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CA 2445864 C 2011/08/02

(11)(21) **2 445 864**

(12) **BREVET CANADIEN  
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2002/04/22

(87) Date publication PCT/PCT Publication Date: 2002/11/07

(45) Date de délivrance/Issue Date: 2011/08/02

(85) Entrée phase nationale/National Entry: 2003/10/29

(86) N° demande PCT/PCT Application No.: GB 2002/001861

(87) N° publication PCT/PCT Publication No.: 2002/088387

(30) Priorité/Priority: 2001/04/30 (GB0110501.4)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2006.01)

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(54) Titre : UN PROCESSUS D'AMPLIFICATION A CHAUD AU MOYEN DE PYROPHOSPHATASE

(54) Title: A HOT START AMPLIFICATION PROCESS USING PYROPHOSPHATASE ENZYME

(57) Abrégé/Abstract:

A method for conducting a nucleic acid amplification reaction, said method comprising forming an amplification reaction mixture in the presence of sufficient of a pyrophosphate salt to prevent primer extension taking place, digesting said pyrophosphate salt with a pyrophosphatase enzyme (PPase), and subjecting said reaction mixture to conditions such that an amplification reaction may proceed. This can be used as a "hot start" amplification. Particular novel pyrophosphatase enzymes for use in the method are also described and claimed.

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
7 November 2002 (07.11.2002)

PCT

(10) International Publication Number  
**WO 02/088387 A2**

(51) International Patent Classification<sup>7</sup>: **C12Q 1/68**

(21) International Application Number: PCT/GB02/01861

(22) International Filing Date: 22 April 2002 (22.04.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
0110501.4 30 April 2001 (30.04.2001) GB

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: AMPLIFICATION PROCESS

(57) Abstract: A method for conducting a nucleic acid amplification reaction, said method comprising forming an amplification reaction mixture in the presence of sufficient of a pyrophosphate salt to prevent primer extension taking place, digesting said pyrophosphate salt with a pyrophosphatase enzyme (PPase), and subjecting said reaction mixture to conditions such that an amplification reaction may proceed. This can be used as a "hot start" amplification. Particular novel pyrophosphatase enzymes for use in the method are also described and claimed.



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## A Hot Start Amplification Process using Pyrophosphatase Enzyme

The present invention relates to processes for carrying out reactions in which nucleic acids are amplified, to means of  
5 controlling these reactions and kits and reagents, in particular enzymes, used for conducting them.

Amplification reactions such as the polymerase chain reaction (PCR) are very well known and widely used in the fields of  
10 biotechnological research, as well as in diagnostics and detection.

PCR is a procedure for generating large quantities of a particular nucleic acid sequence, in particular a DNA sequence,  
15 and is based upon DNA's characteristics of base pairing and precise copying of complementary DNA strands. Typical PCR involves a cycling process of three basic steps.

*Denaturation* : A mixture containing the PCR reagents (including the nucleic acid to be copied, which may be DNA or RNA (the  
20 template), the individual nucleotide bases (A,T,G,C), suitable primers and polymerase enzyme) are heated to a predetermined temperature to separate the two strands of the target DNA.

*Annealing* : The mixture is then cooled to another predetermined temperature and the primers locate their complementary  
25 sequences on the DNA strands and bind to them.

*Extension* : The mixture is heated again to a further predetermined temperature. The polymerase enzyme (acting as a catalyst) joins the individual nucleotide bases to the end of the primer to form a new strand of DNA which is complementary  
30 to the sequence of the target DNA, the two strands being bound together.

Such reactions rely on the sequence of steps occurring in a very precise order and at the precise temperature required for the operation of that step. A problem arises when reagents are  
35 mixed together, even for short periods of time, at different

temperatures, for example prior to the start of the reaction. Primers may interact with nucleic acid template, resulting in primer extension of the template. This can lead to a reduction in the overall yield of the desired product as well as the  
5 production of non-specific products.

Various means of overcoming this problem have been proposed previously. For example, initial attempts to overcome the problem used a wax barrier to separate the various PCR reagents  
10 from each other in a test tube (see for example USP 5,565,339). The wax melted as the reaction mixture was heated to the initial denaturation temperature, allowing the reagents to mix together at the last possible moment, so that the possibility of side-reactions was minimised. Such reactions are known as  
15 "Hot Start" reactions.

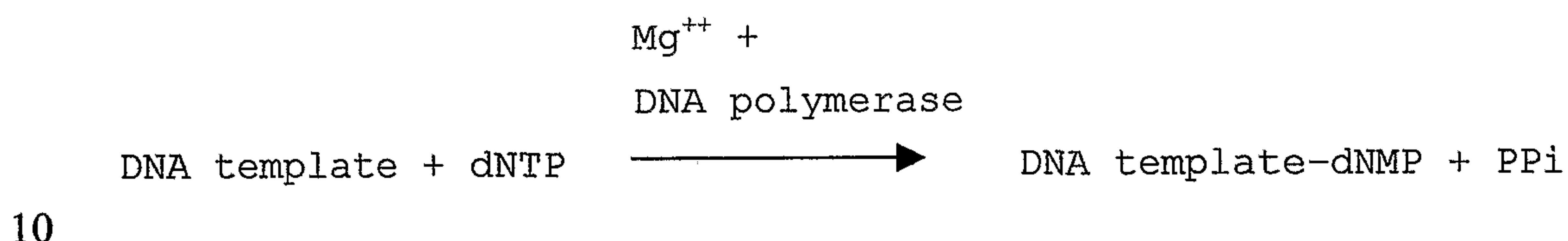
Other chemical methods for achieving the suppression of side-reactions have been attempted. For example, US Patent No. 5,677,152 describes a method in which the DNA polymerase is  
20 chemically modified to ensure that it only becomes active at elevated temperatures. In order to carry out this method however, it is necessary to incubate the reaction mixture at high temperatures for some time in order to generate active enzyme. Such delays, whilst not significant in some instances,  
25 can be detrimental where the results of PCR are required rapidly. For many applications of the PCR technique it is desirable to complete the sequence of cycles in the minimum possible time. In particular for example where respiratory air or fluids or foods for human and animal stock consumption are  
30 suspected of contamination rapid diagnostic methods may save considerable money if not health, even lives.

In other methods, a monoclonal antibody to *Thermus aquaticus* (Taq) DNA polymerase such as the anti-Taq DNA polymerase antibody available from Sigma, is introduced into the reaction  
35 mixture. The antibody binds to the enzyme, so as to inactivate it, at ambient temperature. However, the antibody denatures and dissociates from the enzyme at elevated temperatures used

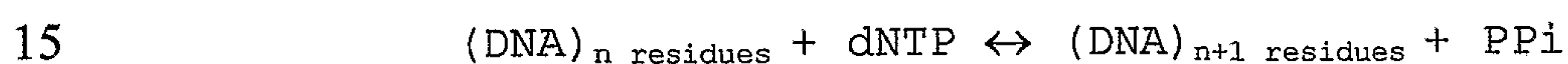
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during the amplification cycles and so the enzyme becomes active. The method however does not appear to eliminate non-specific side-products in some cases.

- 5 Primer extension of a template during a PCR reaction can be represented as:



where dNTP is a deoxyribonucleic acid triphosphates, dNMP is the corresponding deoxyribonucleic acid monophosphate and PPi is an inorganic pyrophosphate. This reaction may also be represented as



- The presence of increased levels of PPi, for example in a DNA sequencing reaction is known to force the reaction shown above in reverse. This is known as pyrophosphorolysis and it is a
- 20 recognised problem in DNA sequencing at 70°C using thermostable DNA polymerases. It has been overcome through the addition of a thermostable PPase to the DNA polymerase formulation used in DNA sequencing.

- 25 The applicants have found that this reaction can form the basis of an advantageous amplification reaction in which the production of non-specific products may be minimised.

- According to the present invention there is provided a method
- 30 for conducting a nucleic acid amplification reaction, said method comprising forming an amplification reaction mixture in the presence of sufficient of a pyrophosphate salt to prevent primer extension taking place, enzymatically digesting said pyrophosphate, and subjecting said reaction mixture to
- 35 conditions such that an amplification reaction may proceed.

Using the method of the invention, accurate amplification reactions, which may be carried out rapidly and with good specificity, can be carried out. It therefore represents a good alternative to existing "Hot Start" amplification technologies.

The initial amplification reaction mixture used in the method of the invention is broadly speaking, a conventional mixture, such as that used in the PCR reaction, to which pyrophosphate salt is added. Thus it will generally comprise: i) a sample which contains or is suspected of containing a target nucleic acid sequence, (ii) at least one primer which hybridises to an end region of said target sequence, iii) a source of magnesium ions, (iv) nucleotide or nucleoside bases which constitute the target sequence (i.e. A, T, C, G and/or U in the case of DNA amplification or A,U,C and G in the case of RNA amplification), and (v) a DNA polymerase which is thermostable at the temperatures at which the amplification reaction is effected. It will also comprise a buffer, as necessary in order to effect the reaction, as is known in the art.

In particular (iv) will comprise nucleotides A, T, G and C in respect of DNA amplification and nucleosides A, U, C and G in respect of RNA amplification.

Other combinations may be used however, where other primer based amplifications reactions such as reverse transcriptase PCR (RT-PCR) are being conducted.

In addition, the reagents may include labelled probes or primers, and/or other labelling means such as intercalating dyes such as Sybr Green, Sybr Gold, ethidium bromide etc. or combinations of these, which might allow the application to be monitored, without the need to examine the product on a gel subsequently. The nature of these depends upon the type of assay being undertaken. Generic intercalator methods use intercalating dyes to monitor the increase in double stranded

DNA which occurs during an amplification process. These are only quasi-strand-specific and therefore other labels are required where strand specific detection is required.

