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(21) International Application Number: PCT/US95/15263 (22) International Filing Date: 29 November 1995 (29.11.95) (30) Priority Data: 08/375,134 18 January 1995 (18.01.95) US (71) Applicant: PHARMACIA BIOTECH INC. [US/US]; 2202 North Bartlett Avenue, Milwaukee, WI 53202 (US). (72) Inventors: LUHM, Robert, A.; 11307 North Rosewood Drive, Mequon, WI 53092 (US). D'HENNEZEL, Olga, B.; 517 Peebles Street, Raleigh, NC 27608 (US). DUFFAUD, Guy, D.; Apartment #1, 323 Oak Run Drive, Raleigh, NC 27606 (US). JOLLY, James, F.; 820 West Clovermook Lane, Glendale, WI 53217 (US). KELLY, Robert, M.; 105 Monarch Way, Cary, NC 27511 (US). TING, Eve, Yi-Fay; 230 Bunker Hill Drive, Brookfield, WI 53005 (US). (74) Agent: BAKER, Jean, C.; Quarles & Brady, Suite 2550, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: PURIFIED THERMOCOCCUS BAROSSII DNA POLYMERASE (57) Abstract A preparation of a purified DNA polymerase substantially identical to native DNA polymerase isolated from <i>Thermococcus barossii</i> is disclosed. In a preferable form of the present invention, the DNA polymerase is approximately 68 kDa in size, retains at least 90 % activity after 8 hours of incubation at 95 °C and retains between 60 % and 80 % proof-reading ability when catalyzing a polymerization reaction with a primer with a 3 base pair mismatch.		

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PURIFIED *THERMOCOCCUS BAROSSII* DNA
POLYMERASE

Field of the Invention

The present invention relates to a thermostable polymerase isolated from *Thermococcus barossii*. Specifically, the present invention relates to a purified thermostable polymerase with a molecular weight of either approximately 68,000 daltons or approximately 90,000 daltons with enhanced thermostability at 95° C.

Background of the Invention

Thermostable polymerases have been isolated and purified from a number of eubacterial and archaeal (archaebacterial) sources. For example, U.S. patent 5,352,778 (Comb, et al.; October 4, 1994) describes a recombinant thermostable DNA polymerase from *Thermococcus litoralis*; U.S. patent 5,079,352 (Gelfand, et al.; January 7, 1992) discloses a thermostable enzyme purified from *Thermus aquaticus*; PCT WO92/09689 (Mathur, E.J.; published 11 June 1992) describes a thermostable polymerase from *Pyrococcus furiosus*; PCT WO92/03556 (Gelfand, et al., published 5 March 1992) describes a DNA polymerase from *Thermotoga maritima*; and WO92/06202 (Gelfand, et al.; published 16 April 1992) describes a DNA polymerase from *Thermosipho africanus*.

Characteristics and properties such as optima of pH, temperature, degree of thermostability, and ionic requirements have been determined for many of these enzymes. Thermostable polymerases initially found great commercial appeal due to their ability to catalyze polymerization of DNA from a DNA template in the polymerase chain reaction (PCR). Other uses of thermostable polymerase include cycle sequencing of small amounts of DNA, sequencing of PCR products, standard sequencing reactions at elevated temperatures, and reverse transcription PCR type reactions. These techniques have been described in detail. (See, for example, G.A. Hawkins, et al., Biotechniques 16:418-20, 1994; M.A. Innis, et al., Proc.

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Natl. Acad. Sci. USA 85:9436-40, 1988; M.C. Krickler, et al., Gene 85:199-204, 1989; J.S. Lee, DNA Cell Biol. 10:67-73, 1991; T.W. Myers, et al., Biochemistry 30:7661-6, 1991; Wolfe, et al., Eur. J. Clin. Chem. Clin. Biochem. 30:717-27, 1992.)

- 5 Currently commercially available polymerases suffer from certain limitations when performing the above described procedures. DNA polymerases from *Thermus aquaticus* (*Taq*) and *Thermus thermophilus* (*Tth*) lack a sufficient proof-reading function as well as having reduced thermostability.
- 10 Additionally, long PCR products are difficult to achieve when using *Taq* and *Tth* polymerases primarily because of the lack of proof-reading function. DNA prepared using these polymerases frequently requires modification before subsequent manipulations can be performed.
- 15 DNA polymerases such as those derived from *Pyrococcus furiosus* and *Thermococcus litoralis* have greater thermostability and exhibit a high degree of proof-reading capability. (See, for example, M. Bergseid, et al., Strategies in Molecular Biology 4(3):34-5, 1991; N.F. Cariello, et al., Nucleic Acids Res. 19:4193-8, 1991; K.A. Eckert, et al., PCR Methods and Applications 1(1):17-24, 1991; H. Kong, et al., J. Biol. Chem. 268:1965-75, 1993; T.A. Kunkel, et al., "Fidelity of DNA polymerase used in polymerase chain reactions," Current Communication in Molecular Biology,
25 pp. 5-10, 1989; K.S. Lundberg, et al., Gene 108:1-6, 1991.) These polymerases, although improvements over the former polymerases, have several drawbacks. Two disadvantages of these polymerases are the higher incubation temperatures to which these polymerases are accustomed as well as an
30 overactive 3' to 5' exonuclease function.

Therefore, it is desirable to develop a thermostable DNA polymerase which functions optimally at approximately 80° C, has increased thermostability, and exhibits proof-reading capabilities that do not overwhelm polymerase function.

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Summary of the Invention

The present invention is a preparation of a purified DNA polymerase substantially identical to native DNA polymerase isolated from *Thermococcus barossii*. In the examples below, we have characterized this DNA polymerase as having two forms, one of which is approximately 90 kDa in size and the other which is approximately 68 kDa in size. We believe that the 68 kDa form result from an N-terminal deletion of approximately 186 amino acids from the 90 kDa form.

In an advantageous embodiment of the present invention, the polymerase retains between 60% and 95% of polymerase proof-reading capability when the polymerase catalyzes a polymerization reaction with a primer with a 3 base pair mismatch. Preferably, the polymerase retains between 60% and 80% proof-reading capability. Most preferably, the polymerase retains between 65% and 75% proof-reading capability.

In another advantageous embodiment of the present invention, a preparation a purified DNA polymerase retains between 25% and 60% of polymerase proof-reading capability when the polymerase catalyzes a polymerization reaction with a primer with a 6 base pair mismatch. More preferably, the polymerase retains between 40% and 50% proof-reading capability. Most preferably, the polymerase retains approximately 46% proof-reading capability.

In another advantageous embodiment of the present invention, the polymerase described above retains at least 70% activity after 2 hours of incubation at 95° C in appropriately buffered solutions. In a preferable embodiment, the polymerase retains at least 80% activity after eight hours of incubation at 95° C. In a more preferable embodiment, the polymerase retains at least 90% activity after 8 hours incubation at 95° C. In a most preferable embodiment, the polymerase retains 100% activity. We refer to the ability to retain enzymatic activity after incubation at 95° C as "thermostability."

In one embodiment of the present invention, the polymerase is isolated directly from *Thermococcus barossii*.

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In another embodiment of the present invention, the polymerase is obtained from expression of the DNA sequence encoding the polymerase that has been inserted into a host organism, such as *E. coli*.

5 The present invention is also a method of elongating or polymerizing a DNA molecule. This method comprises the step of exposing the DNA molecule to a complementary primer molecule, the DNA polymerase of the present invention, and dNTPs under conditions permitting extension of the DNA
10 molecule. The enzyme of the present invention will also be useful in DNA sequencing and polymerase chain reaction (PCR).

It is an object of the present invention to provide a DNA polymerase substantially identical to that isolated from *Thermococcus barossii*.

15 It is another object of the present invention to provide a DNA polymerase retaining at least 90% activity after 8 hours of incubation at 95° C.

It is another object of the present invention to provide a DNA polymerase with an intermediate proof-reading
20 capability. By "intermediate proof-reading capability," we mean that the polymerase of the present invention has proof-reading capability in a range between that exhibited by exonuclease deficient *Taq*-like polymerases, exonuclease minus versions of Vent polymerase and Deep Vent polymerase, and
25 polymerases with overactive exonuclease activity such as Vent polymerase, Deep Vent polymerase, and *Pfu* polymerase.

It is another object of the present invention to provide a polymerase that retains between 60% and 80% of polymerase proof-reading capability when the polymerase catalyzes a
30 polymerization reaction with a primer with a 3 base pair mismatch.

It is another object of the present invention to provide a polymerase that retains between 40% and 50% of polymerase proof-reading capability when the polymerase catalyzes a
35 polymerization reaction with a primer with a 6 base pair mismatch.

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It is another object of the present invention to provide a method for elongating a DNA molecule.

It is another object of the present invention to provide a DNA sequence capable of producing the *Thermococcus barossii* DNA polymerase.

Other objects, advantages and features of the present invention will become obvious after examination of the specification, claims and drawings.

Description of the Drawings

Fig. 1 is a diagram of percentage activity versus pH for the 90 kDa form of *Tba* polymerase.

Fig. 2 is a diagram of percentage activity versus $MgCl_2$ concentration for the 90 kDa form of *Tba* polymerase.

Fig. 3 is a diagram of percentage activity versus KCl concentration of the 90 kDa form of *Tba* polymerase.

Fig. 4 is a diagram of percentage activity versus temperature for both the 68 and 90 kDa forms of *Tba* polymerases, Vent polymerase, *Pfu* polymerase, and *Taq* polymerase.

Fig. 5 is a diagram of activity versus incubation time at 95° C. for both the 68 and 90 kDa forms of *Tba* polymerases, Vent polymerase, *Pfu* polymerase, *Taq* polymerase, Deep Vent polymerase and Deep Vent exo- polymerase.

