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(54) **Title**: DNA POLYMERASES FROM THE RED SEA BRINE POOL ORGANISMS

(57) **Abstract**: A DNA polymerase composition for amplifying nucleic acids can be tolerant of extreme conditions.

DNA POLYMERASES FROM THE RED SEA BRINE POOL ORGANISMS

PRIORITY CLAIM

This application claims priority to U.S. Provisional Application No. 61/978,406, filed April 11, 2014, which is incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to DNA polymerases.

BACKGROUND

Polymerase chain reaction (PCR) is a method for the rapid and exponential amplification of target nucleic acid sequences. It has found numerous applications in gene characterization and molecular cloning technologies including the direct sequencing of PCR amplified DNA, the determination of allelic variation, and the detection of infectious and genetic disease disorders. Various thermostable DNA polymerases have been used for PCR applications; for example, Taq polymerase isolated from *Thermus aquaticus* (Taq), *pfu* polymerase derived from *Pyrococcus furiosus*, KOD polymerase isolated from *Thermococcus kodakaraensis*, and Vent™ DNA polymerase isolated from *Thermococcus litoralis (Tli)*. See, for example, U.S. Patent No. 6,008025, U.S. Patent No. 5,545,552 and U.S. Patent No. 5,489,523, each of which is incorporated by reference in its entirety.

SUMMARY

In one aspect, a DNA polymerase composition for amplifying nucleic acids includes an isolated DNA polymerase having an amino acid residue having at least 80% homology with the sequence of SEQ ID NOS:1-4. The polymerase can be isolated from a brine pool thermophilic archaea species. The polymerase can have about 40% sequence homology to DNA polymerase isolated from *Thermococcus litoralis*.

In certain embodiments, the polymerase retains at least 100% of its optimal DNA polymerase activity in the presence of chloride ion at a concentration as high as 300 mM. The activity increased with increasing the chloride ion concentration with 300 mM being the optimal concentration.

In certain embodiments, the polymerase retains at least 50% of its optimal DNA polymerase activity in the presence of sulfate ion at a concentration as high as 300 mM. The activity increases with increasing the sulfate ion concentration with 100 mM being the optimal concentration and decrease to 50% at sulfate concentration of 300 mM.

In certain embodiments, the polymerase retains 100% of its optimal DNA polymerase activity in the presence of K-glutamate at a concentration as high as 250 mM. The activity increases with increasing the K-glutamate concentration with 250 mM being the optimal concentration.

In certain embodiments, the polymerase retains at least 50% of its optimal DNA polymerase activity in the presence of Zn^{2+} ion at a concentration as high as 1 mM. The activity is optimal at 0.5 mM Zn^{2+} ion concentration and decrease to 50% at 1 mM.

In certain embodiments, the polymerase retains 100% of its DNA polymerase activity in the presence of Mg^{2+} ion at a concentration as high as 100 mM. The activity increases with increasing the Mg^{2+} ion concentration with 100 mM being the optimal concentration.

In certain embodiments, the polymerase has a DNA extension rate of greater than 450 bases per second.

In certain embodiments, the polymerase has a DNA extension processivity of an average of 2000 bases per one cycle of DNA binding event.

In certain embodiments, the polymerase has a DNA proofreading activity that is at least 2 fold more active than *pfu* polymerase.

In certain embodiments, the polymerase retains its stability 100% after being heated at 65°C for 15 minutes.

In certain embodiments, the polymerase is active at room temperature.

In certain embodiments, the polymerase retains 100% of its DNA polymerase activity in pH conditions between 7.5−9.0.

In another aspect, a method for amplifying nucleic acid can include reacting DNA as a template, a primer, dNTP and the DNA polymerase composition, and extending the primer to synthesize a DNA primer extension product.

In other aspect, a method for amplifying nucleic acid where the ability of the polymerase to extend DNA under high salt and metal ion concentration and different type of metal ions, enables its utilization to improve currently available molecular biology, biochemical and biophysical techniques.

In another aspect, a kit for amplifying nucleic acid can include the DNA polymerase composition.

In another aspect, vector can include a gene encoding the DNA polymerase.

In another aspect, a plasmid can include a gene encoding for a recombinant form of the DNA polymerase.

Other aspects, embodiments, and features will be apparent from the following description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is an image comparing BR3 polymerase and *pfu* polymerase activities at different salt concentrations. Salt used in these experiments is NaCl. BR3 and *pfu* concentrations are 50 nM. FIG. 1B is an image of KOD polymerase activities at different salt concentrations. Salt used in this experiment is KCl and KOD concentration is 50 nM.

FIGS. 2A-2C are images comparing BR3 polymerase and *pfu* polymerase activities in the presence of different salts. Salts used in these experiments are: KCl (2A), $NH_4(SO)_4$ (B) and K-Glutamate (C). BR3 and *pfu* concentrations are 50 nM.

FIGS. 3A-3C are images comparing BR3 polymerase and *pfu* polymerase activities in the presence of different metal ions. Concentrations of metal ions used in (A) and (B) is 1 mM for MgCl2, $MnCl_2$, $CaCl_2$, $ZnSO_4$, LiCl. BR3 and *pfu* concentrations are 50 nM.

FIG. 4A is an image comparing BR3 polymerase and *pfu* polymerase activities in the presence of different Mg^{2+} concentrations. BR3 and *pfu* concentrations are 50 nM. FIG. 4B is an image of KOD polymerase activities in the presence of different Mg2+ concentrations.

FIG. 5 is an image comparing BR3 polymerase and *pfu* polymerase proofreading activities.

FIG. 6 is an image and a graph depicting the single molecule assay used to measure rate and processivity of BR3 and *pfu* polymerases and the histograms of rate and processivity of DNA synthesis by BR3 and *pfu* polymerases.

FIG. 7 is an image depicting the thermal stability of BR3 polymerase.

FIG. 8 is an image depicting the ddNTP incorporation efficiency of BR3 polymerase and the strategy to engineer its active site to incorporate ddNTP.

FIG. 9 represents the sequences of BR1, BR2, BR3, and BR6 polymerases.

FIG. 10 represents the open reading frame sequence of the BR3 polymerase (SEQ ID NO:3).

FIG. 11 represents primary sequence alignment of BR3, KOD, *Pfu* and Tli polymerases. Sequences are color-highlighted based on common domain structures in DNA polymerases, key amino acids involved in catalysis are in bold red and blue and cysteine residues involved in thermal stability of the polymerase structure are in bold yellow.

DETAILED DESCRIPTION

The deep-sea anoxic brines of the Red Sea are considered to be one of the most remote, challenging and extreme environments on Earth, while remaining one of the least studied. Approximately 25 such brine-filled pools are currently known, with all of them being anoxic, highly saline deep-sea water bodies with elevated temperatures, heavy metal concentration with different types of metal ions that form characteristically sharp gradient-rich interfaces with the overlaying sea water. See Backer H & Schoell M (1972) New deeps with brines and metalliferous sediments in Red Sea. *Nature-Physical Science* 240(103):153, and Hartmann M, Scholten JC, Stoffers P, & Wehner F (1998) Hydrographic structure of brine-filled deeps in the Red Sea - new results from the Shaban, Kebrit, Atlantis II, and Discovery Deep. *Mar Geol* 144(4):311-330, each of which is incorporated by reference in its entirety. In contrast to frequent geological and geochemical studies, very few studies have focused on the microbiology of the deep-sea brines of the Red Sea, and none have concentrated on their biotechnological applications. Initial cultivation-independent and cultivation-based studies have provided a first glimpse on the unexpected enormous biodiversity of the local microbial communities, with identification of several new groups and the isolation of new extremophilic microorganisms that thrive in these environments. See Antunes A, Eder W, Fareleira P, Santos H, & Huber R (2003) Salinisphaera shabanensis gen. nov., sp nov., a novel, moderately halophilic bacterium from the brine-seawater interface of the Shaban Deep, Red Sea. *Extremophiles* 7(1):29-34, Antunes A, *et al.* (2008) A new lineage of halophilic, wall-less, contractile bacteria from a brine-filled deep of the Red Sea. *J Bacteriol* 190(10):3580-3587, Antunes A, *et al.* (2008) Halorhabdus tiamatea sp nov., a non-pigmented, extremely halophilic archaeon from a deep-sea, hypersaline anoxic basin of the Red Sea, and emended description of the genus Halorhabdus. *Int J Syst Evol Micr*

58:215-220, Eder W, Ludwig W, & Huber R (1999) Novel 16S rRNA gene sequences retrieved from highly saline brine sediments of Kebrit Deep, Red Sea. *Arch Microbiol* 172(4):213-218, Eder W, Jahnke LL, Schmidt M, & Huber R (2001) Microbial diversity of the brine-seawater interface of the Kebrit Deep, Red Sea, studied via 16S rRNA gene sequences and cultivation methods. *Appl Environ Microb* 67(7):3077-3085, and Eder W, Schmidt M, Koch M, Garbe-Schonberg D, & Huber R (2002) Prokaryotic phylogenetic diversity and corresponding geochemical data of the brine-seawater interface of the Shaban Deep, Red Sea. *Environ Microbiol* 4(11):758-763, each of which is incorporated by reference in its entirety. Because of the unusually harsh conditions of this environment, it is highly likely that the residing microbes developed novel metabolic pathways, transport systems across their membranes, enzymes, and chemicals in order to survive.

