Tris–HCl buffer. The λ DNA fragment was found to be amplified optimally at pH 8.0 (Fig. 6a). In addition, PCR was carried out under various concentrations of $MgCl_2$, KCl, and Triton X-100, which resulted in optimal concentrations of 2–3 mM (Fig. 6b), 80–100 mM (Fig. 6c), and 0.01–0.1% (Fig. 6d), respectively. Duplicate experiments established that the optimal

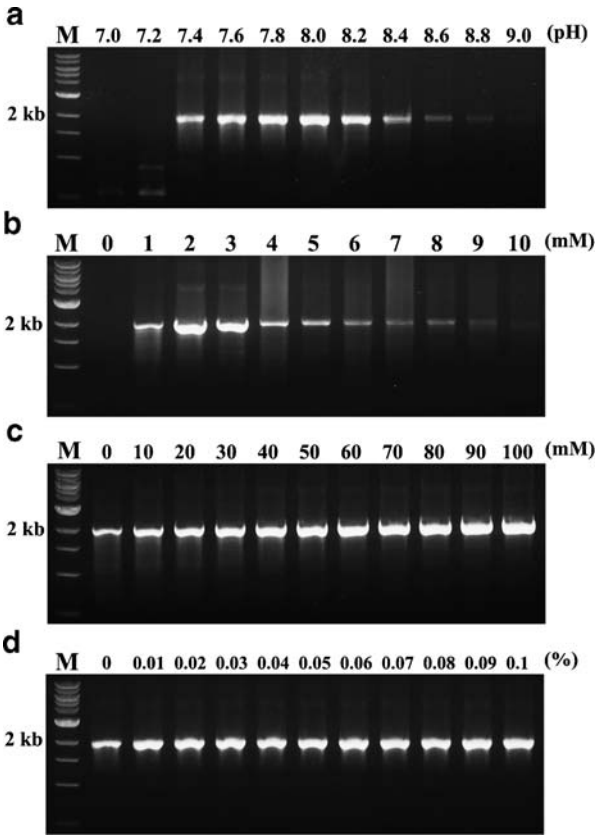

Fig. 6 PCR optimization with *Tpe* DNA polymerase. **a** Effect of pH on PCR amplification with *Tpe* DNA polymerase. The amplification of the 2-kb λ DNA fragment was performed at the indicated pH values. **b** Effect of $MgCl_2$ on PCR amplification with *Tpe* DNA polymerase. **c** Effect of KCl on PCR amplification with *Tpe* DNA polymerase. **d** Effect of Triton X-100 on PCR amplification with *Tpe* DNA polymerase. *Lane M*, DNA molecular size markers

PCR buffer with *Tpe* DNA polymerase consisted of 50 mM Tris–HCl (pH8.0), 2 mM $MgCl_2$, 80 mM KCl, and 0.02% Triton X-100.

Specially, the major divergence between optimal buffer of enzyme activity and optimal PCR buffer of DNA amplification is $MgCl_2$ concentration. Magnesium is a required cofactor for DNA polymerases, and magnesium concentration is a crucial factor that can affect enzyme activity. $MgCl_2$ dissociate to Mg^{2+} and $2Cl^-$ in reaction buffer. The DNA polymerase requires Mg^{2+} to be able to bind also to the negatively charged DNA and Mg^{2+} additionally forms a complex with dNTPs. *Tpe* DNA polymerase is also inactive in the absence of $MgCl_2$ (Figs. 3c and 6b). *Tpe* DNA polymerase had the highest activity with 14 mM, as described above (Fig. 3c). The enzyme activity sharply increases in the concentration of 2 mM $MgCl_2$, but the activity slowly increases up to 14 mM $MgCl_2$.

However, the optimum concentration of $MgCl_2$ for PCR was 2 mM, which was much lower than under standard activity assay. This might be due to the strong base pairing of Mg^{2+}, as they could be released from anionic DNA by repetitive thermal cycle under PCR conditions [31]. When Mg^{2+} is too high in the PCR mixture, primers fail to anneal to the target DNA. When the concentration of $MgCl_2$ is too high in the PCR mixture, the base pairing becomes too strong and the amplicon fails to denature completely when the PCR mixture is heated to 94 °C [32].

