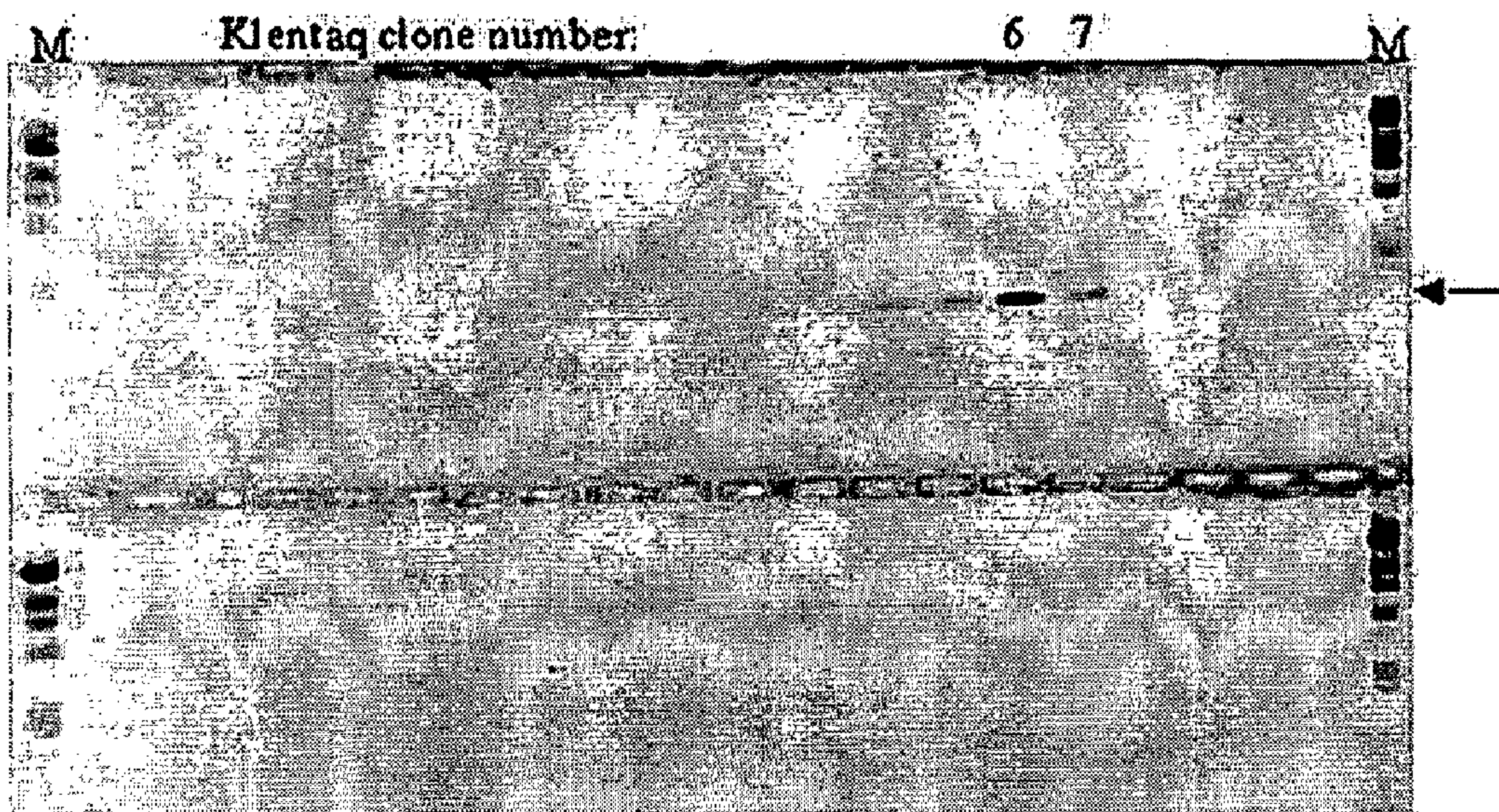




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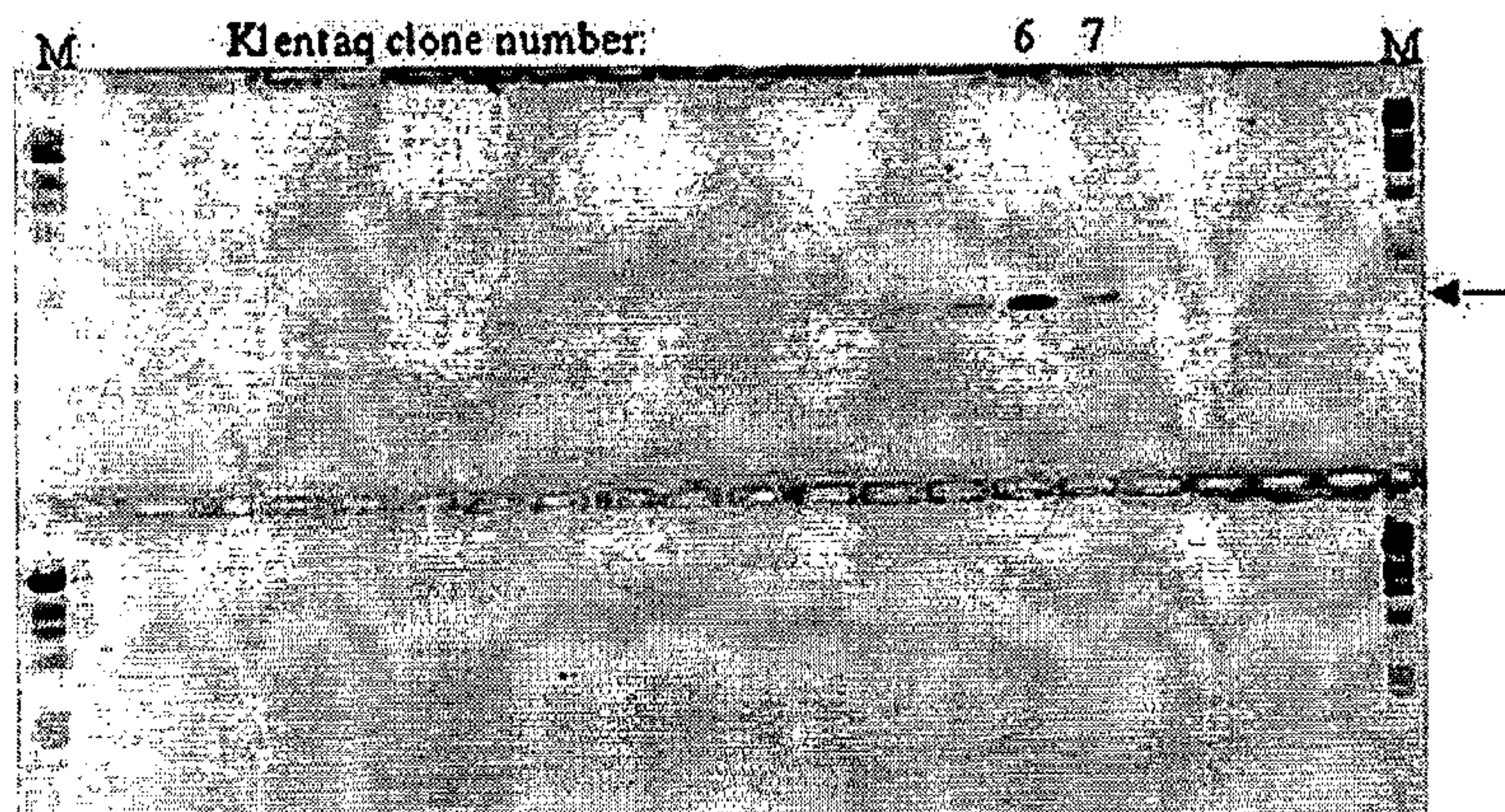
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(54) Title: USE OF WHOLE BLOOD IN PCR REACTIONS



(57) Abstract: A method of obtaining DNA amplification of a nucleic acid target from a volume of whole blood comprising performing DNA amplification in a PCR assay mixture with a blood-resistant polymerase.

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USE OF WHOLE BLOOD IN PCR REACTIONS

[01] BACKGROUND

[02] The polymerase chain reaction (PCR) is a sensitive DNA amplification procedure that permits the selection and detection of specific nucleic acids from a complex mixture.

5 In its most rudimentary form, PCR is employed using a sample that contains a target nucleic acid (DNA), a set of DNA primers that hybridize to the target, and a DNA polymerase that is capable of primer-based synthesis of complementary strands of the target. During the nucleic acid amplification process, the target:primer:polymerase mixture is subjected to successive rounds of heating at different temperatures to facilitate
10 target DNA strand separation (performed at ~90-99° C), primer:target DNA strand annealing (performed at ~40-70° C), and DNA polymerase-mediated primer elongation (performed at ~50-72° C) to create new complementary target strands. Because the reaction may be subjected to ~25-45 rounds of cycling to yield the desired DNA amplification product, PCR is usually conducted using thermal stable DNA polymerases
15 that can withstand the very high temperatures associated with target strand separation without suffering inactivation due to heat-induced protein denaturation. Since its introduction in the mid-1980's, PCR has become the *de facto* standard for detecting minute quantities of nucleic acids in samples, and obtaining specific genes from complex DNA genomes and samples.

20 [03] A major problem with diagnostic and forensic techniques based on PCR is the false-negative reactions or low sensitivity caused by inhibitory substances that interfere with PCR (1, 2, 3). Of particular clinical importance is the PCR analysis of blood samples, which represents the largest fraction of human health related tests for diagnosis of genetic diseases, virus and microbial infections, blood typing, and safe blood banking.
25 Various studies indicate that the inhibitory effect of blood on PCR is primarily associated with direct inactivation of the thermostable DNA polymerase and/or capturing or degradation of the target DNA and primers. It has been reported that the protease activity in blood also contributes to the reduced efficiency of PCR (1-5, 7, 10, 12).

[04] The blood resistance characteristics of the thermostable DNA polymerases vary with the source of the enzyme (6). Widely used thermostable polymerases like *Thermus aquaticus* DNA polymerase (Taq) and AmpliTaq Gold[®] are completely inhibited in the presence of 0.004-0.2% whole human blood (vol/vol; 3, 4, 6). Various agents have been tested for reducing the inhibitory effect of blood on Taq. It was found that an addition of betaine, bovine serum albumin, the single-stranded DNA binding protein of the T4 32 gene (gp 32), or a cocktail of protease inhibitors can partially relieve the blood inhibition and allow Taq to work in up to 2% blood (vol/vol), although this effect could be sample specific (3, 8, 9, 11).

[05] Several major inhibitors of PCR in human blood have been characterized such as immunoglobulin G, hemoglobin, lactoferrin and excess of leukocyte DNA (4, 7, 10). The IgG, hemoglobin, and lactoferin have been purified from plasma, erythrocytes and leukocytes, respectively, using size-exclusion and anion-exchange chromatography (4, 7). The heme has been reported to inactivate the Taq polymerase by binding to its catalytic domain (10), while the mechanism of action of the other inhibitory components is more poorly understood. The inhibitory effect of IgG can be reduced when this plasma fraction is heated at 95° C before adding it to PCR, or with the addition of excess non-target DNA to the PCR mixture. However, heating of IgG together with target DNA at 95° C was found to block amplification. Inhibition by IgG may be due to an interaction with the single-stranded DNA fraction in the target DNA. The inhibitory effect could be removed also by treating the plasma with DNA-agarose beads prior to amplification (4).

[06] Other complicating factors include EDTA and heparin, used as anti-coagulants, which can also inhibit DNA amplification. The addition of heparinase has been shown to counteract the heparin-mediated inhibition (13, 14). Therefore, various laboratory procedures of sample preparation have been developed to reduce the inhibitory effect of blood. The DNA purification methods suitable for PCR can include additional steps like dialysis, treatment with DNA-agarose beads or Chelex 100 resin, multiple DNA washes, or a combination of dilution with buffer which causes lysis of red blood cells, centrifugation to recover the white blood cells, washing with NaOH and the addition of bovine serum albumin (2,3, 15-19).

[07] These pre-treatment steps of the blood samples are generally time-consuming, labor-intensive, and can be sample specific. The guanidinium thiocyanate method for DNA isolation is not suitable for reliable detection of *Mycobacterium tuberculosis* in clinical samples. An alternative method of DNA purification with protease K treatment followed by phenol-chloroform extraction has to be employed to relieve the inhibition (20). Separation with a QIAamp kit followed by dialysis with a Millipore filter are required for eliminating the heme inhibition of hepatitis B virus detection (21). In addition, some the above steps carry a risk of target DNA losses and are not suitable for automation. Moreover, even commercial kits specially formulated for DNA purification from blood samples such as QIAamp or GeneReleaser are not always satisfactory. The reason is due to an incomplete removal of Taq inhibitors, which can result in false-negative results. For example, 14% of the human blood samples tested for hepatitis B virus yielded false-negative results when using such blood kits (21).

[08] The objective of achieving specificity of amplification reactions for samples containing whole blood is further complicated by two types of unwanted DNA synthesis reactions that occur during PCR. Both types of side-reactions are frequently competitive with the desired target and can lead to impure product or failed amplification. This is particularly problematic for PCR assays containing a low copy number of the nucleic acid template target, wherein the PCR conditions are modified to include a greater number of amplification cycles to achieve an adequate yield of the desired amplification product.

[09] The first type of unwanted DNA synthesis is priming on less specific sequences in the template. This is only an issue if the template is contaminated with single-stranded nucleic acid or if the template is single-stranded, which is the case if the DNA preparation has been subjected to melting conditions during its isolation.

[010] The second type of unwanted DNA synthesis is primers acting as templates for themselves and/or each other, with at least the result of modifying their 3' ends by the addition of additional nucleotides. These so-modified primers are able to anneal to the nucleic acid target; however, they do not serve as primers for complementary strand synthesis due to the presence of mismatched nucleotides at the site of elongation between the 3' end of the primer and the desired target. This problem is often referred to as "primer dimer", although this name is not accurately descriptive. This problem can often

be reduced or avoided by careful primer design, and it is more of a problem with multiplex PCR, since there is more opportunity for accidental homology among multiple pairs of primers.

[011] A procedure known as "hot start PCR" avoids the occurrence of both types of unwanted DNA synthesis side-reactions. According to this method, the enzyme DNA polymerase, or a buffer component essential to its activity, such as the magnesium (II) cation and/or the dNTPs, is withheld from the other PCR assay mixture ingredients until the PCR reaction has been heated to at least the normal primer-annealing (or, preferably, the DNA extension) temperature (55-75° C, optimal 68° C). At this temperature the primers can presumably not form stable duplexes with themselves or at unwanted template sequences. After the selective temperature is achieved, the omitted component is added to reaction to reconstitute a functional amplification mixture.

[012] Typical hot start PCR procedures are not only labor-intensive, they expose the PCR reactions to contamination with each other and with molecules that have been previously amplified in the thermal cycler machine.

[013] The more standard ways of executing a hot start consist of formulating the PCR reaction in two parts, such that the DNA polymerase is not able to act on the DNA until the two portions are combined at high temperature, usually 65-85° C. For instance, an initial solution containing all of the magnesium is introduced to the reaction tube encapsulated in a wax bead or sealed under a layer of wax. The rest of the reaction, without Mg, is then added, along with an overlay of oil, if appropriate. While the reaction heats for the first cycle, the wax melts and floats to the surface, allowing the magnesium to mix with the reaction volume. The DNA polymerase activity is therefore reconstituted at a temperature that does not allow non-specific or unwanted primer interactions. A great drawback to the wax method comes after the PCR cycling is complete, and the product must be withdrawn for analysis. The wax then tends to plug the pipette tip, greatly adding to the time and effort of reaction analysis.

[014] Recently, a method of hot start which is not hot at all, but which uses anti-Taq antibodies, has been described, patented and made commercially available (33-35). The antibodies largely neutralize the enzyme activity of the Taq polymerase, and can be added any time prior to the primers, or be conveniently present during storage of the stock

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enzyme. The antibodies are thermolabile, thus permitting the Taq polymerase to resume activity after the first heat step. The antibodies so far developed for this method must be used in 10-fold molar excess and are expensive. Furthermore, the antibodies inhibit some long PCR assays that are conducted with the KlentaqLA polymerase mixture.

5 [015] A chemically inactivated form of the Taq polymerase has been introduced recently, termed AmpliTaq Gold[®]. The nature of the inactivation is proprietary, but the inactivation is reversible by heating the polymerase at 95° C. This method may be even more convenient than the other methods, but it has at least one current disadvantage: the time for reactivation is about 10 minutes at 95° C. This procedure is incompatible with
10 long PCR applications, as this treatment would excessively depurinate nucleic acid targets longer than a few kb.

[016] Thus, the analysis of whole blood samples using PCR would be benefited by the discovery of new reagents and methods that overcome the aforementioned shortcomings of current PCR technologies. The invention disclosed herein addresses and solves many
15 of these shortcomings.

[017] SUMMARY

[018] In a first aspect, the present invention is a method of obtaining DNA amplification of a nucleic acid target from a volume of whole blood comprising performing DNA
20 amplification in a PCR assay mixture with a blood-resistant polymerase.

[019] In a second aspect, the present invention is method of obtaining DNA amplification of a nucleic acid target from a volume of whole blood comprising performing DNA amplification in a PCR assay mixture with KT-1 (SEQ ID NO:2) or Z-TAQ[™].

25 [020] In a third aspect, the present invention is a method of obtaining DNA amplification of a nucleic acid target from a whole blood sample with a DNA amplification cocktail by avoiding mixing of the whole blood sample with the DNA amplification cocktail in a reaction vessel before thermal cycling that includes the following steps: adding the DNA amplification cocktail to the reaction vessel, wherein
30 the DNA amplification cocktail comprises at least one DNA polymerase; adding the

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whole blood sample to the reaction vessel, wherein the whole blood sample is layered beneath the DNA amplification cocktail regardless of the order of addition of the DNA amplification cocktail and the whole blood sample to the reaction vessel; and performing a thermal cycling program to effect DNA amplification of the nucleic acid target.

5 [021] In a fourth aspect, the present invention is a method of obtaining a hot start for DNA amplification of a nucleic acid target that includes the preparation of the reaction cocktail comprising at least a first volume component and a second volume component. The second volume component is heavier than the first volume component. The first volume component comprises a DNA polymerase cocktail lacking an essential constituent
10 required for DNA amplification activity. The second volume component includes the essential constituent required for DNA amplification activity. The second volume component is underlayered below the first volume component without undue mixing before a DNA amplification reaction is initiated.

[022] In a fifth aspect, the present invention is an isolated polypeptide comprising an
15 amino acid sequence having at least 80% amino acid sequence identity with at least one member selected from the group consisting of KT-6 (SEQ ID NO:4), KT-7 (SEQ ID NO:6), KT-10 (SEQ ID NO:20), KT-12 (SEQ ID NO:24), FL-10 (SEQ ID NO:28), and FL-12 (SEQ ID NO:30), wherein the isolated polypeptide comprises a blood-resistant polymerase.

20 [023] In a sixth aspect, the present invention is an isolated polypeptide comprising a nucleotide sequence having at least 80% amino acid sequence identity with at least one member selected from the group consisting of KT-7 (SEQ ID NO:6), KT-11 (SEQ ID NO:22), KT-12 (SEQ ID NO:24), and FL-12 (SEQ ID NO:30), wherein the isolated polypeptide comprises a faster elongating polymerase.

25 [024] In a seventh aspect, the present invention is an isolated polypeptide comprising at least one member selected from the group consisting of KT-6 (SEQ ID NO:4), KT-7 (SEQ ID NO:6), KT-10 (SEQ ID NO:20), KT-11 (SEQ ID NO:22), KT-12 (SEQ ID NO:24), FL-10 (SEQ ID NO:28), and FL-12 (SEQ ID NO:30).

[025] In an eighth aspect, the present invention is an isolated polypeptide comprising
30 KT-1 (SEQ ID NO:2) having at least two amino acid residue substitutions, wherein one of the at least two amino acid residue substitutions comprises amino acid residue position

430 such that the isolated polypeptide encodes a blood-resistant polymerase, a faster elongating polymerase, or a blood-resistant, faster elongating polymerase.

[026] In a ninth aspect, the present invention is an isolated polypeptide comprising Taq DNA polymerase (SEQ ID NO:26) having at least three amino acid residue substitutions, wherein one of the at least three amino acid residue substitutions comprises amino acid residue position 708 such that the isolated polypeptide encodes a blood-resistant polymerase, a faster elongating polymerase, or a blood-resistant, faster elongating polymerase.

[027] In a tenth aspect, the present invention is an isolated nucleic acid comprising a nucleotide sequence having at least 80% nucleotide sequence identity with at least one member selected from the group consisting of KT-1 (SEQ ID NO:1), KT-6 (SEQ ID NO:3), KT-7 (SEQ ID NO:5), KT-10 (SEQ ID NO:19), KT-12 (SEQ ID NO:23), Taq DNA polymerase (SEQ ID NO:25), FL-10 (SEQ ID NO:27), and FL-12 (SEQ ID NO:29), wherein the isolated nucleic acid encodes a blood-resistant polymerase.

[028] In a eleventh aspect, the present invention is an isolated nucleic acid comprising a nucleotide sequence having at least 80% nucleotide sequence identity with at least one member selected from the group consisting of KT-1 (SEQ ID NO:1), KT-7 (SEQ ID NO:5), KT-11 (SEQ ID NO:21), KT-12 (SEQ ID NO:23), Taq DNA polymerase (SEQ ID NO:25), and FL-12 (SEQ ID NO:29), wherein the isolated nucleic acid encodes a faster elongating polymerase.

[029] In a twelfth aspect, the present invention is an isolated nucleic acid comprising at least one member selected from the group consisting of KT-6 (SEQ ID NO:3), KT-7 (SEQ ID NO:5), KT-10 (SEQ ID NO:19), KT-11 (SEQ ID NO:21), KT-12 (SEQ ID NO:23), FL-10 (SEQ ID NO:27), and FL-12 (SEQ ID NO:29).

[030] In a thirteenth aspect, the present invention is an isolated nucleic acid comprising KT-1 (SEQ ID NO:1) having at least two codon substitutions, wherein one of the at least two codon substitutions comprises codon position 430 such that the isolated nucleic acid encodes a blood-resistant polymerase, a faster elongating polymerase, or a blood-resistant, faster elongating polymerase.

[031] In a fourteenth aspect, the present invention is an isolated nucleic acid comprising Taq DNA polymerase (SEQ ID NO:25) having at least three codon substitutions, wherein

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one of the at least three codon substitutions comprises codon position 708 such that the isolated nucleic acid encodes a blood-resistant polymerase, a faster elongating polymerase, or a blood-resistant, faster elongating polymerase.

[032] In a fifteenth aspect, the present invention is a method of obtaining rapid DNA
5 amplification of a nucleic acid target in a PCR assay mixture comprising a faster elongating DNA polymerase.

[033] In a sixteenth aspect, the present invention is a kit for performing PCR assays on samples of whole blood, wherein the kit comprises a blood-resistant polymerase.

[034] In a seventeenth aspect, the present invention is a kit for performing PCR
10 assays on samples of whole blood, wherein the kit comprises KT-1 (SEQ ID NO: 2) or Z-TAQ™.