Fig. 6 is a diagram of polymerase proof-reading capability for various thermostable polymerases.

Description of the Invention

The present invention is a preparation of an enzyme having DNA polymerase activity useful for standard sequencing of DNA, polymerase chain reaction, and cycle sequencing.

Preferably, this enzyme is thermostable. The thermostable DNA polymerase of this invention is an enzyme which catalyzes the polymerization of DNA that is complementary to the template by the incorporation of deoxyribonucleoside triphosphates from a primer annealed to the template. By "thermostable," we mean an enzyme that has a temperature optima of at least 65° C.

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Typically these thermostable enzymes retain at least 20% activity after incubation at 95° C for 4 hours.

This invention provides an improved polymerase with (1) increased thermostability; (2) intermediate proof-reading capability; and (3) greater longevity during extended amplifications. By "intermediate proof-reading capability," we mean that the polymerase of the present invention has a proof-reading capability that is intermediate between thermostable polymerases with a lesser amount of proof-reading ability, such as *Taq* polymerase, and polymerases with a high amount of proof-reading ability, such as Vent polymerase and Deep Vent polymerase. Also provided is an effective method for the production and purification of said polymerase.

The polymerase of the present invention is substantially identical to a polymerase derived from a previously unclassified strain of microorganism of the genus *Thermococcus*, identified as *Thermococcus barossii*. "Substantially identical" means that the candidate polymerase has at least a 95% amino acid sequence homology and at least 95% of the proof-reading capability and thermostability of native *Tba* polymerase. A preferable strain of *Thermococcus barossii* was deposited at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124, Braunschweig, Federal Republic of Germany) on November 1, 1994 at DSM accession number DSM 9535.

In a preferred embodiment, the DNA polymerase is produced directly from *Thermococcus barossii*. The examples below describe a preferable method for isolating the DNA polymerase from *Thermococcus barossii*. The DNA polymerase may also be obtained by expressing a DNA sequence encoding the polymerase in a host organism. Again, the Examples below disclose a preferable method of determining this DNA sequence and a preferable sequence. SEQ ID NO:13 below discloses a preferable partial DNA sequence encoding the 90 kDa form of *Tba* polymerase. From SEQ ID NO:13, one of skill in the art will be able to isolate probes sufficient to isolate the entire *Tba* DNA polymerase sequence from a *T. barossii* genomic

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library. The Examples below disclose a preferable method for achieving this result. Referring to SEQ ID NO:13, the coding region of the polymerase ranges from initiation at bp 233 to termination at bp 2573. We believe that the 68 kDa form of the enzyme is created by a 186 amino acid deletion at the N-terminal end. Therefore, appropriate deletion (by methods known to those of skill in the art) of the SEQ ID NO:12 coding region will produce a sequence encoding the 68 kDa form of the enzyme.

Once one has obtained a suitable sequence for expressing *Thermococcus barossii* polymerase, this sequence should be combined with appropriate regulatory sequences in a vector, such as a plasmid or virus, designed to promote efficient expression of the sequence in a host organism. A particularly advantageous combination of vector and host organism is a λ P_L COL E1 plasmid derivative in a λ CI bacterial strain.

It is envisioned that small changes, deletions, and modifications may be made to a DNA sequence isolated from *Thermococcus barossii* that encodes the DNA polymerase. One of skill in the art would know that certain small modifications to the DNA sequence would not impair DNA polymerase activity. The Examples below describe an assay for DNA polymerase ability that would be suitable for a candidate modified sequence. One would simply express a DNA polymerase from the modified sequence and perform the polymerase assay described above. If the candidate molecule performed similarly (at least 95% activity) to the *Thermococcus barossii* polymerase described in the present invention, then the DNA sequence is suitable. One would want to compare the thermostability of the enzyme with that determined for the 90 kDa and 68 kDa forms of *Thermococcus barossii* DNA polymerase. Fig. 5 diagrams a convenient comparative data set. A successful candidate polymerase will have at least 95% of the DNA polymerase thermostability of the 68 kDa form illustrated in Fig. 5 after 8 hours incubation at 95° C.

A successful candidate polymerase will also have at least 95% of the proof-reading ability of the 68 kDa form, as

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described in Fig. 6. The examples below describe a method of determining proof-reading capability.

The polymerase of this invention consists of two distinctively sized entities, both capable of catalyzing the incorporation of nucleoside triphosphates and causing DNA
5 elongation on a single-stranded template. As determined by comparative migration, the molecular weight of the smaller protein migrates between the albumin and phosphorylase b markers on an SDS polyacrylamide gel within a range of 60,000
10 - 75,000 daltons. The protein exhibits a half-life of 17 - 19 hours at 95° C and retains 100% of its activity when placed at pH 9.3 for 2 hours at 95° C. The approximately 68 kDa protein retains at least 70%, and preferably 90%, of its activity when incubated at 95° C for 8 hours. We refer to this protein as
15 the "68 kDa form."

As determined by comparative migration, the molecular weight of the large protein migrates near the phosphorylase b marker on an SDS polyacrylamide gel within a range of 86,000 - 94,000 d. This 90 kDa protein has an optimal pH of
20 approximately 8.5 - 9.0, an optimal temperature of approximately 70° C - 75° C, requires magnesium ions at an optimal concentration of 5 mM - 10 mM, and has an optimal salt concentration of 20 mM - 40 mM. We refer to this protein as the "90 kDa form."

25 The present invention is also a method of elongating a DNA molecule by exposure of a primer to a complementary DNA template, the DNA polymerase of the present invention, and dNTPs under conditions suitable for DNA elongation. Preferably, this method is accomplished as follows: A
30 reaction mixture consisting of reaction buffer (25 mM glycine, pH 9.3, 50 mM KCl, 1 mM DTT); Mg ions at 5 - 10 mM supplied as MgCl₂; and 0.2 mM of each dGTP, dATP, dCTP, and dTTP at pH 9.0 are incubated with the polymerase, template and primer at 72° C for 2 hours.

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Examples1. Organism

T. barossii was isolated from a deep vent flange, Endeavor Segment, Juan de Fuca Ridge, off the coast of Washington state, USA. As described above, a preferred strain is deposited at DSM at accession number 9535. The organism grows optimally at 82.5° C but has a temperature range of 60° C - 92° C. Cell densities can reach as high as 4×10^8 cells/ml in continuous anaerobic culture. The growth conditions allow the addition of NaCl to a level of 15 - 40 gm/l. Cells have been cultivated in culture pH ranging from 5 - 8. Other characteristics of this organism are an absolute requirement for elemental sulfur, resistance to the antibiotics streptomycin, chloramphenicol and vancomycin, and a GC percentage of approximately 60.

For our work described below, the organism was cultured in a solution of artificial sea water (ASW). ASW consists of (per liter); NaCl 15 - 40 g, $MgCl_2 \cdot 6H_2O$ 2 g, KCl 0.7 g, Na_2SO_4 4.0 g; H_3BO_4 0.03 g; KBr 0.01 g; $SrCl_2 \cdot 6H_2O$ 2 mg; KH_2PO_4 0.05 g and $NaHCO_3$ 0.2 g. The ASW solution is supplemented by the addition of 0.05% yeast extract, and 0.25% tryptone. After sterilization, 3 g of a commercially obtained mixture of maltooligosaccharide is added (Pfanstiehl Laboratories, Waukegan, IL). We also added 0.003 g of $Na_2WO_4 \cdot 2H_2O$ and 0.59 g of $CaCl_2 \cdot 2H_2O$. Elemental sulfur (J. T. Baker Chemical Co., Phillipsburg, NJ) was included in all cultures in the form of powder (5 - 10 g/l).

The sterilized media, supplemented with the solutions described above and elemental sulfur, was dispensed in 100 ml culture bottles. Anaerobic conditions were obtained by heating the medium to 98° C, sparging with pre-purified nitrogen (Ultra High Purity (99.999%), National Specialty Gases, Durham, NC) and adding 0.375 g $Na_2S \cdot 9H_2O$ per liter of medium. Resazurin (1 mg/l) was used as a redox indicator. Once the medium had turned clear (anaerobic conditions), the bottles were sealed and inoculated to a cell density of no

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more than 1×10^6 cells/ml. The organism was grown in continuous culture until a proper biomass amount was achieved.

2. Extraction and purification of the DNA Polymerase

The DNA polymerase from *Thermococcus barossii* was
5 extracted and purified by precipitation with polyethylenimine, followed by chromatography on Q sepharose, Heparin Sepharose, Phosphocellulose, and Mono-Q respectively. All procedures were performed at 4° C.

Fifty grams of cells were suspended in 170 ml of a buffer
10 consisting of 50 mM Tris HCl pH 8.0, 5 mM Na₂EDTA, 1 M KCl, 1 mM DTT and 1 mM PMSF (phenylmethylsulfonyl fluoride). The cell suspension was sonicated to break open the cells. Polyethylenimine to a final concentration of 0.4% was added to the resultant suspension to precipitate the nucleic acids and
15 cell debris. The lysate was centrifuged for 30 minutes at 10,000 RPM and 4° C. Following centrifugation, 75% saturation of ammonium sulfate was added to the supernatant and the protein was precipitated. The suspension was centrifuged at 10,000 RPM in a JA-10 rotor (Beckman) to produce a pellet.
20 The pellet was resuspended in 80 ml of Q Sepharose buffer A (consisting of 50 mM Tris HCl, pH 8.0, 1 mM Na₂EDTA, 1 mM DTT and 1 mM PMSF), so that the conductivity of Fraction A was equal to that of the Q Sepharose Buffer A.