This environment presents the harshest conditions for the DNA replication machinery as well as DNA processing enzymes to copy and to maintain the genomic DNA, indicating the utilization of novel adaptive mechanisms and nucleic acid binding proteins. Archaeal and bacterial species from the brine pool can be used to screen for novel DNA sequencing polymerase and other key DNA modifying enzymes.

The classical chain-termination method used for DNA sequencing, the Sanger method, relies on using a DNA polymerase that has high rate of incorporation of the chain terminator dideoxynucleoside triphosphate (ddNTP). See Tabor S & Richardson CC (1995) A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxyribonucleotides. *Proc Natl Acad Sci U S A* 92(14):6339-6343, which is incorporated by reference in its entirety. DNA polymerases normally catalyze a nucleophilic attack of the 3'-OH group of the primer on the α-phosphate of the incoming dNTP. Two Mg^{2+} ions are required in this reaction to align the primer/template strand and the incoming dNTP and to mediate the substitution nucleophilic attack reaction. See Johnson A & O'Donnell M (2005) Cellular DNA replicases: components and dynamics at the replication fork. *Annu Rev Biochem* 74:283-315, and Hamdan SM & Richardson CC (2009) Motors, switches, and contacts in the replisome, each of which is incorporated by reference in its entirety. The lack of the 3'-OH nuleophilic group in the ddNTP is the reason for its action as a DNA polymerase inhibitor. See Tabor S & Richardson CC (1995) A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between

deoxy- and dideoxyribonucleotides. *Proc Natl Acad Sci U S A* 92(14):6339-6343, which is incorporated by reference in its entirety. During sequencing, DNA synthesis reaction starts from a specific primer and ends upon the incorporation of ddNTP. By using either dye- or radiolabel-based ddNTP, the identity of these products can be mapped. In general, DNA polymerases polymerize the dNTP with very high accuracy (1 mistake per 10^3-10^5 incorporated nucleotides) and encode for a proofreading exonucleases activity to remove misincorporated nucleotide (accuracy increased to 1 mistake per 10^5-10^7 incorporated nucleotides). Therefore, the ideal DNA sequencing polymerase will have high rate and high processivity of DNA synthesis, high accuracy of dNTP incorporation, proofreading exonuclease activity, high thermal stability and high rate of incorporation of ddNTP.

Indeed all these properties have been fulfilled with the introduction of four DNA polymerases to the market that are isolated from archaea species, *Pyrococcus furiosus* DNA polymerase (*pfu* DNA Pol), *Thermococcus litoralis* Vent™ DNA polymerase (Vent DNA Pol), *Thermococcus Kodakarensis* (KOD Pol) and *Thermus Aquaticus* DNA polymerase (Taq Pol) (Table 1).

Table 1. Characteristics of DNA Polymerases. The table is adopted from Takagi M *et al.* (1997) Characterization of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. *Applied and Environmental Microbilogy* 63(11):4504-4510

	KOD	PFU	Taq
Species	Thermococcus kodakaraensis	Pyrococcus furiosus	Thermus aquaticus YT-1
Fidelity	0.0035	0.0039	0.013
Elongation rate (bases/second)	106-138	25	61
Processivity (nucleotide bases)	>300	<20	unavailable

Disclosed herein is a method of utilizing DNA polymerases from the Brine pool with the aim of generating a commercial polymerase that has the robust reaction features of utilizing wide range of salt and metal ion concentration and metal ion types as well as enhanced rate processivity and proofreading activity. Also disclosed is a method and a composition for amplifying nucleic acid where the ability of the polymerase to extend DNA under high salt and high metal ion concentrations, in the presence of different type of metal ions, and high temperature conditions enables its utilization to improve currently available molecular biology, biochemical and biophysical techniques. None of the

conventional polymerases are ideal for the harsh conditions like high salt concentrations, high metal concentrations, and high temperature, let alone combinations of two or more of these conditions. Disclosed herein is a polymerase that can not only tolerate under one of these harsh conditions, but also can tolerate various combinations of those conditions, e.g. high salt and metal concentrations, high metal concentration and high temperate, high salt concentration and high temperate, or high salt and metal concentrations and high temperate.

Robust DNA sequencing enzymes isolated from the Brine pool can sustain wide range of salt and metal ion concentrations, different type of metal ions and wide range of pH during PCR. Four clones of DNA polymerase have been identified from the brine pool (FIG. 9, Table 2). These polymerases from microorganisms from the Brine pool can be used for conducting a PCR reaction at wide range of buffer conditions and metal ions concentration and types. Optimization of PCR remains tricky as it might require screening for the appropriate salt and metal ion concentrations that lead to high yield, high processivity and accuracy of the amplified DNA fragment. The ability of thermal archaea species from the brine pool to replicate their genome at high salt concentration indicates that their DNA polymerases binds to the DNA with relatively high affinity, which could potentially enhance the sensitivity of the PCR, and could therefore tolerate wide range of salt concentrations. Furthermore, the ability of these DNA polymerases to tolerate high metal ion concentrations indicates that they can work at wide range of metal ion concentrations. Finally, the ability of these DNA polymerases to tolerate different type of metal ions indicates that they can work at wide range of metal ion types. DNA polymerases from the brine pool thermophilic archaea species were cloned, expressed, purified and characterized.

Table 2. Identification of DNA Polymerases from the Brine Pool.

	size	Homologous species	Enzyme	% homology
Clone 1 (SEQ ID NO:1)	2100bp, 700aa	*Candidatus Nitrososphaera gargensis*	DNA Polymerase B	25%
Clone 2 (SEQ ID NO:2)	2352bp, 784aa	*Thermococcus celer*	DNA dependent Polymerase	42%
Clone 3 (SEQ ID NO:3)	2457bp, 819aa	*Thermococcus litoralis*	DNA dependent Polymerase	42%
Clone 6 (SEQ ID NO:4)	2400bp, 800aa	*Candidatus Nanosalinarum*	DNA dependent Polymerase	42%

Especially, Clone 3 (termed BR3, FIG. 10) which is 42% homologous to DNA dependent polymerase of *Thermococcus litoralis* shows much more robust properties than any known commercially available DNA polymerases that are used in PCR and DNA sequencing.

BR3 tolerates extremely versatile buffer conditions. FIGS. 1A and 1B show that BR3 polymerase retains its optimal activity up to 300 mM NaCl whereas *pfu* and KOD polymerases retains their optimal activity only up to 10 mM. BR3 polymerase also tolerates different types of salts. FIG.2 shows that BR3 polymerase tolerates up to 300 mM KCl (FIG. 2A), up to 100 mM $(NH_4)_2SO_4$ (FIG. 2B), and up to 250 mM K-Glutamate (FIG. 2C). The range is at least 15-30 fold higher than *pfu* polymerase.