5 Strand specific methods utilise additional nucleic acid reaction components to monitor the progress of amplification reactions. These methods often use fluorescence energy transfer (FET) as the basis of detection. One or more nucleic acid probes are labelled with fluorescent molecules, one of  
10 which is able to act as an energy donor and the other of which is an energy acceptor molecule. These are sometimes known as a reporter molecule and a quencher molecule respectively. The donor molecule is excited with a specific wavelength of light which falls within its excitation spectrum and subsequently it  
15 will emit light within its fluorescence emission wavelength. The acceptor molecule is also excited at this wavelength by accepting energy from the donor molecule by a variety of distance-dependent energy transfer mechanisms. A specific example of fluorescence energy transfer which can occur is  
20 Fluorescence Resonance Energy Transfer or "FRET". Generally, the acceptor molecule accepts the emission energy of the donor molecule when they are in close proximity (e.g. on the same, or a neighbouring molecule). The basis of fluorescence energy transfer detection is to monitor the changes at donor and  
25 acceptor emission wavelengths.

There are two commonly used types of FET or FRET probes, those using hydrolysis of nucleic acid probes to separate donor from acceptor, and those using hybridisation to alter the spatial  
30 relationship of donor and acceptor molecules.

Hydrolysis probes are commercially available as TaqMan™ probes. These consist of DNA oligonucleotides that are labelled with donor and acceptor molecules. The probes are  
35 designed to bind to a specific region on one strand of a PCR product.

Following annealing of the PCR primer to this strand, *Taq* enzyme extends the DNA with 5' to 3' polymerase activity. *Taq* enzyme also exhibits 5' to 3' exonuclease activity. TaqMan™ probes are protected at the 3' end by phosphorylation to prevent them from priming *Taq* extension. If the TaqMan™ probe is hybridised to the product strand, an extending *Taq* molecule may also hydrolyse the probe, liberating the donor from acceptor as the basis of detection. The signal in this instance is cumulative, the concentration of free donor and acceptor molecules increasing with each cycle of the amplification reaction.

US Patent No. 5,491,063 describes a method for in-solution quenching of fluorescently labelled probes which relies on modification of the signal from a labelled single stranded oligonucleotide by a DNA binding agent. The difference in this signal which occurs as a result of a reduced chain length of the probe following probe cleavage (hydrolysis) during a polymerase chain reaction is suggested for providing a means for detecting the presence of a target nucleic acid.

Hybridisation probes are available in a number of forms. Molecular beacons are oligonucleotides that have complementary 5' and 3' sequences such that they form hairpin loops. Terminal fluorescent labels are in close proximity for FRET to occur when the hairpin structure is formed. Following hybridisation of molecular beacons to a complementary sequence the fluorescent labels are separated, so FRET does not occur, and this forms the basis of detection.

Pairs of labelled oligonucleotides may also be used. These hybridise in close proximity on a PCR product strand bringing donor and acceptor molecules together so that FRET can occur. Enhanced FRET is the basis of detection. Variants of this type include using a labelled amplification primer with a single adjacent probe.

US Patent No. 4,868,103 describes in general terms, a FRET system for detecting the presence of an analyte, which utilises an intercalating dye as the donor molecule. The process does not involve an amplification stage.

5

Other examples of assays which utilise FET or FRET detection are described in WO 99/28500, which utilises a combination of an intercalating dye and a single labelled probe as a signalling system, WO 99/28501 which utilises a combination of a labelled primer and an enzyme to generate a detectable fluorescent signal, and WO 99/42611 which uses a combination of an intercalating dye and a fluorescently labelled nucleotide as the basis of the signal. Yet further assays which utilise complex primers including labels and chemical blocking agents and which are complementary are described for example in WO 99/66071.

Reaction mixtures used in the method of the invention may include any of the labelling reagents necessary to conduct assays as described above. In particular, such reaction mixtures may advantageously be used in genotyping and, more especially, in SNP evaluation. In these instances, the method of the present invention is used in combination with dual Taqman<sup>TM</sup> probes, one specific for the basic sequence and one specific for the mutant. Each probe preferably contains a different flurophore and therefore different signals are generated depending on the amount of the various forms of the gene present. A single signal is generated from a homozygote and a mixed signal is generated from a heterozygote.

30

Examples of suitable DNA polymerases which may be used in the context of the invention are thermostable polymerases such as *Thermus aquaticus* polymerase (Taq), *Thermus thermophilus* polymerase (Tth), *Thermus species NH* polymerase (TspNH), *Thermus brockianus* polymerase (Tbr) (all obtainable for example from GeneSys Limited, Farnborough, U.K.), *Pyrococcus furiosus* polymerase (Pfu) (obtainable from Stratagene), 9°N7 exo-DNA

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polymerase, and *Thermococcus litoralis* DNA polymerase (obtainable from New England Biolabs as VENT™ DNA polymerase).

The pyrophosphate used in the method of the invention may be  
5 any soluble pyrophosphate including soluble metal and non-metal (e.g. ammonium salts). Such compounds are often generically known as "inorganic pyrophosphate" or PPi and this nomenclature is used in the present application. In particular, the pyrophosphate will be an alkali metal pyrophosphate, such as  
10 sodium or potassium pyrophosphates including disodium pyrophosphate ( $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ ), anhydrous tetrasodium pyrophosphate ( $\text{Na}_4\text{P}_2\text{O}_7$ ), tetrasodium pyrophosphate decahydrate ( $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) and tetrapotassium pyrophosphate (anhydrous). Other soluble pyrophosphates which may be used include iron pyrophosphates  
15 such as ferric pyrophosphate ( $\text{Fe}_4(\text{P}_2\text{O}_7)_3$ ), and soluble ammonium salts such as anhydrous tributylammonium pyrophosphate. Other soluble pyrophosphates are available from commercial sources.

A preferred inorganic pyrophosphate is tetrasodium  
20 pyrophosphate of formula  $\text{Na}_4\text{P}_2\text{O}_7$ .

The concentration of pyrophosphate used in the reaction mixture should be sufficient to prevent primer extension taking place. This will depend to a large extent upon the particular nature  
25 and concentration of the sequences being amplified, the primers and the polymerase enzymes being used, as well as their concentrations, and may be determined in any particular case by routine methods.

30 The reaction mixture formed initially suitably contains pyrophosphate at a concentration of at least 0.5mM, suitably at a concentration of least 1mM, for example from 1-10mM and preferably from 1-5mM.

35 Enzymatic digestion of the inorganic pyrophosphate is suitably effected immediately prior to or during first phase of the amplification reaction. This may be achieved by addition of an

pyrophosphatase enzyme (PPase) (which may be known as an inorganic pyrophosphatase enzyme - PPIase) immediately prior to the start of the amplification reaction.

- 5 Preferably, however, the enzymatic digestion is effected using a thermostable PPase, which is active at elevated temperatures, for example at temperatures in excess of 50°C. Preferably the enzyme is only significantly active at these elevated temperatures. This means that the PPase may be included in the  
10 reaction mixture on formation, but it will not or not significantly digest the inhibitory pyrophosphate at ambient temperature. It will only become properly active when the reaction mixture is heated as will be necessary for example during the initial denaturation phase of a PCR reaction.
- 15 However, a short preliminary incubation at elevated temperature, for example at from 50 to 100°C, and, preferably, at from 80 to 95°C, may be carried out.

- Examples of thermostable PPase include *Sulfolobus acidicaldarius*  
20 pyrophosphatase, (Sac PPase - Meyer et al. Archives of Biochem. and Biophys. (1995) 319, 1, 149-156) obtainable from GeneSys Limited, Farnborough UK., or *Thermococcus litoralis* pyrophosphatase, available from New England Biolabs (Catalogue nos #M0296S and #M0296L). Preferably the thermostable PPase is  
25 *Aeropyrum pernix* inorganic pyrophosphatase obtainable from Genesys Limited, Farnborough UK.

- Aeropyrum pernix* K1, the first strictly aerobic  
hyperthermophilic archaeon, was isolated in 1993 from a coastal  
30 solfataric thermal vent at Kodakara-Jima Island, Japan, (Sako et al, Int. J. Syst. Bacteriol. 46 (1996): 1070-1077. It is deposited in the Japan Collection of Microorganisms, JCM 9820.

- The applicants have for the first time isolated a thermostable  
35 PPase from *Aeropyrum pernix* and this forms a further aspect of the present invention. The genomic sequence comprising this pyrophosphatase is shown in SEQ ID NO. 1 and the corresponding

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amino acid sequence is shown in SEQ ID NO. 2 (Figure 11 hereinafter). In particular the enzyme of the invention has the amino acid sequence as shown as SEQ ID NO 25, which is encoded by the region of SEQ ID NO 1 shown in bold type in  
5 Figure 11, and represented also as SEQ ID NO 26.

The present invention, therefore, includes a polynucleotide comprising SEQ ID NO 26 and variants or fragments thereof. For example, the invention provides a polynucleotide of SEQ ID NO  
10 1.

The present invention further includes an amino acid sequence comprising SEQ ID NO 25 and variants or fragments thereof. For example, the amino acid sequence may comprise SEQ ID NO 2.  
15

The term "fragment thereof" as used herein in relation to a polynucleotide sequence refers to any portion of the given polynucleotide sequence which has the same activity as the complete polynucleotide sequence. Fragments will suitably  
20 comprise at least 300 and preferably at least 450 consecutive bases from the basic sequence.

The term "variant thereof" in relation to a polynucleotide sequences means any substitution of, variation of, modification  
25 of, replacement of deletion of, or the addition of one or more nucleic acid(s) from or to a polynucleotide sequence providing the resultant protein sequence encoded by the polynucleotide exhibits the same properties as the protein encoded by the basic sequence. The term therefore includes allelic variants  
30 and also includes a polynucleotide which substantially hybridises to the polynucleotide sequence of the present invention. Preferably, such hybridisation occurs at, or between low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SSC at about  
35 ambient temperature to about 55°C and high stringency condition as 0.1 x SSC at about 65°C. SSC is the name of the buffer of 0.15M NaCl. 0.015M tri-sodium citrate. 3 x SSC is three times

as strong as SSC and so on.