Fraction A was applied onto a Q Sepharose column (2.5 x
25 15.0 cm, Pharmacia). The column was washed and the enzyme was eluted with Q sepharose Buffer (50 mM Tris HCl pH 8.0, 1 mM Na₂EDTA, 100 mM KCl and 1 mM PMSF). The fractions collected were assayed for DNA polymerase activity. Those exhibiting polymerase activity were pooled and given the designation
30 fraction B.

Fraction B was then applied to a Heparin Sepharose column (1.25 x 16.0 cm, Pharmacia) and washed with the same Q
Sepharose buffer. The enzyme was eluted with a linear salt gradient from 0.1 M to 0.7 M KCl in Q Sepharose buffer. The
35 polymerase activity was assayed and the active fractions with no detectable nuclease(s) were pooled as Fraction C.

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Fraction C was dialyzed overnight in phosphocellulose buffer (20mM KPO₄, pH 7.5, 0.5 mM Na₂EDTA, 50 mM KCl, 1 mM DTT, 5% glycerol, 0.1% NP-40, and 0.1% Tween 20). The dialysate was applied onto a phosphocellulose p-11 column (1.25 x 8.0 cm, Whatman) equilibrated with the same buffer. The column was washed with the phosphocellulose buffer and the enzyme was eluted with a linear salt gradient from 50 mM to 500 mM KCl. The activity was assayed and the active fractions with undetectable nuclease(s) were pooled as Fraction D.

Fraction D was dialyzed overnight against a buffer of 20 mM Tris-HCl, pH 8.0, 1 mM Na₂EDTA, 1 mM DTT, 50 mM KCl, 5% glycerol, 0.1% NP-40, and 0.1% Tween 20. The dialysate was applied to a 1.0 ml Mono-Q column (Pharmacia) equilibrated and washed with the above described buffer. The enzyme was eluted with a linear salt gradient from 50 mM to 300 mM KCl in the same buffer. Each fraction containing polymerase activity was run on a Phast Gel 8% - 25% gradient (Pharmacia) and stained with coomassie brilliant blue. Certain fractions showed a single band in the range of 60,000 - 75,000 d. This band has been identified as a truncated form of full-length *Tba* DNA polymerase. Other fractions showed this band as well as a major band in the range 85,000 to 98,000 d as determined by comparative migration relative to molecular weight standards. These bands consist of the truncated form as well as the full-length version of *Tba* DNA polymerase.

The molecular weight standards employed were the low molecular SDS gel makers (Pharmacia). This marker consists of 6 proteins as follows: Phosphorylase b (94,000 d), Albumin (67,000 d), Ovalbumin (43,000 d), Carbonic anhydrase (30,000 d), Trypsin inhibitor (20,100 d), and α lactalbumin (14,400 d).

3. DNA Polymerase Assay

Assay Mixture:

25 mM Glycine, pH 9.3 (25° C)
2 mM Magnesium Chloride
50 mM Potassium Chloride
1 mM DTT
0.2 mM dCTP, dGTP, and dTTP
0.1 mM α ³²P-dATP

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5.0 µg BSA
1.0 µg M13mpl8 ssDNA
0.3 µg single strand primer

5 µl of the enzyme pool containing 1.25 U in dilution
5 buffer was added to 45 µl of the assay mixture described
above. 1.25 U of the other commercially available polymerases
were diluted in the same dilution buffer and treated similarly.
The commercially available polymerases we used in the Examples
below were Vent (*Thermococcus litoralis*, available from New
10 England BioLabs, Beverly, MA); Taq (*Thermus aquaticus*,
available from Perkin Elmer); Pfu (*Pyrococcus furiosus*,
available from Stratagene, La Jolla, CA) and Deep Vent and Deep
Vent exo⁻ (both *Pyrococcus* species GB-D obtained from New
England BioLabs Inc., Beverly, MA).

15 Following gentle mixing, the reaction was incubated at 72°
C for 10 minutes. 50 µl of RNA coprecipitant and 1 ml of cold
10% TCA were added. The suspension was well mixed and
incubated on ice for at least 10 minutes, but no longer than 30
minutes. After filtration through glass fiber filters
20 (Whatman), the samples were measured by liquid scintillation
counting (Packard Tricarb 2000 CA). In this manner, the
quantity of ³²P labeled dATP incorporated into the acid-soluble
fraction was calculated. The assay was used in the Examples
below to determine DNA polymerase ability.

25 4. Optimal pH

The effect of reaction pH on the 90 kDa form of *Tba*
polymerase was examined using the assay buffer. Assay buffer
(see supra in Examples) was made at the following pH; 6.0, 7.0,
8.0, 9.3, and 10.5. Aliquots of the diluted polymerases were
30 incubated for 10 minutes at 72° C in assay buffers representing
each pH. Polymerase activity was assayed under standard
conditions with only the pH of the assay buffer being altered.
The optimal pH of the 90 kDa form of *Tba* DNA polymerase was
approximately 8.5 to 9. The results of measuring activity at
35 various pH values are as shown in Fig. 1. The optimal pH is
approximately from 8.5 to 9.0.

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5. Optimal MgCl₂ Concentration

The magnesium chloride concentration of the assay mixture used for measuring the activity of the 90 kDa form of the enzyme was varied, while the other conditions were maintained unchanged. The results are shown in Fig. 2. An optimal MgCl₂ concentration is between 5 and 10 mM.

6. Optimal KCl Concentration

The effect of potassium chloride concentration on the enzymatic activity was measured as described above for MgCl₂. The results are shown in Fig. 3. The optimal KCl concentration for the 90 kDa form of the DNA polymerase is between 20 and 40 mM.

7. Optimal Temperature

The results of measuring enzymatic activity at various temperature are shown in Fig. 4. Both the 68 kDa and 90 kDa form of the polymerase are compared to other commercially available enzymes. The commercially available thermostable polymerases we analyzed were Vent polymerase, *Pfu* polymerase, and *Taq* polymerases.

The enzymes were diluted in a dilution buffer consisting of the following components:

50 mM Tris-HCl (pH 7.6)
100 mM NaCl
0.1 mM EDTA
1 mM DTT
1% (v/v) Tween-20 (Bio-Rad)
1% (v/v) NP-40 (Calbiochem)
50% Glycerol

The effect of temperature on each polymerase was assayed utilizing assay conditions described above. 1.25 units in 5 µl dilution buffer was added to 45 µl assay buffer. Aliquots from each polymerase-containing reaction were placed at 37° C, 65° C, 75° C, 85° C, and 95° C and incubated for 10 minutes. The amount of activity exhibited was determined by the percent of labeled nucleotide incorporated when performing a standard activity assay.

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Fig. 4 demonstrates that the optimal temperature for all enzymes is between 70° C and 75° C.

8. Thermostability Comparison of Different DNA Polymerases

About 25 units of both the 68 kDa and 90 kDa enzymes and other commercially available thermostable DNA polymerases were diluted to a final volume of 100 ul. The enzymes were diluted in the dilution buffer described above.

The diluted polymerases were incubated for 8 hours at 95° C. At 0, 1, 2, 4, and 8 hour time points, 5 µl aliquots of each DNA polymerase solution were assayed for polymerase activity at 72° C under standard conditions. The results are shown in Fig. 5. The 68 kDa form of *Tba* polymerase is the most thermostable, having a half-life of 17 hours at 95° C, while the Vent polymerase has a half-life of only 8 hours [J. Bio. Chem. 268:1965-75 (1993)]. The 90 kDa *Tba* enzyme is the least thermostable of the test polymerases. This finding might be due to contaminants in the 90 kDa preparation. (The half life value for the 68 kDa form of *Tba* polymerase was determined from data not presented in Fig. 5. The DNA polymerase thermostability data was determined from experiments with time intervals out to 24 hours.)

9. Molecular Weight

The molecular weights of the two polymerase forms measured by 8% - 25% SDS Phast gel electrophoresis were approximately 68,000 daltons and 90,000 daltons, respectively.

10. Exonuclease Activity

The exonuclease activity of *Tba* enzyme is summarized in Table 1 and diagrammed in Fig. 6. We examined the different polymerases for the ability to correct synthesis errors by determining whether a primer with various mismatches can be extended from a template. The retention of high polymerization activity is indicative of the strength of the 3' to 5' exonuclease.

Three primers were employed - RL-150 (SEQ ID NO:9), RL-151 (SEQ ID NO:10), and RL-152 (SEQ ID NO:11). RL-150 is a 29 bp

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oligonucleotide that has complete homology to M13mp18 (+) strand. The sequence of RL-150 is as follows:

CGC CAG GGT TTT CCC AGT CAC GAC GTT GT

Sequences RL-151 and RL-152 are 32 bp and 35 bp

5 oligonucleotides respectively. The oligonucleotides are identical to RL-150 for the first 29 bp. RL-151 differs from RL-150 in that it incorporates 3 bp of mismatch on the 3' end of the oligonucleotide. RL-152 incorporates 6 bp of mismatch on the 3' end. These sequences are as follows:

10 CGC CAG GGT TTT CCC AGT CAC GAC GTT GTT TT RL-151
CGC CAG GGT TTT CCC AGT CAC GAC GTT GTT TTT TT RL-152

The percent incorporation shown in Table 1 is determined relative to the values obtained using RL-150 in a standard polymerase assay. Due to the lack of complete annealing on the 15 3' end of the duplex when using oligos RL-151 and RL-152 in a polymerase reaction, exonuclease deficient polymerases would not be able to catalyze polymerization of nascent DNA from the primer-template duplex. This would be manifested as little or no incorporation of nucleotide relative to the results obtained 20 using RL-150. Polymerases that possess 3' to 5' exonuclease function would be capable of removing the 3' mismatches and thus continue polymerization. The greater the exonuclease activity, the more mismatched bases removed. Those polymerases with a great deal of exonuclease function would exhibit values 25 equivalent to RL-150 when employing RL-151 and RL-152. As exonuclease function decreased, the results relative to RL-150 would decrease as the number of mismatches increased.