FIG. 3 shows that BR3 polymerase shows much higher activity than *pfu* polymerase in the presence of $MgCl_2$ and $MnCl_2$ when the salt concentration is high (FIG. 3B). Moreover, BR3 polymerase shows high metal ion resistance, for example 0.1-100 mM $MgCl_2$ (FIGS. 4A and 4B). This range is 10-fold higher than *pfu* polymerase and KOD polymerase. When the salt concentration is low (FIG. 3A), *pfu* polymerase shows higher activity in the presence of $MgCl_2$ and $MnCl_2$. Neither polymerases show a significant activity when calcium or lithium ions are present. It is notable that in the high salt concentration, BR3 polymerase retains activity in the presence of zinc ions (FIGS. 3B and 3C). BR3 polymerase is the first known polymerase to use zinc ions or any metal ions other than Mg^{2+} and Mn^{2+}.

BR3 polymerase has at least 2-fold higher proofreading activity than *pfu* polymerase (FIG. 5). FIG.5 shows that BR3 polymerase only requires half the concentration of *pfu* polymerase to produce the same activity level in the presence of up to three mismatches on the primer strand.

FIG. 6 shows the single molecule assay that was used to measure the rate and processivity of BR3 and compare it with *pfu* polymerase and Table 3 shows the results from this measurement, where BR3 polymerase displays at least 1.5-fold higher rate and processivity than *pfu* polymerase.

Table 3. Comparison of rate and processivity of BR3 polymerase and *pfu* polymerase

	Rate (base/sec)	Processivity (kb)
BR3 polymerase	463.34±34.73	2.0±0.3
PFU polymerase	305.5±40.46	1.3±0.1

BR3 polymerase retains the same polymerization activity at pH range between 7.5−9.0. It also showed high thermal stability up to 65 °C (FIG. 7). Its thermal stability can be likely increased by inducing the formation of a highly conserved disulfide bond in the active site of extreme thermophilic polymerases. BR3 polymerase also discriminates well against the incorporation of ddNTP (FIG. 8). It is highly likely to increase the incorporation efficiency of ddNTP by mutating F residue in active site to Y in BR3 (FIG. 8).

These properties make this polymerase ideal to be used in DNA sequencing and molecular biology techniques with minimal reaction optimization and with different sample types and preparations.

The BR3 polymerase can be produced using recombinant techniques.

As used herein, the terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term "recombinant polypeptide" refers to a polypeptide that is produced by recombinant techniques, wherein generally DNA or RNA encoding the expressed protein is inserted into a suitable expression vector that is in turn used to transform a host cell to produce the polypeptide.

As used herein, the terms "homolog," and "homologous" refer to a polynucleotide or a polypeptide comprising a sequence that is at least about 50% identical to the corresponding polynucleotide or polypeptide sequence. Preferably homologous polynucleotides or polypeptides have polynucleotide sequences or amino acid sequences that have at least about 80%, at least about 85%, at least about 90%, at least about 91 %, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% homology to the corresponding amino acid sequence or polynucleotide sequence. As used herein the terms sequence "homology" and sequence "identity" are used interchangeably. One of ordinary skill in the art is well aware of methods to determine homology between two or more sequences, for example, using BLAST.

A mutant or variant polypeptide refers to a polypeptide having an amino acid sequence that differs from the corresponding wild-type polypeptide by at least one amino acid. In some embodiments, the mutant polypeptide has about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more amino acid substitutions, additions, insertions, or deletions. For example, the mutant can comprise one or more conservative amino acid substitutions. As used herein, a "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Preferred variants of a polypeptide or fragments a polypeptide retain some or all of the biological function (e.g., enzymatic activity) of the corresponding wild-type polypeptide. In some embodiments, the variant or fragment retains at least about 75% (e.g., at least about 80%, at least about 90%, or at least about 95%) of the biological function of the corresponding wild-type polypeptide. In other embodiments, the variant or fragment retains about 100% of the biological function of the corresponding wild-type polypeptide. In still further embodiments, the variant or fragment has greater than 100% of the biological function of the corresponding wild-type polypeptide. It is understood that the polypeptides described herein may have additional conservative or non-essential

amino acid substitutions, which do not have a substantial effect on the polypeptide function.

EXAMPLES

Enzymes: The cDNA fragment corresponding to BR3 (see FIG. 9 SEQ ID NO:3) was amplified by PCR using primers (5'-CACCATGGCAAATCAGACAACAAATGG-3' and 5'-TTATTTGAATTTTCCGAGTTTTACTTGTCG-3') and cloned into the pENTR-D/TOPO vector (Life Technology). The ORF of BR3 was transferred to pDEST17 vector (Life Technology) by using LR Clonase II enzyme mix (Life Technology). BR3 is overexpressed in *E. coli* Rozetta2 (DE3) (Novagen) after transformation with plasmids pDEST17/BR3. The overexpression was induced by addition of Isopropyl ß-D-1-thiogalactopyranoside (final concentration, 1 mM), and cells were harvested after 3 h of incubation. The collected cells were dissolved in Lysis buffer (10 mM Tris-HCl pH 8.0, 80 mM KCl, 5 mM 2-Mercaptoethanol, 1mM EDTA) and incubated on ice with Lysozyme (final concentration, 1mM) for 30 m, then disrupted by sonication. The crude extract was centrifuged to remove cell debris, the supernatant was collected and ammonium precipitation was performed with 80% saturation. The pellet obtained from ammonium precipitation was dissolved in Buffer A (10 mM Tris-HCl pH 8.0, 1 mM EDTA and loaded onto the Sephacryl Sepharose (GE Healthcare) column. The flow through fraction from Sephacryl Sepharose was collected and diluted enough to reduce EDTA concentration, then loaded onto HisTrap HP 5ml (GE Healthcare) and the bound proteins were eluted by Buffer B (10 mM Tris-HCl pH 8.0, 50 mM KCl, 500 mM Imidazole). The peak fractions were collected and passed through HiTrap Heparin 1ml (GE Healthcare) and the fractions containing pure proteins were eluted by making a gradient against Buffer C (10 mM Tris-HCl pH 8.0, 50 mM KCl, 1 M KCl). The purified BR3 proteins were dialyzed against Buffer D (50 mM Tris-HCl pH 7.5, 50 mM KCl, 1mM DTT, 0.1 % Tween20, 50 % Glycerol). The protein concentration was determined by absorbance at 280 nm with the extinction coefficient and molecular weight were calculate based on the amino acid sequence of BR3 protein.

Primer extension and proofreading activity assay: The polymerase and proofreading activities were characterized as published (see Lundberge K.S. *et al.* (1991) High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus* (Polymerase chain reaction; mutation archaebacteria) frequency; lack; proofreading;

3'40-5 exonuclease; recombinant DNA. *Gene* (108): 1-6 with following modifications for the proofreading assay. The 35-mer template containing an internal *Eco*RI site is annealed to 15-mer Cy3-labeled primers with 0, 1, and 3 mismatch nt at the 3' terminus. Reactions were carried out at 45 C° in 22 µl for 5 min and contained basic buffer (20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1 % TritonX-100), 200 µM dNTPs and 1 mM $MgCl_2$. Of each reaction 10 µl was removed and reactions were stopped by adding 4 µl of stop solution (100 mM EDTA pH 8.0), the remaining 10 µl of each reaction was digested with 5 u of *Eco*RI at 37 C° for 30 min. The reactions were terminated by adding 4 µl of stop solution. The synthesized product was loaded to 15 % polyacrylamide/7.5 M urea/1x TBE denaturing gel. The gel was visualized by Typhoon TRIO (GE Healthcare). The polymerization activity of KOD was tested by conventional PCR on primed ssDNA pUC19 plasmid as a template.