Long-Range PCR by Various DNA Polymerases

Despite the utility of PCR, the technique is limited by the amplification of long DNA targets over 5 kb. Recently, successful protocols have identified the conditions for effective amplification of longer DNA targets [33]: the combined modification of standard PCR buffers and thermal cycling profiles with a two polymerase system in order to provide optimal levels of both processive and proofreading polymerase activities [22, 33]. Long-range PCR is based on this simple principle: DNA synthesis is catalyzed by means of exonuclease-free DNA polymerase (*Taq* DNA polymerase) at relatively high concentration in PCR mixture, and misincorporated nucleotides during an amplification reaction by *Taq* DNA polymerase are repaired by a DNA polymerase with $3' \rightarrow 5'$ exonuclease activity, which is present at low concentration [22, 34]. This combination of two DNA polymerases allows longer primer extension than can be achieved with *Taq* DNA polymerase alone. For further investigation, the ratio of *Taq* DNA polymerase to *Tpe* DNA polymerase was varied from 1:1 to 255:1 and their characteristics in PCR were examined. The most efficient DNA amplification was observed at a ratio of 31:1 (Fig. 7a). We named the enzyme mixture with *Taq* and *Tpe* DNA polymerase as *Tpe* plus DNA polymerase. To investigate the extension efficiency of the *Tpe* plus DNA polymerase, PCR was conducted using various fragment sizes. *Tpe* DNA polymerases could amplify the λ DNA fragment up to 4 kb. *Taq* DNA polymerase barely amplified the λ DNA fragment at 10 kb (Fig. 7b) and was incapable of amplifying any longer fragment. On the contrary, *Tpe* plus DNA polymerase clearly demonstrated enhanced efficiency in successfully amplifying the 15-kb fragment using 23 ng of λ DNA as a template (Fig. 7b). Commercial "LA (long and accurate) PCR" kit (Takara, Japan) typically consists of *Taq* DNA polymerase along with a lesser amount of an archaeal proofreading DNA polymerase [21]. The direct amplification and DNA sequencing of whole mitochondrial genomes also have been used by long-range PCR [35, 36]. *Tpe* plus DNA polymerase might be useful in long-range DNA amplification and various PCR-based applications.

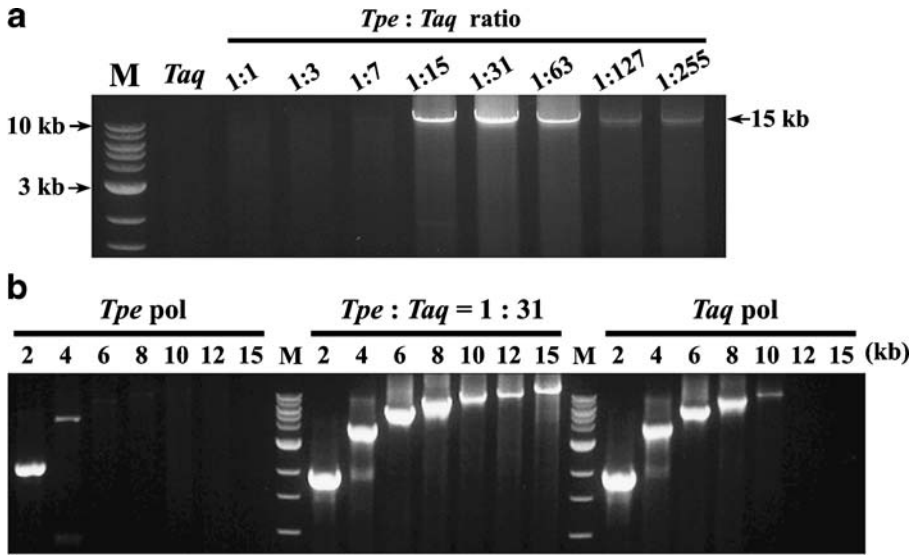

Fig. 7 Comparison of long-range PCR amplification abilities. **a** Target of 15 kb was amplified using mixtures of *Tpe* and *Taq* DNA polymerases (*Tpe/Taq*) at the indicated ratios. **b** Amplification of 2–15 kb was conducted by *Tpe* (*lanes 1–7*), *Tpe* plus (*lanes 9–15*), and *Taq* (*lanes 17–23*) DNA polymerases. Target product sizes (kb) are indicated above each lane. *Lane M*, DNA molecular size marker

PCR Amplification Efficiency Assay

In vitro amplification of DNA fragments and determination of DNA sequence are the major uses of thermostable DNA polymerases [6]. *Taq* DNA polymerase is most widely used, but the high error rate causes amplicons to be prone to unexpected mutations.