Table 1

	DNA Polymerase	% Activity Remaining	
		RL 151*	RL 152**
30	Vent exo ⁻	7.6	2.3
	<i>Taq</i>	9.7	1.3
	Deep Vent exo ⁻	55	20
	68 kDa form of <i>Tba</i> enzyme	67	46
	Vent	100	50
35	Deep Vent	100	96

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*29 basepair oligo + 3 mismatches at the 3' terminus

**29 basepair oligo + 6 mismatches at the 3' terminus

The results listed in Table 1 and Fig. 6 indicate that significant levels of intermediate exonuclease activity for the 68 kDa form of the *Tba* DNA polymerase, which falls between the high exonuclease capability of Deep Vent and Vent and the low exonuclease ability of Deep Vent exo^- , Vent exo^- and *Taq*. The results determined with the 3 base pair mismatch were intermediate between the low exonuclease ability of Deep Vent exo^- , Vent exo^- and *Taq* (55% or less) and the high exonuclease capability of Deep Vent and Vent polymerase (100%). The proof-reading ability of 68 kDa form of *Tba* polymerase with a 6 base pair mismatch primer is intermediate between the low exonuclease ability of Deep Vent exo^- , *Taq*, and Vent exo^- (less than 20%) and the high exonuclease capability of Deep Vent (at least 96%). The proof-reading ability is similar to that obtained with Vent polymerase. However, Vent polymerase is not as thermostable after incubation at 95° C as the 68 kDa form of *Tba* polymerase (see Fig. 5).

11. Purification of DNA from culture

A quantity of *Tba* cell culture, sufficient to produce a pellet of 1.0 g wet weight, was centrifuged and the resultant cell pellet was resuspended in 4.0 ml 50 mM glucose, 10 mM EDTA (pH 8.0) and 25 mM Tris • HCl (pH 8.0). 600 μl of 8 mg/ml lysozyme in 0.25 mM EDTA was added. The tube was incubated with gentle shaking at 37° C for 1 hour. 200 μl 20% Sodium dodecyl sulfate was added and incubation was continued for 30 minutes without additional shaking. 160 μl of a solution of 1 mg/ml RNase A was added with incubation at 37° C for 15 minutes. Finally, 320 μl of Proteinase K (10 mg/ml) was added and the suspension was incubated for an additional 30 minutes at 37° C.

The DNA was extracted several times with Phenol, followed by phenol:chloroform, followed by chloroform alone, with intervening centrifugation at 6000 x g, until there was no

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further interface. The suspension was centrifuged at 6,000 x g for 30 minutes in order to separated the phases. The supernatant was transferred to clean sterile tubes. One tenth volume of 3 M sodium acetate (pH 5.2) was added and the DNA was precipitated by addition of 2 volumes of ice cold ethanol. The phases were not allowed to mix. The DNA was spooled by disturbing the interface with a glass micropipette. The spooled DNA was transferred to 400 µl of TE.

12. Screening for the presence of the polymerase gene

Degenerate primers were made to potentially related thermostable polymerases. The primers sequences were obtained by looking for regions of consensus between known polymerases. In our particular case, these primers were derived from regions exhibiting a high degree of homology between Vent polymerase and *Pfu* polymerase. Homologies were determined by performing a direct comparison between Vent polymerase and *Pfu* polymerase utilizing the DNAsis program produced by Hitachi software. Where differences in the nucleotide sequences of Vent and *Pfu* existed, degeneracies were built into the primers providing for any combination of codons that would code for that amino acid. Two primers were chosen as potential PCR primers. Primer 1 (RL-155) was CTCAT(ACT)GACAA(AG)GGC(CT)T(AG)AT(ACT)CCNATG GAGGG and primer2 (RL-156) was TGCTTCATANACNGCCTCNAGNGT (AG)TANGTTGG. (Primer 1 is described below at SEQ ID NO:1 and primer 2 is described at SEQ ID NO:2.) The nucleotides in parenthesis or represented by N denote degeneracy. These primers were used to perform standard polymerase chain reaction (PCR) according to the manufacturer's direction Perkin Elmer Cetus). The product of the reactions was purified on low melting point agarose (SeaPlaque, FMC) using the gelase protocol (Epicentre). The purified DNA fragment was cloned into pUC18 SmaI/Bap utilizing the SureClone PCR cloning kit (Pharmacia). The insert was sequenced using T7 in a modified Sanger chain termination procedure.

Sequence confirmed this fragment as a fragment of a polymerase gene due to sequence similarity with other known

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polymerases. Other degenerate primers were constructed in an attempt to "walk" further out on the gene utilizing sequence similarity. Two other overlapping fragments of the gene were obtained using the following degenerate primers: Primer 3 (RL-
5 205) CGGATGAT(AT)(CT)T(GA)GA(CT)N NNGATTACATAAC(AT)(AG)AAG
A(AT)GG and primer 4 (RL-206) AATGC
C(CT)TTGTC(GT)ATGAG(AG)TA(TA)C (GT)CTT(GT)GCAAA(GT)GG as one
pair and primer 5 (RL-211) CCCGCGCATAAGGCGCACCATAAACCTCCC and
primer 6 (RL-212) CCCCANGCNGTAACGCT(CT)TCAGCACA(CT)TCC as the
10 other pair. (Primers 3 - 6 are described below in SEQ ID NOs:
3 - 6, respectively.) These regions were amplified by PCR and
purified, cloned, and sequenced as previously described. The
entirety of the three overlapping fragments was sequenced in
both directions.

15 A library from *T. barossii* was prepared by partial
digestion of genomic DNA with Sau3A I. This was done in order
to obtain those portions of the gene not obtainable by the
previous method. Pilot digestions were performed to establish
the proper amounts of enzyme per unit of time. The established
20 conditions were used to digest 400 µg of genomic DNA. Once
digested, the DNA was phenol:chloroform extracted and
precipitated. The resultant pellet was resuspended in 400 µl
of TE (pH 8.0) and fractionated over a 10% - 40% sucrose step
gradient.

25 DNA was banded by centrifugation in an SW-28 rotor
(Beckman) at 26,000 RPM for 24 hours at 4° C. The DNA was
recovered from the tube by needle puncture at the bottom of the
tube. 500 µl fractions were collected and every third fraction
was examined for DNA size. Fractions containing DNA of a size
30 ranging from 6,000 bp to 15,000 bp were pooled and dialyzed
overnight against three changes of TE (pH 8.0). The dialysate
was removed and one tenth volume of 3 M sodium acetate (pH 5.2)
was added. The DNA was precipitated by the addition of 2
volumes of ice cold ethanol. The well-mixed suspension was
35 incubated for 30 m at -70° C in a tube suitable for the SW-28
rotor. The DNA was pelleted by centrifugation for 1 hour at
23,000 RPM and 4° C. The purified DNA was allowed to dry

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following decanting of the ethanol. The dry DNA pellet was resuspended in 400 µl of TE (pH 8.0).

The DNA obtained was ligated to pUC 18 BamHI/Bap and transformed into DH10B competent cells (Life Technologies) in order to produce a library. The resultant library was screened for the presence of positive clones by sequential pool reduction using PCR and two primers internal to the originally amplified overlapping DNA polymerase fragments. These two primers were primer 7 (RL-217) GGTTCATCTTCCTCTTTATCTTCTGCC and primer 8 (RL-218) GGA AGAAGATCGACCTGCCCTACGTCG. (Primers 7 and 8 are described below in SEQ ID NOs:7 and 8.) By this method, a large number of clones were screened with increasingly reduced numbers remaining in the pool. The original screening, which resulted in several positive signals, has since been reduced in pool numbers from 6,000 clones, to 400 clones, to several pools of 24 clones. The final 24 clone pools were screened and individual clones representing the entire gene were isolated as requested.

SEQ ID NO:13 is the DNA sequence generated from the PCR product produced from the degenerated primers. The PCR products were subcloned into pUC18 and double stranded sequencing was performed using a modified Sanger Chain termination method. The protein sequence was the direct translation of the nucleotide sequence (SEQ ID NO:12) using DNAsis software. Referring to SEQ ID NO:13, the coding region of the polymerase ranges from initiation at bp 233 to termination at bp 2573.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Luhm, Robert A.
d'Hennezel, Olga B.
Duffaud, Guy D.
Jolly, James F.
Kelly, Robert M.
Ting, Eve Yi-Fay
- (ii) TITLE OF INVENTION: PURIFIED THERMOCOCCUS BAROSSII
DNA POLYMERASE
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Quarles & Brady
 - (B) STREET: 411 East Wisconsin Avenue
 - (C) CITY: Milwaukee
 - (D) STATE: Wisconsin
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 53202-4497
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Baker, Jean C.
 - (B) REGISTRATION NUMBER: 35,433
 - (C) REFERENCE/DOCKET NUMBER: 740211.90440
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (414) 277-5709
 - (B) TELEFAX: (414) 271-3552

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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCATHGACA ARGGCYTRAT HCCNATGGAG GG

32

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGCTTCATAN ACNGCCTCNA GNGTRTANGT TGG

33

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGATGATWY TRGAYNNNGA TTACATAACW RAAGAWGG

38

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATGCCYTTG TCKATGAGRT AWCKCTTKGC AAAKGG

36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCGCGCATA AGGCGCACCA TAAACCTCCC

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCCANGCNG TAACGCTYTC AGCACAYTCC

30

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGTTCATCTT CCTCTTTATC TTCTGCC

27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAAGAAGAT CGACCTGCCC TACGTCG

27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCCAGGGTT TTCCCAGTCA CGACGTTGT