Typically, variants have 62% or more of the nucleotides in common with the polynucleotide sequence of the present invention, more typically 65%, preferably 70%, even more preferably 80% or 85% and, especially preferred are 90%, 95%, 98% or 99% or more identity.

When comparing nucleic acid sequences for the purposes of determining the degree of identity, programs such as BESTFIT and GAP (both from Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention when discussing identity of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

The term "fragment thereof" as used herein in relation to an amino acid sequence refers to any portion of the given amino acid sequence which has the same activity as the complete amino acid sequence. Fragments will suitably comprise at least 100 and preferably at least 150 consecutive amino acids from the basic sequence.

The term "variant thereof" as used herein in relation to an amino acid sequence means sequences of amino acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering

the biological activity of the polypeptide. Suitably variants will be at least 60% identical, preferably at least 75% identical, and more preferably at least 90% identical to the base sequence.

5

Homology in this instance can be judged for example using the algorithm of Lipman-Pearson, with Ktuple:2, gap penalty:4, Gap Length Penalty:12, standard PAM scoring matrix (Lipman, D.J. and Pearson, W.R., Rapid and Sensitive Protein Similarity

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Searches, Science, 1985, vol. 227, 1435-1441). Preferably, the polynucleotide of the present invention comprises SEQ ID NO 26 and sequences having greater than 62% identity thereto.

15

These enzymes may be obtained from the natural source, or may be expressed in recombinant host cells, such as E. coli cells, using conventional methods.

20 Removal of pyrophosphate for example, at >50°C by the action of a thermostable pyrophosphatase enzyme (PPase) then allows primer extension (and therefore amplification) to proceed as normal. During this process, 1mole of pyrophosphate is converted to 2moles of inorganic phosphate (Pi), which does not

25

The amount of pyrophosphatase included should be sufficient to digest excess pyrophosphate salt present in the reaction mixture. Generally speaking, this will be greater than the

30 amounts of these enzymes used conventionally in an equivalent cycle reaction to prevent pyrophosphorolysis, for example some 5 fold more. The precise amounts will depend upon various factors including the particular enzyme being used, the concentration of the pyrophosphate etc. Typically, PPase and

35 particularly thermostable PPase enzymes will be included in the amplification reaction mixture at concentrations of at least 0.04 units per 50µL PCR reaction mixture, preferably at least

0.08 units per 50 $\mu$ L PCR reaction mixture and more preferably from about 0.2-10 units per 50 $\mu$ L PCR reaction mixture. In this case, one unit is defined as the amount of enzyme catalysing the conversion of 1 $\mu$ mol pyrophosphate into 2 $\mu$ mol orthophosphate  
5 in one minute at 75°C under the following reaction conditions:  
1mM K<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2mM MgCl<sub>2</sub>, 50mM Tris-HCl, pH 9.0 (25°C).

Enzymes used in the method of the invention can result in rapid removal of inorganic pyrophosphate, depending upon the  
10 temperature being used. Generally complete removal can be achieved in less than 5 minutes, more often, in less than 2 minutes and as little as 15 seconds if required.

Once the inorganic pyrophosphate has been enzymatically removed  
15 from the reaction mixture, the amplification reaction can proceed, for example using a conventional thermal cycling procedure.

The mechanism by which the method of the invention achieves the  
20 desired result is not clear. It is probable that the presence of excess pyrophosphate inhibits the primer extension reaction. There appears, however, to be no noticeable decrease in PCR sensitivity or product yield.

25 The method of the invention can be conducted in any conventional apparatus for conducting application reactions. These include conventional block heating devices as described for example in EP-A-0810030 and supplied by The Perkin-Elmer Corporation, or rapid hot air thermal cyclers such as the  
30 RapidCycler™ and LightCycler™ from Idaho Technologies Inc. or other types of thermal cycler such as those described in WO98/24548.

According to a further aspect, the invention provides a kit for  
35 conducting an amplification reaction; said kit comprising an inorganic pyrophosphate, an inorganic pyrophosphatase enzyme,

and optionally one or more reagents required for use in an amplification reaction. The inorganic pyrophosphate is suitably present in a sufficient amount to inhibit an amplification reaction, as described above. Preferably the amount of inorganic pyrophosphatase enzyme present in the kit is sufficient to digest all of the said inorganic pyrophosphate.

The one or more reagents include any one of reagents (ii) to (v) listed above, and may also include buffers. Particular examples of inorganic pyrophosphatase enzymes are thermostable inorganic pyrophosphatase enzymes as described above.

In particular, the kits may suitably comprise as an optional additional reagent, one or more primers required to conduct amplification of a particular target DNA sequence, for example, a sequence, which is diagnostic of a particular disease condition or the presence of a particular pathogen in a sample. The methods may also be used in the detection of polymorphisms or allelic variations in genetic analysis.

Furthermore, the kits may comprise one or more labelled reagents such as intercalating dyes, or fluorescently labelled probes, primers or nucleotides, which may be useful in detecting or monitoring the amplification reaction in situ.

In a further aspect, the invention provides the use of an inorganic pyrophosphate as described above, in a method for carrying out amplification reactions as described above. Preferably, the inorganic pyrophosphatase enzyme is from *Aeropyrum pernix*.

Finally, in yet a further aspect, the invention provides the use of an inorganic pyrophosphatase enzyme as described above, in a method for carrying out amplification reactions as described above.

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In one aspect, the present invention relates to a method for inhibiting production of non-specific products in a nucleic acid amplification reaction, said method comprising forming an amplification reaction mixture in the presence of sufficient amount of a pyrophosphate salt to prevent primer extension taking  
5 place, digesting said pyrophosphate salt with a thermostable pyrophosphatase enzyme (PPase) which is only significantly active at temperatures in excess of 50°C, and subjecting said reaction mixture to conditions such that an amplification reaction may proceed.

In another aspect, the present invention relates to a kit for inhibiting  
10 production of non-specific products when conducting an amplification reaction, said kit comprising a pyrophosphate salt, a thermostable pyrophosphatase enzyme which is only significantly active at temperatures in excess of 50°C, and optionally one or more reagents required for use in an amplification reaction.

In still another aspect, the present invention relates to use of a  
15 thermostable pyrophosphatase enzyme which is only significantly active at temperatures in excess of 50°C, in the method as defined above.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which

5 Figure 1 shows the results of conducting a PCR in the presence of various amounts of PPI where PPI is tetrasodium pyrophosphate;

10 Figure 2 shows the effect of increasing  $MgCl_2$  in the absence and presence of 3mM PPI;

Figure 3 shows the results obtained using the method of the invention and conventional PCR reaction;

15 Figure 4 shows the results obtained using the method of the invention in an assay compared to a conventional PCR assay;

20 Figure 5 shows the results of an experiment to test the storage stability of PCR reaction mixtures used in the method of the invention, as compared to conventional mixtures;

Figure 6 shows the results of the use of a different PPase in the method of the invention;

25 Figure 7a and 7b and Figures 8a and 8b show the results of PCR experiments using the method of the invention and a variety of different DNA polymerases;

30 Figure 9 shows the results of an experiment comparing a conventional "Hot Start" PCR with the method of the invention;

Figure 10 shows the results obtained by carrying out a similar assay but using an alternative conventional PCR;

35 Figure 11 shows the genomic sequence of *Aeropyrum pernix* shown as SEQ ID NO. 1 (Referenced as NC 000854 in GenBank BA000002),

the corresponding amino acid sequence SEQ ID NO.2 and the sequence of the enzyme (SEQ ID NO 25);

Figure 12 shows an alignment of different PPase sequences (SEQ ID NOS 2 to 9), including the protein sequence of *Aeropyrum pernix* shown as SEQ ID NO. 2;

Figure 13 shows the 686 base pair PCR product (SEQ ID NO 10) produced during isolation of the pyrophosphatase enzyme from *Aeropyrum pernix*;

Figure 14 shows the polylinker sequence (SEQ ID NO 11) used in the isolation of the pyrophosphatase from *Aeropyrum pernix*;

Figure 15 shows the sequence of the pTTQ18NHK vector (SEQ ID NO 12) used in the isolation of the pyrophosphatase from *Aeropyrum pernix*;

Figure 16 shows the sequence (Z = stop) of the pTTQ18NHK vector including the PPase sequence used in the isolation of the pyrophosphatase from *Aeropyrum pernix* (SEQ ID NO 13); and

Figure 17 shows the results of the method of the invention using the inorganic pyrophosphatase from *Aeropyrum pernix*.

#### Example 1

##### Effect of PPi on PCR

Using *Taq* DNA polymerase, a standard 500bp lambda template PCR using the following reagents, was conducted in the presence of differing quantities of the inorganic pyrophosphate, tetrasodium pyrophosphate decahydrate (PPi).

| Reagent                | Volume | Final concn. |
|------------------------|--------|--------------|
| 10 x Reaction Buffer   | 5µl    | 1x           |
| 25mM MgCl <sub>2</sub> | 3µl    | 1.5mM        |
| 5mM dNTPs              | 2µl    | 200µM        |

17

|                              |              |            |
|------------------------------|--------------|------------|
| 5' primer (10pm/ $\mu$ l)    | 5 $\mu$ l    | 1 $\mu$ M  |
| 3' primer (10pm/ $\mu$ l)    | 5 $\mu$ l    | 1 $\mu$ M  |
| Template                     | 1ng          | Lambda DNA |
| DNA polymerase (5u/ $\mu$ l) | 0.25         | 1.25u      |
| Water to Total volume        | 50.0 $\mu$ l |            |

# Lambda 500bp Primer sequences

5' Primer GAT GAG TTC GTG TCC GTA CAA CTG G (SEQ ID NO 14)  
5 3' Primer GGT TAT CGA AAT CAG CCA CAG CGC C (SEQ ID NO 15)

1 x Reaction Buffer: 10mM Tris. pH 8.0, 50mM KCl.