To minimize any possible misincorporations that may occur during PCR, several family B DNA polymerases were isolated from the hyperthermophilic archaea. Among them, *Pfu* DNA polymerase was found to possess high $3' \rightarrow 5'$ exonuclease proofreading activity that subsequently lowered amplification efficiency. In this study, we confirmed that *Tpe* DNA polymerase has a higher amplification efficiency compared to *Taq* DNA polymerase, despite its possession of $3' \rightarrow 5'$ exonuclease activity. *Pfu* DNA polymerase is able to amplify a 1-kb target but with a lower efficiency compared to *Taq* DNA polymerase (Fig. 8c). *Tpe* DNA polymerase with 1 min/cycle extension time could amplify target DNA up to 4 kb, outperforming both *Taq* and *Pfu* DNA polymerase (Fig. 8b). This suggests *Tpe* DNA polymerase might produce a more efficient DNA amplification in a shorter reaction time.

PCR Fidelity of *Tpe* DNA Polymerase

The use of high fidelity DNA polymerases in PCR is essential for reducing amplification errors in PCR products that are to be used in cloning and site-directed mutagenesis [37]. The high fidelity is achieved by selection of the correct nucleotide in the polymerase (Pol) domain and subsequent proofreading in the exonuclease (Exo) domain. Proofreading by the associated $3' \rightarrow 5'$ exonuclease activity removes most misincorporated nucleotides [38]. The importance of proofreading activity to replication fidelity has been demonstrated for

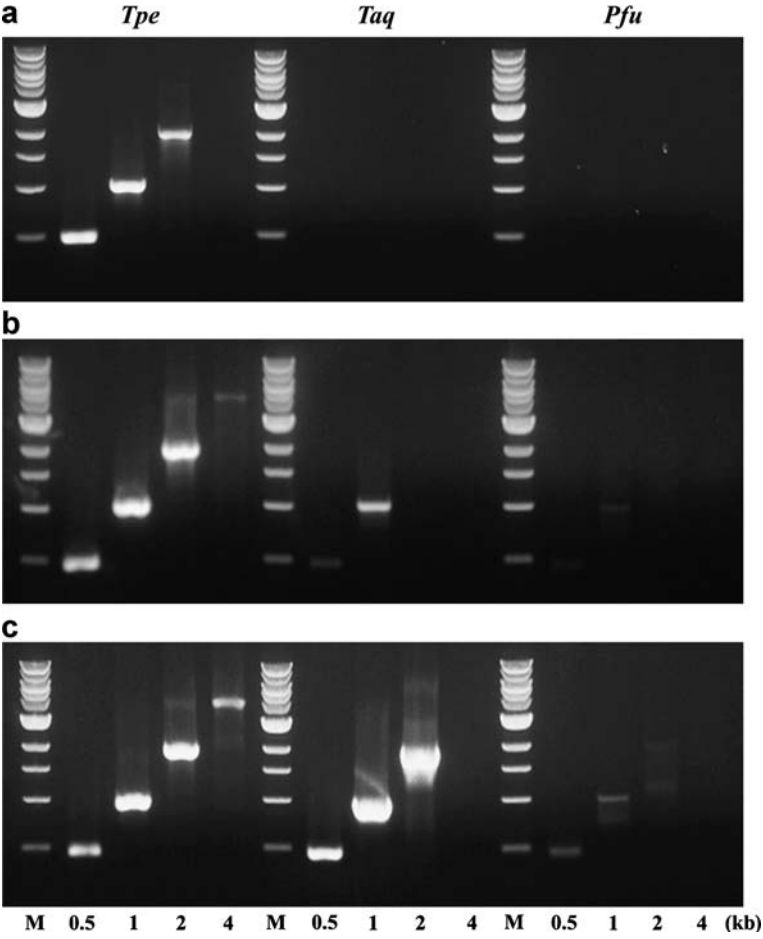

Fig. 8 Comparison of PCR efficiency of *Tpe*, *Taq*, and *Pfu* DNA polymerase. λ DNA was used as the template and the amplicon sizes are *indicated at the bottom*. The cycling protocol was: 94 °C for 20 s; 30 cycles of 94 °C for 20 s and 72 °C for 30 s (**a**) or for 60 s (**b**) or for 2 min (**c**); 72 °C for 30 s. *Lane M*, DNA molecular size marker

Table 2 Comparison of *Tpe*, *Tpe* plus, *Taq*, and *Pfu* DNA polymerase fidelities.