Primer extension assay at the single molecule level: DNA synthesis was measured by monitoring the length of individual DNA molecules in real time as described previously (See Tanner, N. A. *et al.* (2008) Single-molecule studies of fork dynamics in Escherichia coli DNA replication. *Nature structural & molecular biology* (15): 170-176, Jergic, S. *et al.* (2013) A direct proofreader-clamp interaction stabilizes the Pol III replicase in the polymerization mode. *The EMBO journal* (32):1322-1333, and Lee, J. B. *et al.* (2006) DNA primase acts as a molecular brake in DNA replication. *Nature* (439):621-624, each of which is incorporated by reference in its entirety. Briefly, ssDNA template containing a biotinylated primer was attached to the surface of a glass coverslip via one end and to a magnetic bead via the other end in microfluidic flow cell (FIG. 6). The DNA molecules were stretched by a laminar flow that exerted a 2.6 piconewten (pN) drag force on the beads. Primer extension converts the surface tethered ssDNA (short) to dsDNA (long) and increase the length of the DNA as schematically illustrated in FIG. 6 and shown in the trajectories in FIG. 6. The assay was performed at 25 °C in buffer containing (20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % TritonX-100), 200 µM dNTPs, 1 mM $MgCl_2$, and either 250 mM KCl in case of BR3 polymerase or 50 mM KCl in case of *pfu* polymerase. BR3 and *pfu* polymerases were used at 50 nM.

Other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A DNA polymerase composition for amplifying nucleic acids comprising:
an isolated DNA polymerase having at least 80% homology with the sequence of SEQ ID NO:1-4.

2. A method for amplifying nucleic acid comprising:
reacting DNA as a template, a primer, dNTP and the DNA polymerase composition of claim 1, and extending the primer to synthesize a DNA primer extension product.

3. A kit for amplifying nucleic acid comprising the DNA polymerase composition of claim

4. A vector comprising a gene encoding the DNA polymerase of claim 1.

5. A method of utilizing the Brine pool to isolate a DNA polymerase,
wherein the polymerase retains its activity in the presence of high salts and metal ions concentrations and different type of metal ions including Zn^{2+}.

6. The DNA polymerase composition of claim 1 whereing the isolated DNA polymerase has at least 80% homology with the sequence of SEQ ID NO: 1.

7. The DNA polymerase composition of claim 1 whereing the isolated DNA polymerase has at least 80% homology with the sequence of SEQ ID NO: 2.

8. The DNA polymerase composition of claim 1 whereing the isolated DNA polymerase has at least 80% homology with the sequence of SEQ ID NO: 3.

9. The DNA polymerase composition of claim 1 whereing the isolated DNA polymerase has at least 80% homology with the sequence of SEQ ID NO: 4.

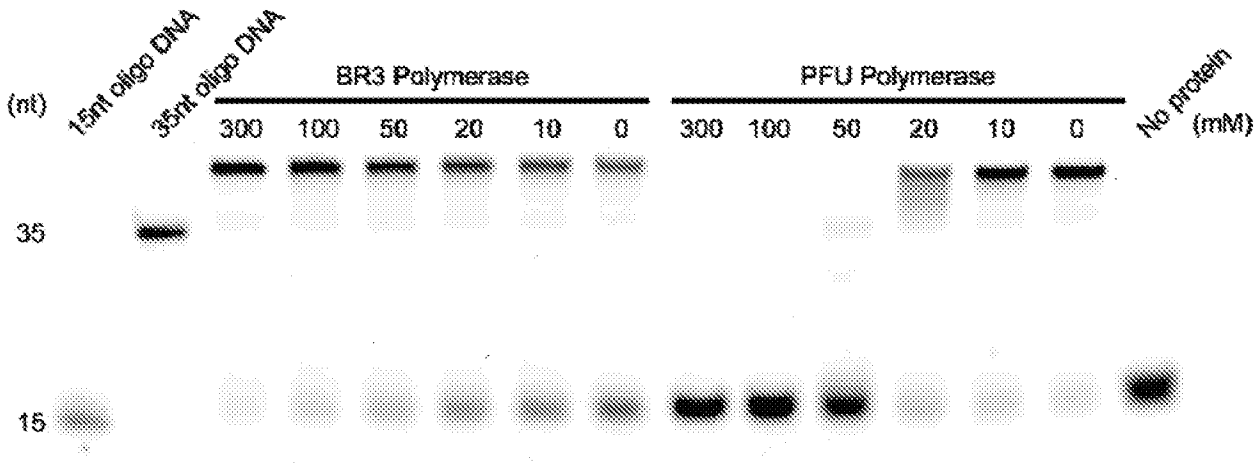

(nt)
15nt oligo DNA
35nt oligo DNA
BR3 Polymerase
300 100 50 20 10 0
PFU Polymerase
300 100 50 20 10 0
No protein
(mM)
35
15

FIG. 1A

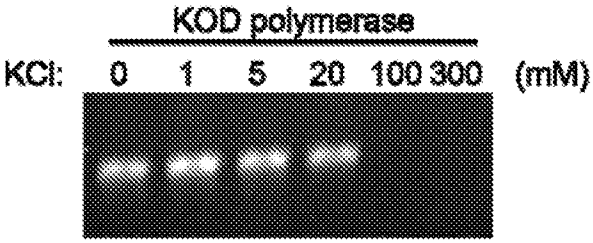

KOD polymerase
KCl: 0 1 5 20 100 300 (mM)