PCR conditions for the assay were as follows:

- i) 94°C 3.00 min  
10 ii) 20 cycles of 94°C for 10 secs  
50°C for 10 secs  
72°C for 30 secs  
iii) 72°C for 7 mins  
iv) 25°C hold,

15

The PPI was added such that the final concentration in the reaction mixture was 0, 1, 2, 3, 4 and 5mM. The results are shown in Figure 1. In this Figure, the lanes correspond to the following concentrations of PPI

20

## Lanes

1 + 2 0 PPI  
3 + 4 1mM PPI  
5 + 6 2mM PPI  
7 + 8 3mM PPI  
9 + 10 4mM PPI  
11 5mM PPI

At all levels of PPI tested, no PCR product was produced.

Example 2Effect of increasing magnesium ion concentration

Mg binds to PPI and therefore it is possible that the observations of Example 1 are due to chelation of Mg by excess PPI. This would lead to insufficient Mg being present to allow primer extension to proceed. In order to eliminate this possibility, the procedure of Example 1 with 3mM PPI was repeated in the presence of various concentrations of magnesium ions.

10

The results are shown in Figure 2. In that Figure the lanes represent the following reactions:

**Lanes**

|         |                                   |
|---------|-----------------------------------|
| 1 + 2   | 1.5mM MgCl <sub>2</sub>           |
| 3 + 4   | 5mM MgCl <sub>2</sub>             |
| 5 + 6   | 7.5mM MgCl <sub>2</sub>           |
| 7 + 8   | 10mM MgCl <sub>2</sub>            |
| 9 + 10  | 1.5mM MgCl <sub>2</sub> + 3mM PPI |
| 11 + 12 | 5mM MgCl <sub>2</sub> + 3mM PPI   |
| 13 + 14 | 7.5mM MgCl <sub>2</sub> + 3mM PPI |
| 15      | 10mM MgCl <sub>2</sub> + 3mM PPI  |

The results show that the addition of Mg<sup>++</sup> up to 10mM final concentration (1.5mM is standard in a PCR) does not allow PCR to occur, suggesting that it is the PPI which is blocking primer extension.

Example 320 PCR reactions in the presence of Ppi and PPase

The 500bp lambda PCR of Example 1 was repeated, but this time, 0.2u of *Sulfolobus acidocaldarius* PPase (Sac PPase) was included in reactions containing pyrophosphate (PPI). Incubating the reaction at 95°C for 5 mins in the presence of 0.2u of Sac PPase was sufficient to destroy the pyrophosphate so that the PCR reaction could proceed.

25

19

The results are shown in Figure 3 where the lanes represent the following reactions:

**Lanes****Top Row**

|        |                      |
|--------|----------------------|
| 1 + 2  | 1mM PPI + 0.2u PPase |
| 3 + 4  | 2mM PPI + 0.2u PPase |
| 5 + 6  | 3mM PPI + 0.2u PPase |
| 7 + 8  | 4mM PPI + 0.2u PPase |
| 9 + 10 | 5mM PPI + 0.2u PPase |

**Bottom Row**

|         |         |
|---------|---------|
| 1 + 2   | 1mM PPI |
| 3 + 4   | 2mM PPI |
| 5 + 6   | 3mM PPI |
| 7 + 8   | 4mM PPI |
| 9 + 10  | 5mM PPI |
| 11 + 12 | 0mM PPI |

5

A comparable level of PCR product was generated when compared to the reaction without both PPI and PPase.

10 The example was repeated using concentrations of PPI of less than 1mM. Results (not shown) indicated that 0.4mM PPI did not completely suppress the PCR, but no PCR occurred at concentrations of 0.6mM

Example 415 PCR Assay

The method of the invention was then applied to an assay system that requires a "HotStart" reaction in order to generate a PCR product of the correct size.

20 The assay is based around the amplification of a 321bp fragment of the human angiotensin gene. It has been recognised that the assay will only generate the correct amplification product in

20

the presence of betaine (EP-A-0962526 - see in particular Example 8).

Without betaine a HotStart DNA polymerase generates few non-specific amplification products or no products at all whereas a  
5 non-HotStart DNA polymerase PCR generates a large number of non-specific amplification products.

The PCR conditions used in the Angiotensin assay can be  
10 summarised as follows.

| Reagent                      | Volume                       | Final concn.     |
|------------------------------|------------------------------|------------------|
| 10x Reaction Buffer          | 5 $\mu$ l                    | 1x               |
| 25mM MgCl <sub>2</sub>       | 3 $\mu$ l                    | 1.5mM            |
| 5mM dNTPs                    | 2 $\mu$ l                    | 200 $\mu$ M      |
| 5' primer (100 $\mu$ M)      | 0.25                         | 0.5/ $\mu$ M     |
| 3' primer (100 $\mu$ M)      | 0.25                         | 0.5/ $\mu$ M     |
| Template 100ng/ $\mu$ l      | 50ng                         | Human xsomal DNA |
| 5M Betaine                   | 10.0                         | 1M               |
| DNA polymerase (5u/ $\mu$ l) | 0.25                         | 1.25u            |
| <b>Water to Total volume</b> | <b>50.0<math>\mu</math>l</b> |                  |

Angiotensin primer sequences

15 5' Primer GCA ACG CCC CTC ACT ATA AA (SEQ ID NO 16)  
3' Primer GCA CCC CGC CCT TGA AGT CC (SEQ ID NO 17)

1 x Reaction Buffer: 10mM Tris. pH 8.0, 50mM KCl.

PCR conditions for the assay were as follows:

20 i) 95°C 2.00 min or less  
ii) 35 cycles of 95°C for 15 secs  
50°C for 30 secs  
72°C for 30 secs  
iii) 72°C for 7 mins  
25 iv) 25°C hold

The reaction was conducted using a PE9700 Instrument in the presence of 3mM PPI and 0.2u PPase as described in Example 3.

5 The results are shown in Figure 4 in which the Lanes shown represent the following reactions.

#### Lanes

- 1 Standard *Taq* polymerase PCR - without betaine - lots of false priming
- 2 Standard *Taq* polymerase PCR - with betaine - bright band is correct product - with some false priming
- 3 Standard *Taq* polymerase PCR - without betaine but plus 3mM PPI and 0.2u Sac PPase - No false priming at all - 5mins denaturation at 95°C
- 4 Standard *Taq* polymerase PCR - with betaine but plus 3mM PPI and 0.2u Sac PPase - only correct product - 5mins denaturation at 95°C
- 5 + 6 As per 3 but only 2 mins denaturation at 95°C
- 7 + 8 As per 4 but only 2 mins denaturation at 95°C

10 It is clear that using the method of the invention, an effective "HotStart" reaction is achieved. A clear single product band was obtained using PPI and Sac PPase in the presence of betaine. In addition, no false priming was seen, even in the absence of betaine.

#### Example 5

##### 15 Effects of Storage at Ambient Temperature

The effect of leaving a PCR mixture containing 0.2u Sac PPase and 3mM PPI at room temperature 20°C for various lengths of time prior to conducting the Angiotensin assay, was investigated. Although Sac PPase is a thermostable enzyme, it was possible that there would be a small level of enzyme activity at ambient temperatures. This might lead to insufficient PPI in the reaction to inhibit/stop the DNA polymerase leading to primer extension and lack of "HotStart" functionality.

20

The method of Example 4 was repeated but the reaction mixtures were stored at ambient temperature for various lengths of time up to 2 hours prior to conducting the assay.

5 The results are shown in Figure 5 in which:

The Top Row - shows the results of a conventional *Taq* polymerase PCR of angiotensin (with and without betaine present) following incubation of reagents at room temperature  
10 for the time shown; and

The Bottom Row shows the results of a similar set of assays in accordance with the method of the invention where, in all cases, the assay mix contained 3mM PPI and 0.2u PPase per 50µl  
15 PCR.

| Lanes   | Presence of betaine | Time at 22°C (Room Temp) |
|---------|---------------------|--------------------------|
| 1 + 2   | -                   | 0                        |
| 3 + 4   | +                   | 0                        |
| 5 + 6   | -                   | 30 mins                  |
| 7 + 8   | +                   | 30 mins                  |
| 9 + 10  | -                   | 60 mins                  |
| 11 + 12 | +                   | 60 mins                  |
| 13 + 14 | -                   | 120 mins                 |
| 15 + 16 | +                   | 120 mins                 |

Even after two hours, assay conducted in accordance with the present invention functioned as expected, suggesting there is  
20 insufficient ambient temperature digestion of the PPI by the Sac PPase.

The result shown in Figure 5 showed that a 2 hour incubation of the PCR mix at room temperature, prior to PCR, had no effect on  
25 the specificity providing PPI and Sac PPase was used.

Example 6Use of other thermostable PPase enzymes in the method of the invention

The assay described in Example 4 was repeated alongside a  
 5 similar reaction using a different commercially available  
 thermostable PPase (with different unit definition of activity)  
 in place of Sac PPase. The results are shown in Figure 6 in  
 which the lanes represent the following reactions:

**Lanes**

|         |                                                                                                                       |
|---------|-----------------------------------------------------------------------------------------------------------------------|
| 1 + 2   | Standard <i>Taq</i> polymerase PCR - without betaine                                                                  |
| 3 + 4   | Standard <i>Taq</i> polymerase PCR - with betaine                                                                     |
| 5 + 6   | Standard <i>Taq</i> polymerase PCR - without betaine but plus<br>3mM PPI and 0.2u Sac PPase                           |
| 7 + 8   | Standard <i>Taq</i> polymerase PCR - with betaine plus 3mM PPI<br>and 0.2u Sac PPase                                  |
| 9 + 10  | Standard <i>Taq</i> polymerase PCR - without betaine but plus<br>3mM PPI and 10u* <i>Thermococcus litoralis</i> PPase |
| 11 + 12 | Standard <i>Taq</i> polymerase PCR - with betaine plus 3mM PPI<br>and 10u* <i>Thermococcus litoralis</i> PPase        |

10

\* Units used in this case were as supplied by the manufacturer  
 and are defined as the amount of enzyme that will generate  
 40nmols of phosphate per minute under standard reaction  
 conditions (10 minute reaction at 75°C in 50mM Tricine [pH  
 15 8.5], 1mM MgCl<sub>2</sub>, 0.32mM PPI, reaction volume of 0.5ml).