	Blue	White	Mutation frequency[a]	Template doublings[b]	Error rate[c] ($\times 10^{-6}$)	Fold improvement over *Taq*
Tpe	5,423	120	0.0216	7.72	3.37	3.6
Tpe plus	4,808	155	0.0312	6.91	5.43	2.2
Pfu	6,175	84	0.0134	7.41	2.18	5.6
Taq	5,810	526	0.0831	8.23	12.13	1

[a] The mutation frequency is expressed as a percentage of mutant colonies in relation to the total number of colonies

[b] Template doublings are calculated using the equation 2^d =(amount of PCR product)/(amount of starting target)

[c] Error rate is determined using the equation ER=mf/(bp×*d*), where mf is the mutation frequency, bp is the *lacZ* target size(=832), and *d* is the number of template doublings

both the Klenow fragment [39] and for *Vent* polymerase [13], which exhibit 10- and 5-fold increases in error rates, respectively, when the associated 3′ → 5′ exonuclease activity is inactivated. The contribution of proofreading activity to DNA polymerase fidelity is also evident when the error rates of proofreading and non-proofreading enzymes are compared [21]. The fidelity of DNA polymerases has been assigned to the presence of a 3′ → 5′ proofreading exonuclease activity as described above. The fidelity of *Tpe* DNA polymerase in PCR was compared with that of *Taq* and *Pfu* DNA polymerases by measuring the error frequency of each enzyme in the *lacZ* forward mutation assay [19]. *Tpe* DNA polymerase showed high fidelity with an error rate of 3.37×10^{-6} (error rate = mutation frequency/bp/duplication [12, 21]). Moreover, a *Tpe* plus DNA polymerase was compared with other DNA polymerases. *Tpe* plus DNA polymerase exhibited an error rate of 5.43×10^{-6} in the *Taq* buffer. Error rates of DNA polymerases increased in following order: *Pfu* (2.18×10^{-6}) < *Tpe* (3.37×10^{-6}) < *Tpe* plus (5.43×10^{-6}) < *Taq* (12.13×10^{-6}) (Table 2). The fidelity of *Tpe* DNA polymerase was approximately 3.6-fold better than *Taq* DNA polymerase, yet worse than *Pfu* DNA polymerase. *Tpe* plus DNA polymerase also had superior fidelity than *Taq* DNA polymerase.

Acknowledgment This work was supported by the Extreme Genome Research Center Program of the Ministry of Land, Transportation and Maritime Affairs, Republic of Korea.

References

1. Woese, C. R., & Fox, G. E. (1977). *Proceedings of the National Academy of Sciences of the United States of America, 74*, 5088–5090. doi:10.1073/pnas.74.11.5088.
2. Barns, S. M., Delwiche, C. F., Palmer, J. D., & Pace, N. R. (1996). *Proceedings of the National Academy of Sciences of the United States of America, 93*, 9188–9193. doi:10.1073/pnas.93.17.9188.
3. Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C., & Stetter, K. O. (2002). *Nature, 417*, 63–67. doi:10.1038/417063a.
4. Cambon-Bonavita, M. A., Schmitt, P., Zieger, M., Flaman, J. M., Lesongeur, F., Raguenes, G., et al. (2000). *Extremophiles, 4*, 215–225. doi:10.1007/PL00010714.
5. Saiki, R. K., Bugawan, T. L., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1986). *Nature, 324*, 163–166. doi:10.1038/324163a0.
6. Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., et al. (1997). *Applied and Environmental Microbiology, 63*, 4504–4510.
7. Marsic, D., Flaman, J. M., & Ng, J. D. (2008). *Extremophiles, 12*, 775–788. doi:10.1007/s00792-008-0181-7.
8. Lee, Y. J., Choi, J. J., & Kwon, S.-T. (2006). *Enzyme and Microbial Technology, 38*, 821–830. doi:10.1016/j.enzmictec.2005.08.010.
9. Kahler, M., & Antranikian, G. (2000). *Journal of Bacteriology, 182*, 655–663. doi:10.1128/JB.182.3.655-663.2000.
10. Griffiths, K., Nayak, S., Park, K., Mandelman, D., Modrell, B., Lee, J., et al. (2007). *Protein Expression and Purification, 52*, 19–30. doi:10.1016/j.pep. 2006.07.022.
11. Kong, H., Kucera, R. B., & Jack, W. E. (1993). *The Journal of Biological Chemistry, 268*, 1965–1975.
12. Lundberg, K. S., Shoemaker, D. D., Adams, M. W., Short, J. M., Sorge, J. A., & Mathur, E. J. (1991). *Gene, 108*, 1–6. doi:10.1016/0378-1119(91)90480-Y.
13. Mattila, P., Korpela, J., Tenkanen, T., & Pitkanen, K. (1991). *Nucleic Acids Research, 19*, 4967–4973. doi:10.1093/nar/19.18.4967.
14. Southworth, M. W., Kong, H., Kucera, R. B., Ware, J., Jannasch, H. W., & Perler, F. B. (1996). *Proceedings of the National Academy of Sciences of the United States of America, 93*, 5281–5285. doi:10.1073/pnas.93.11.5281.
15. Kim, Y. J., Lee, H. S., Bae, S. S., Jeon, J. H., Lim, J. K., Cho, Y., et al. (2007). *Journal of Microbiology and Biotechnology, 17*, 1090–1097.
16. Shin, H. J., Lee, S. K., Choi, J. J., Koh, S., Lee, J. H., Kim, S. J., et al. (2005). *Journal of Microbiology and Biotechnology, 15*, 1359–1367.