29

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(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCCAGGGTT TTCCAGTCA CGACGTTGTT TT

32

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCCAGGGTT TTCCAGTCA CGACGTTGTT TTTT

35

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 779 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met	Ile	Leu	Asp	Val	Asp	Tyr	Ile	Thr	Glu	Asp	Gly	Lys	Pro	Val	Ile
1				5					10					15	

Arg	Val	Phe	Lys	Lys	Asp	Lys	Gly	Glu	Phe	Lys	Ile	Glu	Tyr	Asp	Arg
			20				25						30		

Glu	Phe	Glu	Pro	Tyr	Ile	Tyr	Ala	Leu	Leu	Arg	Asp	Asp	Ser	Ala	Ile
		35					40					45			

Glu	Glu	Ile	Glu	Lys	Ile	Thr	Ala	Glu	Arg	His	Gly	Lys	Val	Val	Lys
						55					60				

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Val Lys Arg Ala Glu Lys Val Lys Lys Lys Phe Leu Gly Arg Ser Val
 65 70 75 80
 Glu Val Trp Val Leu Tyr Phe Thr His Pro Gln Asp Val Pro Ala Ile
 85 90 95
 Arg Pro Asp Lys Ile Arg Lys His Pro Ala Val Ile Asp Ile Tyr Glu
 100 105 110
 Tyr Asp Ile Pro Phe Ala Lys Arg Tyr Leu Ile Asp Lys Gly Leu Ile
 115 120 125
 Pro Met Glu Gly Asp Glu Glu Leu Lys Leu Met Ser Phe Asp Ile Glu
 130 135 140
 Thr Leu Tyr His Glu Gly Glu Glu Phe Gly Thr Gly Pro Ile Leu Met
 145 150 155 160
 Ile Ser Tyr Ala Asp Glu Ser Glu Ala Arg Val Ile Thr Trp Lys Lys
 165 170 175
 Ile Asp Leu Pro Tyr Val Asp Val Val Ser Thr Glu Lys Glu Met Ile
 180 185 190
 Lys Arg Phe Leu Lys Val Val Lys Glu Lys Asp Pro Asp Val Leu Ile
 195 200 205
 Thr Tyr Asp Gly Asp Asn Phe Asp Phe Ala Tyr Leu Lys Lys Arg Cys
 210 215 220
 Glu Lys Leu Gly Val Ser Phe Thr Leu Gly Arg Asp Gly Ser Leu Pro
 225 230 235 240
 Lys Ile Gln Arg Met Gly Asp Arg Phe Ala Val Glu Val Lys Gly Arg
 245 250 255
 Ile His Phe Asp Leu Tyr Pro Ala Ile Arg Arg Thr Ile Asn Leu Pro
 260 265 270
 Thr Tyr Thr Leu Glu Ala Val Tyr Glu Ala Val Phe Gly Lys Pro Lys
 275 280 285
 Glu Lys Val Tyr Ala Glu Glu Ile Ala Thr Ala Trp Glu Thr Gly Glu
 290 295 300
 Gly Leu Glu Gly Val Ala Arg Tyr Ser Met Glu Asp Ala Arg Val Thr
 305 310 315 320
 Tyr Glu Leu Gly Arg Glu Phe Phe Pro Met Glu Ala Gln Leu Ser Arg
 325 330 335
 Leu Ile Gly Gln Gly Leu Trp Asp Val Ser Arg Ser Ser Thr Gly Asn
 340 345 350
 Leu Val Glu Trp Phe Leu Leu Arg Lys Ala Tyr Glu Arg Asn Glu Leu
 355 360 365

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Ala	Pro	Asn	Lys	Pro	Asp	Glu	Arg	Glu	Leu	Ala	Arg	Arg	Arg	Gly	Gly				
	370					375					380								
Tyr	Ala	Gly	Gly	Tyr	Val	Lys	Glu	Pro	Glu	Arg	Gly	Leu	Trp	Asp	Asn				
385					390					395					400				
Ile	Val	Tyr	Leu	Asp	Phe	Arg	Ser	Leu	Tyr	Pro	Ser	Ile	Ile	Ile	Thr				
				405					410					415					
His	Asn	Val	Ser	Pro	Asp	Thr	Leu	Asn	Arg	Glu	Gly	Cys	Lys	Ser	Tyr				
			420					425					430						
Asp	Val	Ala	Pro	Gln	Val	Gly	His	Lys	Phe	Cys	Lys	Asp	Phe	Pro	Gly				
		435					440					445							
Phe	Ile	Pro	Ser	Leu	Leu	Gly	Asn	Leu	Leu	Glu	Glu	Arg	Gln	Lys	Ile				
	450					455					460								
Lys	Arg	Lys	Met	Lys	Ala	Thr	Leu	Asp	Pro	Leu	Glu	Arg	Lys	Leu	Leu				
465					470					475					480				
Asp	Tyr	Arg	Gln	Arg	Ala	Ile	Lys	Ile	Leu	Ala	Asn	Ser	Phe	Tyr	Gly				
				485					490					495					
Tyr	Tyr	Gly	Tyr	Ala	Arg	Ala	Arg	Trp	Tyr	Cys	Lys	Glu	Cys	Ala	Glu				
			500					505					510						
Ser	Val	Thr	Ala	Trp	Gly	Arg	Glu	Tyr	Ile	Glu	Met	Val	Ile	Arg	Glu				
		515					520					525							
Leu	Glu	Glu	Lys	Phe	Gly	Phe	Lys	Asp	Leu	Tyr	Ala	Asp	Thr	Asp	Gly				
	530					535					540								
Leu	His	Ala	Thr	Ile	Pro	Gly	Ala	Asp	Arg	Glu	Thr	Val	Lys	Lys	Lys				
545					550					555					560				
Asp	Leu	Glu	Phe	Leu	Asn	Tyr	Ile	Asn	Pro	Lys	Leu	Pro	Gly	Leu	Leu				
				565					570					575					
Glu	Leu	Glu	Tyr	Glu	Gly	Phe	Tyr	Ser	Arg	Gly	Phe	Phe	Val	Thr	Lys				
			580					585					590						
Lys	Lys	Tyr	Ala	Val	Ile	Asp	Glu	Glu	Gly	Lys	Ile	Thr	Thr	Arg	Gly				
		595					600					605							
Leu	Glu	Ile	Val	Arg	Arg	Asp	Trp	Ser	Glu	Ile	Ala	Lys	Glu	Thr	Leu				
	610					615					620								
Ala	Arg	Val	Leu	Glu	Ala	Ile	Leu	Arg	His	Gly	Asp	Val	Glu	Glu	Ala				
625					630					635					640				
Val	Arg	Ile	Val	Lys	Glu	Glu	Thr	Glu	Lys	Leu	Ser	Lys	Tyr	Glu	Val				
				645					650					655					
Pro	Pro	Glu	Lys	Leu	Val	Ile	Thr	Glu	Gln	Ile	Thr	Arg	Glu	Leu	Lys				
			660					665					670						

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Asp Tyr Lys Ala Thr Gly Pro His Val Ala Ile Ala Lys Arg Leu Ala
 675 680 685
 Ala Arg Gly Ile Lys Ile Arg Pro Gly Thr Val Ile Ser Tyr Ile Val
 690 695 700
 Leu Lys Gly Ser Gly Arg Ile Gly Asp Arg Ala Ile Pro Phe Asp Glu
 705 710 715 720
 Phe Asp Pro Thr Lys His Arg Tyr Asp Ala Asp Tyr Tyr Ile Glu Asn
 725 730 735
 Gln Val Leu Pro Ala Val Glu Arg Ile Leu Arg Ala Phe Gly Tyr Lys
 740 745 750
 Lys Glu Asp Glu Arg Tyr Gln Lys Thr Arg Gln Val Gly Leu Gly Ala
 755 760 765
 Trp Leu Gly Met Gly Gly Glu Arg Leu Lys Leu
 770 775

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2734 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGCCAGTCC GCGGGCCTTG TCCCTATCCC TACCAGCTAC CCGCTTAAAA ACTCTCTCAA	60
GGGCGTGCTT CGACGACAGC TCGCGCTCCT CAACCAGGCT TAAAGCGTCG GCCACAACCTT	120
CCTGAAAGCT CACCCTGTAA AACAGCTCCA TGGACGGGGC TTTGAGCGGG AGTTTAAAAA	180
GGTGGTGGTG AGGTTTATTA GCTCTGGGTT CGATAATCAA GCAGGTGTTG GTCATGATCC	240
TTGACGTTGA TTACATCACA GAGGACGGAA AGCCCGTCAT CAGGGTCTTC AAGAAGGATA	300
AAGGAGAGTT CAAAATTGAG TACGACAGAG AATTCGAGCC CTACATCTAT GCTCTTCTCA	360
GGGACGACTC TGCCATCGAA GAAATCGAAA AGATAACCGC AGAGAGGCAC GGCAAGGTCG	420
TTAAGGTAA GCGCGCGGAG AAGGTGAAGA AAAAGTTCCT CGGCAGGTCT GTGGAGGTCT	480
GGGTCTCTA CTTACGCAC CCGCAGGACG TTCCGGCAAT CCGCCCGGAC AAAATAAGGA	540
AGCACCCCGC GGTCATCGAC ATCTACGAGT ACGACATACC CTTTGCCAAG CGATATCTCA	600