[034A] The invention as claimed relates to:

- an isolated polypeptide comprising an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID NO: 4 (KT-6), wherein amino residue
15 position 430 of the isolated polypeptide is valine (V) and amino residue position 429 of the isolated polypeptide is leucine (L); SEQ ID NO: 6 (KT-7), wherein amino residue position 430 of the isolated polypeptide is tryptophan (W), amino residue position 348 of the isolated polypeptide is lysine (K), and amino residue position 429 of the isolated polypeptide is leucine (L); SEQ ID NO: 20 (KT-10), wherein amino residue position 430 of the isolated
20 polypeptide is lysine (K), amino residue position 348 of the isolated polypeptide is lysine (K), and amino residue position 429 of the isolated polypeptide is leucine (L); SEQ ID NO: 24 (KT-12), wherein amino residue position 430 of the isolated polypeptide is leucine (L), amino residue position 331 of the isolated polypeptide is proline (P), amino residue position 348 of the isolated polypeptide is lysine (K), and amino residue position 429 of the isolated
25 polypeptide is leucine (L); SEQ ID NO: 28 (FL-10), wherein amino residue position 708 of the isolated polypeptide is lysine (K), amino acid residue position 626 of the isolated polypeptide is lysine (K), and amino residue position 707 of the isolated polypeptide is

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leucine (L); or SEQ ID NO: 30 (FL-12), wherein amino residue position 708 of the isolated polypeptide is leucine (L), amino residue position 609 of the isolated polypeptide is proline (P), amino residue position 626 of the isolated polypeptide is lysine (K), and amino residue position 707 of the isolated polypeptide is leucine (L); wherein the isolated polypeptide

5 comprises a DNA polymerase having a blood-resistant phenotype and a cold sensitive phenotype; the blood-resistant phenotype displaying amplification activity in a Polymerase Chain Reaction assay solution containing 3% to 25% whole blood; the cold sensitive phenotype displaying lower amplification than wild-type Taq at temperatures lower than 72°C in a Polymerase Chain Reaction assay;

10 - an isolated polypeptide comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 4 (KT-6), SEQ ID NO: 6 (KT-7), SEQ ID NO: 20 (KT-10), SEQ ID NO: 24 (KT-12), SEQ ID NO: 28 (FL-10), and SEQ ID NO: 30 (FL-12);

- a method of amplifying a deoxyribonucleic acid (DNA) target comprising: performing a polymerase chain reaction (PCR) with a reaction mixture comprising a
15 deoxyribonucleic acid (DNA) target, deoxynucleotide triphosphates, a magnesium salt, and an isolated polypeptide as described herein; and

- a kit for performing PCR assays on samples comprising whole blood or blood fraction, wherein the kit comprises (a) the isolated polypeptide as described herein and (b) an oligonucleotide primer, a buffer, a control solution, or instructions to perform said PCR
20 assays.

[035] Definitions

[036] The term "amplicon" refers to the nucleic acid that is the target of DNA amplification of a PCR assay.

[037] The phrase "amplification activity" refers to the functional ability of a DNA
25 polymerase to synthesize copies of a nucleic acid target under the PCR conditions disclosed

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herein to yield a quantity of amplified DNA product that is discernable by intercalative dye (*e.g.*, ethidium bromide) staining methods that are well known in the art.

[038] The phrase "homogeneous PCR assay solution" as used herein refers to a solution that is homogenous with respect to the absence of discrete phases. A homogeneous
5 PCR assay solution is one that is typically prepared by mixing the contents of a reaction vessel using a vortexer or comparable mixing apparatus. In the context of heavy hot start PCR assays, the PCR assay solution is composed of two phases prior to initiating the thermal cycling program; that is, the PCR assay solution of a heavy hot start PCR assay is not premixed prior to initiating a thermal cycling program and is not considered a homogenous
10 PCR assay solution.

[039] The phrase "blood-resistant polymerase" as used herein refers to a mutant form of either KlenTaq-278 DNA polymerase or full-length Taq DNA polymerase wherein the mutant enzyme is cold sensitive and displays amplification activity in a homogeneous PCR assay solution containing whole blood in the range from about 3% (vol/vol) to about

25% (vol/vol). By “cold sensitive,” the mutant enzyme displays lower amplification activity than wild-type Taq DNA polymerase at reduced temperatures relative to the normal temperature at which DNA extension reactions are performed (~72 °C). Such a mutant enzyme displays DNA amplification activity under hot start PCR conditions. A

5 mutant form of Klentaq-278 DNA polymerase includes a polypeptide that does not encode the identical amino acid sequence of Klentaq-278 DNA polymerase (SEQ ID NO:2). Examples of such mutant forms include a deletion of at least one amino acid, an insertion of additional amino acids, or a change of at least one amino acid relative to the amino acid sequence of the Klentaq-278 DNA polymerase (SEQ ID NO:2). A mutant
10 form of full-length Taq DNA polymerase includes a polypeptide that does not encode the identical amino acid sequence of the full-length Taq DNA polymerase (GenBank Accession No. J04639; SEQ ID NO:25). Examples of such mutant forms include a deletion of at least one amino acid, an insertion of additional amino acids, or a change of at least one amino acid relative to the amino acid sequence of the full-length Taq DNA
15 polymerase (SEQ ID NO:25).

[040] The phrase “faster elongating polymerase” as used herein refers to a derivative of Taq DNA polymerase that displays amplification activity in PCR assays conducted with extension times in the range from about 12 seconds to about 50 seconds to complete up to 2 kb.

20 [041] The phrase “physiologically compatible buffer” as used herein refers to any solution that is compatible with the function of enzyme activities and enables cells and biological macromolecules to retain their normal physiological and biochemical functions. Typically, a physiologically compatible buffer will include a buffering agent (*e.g.*, TRIS, MES, PO₄, HEPES, *etc.*), a chelating agent (*e.g.*, EDTA, EGTA, or the like),
25 a salt (*e.g.*, NaCl, KCl, MgCl₂, CaCl₂, NaOAc, KOAc, Mg(OAc)₂, *etc.*) and optionally a stabilizing agent (*e.g.*, sucrose, glycerine, Tween20, *etc.*).

[042] The polymerases referred to throughout this description have the following structures and properties: (1) Taq refers to the wild-type, full-length DNA Polymerase from *Thermus aquaticus* (GenBank Accession No. J04639) and also used for chemically
30 modified variants thereof, such as AmpliTaq Gold®; (2) Klentaq-235 refers to an N-terminal deletion of the first 235 amino acids of Taq. Klentaq-235 is also known in

commerce as DeltaTaq, Δ Taq, Klentaq, and Klentaq5; (3) Klentaq-278 refers to an N-terminal deletion of the first 278 amino acids of Taq (Klentaq-278 is also referred to as “Klentaq1” or “KT-1” or wild-type Klentaq1) and is described in claims 1-5 of U.S. Patent No. 5,436,149; (4) Klentaq6 (abbreviated as KT-6) refers to Klentaq-278 with two amino-acid changes; (5) Klentaq7 (abbreviated as KT-7) refers to Klentaq-278 with three amino-acid changes; (6) Klentaq10 (abbreviated as KT-10) refers to Klentaq-278 with three amino acid changes; Klentaq11 (abbreviated as KT-11) refers to Klentaq-278 with four amino acid changes; Klentaq12 (abbreviated as KT-12) refers to Klentaq-278 with four amino acid changes; FL-10 refers to full-length Taq polypeptide with three amino acid changes; and FL-12 refers to full-length Taq polypeptide with four amino acid changes. These codon changes of the relevant Taq polymerase mutants are summarized in abbreviated form in Table I.

Table I. Codon changes in Taq DNA polymerase mutants

SEQ ID NO: ¹	Moniker	DNA Change ² <WT>nuc<MUT>	Codon Change(s)	Amino Acid Change(s) ²	Phenotype ³
3 4	KT-6	A2119C; (1285) A2123T; (1289)	ATT to CTT; GAG to GTG	I707L; (429) E708V (430)	CS BR
5 6	KT-7	G1876A; (1042) A2119C; (1285) G2122T/A2123G (1288) (1289)	GAG to AAG; ATT to CTT; GAG to TGG	E626K; (348) I707L; (429) E708W (430)	CS CS BR*, FAST*
19 20	KT-10	G1876A; (1042) A2119C; (1285) G2122A (1288)	GAG to AAG; ATT to CTT; GAG to AAG	E626K; (348) I707L; (429) E708K (430)	CS CS BR
21 22	KT-11	G1876A; (1042) G1945A; (1111) A2119C; (1285) G2122A/A2123C (1288) (1289)	GAG to AAG; GTC to ATC; ATT to CTT; GAG to TCG	E626K; (348) V649I; (371) I707L; (429) E708S (430)	CS FAST CS FAST
23 24	KT-12	T1826C; (992) G1876A; (1042) A2119C; (1285) G2122T/A2123T (1288) (1289)	CTG to CCG; GAG to AAG; ATT to CTT; GAG to TTG	L609P; (331) E626K; (348) I707L; (429) E708L (430)	BR*, FAST* CS CS BR*, FAST*
27 28	FL-10	G1876A; A2119C; G2122A	GAG to AAG; ATT to CTT; GAG to AAG	E626K; I707L; E708K	CS CS BR
29 30	FL-12	T1826C; G1876A; A2119C; G2122T/A2123T	CTG to CCG; GAG to AAG; ATT to CTT; GAG to TTG	L609P; E626K; I707L; E708L	BR*, FAST* CS CS BR*, FAST*

¹Odd- and even-numbered SEQ ID NOs refer to nucleic acid and polypeptide sequences, respectively, as illustrated in the Sequence Listing. ²Wild-type ("WT") base of top (codon) strand on the left, mutant ("MUT") base on the right, of numerical positions of changes ("nuc") which numbers are in reference to the full-length

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Taq DNA polymerase encoding nucleic acid and polypeptide (herein SEQ ID NOs: 25 & 26, respectively; disclosed in GenBank Acc. No. J04639); parenthetical numbers refer to the corresponding KlenTaq-278 sequence positions (herein SEQ ID NOs: 1 & 2, respectively; disclosed in U.S. Patent No. 5,436,149).

5 ³Phenotype that was conferred when this mutation was added to its parent; CS, cold sensitive; BR, blood-resistant; FAST, fast DNA extension.

* In the cases of KT-7, KT-12, and its respective FL-version, both BR and FAST phenotypes are present, presuming a possible double effect of these changes. Testing each mutation individually will clarify the linkage between the phenotypes.

[043] The suffix "LA" means "Long and Accurate" and refers to a mixture of thermostable DNA polymerases, after claims 6-16 of US Patent 5,436,149 and Barnes (1994). Major component is usually Taq or Klentaq1. A minor component is usually an archaebacterial DNA polymerase such as Pfu polymerase, Pwu polymerase, Vent polymerase, or Deep Vent polymerase.

[044] KlentaqLA is a mixture of 47:1::Klentaq1:Deep Vent by volume of commercially available enzymes. This mixture also may be modified to 24:1 as noted in the text. Since commercially distributed Klentaq1 is about 15-20 times more concentrated than commercially distributed Deep Vent, the true ratio, by units or protein, is approximately 15-20 times higher, *i.e.*, 705:1 or 360:1

[045] TaqLA is a mixture of 47:1::Taq:Deep Vent, or 16:1::Taq:Pfu, or an unspecified mixture of Taq:Pfu that is commercially known as "TaqPlus."

[046] Control sequences are DNA sequences that enable the expression of an operably-linked coding sequence in a particular host organism. Prokaryotic control sequences include promoters, operator sequences, and ribosome binding sites. Eukaryotic cells utilize promoters, polyadenylation signals, and enhancers.

[047] The phrase "a reaction vessel" refers to any container that may be used for performing a biological, biochemical, or chemical reaction. In the context of PCR assays, a reaction vessel is any suitable container that can withstand the temperatures carried out during a typical DNA amplification reaction. Preferably, a reaction vessel that is used for PCR assays includes a tube fitted with a closure, wherein both the tube and the closure are made of polymeric material such as polypropylene or similar material commonly employed in the art.

[048] The phrase "isolated nucleic acid molecule" is purified from the setting in which it is found in nature and is separated from at least one contaminant nucleic acid molecule.

[049] The phrase "isolated polypeptide molecule" is purified from the setting in which it is found in nature and is separated from at least one contaminant polypeptide molecule.

[050] The phrase “purified polypeptide” refers to a polypeptide molecule that has been purified to greater than 80% homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or silver stain. Isolated polypeptides include those expressed heterologously in genetically engineered
5 cells or expressed *in vitro*. Ordinarily, isolated polypeptides are prepared by at least one purification step.

[051] BRIEF DESCRIPTION OF THE DRAWINGS

[052] Figure 1 depicts results of PCR assays with different forms of Klentaq
10 polymerase (40 mutants and wild-type form of Klentaq) conducted in the presence of inhibitory amounts of blood. Clones KT-6 and KT-7 were capable of amplifying a 1.65 kbp target DNA from added plasmid template in the presence of 10% whole human blood;

[053] Figure 2A depicts results of PCR amplification of a 0.32 kbp endogenous
15 target DNA directly from whole blood with two mutant forms of Klentaq (KT-6 and KT-7) in the presence of increasing amounts of whole human blood (lane 1: 0%; lane 2: 5%; lane 3: 10%; lane 4: 15%);

[054] Figure 2B depicts results of PCR assays directed toward the amplification
20 of a 0.32 kbp endogenous human Dystrophin gene fragment in the presence of the indicated percentages of whole blood (vol/vol) in homogeneous PCR assay solutions with two mutant forms of Klentaq (KT-10 and KT-12), as shown above the figure;

[055] Figure 2C depicts results of PCR assays directed toward amplification of a
25 1.1 kbp endogenous CCR5 gene fragment in the presence of the indicated percentages of whole blood (vol/vol) in homogeneous PCR assay solutions with two mutant forms of Klentaq (KT-10 and KT-12), as shown above the figure;

[056] Figure 3A depicts results of PCR assays directed toward the amplification
of a 0.32 kbp fragment of the endogenous human Dystrophin gene, 1.1 kbp and 2.5 kbp fragments of the endogenous human CCR5 gene, or a 4.3 kbp endogenous

human Tissue Plasminogen Activator (TPA) gene fragment in the presence of the indicated amounts of whole blood (vol/vol) (as shown above the figure) in homogeneous PCR assay solutions using a blood-resistant mutant form of full-length Taq DNA polymerase (FL-10) in comparison to blood-inactive commercial
5 Taq enzymes (JumpStart™ Taq (Sigma), AmpliTaq Gold® (Applied Biosystems) and Ex Taq™ (Takara)) (lanes denoted by “0” are PCR assays conducted in the absence of blood and lanes indicated by “0+” refer to PCR assays conducted in the presence of 10 ng of human DNA);

[057] Figure 3B depicts results of DNA amplification of a 1.1 kbp endogenous
10 CCR5 human gene fragment (indicated by the arrow) in reactions of homogeneous PCR assay solutions containing whole blood at the indicated percentages (vol/vol) (as shown below the figure) using FL-12 and Z-TAQ™ (Takara) Taq DNA polymerases;

[058] Figure 4A depicts results of PCR amplification of a 1.65 kbp target DNA
15 as a function of elongation time for reactions containing Klentaq1 polymerase (lane 1), two mutant Klentaq polymerases (KT-6 (lane 2) and KT-7 (lane 3)), and another commercially available Taq polymerase (lane 4). The extension times are indicated below the panel;

[059] Figure 4B depicts results of PCR amplification of a 1.65 kbp target DNA as
20 a function of exogenous template concentration and elongation time for reactions containing a mutant Klentaq polymerase (KT-7) and a DNA polymerase that possesses the highest prior art elongation rates (Z-TAQ™). The added nucleic acid target amounts were as follows: 0.5 ng (lane 1); 0.25 ng (lane 2); 0.125 ng (lane 3); and 0.06 ng (lane 4). The extension times were as follows: 60 sec (upper
25 panel); 15 sec (central panel); and 12 sec (lower panel);

[060] Figure 4C depicts results of PCR amplification of a 1.65 kbp target DNA (denoted by arrow) using either mutant Klentaq DNA polymerases KT-7 (lane 1), KT-11 (lane 2), or KT-12 (lane 3), the wild-type Klentaq DNA polymerase (lane 4), the mutant full-length Taq DNA polymerase FL-12 (lane 5), or Z-TAQ™

(Takara; lane 6) performed with PCR cycles having extension steps reduced to 30 sec.;

[061] Figure 5A illustrates the results of heavy hot start PCR assays (100 μ l reaction volumes) conducted with KT-1 (SEQ ID NO:2), KT-6 (SEQ ID NO:4) and KT-7 (SEQ ID NO:6) in the presence of whole blood and under different conditions of pre-treatment of the reaction samples prior to initiating the thermal cycling program. The asterisks indicate those reaction vessels wherein the heavy and light volume component layers were premixed by vortexing, *i.e.*, reactions that contain homogeneous PCR assay solution and that were not subjected to a heavy hot start procedure as described herein. Lanes 1-13, 15 and 17 are PCR assays directed toward the amplification of a 1.1kbp target from the human CCR5 gene whereas lanes 14, 16, and 18 are PCR assays directed toward the amplification of a 2.5kbp target from the human CCR5 gene;

[062] Figure 5B illustrates an example of PCR assay tubes from reaction mixtures 9-14 of Fig. 5A that were not mixed prior to initiating the thermal cycling reaction (heavy hot start reactions; reaction nos. 9,10, 13, and 14) or mixed by vortexing briefly prior to initiating the thermal cycling reaction (non-heavy hot start reactions; reaction nos. 11 and 12);

[063] Figure 6 depicts results of heavy hot start PCR amplification of a 0.5 kbp target from the human CCR5 gene of cells present in whole blood. The reactions were conducted in the presence of whole blood in the lower layer at the percentages indicated below each lane (vol/vol; adjusted for total volume of both layers), in the absence of whole blood (indicated by "0"), or in absence of blood and in the presence of 10 ng of human DNA (indicated by "0+");

[064] Figure 7 depicts results of PCR assays directed toward the amplification of a 2.5kbp target from the human CCR5 gene derived from 2 ng of genomic DNA (designated "DNA") or from 3% whole blood (vol/vol) (designated "Blood") using either KT-1 (SEQ ID NO:2), KT-6 (SEQ ID NO:4), or KT-7 (SEQ ID NO:7) in the absence of Deep Vent polymerase (lanes 1-3 and 7-9) or in the

presence of Deep Vent polymerase (lanes 4-6 and 10-12), wherein the ratio of the KT enzyme to Deep Vent polymerase is about 360 to 1.

[065] DETAILED DESCRIPTION

5 [066] The present invention makes use of the discovery that Taq polymerases bearing certain N-terminal deletions are unusually resistant to whole blood, making them ideally suited for use in analytical PCR assays of nucleic acid targets from human blood. Furthermore, mutant(s) of full-length Taq DNA polymerase with even higher resistance to blood inhibitors have been developed that remain
10 fully functional in the presence of at least about 20-25% blood or the equivalent of blood fractions. This level of blood tolerance exceeds that of the existing thermostable DNA polymerases (and even exceeds the amount of blood that can be practically or conveniently handled in the PCR analysis due to physical clumping). Moreover, mutants that display a high resistance to blood inhibitors
15 have been identified that possess faster elongation rates. The use of these novel enzymes is expected to simplify and accelerate the performance of clinical and forensic tests as well as render such tests more sensitive and economical. Finally, the present invention provides methods for enhancing DNA amplification specificity using these polymerases with samples from whole blood. These Taq
20 polymerase mutants and methods for their use are described below.

[067] *Identification of Klentaq mutants that are highly resistant to blood inhibition.*

[068] Klentaq1 polymerase (SEQ ID NO: 1 (nucleic acid) and SEQ ID NO:2 (polypeptide)) is an improved and more robust version of the Taq polymerase that
25 bears an N-terminal deletion of 278 amino acids from the full-length (832 amino acids) enzyme. Klentaq1 displays higher fidelity and greater thermostability than Taq. Klentaq1 is also inhibited to a lesser extent than Taq when the polymerase is used in PCR assays carried out in the presence of blood products. For example, the purified Klentaq1 enzyme easily amplifies a nucleic acid target in the presence

of about 5% whole blood in reaction mixture (vol/vol). This was a highly unexpected result, as the full-length Taq enzyme is completely inhibited in a blood concentration range of about 0.004% to about 0.2% whole blood in the reaction mixture (vol/vol). No correlation between the N-terminal deletion of Taq, which
5 generates Klentaq1, and the blood resistance feature of the enzyme has been reported.

[069] Several mutant Klentaq clones were analyzed by PCR assays for their ability to tolerate whole blood. About 40 mutagenized, yet PCR-functional Klentaq clones were constructed and tested in PCR assay mixtures containing
10 about 10% whole human blood (vol/vol). These 40 clones are cold sensitive or are mutants of clones whose enzyme product exhibited the cold sensitive phenotype. The cold sensitivity of the additional mutant clones has not yet been determined. Remarkably, two mutants of this small collection, KT-6 (SEQ ID NO: 3 (nucleic acid); SEQ ID NO: 4 (polypeptide)) and KT-7 (SEQ ID: 5 (nucleic acid); SEQ ID
15 NO:6 (polypeptide)), clearly outperformed the rest of the clones and the wild-type Klentaq1 protein under these conditions (Fig. 1).

[070] These results were confirmed by performing PCR assays in the presence of increasing amounts of whole blood. As shown in Figure 2A, clones KT-6 and KT-7 remained functionally active in the presence of whole blood, being able to
20 amplify an endogenous gene target directly from blood cells present in reactions containing about 15% whole human blood (vol/vol) without any DNA purification step. The presence of as little as 1% whole blood (vol/vol) in PCR assays was inhibitory for Taq (Roche) (*see* Example 4). Two additional mutant forms of Klentaq-278, clones KT-10 (SEQ ID NO:19 (nucleic acid) and SEQ ID NO:20
25 (polypeptide)) and KT-12 (SEQ ID NO:23 (nucleic acid) and SEQ ID NO:24 (polypeptide)) also displayed the ability to amplify endogenous gene targets from whole blood samples (Figs. 2B and 2C).

[071] The foregoing results reveal that whole blood may be used directly in screening assays to identify mutants of Klentaq-278 that are even more resistant to
30 blood. The present invention is drawn in part to mutant forms of the Klentaq-278

DNA polymerase that display activity in PCR assays containing from about 5% whole blood to about 25% whole blood in the reaction mixture (vol/vol). More preferably, the invention is drawn to mutant forms of the Klentaq DNA polymerase that display amplification activity in PCR assays containing from about 5% whole blood to about 20% whole blood in the reaction mixture (vol/vol), including 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, and 19% whole blood in the reaction mixture (vol/vol).

[072] *Derivation of full-length Taq mutants that are highly resistant to blood inhibition.*

[073] Because the mutant forms of Klentaq-278 were more robust polymerases in whole blood PCR assays than Klentaq-278, we considered it likely that the additional amino acid changes within the structure of this truncated Taq polypeptide might confer similar blood-resistant activities when incorporated into the full-length Taq enzyme. To test this hypothesis, the region of the KT-10 gene (SEQ ID NO:19) that contains the relevant codon substitutions was re-introduced into the background of a wild-type full-length Taq (SEQ ID NO: 25 (nucleic acid) and SEQ ID NO:26 (polypeptide)) using standard recombinant DNA methods to yield the resultant mutant Taq gene, termed FL-10 (SEQ ID NO:27 (nucleic acid) and SEQ ID NO:28 (polypeptide)). The resultant polypeptide was expressed and tested in conjunction with other commercially available Taq polymerases in homogenous PCR assay solutions containing varying amounts of whole blood (0%, 10%, or 20% (vol/vol)). As is shown in Figure 3A, FL-10 displays remarkably robust, blood-resistant, DNA amplification activity in comparison with JumpStart™ Taq, AmpliTaq Gold®, or Ex Taq™.

[074] Similar to that found for FL-10, another mutant form of full-length Taq DNA polymerase was identified that displayed high blood-resistant DNA amplification activity. This mutant was derived by cloning the region of the KT-12 (SEQ ID NO:23) that contains the relevant codon substitutions that impart blood-resistant DNA amplification activity to the KT-12 polymerase (SEQ ID NO:24) into the background of the wild-type full-length Taq (SEQ ID NO:25)

using standard recombinant DNA methods to yield the resultant mutant Taq polymerase, termed FL-12 (SEQ ID NO:29 (nucleic acid) and SEQ ID NO:30 (polypeptide)). This full-length Taq polymerase mutant displayed blood-resistant DNA amplification activity that mirrors the activity observed for the KT-12 polymerase mutant (SEQ ID NO:24) (Fig. 3B). These findings provide evidence that the region of any KlenTaq-278 mutant that encodes blood-resistant DNA amplification activity will impart similar properties to the full-length Taq DNA polymerase when re-introduced into the context of the wild-type gene background.

[075] Although both FL-10 and FL-12 Taq polymerases displayed high blood-resistant DNA amplification activity, only the FL-12 Taq polymerase displays both faster-elongating activity and high blood-resistant activity. Because these two properties are discrete attributes, we tested whether faster-elongating activity correlated with high blood-resistant activity. As described herein, Z-TAQ™ (Takara) is a proprietary form of the full-length Taq DNA polymerase that displays 5-fold faster elongation rates relative to Taq DNA polymerase. The nature of the alteration of Z-TAQ™ that is responsible for its enhanced elongation activity is unknown in the art, owing to the fact that the manufacturer of the enzyme regards Z-TAQ™ as a proprietary product. For this experiment, FL-12 Taq and Z-TAQ™ were evaluated for their respective blood-resistant DNA amplification activities. As shown in Figure 3B, both FL-12 and Z-TAQ™ displayed blood-resistant DNA amplification activity in homogeneous PCR assay solutions, albeit the FL-12 enzyme was more robust than Z-TAQ™ in reactions containing significant amounts of whole blood (20% (vol/vol)).