*Thermococcus litoralis* PPase (available from New England  
 Biolabs) appears to have the same effect as Sac PPase in this  
 assay.

20

Example 7Use of different thermostable DNA polymerases in the method of the invention

A variety of thermostable DNA polymerases were employed in the  
 25 method of the invention and some comparative assays. These  
 included several non-proofreading *Thermus* sp. DNA polymerases,

proof-reading hyperthermophilic archael DNA polymerases and mixes of non-proofreading and proofreading DNA polymerases.

They were all tested using the 500bp lambda PCR as described in Example 1 (Figure 7a and 7b), and several using the Angiotensin assay as described in Example 4 (Figure 8a and 8b).

Details of the assay conditions are summarised as follows:

#### 10 Figure 7a - *Thermus* DNA polymerases

##### Lanes

##### Top Row

|         |                                                  |
|---------|--------------------------------------------------|
| 1 + 2   | <i>Taq</i> polymerase 0mM PPi and no PPase       |
| 3 + 4   | <i>Taq</i> polymerase 3mM PPi and no PPase       |
| 5 + 6   | <i>Taq</i> polymerase 3mM PPi and 0.2u Sac PPase |
| 7 + 8   | <i>Tbr</i> polymerase 0mM PPi and no PPase       |
| 9 + 10  | <i>Tbr</i> polymerase 3mM PPi and no PPase       |
| 11 + 12 | <i>Tbr</i> polymerase 3mM PPi and 0.2u Sac PPase |

##### Bottom Row

|         |                                                    |
|---------|----------------------------------------------------|
| 1 + 2   | <i>Tth</i> polymerase 0mM PPi and no PPase         |
| 3 + 4   | <i>Tth</i> polymerase 3mM PPi and no PPase         |
| 5 + 6   | <i>Tth</i> polymerase 3mM PPi and 0.2u Sac PPase   |
| 7 + 8   | <i>TspNH</i> polymerase 0mM PPi and no PPase       |
| 9 + 10  | <i>TspNH</i> polymerase 3mM PPi and no PPase       |
| 11 + 12 | <i>TspNH</i> polymerase 3mM PPi and 0.2u Sac PPase |

Figure 7b - Archaeal Proof-reading DNA polymerases

**Lanes****Top Row**

|         |                                                       |
|---------|-------------------------------------------------------|
| 1 + 2   | <i>Pfu</i> polymerase 0mM PPi and no PPase            |
| 3 + 4   | <i>Pfu</i> polymerase 3mM PPi and no PPase            |
| 5 + 6   | <i>Pfu</i> polymerase 3mM PPi and 0.2u Sac PPase      |
| 7 + 8   | 9°N <i>exo-</i> polymerase 0mM PPi and no PPase       |
| 9 + 10  | 9°N <i>exo-</i> polymerase 3mM PPi and no PPase       |
| 11 + 12 | 9°N <i>exo-</i> polymerase 3mM PPi and 0.2u Sac PPase |

**Bottom Row**

|       |                                            |
|-------|--------------------------------------------|
| 1 + 2 | VENT polymerase 0mM PPi and no PPase       |
| 3 + 4 | VENT polymerase 3mM PPi and no PPase       |
| 5 + 6 | VENT polymerase 3mM PPi and 0.2u Sac PPase |

Fig 8a Angiotensin assay without PPi and without Sac PPase  
(with and without Betaine)**Lanes**

|         |                                       |
|---------|---------------------------------------|
| 1 + 2   | <i>Taq</i> polymerase without betaine |
| 3 + 4   | <i>Taq</i> polymerase with betaine    |
| 5 + 6   | Accurase polymerase without betaine   |
| 7 + 8   | Accurase polymerase with betaine      |
| 9 + 10  | <i>Tbr</i> polymerase without betaine |
| 11+ 12  | <i>Tbr</i> polymerase with betaine    |
| 13 + 14 | <i>Tth</i> polymerase without betaine |
| 15 + 16 | <i>Tth</i> polymerase with betaine    |

Fig 8b Angiotensin assay with PPi and Sac PPase (with and  
5 without Betaine)

Control Lanes 1-4 (Top Row) and 12-16 (Bottom Row)

# **Lanes**

## **Top Row**

1 + 2      *Taq* polymerase without betaine but plus 3mM PPi -

## **No Sac PPase**

3 + 4      *Taq* polymerase with betaine but plus 3mM PPi - -

## **No Sac PPase**

## **All below with 3mM PPi and 0.2u Sac PPase**

5 + 6      *Taq* polymerase without betaine

7 + 8      *Taq* polymerase with betaine

9 + 10      Accurase polymerase without betaine

11 + 12      Accurase polymerase with betaine

13 + 14      *Tbr* polymerase without betaine

15 + 16      *Tbr* polymerase with betaine

## **Bottom Row**

## **All below with 3mM PPi and 0.2u Sac PPase**

1 + 2      *Tth* polymerase without betaine

3 + 4      *Tth* polymerase with betaine

5 + 6      *TspNH* polymerase without betaine

7 + 8      *TspNH* polymerase with betaine

9 + 10      *Pfu* polymerase without betaine

11 + 12      *Pfu* polymerase with betaine

13 + 14      *Taq* polymerase control without betaine and no PPi  
or PPase

15 + 16      *Taq* polymerase control with betaine and no PPi or  
PPase

All DNA polymerases tested were inhibited by PPi and that inhibition could be overcome with Sac PPase.

Comparative Example 8Comparison of method of invention with conventional "Hotstart" methodologies

We have some initial results (Figure 9 and 10) that show that a  
 5 chemically modified *Taq* polymerase (modified as described in US  
 Patent No 5,677,152) does generate some false PCR products in  
 the absence of betaine but gives the correct product in the  
 presence of betaine.

## 10 Figure 9 Angiotensin assay

**Lanes**

|         |                                                                       |
|---------|-----------------------------------------------------------------------|
| 1 + 2   | <i>Taq</i> polymerase without betaine                                 |
| 3 + 4   | <i>Taq</i> polymerase with betaine                                    |
| 5 + 6   | Chemically modified <i>Taq</i> without betaine                        |
| 7 + 8   | Chemically modified <i>Taq</i> with betaine                           |
| 9 + 10  | Method of the invention (3mM PPI and 2u Sac PPase)<br>without betaine |
| 11 + 12 | Method of the invention (3mM PPI and 2u Sac PPase)<br>with betaine    |

It appears that under these circumstances, the chemically  
 modified enzyme is inactive until it has a 10 min activation at  
 15 95°C. Without this preliminary incubation, negligible PCR  
 product was generated. The apparent false priming and  
 generation of wrong PCR products in the absence of betaine is  
 difficult to explain however, since the chemically modified *Taq*  
 is inactive at room temperature.

20

Figure 10 Angiotensin assay with *Taq* and anti-*Taq* antibody**Lanes**

|       |                                                                      |
|-------|----------------------------------------------------------------------|
| 1 + 2 | Anti- <i>Taq</i> antibody plus <i>Taq</i> polymerase without betaine |
| 3 + 4 | Anti- <i>Taq</i> antibody plus <i>Taq</i> polymerase with betaine    |

In an anti-*Taq* DNA polymerase antibody mediated HotStart, a  
 25 substantial number of false products are generated in the

absence of betaine (similar to a standard *Taq* polymerase PCR without betaine) and a minor false product is also generated along with the correct product in the presence of betaine.

- 5 The method of the invention appears to give a rapid PCR reaction which is more specific than both of these commercial HotStart methodologies.

#### Example 9

- 10 Isolation of inorganic pyrophosphatase from *Aeropyrum pernix*  
*Aeropyrum pernix* was obtained from the J.C.M. culture collection. The inorganic pyrophosphatase enzyme was cloned, expressed and purified.

- 15 Cloning and expression of inorganic pyrophosphatase from *A. pernix*

The genome sequence comprising the pyrophosphatase gene of *Aeropyrum pernix* is shown in Figure 11. The primers used were designed from the genome sequence of *Aeropyrum pernix*. These  
 20 are shown below as 5' to 3' with the restriction sites shown in bold.

|                                                                 |
|-----------------------------------------------------------------|
| Upper primer, introducing the <i>Nde</i> I site: (SEQ ID NO 18) |
|-----------------------------------------------------------------|

|                                          |
|------------------------------------------|
| TGCATG <b>CATATG</b> ACAGGCTGTCTGAAAATTG |
|------------------------------------------|

|                                                                    |
|--------------------------------------------------------------------|
| Lower primer, introducing the <i>Hind</i> III site: (SEQ ID NO 19) |
|--------------------------------------------------------------------|

|                                            |
|--------------------------------------------|
| TAAGTGT <b>AAGCTT</b> GACTGTGGGGGCGGTGAAAG |
|--------------------------------------------|

- Aligning the putative sequence from the genome with other  
 25 pyrophosphates genes suggested that a later ATG should be the start methionine and not the one shown in the databank (shown in italics in SEQ ID NO.1 in Figure 11) and that the amino acid sequence of the enzyme is, in fact, as shown in SEQ ID NO 25. Primers were therefore designed corresponding to the later  
 30 methionine (shown in bold in SEQ ID NO.1 in Figure 11).