FIG. 1B

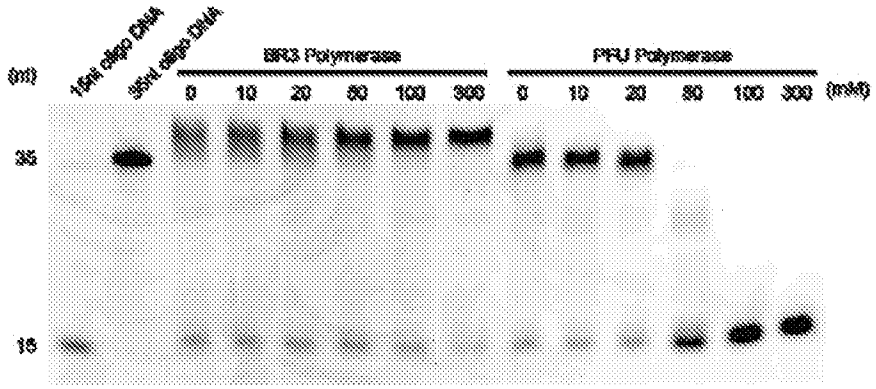


FIG. 2A

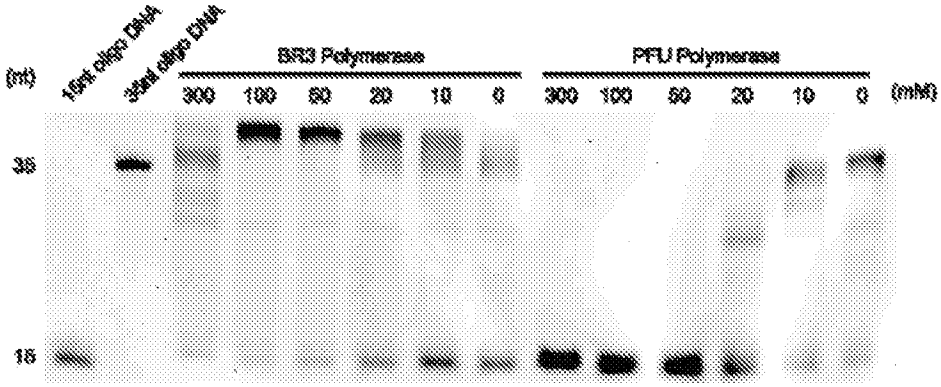


FIG. 2B

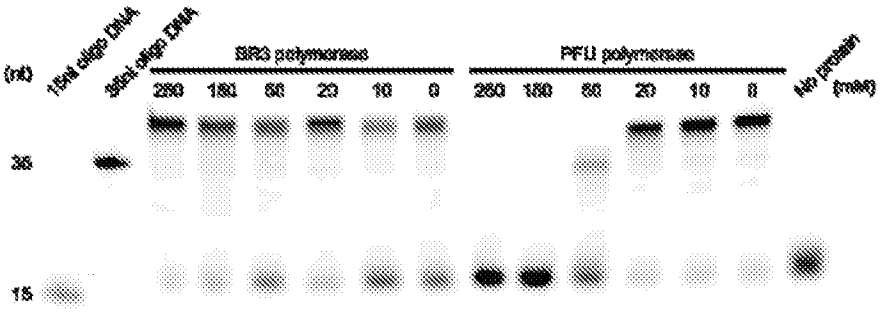


FIG. 2C

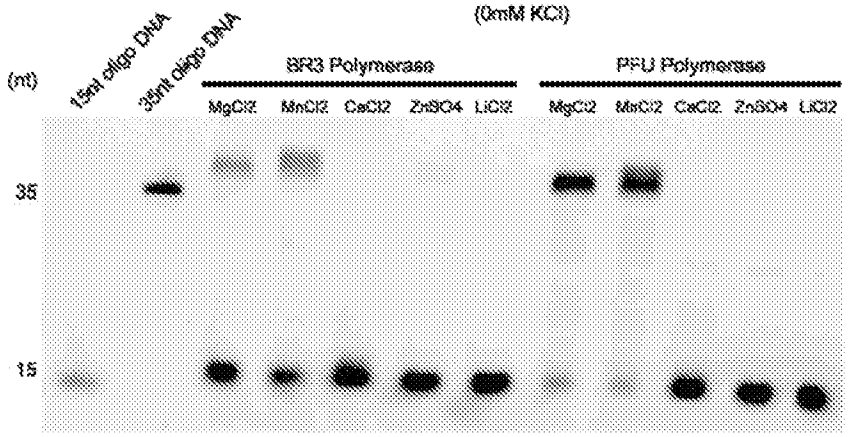


FIG. 3A

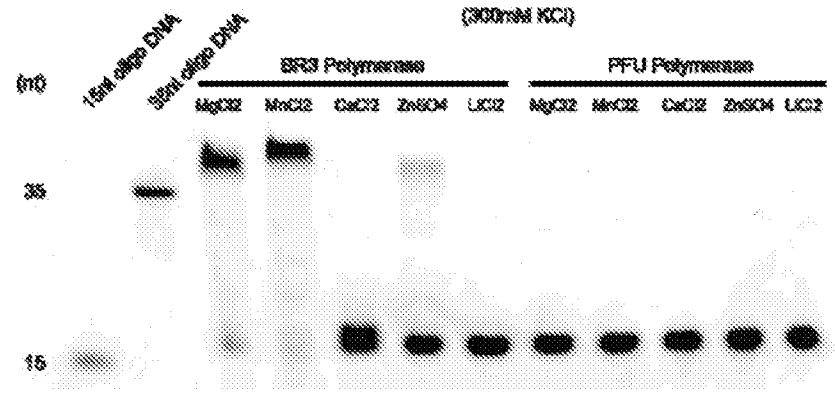


FIG. 3B

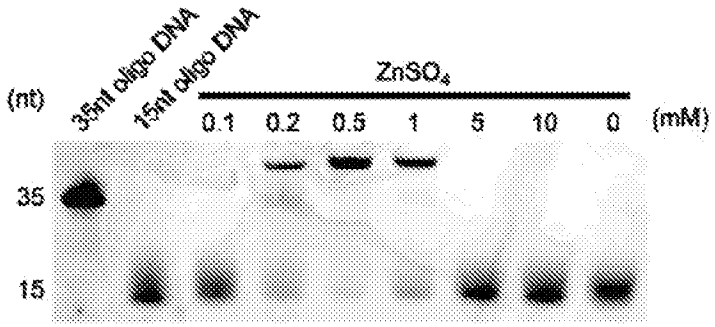


FIG. 3C

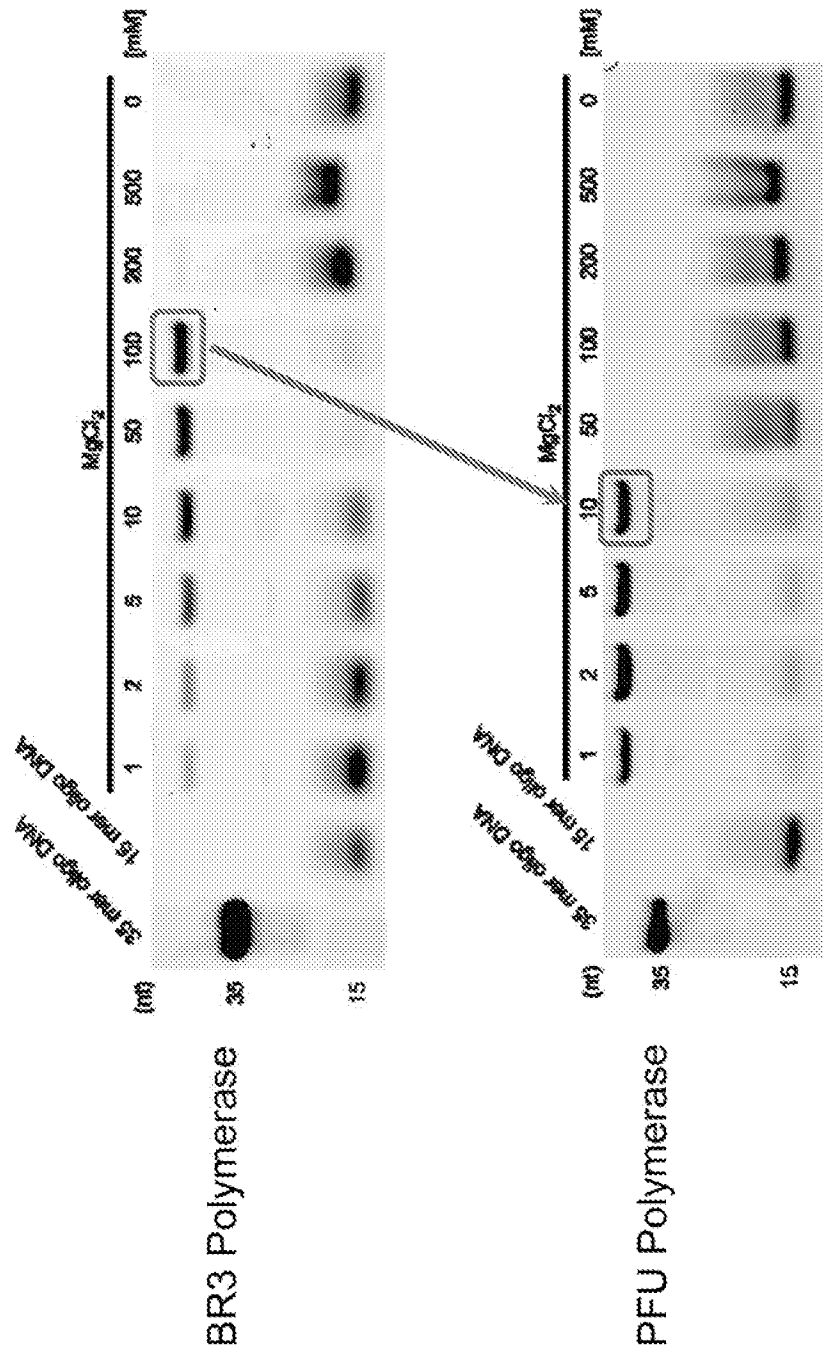

BR3 Polymerase
PFU Polymerase
35 mer oligo DNA
15 mer oligo DNA
MgCl2
[mM]
0
500
200
100
50
10
5
2
1
(nt)
35
15