[076] One functional characteristic that distinguishes the aforementioned FL mutants (*i.e.*, FL-10 and FL-12) from Z-TAQ™ is that the FL mutants display a cold sensitive phenotype whereas Z-TAQ™ does not. One additional functional attribute that distinguishes the aforementioned FL mutants from Z-TAQ™ is that the FL mutants are capable of carrying out DNA amplifications under hot start conditions whereas the Z-TAQ™ polymerase lacks this capability. Thus, whatever chemical or genetic attribute that endows Z-TAQ™ with its unusually

high activity in whole blood PCR assays, it is not the identical modifications that render the FL mutants blood-resistant. For the purposes of this disclosure, blood-resistant DNA polymerases are defined to have three attributes: (1) display a cold sensitive phenotype in PCR assays relative to the wild-type Taq DNA polymerase; (2) display DNA amplification activity under hot start PCR conditions; and (3) display DNA amplification activity in PCR assays containing whole blood in the range from about 3% to about 25% (vol/vol).

[077] The foregoing results reveal that whole blood may be used directly in screening assays to identify mutants of KlenTaq-278 that are even more resistant to blood and that the methods are readily extendable to identifying mutants of full-length Taq that display blood-resistant DNA amplification activity. The present invention is drawn in part to mutant forms of the full-length Taq DNA polymerase that display activity in PCR assays containing from about 5% whole blood to about 25% whole blood in the reaction mixture (vol/vol). More preferably, the invention is drawn to mutant forms of the full-length Taq DNA polymerase that display amplification activity in PCR assays containing from about 5% whole blood to about 20% whole blood in the reaction mixture (vol/vol), including 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, and 19% whole blood in the reaction mixture (vol/vol).

[078] The presently preferred procedure for identifying blood-resistant Taq DNA polymerases is to perform two screening operations on a collection of mutants: (1) identifying those mutants that display a cold sensitive phenotype in modified PCR assays; followed by (2) characterizing the subset of cold sensitive Taq DNA polymerase mutants for DNA amplification activity in whole blood PCR assays. Even more preferably, one may initially identify blood-resistant polymerases using an adaptation of a selection procedure termed compartmentalized self-replication (25, 26) for obtaining DNA polymerase mutants with a predetermined activity. As illustrated in the Prophetic Example, one would initially select the Taq DNA polymerase mutant for its blood-resistant activity, followed secondarily by a screening procedure to characterize its cold sensitive phenotype (*e.g.*, DNA

amplification activity under hot start PCR conditions). All mutants that are blood-resistant and display a cold sensitive phenotype would comprise members of the group of blood-resistant polymerases as defined herein.

[079] *Identification of Klentaq and Taq mutants with faster DNA elongation rates*

5 [080] "Rapid" thermostable DNA polymerase mutants have been discovered that display a faster DNA elongation rate than found for the wild-type Klentaq-278 polymerase. By lowering the DNA extension times during PCR, certain PCR conditions have been determined where the elongation step in the cycle becomes limiting for successful amplification by the wild-type Klentaq-278 enzyme. In the
10 case of using the Klentaq-278 gene as a target (1.65 kb long), the minimum extension time required was about 1 minute. For example, the Klentaq-278 polymerase did not possess amplification activity in PCR assays performed under conditions that employ extension times of 50 seconds. Similar results were obtained with Taq enzyme.

15 [081] About 40 functional mutant Klentaq clones were evaluated as a function of elongation rate. A 30 sec extension time was initially employed in the PCR assays, which reflect conditions that were found ineffective for the wild-type Klentaq and AmpliTaq Gold[®]. Interestingly, the mutants KT-6 (SEQ ID NO:4) and KT-7 (SEQ ID NO:6) were able to efficiently amplify the target with this
20 shorter extension time (Fig. 4). This feature of the two mutant enzymes was confirmed in further tests, wherein one of them (clone KT-7 (SEQ ID NO:6)) yielded amplification products with a 20 sec extension time (Fig. 4A). This enzyme feature was characterized further and yielded good amplification products even with 15 and 12 sec extension times (Fig. 4B). Remarkably, the selected
25 mutant completely outperformed the Z-TAQ[™] (Takara) at these low extension times (Fig. 4B, central and lower panels). Similar results were obtained with two additional mutant forms of Klentaq-278, clone KT-11 (SEQ ID NO:21 (nucleic acid) and SEQ ID NO:22 (polypeptide)) and clone KT-12 (SEQ ID NO:23 (nucleic acid) and SEQ ID NO:24 (polypeptide)) (Fig. 4C). This is noteworthy

because Z-TAQ™, a proprietary Taq enzyme, is one of the fastest DNA elongating PCR enzymes that is commercially available.

[082] Because some of the mutant forms of Klentaq-278 were faster-elongating polymerases than observed for Klentaq-278, we considered it likely that the
5 additional amino acid changes within the structure of this truncated polypeptide might confer similar faster elongating activities when incorporated into the full-length Taq enzyme. To test this hypothesis, the region of KT-12 gene (SEQ ID NO:23) that contains the relevant codon substitutions was re-introduced into the background of a wild-type full-length Taq (SEQ ID NO:25 (nucleic acid) and SEQ
10 ID NO:26 (polypeptide)) using standard recombinant DNA methods to yield the resultant mutant Taq gene, termed FL-12 (SEQ ID NO:29 (nucleic acid) and SEQ ID NO:30 (polypeptide)). The resultant polypeptide was expressed and tested in conjunction with other commercially available Taq polymerases in homogenous PCR assay solutions using PCR conditions wherein the extension time was
15 reduced to 30 sec. As is shown in Figure 4C, FL-12 displays remarkably robust, faster-elongating, DNA amplification activity in comparison to Z-TAQ™.

[083] These results demonstrate that the elongation speed of the Klentaq DNA polymerase and the full-length Taq DNA polymerase can be improved by mutagenesis. The present invention is drawn in part to mutant forms of the
20 Klentaq and full-length Taq DNA polymerases that display increased elongation rate in PCR assays under conditions where the respective enzymes fail to display successful amplification activity. Preferably, the invention is drawn to mutant forms of the Klentaq-278 and full-length Taq DNA polymerases that display amplification activity in PCR assays under conditions where the elongation step is
25 time-limiting for the reaction with the wild-type Klentaq-278 polymerase. Even more preferably, the invention is drawn to mutant forms of Klentaq-278 and full-length Taq DNA polymerases that display amplification activity under PCR conditions disclosed herein and having extension times in the range from about 12 sec to about 50 sec, including 15 sec, 18 sec, 20 sec, 22 sec, 24 sec, 25 sec, 26 sec,

28 sec, 30 sec, 32 sec, 34 sec, 36 sec, 38 sec, 40 sec, 42 sec, 44 sec, 45 sec, 46 sec, and 48 sec.

[084] *Heavy hot start PCR procedures and applications to whole blood PCR*

[085] The new protocol described here uses no wax or antibodies, and requires no
5 manipulations once the thermal cycling program has commenced. This protocol
uses two aqueous layers at the time of setup of the PCR assay. The lower layer,
which represents about 1/10 to about 1/4 of the final volume, includes the dNTPs
and magnesium(II) that is required for the reaction. The upper layer contains the
polymerase enzyme, the primers, and the nucleic acid target. Both layers contain
10 equivalent concentrations of other buffer components at the concentrations
required for amplification. The lower layer also contains a constituent to make it
heavy, such as about 10-20% (wt/vol) sucrose, sorbitol or DMSO (or a suitable
combination of similar reagents compatible with PCR up to about 10-20%
(wt/vol)).

15 [086] Optionally, other components that impart greater density to the lower layer
may substitute for or supplement the items described above. For instance,
Baskaran and co-workers have demonstrated that 1.4 M betaine, 5% DMSO is
good for PCR assays involving nucleic acid targets possessing high GC content
(36). These results suggest that inclusion of 2.8 M betaine, 10% DMSO is
20 feasible as the heavy start component of the lower layer containing the $MgCl_2$ and
the dNTPs. Optionally and routinely, color in the form of 0.05% cresol red is also
included in the lower, heavy layer.

[087] In reactions that include whole blood, the addition of components that
impart greater density to the lower layer and a color agent are not required. These
25 features are superfluous because whole blood imparts a density to the lower layer
that approximates that of the aforementioned heavy layer components and because
the hemoglobin of blood provides color. In reactions containing whole blood, the
template is included in the heavy layer, and all other components of the reaction
are in the upper layer. The range of volumes appropriate to the use of whole blood
30 in the heavy layer comprises 1% to 25%.

[088] Some adverse components of blood attack various components of the PCR reaction, such as the enzyme or the primers, yet the adverse components may be heat labile. Thus, the addition of the blood carefully as an unmixed underlay allows it to be added without significant contact with the putatively sensitive PCR reaction components. Upon heating to normal PCR thermal cycling temperatures of 90-95° C, many of the blood components appeared denatured and aggregated *in place*, were visible as brown after the cycling, and either did not mix with the PCR components before being inactivated by the heat, or never did mix appreciably with the PCR reaction components. Nevertheless, the genomic DNA template, and presumably other target templates such as viral and other microbial genomes, become timely available to the amplification reaction by convective mixing.

[089] This principal of segregating heat labile inhibitors during reaction setup may have application to other situations of complex or environmental samples that do not involve blood.

[090] The order of addition of the DNA polymerase cocktail and the whole blood sample to the PCR reaction vessel is not the critical aspect to the heavy hot start PCR procedure. Rather, the important aspect to the set-up of the heavy hot start PCR reaction is the careful addition of the DNA polymerase cocktail and the lower, heavier solution (*e.g.*, a whole blood sample) to the PCR reaction vessel so as to avoid as little mixing of the individual layers of solutions as possible before thermal cycling begins. Thus, the lower, heavier solution can be initially added to the PCR reaction vessel, followed by the careful addition of the DNA polymerase cocktail as an overlayer. More preferably, however, the DNA polymerase cocktail is initially added to the PCR reaction vessel, followed by the careful addition of the lower, heavy solution to the PCR reaction vessel as an underlayer.

[091] In the preferred embodiment, mixing of the layers occurs by diffusion and/or convection after the thermal cycler has warmed and cooled the reaction to begin the PCR process. Layered reaction tubes containing whole blood that are experimentally premixed by vortex treatment are variably unable to support PCR amplification activity, depending on the resistance of the reaction components, and

the most sensitive component was discovered to be the DNA polymerase enzyme (Fig. 5A). Figure 5B illustrates an example of PCR assay tubes that contain discrete layers prior to reaction and the mixing of the layers during reaction.

[092] It is well understood to one of ordinary skill in the art that the combinations
5 of components in the separate layers may be formulated in a variety of permutations. The only criteria that must be met in the present invention is that the polymerase is separated from at least one component essential to the amplification reaction (*e.g.*, the primers, and/or the template, and/or Mg^{2+}), that the lower layer contains a component that imparts greater density to the solution,
10 and that the mixing of the two layers results in reconstitution of the PCR assay conditions to permit amplification activity.

[093] Because the inclusion of heavy reagents, such as sucrose, sorbitol or DMSO will decrease slightly the melting temperature of the nucleic acid target, the denaturation step of the PCR cycle may have to be reduced by about 1-2 °C to
15 compensate for this effect.

[094] Mutant forms of Taq DNA polymerase include full-length Taq DNA polymerases that contain at least one amino acid change relative to the wild-type polypeptide (SEQ ID NO:26) encoded by the nucleic acid (SEQ ID NO:25) that are illustrated in the Sequence Listing. Examples of such mutant forms of Taq
20 DNA polymerase include FL-10 (SEQ ID NO:28) and FL-12 (SEQ ID NO:30). Additional mutant forms of Taq DNA polymerase used in the invention include truncation mutants, such as Klentaq-278 that comprises the amino acid sequence (SEQ ID NO:2) encoded by the nucleic acid (SEQ ID NO:1) whose sequences are illustrated in the Sequence Listing, or other codons that encode those amino acids,
25 or those amino acids with a few extra codons on the amino terminus thereof. The invention also uses a mutant or variant gene encoding full-length *Taq* or *Klentaq*-278, any of whose bases may be changed from the corresponding base shown in Tables 1-6 and 8-19 while still encoding a protein that maintains the activities and physiological functions of full-length Taq or of Klentaq-278, or a slightly longer
30 or shorter version of Klentaq-278 at the N-terminus. Further included are nucleic

acids whose sequences are complementary to those just described, including complementary nucleic acid fragments. Additionally, nucleic acids or nucleic acid fragments, or complements thereto, whose structures include chemical modifications, are also included. Such modifications include, by way of nonlimiting example, modified bases, and nucleic acids whose sugar phosphate backbones are modified or derivatized. In the mutant or variant nucleic acids, and their complements, up to 20% or more of the bases may be so changed.

[095] The invention also includes the use of polypeptides and nucleotides having 80-100% sequence identity to SEQ ID NOS:1-6 and 19-30, including 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, and 99% sequence identity to SEQ ID NOS:1-6 and 19-24, as well as nucleotides encoding any of these polypeptides, and complements of any of these nucleotides. In the case of Klentaq1 (SEQ ID NO:1), the invention includes mutant forms that contain at least one codon change in the open reading frame of Klentaq1 (SEQ ID NO:2). In the case of Taq DNA polymerase (SEQ ID NO:25), the invention includes mutant forms that contain at least one codon change in the open reading frame of Taq DNA polymerase (SEQ ID NO:26).

[096] *Percentage Sequence Identity*

[097] "Percent (%) nucleic acid sequence identity" with respect to Klentaq-278-encoding nucleic acid sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the Klentaq-278 sequence of interest, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining % nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. The same methods and principles apply to ascertain "percent (%) nucleic sequence

identity with respect to Taq DNA polymerase-encoding nucleic acid sequences in a candidate nucleic acid sequence when the two sequences are aligned.

[098] When nucleotide sequences are aligned, the percent (%) nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) can be calculated as follows:

[099] $\% \text{ nucleic acid sequence identity} = W/Z \cdot 100$

[0100] where

10 [0101] W is the number of nucleotides scored as identical matches by the sequence alignment program's or algorithm's alignment of C and D

[0102] and

[0103] Z is the total number of nucleotides in D.

15 [0104] When the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

[0105] "Percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues that are identical with amino acid residues in the disclosed Klentaq-278 DNA polymerase polypeptide sequences in a candidate sequence
20 when the two sequences are aligned. To determine % amino acid identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum % sequence identity; conservative substitutions are not considered as part of the sequence identity. Amino acid sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often
25 publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align peptide sequences. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. The same methods and principles apply
30 to ascertain "percent (%) amino acid sequence identity with respect to Taq DNA

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polymerase-encoding polypeptide sequences in candidate sequences when the two sequences are aligned.

[0106] When amino acid sequences are aligned, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as:

[0107] $\% \text{ amino acid sequence identity} = X/Y \cdot 100$

[0108] where

10 [0109] X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B

[0110] and

[0111] Y is the total number of amino acid residues in B.

[0112] If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

[0113] A nucleic acid molecule used in the invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NOS:1, 3, 5, 19, 21, 23, 25, 27, or 29 or a complement of this aforementioned nucleotide sequence, can be isolated using standard molecular biology techniques and the provided sequence information.

20 Using all or a portion of the nucleic acid sequence of SEQ ID NOS:1, 3, 5, 19, 21, 23, 25, 27, or 29 as a hybridization probe, *Klentaq-278* or *Taq* gene molecules can be isolated using standard hybridization and cloning techniques (29, 30).

[0114] PCR amplification techniques can be used to amplify *Klentaq-278*- or *Taq*-encoding DNA using *Thermus aquaticus* genomic DNA as a template and appropriate oligonucleotide primers. Furthermore, oligonucleotides corresponding to *Klentaq-278* or *Taq* gene sequences can be prepared by standard synthetic techniques, *e.g.*, an automated DNA synthesizer.

[0115] *Klentaq-278* is the subject of U.S. Patent No. 5,436,149 (31).

[0116] Klentaq-235 is the subject of U.S. Patent No. 5,616,494 (32).

[0117] *Medical Applications*

[0118] The applications of the present invention include diagnostic evaluations of
5 whole blood samples for the presence and status of genetic disorders (*e.g.*, cancer, blood disorders, diabetes, *etc.*) and diseases caused by blood borne microbial agents (*e.g.*, viruses, bacteria, fungi, *etc.*); tissue-typing using polymorphisms, and forensic research. One of ordinary skill would recognize the utilities of blood-resistant polymerases and high elongating polymerases of the present invention
10 toward advancing the application of PCR to whole blood samples directed to these objectives.

[0119] *Kits*

[0120] The present invention also contemplates kits that may be employed in the clinical setting or in the field for permitting a simplified set of reagents for rapid
15 PCR analysis of whole blood samples using the blood-resistant polymerases and high elongating polymerases of the present invention. Kits would typically include suitable oligonucleotide primers, PCR reaction buffer components, control solutions, and a suitable DNA polymerase, as well as instructions for the kit's use. Preferred DNA polymerase include the disclosed blood-resistant polymerases as
20 defined herein (*e.g.*, KT mutants that are blood-resistant and display a cold sensitive phenotype) as well as the Z-TAQ™ enzyme and the KT-1 enzyme (each of which displayed moderate blood resistance, but not cold sensitive).

[0121] **EXAMPLES**

25 [0122] *Example 1. Screening of mutagenized Klentaq clones for blood-resistant mutant enzyme activity.*

[0123] In order to functionally characterize new mutants, it is desirable to produce highly-purified enzyme from expression systems. The procedure, which included PEI treatment, BioRex-70 chromatography, and Heparin-Agarose
30 chromatography, yielded DNA-free and nuclease-free Klentaq enzyme purified to

homogeneity, as judged by a single band in Coomassie stained protein gel (23). The same purification procedure also worked very well for purification of cold sensitive Klentaq mutants (23). This procedure was readily adaptable to accommodate purification of mutant polymerases that display unusual features such as changed affinity and elution profile on a particular chromatography resin. The efficiency of each step in the purification scheme was monitored easily by a standard DNA incorporation assay.

[0124] The amplification activity of the obtained mutant enzymes were extensively evaluated in PCR amplification of various gene targets. The new enzymes were tested both in conventional and real-time PCR with SYBR green fluorescent detection. These tests included at least about 20% whole human blood (untreated, EDTA-treated, or heparinized), or blood IgG and hemoglobin fractions equivalent. Optionally, the differential sensitivities that the polymerase mutants display toward whole blood were evaluated by performing an amplification activity titration experiment with increasing incremental amounts of whole blood added to the assay mixtures from about 5% whole blood (vol/vol) to about 25% whole blood (vol/vol).

[0125] Figure 1 illustrates the results of screening of a collection of 40 KT mutants by PCR assay with homogeneous PCR assay solutions containing 10% whole blood (vol/vol), wherein the Klentaq gene represented the target nucleic acid. The primers used in the PCR assays comprise KT1 (SEQ ID NO:11) and RevTaqH (SEQ ID NO:12), which resulted in the specific amplification of a 1.65kbp target fragment.

[0126] Figure 2A illustrates the results of typical PCR assays with homogeneous PCR assay solutions containing different amounts of whole blood (vol/vol) in the reaction, wherein an endogenous human gene from blood represents the target nucleic acid. The primers used in the PCR assays comprise DMDex21f (SEQ ID NO:13) and DMDex21r (SEQ ID NO:14), which resulted in the specific amplification of a 0.32kbp target fragment of the endogenous human Duchenne muscular dystrophy gene (Dystrophin).

[0127] In order to confirm the blood resistance feature of the Klentaq mutant enzymes, numerous exogenous and endogenous test gene targets were used. Two-three ng plasmid pWB254 DNA or human DNA were used as exogenous targets to amplify the Klentaq gene itself (1.65 kb fragment, which was obtained with
5 primers KT1 (SEQ ID NO:11) and RevTaqH (SEQ ID NO:12)) or a 4.3 kb fragment of the human TPA gene (obtained with primers TPA forward (SEQ ID NO:17) and TPA reverse (SEQ ID NO:18)), respectively. The endogenous targets (from DNA present in the blood cells) included a 0.32 kb amplicon of the human Dystrophin gene (obtained with primers DMDex21f (SEQ ID NO:13) and
10 DMDex21r (SEQ ID NO:14) and 1.1 kb or 2.5 kb amplicons of the human CCR5 gene (obtained with primer pairs ccr5+1kb (SEQ ID NO:9)/CCR5-KOZ (SEQ ID NO:7) and CCR5-2kb (SEQ ID NO:8)/ccr5deltaRT (SEQ ID NO:10), respectively). Whole or EDTA-treated (4.8 mM EDTA) human blood was added at concentrations 0%-20% to the PCR cocktail prior to PCR (homogeneous PCR
15 setup). As illustrated in Figs. 2B and 2C, KT-10 and KT-12 mutants easily amplified the targets in at least 20% whole blood. The wild-type Taq enzyme failed under comparable conditions. The amplification signal obtained with the mutants when detecting endogenous blood genes was gene-dose-responsive.

[0128] *Example 2. Full-length Taq DNA polymerase mutants display blood-resistant activity.*
20

[0129] Importantly, the amino acid changes responsible for the blood-resistant phenotype of the Klentaq, were also sufficient to render the full-length Taq blood-resistant when these amino acid changes were incorporated into the full-length gene. For example, the amino acid changes of KT-10 and KT-12 mutants were
25 incorporated into the full-length Taq gene to generate the analogous Taq-mutants FL-10 and FL-12. As shown in Figure 3A (for FL-10) and Figure 3B (for FL-12), both full-length Taq mutants exhibited very high resistance to blood inhibition, and successfully amplified the endogenous human Dystrophin and CCR5 genes in homogeneous PCR solutions containing 20% blood. The observed high blood
30 resistance of these mutants reflects dramatic change in the property of the Taq

enzyme, considering the fact that the wild-type Taq is typically inactivated in homogeneous PCR assay solutions containing as little as 0.1-0.5 % whole blood. Various commercial Taq enzymes, including AmpliTaq Gold®, JumpStart™ Taq, and Ex Taq™ failed to detect endogenous blood genes even at the lowest blood concentrations tested. One surprising exception was the enzyme Z-TAQ™, which showed a significant blood resistance at 5% and 10% blood; however, the FL-12 polymerase mutant outperformed Z-TAQ™ when used in homogeneous PCR assay solutions containing 20% blood to amplify a 1.1kbp fragment of the endogenous CCR5 gene with primers ccr5+1kb (SEQ ID NO:9) and CCR5-KOZ (SEQ ID NO:7)) (Fig. 3B). The molecular change in the Z-TAQ™ enzyme responsible for its blood-resistant property is unknown, as the manufacturer (Takara) maintains its composition as a proprietary secret.

[0130] *Example 3. Mutagenized Klentaq mutants with a faster DNA elongation rate.*

[0131] The screening factor here is to simply shorten the DNA extension step of the PCR cycle beyond the point where the wild-type or prior art enzyme stops working. In the case when wild-type Klentaq amplified its own gene, the amplification efficiency was significantly lower at 60 seconds extension step (Fig. 4A, lane 1 at 1 min). Additional tests with discrete extension times showed that the Klentaq polymerase did not display amplification activity in PCR assays performed under conditions that employ an extension time of about 50 sec or less (*e.g.*, see Fig. 4A, lane 1 at 30 sec and 20 sec). On the other hand, mutant Klentaq clone KT-7 displayed amplification activity with the same target in PCR assays under conditions having an extension step of as little as about 12 sec. (Fig. 4B, lower panel). For the evaluation of fast-elongating mutants, extension times in the PCR cycle not exceeding 20 sec per 2 kb amplicon were used. The KT mutants, KT-7 (SEQ ID NO:6), KT-11 (SEQ ID NO:21), and KT-12 (SEQ ID NO:24) were markedly faster elongating polymerases than KT-1 (SEQ ID NO:2), whereas the full-length Taq mutant, FL-12 (SEQ ID NO:30), displayed increased elongation activity relative to Z-TAQ™ (Fig. 4C). For these experiments, the PCR assays

were conducted using homogeneous PCR assay solutions with KT1 (SEQ ID NO:11) and RevTaqH (SEQ ID NO:12), which resulted in the specific amplification of a 1.65kbp target fragment from the Klentaq1 gene.