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A PCR was run using 100ng of the *Aeropyrum pernix* DNA in a 100µl volume with 50pM of the above primers. 20 cycles were run with 55°C annealing and a 45 second extension time.

5

Initial hold of 3mins at 94°C

20 Cycles of 94°C, 10 secs, 55°C, 10 secs, 68°C, 45 secs.

10 Final hold of 72°C 7 minutes

PCR conditions.

50pM Upper Primer (5'..TGCATGCATATGACAGGCTGTCTGAAAATTG..3'-SEQ ID NO 18)

15 50pM Lower Primer (5'..TAAGTGTAAGCTTGACTGTGGGGGCGGTGAAAG..3' - SEQ ID NO 19)

1.5mM MgCl<sub>2</sub>

1.25u Accurase DNA polymerase (Cat. No. AC001, GeneSys Ltd.)

75mM Tris, pH 8.8

20 20mM Ammonium sulphate

0.1% (w/v) Tween20\*

100ng *Aeropyrum pernix* genomic DNA

The PCR product was 686 base pairs long as shown in Figure 13.

25 The PCR product was Prepanol™ (Cat. No. P001, GeneSys Ltd.) precipitated following the manufacturers recommended conditions and finally re-suspended in 10mM Tris, 0.1mM EDTA.

The PCR product was digested with restriction enzymes Nde I and

30 Hind III, phenol extracted, precipitated with ethanol and re-suspended in 10mM Tris, 0.1mM EDTA.

pTTQ18NHK vector (shown in Figure 15) had also been digested with Nde I and Hind III, phenol extracted, ethanol precipitated  
35 and re-suspended in 10mM Tris, 0.1mM EDTA.

100ng cut PCR sequence was ligated with 1µg of cut pTTQ18NHK

\* Trade-mark

vector (see Figure 16) in a total volume of 10 $\mu$ l, overnight at 16°C in 1x NEB ligation buffer using 200u of New England Biolabs T4 DNA ligase. The plasmid vector was pTTQ18N HK, a modified form of vector pTTQ18 (Stark MJ, Gene, 1987; 51(2-3):255-67) containing a kanamycin antibiotic gene inserted at the unique Eco0109 I restriction enzyme site and a replacement polylinker (see Figure 14) inserted between the EcoR I site and Hind III site of the original vector.

20 $\mu$ l of water was added and the reaction heated to 70°C for 20mins. 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volumes of ethanol added. It was mixed and stored at -20°C for 1 hour. After microfuging at 10,000g for 10mins, the supernatant was removed from the pelleted DNA and the DNA re-suspended in 5 $\mu$ l water.

0.5 $\mu$ l was electroporated into *E.coli* TOP10F' cells and following 1 hour recovery at 37°C, aliquots of the cells were plated on Kanamycin Luria Broth agar plates. The plates were incubated at 37°C overnight.

Colonies were gridded in duplicate on both a fresh Kanamycin Luria Broth agar plate and a Kanamycin Luria Broth agar plate prepared by addition of 1 $\mu$ l of 20mg/ml XGAL and 1 $\mu$ l of 0.5M IPTG per ml of agar gel (KIX plate).

Following overnight incubation at 37°C, white colonies on the KIX plate were screened by PCR with M13 forward and reverse primers for the presence of an insert corresponding to the *Aeropyrum pernix* PCR product.

9 colonies containing a 701bp product were grown up in 20ml LB plus 100 $\mu$ g/ml Kanamycin to an OD600 of 1.0 then expression was induced by addition of IPTG to 0.5mM final. Cells were grown for a further 4hours and then the cells harvested and stored frozen at -20°C.

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Cells were lysed by addition of 0.5ml 50mM Tris-HCl, pH 7.9, 50mM dextrose, 1mM EDTA and 0.5ml 10mM Tris-HCl, pH7.9, 50mM KCl, 1mM EDTA, 0.5% v/v) Tween 20, 0.5% (v/v) Nonidet-P40 and incubation at 80°C for 15 minutes.

5

Following centrifugation at 10,000g for 10 minutes at room temperature, an aliquot from each lysed cells were analysed by SDS polyacrylamide gel electrophoresis using a 12% gel. The gel was run then stained with Coomassie blue R250. All samples showed a band of approx 23kDa, which corresponds to the size of the putative PPase.

The same samples were then assayed for PPase activity at 75°C using the colorimetric assay of Jukka K. Heinonen, Reijo J. Lahti. (1981) Analytical Biochemistry, Vol.113, pp313-317.

All samples showed as positive, confirming that the expressed protein possessed thermophilic inorganic pyrophosphatase activity.

20

The first clone was subsequently used for larger scale production of the protein.

#### Purification of the Pyrophosphatase

This clone was in 24 litres of LB. Once the OD<sub>600</sub> reached approximately 1.5, the culture was induced with 0.5mM IPTG and left to grow for a further 4 hours. The cells were then harvested and the cell pellet lysed. The expressed enzyme was purified by standard column chromatography on phenyl-sepharose CL4B (Amersham Pharmacia Biotech), hydroxylapatite (Bio-rad Laboratories) and Hi-Performance Q Sepharose\* (Amersham Pharmacia Biotech), finally being stored at -20°C in 20mM Tris-HCl, pH 8.0, 100mM NaCl, 0.5% (v/v) Tween 20, 0.5% (v/v) Nonidet P40, 0.1mM EDTA, 1mM dithiothreitol and 50% glycerol.

\* Trade-mark

Example 10PCR Assay using the *A. pernix* inorganic pyrophosphatase enzyme

The method of the present invention was carried out using the *A. pernix* inorganic pyrophosphatase enzyme. The assay is based  
 5 around the amplification of the human B-actin gene.

In this assay, a kit was used which was obtained from from Eurogentec S.A., Parc Scientifique du Sart-Tilman, rue Bois Saint-Jean 14, 4102 SERAING, Belgium (Cat. No. RT-QP73-05).  
 10 The standard Taq polymerase was substituted for the HotStart Taq polymerase provided with the kit.

**PCR reaction mixture**

1x Reaction Buffer  
 15 200μM, dATP, dCTP, dGTP and 400μM dUTP  
 0.025u/μl unmodified Taq polymerase  
 0.002u/μl Aeropyrum pernix inorganic pyrophosphatase  
 0.3μM 5' Primer (5' GAC TCG TCA TAC TCC TGC TTG CT 3' - SEQ ID NO 22)  
 20 0.3μM 3' Primer (5' CAT TGC CGA CAG GAT GCA GAA 3' - SEQ ID NO 23)  
 0.15μM Taqman probe (FAM-ATCCACATCTGCTGGAAGGTGGACAGT-TAMRA - SEQ ID NO 24)  
 5mM MgCl<sub>2</sub>  
 25 2mM NaPPi  
 Passive Reference  
 1 in 4 dilutions of Human genomic DNA starting with 7.5ng (2500 copies)

**30 Cycling conditions**

Initial denaturation of 94°C 3 minutes

40 cycles of 94°C, 15 seconds and 60°C, 60 seconds

35

The results are shown in Figure 19.

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33

In conclusion we believe that using the method of the invention, by using pyrophosphate to inhibit a PCR and then removing that inhibition, for example at 80°C-95°C through the use of a thermostable PPase, behaves in the same manner as  
5 HotStart PCR but at a rapid rate with the additional benefit of increased specificity.

Other modifications of the present  
10 invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with the specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such  
15 specific embodiments. Indeed, various modifications of the described modes for carrying out the invention, which are obvious to those skilled in the art, are intended to be within the scope of the following claims.

## SEQUENCE LISTING

<110> The Secretary of State for Defence in Her Britannic Majesty's  
Government of the United Kingdom of Great Britain and  
Northern Ireland  
Clark, Duncan Roy  
Vincent, Suzanne P

<120> Amplification process

<130> CG/P/133/WOD

<140>

<141>

<150> GB 0110501.4

<151> 2001-04-30

<160> 26

<170> PatentIn Ver. 2.1

<210> 1

<211> 960

<212> DNA

<213> Aeropyrum pernix

<400> 1

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gtctgaaaat tggtcctgga gatgaggctc cagatgttgt gaatgtcgtt atagagatac 180  
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2

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&lt;210&gt; 2

&lt;211&gt; 207

&lt;212&gt; PRT

&lt;213&gt; Aeropyrum pernix

&lt;400&gt; 2

Met Trp Thr Ile Leu Pro Ser Lys Thr Gly Phe Val Asn Ser Leu Ser  
1 5 10 15

Phe Ile Thr Arg Leu Ala Lys Leu Ser Val Arg Arg Val His Ala Met  
20 25 30

Thr Gly Cys Leu Lys Ile Gly Pro Gly Asp Glu Ala Pro Asp Val Val  
35 40 45

Asn Val Val Ile Glu Ile Pro Met Asn Ser Ser Val Lys Tyr Glu Phe  
50 55 60

Asp Lys Glu Ala Cys Ile Val Lys Val Asp Arg Phe Leu Tyr Thr Ser  
65 70 75 80

Met Val Tyr Pro Phe Asn Tyr Gly Phe Ile Pro Gly Thr Leu Glu Glu  
85 90 95

Asp Gly Asp Pro Val Asp Val Leu Val Ile Ser Arg Glu Pro Val Ala  
100 105 110

Pro Gly Ser Leu Ile Glu Ala Val Pro Val Ala Val Leu Asp Met Glu  
115 120 125

3

Asp Glu Glu Gly Pro Asp Ser Lys Val Val Ala Val Pro Lys Ala Lys  
130 135 140

Leu Asp Pro Leu Phe Ala Ser Tyr Lys Asp Val Gly Asp Ile Pro Asp  
145 150 155 160