17. Henneke, G., Flament, D., Hubscher, U., Querellou, J., & Raffin, J. P. (2005). *Journal of Molecular Biology, 350*, 53–64. doi:10.1016/j.jmb.2005.04.042.
18. Choi, J. J., Nam, K. H., Min, B., Kim, S. J., Soll, D., & Kwon, S. T. (2006). *Journal of Molecular Biology, 356*, 1093–1106. doi:10.1016/j.jmb.2005.12.036.
19. Song, J. M., Choi, J. J., Kim, T. O., Seo, M. S., Lee, M. S., Kim, H. K., et al. (2007). *Enzyme and Microbial Technology, 40*, 1475–1483. doi:10.1016/j.enzmictec.2006.10.025.
20. Cann, I. K., & Ishino, Y. (1999). *Genetics, 152*, 1249–1267.
21. Cline, J., Braman, J. C., & Hogrefe, H. H. (1996). *Nucleic Acids Research, 24*, 3546–3551. doi:10.1093/nar/24.18.3546.
22. Barnes, W. M. (1994). *Proceedings of the National Academy of Sciences of the United States of America, 91*, 2216–2220. doi:10.1073/pnas.91.6.2216.
23. Weissensteiner, T., Griffin, H. G., & Griffin, A. M. (2004). *Current innovations*. Boca Raton: CRC.
24. Gonzalez, J. M., Kato, C., & Horikoshi, K. (1995). *Archives of Microbiology, 164*, 159–164. doi:10.1007/BF02529966.
25. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbor Laboratory.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *The Journal of Biological Chemistry, 193*, 265–275.
27. Sanger, F., Coulson, A. R., Hong, G. F., Hill, D. F., & Petersen, G. B. (1982). *Journal of Molecular Biology, 162*, 729–773. doi:10.1016/0022-2836(82)90546-0.
28. Kwon, S.-T., Kim, J. S., Park, J. H., Kim, H. K., & Lee, D. S. (1997). *Molecules and Cells, 7*, 264–271.
29. Choi, J. J., Song, J. G., Nam, K. H., Lee, J. I., Bae, H., Kim, G. A., et al. (2008). *Applied and Environmental Microbiology, 74*, 6563–6569. doi:10.1128/AEM.00624-08.
30. Hamilton, S. C., Farchaus, J. W., & Davis, M. C. (2001). *BioTechniques, 31,* 370–376, 378–380, 382–383.
31. Park, J. H., Kim, J. S., Kwon, S.-T., & Lee, D.-S. (1993). *European Journal of Biochemistry, 214*, 135–140. doi:10.1111/j.1432-1033.1993.tb17905.x.
32. Ellsworth, D. L., Eittenhouse, K. D., & Honeycutt, R. L. (1993). *BioTechniques, 14*, 214–217.
33. Cheng, S., Chang, S. Y., Gravitt, P., & Respess, R. (1994). *Nature, 369*, 684–685. doi:10.1038/369684a0.
34. Nishioka, M., Mizuguchi, H., Fujiwara, S., Komatsubara, S., Kitabayashi, M., Uemura, H., et al. (2001). *Journal of Biotechnology, 88*, 114–119. doi:10.1016/S0168-1656(01)00275-9.
35. Burger, G., Lavrov, D. V., Forget, L., & Lang, B. F. (2007). *Nature Protocols, 2*, 603–614. doi:10.1038/nprot.2007.59.
36. Hu, M., Chilton, N. B., & Gasser, R. B. (2002). *Molecular and Cellular Probes, 16*, 261–267. doi:10.1006/mcpr.2002.0422.
37. Zheng, R., Matsui, E., Shen, Y., Musti, K. V., Feng, Y., Darnis, S., et al. (2001). *Extremophiles, 5*, 111–117. doi:10.1007/s007920100178.
38. Jacewicz, A., Makiela, K., Kierzek, A., Drake, J. W., & Bebenek, A. (2007). *Journal of Molecular Biology, 368*, 18–29. doi:10.1016/j.jmb.2007.01.067.
39. Kunkel, T. A. (1988). *Cell, 53*, 837–840. doi:10.1016/S0092-8674(88)90189-4.