[0132] *Example 4. Hot start achieved by underlay of heavy liquid component can enhance yield of specific amplification products – “heavy hot start” amplification*

[0133] This amplification procedure permits one to obtain an enhanced specificity and reliability from a PCR assay. The strategy is also amenable to PCR assays involving whole blood, as described below. In two preferred embodiments, two heavy hot start mixes are disclosed that differ mainly in the amounts of Mg^{2+} and dNTPs present in the reaction mixture, since the optimum Mg^{2+} and dNTP concentrations for Klentaq1 and KlentaqLA is higher than for Taq and TaqLA. These heavy hot start mixes can be stored for at least a month at 4° C.

[0134] 10× TCA is 500 mM Tris-HCl pH 9.2, 160 mM ammonium sulfate. When the pH of the Tris-HCl stock was adjusted to pH 9.2, the pH of the aliquots was measured at a buffer concentration of 50 mM in water at room temperature. The concentration of the 1 M $MgCl_2$ stock was confirmed by determining the refractive index of the solution using a refractometer and by reference to Refractive Index-Concentration Data in a technical manual, such as THE HANDBOOK OF CHEMISTRY AND PHYSICS by Chemical Rubber Company.

[0135] The heavy mix recipe for the KlentaqLA yielded a final Mg(II) cation concentration that was 2.5 mM greater than the total concentration of the dNTP. This heavy mix recipe consists of the following components: 100 µl of 10× TCA; 100 µl of a dNTP mix consisting of 10 mM dATP, 10 mM dGTP, 10 mM dCTP, and 10 mM dTTP; 140 µl of 100 mM $MgCl_2$, 67 µl of 0.75 mM Cresol Red, 4.25 mM Tris Base, 400 µl of 50% Sucrose or Sorbitol; and 193 µl of water to 1 ml.

[0136] The heavy mix recipe for Taq or TaqLA yielded final Mg(II) cation concentration that was 0.75 mM greater than the total concentration of the dNTPs. This heavy mix recipe consists of the following components: 100 µl of 10× TCA;

94 μ l of 100 mM $MgCl_2$, 16 μ l of 100 mM dATP; 16 μ l of 100 mM dGTP; 16 μ l of 100 mM dCTP; 16 μ l of 100 mM dTTP; 67 μ l of 0.75 mM Cresol Red, 4.25 mM Tris Base 400 μ l of 50% Sucrose or Sorbitol; and 275 μ l of water to 1 ml.

[0137] Typical reaction mixtures were assembled with the following components:

- 5 3.75 μ l 10 $\times$ TCA; 1.0 ng target DNA; 1.0 μ l (each) 10 μ M primers; 0.25 to 0.50 μ l enzyme; 30.25 μ l water to a final volume of 37.5 μ l. This initial mixture represented the top layer. The top layer was added to the PCR assay tube, followed by the addition of oil (if desired or necessary). The PCR tube was subjected to a brief centrifugation step to resolve the aqueous and oil layers.
- 10 Finally, 13.0 μ l of heavy mix was added as an underlayer of the PCR tube contents without mixing. The tubes were closed and carefully carried to and installed into the thermal cycler without undue agitation. The thermal cycler was set to start with a 5 min heating step from 60 $^{\circ}$ C to 68 $^{\circ}$ C before the first heat denaturation step. A visual inspection of the tubes thereafter confirmed that the two layers had
- 15 already mixed during this time.

- [0138] For heavy hot start PCR assays that included whole blood in the heavy layer, the following experiment was performed. One hundred microliter reactions were assembled with the whole blood being added last. The top layer consisted of 80 μ l mixtures, wherein each mixture contained 0.25 μ l of polymerase selected
- 20 from the group consisting of Klentaq1 (Klentaq-278), Klentaq5 (Klentaq-235), Klentaq6, Klentaq7, additional mutants, and Taq. Before the blood was added, water was added to complement the blood volume, so that at the final volume would be 100 μ l, even though the volume of the heavy, whole blood underlay ranged from 0.5 μ l to 20 μ l. The blood was carefully added at the bottom of the
- 25 tubes, underneath the 80 μ l top layer. For example, in PCR assays that contained 0.5 μ l of blood, 19.5 μ l of water was added to the upper layer before the blood was added as an underlay at the bottom of the tube. The layers were not manually mixed before the PCR assay was performed. The primers were present at 20 pmoles each per 100 μ l reaction. The buffer was KLA pH 9, the concentration of

dNTPs was 100 μ M each, and 1.3 M betaine was present (all concentrations as final in the 100 μ l). Ten nanograms of human DNA (from Novagen) was included in the two of the no-blood reactions (the ones catalyzed by Klentaq-235 and Taq) (indicated by lanes denoted by "0+") to provide a positive control for the

5 polymerase activity. The thermal cycling program was 3 min preheat at 60° C, 35 cycles of (71 sec at 93° C, 60 sec at 60° C, and 5 min at 68° C).

[0139] Figure 5A illustrates the results of heavy hot start PCR assays (100 μ l reaction volumes) conducted with KT-1 (SEQ ID NO:2), KT-6 (SEQ ID NO:4) and KT-7 (SEQ ID NO:6) in the presence of whole blood and under different

10 conditions of pre-treatment of the reaction samples prior to initiating the thermal cycling program. The asterisks indicate those reaction vessels wherein the heavy and light volume component layers were premixed by vortexing, *i.e.*, reactions that contain homogeneous PCR assay solution and that were not subjected to a heavy hot start procedure as described herein. Lanes 1-13, 15 and 17 are PCR

15 assays directed toward the amplification of a 1.1kbp target from the human CCR5 gene using ccr5+1kb (SEQ ID NO:9) and CCR5-KOZ (SEQ ID NO:7). Lanes 14, 16, and 18 are PCR assays directed toward the amplification of a 2.5kbp target from the human CCR5 gene using CCR5-2kb (SEQ ID NO:8) and ccr5deltaRT (SEQ ID NO:10).

20 [0140] Figure 6 depicts the additional results of this type of experiment. The amplification activity was revealed by the specific amplification of a 0.5 kbp DNA product from the CCR5 gene endogenous to the human cells in the blood (except for the lanes indicated by "0+", which indicates the presence 10 ng of exogenous human DNA template without whole blood). KT-1 (SEQ ID NO:2), KT-6 (SEQ

25 ID NO:4), and KT-7 (SEQ ID NO:6) displayed DNA amplification activity in reaction containing from about 1% whole blood (vol/vol) to about 20% whole blood (vol/vol) whereas Klentaq5 and Taq did not display amplification activity in reactions containing as little as about 1% whole blood (vol/vol). The primers used to generate this amplification product were CCR5-D5 (SEQ ID NO:15) and

30 CCR5-D3 (SEQ ID NO:16).

[0141] *Example 5. Whole blood PCR assays that employ KT mutant polymerases with a second thermostable DNA polymerase having a 3'-exonuclease activity.*

[0142] This example shows that long and accurate PCR works with whole blood
5 as the source of the target template. Since long and accurate PCR (US Patent 5,436,149, claims 6-16) comprises the use of a mixture of DNA polymerases, this example also illustrates that the minor component of the mixture, an archaeobacterial DNA polymerase which is thermostable and which exhibits 3'-exonuclease activity, is surprisingly active with whole blood.

10 [0143] The master PCR cocktail was assembled as follows:

[0144] 200 μ l 10 $\times$ KLA pH 9

[0145] 20 μ l 10/40 (mix of 10 mM each dNTP and 40 mM MgCl₂)

[0146] 520 μ l 5 M Betaine

[0147] 40 μ l primer CCR5-2kb (SEQ ID NO:8)

15 [0148] 40 μ l primer ccr5deltaRT (SEQ ID NO:10)

[0149] 1120 μ l water to make 20 $\times$ 97 μ l reaction mixture aliquots

[0150] 1940 μ l total cocktail volume

[0151] It is worth noting that the PCR cocktail lacked target nucleic acid template and the DNA polymerase at this stage.

20 [0152] Enzyme dilutions were prepared on ice by mixing them with a portion of the master mix as follows: six aliquots (75 μ l each) of master mix were withdrawn and added to an aliquot (0.75 μ l) of enzymes KT-1(SEQ ID NO:2), KT-6 (SEQ ID NO:4), or KT-7 (SEQ ID NO:6) each at about 30 U/ μ l, and the same three enzymes that have been previously mixed with 1:24 dilution volume of
25 the archaeobacterial enzyme Deep Vent, which is available commercially at 2 U/ μ l. These latter enzyme mixtures possessed a ratio of KT enzyme to Deep Vent enzyme of about 1:360.

[0153] Aliquots of the master mix (72 μ l) were dispensed to reaction tubes, then aliquots of the appropriate enzyme dilution mix (25 μ l) were dispensed into the reaction tubes to provide for a total volume of 97 μ l.

5 [0154] Pure human DNA (Novagen), stored at a temperature of 4° C and at a concentration of 3 ng/ μ l, was diluted 3-fold with standard TEN buffer (10 mM Tris pH 7.9, 10 mM NaCl, 0.1 mM EDTA) to make 1 ng/ μ l, and then an aliquot of this solution (3 μ l) was pipetted into the aforementioned 97 μ l mixture to yield the final PCR assay mastermix.

10 [0155] Whole blood, which is typically stored in an aliquot of 0.5 ml with 4.5 mM EDTA at -80° C, was thawed at room temperature for about 15 to 30 minutes and mixed by gentle inversion before 3 μ l was pipetted underneath the aforementioned 97 μ l mixture in additional PCR reaction tubes, avoiding mixing. The pipettor was set to 3.2 μ l, and care was exercised not eject the last small amount of blood volume (~0.2 μ l), so as to avoid injecting a bubble of air into the PCR assay
15 solution and thereby disturb the heavy phase at the bottom of the tube.

[0156] Thermal cycling for the PCR amplification was carried out using a similar program as described above (2 minutes at 93° C, followed by 33 cycles of (71 seconds at 93° C, 1 minute at 60° C, 10 minutes at 68° C). After the PCR assays were completed, aliquots of the reactions (18 μ l) were mixed with 4.4 μ l of blue
20 dye mix, and analyzed by electrophoresis on a 1.4% agarose gel.

[0157] Figure 7 illustrates that little or no PCR product of the expected size (2.5 kbp) is obtained unless an amount of Deep Vent polymerase is present to complement the major DNA polymerase Klentaq1 (SEQ ID NO:2), Klentaq6 (SEQ ID NO:4), or Klentaq7 (SEQ ID NO:6), all of which perform equally well
25 under these conditions.

[0158] *Prophetic Example. Selection of blood-resistant Klentaq DNA polymerase mutants using compartmentalized self-replication.*

[0159] The recently described highly effective compartmentalized self-replication (CSR) strategy for directed evolution of enzymes (25, 26) could be adapted to

select for blood-resistant Klentaq mutants. The existence of blood-resistant mutant(s) of Klentaq should be evident in the library as a manifestation of detectable self-replication of the Klentaq gene in the presence of 10% blood, a concentration that is inhibitory for the wild-type Klentaq. Blood-resistant Klentaq clones could be isolated and Klentaq mutant proteins prepared according to the procedures set forth in Example 1. Individual Klentaq polymerase mutants could then subjected to screening procedures to ascertain whether each displays a cold sensitive phenotype. Those Klentaq mutants that are blood-resistant and display a cold sensitive phenotype would be expected to conform to the group of blood-resistant DNA polymerases as defined herein. The aforementioned selection/screening procedure should also be amenable to identifying full-length Taq DNA polymerase mutants that are blood-resistant DNA polymerases as defined herein.

[0160] The nucleic acids and polypeptides of the various DNA polymerases and the oligonucleotide primers described in this application include the sequences shown in the Sequence Listing. Table II provides the nucleic acid sequences for the specific oligonucleotide primers used in the various examples disclosed in this application.

SEQ ID NO.:	Name	Primer Sequence (5'→3')
7	CCR5-KOZ	TGGAACAAGATGGATTATCAAGTGTC AAGTCCA 1 10 20 30
8	CCR5-2kb	AGAAGAGCTGAGACATCCGTTCCCCTACAAGAA 1 10 20 30
9	ccr5+1kb	AGGCTGTGTATGAAAATAAGCCATGTGCACAA 1 10 20 30
10	ccr5deltaRT	GCAGCGGCAGGACCAGCCCCAAGATGACTATCT 1 10 20 30
11	KT1	GAGCCATGGTCCTCCTCCACGAGTTCGGCCTTCTGG 1 10 20 30
12	RevTaqH	CGGTCCGAAAGCTTCTATCACTCCTTGGCGG 1 10 20 30
13	DMDex21f	GGCTGTGATAGAGGCTTGTCTATA 1 10 20
14	DMDex21r	CTGGCCTGCACATCAGAAAAGACT 1 10 20
15	CCR5-D5	AGGTACCTGGCTGTCGTCCATGCTGTGTTT 1 10 20 30
16	CCR5-D3	GATGATGGGGTTGATGCAGCAGTGCATGTCAT 1 10 20 30
17	TPA forward	GGAAGTACAGCTCAGAGTTCTGCAGCACCCCTGC 1 10 20 30
18	TPA reverse	GATGCGAAACTGAGGCTGGCTGTACTGTCTC 1 10 20 30

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SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 77586-69 Seq 30-05-12 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

<110> KERMEKCHIEV, MILKO B.
BARNES, WAYNE B.

<120> USE OF WHOLE BLOOD IN PCR REACTIONS

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<141> 2004-12-06

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aggcttgagg gggaggagag gctcctttgg ctttaccggg aggtggagag gccctttcc 480
gctgtcctgg cccacatgga ggccacgggg gtgcgcctgg acgtggccta tctcagggcc 540
ttgtccctgg aggtggccga ggagatcgcc cgcctcgagg ccgaggtctt ccgcctggcc 600
ggccaccctc tcaacctcaa ctcccgggac cagctggaaa gggtcctctt tgacgagcta 660
gggcttcccg ccateggcaa gacggagaag accggcaagc gctccaccag cgccgccgtc 720
ctggaggccc tccgcgaggc ccaccccatc gtggagaaga tcctgcagta ccgggagctc 780
accaagctga agagcaccta cattgacccc ttgccggacc tcattccacc caggacgggc 840
cgctccaca cccgcttcaa ccagacggcc acggccacgg gcaggctaag tagctccgat 900

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44b

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cccaacctcc agaacatccc cgtccgcacc ccgcttgggc agaggatccg ccgggccttc 960
atcgccgagg aggggtggct attggtggcc ctggactata gccagataga gctcagggtg 1020
ctggcccacc tctccggcga cgagaacctg atccgggtct tccaggaggg gcgggacatc 1080
cacacggaga ccgccagctg gatgttcggc gtcccccggg aggccgtgga cccctgatg 1140
cgccgggagg ccaagaccat caacttcggg gtctcttacg gcatgtcggc ccaccgcctc 1200
tcccaggagc tagccatccc ttacgaggag gcccaggcct tcattgagcg ctactttcag 1260
agcttcccca aggtgcgggc ctggattgag aagaccctgg aggagggcag gaggcggggg 1320
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agcgtgcggg aggcggccga gcgcatggcc ttcaacatgc ccgtccaggg caccgccgcc 1440
gacctcatga agctggctat ggtgaagctc ttccccaggc tggaggaaat gggggccagg 1500
atgtctcttc aggtccacga cgagctggtc ctcgaggccc caaaagagag ggcggaggcc 1560
gtggcccggc tggccaagga ggtcatggag ggggtgtatc ccctggccgt gccctggag 1620
gtggagggtg ggatagggga ggactggctc tccgccaagg agtagtaagc ttatcgatga 1680
ta 1682

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<210> 2
 <211> 554
 <212> PRT
 <213> *Thermus aquaticus*

<400> 2
 Met Gly Leu Leu His Glu Phe Gly Leu Leu Glu Ser Pro Lys Ala Leu
 1 5 10 15
 Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly Ala Phe Val Gly Phe Val
 20 25 30
 Leu Ser Arg Lys Glu Pro Met Trp Ala Asp Leu Leu Ala Leu Ala Ala
 35 40 45
 Ala Arg Gly Gly Arg Val His Arg Ala Pro Glu Pro Tyr Lys Ala Leu
 50 55 60
 Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu Ala Lys Asp Leu Ser Val
 65 70 75 80
 Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro Pro Gly Asp Asp Pro Met
 85 90 95
 Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val
 100 105 110
 Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu Glu Ala Gly Glu Arg Ala
 115 120 125
 Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu Trp Gly Arg Leu Glu Gly
 130 135 140
 Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu Arg Pro Leu Ser
 145 150 155 160
 Ala Val Leu Ala His Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala
 165 170 175
 Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala Glu Glu Ile Ala Arg Leu
 180 185 190
 Glu Ala Glu Val Phe Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser
 195 200 205
 Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu Gly Leu Pro Ala
 210 215 220
 Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val
 225 230 235 240
 Leu Glu Ala Leu Arg Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln
 245 250 255
 Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr Tyr Ile Asp Pro Leu Pro
 260 265 270

44c

Asp	Leu	Ile	His	Pro	Arg	Thr	Gly	Arg	Leu	His	Thr	Arg	Phe	Asn	Gln
	275						280					285			
Thr	Ala	Thr	Ala	Thr	Gly	Arg	Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln
	290					295					300				
Asn	Ile	Pro	Val	Arg	Thr	Pro	Leu	Gly	Gln	Arg	Ile	Arg	Arg	Ala	Phe
305					310					315					320
Ile	Ala	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala	Leu	Asp	Tyr	Ser	Gln	Ile
				325					330					335	
Glu	Leu	Arg	Val	Leu	Ala	His	Leu	Ser	Gly	Asp	Glu	Asn	Leu	Ile	Arg
			340					345					350		
Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr	Glu	Thr	Ala	Ser	Trp	Met
	355						360					365			
Phe	Gly	Val	Pro	Arg	Glu	Ala	Val	Asp	Pro	Leu	Met	Arg	Arg	Ala	Ala
	370					375					380				
Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly	Met	Ser	Ala	His	Arg	Leu
385					390					395					400
Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu	Ala	Gln	Ala	Phe	Ile	Glu
				405					410					415	
Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg	Ala	Trp	Ile	Glu	Lys	Thr
			420					425					430		
Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val	Glu	Thr	Leu	Phe	Gly	Arg
	435						440				445				
Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	Arg	Val	Lys	Ser	Val	Arg	Glu
	450					455					460				
Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	Met	Pro	Val	Gln	Gly	Thr	Ala	Ala
465					470					475					480
Asp	Leu	Met	Lys	Leu	Ala	Met	Val	Lys	Leu	Phe	Pro	Arg	Leu	Glu	Glu
			485					490						495	
Met	Gly	Ala	Arg	Met	Leu	Leu	Gln	Val	His	Asp	Glu	Leu	Val	Leu	Glu
			500					505					510		
Ala	Pro	Lys	Glu	Arg	Ala	Glu	Ala	Val	Ala	Arg	Leu	Ala	Lys	Glu	Val
	515						520					525			
Met	Glu	Gly	Val	Tyr	Pro	Leu	Ala	Val	Pro	Leu	Glu	Val	Glu	Val	Gly
	530					535					540				
Ile	Gly	Glu	Asp	Trp	Leu	Ser	Ala	Lys	Glu						
545						550									

<210> 3
 <211> 1682
 <212> DNA
 <213> Thermus aquaticus

<400> 3
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 gccgatcttc tggccctggc cgccgccagg gggggccggg tccaccgggc ccccgagcct 180
 tataaagccc tcagggacct gaaggaggcg cgggggcttc tcgccaaaga cctgagcggt 240
 ctggccctga gggaaggcct tggcctcccg cccggcgacg accccatgct cctcgcctac 300
 ctctggacc cttccaacac ccccccgag ggggtggccc ggcgctacg cggggagtgg 360
 acggaggagg cgggggagcg ggccgccctt tccgagaggc tcttcgccaa cctgtggggg 420
 aggccttgagg gggaggagag gctcctttgg ctttaccggg aggtggagag gcccttttcc 480
 gctgtcctgg ccacatgga ggccacgggg gtgcgcctgg acgtggccta tctcagggcc 540
 ttgtccctgg aggtggccga ggagatcgcc cgcctcgagg ccgaggtctt ccgcctggcc 600
 ggccaccctt tcaacctcaa ctcccgggac cagctggaag gggctctctt tgacgagcta 660
 gggcttcccg ccatcggcaa gacggagaag accggcaagc gctccaccag cgccgccgtc 720

44d

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ctggaggccc tccgcgaggc ccaccccatc gtggagaaga tcctgcagta ccgggagctc 780
accaagctga agagcaccta cattgacccc ttgccggacc tcatccaccc caggacgggc 840
cgctccaca cccgcttcaa ccagacggcc acggccacgg gcaggctaag tagctccgat 900
cccaacctcc agaacatccc cgtccgcacc ccgcttgggc agaggatccg ccgggccttc 960
atcgccgagg aggggtggct attggtggcc ctggactata gccagataga gctcaggggtg 1020
ctggcccacc tctccggcga cgagaacctg atccgggtct tccaggaggg gcgggacatc 1080
cacacggaga ccgccagctg gatgttcggc gtcccccggg aggccgtgga cccctgatg 1140
cgccgggagg ccaagaccat caacttcggg gtccctctac gcattgtcggc ccaccgcctc 1200
tcccaggagc tagccatccc ttacgaggag gcccaggcct tcattgagcg ctactttcag 1260
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agcgtgcggg aggcggccga gcgcatggcc ttcaacatgc ccgtccaggg caccgccgcc 1440
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atgtccttc aggtccacga cgagctggtc ctcgaggccc caaaagagag ggcggaggcc 1560
gtggcccggc tggccaagga ggtcatggag ggggtgtatc ccctggccgt gccctggag 1620
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ta

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<210> 4
 <211> 554
 <212> PRT
 <213> Thermus aquaticus

<400> 4
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 Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly Ala Phe Val Gly Phe Val
 20 25 30
 Leu Ser Arg Lys Glu Pro Met Trp Ala Asp Leu Leu Ala Leu Ala Ala
 35 40 45
 Ala Arg Gly Gly Arg Val His Arg Ala Pro Glu Pro Tyr Lys Ala Leu
 50 55 60
 Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu Ala Lys Asp Leu Ser Val
 65 70 75 80
 Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro Pro Gly Asp Asp Pro Met
 85 90 95
 Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val
 100 105 110
 Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu Glu Ala Gly Glu Arg Ala
 115 120 125
 Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu Trp Gly Arg Leu Glu Gly
 130 135 140
 Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu Arg Pro Leu Ser
 145 150 155 160
 Ala Val Leu Ala His Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala
 165 170 175
 Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala Glu Glu Ile Ala Arg Leu
 180 185 190
 Glu Ala Glu Val Phe Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser
 195 200 205
 Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu Gly Leu Pro Ala
 210 215 220
 Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val
 225 230 235 240

44e

Leu Glu Ala Leu Arg Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln
 245 250 255
 Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr Tyr Ile Asp Pro Leu Pro
 260 265 270
 Asp Leu Ile His Pro Arg Thr Gly Arg Leu His Thr Arg Phe Asn Gln
 275 280 285
 Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp Pro Asn Leu Gln
 290 295 300
 Asn Ile Pro Val Arg Thr Pro Leu Gly Gln Arg Ile Arg Arg Ala Phe
 305 310 315 320
 Ile Ala Glu Glu Gly Trp Leu Leu Val Ala Leu Asp Tyr Ser Gln Ile
 325 330 335
 Glu Leu Arg Val Leu Ala His Leu Ser Gly Asp Glu Asn Leu Ile Arg
 340 345 350
 Val Phe Gln Glu Gly Arg Asp Ile His Thr Glu Thr Ala Ser Trp Met
 355 360 365
 Phe Gly Val Pro Arg Glu Ala Val Asp Pro Leu Met Arg Arg Ala Ala
 370 375 380
 Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly Met Ser Ala His Arg Leu
 385 390 395 400
 Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu Ala Gln Ala Phe Ile Glu
 405 410 415
 Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg Ala Trp Leu Val Lys Thr
 420 425 430
 Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val Glu Thr Leu Phe Gly Arg
 435 440 445
 Arg Arg Tyr Val Pro Asp Leu Glu Ala Arg Val Lys Ser Val Arg Glu
 450 455 460
 Ala Ala Glu Arg Met Ala Phe Asn Met Pro Val Gln Gly Thr Ala Ala
 465 470 475 480
 Asp Leu Met Lys Leu Ala Met Val Lys Leu Phe Pro Arg Leu Glu Glu
 485 490 495
 Met Gly Ala Arg Met Leu Leu Gln Val His Asp Glu Leu Val Leu Glu
 500 505 510
 Ala Pro Lys Glu Arg Ala Glu Ala Val Ala Arg Leu Ala Lys Glu Val
 515 520 525
 Met Glu Gly Val Tyr Pro Leu Ala Val Pro Leu Glu Val Glu Val Gly
 530 535 540
 Ile Gly Glu Asp Trp Leu Ser Ala Lys Glu
 545 550