FIG. 4A

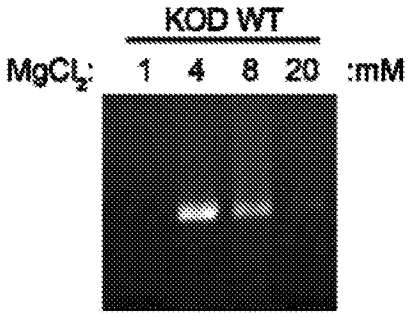


FIG. 4B

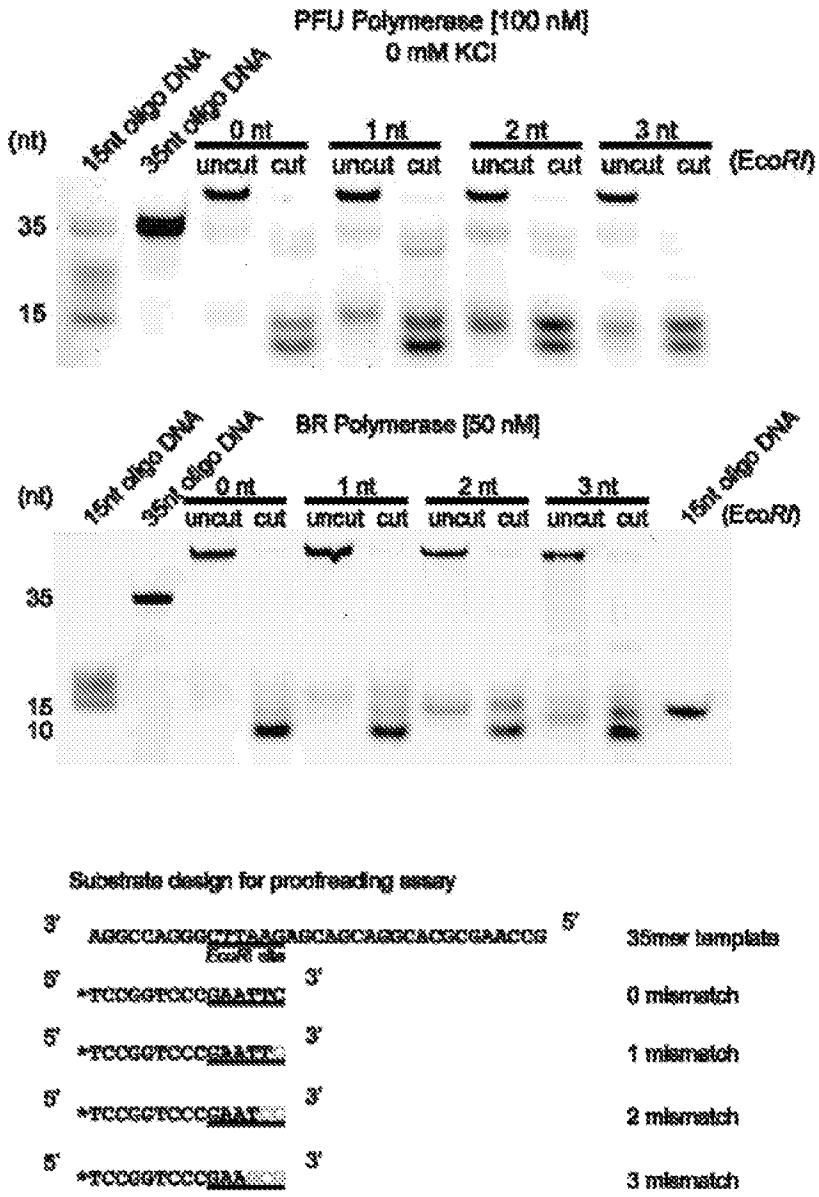
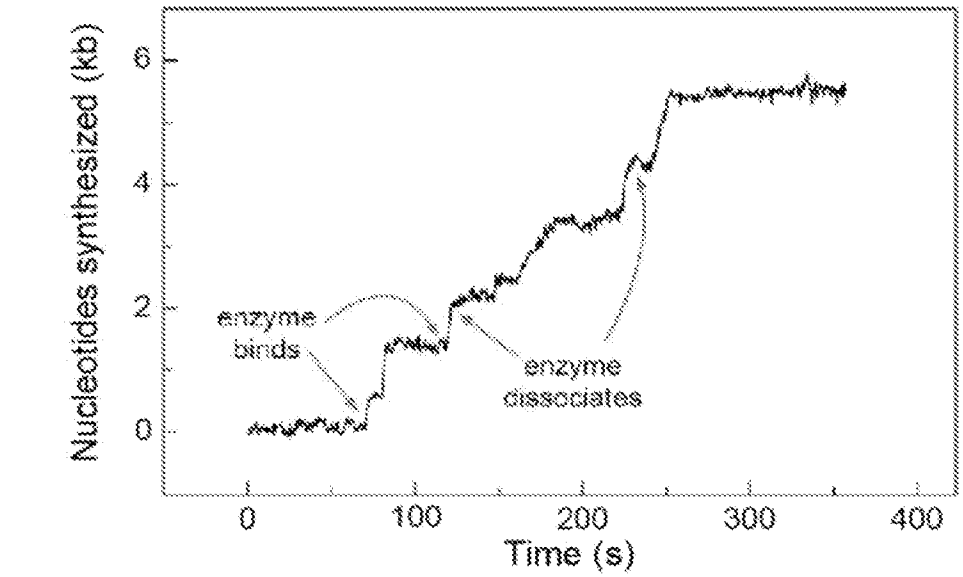

PFU Polymerase [100 nM]
0 mM KCl
15nt oligo DNA
35nt oligo DNA
0 nt
1 nt
2 nt
3 nt
uncut cut
(nt)
(EcoRI)
35
15
BR Polymerase [50 nM]
10
Substrate design for proofreading assay
35mer template
0 mismatch
1 mismatch
2 mismatch
3 mismatch

FIG. 5


Nucleotides synthesized (kb)
0
2
4
6
enzyme binds
enzyme dissociates
0
100
200
300
400
Time (s)

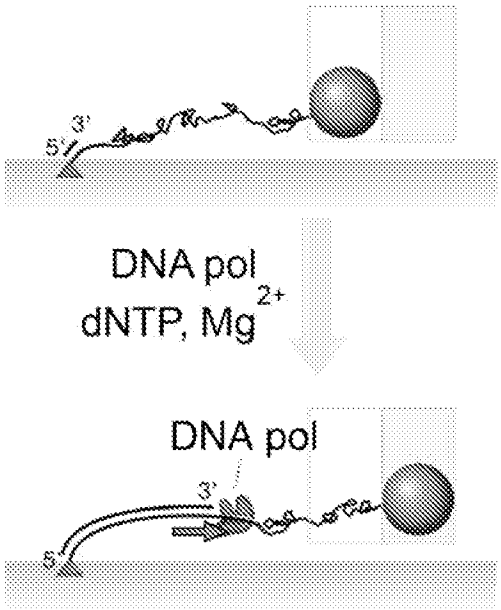

5'
3'
DNA pol
dNTP, Mg2+
DNA pol
3'
5'