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TAGACAAGGG	CTTGATTCT	ATGGAGGGCG	ATGAGGAGCT	TAAACTCATG	TCCTTCGACA	660
TCGAGACGCT	CTACCACGAG	GGAGAAGAGT	TCGGAACCGG	GCCGATTCTG	ATGATAAGCT	720
ACGCAGATGA	AAGCGAGGCG	CGTGTGATAA	CCTGGAAGAA	GATCGACCTG	CCCTACGTCG	780
ACGTTGTCTC	CACCGAGAAG	GAGATGATAA	AGCGCTTCCT	TAAGGTCGTT	AAGGAGAAGG	840
ACCCGGACGT	GCTGATAACA	TACGACGGCG	ACAACTTCGA	CTTCGCCTAC	CTCAAAAAGC	900
GGTGTGAGAA	GCTTGGCGTG	AGCTTTACCC	TCGGCAGGGA	CGGGAGCGAG	CCGAAGATAC	960
AGCGCATGGG	CGACCGCTTC	GCCGTTGAGG	TGAAGGGCAG	GATCCACTTC	GACCTGTACC	1020
CCGCCATAAG	GCGCACCATA	AACCTCCCGA	CCTACACCCT	TGAGGCTGTA	TACGAGGCGG	1080
TTTTCGGCAA	GCCCAAGGAG	AAGGTCTACG	CCGAGGAGAT	AGCCACCGCT	TGGGAGACCG	1140
GTGAGGGGCT	TGAGGGGGTC	GCGCGCTACT	CGATGGAGGA	CGCGAGGGTT	ACCTACGAGC	1200
TTGGCAGGGA	GTTCTTCCCG	ATGGAGGCC	AGCTTTCCAG	GCTCATCGGC	CAGGGTCTCT	1260
GGGACGTTTC	CCGCTCCAGC	ACCGGCAACC	TTGTTGAGTG	GTTTTTGCTC	AGGAAAGCCT	1320
ACGAGAGGAA	CGAACTCGCT	CCCAACAAGC	CCGACGAGAG	GGAGCTGGCG	AGGAGAAGGG	1380
GGGGCTACGC	CGGTGGCTAC	GTCAAGGAGC	CGGAGCGGGG	ACTGTGGGAT	AATATTGTGT	1440
ACCTCGATTT	TCGCTCGCTG	TACCCCTCCA	TTATCATCAC	CCACAACGTC	TCGCCAGATA	1500
CGCTCAACCG	CGAGGGATGT	AAGAGCTACG	ACGTTGCCCC	GCAGGTCGGT	CACAAGTTCT	1560
GCAAAGACTT	CCCCGGCTTC	ATTCCGAGCC	TGCTCGGAAA	CCTGCTGGAG	GAGAGGCAGA	1620
AGATAAAGAG	GAAGATGAAG	GCAACGCTCG	ACCCGCTGGA	GAGGAAGCTT	CTCGATTATC	1680
GCCAGCGCGC	TATCAAAATC	CTGGCGAACA	GCTTCTACGG	CTATTACGGC	TACGCCAGGG	1740
CAAGATGGTA	CTGCAAGGAG	TGCGCCGAGA	GCGTTACGGC	ATGGGGCAGG	GAGTACATCG	1800
AAATGGTTAT	CAGAGAGCTT	GAGGAAAAGT	TCGGTTTTAA	AGACCTCTAT	GCAGACACAG	1860
ACGGTCTTCA	TGCCACCATT	CCTGGAGCGG	ACCGTGAAAC	AGTCAAGAAA	AAGGACCTGG	1920
AGTTCTTAAA	CTATATCAAT	CCCAAACGTC	CCGGCCTTCT	CGAACTCGAA	TACGAGGGCT	1980
TCTACTCGAG	GGGCTTCTTC	GTCACCAAGA	AGAAGTACGC	GGTTATAGAC	GAGGAGGGCA	2040
AGATAACCAC	GCGCGGGCTT	GAGATAGTTA	GGAGGGACTG	GAGCGAGATA	GCGAAGGAGA	2100
CGCTGGCGAG	GGTTCTTGAG	GCGATACTCA	GGCACGGTGA	CGTTGAGGAG	GCCGTCAGAA	2160
TCGTCAAGGA	AGAGACGGAA	AAGCTGAGCA	AGTACGAGGT	TCCGCCGGAG	AAGCTGGTTA	2220
TCACCGAGCA	GATAACGCGC	GAGCTCAAAG	ACTACAAGGC	CACCGGCCCC	CACGTGGCCA	2280
TAGCGAAGCG	CCTCGCCGCG	AGGGGAATAA	AGATACGCCC	CGGGACGGTG	ATAAGCTACA	2340

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TCGTCCTCAA GGGCTCGGGG AGAATAGGCG ACAGGGCCAT TCCCTTCGAC GAGTTCGATC	2400
CGACGAAGCA CAGGTACGAC GCTGACTACT ACATCGAGAA CCAGGTTCTT CCAGCGGTGG	2460
AGAGAATCCT CAGGGCCTTC GGCTACAAGA AGGAAGACCT GCGCTACCAG AAGACGAGGC	2520
AGGTTGGGCT TGGCGCGTGG CTCGGAATGG GAGGAGAAAG ACTTAACTT TAGGAATCAT	2580
CTTTGCTCCT ATTGTTTTTA TACTTTATAC TGTTTAGATC GCTTTCTAAT AGTTTCACCA	2640
CTGGATTGTC TGGTCCAGTT TTGTATTTCA AGAGGGTTAT GATATCCTCC CCATAGTTTC	2700
TAATCAGATA CATGTATAGC TTGTAGTGGT TCTT	2734

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Claims

We claim:

1. A preparation of a purified DNA polymerase substantially identical to native DNA polymerase isolated from *Thermococcus barossii*.
2. The preparation of claim 1 wherein the polymerase is approximately 90 kDa.
3. The preparation of claim 1 wherein the polymerase is approximately 68 kDa.
4. The preparation of claim 1 wherein the polymerase is present in both an approximately 90 kDa and approximately 68 kDa form.
5. The preparation of claim 3 wherein the polymerase retains at least 70% activity after eight hours of incubation at 95° C.
6. The preparation of claim 5 wherein the polymerase retains at least 80% activity after eight hours of incubation at 95° C.
7. The preparation of claim 5 wherein the polymerase retains at least 90% activity after eight hours of incubation at 95° C.
8. The preparation of claim 1 wherein the polymerase is isolated from *Thermococcus barossii*.
9. The preparation of claim 1 wherein the polymerase is obtained from expression of a DNA sequence encoding the polymerase wherein the DNA sequence is inserted into a host organism other than *Thermococcus barossii*.

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10. A preparation of a purified DNA polymerase wherein the polymerase retains between 60% and 95% of polymerase proof-reading capability when the polymerase catalyzes a polymerization reaction with a primer with a 3 bp mismatch.

11. The preparation of claim 10 wherein the polymerase is thermostable.

12. The preparation of claim 10 wherein the polymerase retains between 60% and 80% proof-reading capability.

13. The preparation of claim 10 wherein the polymerase retains between 65% and 75% proof-reading capability.

14. The preparation of claim 10 wherein the polymerase retains at least 70% activity after eight hours of incubation at 95° C.

15. The preparation of claim 14 wherein the polymerase retains at least 80% activity after eight hours of incubation at 95° C.

16. The preparation of claim 15 wherein the polymerase retains at least 90% activity after eight hours of incubation at 95° C.

17. The preparation of claim 16 wherein the polymerase retains approximately 100% activity after eight hours of incubation at 95° C.

18. The preparation of claim 13 wherein the polymerase retains at least 90% activity after eight hours of incubation at 95° C.

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19. A preparation of a purified DNA polymerase wherein between 25% and 60% of polymerase proof-reading capability is retained when the polymerase catalyzes a polymerization reaction with a primer with a 6 bp mismatch.

20. The preparation of claim 19 wherein between 40% and 50% of the proof-reading capability is retained.

21. A method of elongating a DNA molecule comprising the step of exposing the DNA molecule to a complementary primer molecule, the DNA polymerase of claim 1 and dNTPs under conditions permitting extension of the DNA molecule.

22. A DNA construct comprising a region encoding a DNA polymerase substantially identical to native DNA polymerase isolated from *Thermococcus barossii*.

23. The construct of claim 22 wherein the encoding region comprises SEQ ID NO:13.

24. The construct of claim 22 wherein the encoding region comprises a portion of SEQ ID NO:13 sufficient to produce the 68 kDa form of the polymerase.

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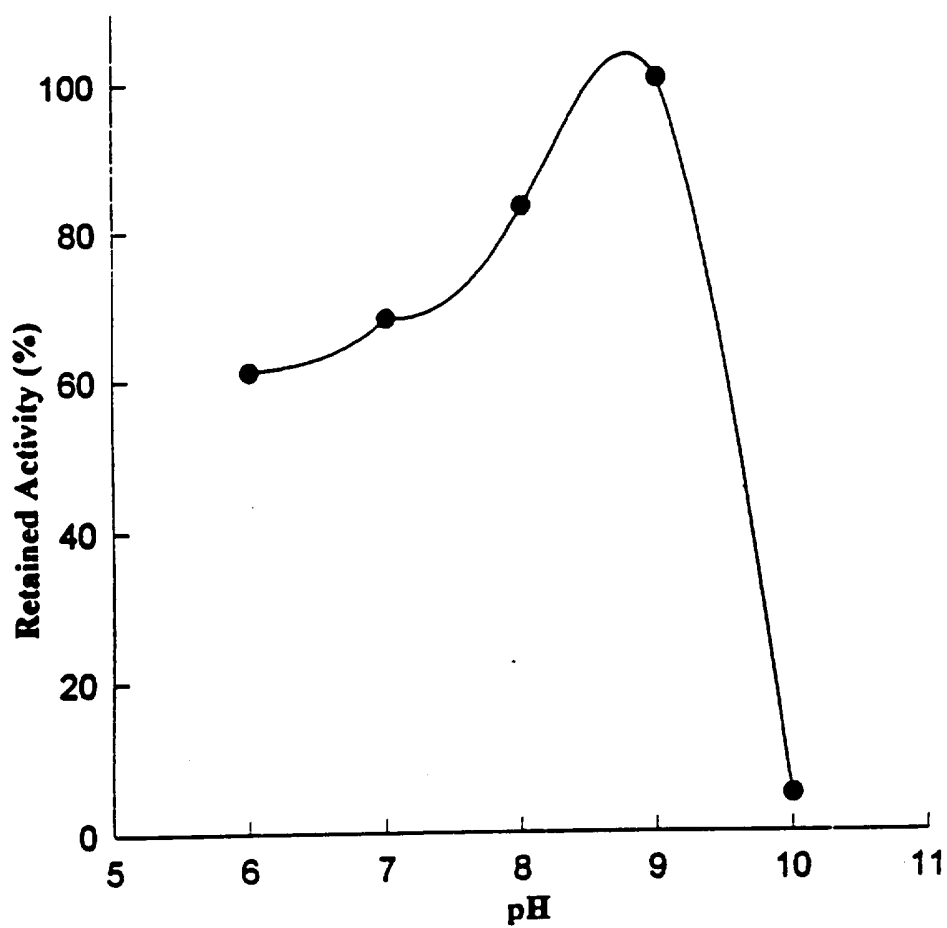