<210> 5

<211> 1682

<212> DNA

<213> *Thermus aquaticus*

<400> 5

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 gccgatcttc tggccctggc cgccgccagg gggggccggg tccaccgggc ccccgagcct 180
 tataaagccc tcagggacct gaaggaggcg cgggggcttc tcgccaaaga cctgagcggt 240
 ctggccctga gggaaggcct tggcctcccg cccggcgacg accccatgct cctcgcctac 300
 ctcttgacc cttccaacac ccccccgag ggggtggccc ggcgctacgg cggggagtgg 360
 acggaggagg cgggggagcg ggccgccctt tccgagaggc tcttcgcca cctgtggggg 420
 aggcttgagg gggaggagag gctcctttgg ctttaccggg aggtggagag gccctttcc 480

44f

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gctgtcctgg cccacatgga ggccacgggg gtgcgcctgg acgtggccta tctcagggcc 540
ttgtccctgg aggtggccga ggagatcgcc cgcctcgagg ccgaggtctt ccgcctggcc 600
ggccacccct tcaacctcaa ctcccgggac cagctggaaa gggtcctctt tgacgagcta 660
gggttcccg ccatcggaac gacggagaag accggcaagc gctccaccag cgcgcgcgtc 720
ctggaggccc tccgcgaggc ccaccccata gtggagaaga tcctgcagta ccgggagctc 780
accaagctga agagcaccta cattgacccc ttgccggacc tcataccacc caggacgggc 840
cgctccaca cccgcttcaa ccagacggcc acggccacgg gcaggctaag tagctccgat 900
cccaacctcc agaacatccc cgtccgcacc ccgcttgggc agaggatccg ccgggccttc 960
atcgccgagg aggggtggct attggtggcc ctggactata gccagataga gctcagggtg 1020
ctggcccacc tctccggcga caagaacctg atccgggtct tccaggaggg gcgggacata 1080
cacacggaga ccgccagctg gatgttcggc gtcccccggg aggccgtgga cccctgatg 1140
cgccggggcg ccaagaccat caacttcggg gtcctctacg gcatgtcggc ccaccgcctc 1200
tcccaggagc tagccatccc ttacgaggag gccaggcct tcattgagcg ctactttcag 1260
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tacgtggaga ccctcttcgg ccgcgcgcgc tacgtgccag acctagaggc ccgggtgaag 1380
agcgtgcggg aggcggccga gcgcattggc ttcaacatgc ccgtccaggg caccgccgcc 1440
gacctcatga agctggctat ggtgaagctc ttccccaggc tggaggaaat gggggccagg 1500
atgctccttc aggtccacga cgagctggtc ctcgaggccc caaaagagag ggcggaggcc 1560
gtggcccggc tggccaagga ggtcatggag ggggtgtatc ccctggccgt gccctggag 1620
gtggagggtg ggatagggga ggactggctc tccgccaagg agtagtaagc ttatcgatga 1680
ta

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<210> 6

<211> 554

<212> PRT

<213> *Thermus aquaticus*

<400> 6

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Met Gly Leu Leu His Glu Phe Gly Leu Leu Glu Ser Pro Lys Ala Leu
  1              5              10              15
Glu Glu Ala Pro Trp Pro Pro Pro Glu Gly Ala Phe Val Gly Phe Val
              20              25              30
Leu Ser Arg Lys Glu Pro Met Trp Ala Asp Leu Leu Ala Leu Ala Ala
              35              40              45
Ala Arg Gly Gly Arg Val His Arg Ala Pro Glu Pro Tyr Lys Ala Leu
              50              55              60
Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu Ala Lys Asp Leu Ser Val
              65              70              75              80
Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro Pro Gly Asp Asp Pro Met
              85              90              95
Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val
              100              105              110
Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu Glu Ala Gly Glu Arg Ala
              115              120              125
Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu Trp Gly Arg Leu Glu Gly
              130              135              140
Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu Arg Pro Leu Ser
              145              150              155              160
Ala Val Leu Ala His Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala
              165              170              175
Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala Glu Glu Ile Ala Arg Leu
              180              185              190
Glu Ala Glu Val Phe Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser
              195              200              205

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44g

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Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu Gly Leu Pro Ala
 210                215                220
Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val
225                230                235                240
Leu Glu Ala Leu Arg Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln
                245                250                255
Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr Tyr Ile Asp Pro Leu Pro
                260                265                270
Asp Leu Ile His Pro Arg Thr Gly Arg Leu His Thr Arg Phe Asn Gln
                275                280                285
Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp Pro Asn Leu Gln
                290                295                300
Asn Ile Pro Val Arg Thr Pro Leu Gly Gln Arg Ile Arg Arg Ala Phe
305                310                315                320
Ile Ala Glu Glu Gly Trp Leu Leu Val Ala Leu Asp Tyr Ser Gln Ile
                325                330                335
Glu Leu Arg Val Leu Ala His Leu Ser Gly Asp Lys Asn Leu Ile Arg
                340                345                350
Val Phe Gln Glu Gly Arg Asp Ile His Thr Glu Thr Ala Ser Trp Met
                355                360                365
Phe Gly Val Pro Arg Glu Ala Val Asp Pro Leu Met Arg Arg Ala Ala
                370                375                380
Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly Met Ser Ala His Arg Leu
385                390                395                400
Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu Ala Gln Ala Phe Ile Glu
                405                410                415
Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg Ala Trp Leu Trp Lys Thr
                420                425                430
Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val Glu Thr Leu Phe Gly Arg
                435                440                445
Arg Arg Tyr Val Pro Asp Leu Glu Ala Arg Val Lys Ser Val Arg Glu
                450                455                460
Ala Ala Glu Arg Met Ala Phe Asn Met Pro Val Gln Gly Thr Ala Ala
465                470                475                480
Asp Leu Met Lys Leu Ala Met Val Lys Leu Phe Pro Arg Leu Glu Glu
                485                490                495
Met Gly Ala Arg Met Leu Leu Gln Val His Asp Glu Leu Val Leu Glu
                500                505                510
Ala Pro Lys Glu Arg Ala Glu Ala Val Ala Arg Leu Ala Lys Glu Val
                515                520                525
Met Glu Gly Val Tyr Pro Leu Ala Val Pro Leu Glu Val Glu Val Gly
                530                535                540
Ile Gly Glu Asp Trp Leu Ser Ala Lys Glu
545                550

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<210> 7
 <211> 33
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> OTHER INFORMATION: Synthetic
 primer

44h

<400> 7
tggaacaaga tggattatca agtgtcaagt cca 33

<210> 8
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic
primer

<400> 8
agaagagctg agacatccgt tcccctacaa gaa 33

<210> 9
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 9
aggctgtgta tgaaaactaa gccatgtgca caa 33

<210> 10
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 10
gcagcggcag gaccagcccc aagatgacta tct 33

<210> 11
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 11
gagccatggt cctcctccac gagttcggcc ttctgg 36

<210> 12
<211> 31

<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 12
cggtccgaaa gcttctatca ctccttggcg g 31

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 13
ggctgtgata gaggttggtc tata 24

<210> 14
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 14
ctggcctgca catcagaaaa gact 24

<210> 15
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 15
aggtacctgg ctgtcgtcca tgctgtgttt 30

<210> 16
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic primer

<400> 16
gatgatgggg ttgatgcagc agtgcgtcat 30

44j

<210> 17
 <211> 34
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Synthetic primer

<400> 17
 ggaagtacag ctcagagttc tgcagcaccc ctgc 34

<210> 18
 <211> 31
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Synthetic primer

<400> 18
 gatgcgaaac tgaggctggc tgtactgtct c 31

<210> 19
 <211> 1682
 <212> DNA
 <213> *Thermus aquaticus*

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 gccgatcttc tggccctggc cgccgccagg gggggccggg tccaccgggc ccccgagcct 180
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 tacgtggaga ccctcttcgg ccgcgcgcgc tacgtgccag acctagaggc ccgggtgaag 1380
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ta 1682
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<210> 20
<211> 554
<212> PRT
<213> Thermus aquaticus
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	Leu	Ser	Arg	Lys	Glu	Pro	Met	Trp	Ala	Asp	Leu	Leu	Ala	Leu	Ala	Ala
			35					40					45			
	Ala	Arg	Gly	Gly	Arg	Val	His	Arg	Ala	Pro	Glu	Pro	Tyr	Lys	Ala	Leu
		50					55					60				
	Arg	Asp	Leu	Lys	Glu	Ala	Arg	Gly	Leu	Leu	Ala	Lys	Asp	Leu	Ser	Val
	65					70					75					80
	Leu	Ala	Leu	Arg	Glu	Gly	Leu	Gly	Leu	Pro	Pro	Gly	Asp	Asp	Pro	Met
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	Leu	Leu	Ala	Tyr	Leu	Leu	Asp	Pro	Ser	Asn	Thr	Thr	Pro	Glu	Gly	Val
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	Glu	Glu	Arg	Leu	Leu	Trp	Leu	Tyr	Arg	Glu	Val	Glu	Arg	Pro	Leu	Ser
	145					150					155					160
	Ala	Val	Leu	Ala	His	Met	Glu	Ala	Thr	Gly	Val	Arg	Leu	Asp	Val	Ala
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	Tyr	Leu	Arg	Ala	Leu	Ser	Leu	Glu	Val	Ala	Glu	Glu	Ile	Ala	Arg	Leu
			180						185					190		
	Glu	Ala	Glu	Val	Phe	Arg	Leu	Ala	Gly	His	Pro	Phe	Asn	Leu	Asn	Ser
		195						200					205			
	Arg	Asp	Gln	Leu	Glu	Arg	Val	Leu	Phe	Asp	Glu	Leu	Gly	Leu	Pro	Ala
	210						215					220				
	Ile	Gly	Lys	Thr	Glu	Lys	Thr	Gly	Lys	Arg	Ser	Thr	Ser	Ala	Ala	Val
	225					230					235					240
	Leu	Glu	Ala	Leu	Arg	Glu	Ala	His	Pro	Ile	Val	Glu	Lys	Ile	Leu	Gln
				245						250					255	
	Tyr	Arg	Glu	Leu	Thr	Lys	Leu	Lys	Ser	Thr	Tyr	Ile	Asp	Pro	Leu	Pro
			260						265					270		
	Asp	Leu	Ile	His	Pro	Arg	Thr	Gly	Arg	Leu	His	Thr	Arg	Phe	Asn	Gln
		275						280					285			
	Thr	Ala	Thr	Ala	Thr	Gly	Arg	Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln
		290				295						300				
	Asn	Ile	Pro	Val	Arg	Thr	Pro	Leu	Gly	Gln	Arg	Ile	Arg	Arg	Ala	Phe
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	Ile	Ala	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala	Leu	Asp	Tyr	Ser	Gln	Ile
				325												

441

Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr	Glu	Thr	Ala	Ser	Trp	Met
		355					360					365			
Phe	Gly	Val	Pro	Arg	Glu	Ala	Val	Asp	Pro	Leu	Met	Arg	Arg	Ala	Ala
	370					375					380				
Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly	Met	Ser	Ala	His	Arg	Leu
385					390					395					400
Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu	Ala	Gln	Ala	Phe	Ile	Glu
				405					410					415	
Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg	Ala	Trp	Leu	Lys	Lys	Thr
			420					425					430		
Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val	Glu	Thr	Leu	Phe	Gly	Arg
		435					440					445			
Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	Arg	Val	Lys	Ser	Val	Arg	Glu
	450					455					460				
Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	Met	Pro	Val	Gln	Gly	Thr	Ala	Ala
465					470					475					480
Asp	Leu	Met	Lys	Leu	Ala	Met	Val	Lys	Leu	Phe	Pro	Arg	Leu	Glu	Glu
			485						490					495	
Met	Gly	Ala	Arg	Met	Leu	Leu	Gln	Val	His	Asp	Glu	Leu	Val	Leu	Glu
		500						505					510		
Ala	Pro	Lys	Glu	Arg	Ala	Glu	Ala	Val	Ala	Arg	Leu	Ala	Lys	Glu	Val
	515					520					525				
Met	Glu	Gly	Val	Tyr	Pro	Leu	Ala	Val	Pro	Leu	Glu	Val	Glu	Val	Gly
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Ile	Gly	Glu	Asp	Trp	Leu	Ser	Ala	Lys	Glu						
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<210> 21

<211> 1682

<212> DNA

<213> Thermus aquaticus

<400> 21

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44m

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atgctccttc aggtccacga cgagctggtc ctcgaggccc caaaagagag ggcggaggcc 1560
gtggccccgc tggccaagga ggtcatggag ggggtgtatc ccctggccgt gccctggag 1620
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ta 1682

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<210> 22
 <211> 554
 <212> PRT
 <213> *Thermus aquaticus*

<400> 22
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 35 40 45
 Ala Arg Gly Gly Arg Val His Arg Ala Pro Glu Pro Tyr Lys Ala Leu
 50 55 60
 Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu Ala Lys Asp Leu Ser Val
 65 70 75 80
 Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro Pro Gly Asp Asp Pro Met
 85 90 95
 Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val
 100 105 110
 Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu Glu Ala Gly Glu Arg Ala
 115 120 125
 Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu Trp Gly Arg Leu Glu Gly
 130 135 140
 Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu Arg Pro Leu Ser
 145 150 155 160
 Ala Val Leu Ala His Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala
 165 170 175
 Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala Glu Glu Ile Ala Arg Leu
 180 185 190
 Glu Ala Glu Val Phe Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser
 195 200 205
 Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu Gly Leu Pro Ala
 210 215 220
 Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val
 225 230 235 240
 Leu Glu Ala Leu Arg Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln
 245 250 255
 Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr Tyr Ile Asp Pro Leu Pro
 260 265 270
 Asp Leu Ile His Pro Arg Thr Gly Arg Leu His Thr Arg Phe Asn Gln
 275 280 285
 Thr Ala Thr Ala Thr Gly Arg Leu Ser Ser Ser Asp Pro Asn Leu Gln
 290 295 300
 Asn Ile Pro Val Arg Thr Pro Leu Gly Gln Arg Ile Arg Arg Ala Phe
 305 310 315 320

44n

Ile	Ala	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala	Leu	Asp	Tyr	Ser	Gln	Ile
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Glu	Leu	Arg	Val	Leu	Ala	His	Leu	Ser	Gly	Asp	Lys	Asn	Leu	Ile	Arg
			340					345					350		
Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr	Glu	Thr	Ala	Ser	Trp	Met
		355					360					365			
Phe	Gly	Ile	Pro	Arg	Glu	Ala	Val	Asp	Pro	Leu	Met	Arg	Arg	Ala	Ala
	370					375					380				
Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly	Met	Ser	Ala	His	Arg	Leu
385					390					395					400
Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu	Ala	Gln	Ala	Phe	Ile	Glu
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Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg	Ala	Trp	Leu	Ser	Lys	Thr
			420					425					430		
Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val	Glu	Thr	Leu	Phe	Gly	Arg
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Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	Arg	Val	Lys	Ser	Val	Arg	Glu
	450					455					460				
Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	Met	Pro	Val	Gln	Gly	Thr	Ala	Ala
465					470					475					480
Asp	Leu	Met	Lys	Leu	Ala	Met	Val	Lys	Leu	Phe	Pro	Arg	Leu	Glu	Glu
			485						490					495	
Met	Gly	Ala	Arg	Met	Leu	Leu	Gln	Val	His	Asp	Glu	Leu	Val	Leu	Glu
			500					505					510		
Ala	Pro	Lys	Glu	Arg	Ala	Glu	Ala	Val	Ala	Arg	Leu	Ala	Lys	Glu	Val
		515					520					525			
Met	Glu	Gly	Val	Tyr	Pro	Leu	Ala	Val	Pro	Leu	Glu	Val	Glu	Val	Gly
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Ile	Gly	Glu	Asp	Trp	Leu	Ser	Ala	Lys	Glu						
545						550									

<210> 23
 <211> 1682
 <212> DNA
 <213> Thermus aquaticus

<400> 23
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440

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ta 1682

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<210> 24

<211> 554

<212> PRT

<213> *Thermus aquaticus*

<400> 24

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          20             25             30
Leu Ser Arg Lys Glu Pro Met Trp Ala Asp Leu Leu Ala Leu Ala Ala
          35             40             45
Ala Arg Gly Gly Arg Val His Arg Ala Pro Glu Pro Tyr Lys Ala Leu
          50             55             60
Arg Asp Leu Lys Glu Ala Arg Gly Leu Leu Ala Lys Asp Leu Ser Val
          65             70             75             80
Leu Ala Leu Arg Glu Gly Leu Gly Leu Pro Pro Gly Asp Asp Pro Met
          85             90             95
Leu Leu Ala Tyr Leu Leu Asp Pro Ser Asn Thr Thr Pro Glu Gly Val
          100             105             110
Ala Arg Arg Tyr Gly Gly Glu Trp Thr Glu Glu Ala Gly Glu Arg Ala
          115             120             125
Ala Leu Ser Glu Arg Leu Phe Ala Asn Leu Trp Gly Arg Leu Glu Gly
          130             135             140
Glu Glu Arg Leu Leu Trp Leu Tyr Arg Glu Val Glu Arg Pro Leu Ser
          145             150             155             160
Ala Val Leu Ala His Met Glu Ala Thr Gly Val Arg Leu Asp Val Ala
          165             170             175
Tyr Leu Arg Ala Leu Ser Leu Glu Val Ala Glu Glu Ile Ala Arg Leu
          180             185             190
Glu Ala Glu Val Phe Arg Leu Ala Gly His Pro Phe Asn Leu Asn Ser
          195             200             205
Arg Asp Gln Leu Glu Arg Val Leu Phe Asp Glu Leu Gly Leu Pro Ala
          210             215             220
Ile Gly Lys Thr Glu Lys Thr Gly Lys Arg Ser Thr Ser Ala Ala Val
          225             230             235             240
Leu Glu Ala Leu Arg Glu Ala His Pro Ile Val Glu Lys Ile Leu Gln
          245             250             255
Tyr Arg Glu Leu Thr Lys Leu Lys Ser Thr Tyr Ile Asp Pro Leu Pro
          260             265             270
Asp Leu Ile His Pro Arg Thr Gly Arg Leu His Thr Arg Phe Asn Gln
          275             280             285

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44p

Thr	Ala	Thr	Ala	Thr	Gly	Arg	Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln
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Asn	Ile	Pro	Val	Arg	Thr	Pro	Leu	Gly	Gln	Arg	Ile	Arg	Arg	Ala	Phe
305					310					315					320
Ile	Ala	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala	Pro	Asp	Tyr	Ser	Gln	Ile
				325					330					335	
Glu	Leu	Arg	Val	Leu	Ala	His	Leu	Ser	Gly	Asp	Lys	Asn	Leu	Ile	Arg
			340					345					350		
Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr	Glu	Thr	Ala	Ser	Trp	Met
		355					360					365			
Phe	Gly	Val	Pro	Arg	Glu	Ala	Val	Asp	Pro	Leu	Met	Arg	Arg	Ala	Ala
	370					375					380				
Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly	Met	Ser	Ala	His	Arg	Leu
385					390					395					400
Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu	Ala	Gln	Ala	Phe	Ile	Glu
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Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg	Ala	Trp	Leu	Leu	Lys	Thr
			420					425					430		
Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val	Glu	Thr	Leu	Phe	Gly	Arg
		435					440					445			
Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	Arg	Val	Lys	Ser	Val	Arg	Glu
	450					455					460				
Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	Met	Pro	Val	Gln	Gly	Thr	Ala	Ala
465					470					475					480
Asp	Leu	Met	Lys	Leu	Ala	Met	Val	Lys	Leu	Phe	Pro	Arg	Leu	Glu	Glu
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Met	Gly	Ala	Arg	Met	Leu	Leu	Gln	Val	His	Asp	Glu	Leu	Val	Leu	Glu
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Ala	Pro	Lys	Glu	Arg	Ala	Glu	Ala	Val	Ala	Arg	Leu	Ala	Lys	Glu	Val
		515					520					525			
Met	Glu	Gly	Val	Tyr	Pro	Leu	Ala	Val	Pro	Leu	Glu	Val	Glu	Val	Gly
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Ile	Gly	Glu	Asp	Trp	Leu	Ser	Ala	Lys	Glu						
545						550									

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 <211> 2506
 <212> DNA
 <213> Thermus aquaticus

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44q

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cggctggcca aggaggtcat ggaggggggtg tatccctgg ccggtgccct ggaggtggag 2460
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<210> 26

<211> 832

<212> PRT

<213> *Thermus aquaticus*

<400> 26

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Val Asp Gly His His Leu Ala Tyr Arg Thr Phe His Ala Leu Lys Gly
          20             25             30
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
          35             40             45
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Ala Val Ile Val
          50             55             60
Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Gly Gly
          65             70             75             80
Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu
          85             90             95
Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Ala Arg Leu Glu
          100            105            110
Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Ser Leu Ala Lys Lys
          115            120            125
Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Lys Asp
          130            135            140
Leu Tyr Gln Leu Leu Ser Asp Arg Ile His Val Leu His Pro Glu Gly
          145            150            155            160

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Tyr	Leu	Ile	Thr	Pro	Ala	Trp	Leu	Trp	Glu	Lys	Tyr	Gly	Leu	Arg	Pro	
				165					170					175		
Asp	Gln	Trp	Ala	Asp	Tyr	Arg	Ala	Leu	Thr	Gly	Asp	Glu	Ser	Asp	Asn	
			180					185					190			
Leu	Pro	Gly	Val	Lys	Gly	Ile	Gly	Glu	Lys	Thr	Ala	Arg	Lys	Leu	Leu	
		195					200					205				
Glu	Glu	Trp	Gly	Ser	Leu	Glu	Ala	Leu	Leu	Lys	Asn	Leu	Asp	Arg	Leu	
	210					215					220					
Lys	Pro	Ala	Ile	Arg	Glu	Lys	Ile	Leu	Ala	His	Met	Asp	Asp	Leu	Lys	
225					230					235					240	
Leu	Ser	Trp	Asp	Leu	Ala	Lys	Val	Arg	Thr	Asp	Leu	Pro	Leu	Glu	Val	
			245					250						255		
Asp	Phe	Ala	Lys	Arg	Arg	Glu	Pro	Asp	Arg	Glu	Arg	Leu	Arg	Ala	Phe	
			260					265					270			
Leu	Glu	Arg	Leu	Glu	Phe	Gly	Ser	Leu	Leu	His	Glu	Phe	Gly	Leu	Leu	
		275				280						285				
Glu	Ser	Pro	Lys	Ala	Leu	Glu	Glu	Ala	Pro	Trp	Pro	Pro	Pro	Glu	Gly	
	290					295				300						
Ala	Phe	Val	Gly	Phe	Val	Leu	Ser	Arg	Lys	Glu	Pro	Met	Trp	Ala	Asp	
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Leu	Leu	Ala	Leu	Ala	Ala	Ala	Arg	Gly	Gly	Arg	Val	His	Arg	Ala	Pro	
				325					330					335		
Glu	Pro	Tyr	Lys	Ala	Leu	Arg	Asp	Leu	Lys	Glu	Ala	Arg	Gly	Leu	Leu	
			340				345						350			
Ala	Lys	Asp	Leu	Ser	Val	Leu	Ala	Leu	Arg	Glu	Gly	Leu	Gly	Leu	Pro	
		355				360						365				
Pro	Gly	Asp	Asp	Pro	Met	Leu	Leu	Ala	Tyr	Leu	Leu	Asp	Pro	Ser	Asn	
	370					375					380					
Thr	Thr	Pro	Glu	Gly	Val	Ala	Arg	Arg	Tyr	Gly	Gly	Glu	Trp	Thr	Glu	
385					390					395					400	
Glu	Ala	Gly	Glu	Arg	Ala	Ala	Leu	Ser	Glu	Arg	Leu	Phe	Ala	Asn	Leu	
				405					410					415		
Trp	Gly	Arg	Leu	Glu	Gly	Glu	Glu	Arg	Leu	Leu	Trp	Leu	Tyr	Arg	Glu	
			420				425						430			
Val	Glu	Arg	Pro	Leu	Ser	Ala	Val	Leu	Ala	His	Met	Glu	Ala	Thr	Gly	
		435					440					445				
Val	Arg	Leu	Asp	Val	Ala	Tyr	Leu	Arg	Ala	Leu	Ser	Leu	Glu	Val	Ala	
	450					455					460					
Glu	Glu	Ile	Ala	Arg	Leu	Glu	Ala	Glu	Val	Phe	Arg	Leu	Ala	Gly	His	
465					470					475					480	
Pro	Phe	Asn	Leu	Asn	Ser	Arg	Asp	Gln	Leu	Glu	Arg	Val	Leu	Phe	Asp	
				485					490					495		