FIG. 6

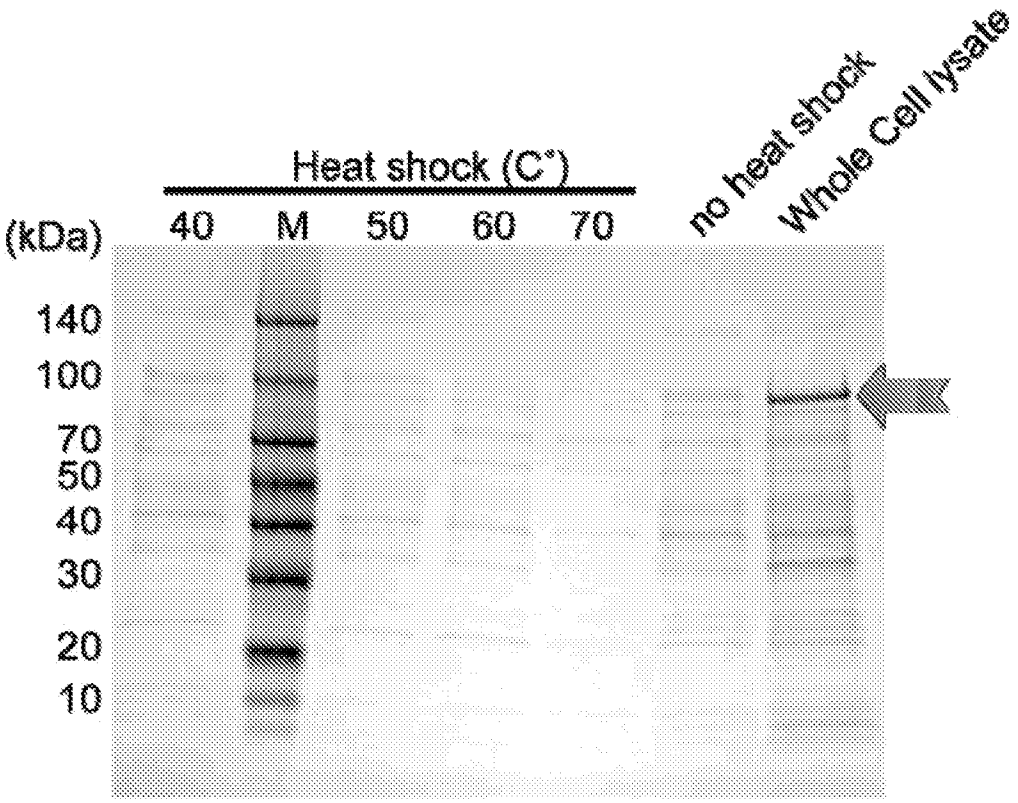
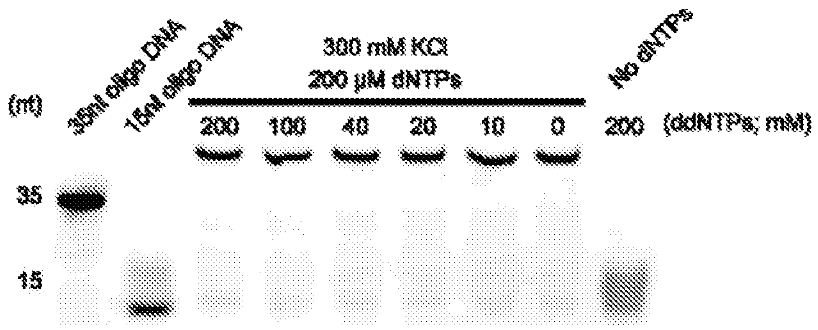
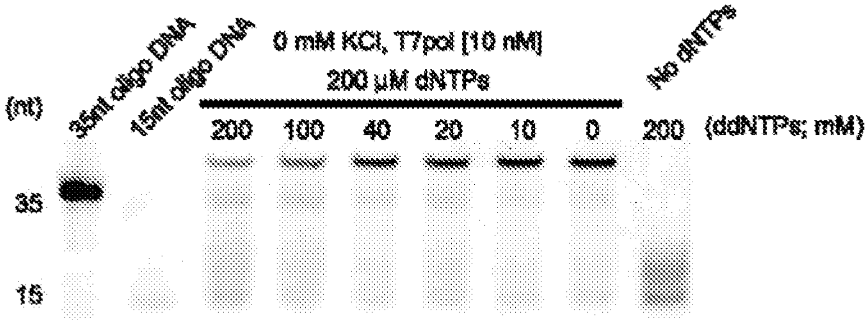

Heat shock (C°)
no heat shock
Whole Cell lysate
(kDa)
40
M
50
60
70
140
100
70
50
40
30
20
10

FIG. 7





amino acids sequence alignment

```
                491      500       510                 520      530
gp5     ----FDNGSYAHEILNGDIHTKNQIAASLPTR---------DNAKTPI[illegible]GFLYGAGDEKI
clone3  ALKIIANSFYGMLGYPRARWYSKECAESVTSFGREYIKDTIEMAKDEG[illegible]EVIYGDTDSLF
            : *. *.          .:: * .:.:           : **    : .:**  *. :
```

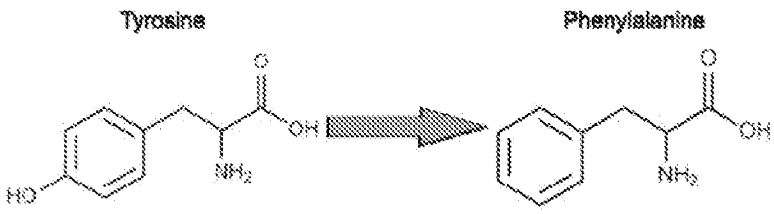


FIG. 8

SEQ ID NO:1- Clone1 (BR1)
MRETSEGWLLDAYIEGRYAVLWLKSIDGTVIHRLRERYRPCFYAEPRDIDCRIEDAASTIETHPAVHSALE
VERYATLRRREVKRVVKVSVESTDELDQAVAFARRHQMVRELYNVGLTPVQWYLFQLDAAPSSHVE
WTRRGGVLESTVLDGGLRVEPPPFKPTIIQTSKPPIEEVDLYDDCGSHLAALRGCEREVLSELQGAVTEI
DPDIVAMVDGVDTIRRLRQRAGAKGVDLCVGRLGDASHGRVALENRWFRDLGVVGLVERARFAMAP
MGVCAGWAAGRTVDSRQCYEADRLGVLVSEMKGGYAYAATAWELLFRDRGGMVLSPEMGLHENV
GVLDFESMYPNIIVTRNVSYENITPNGVERGPQGFLGGFTRRFLRRRLHYKHLRSSYPTDSREWRWCEQ
RQRSLKLMLVVIYGYSGCYANRFGNVRVFQEINRVARQALVESLNTALSRGYRVVYGDSDSLFTAKQ
GATREDYLGLAEEIAEATGLPITLDRHFKYLVLLPQAGDPEMGAARRYYGKLTDGTLFYRGIELRRRDT
PPYIRRLQRRVMETLFNADTAEEVRGRQLPKALELVKAACAELLRGQVDPRELVVSKRLRRRPGDYAS
KQPHVVAAQLEGLEEGYSEFLYVNSERRNPYIRVMPASMVNGGHHTIDRAWYSSMARRAAENILRPFL
DEGSNGGGKLRVSRLDTFFSRR

SEQ ID NO:2 - Clone2 (BR2)
MKAYLLDIDYTIVKDRAEVKLYLRGEEGRLEVYDRNFLPYFYVLGDEVEEKLLEEGALKVEGRRKKL
LGREVKALQVFASHPQEVPELRNKVKKIEGVDLTLEDDILFTRRYLIDRGMKPLTWYDFDVREEGGKY
YLQGFKEIEGGSPGLRTVALDIEVYNPGGVPRPEEDPHMVSLAGSGGLKKVLTWKDEGEVPGFVEVLS
SEGAMLGRLEEIFKEEEIDVVVGYNTDNFDFPYIKKRLQTLDMELQLGGDNIKIKGRKSLPQAALGGLP
HLDLYPIVRRNVRLNSYVLENVLKEVLKEEKEKIPNEKIWEYWDAGGEKLEKLFHYSLEDAEGTLRLS
QRFVPLYVQLSAIVGQCLYDTSRMTTGQMVEWYLMRIASRASELIPNRPKGEELKGRFSTTYAGGYVH
QPRKGMVRDIAVFDFRSLYPSIIVTHNIDPSTLREGVGCEENKAPSLDYCFSREEKGFIPSILEGLVNWRG
EVKKKMEGKGGEELRTLDFTQKALKILSNSFYGYMGYPRARWYRRECAESVASFAREYIKKVMATAK
EEFGLEVVYGDTDSLFVLLPGKEKARAEEFLEHVNRSMPGIQLELEGFYLRGLFVSKKRYALLDEKGK
ITVKGLEFVRRDWAPIARETQQKVLEILLKEGDEGKALRLVREVIENIKRREVTLNQISIYTQLTRKVES
YEGKEPHVGAAKKLQDEGYKVKAGSIIGYIVAKGRKGEKISERTLPVELASVEDYDPNYYIENQILPAV
GRIFDALGYRRIDYIKTGVEQRSLGKWIS

SEQ ID NO:3 - Clone3 (BR3)
MANQTTNGDHMEGLLLDSDYLKTRKPPAMRLFIKKDGGIVTVLDPHFTHYFYVESENPQKIAKAIERV
EAEKYGKKVSPKSTKVVERKPLGEEKKVIKVLADSPRDITPLRKEIKDFPEVKGFYEHDIPPARRYLIEH
ELTPMSGVKAEGESQKGDYGEELVLTKPPESIEGADEELNILAFDIETYSPTGNPRAEKDPIVMISVSDN
QGLEKILTWKDFDLNLDYVEVLDDEKSMIERFIQLVQECDADIIMGYNTDLFDFPYLTQRAEKLDIKLE
LGRDGSEPSTKKRRFATVTKIAGRVHADVYAMVEFLSRIGAIRLIDYTLENVYKHVIGKEKPDLEYSDIP
KAWDEGGEKARELVEYSLSDAKATLELGTEILPLFTELSRTVKQSLFDVSRMTPGQLVEWLLIFNAHKI
NELILPRPLGREYKRRRGETYIGGYVKEPTPGLIHEDLVVFDFRSLYPTIITHNIDPATLDGERCPSEETVT
APDLEYEFCQDRKGFIPETLKGLVEGRAKLKQEMSQLDEESREYQSLYNRQWALKIIANSFYGMLGYP
RARWYSKECAESVTSFGRHYIKDTIEMAKDEGFEVIYGDTDSLFAKLNGKSREDVENFLNKVNESLPGI
MKLELEDYYKRGVFVTKKRYAMISEDDKIVVKGLEFVRRDWAALAKRTQEQVIEAILHDASPEKAAKI
VLETTKAIKQGEVDLDDLVIHTQLKKPLDEYKARGPHVAAAERLQKLGEEVEPGMTITYIVEKGSGSIS
DRAIPPSDFEGRDYDPDYYVENQVLPAVMRIMEVLDYGEEDLRHEETRQVKLGKFK