FIG. 1

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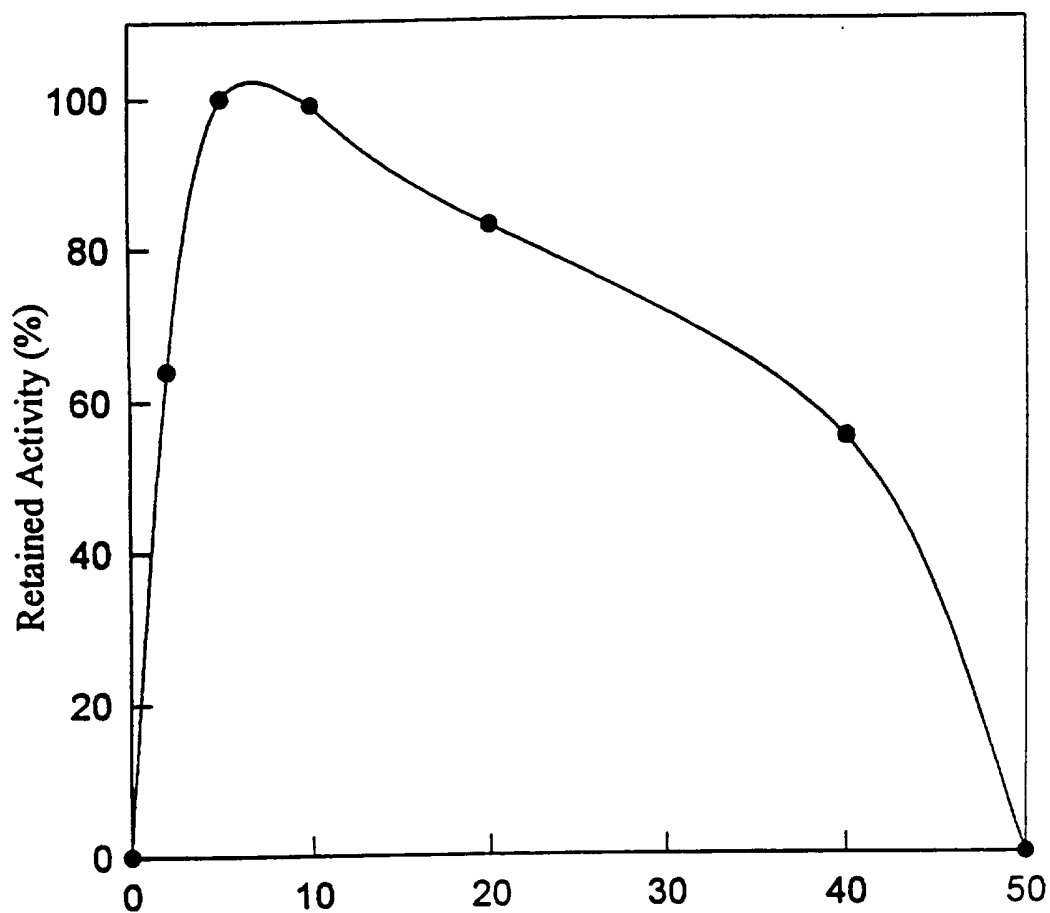


FIG. 2

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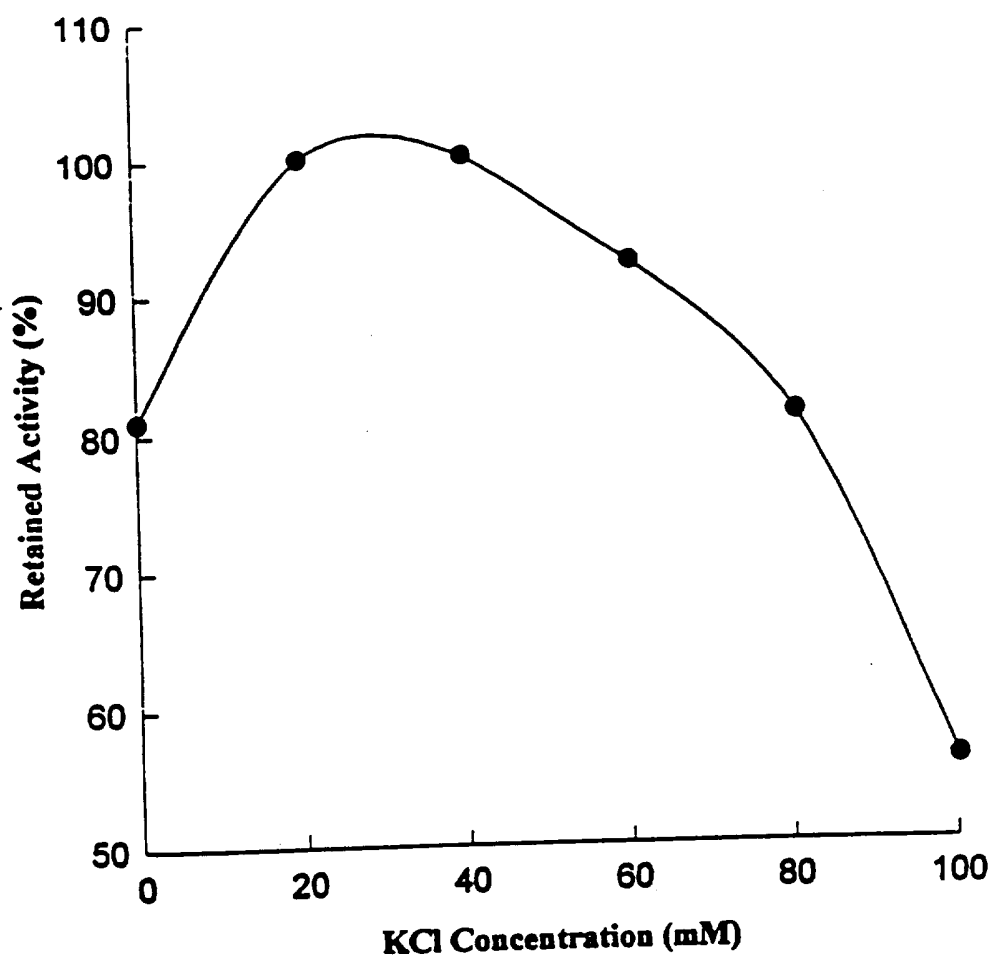


FIG. 3

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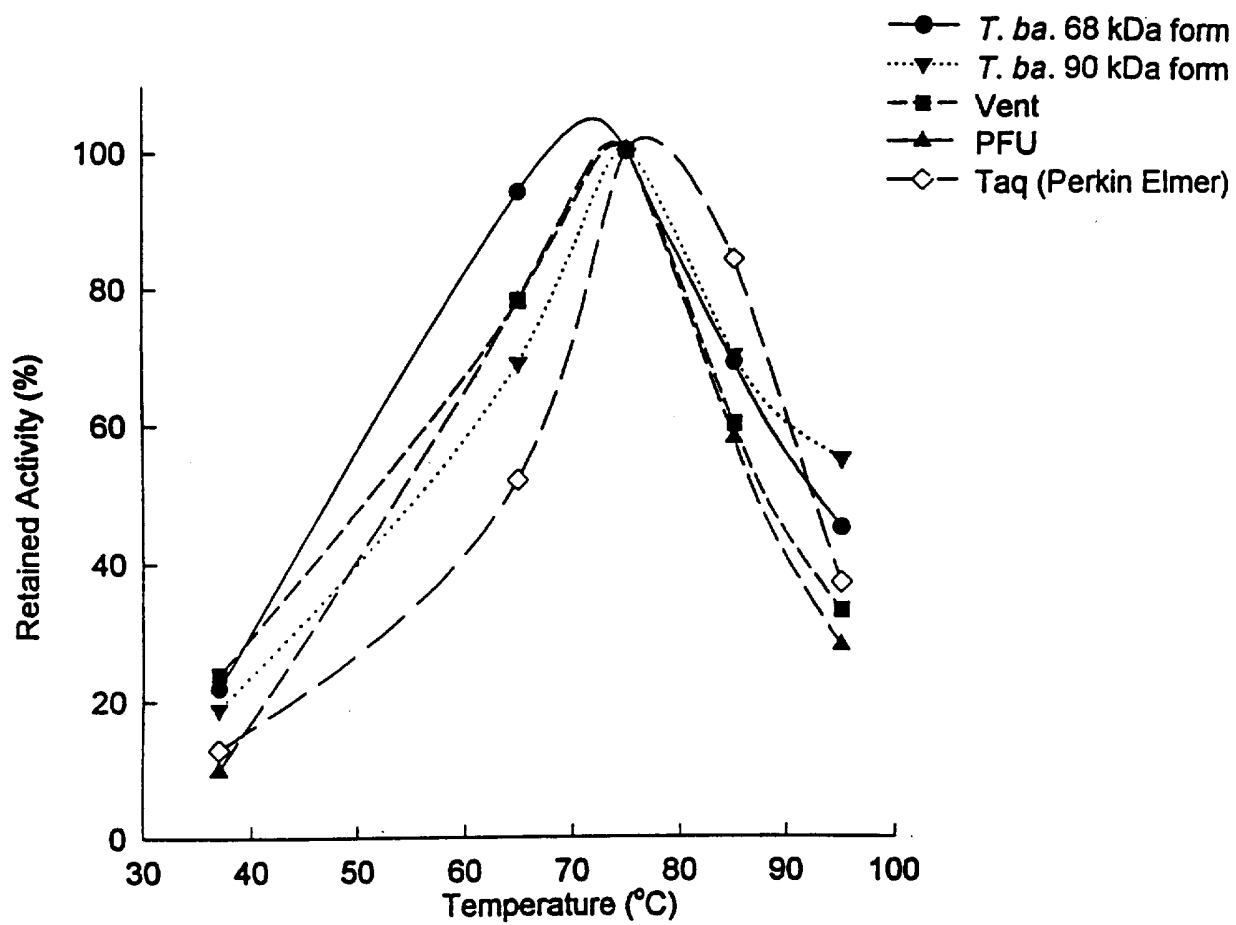