Ala Leu Lys Ser Lys Ile Lys His Phe Phe Glu His Tyr Lys Glu Leu  
165 170 175

Glu Pro Gly Lys Trp Val Arg Val Thr Gly Trp Arg Pro Ala Ala Asp  
180 185 190

Ala Lys Glu Ile Ile Arg Arg Ala Ile Glu Arg Tyr Lys Gly Ala  
195 200 205

&lt;210&gt; 3

&lt;211&gt; 173

&lt;212&gt; PRT

&lt;213&gt; Sulfolobus solfataricus

&lt;400&gt; 3

Met Lys Leu Ser Pro Gly Lys Asn Ala Pro Asp Val Val Asn Val Leu  
1 5 10 15

Val Glu Ile Pro Gln Gly Ser Asn Ile Lys Tyr Glu Tyr Asp Asp Glu  
20 25 30

Glu Gly Val Ile Lys Val Asp Arg Val Leu Tyr Thr Ser Met Asn Tyr  
35 40 45

Pro Phe Asn Tyr Gly Phe Ile Pro Gly Thr Leu Glu Glu Asp Gly Asp  
50 55 60

Pro Leu Asp Val Leu Val Ile Thr Asn Tyr Gln Leu Tyr Pro Gly Ser  
65 70 75 80

4

Val Ile Glu Val Arg Pro Ile Gly Ile Leu Tyr Met Lys Asp Glu Glu  
85 90 95

Gly Glu Asp Ala Lys Ile Val Ala Val Pro Lys Asp Lys Thr Asp Pro  
100 105 110

Ser Phe Ser Asn Ile Lys Asp Ile Asn Asp Leu Pro Gln Ala Thr Lys  
115 120 125

Asn Lys Ile Val His Phe Phe Glu His Tyr Lys Glu Leu Glu Pro Gly  
130 135 140

Lys Tyr Val Lys Ile Ser Gly Trp Gly Ser Ala Thr Glu Ala Lys Asn  
145 150 155 160

Arg Ile Gln Leu Ala Ile Lys Arg Val Ser Gly Gly Gln  
165 170

&lt;210&gt; 4

&lt;211&gt; 176

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 4

Met Ser Leu Leu Asn Gly Pro Ala Gly Lys Asp Leu Pro Glu Asp Ile  
1 5 10 15

Tyr Val Val Ile Glu Ile Pro Ala Asn Ala Asp Pro Ile Lys Tyr Glu  
20 25 30

Ile Asp Lys Glu Ser Gly Ala Leu Phe Val Asp Arg Phe Met Ser Thr  
35 40 45

Ala Met Phe Tyr Pro Cys Asn Tyr Gly Tyr Ile Asn His Ile Leu Ser  
50 55 60

5

Leu Asp Gly Asp Pro Val Asp Val Leu Val Pro Thr Pro Tyr Pro Leu  
65 70 75 80

Gln Pro Gly Ser Val Ile Arg Cys Arg Pro Val Gly Val Leu Lys Met  
85 90 95

Thr Asp Glu Ala Gly Glu Asp Ala Lys Leu Val Ala Val Pro His Ser  
100 105 110

Lys Leu Ser Lys Glu Tyr Asp His Ile Lys Asp Val Asn Asp Leu Pro  
115 120 125

Glu Leu Leu Lys Ala Gln Ile Ala His Phe Phe Glu His Tyr Lys Asp  
130 135 140

Leu Glu Lys Gly Lys Trp Val Lys Val Glu Gly Trp Glu Asn Ala Glu  
145 150 155 160

Ala Ala Lys Ala Glu Ile Val Ala Ser Phe Glu Arg Ala Lys Asn Lys  
165 170 175

&lt;210&gt; 5

&lt;211&gt; 178

&lt;212&gt; PRT

&lt;213&gt; Aquifex aeolicus

&lt;400&gt; 5

Met Gly Tyr Asp Gln Leu Pro Pro Gly Lys Asn Pro Pro Glu Asp Ile  
1 5 10 15

Tyr Val Val Ile Glu Ile Pro Gln Gly Ser Ala Val Lys Tyr Glu Leu  
20 25 30

Asp Lys Asp Thr Gly Val Ile Phe Val Asp Arg Phe Leu Phe Thr Ala  
35 40 45

6

Met Tyr Tyr Pro Phe Asn Tyr Gly Phe Val Pro Gln Thr Leu Ala Asp  
50 55 60

Asp Gly Asp Pro Val Asp Val Leu Val Ile Ser Arg Glu Pro Val Val  
65 70 75 80

Pro Gly Ala Val Met Arg Cys Arg Pro Ile Gly Met Leu Glu Met Arg  
85 90 95

Asp Glu Ala Gly Ile Asp Thr Lys Val Ile Ala Val Pro His Glu Lys  
100 105 110

Leu Asp Pro Ser Tyr Ser Asn Ile Lys Thr Val Asp Asn Leu Pro Glu  
115 120 125

Ile Val Arg Glu Lys Ile Lys His Phe Phe Glu His Tyr Lys Glu Leu  
130 135 140

Glu Pro Gly Lys Trp Val Lys Val Glu Asn Trp Lys Gly Leu Gln Asp  
145 150 155 160

Ala Ile Glu Glu Ile Lys Lys Gly Ile Glu Asn Tyr Lys Lys Asn Lys  
165 170 175

Glu Gly

<210> 6

<211> 178

<212> PRT

<213> Pyrococcus horikoshii

<400> 6

Met Asn Pro Phe His Asp Leu Glu Pro Gly Pro Asn Val Pro Glu Val  
1 5 10 15

Val Tyr Ala Leu Ile Glu Ile Pro Lys Gly Ser Arg Asn Lys Tyr Glu  
20 25 30

7

Leu Asp Lys Glu Thr Gly Leu Leu Lys Leu Asp Arg Val Leu Tyr Thr  
35 40 45

Pro Phe His Tyr Pro Val Asp Tyr Gly Ile Ile Pro Arg Thr Trp Tyr  
50 55 60

Glu Asp Gly Asp Pro Phe Asp Ile Met Val Ile Met Arg Glu Pro Thr  
65 70 75 80

Tyr Pro Leu Thr Ile Ile Glu Ala Arg Pro Ile Gly Leu Phe Lys Met  
85 90 95

Ile Asp Ser Gly Asp Lys Asp Tyr Lys Val Leu Ala Val Pro Val Glu  
100 105 110

Asp Pro Tyr Phe Lys Asp Trp Lys Asp Ile Ser Asp Val Pro Lys Ala  
115 120 125

Phe Leu Asp Glu Ile Ala His Phe Phe Lys Arg Tyr Lys Glu Leu Glu  
130 135 140

Gly Lys Glu Ile Ile Val Glu Gly Trp Glu Gly Ala Glu Ala Ala Lys  
145 150 155 160

Arg Glu Ile Leu Arg Ala Ile Glu Met Tyr Lys Glu Lys Phe Gly Lys  
165 170 175

Lys Glu

<210> 7

<211> 178

<212> PRT

<213> Pyrococcus abyssi

<400> 7

Met Asn Pro Phe His Asp Leu Glu Pro Gly Pro Asn Val Pro Glu Val  
1 5 10 15

8

Val Tyr Ala Leu Ile Glu Ile Pro Lys Gly Ser Arg Asn Lys Tyr Glu  
20 25 30

Leu Asp Lys Lys Thr Gly Leu Leu Lys Leu Asp Arg Val Leu Tyr Ser  
35 40 45

Pro Phe Phe Tyr Pro Val Asp Tyr Gly Ile Ile Pro Arg Thr Trp Tyr  
50 55 60

Asp Asp Asp Asp Pro Phe Asp Ile Met Val Ile Met Arg Glu Pro Thr  
65 70 75 80

Tyr Pro Leu Thr Ile Ile Glu Ala Arg Pro Ile Gly Leu Phe Lys Met  
85 90 95

Ile Asp Ser Gly Asp Lys Asp Tyr Lys Val Leu Ala Val Pro Val Glu  
100 105 110

Asp Pro Tyr Phe Lys Asp Trp Lys Asp Ile Asp Asp Val Pro Lys Ala  
115 120 125

Phe Leu Asp Glu Ile Ala His Phe Phe Lys Arg Tyr Lys Glu Leu Gln  
130 135 140

Gly Lys Glu Ile Ile Val Glu Gly Trp Glu Gly Ala Glu Ala Ala Lys  
145 150 155 160