SEQ ID NO:4 - Clone6 (BR6)
MEEKIYLLDLDYIEEETERGMEATVRLWGKNGEGKSVVAWDRAFDPYFYYVPGDFPLAKERLEGVDE
PRIKGVEEAEKILGKEEVKALRVYGSRPSDLPKLRDKLKGEGFDGERFYEYGMSFYRQYVVSKGLLPA
SWVLVKGKEVEKEGFDLAFEAGEVQALEGEEEAPLKTLAFDLETYESQEGRGIIMLSLAGDKKGYRKV
LTYKGEGYGDEVEVVGGEKELLQRFLEIVEEEDPDILLTYNGDGYDFRVLRERAEELGVELTMGRGGS
RLEFARRGRVSSARLGGRVHIDLFSFVNLALAGHLETEVLTLDAVAAELLGERKIEMEMEEMLEKWRR
EEDLGKLARYSLKDSGITCRLGEQLLPQIYALCNLTAQTPYDCSRMSYGQLVEWFLIKEAHGERIVPNR
PKWKELQKRRELEPYKGGFVREPVVGMHENLAVLDFQSLYPSIIASYNIAPETVNCDCCKGGEVEGVR
LCREKRGFIPSLLRGLIEERSRIKEKLEGVEEPLEHRTLDNRQYALKILANSTYGYFGYVGARWYCRDC
ARVTSALGREWIKKVMGMAEEEGFRVIYGDTDSLIIKDGEPRSFLEKVNSQLPGIMNLEMEGRFARGLF
VREKKGRGAKKRYALLDGKGGMKVRGFETVRRDWCSLAKRAQREILYILLSENSVPRATRHARRVIE
RLESKDVSLRDLIIYTSLTKAPGDYETTSPHVSAARKLEEKGRVVKPGSVIMYVVVEGKGSISERALPVE
FATIEEVDSEYYIENQIVPAALRVLGVMGVDERELRGGGTQETIEEFF

FIG. 9

SEQ ID NO:3

BR3 polymerase open reading frame sequence

atggcaaatcagacaacaaatggtgatcatatggaaggcctgctcctagatagcgattatctcaaa
actcgcaagccccagcaatgagactattcatcaaaaaagatgggggaatagtcacgtcctagat
ccacatttcactcattatttttatgtagaatctgaaaatcctcaaaaaatagctaaagcgatagag
agggtcgaagcggaaaagtatgggaaaaaagtaagcccaaagtcaactaaggttgtcgaacgcaag
tttctcggtgaagagaagaaagtcattaaagtcctagcagacagtccccgagatataactccatta
agaaaagaaatcaaagattttcctgaagtcaagggattttacgagcacgacattcctccagccaga
cgatacctcatagaacacgaattaaccccaatgagcggggtaaaggcagagggagaatcacaaaaa
ggtgattatggcgaggaattagtactcaccaaaccgcctgagtcaatcgaaggagcagacgaagaa
ctcaatatcctcgcctttgacatagaaacctacagtcccacaggcaatcctcgcgccgaaaaagat
ccaatagtaatgataagtgtttcagataatcaaggcttagagaagatccttacatggaaagatttt
gacctaaatctagattatgtggaagttttagatgatgaaaaatcaatgattgagagatttatccaa
ttagttcaagaatgcgatgcagacatcataatgggctacaacacagacctctttgacttcccatac
ctaactcaacgagcagaaaaactagacatcaagctagaactcggtagagacggttcagaaccctca
actaagaaaaggcgattcgctacagtaaccaaaattgctggcagagtccacgcggacgtttatgca
atggtcgaattcctttcgcgaattggagcaattagattgatagattacaccttgaaaatgtttac
aagcacgtgatagggaaggaaaaacccgatttagaatacagtgacattccaaaagcttgggatgaa
ggagggaaaaagctagagagttagtagagtactcgttatctgacgctaaggcaactctagagcta
ggcactgaaatacttccattattcactgaactgagtcgaaccgtgaaacaatcactctttgatgtt
tcgcgaatgactccaggccaattggtagagtggctcctaatcttcaatgctcataagatcaacgaa
ctcatcctcccgcgcccgctaggacgagaatacaagagacggcgtggtgagacttatattggtggt
tatgtaaaggaaccgacgccaggtcttcatgaggatctcgtagtctttgattttcgctctctatac
ccgaccataatcattactcacaatattgatccagcgacactcgatggggagcgttgtccctcagaa
gaaactgtgacagctccagatcttgaatacgagttctgtcaagatcggaagggtttcattccggag
acattgaaagggcttgttgaaggaagagcaaaattaaagcaggagatgagtcaacttgatgaggag
agtagagaataccaatccctctataatagacaatgggcactcaagatcatagcgaactcattctat
gggatgcttggataccctcgagccagatggtattctaaagaatgtgcagaaagcgttacgagcttc
ggccgtcactatattaaagacacgattgagatggcgaaagacgaaggatttgaagtcatctatggg
gatactgattccctcttcgctaagctcaatgggaaaagtcgagaagatgtcgaaaatttcctgaat
aaggtcaatgagagcttgccagggataatgaaactcgagctggaggattactacaagcgaggagta
ttcgtcaccaaaaaaaagatacgcaatgatcagcgaggatgacaaaatagtcgttaagggactcgag
ttcgtcaggcgtgactgggcagctctggcgaaaagaactcaagagcaagtcatcgaag

BR3 polymerase amino acid sequence

MANQTTNGDHMEGLLLDSDYLKTRKPPAMRLFIKKDGGIVTVLDPHFTHYFYVESEKPQKIAKAIE
KVEAEKYGKKVSPKSTKVVERKFLGEEKKVIKVLADSPRDITPLRKEIKDFPEVKGFYEHDIPPAR
RYLIEHELTPMSGVKARGESQKGDYGEELVLTKPPESIEGADEELNILAFDIETYSPTGNPRAEKD
PIVMISVSDNQGLEKILTWKDFDLNLDYVEVLDDEKSMIERFIQLVQECDADIINGYNFDLFDFPY
LFQRAEKLDIKLELGRDGSEPSTKKRRFATVTKIAGRVHADVYAMVEFLSRIGAIRLIDYTLENVY
KHVIGKEKPDLEYSDIPKAWDEGGEKARELVEYSLSDAKATLELGTKILPLFTELSRTVKQSLFDV
SRMTPGQLVEWLLIFKAHKINELILPRPLGREYKRRRGETYIGGYVKEPTPGLHEDLVVFDFRSLY
PTIIITHNIDPATLDGERCPSEETVTAPDLEYKFCQDRKGFIPETLKGLVEGRAKLKQEMKSQLDEE
SREYQSLYNRQWALKIIANSFYGNLGYPRARWYSKECAESVTSFGRHYIKDTIEMAKDEGFEVIYG
DTDSLFAKLNGKSREDVENFLNKVNESLPGIMKLELEDYYKRGVFVTKKRYAMISEDDKIVVKGLE
FVRRDWAALAKRTQEQVIEAILHDASPEKAAKIVLETTKAIKQGEVDLDDLVIHTQLKKPLDEYKA
RGPHVAAAERLQKLGEEVEPGMTITYIVEKGSGSISDRAIPPSDFEGRDYDPDYYVENQVLPAVMR
IMEVLDYGEEDLRHEETRQVKLGKFK

FIG. 10

Figure 1