FIG. 4

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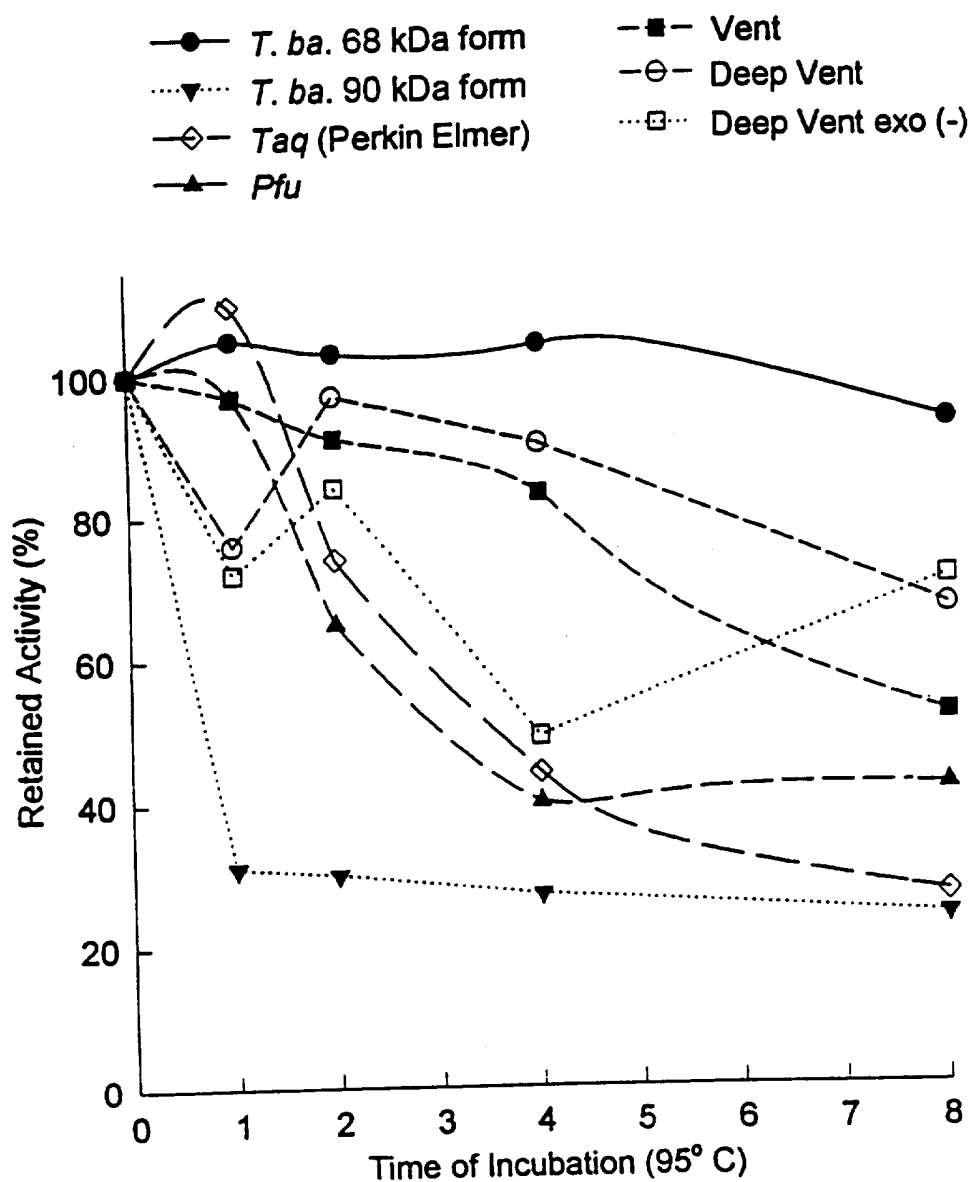


FIG. 5

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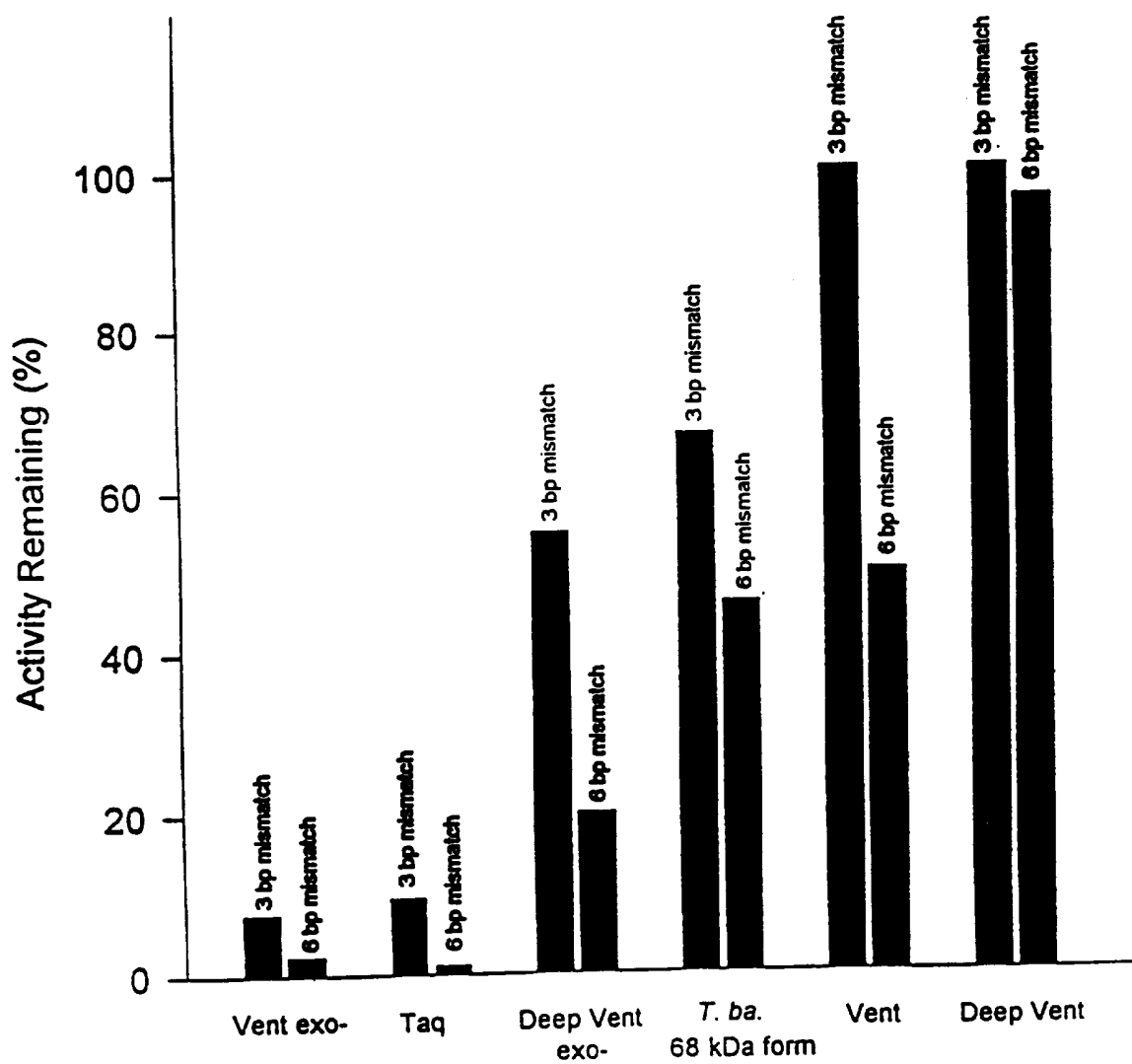


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/15263

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12N 9/12, 15/54

US CL : 435/194, 6; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/194, 6; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: Thermococcus barossii, DNA polymerase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,889,818 (GELFAND ET AL.) 26 December 1989.	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 FEBRUARY 1996

Date of mailing of the international search report

06 MAR 1996

Name and mailing address of the ISA/US
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Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

KEITH D. HENDRICKS

Telephone No. (703) 308-0196