44s

```

Leu Asp Tyr Ser Gln Ile Glu Leu Arg Val Leu Ala His Leu Ser Gly
 610                      615                      620
Asp Glu Asn Leu Ile Arg Val Phe Gln Glu Gly Arg Asp Ile His Thr
625                      630                      635                      640
Glu Thr Ala Ser Trp Met Phe Gly Val Pro Arg Glu Ala Val Asp Pro
                      645                      650                      655
Leu Met Arg Arg Ala Ala Lys Thr Ile Asn Phe Gly Val Leu Tyr Gly
                      660                      665                      670
Met Ser Ala His Arg Leu Ser Gln Glu Leu Ala Ile Pro Tyr Glu Glu
                      675                      680                      685
Ala Gln Ala Phe Ile Glu Arg Tyr Phe Gln Ser Phe Pro Lys Val Arg
                      690                      695                      700
Ala Trp Ile Glu Lys Thr Leu Glu Glu Gly Arg Arg Arg Gly Tyr Val
705                      710                      715                      720
Glu Thr Leu Phe Gly Arg Arg Arg Tyr Val Pro Asp Leu Glu Ala Arg
                      725                      730                      735
Val Lys Ser Val Arg Glu Ala Ala Glu Arg Met Ala Phe Asn Met Pro
                      740                      745                      750
Val Gln Gly Thr Ala Ala Asp Leu Met Lys Leu Ala Met Val Lys Leu
                      755                      760                      765
Phe Pro Arg Leu Glu Glu Met Gly Ala Arg Met Leu Leu Gln Val His
                      770                      775                      780
Asp Glu Leu Val Leu Glu Ala Pro Lys Glu Arg Ala Glu Ala Val Ala
785                      790                      795                      800
Arg Leu Ala Lys Glu Val Met Glu Gly Val Tyr Pro Leu Ala Val Pro
                      805                      810                      815
Leu Glu Val Glu Val Gly Ile Gly Glu Asp Trp Leu Ser Ala Lys Glu
                      820                      825                      830

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<210> 27

<211> 2506

<212> DNA

<213> *Thermus aquaticus*

<400> 27

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gtgcaggcgg tctacggctt cgccaagagc ctctcaagg ccctcaagga ggacggggac 180
gcggtgatcg tggcttttga cgccaaggcc ccctccttcc gccacgaggc ctacgggggg 240
tacaaggcgg gccgggcccc cacgccggag gactttcccc ggcaactcgc cctcatcaag 300
gagctggtgg acctcctggg gctggcgcgc ctcgaggtec cgggctacga ggcggacgac 360
gtcctggcca gcctggccaa gaaggcggaa aaggagggtt acgagggtcc catcctcacc 420
gccgacaaag acctttacca gctcctttcc gaccgcatcc acgtcctcca ccccgagggg 480
tacctcatca ccccggcctg gctttgggaa aagtacggcc tgaggcccga ccagtgggcc 540
gactaccggg ccctgaccgg ggacgagtec gacaaccttc ccgggggtcaa gggcatcggg 600
gagaagacgg cgaggaagct tctggaggag tgggggagcc tggaagccct cctcaagaac 660
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ctgaggggaag gccttggcct cccgccggc gacgaccca tgctcctcgc ctacctctg 1140
gaccctcca acaccacccc cgaggggggtg gcccggcgct acggcgggga gtggacggag 1200

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44t

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gagggggagg agaggctcct ttggctttac cgggagggtg agaggccctt ttccgctgtc 1320
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ctggagggtg ccgaggagat cggccgcctc gagggcgagg tcttccgcct ggccggccac 1440
cccttcaacc tcaactcccg ggaccagctg gaaaggggtc tctttgacga gctagggctt 1500
cccgccatcg gcaagacgga gaagaccggc aagcgtcca ccagcgccgc cgtcctggag 1560
gccctccgcg agggccaccc catcgtggag aagatcctgc agtaccggga gctcaccaag 1620
ctgaagagca cctacattga ccccttgccg gacctcatcc accccaggac gggccgcctc 1680
cacaccgct tcaaccagac ggccacggcc acgggcaggc taagtagctc cgatcccaac 1740
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gaggaggggt ggctattggt ggccctggac tatagccaga tagagctcag ggtgctggcc 1860
cacctctccg gcgacaagaa cctgatccgg gtcttccagg aggggcggga catccacacg 1920
gagaccgcca gctggatgtt cggcgctccc cgggaggccg tggacccctt gatgcgccgg 1980
gcggccaaga ccatcaactt cggggtcctc tacggcatgt cggcccaccg cctctcccag 2040
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cccaagggtg gggcctggct taagaagacc ctggaggagg gcaggaggcg ggggtacgtg 2160
gagaccctct tcggccgcg cgcctacgtg ccagacctag agggccgggt gaagagcgtg 2220
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<210> 28

<211> 832

<212> PRT

<213> *Thermus aquaticus*

<400> 28

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Met Arg Gly Met Leu Pro Leu Phe Glu Pro Lys Gly Arg Val Leu Leu
  1             5             10             15
Val Asp Gly His His Leu Ala Tyr Arg Thr Phe His Ala Leu Lys Gly
             20             25             30
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
             35             40             45
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Ala Val Ile Val
             50             55             60
Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Gly Gly
             65             70             75             80
Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu
             85             90             95
Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Ala Arg Leu Glu
             100            105            110
Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Ser Leu Ala Lys Lys
             115            120            125
Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Lys Asp
             130            135            140
Leu Tyr Gln Leu Leu Ser Asp Arg Ile His Val Leu His Pro Glu Gly
             145            150            155            160
Tyr Leu Ile Thr Pro Ala Trp Leu Trp Glu Lys Tyr Gly Leu Arg Pro
             165            170            175
Asp Gln Trp Ala Asp Tyr Arg Ala Leu Thr Gly Asp Glu Ser Asp Asn
             180            185            190
Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Arg Lys Leu Leu
             195            200            205

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44u

Glu	Glu	Trp	Gly	Ser	Leu	Glu	Ala	Leu	Leu	Lys	Asn	Leu	Asp	Arg	Leu
210						215					220				
Lys	Pro	Ala	Ile	Arg	Glu	Lys	Ile	Leu	Ala	His	Met	Asp	Asp	Leu	Lys
225					230					235					240
Leu	Ser	Trp	Asp	Leu	Ala	Lys	Val	Arg	Thr	Asp	Leu	Pro	Leu	Glu	Val
				245					250					255	
Asp	Phe	Ala	Lys	Arg	Arg	Glu	Pro	Asp	Arg	Glu	Arg	Leu	Arg	Ala	Phe
			260					265					270		
Leu	Glu	Arg	Leu	Glu	Phe	Gly	Ser	Leu	Leu	His	Glu	Phe	Gly	Leu	Leu
		275					280					285			
Glu	Ser	Pro	Lys	Ala	Leu	Glu	Glu	Ala	Pro	Trp	Pro	Pro	Pro	Glu	Gly
	290					295					300				
Ala	Phe	Val	Gly	Phe	Val	Leu	Ser	Arg	Lys	Glu	Pro	Met	Trp	Ala	Asp
305					310					315					320
Leu	Leu	Ala	Leu	Ala	Ala	Ala	Arg	Gly	Gly	Arg	Val	His	Arg	Ala	Pro
				325					330					335	
Glu	Pro	Tyr	Lys	Ala	Leu	Arg	Asp	Leu	Lys	Glu	Ala	Arg	Gly	Leu	Leu
			340					345					350		
Ala	Lys	Asp	Leu	Ser	Val	Leu	Ala	Leu	Arg	Glu	Gly	Leu	Gly	Leu	Pro
		355					360					365			
Pro	Gly	Asp	Asp	Pro	Met	Leu	Leu	Ala	Tyr	Leu	Leu	Asp	Pro	Ser	Asn
	370					375					380				
Thr	Thr	Pro	Glu	Gly	Val	Ala	Arg	Arg	Tyr	Gly	Gly	Glu	Trp	Thr	Glu
385					390					395					400
Glu	Ala	Gly	Glu	Arg	Ala	Ala	Leu	Ser	Glu	Arg	Leu	Phe	Ala	Asn	Leu
				405					410					415	
Trp	Gly	Arg	Leu	Glu	Gly	Glu	Glu	Arg	Leu	Leu	Trp	Leu	Tyr	Arg	Glu
			420					425					430		
Val	Glu	Arg	Pro	Leu	Ser	Ala	Val	Leu	Ala	His	Met	Glu	Ala	Thr	Gly
		435					440					445			
Val	Arg	Leu	Asp	Val	Ala	Tyr	Leu	Arg	Ala	Leu	Ser	Leu	Glu	Val	Ala
	450					455					460				
Glu	Glu	Ile	Ala	Arg	Leu	Glu	Ala	Glu	Val	Phe	Arg	Leu	Ala	Gly	His
465					470					475					480
Pro	Phe	Asn	Leu	Asn	Ser	Arg	Asp	Gln	Leu	Glu	Arg	Val	Leu	Phe	Asp
				485					490					495	
Glu	Leu	Gly	Leu	Pro	Ala	Ile	Gly	Lys	Thr	Glu	Lys	Thr	Gly	Lys	Arg
			500					505					510		
Ser	Thr	Ser	Ala	Ala	Val	Leu	Glu	Ala	Leu	Arg	Glu	Ala	His	Pro	Ile
		515					520					525			
Val	Glu	Lys	Ile	Leu	Gln	Tyr	Arg	Glu	Leu	Thr	Lys	Leu	Lys	Ser	Thr
	530					535					540				
Tyr	Ile	Asp	Pro	Leu	Pro	Asp	Leu	Ile	His	Pro	Arg	Thr	Gly	Arg	Leu
545					550					555					560
His	Thr	Arg	Phe	Asn	Gln	Thr	Ala	Thr	Ala	Thr	Gly	Arg	Leu	Ser	Ser
				565					570					575	
Ser	Asp	Pro	Asn	Leu	Gln	Asn	Ile	Pro	Val	Arg	Thr	Pro	Leu	Gly	Gln
			580					585					590		
Arg	Ile	Arg	Arg	Ala	Phe	Ile	Ala	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala
		595					600					605			
Leu	Asp	Tyr	Ser	Gln	Ile	Glu	Leu	Arg	Val	Leu	Ala	His	Leu	Ser	Gly
	610					615					620				
Asp	Lys	Asn	Leu	Ile	Arg	Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr
625					630					635					640
Glu	Thr	Ala	Ser	Trp	Met	Phe	Gly	Val	Pro	Arg	Glu	Ala	Val	Asp	Pro
				645					650					655	

44v

Leu	Met	Arg	Arg	Ala	Ala	Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly		
			660					665					670				
Met	Ser	Ala	His	Arg	Leu	Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu		
		675					680					685					
Ala	Gln	Ala	Phe	Ile	Glu	Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg		
	690					695					700						
Ala	Trp	Leu	Lys	Lys	Thr	Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val		
705					710					715					720		
Glu	Thr	Leu	Phe	Gly	Arg	Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	Arg		
			725					730					735				
Val	Lys	Ser	Val	Arg	Glu	Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	Met	Pro		
		740						745					750				
Val	Gln	Gly	Thr	Ala	Ala	Asp	Leu	Met	Lys	Leu	Ala	Met	Val	Lys	Leu		
	755					760						765					
Phe	Pro	Arg	Leu	Glu	Glu	Met	Gly	Ala	Arg	Met	Leu	Leu	Gln	Val	His		
	770					775					780						
Asp	Glu	Leu	Val	Leu	Glu	Ala	Pro	Lys	Glu	Arg	Ala	Glu	Ala	Val	Ala		
785					790					795					800		
Arg	Leu	Ala	Lys	Glu	Val	Met	Glu	Gly	Val	Tyr	Pro	Leu	Ala	Val	Pro		
			805					810					815				
Leu	Glu	Val	Glu	Val	Gly	Ile	Gly	Glu	Asp	Trp	Leu	Ser	Ala	Lys	Glu		
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<210> 29

<211> 2506

<212> DNA

<213> *Thermus aquaticus*

<400> 29

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gtgcaggcgg tctacggctt cgccaagagc ctctcaagg ccctcaagga ggacggggac 180
gcggtgatcg tgggtctttga cgccaaggcc ccctccttcc gccacgagge ctacggggggg 240
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gccgacaaag acctttacca gctcctttcc gaccgcctcc acgtcctcca ccccgagggg 480
tacctcatca ccccggcctg gctttgggaa aagtaaggcc tgaggcccga ccagtggggc 540
gactaccggg ccctgaccgg ggacgagtc gacaaccttc ccgggggtcaa gggcatcggg 600
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cccgccatcg gcaagacgga gaagaccggc aagcgctcca ccagcgccgc cgtcctggag 1560

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44w

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cacacccgct tcaaccagac ggccacggcc acgggcaggc taagtagctc cgatcccaac 1740
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<210> 30

<211> 832

<212> PRT

<213> *Thermus aquaticus*

<400> 30

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          20          25          30
Leu Thr Thr Ser Arg Gly Glu Pro Val Gln Ala Val Tyr Gly Phe Ala
      35          40          45
Lys Ser Leu Leu Lys Ala Leu Lys Glu Asp Gly Asp Ala Val Ile Val
      50          55          60
Val Phe Asp Ala Lys Ala Pro Ser Phe Arg His Glu Ala Tyr Gly Gly
      65          70          75          80
Tyr Lys Ala Gly Arg Ala Pro Thr Pro Glu Asp Phe Pro Arg Gln Leu
          85          90          95
Ala Leu Ile Lys Glu Leu Val Asp Leu Leu Gly Leu Ala Arg Leu Glu
      100         105         110
Val Pro Gly Tyr Glu Ala Asp Asp Val Leu Ala Ser Leu Ala Lys Lys
      115         120         125
Ala Glu Lys Glu Gly Tyr Glu Val Arg Ile Leu Thr Ala Asp Lys Asp
      130         135         140
Leu Tyr Gln Leu Leu Ser Asp Arg Ile His Val Leu His Pro Glu Gly
      145         150         155         160
Tyr Leu Ile Thr Pro Ala Trp Leu Trp Glu Lys Tyr Gly Leu Arg Pro
          165         170         175
Asp Gln Trp Ala Asp Tyr Arg Ala Leu Thr Gly Asp Glu Ser Asp Asn
          180         185         190
Leu Pro Gly Val Lys Gly Ile Gly Glu Lys Thr Ala Arg Lys Leu Leu
          195         200         205
Glu Glu Trp Gly Ser Leu Glu Ala Leu Leu Lys Asn Leu Asp Arg Leu
      210         215         220
Lys Pro Ala Ile Arg Glu Lys Ile Leu Ala His Met Asp Asp Leu Lys
      225         230         235         240
Leu Ser Trp Asp Leu Ala Lys Val Arg Thr Asp Leu Pro Leu Glu Val
          245         250         255

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44x

Asp	Phe	Ala	Lys	Arg	Arg	Glu	Pro	Asp	Arg	Glu	Arg	Leu	Arg	Ala	Phe	
			260					265					270			
Leu	Glu	Arg	Leu	Glu	Phe	Gly	Ser	Leu	Leu	His	Glu	Phe	Gly	Leu	Leu	
		275					280					285				
Glu	Ser	Pro	Lys	Ala	Leu	Glu	Glu	Ala	Pro	Trp	Pro	Pro	Pro	Glu	Gly	
	290					295					300					
Ala	Phe	Val	Gly	Phe	Val	Leu	Ser	Arg	Lys	Glu	Pro	Met	Trp	Ala	Asp	
305					310					315					320	
Leu	Leu	Ala	Leu	Ala	Ala	Ala	Arg	Gly	Gly	Arg	Val	His	Arg	Ala	Pro	
			325					330						335		
Glu	Pro	Tyr	Lys	Ala	Leu	Arg	Asp	Leu	Lys	Glu	Ala	Arg	Gly	Leu	Leu	
			340					345					350			
Ala	Lys	Asp	Leu	Ser	Val	Leu	Ala	Leu	Arg	Glu	Gly	Leu	Gly	Leu	Pro	
		355					360					365				
Pro	Gly	Asp	Asp	Pro	Met	Leu	Leu	Ala	Tyr	Leu	Leu	Asp	Pro	Ser	Asn	
	370					375					380					
Thr	Thr	Pro	Glu	Gly	Val	Ala	Arg	Arg	Tyr	Gly	Gly	Glu	Trp	Thr	Glu	
385					390					395					400	
Glu	Ala	Gly	Glu	Arg	Ala	Ala	Leu	Ser	Glu	Arg	Leu	Phe	Ala	Asn	Leu	
			405						410					415		
Trp	Gly	Arg	Leu	Glu	Gly	Glu	Glu	Arg	Leu	Leu	Trp	Leu	Tyr	Arg	Glu	
			420					425						430		
Val	Glu	Arg	Pro	Leu	Ser	Ala	Val	Leu	Ala	His	Met	Glu	Ala	Thr	Gly	
		435					440					445				
Val	Arg	Leu	Asp	Val	Ala	Tyr	Leu	Arg	Ala	Leu	Ser	Leu	Glu	Val	Ala	
	450					455					460					
Glu	Glu	Ile	Ala	Arg	Leu	Glu	Ala	Glu	Val	Phe	Arg	Leu	Ala	Gly	His	
465					470					475					480	
Pro	Phe	Asn	Leu	Asn	Ser	Arg	Asp	Gln	Leu	Glu	Arg	Val	Leu	Phe	Asp	
			485					490						495		
Glu	Leu	Gly	Leu	Pro	Ala	Ile	Gly	Lys	Thr	Glu	Lys	Thr	Gly	Lys	Arg	
			500					505					510			
Ser	Thr	Ser	Ala	Ala	Val	Leu	Glu	Ala	Leu	Arg	Glu	Ala	His	Pro	Ile	
		515					520					525				
Val	Glu	Lys	Ile	Leu	Gln	Tyr	Arg	Glu	Leu	Thr	Lys	Leu	Lys	Ser	Thr	
	530					535					540					
Tyr	Ile	Asp	Pro	Leu	Pro	Asp	Leu	Ile	His	Pro	Arg	Thr	Gly	Arg	Leu	
545					550					555					560	
His	Thr	Arg	Phe	Asn	Gln	Thr	Ala	Thr	Ala	Thr	Gly	Arg	Leu	Ser	Ser	
			565					570						575		
Ser	Asp	Pro	Asn	Leu	Gln	Asn	Ile	Pro	Val	Arg	Thr	Pro	Leu	Gly	Gln	
			580					585					590			
Arg	Ile	Arg	Arg	Ala	Phe	Ile	Ala	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala	
		595					600					605				
Pro	Asp	Tyr	Ser	Gln	Ile	Glu	Leu	Arg	Val	Leu	Ala	His	Leu	Ser	Gly	
	610					615					620					
Asp	Lys	Asn	Leu	Ile	Arg	Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr	
625					630					635					640	
Glu	Thr	Ala	Ser	Trp	Met	Phe	Gly	Val	Pro	Arg	Glu	Ala	Val	Asp	Pro	
			645					650						655		
Leu	Met	Arg	Arg	Ala	Ala	Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly	
			660					665					670			
Met	Ser	Ala	His	Arg	Leu	Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu	
		675					680					685				
Ala	Gln	Ala	Phe	Ile	Glu	Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg	
	690					695					700					

44y

Ala	Trp	Leu	Leu	Lys	Thr	Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val
705					710					715					720
Glu	Thr	Leu	Phe	Gly	Arg	Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	Arg
				725					730						735
Val	Lys	Ser	Val	Arg	Glu	Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	Met	Pro
			740					745					750		
Val	Gln	Gly	Thr	Ala	Ala	Asp	Leu	Met	Lys	Leu	Ala	Met	Val	Lys	Leu
		755					760					765			
Phe	Pro	Arg	Leu	Glu	Glu	Met	Gly	Ala	Arg	Met	Leu	Leu	Gln	Val	His
	770					775					780				
Asp	Glu	Leu	Val	Leu	Glu	Ala	Pro	Lys	Glu	Arg	Ala	Glu	Ala	Val	Ala
785					790					795					800
Arg	Leu	Ala	Lys	Glu	Val	Met	Glu	Gly	Val	Tyr	Pro	Leu	Ala	Val	Pro
			805						810						815
Leu	Glu	Val	Glu	Val	Gly	Ile	Gly	Glu	Asp	Trp	Leu	Ser	Ala	Lys	Glu
			820					825					830		

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CLAIMS:

1. An isolated polypeptide comprising an amino acid sequence having at least 80% amino acid sequence identity to

5 SEQ ID NO: 4 (KT-6), wherein amino residue position 430 of the isolated polypeptide is valine (V) and amino residue position 429 of the isolated polypeptide is leucine (L);

SEQ ID NO: 6 (KT-7), wherein amino residue position 430 of the isolated polypeptide is tryptophan (W), amino residue position 348 of the isolated polypeptide is lysine (K), and amino residue position 429 of the isolated polypeptide is leucine (L);

10 SEQ ID NO: 20 (KT-10), wherein amino residue position 430 of the isolated polypeptide is lysine (K), amino residue position 348 of the isolated polypeptide is lysine (K), and amino residue position 429 of the isolated polypeptide is leucine (L);

15 SEQ ID NO: 24 (KT-12), wherein amino residue position 430 of the isolated polypeptide is leucine (L), amino residue position 331 of the isolated polypeptide is proline (P), amino residue position 348 of the isolated polypeptide is lysine (K), and amino residue position 429 of the isolated polypeptide is leucine (L);

SEQ ID NO: 28 (FL-10), wherein amino residue position 708 of the isolated polypeptide is lysine (K), amino acid residue position 626 of the isolated polypeptide is lysine (K), and amino residue position 707 of the isolated polypeptide is leucine (L); or

20 SEQ ID NO: 30 (FL-12), wherein amino residue position 708 of the isolated polypeptide is leucine (L), amino residue position 609 of the isolated polypeptide is proline (P), amino residue position 626 of the isolated polypeptide is lysine (K), and amino residue position 707 of the isolated polypeptide is leucine (L);

wherein

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the isolated polypeptide comprises a DNA polymerase having a blood-resistant phenotype and a cold sensitive phenotype;

the blood-resistant phenotype displaying amplification activity in a Polymerase Chain Reaction assay solution containing 3% to 25% whole blood;

5 the cold sensitive phenotype displaying lower amplification than wild-type Taq at temperatures lower than 72°C in a Polymerase Chain Reaction assay.

2. The isolated polypeptide of claim 1, wherein the sequence identity is at least 85%.

3. The isolated polypeptide of claim 1, wherein the sequence identity is at
10 least 90%.

4. The isolated polypeptide of claim 1, wherein the sequence identity is at least 95%.

5. The isolated polypeptide of claim 1, wherein the sequence identity is at least 99%.

15 6. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of: SEQ ID NO: 4 (KT-6), SEQ ID NO: 6 (KT-7), SEQ ID NO: 20 (KT-10), SEQ ID NO: 24 (KT-12), SEQ ID NO: 28 (FL-10), and SEQ ID NO: 30 (FL-12).

7. An isolated polynucleic acid encoding the isolated polypeptide of any one of claims 1-6.

20 8. A method of amplifying a deoxyribonucleic acid (DNA) target comprising:

performing a polymerase chain reaction (PCR) with a reaction mixture comprising a deoxyribonucleic acid (DNA) target, deoxynucleotide triphosphates, a magnesium salt, and an isolated polypeptide of any one of claims 1-6.

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9. The method of claim 8, wherein the reaction mixture comprises a volume of whole blood or blood fraction.
10. The method of any one of claims 8-9, wherein the volume of whole blood or blood fraction is from 3% to 25% of the reaction mixture (vol/vol).
- 5 11. The method of any one of claims 8-9, wherein the volume of whole blood or blood fraction is from 5% to 20% of the reaction mixture (vol/vol).
12. The method of any one of claims 8-9, wherein the volume of whole blood or blood fraction is from 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% of the reaction mixture (vol/vol).
- 10 13. The method of any one of claims 8-12, wherein the reaction mixture further comprises betaine.
14. The method of any one of claims 8-13, further comprising:

forming the reaction mixture by (a) adding an amplification cocktail to a reaction vessel, wherein the amplification cocktail comprises the isolated polypeptide of any
15 one of claims 1-6; and (b) adding a sample comprising whole blood or blood fraction to the reaction vessel, wherein the sample is layered beneath the amplification cocktail; and

wherein performing the PCR comprises performing a thermal cycling program to effect amplification of the nucleic acid target, and the sample is not mixed with the amplification cocktail in the reaction vessel before thermal cycling.
- 20 15. The method of any one of claims 8-13 further comprising:

combining a first volume component and a second volume component to form the reaction mixture; and

performing the PCR comprises performing a thermal cycling program to effect amplification of the nucleic acid target;

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wherein

the second volume component is heavier than the first volume component;

the first volume component comprises the isolated polypeptide of any one of claims 1-6 but does not contain the nucleic acid target;

5 the second volume component comprises the nucleic acid target;

the second volume component is underlaid below the first volume component and the first volume component and the second volume component are not mixed before the PCR is initiated.

16. The method of claim 15, wherein the second volume component further
10 comprises a volume of whole blood or blood fraction.

17. The method of any one of claims 15-16, wherein the second volume component further comprises a magnesium salt and deoxynucleotide triphosphates.

18. The method of any one of claims 15-17, wherein the second volume component further comprises betaine.

15 19. The method of any one of claims 8-18, wherein the reaction mixture further comprises a second DNA polymerase enzyme having a 3'-exonuclease activity selected from the group consisting of Vent DNA polymerase, Deep Vent DNA polymerase, Pfu DNA polymerase, and Pwu DNA polymerase.

20 20. A kit for performing PCR assays on samples comprising whole blood or blood fraction, wherein the kit comprises (a) the isolated polypeptide of any one of claims 1-6 and (b) an oligonucleotide primer, a buffer, a control solution, or instructions to perform said PCR assays.

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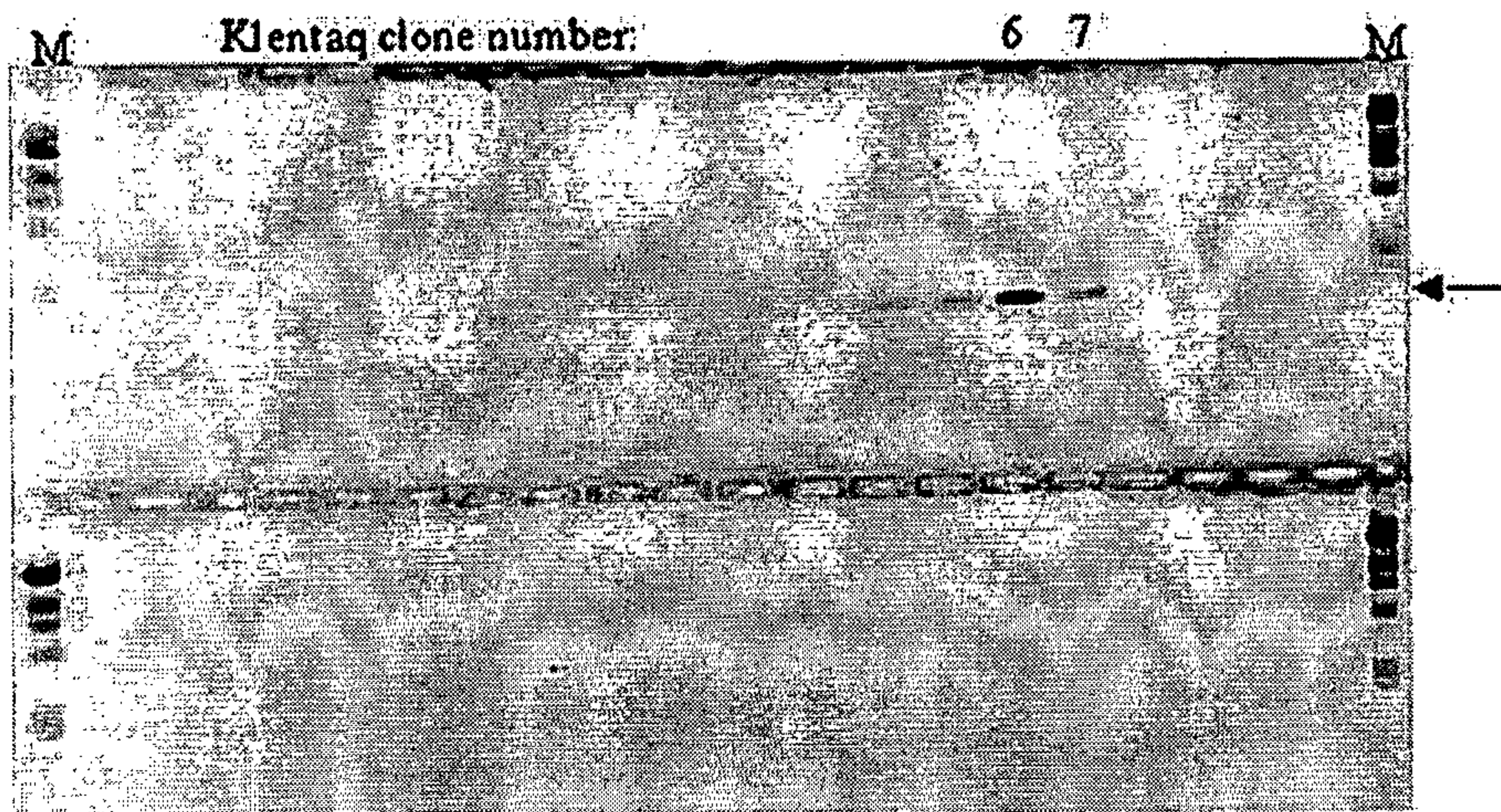


Fig. 1

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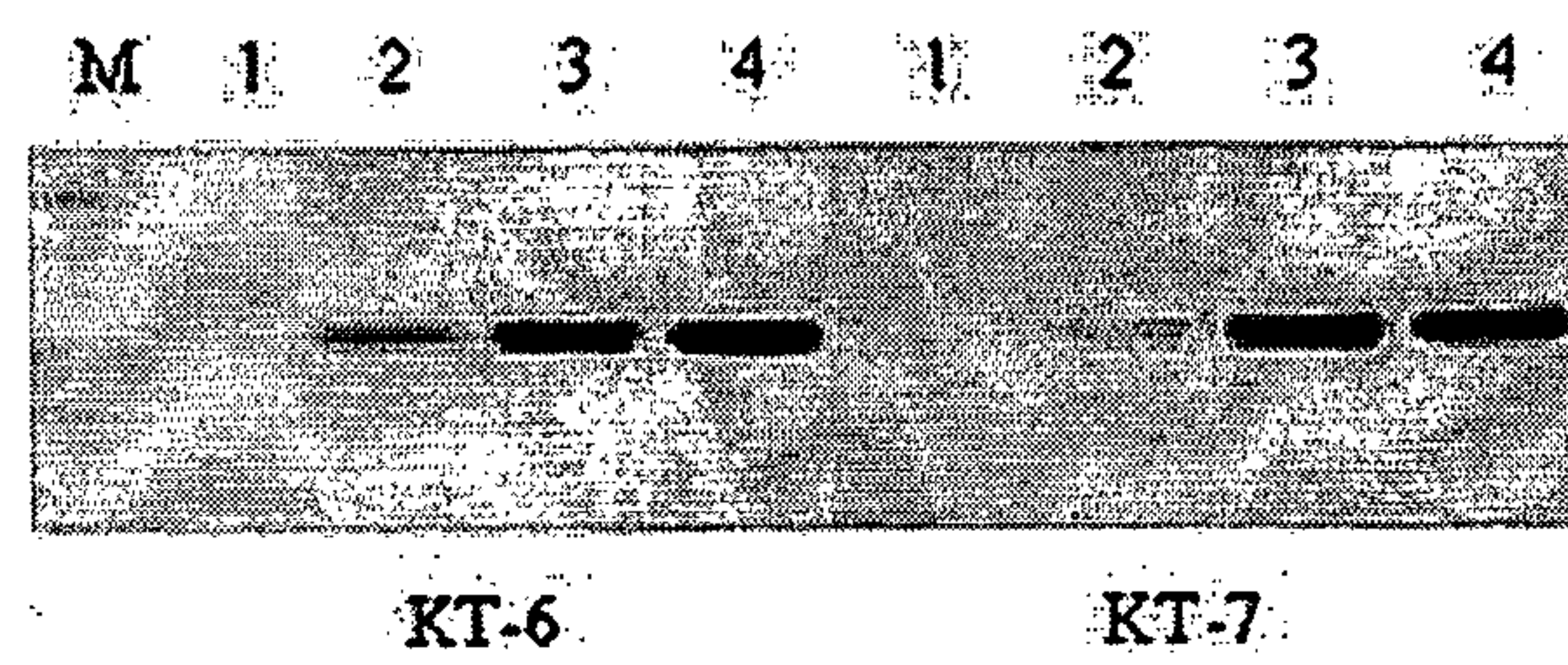


Fig. 2A

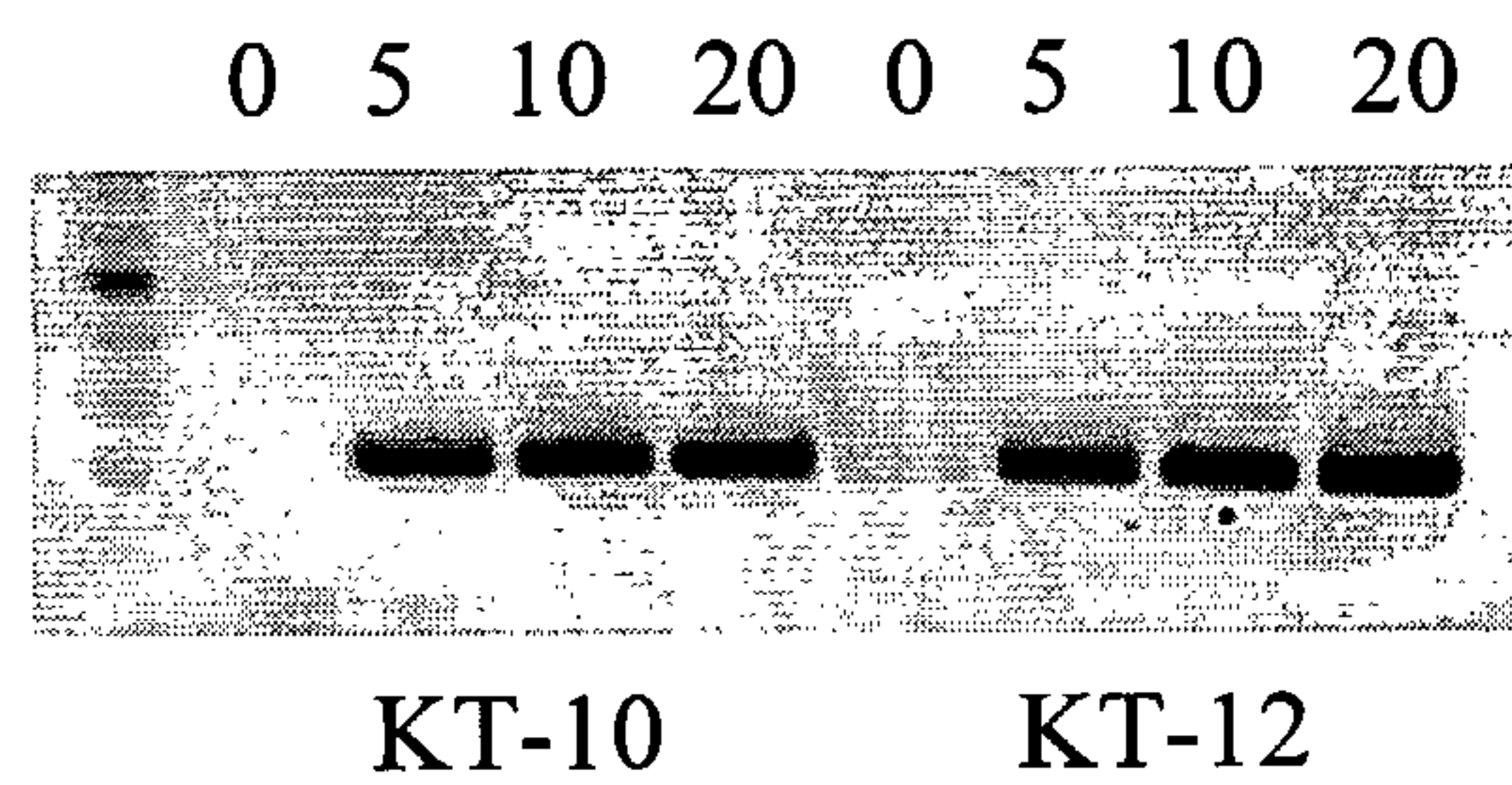


Fig. 2B

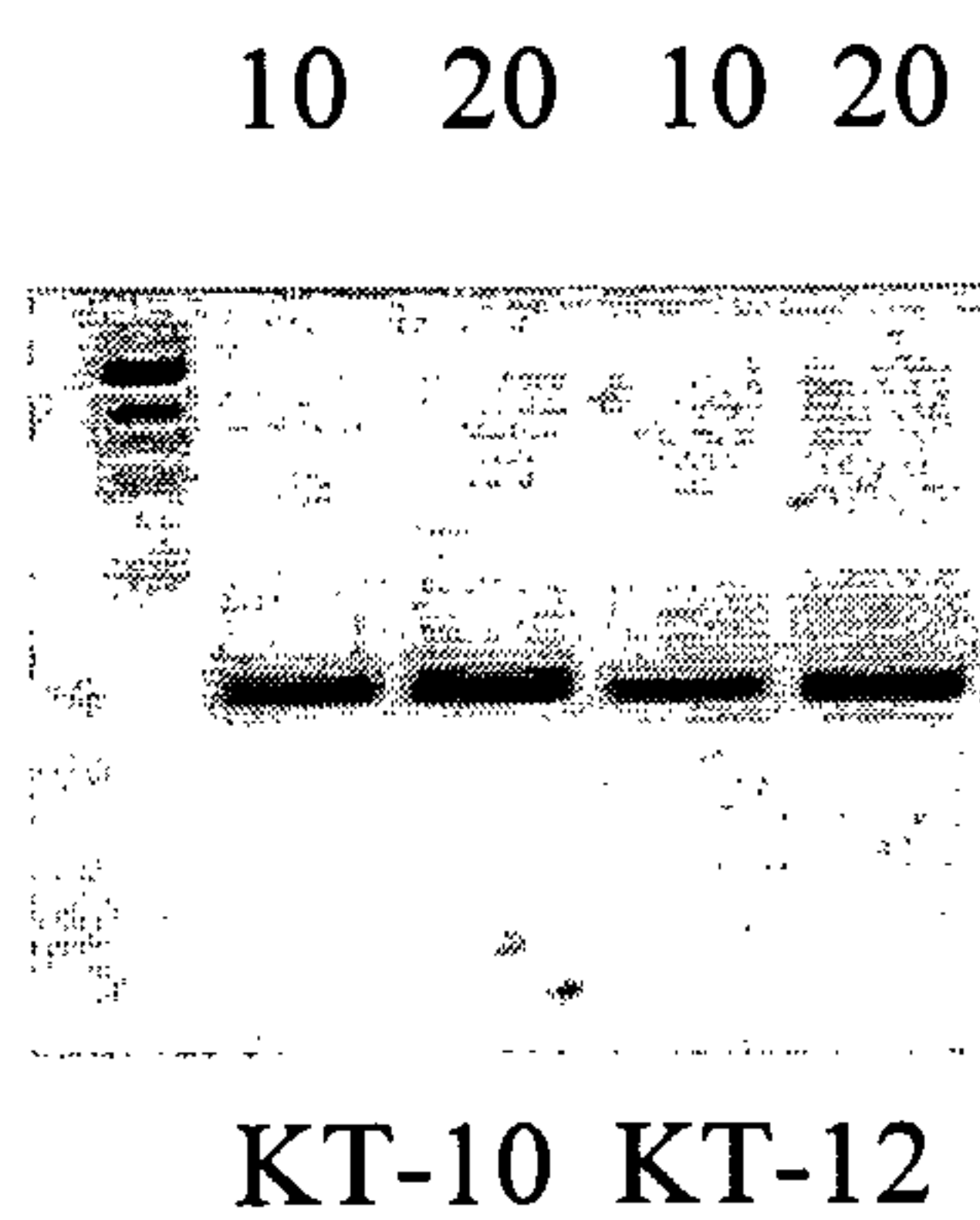


Fig. 2C

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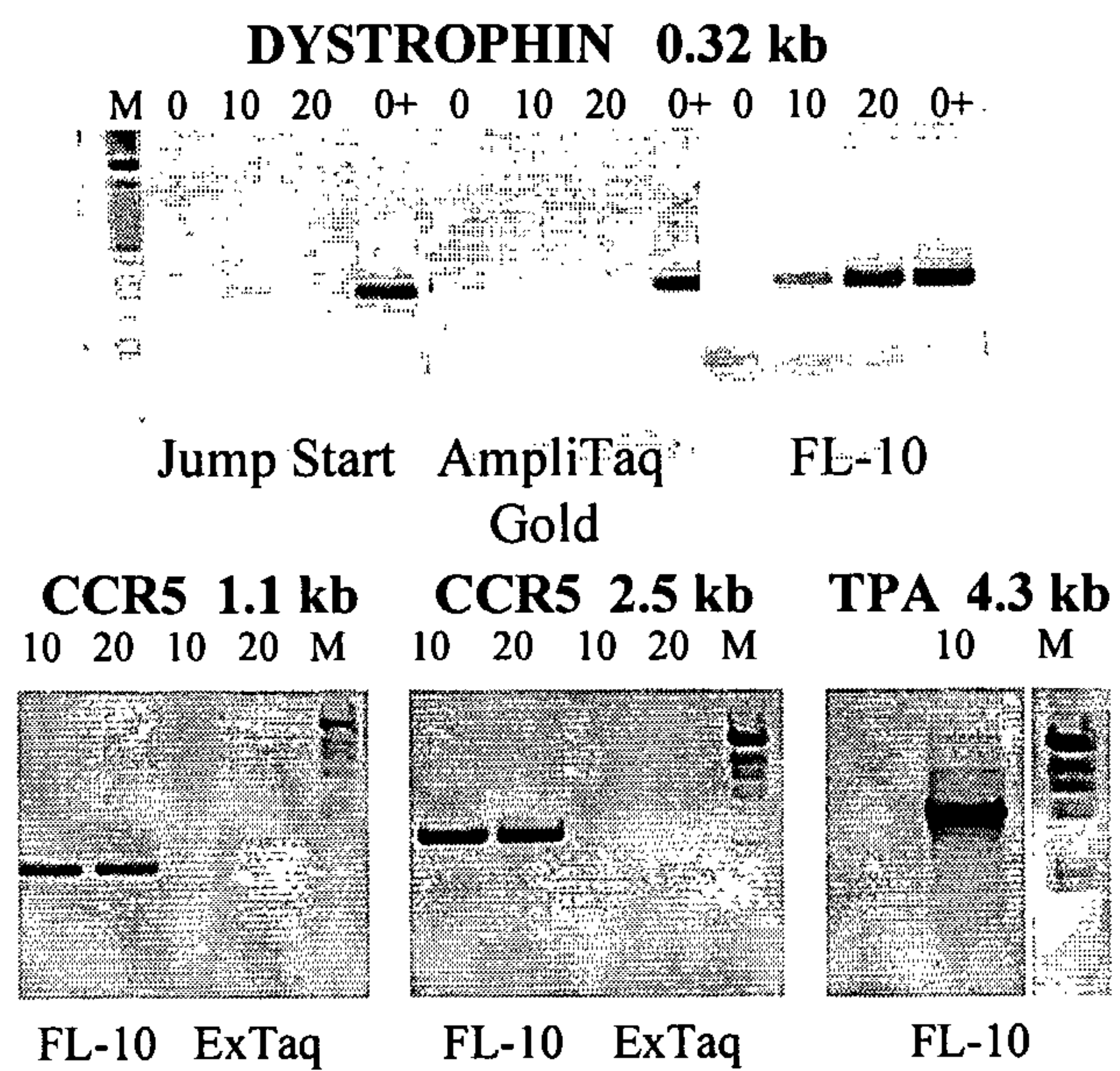


Fig. 3A

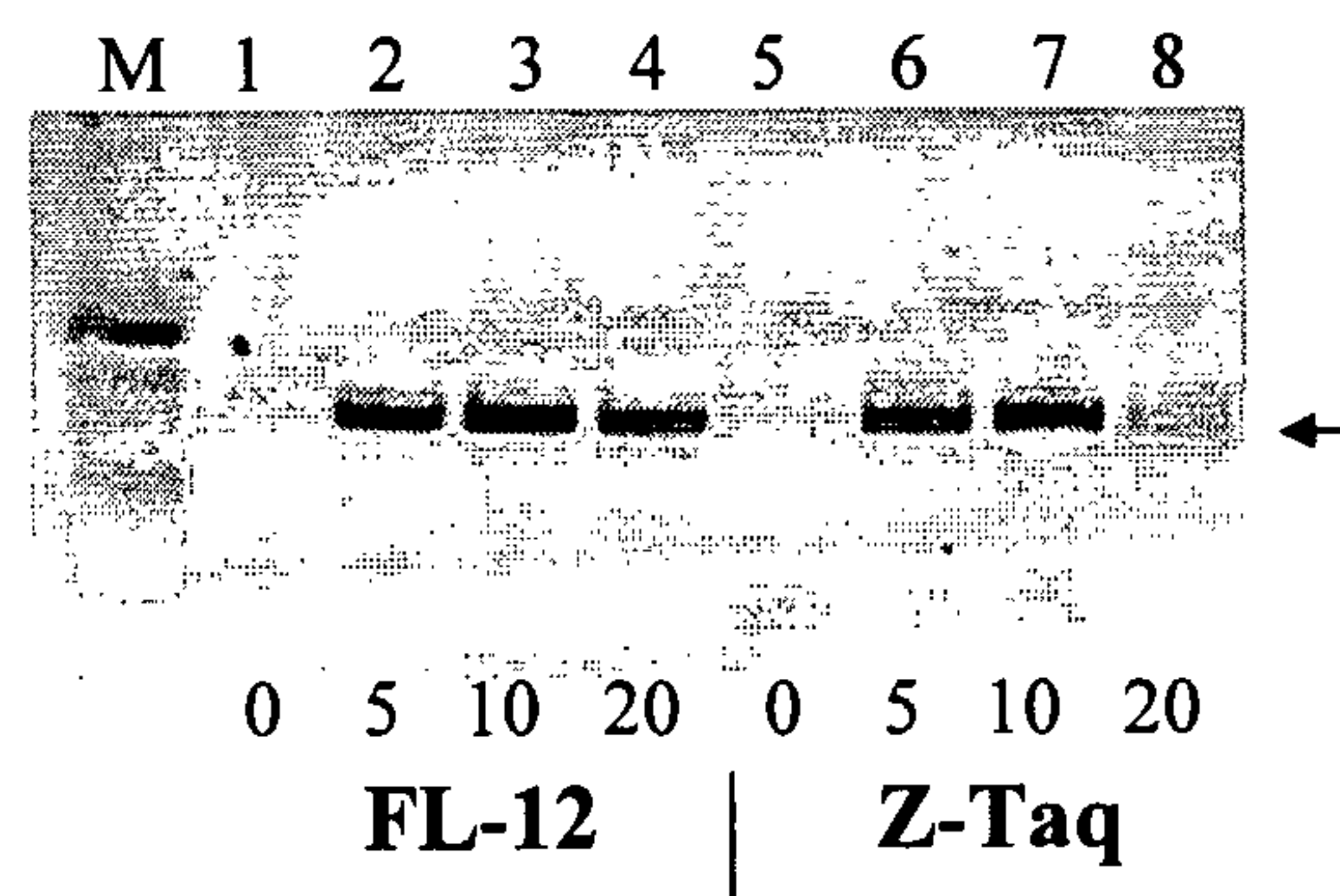


Fig. 3B

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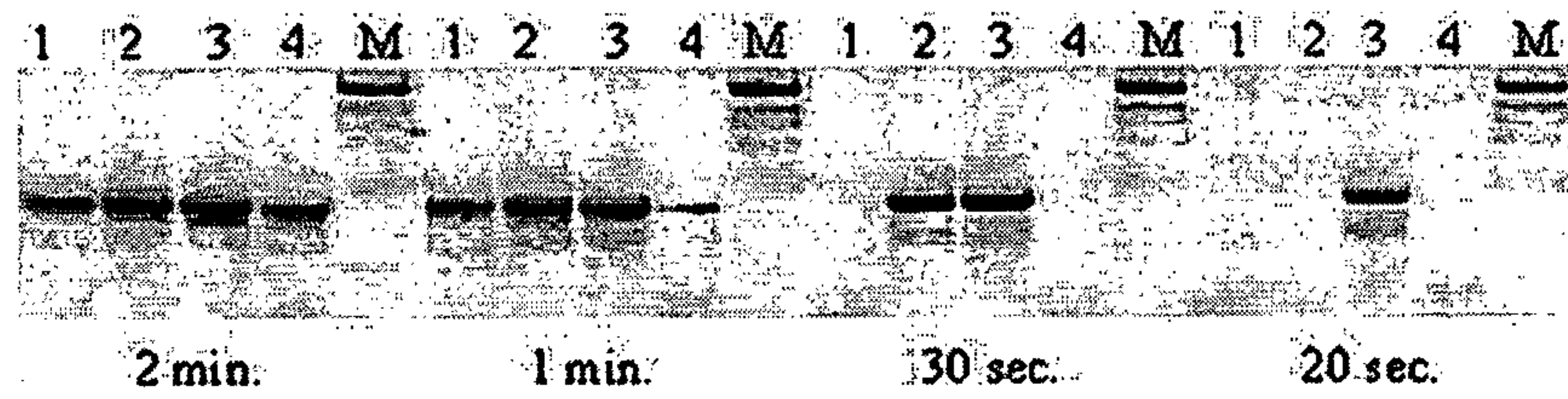


Fig. 4A

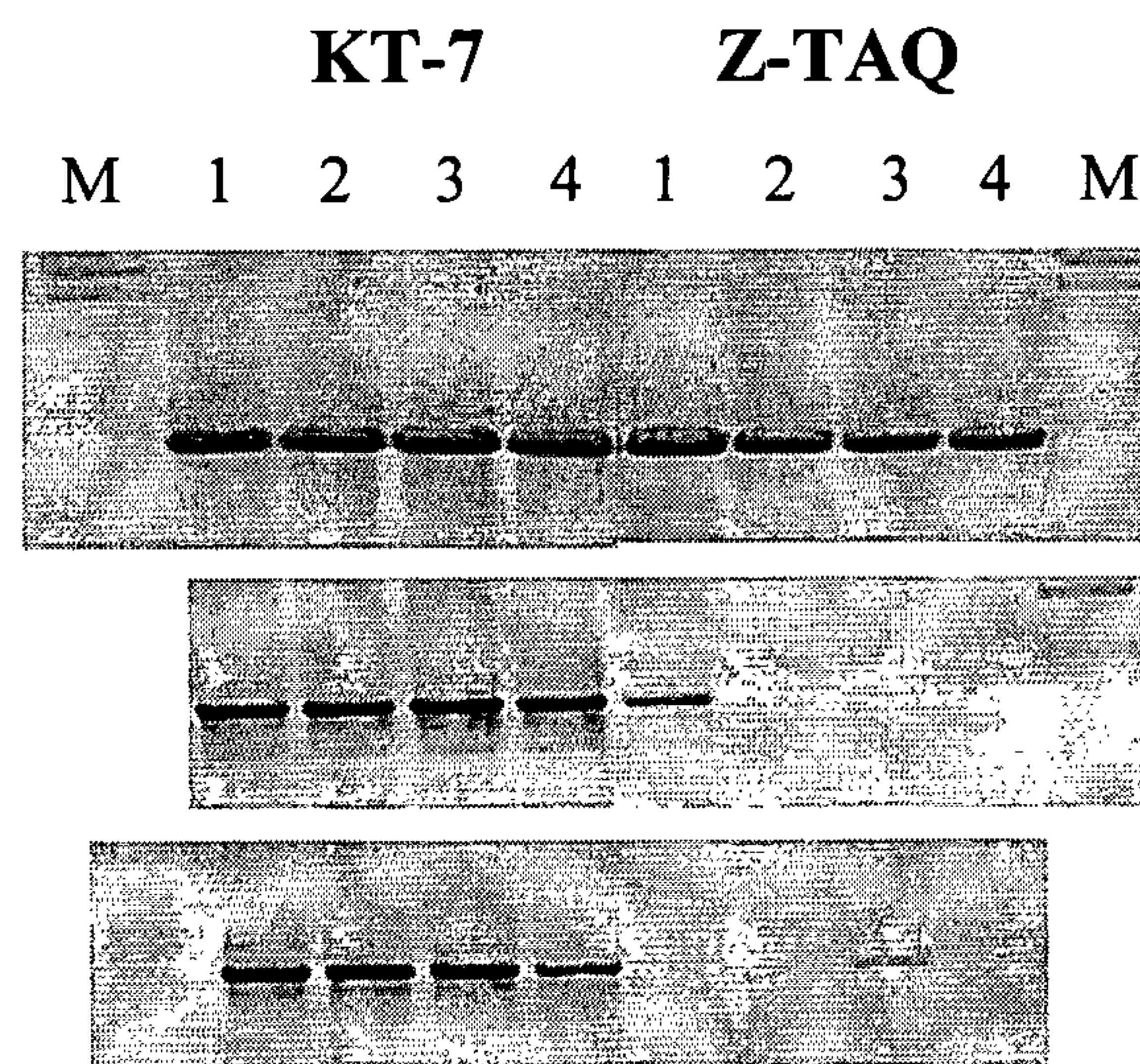


Fig. 4B

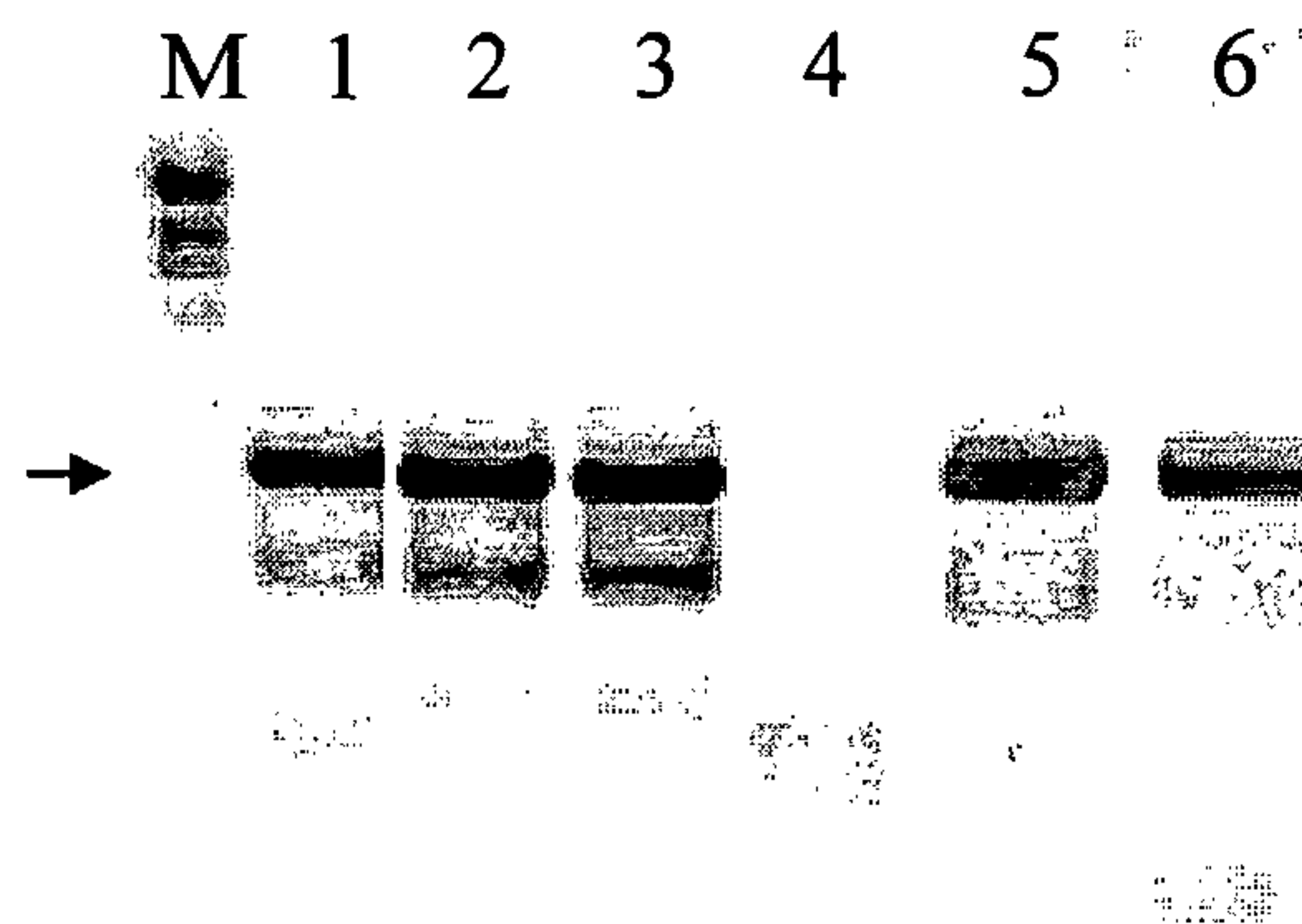


Fig. 4C

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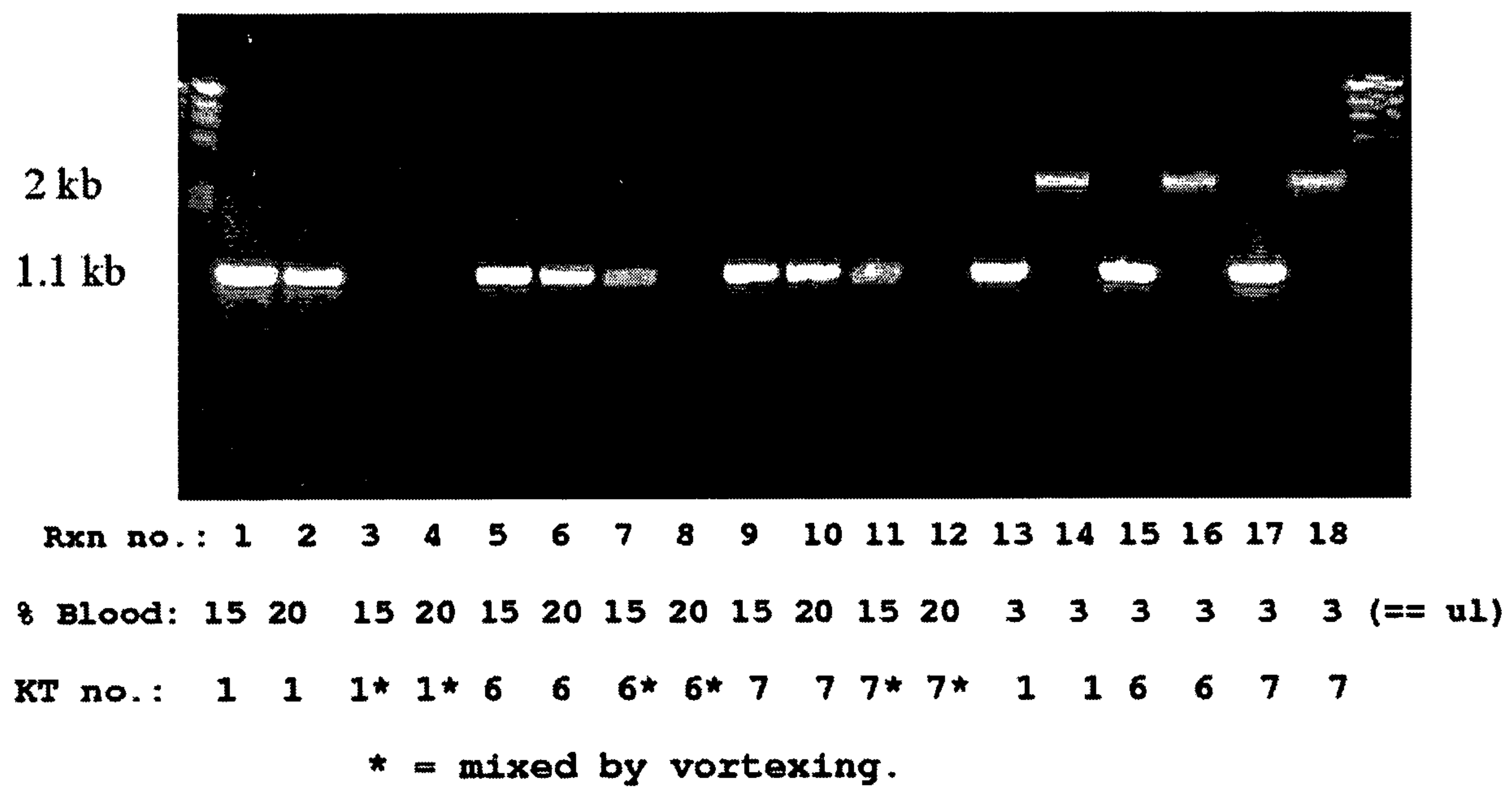


Fig. 5A

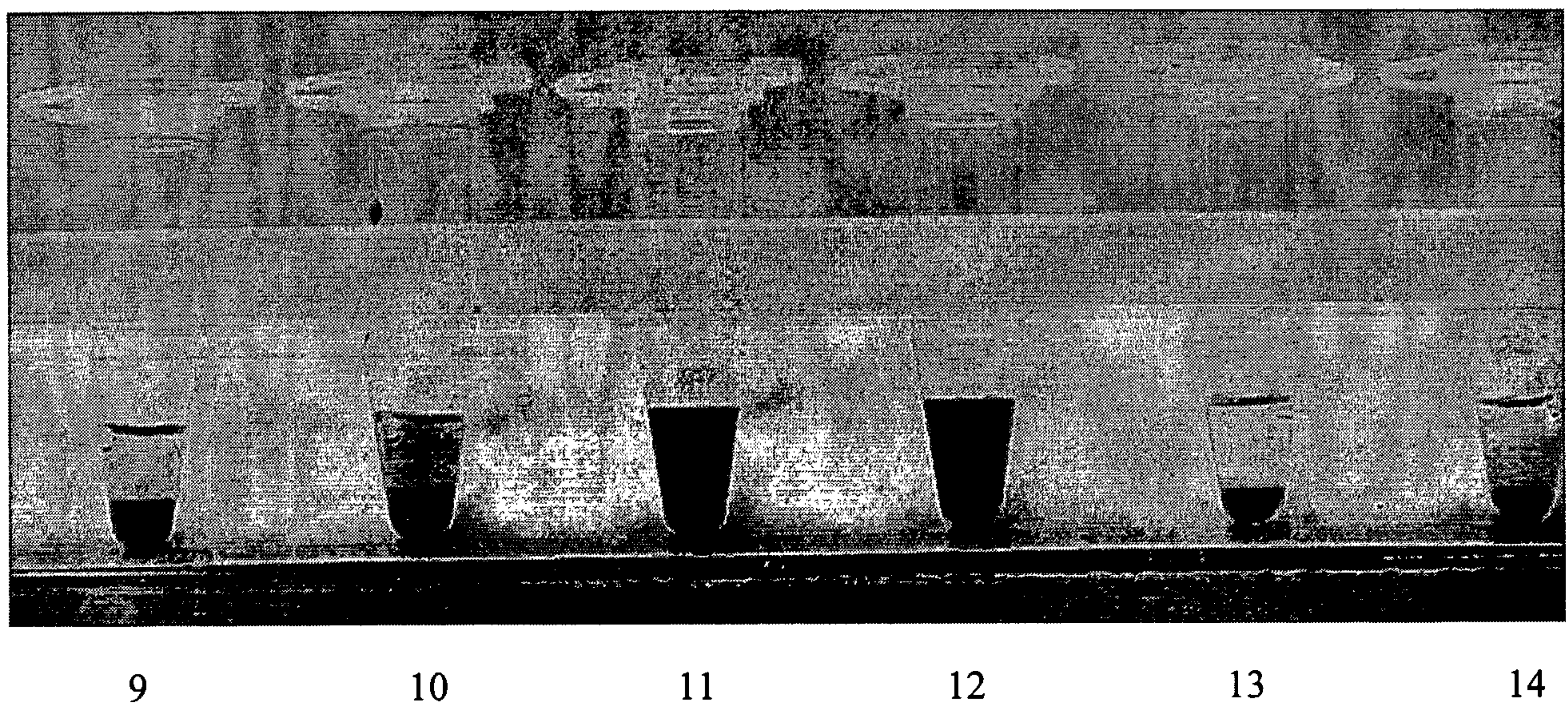


Fig. 5B

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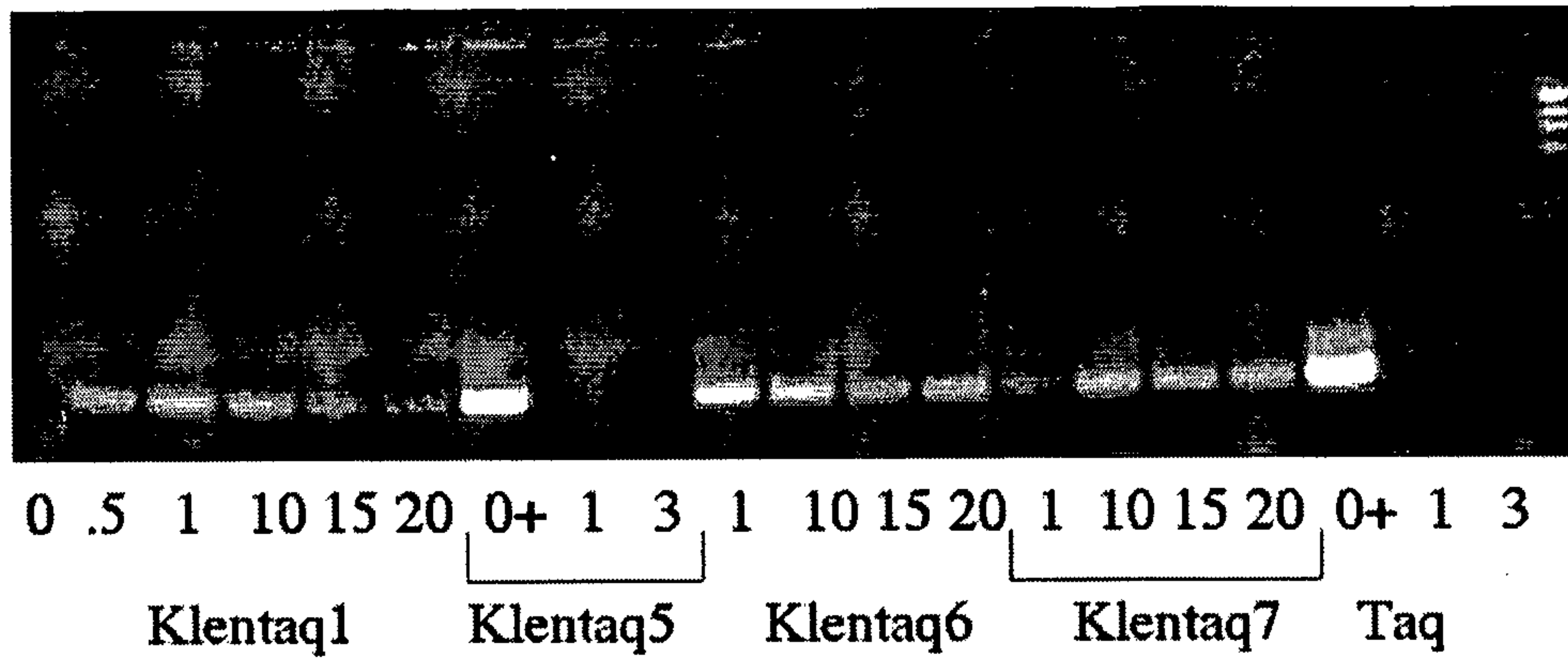


Fig. 6

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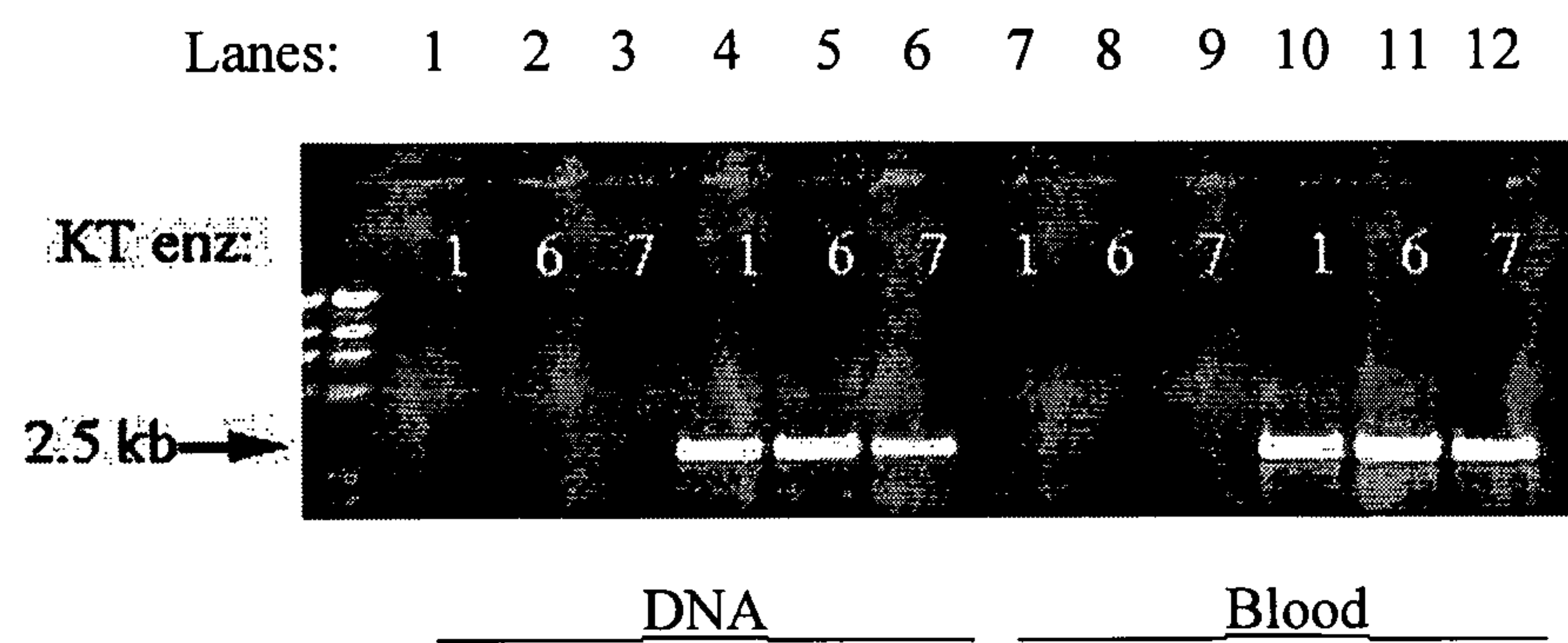


Fig. 7

M

Klentag clone number:

6 7

M