Arg Glu Ile Leu Arg Ala Ile Glu Leu Tyr Lys Glu Lys Phe Gly Ser  
165 170 175

Lys Glu

9

&lt;210&gt; 8

&lt;211&gt; 176

&lt;212&gt; PRT

&lt;213&gt; Thermococcus litoralis

&lt;400&gt; 8

Met Asn Pro Phe His Asp Leu Glu Pro Gly Pro Glu Val Pro Glu Val

1

5

10

15

Val Tyr Ala Leu Ile Glu Ile Pro Lys Gly Ser Arg Asn Lys Tyr Glu

20

25

30

Leu Asp Lys Lys Thr Gly Leu Ile Lys Leu Asp Arg Val Leu Tyr Ser

35

40

45

Pro Phe His Tyr Pro Val Asp Tyr Gly Ile Ile Pro Gln Thr Trp Tyr

50

55

60

Asp Asp Asp Asp Pro Phe Asp Ile Met Val Ile Met Arg Glu Pro Thr

65

70

75

80

Tyr Pro Gly Val Leu Ile Glu Ala Arg Pro Ile Gly Leu Phe Lys Met

85

90

95

Ile Asp Ser Gly Asp Lys Asp Tyr Lys Val Leu Ala Val Pro Val Glu

100

105

110

Asp Pro Tyr Phe Asn Asp Trp Lys Asp Ile Ser Asp Val Pro Lys Ala

115

120

125

Phe Leu Asp Glu Ile Ala His Phe Phe Gln Arg Tyr Lys Glu Leu Gln

130

135

140

Gly Lys Glu Ile Ile Val Glu Gly Trp Glu Asn Ala Glu Lys Ala Lys

145

150

155

160

Gln Glu Ile Leu Arg Ala Ile Glu Leu Tyr Lys Glu Lys Phe Lys Lys

165

170

175

10

&lt;210&gt; 9

&lt;211&gt; 179

&lt;212&gt; PRT

&lt;213&gt; Thermoplasma acidophilum

&lt;400&gt; 9

Met Glu Ser Phe Tyr His Ser Val Pro Val Gly Pro Lys Pro Pro Glu  
1 5 10 15

Glu Val Tyr Val Ile Val Glu Ile Pro Arg Gly Ser Arg Val Lys Tyr  
20 25 30

Glu Ile Ala Lys Asp Phe Pro Gly Met Leu Val Asp Arg Val Leu Tyr  
35 40 45

Ser Ser Val Val Tyr Pro Val Asp Tyr Gly Leu Ile Pro Arg Thr Leu  
50 55 60

Tyr Tyr Asp Gly Asp Pro Met Asp Val Met Val Leu Ile Ser Gln Pro  
65 70 75 80

Thr Phe Pro Gly Ala Ile Met Lys Val Arg Pro Ile Gly Met Met Lys  
85 90 95

Met Val Asp Gln Gly Glu Thr Asp Asn Lys Ile Leu Ala Val Phe Asp  
100 105 110

Lys Asp Pro Asn Val Ser Tyr Ile Lys Asp Leu Lys Asp Val Asn Ala  
115 120 125

His Leu Leu Asp Glu Ile Ala Asn Phe Phe Ser Thr Tyr Lys Ile Leu  
130 135 140

Glu Lys Lys Glu Thr Lys Val Leu Gly Trp Glu Gly Lys Glu Ala Ala  
145 150 155 160

Leu Lys Glu Ile Glu Val Ser Ile Lys Met Tyr Glu Glu Lys Tyr Gly  
165 170 175

11

Lys Lys Asn

&lt;210&gt; 10

&lt;211&gt; 686

&lt;212&gt; DNA

<213> *Aeropyrum pernix*

&lt;400&gt; 10

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tgtattgtta aggttgatag gttcctttac accagcatgg tctaccctt caactacggg 180
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cgccccaca gtcaagctta cactta 686
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&lt;210&gt; 11

&lt;211&gt; 89

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Modified  
polylinker sequence of pTTQ18NHK

&lt;400&gt; 11

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ctagagtcga cctgcaggca tgcaagctt 89
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12

&lt;210&gt; 12

&lt;211&gt; 5503

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pTTQ18NHK

vector

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (3279, 3525)

&lt;223&gt; n is a or g or c or t

&lt;400&gt; 12

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15

&lt;210&gt; 13

&lt;211&gt; 6105

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: pTTQ18NHK  
vector including the PPase sequence

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (3881, 4127)

&lt;223&gt; n is a or g or c or t

&lt;400&gt; 13

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18

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&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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&lt;400&gt; 14

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&lt;210&gt; 15

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Primer

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&lt;210&gt; 16

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&lt;212&gt; DNA

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&lt;223&gt; Description of Artificial Sequence: Primer

19

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20

&lt;210&gt; 17

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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&lt;223&gt; Description of Artificial Sequence: Primer

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&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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&lt;223&gt; Description of Artificial Sequence: Primer

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31

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&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Primer

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23

&lt;210&gt; 23

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Primer

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21

21

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&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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27

&lt;210&gt; 25

&lt;211&gt; 176

&lt;212&gt; PRT

&lt;213&gt; Aeropyrum pernix

&lt;400&gt; 25

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

Val Asn Val Val Ile Glu Ile Pro Met Asn Ser Ser Val Lys Tyr Glu  
20 25 30

Phe Asp Lys Glu Ala Cys Ile Val Lys Val Asp Arg Phe Leu Tyr Thr  
35 40 45

Ser Met Val Tyr Pro Phe Asn Tyr Gly Phe Ile Pro Gly Thr Leu Glu  
50 55 60

Glu Asp Gly Asp Pro Val Asp Val Leu Val Ile Ser Arg Glu Pro Val  
65 70 75 80

Ala Pro Gly Ser Leu Ile Glu Ala Val Pro Val Ala Val Leu Asp Met  
85 90 95

Glu Asp Glu Glu Gly Pro Asp Ser Lys Val Val Ala Val Pro Lys Ala  
100 105 110

22

Lys Leu Asp Pro Leu Phe Ala Ser Tyr Lys Asp Val Gly Asp Ile Pro  
115 120 125

Asp Ala Leu Lys Ser Lys Ile Lys His Phe Phe Glu His Tyr Lys Glu  
130 135 140

Leu Glu Pro Gly Lys Trp Val Arg Val Thr Gly Trp Arg Pro Ala Ala  
145 150 155 160

Asp Ala Lys Glu Ile Ile Arg Arg Ala Ile Glu Arg Tyr Lys Gly Ala  
165 170 175

&lt;210&gt; 26

&lt;211&gt; 531

&lt;212&gt; DNA

&lt;213&gt; Aeropyrum pernix

&lt;400&gt; 26

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CLAIMS:

1. A method for inhibiting production of non-specific products in a nucleic acid amplification reaction, said method comprising forming an amplification reaction mixture in the presence of sufficient amount of a pyrophosphate salt to prevent primer extension taking place, digesting said pyrophosphate salt with a thermostable pyrophosphatase enzyme (PPase) which is only significantly active at temperatures in excess of 50°C, and subjecting said reaction mixture to conditions such that an amplification reaction may proceed.
2. The method according to claim 1 wherein the thermostable pyrophosphatase enzyme (PPase) is not active below 50°C.
3. The method according to claim 1 or claim 2 wherein the amplification reaction is a polymerase chain reaction (PCR) and the reaction mixture contains reagents suitable for conducting such a reaction.
4. The method according to any one of claims 1 to 3 wherein the reaction mixture contains a DNA polymerase which is selected from *Thermus aquaticus* polymerase (*Taq*), *Thermus thermophilus* polymerase (*Tth*), *Thermus species NH* polymerase (*TspNH*), *Thermus brockianus* polymerase (*Tbr*), *Pyrococcus furiosus* polymerase (*Pfu*), 9°N7 exo-DNa polymerase, and *Thermococcus literalis* DNA polymerase.
5. The method according to any one of claims 1 to 4 wherein the pyrophosphate is an alkali earth metal pyrophosphate.
6. The method according to claim 5 wherein the pyrophosphate is tetrasodium pyrophosphate of formula  $\text{Na}_4\text{P}_2\text{O}_7$ .
7. The method according to any one of claims 1 to 6 wherein the pyrophosphate is present in the reaction mixture at a concentration of at least 0.5mM.
8. The method according to claim 7 wherein the pyrophosphate is present at a concentration of from 1 to 10mM.

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9. The method according to any one of claims 1 to 8 wherein the thermostable PPase is *Sulfolobus acidicaldarius* inorganic pyrophosphatase (Sac PPase), *Thermococcus litoralis* inorganic pyrophosphatase or *Aeropyrum pernix* inorganic pyrophosphatase.

5 10. The method according to any one of claims 1 to 9 wherein the thermostable PPase comprises the amino acid sequence shown in SEQ ID NO. 25 or a variant or fragment thereof wherein the variant or fragment thereof has PPase activity.

11. The method according to claim 9 or claim 10 wherein the  
10 thermostable PPase is added to the reaction mixture on formation thereof.

12. The method according to claim 11 which includes an incubation step prior to the amplification reaction at a temperature of 50°C or more, in order to allow the PPase to digest inorganic pyrophosphate present.

13. The method according to any one of claims 1 to 12 wherein the  
15 PPase is added to the reaction mixture at a concentration of at least 0.04u per 50µL PCR reaction mixture.

14. The method according to claim 13 wherein the PPase is added to the reaction mixture at a concentration of 0.08u per 50µL PCR reaction mixture.

15. The method according to claim 13 or claim 14 wherein the PPase is  
20 added to the reaction mixture at a concentration of from 0.2 to 10u per 50µL PCR reaction mixture.

16. A kit for inhibiting production of non-specific products when conducting an amplification reaction, said kit comprising a pyrophosphate salt, a thermostable pyrophosphatase enzyme which is only significantly active at  
25 temperatures in excess of 50°C, and optionally one or more reagents required for use in an amplification reaction.

17. The kit according to claim 16 wherein the thermostable pyrophosphatase enzyme is not active below 50°C.

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18. The kit according to claim 16 or claim 17 which further comprises one or more primers necessary to carry out amplification of a particular target nucleic acid.
19. The kit according to any one of claims 16 to 18 which further  
5 includes one or more fluorescently labelled reagents.
20. The kit according to claim 19 wherein the fluorescently labelled reagents are selected from one or more of an intercalating dye, a fluorescently labelled probe, a fluorescently labelled primer and a fluorescently labelled nucleotide.
- 10 21. Use of a thermostable pyrophosphatase enzyme which is only significantly active at temperatures in excess of 50°C, in the method of any one of claims 1 to 15.
22. The use according to claim 21 wherein the thermostable pyrophosphatase enzyme is not active below 50°C.
- 15 23. The use according to claim 21 or claim 22 wherein the pyrophosphatase enzyme is an enzyme isolated from *Aeropyrum pernix*.
24. The use according to claim 21 or claim 22 wherein the pyrophosphatase enzyme is encoded by the polynucleotide sequence as shown in SEQ ID NO. 26 or a variant or fragment thereof wherein the variant or fragment  
20 thereof encodes a polypeptide having PPase activity.
25. The use according to claim 21 wherein the pyrophosphatase enzyme comprises the amino acid sequence as shown in SEQ ID NO. 25 or a variant or fragment thereof wherein the variant or fragment thereof has PPase activity.



FIGURE 1

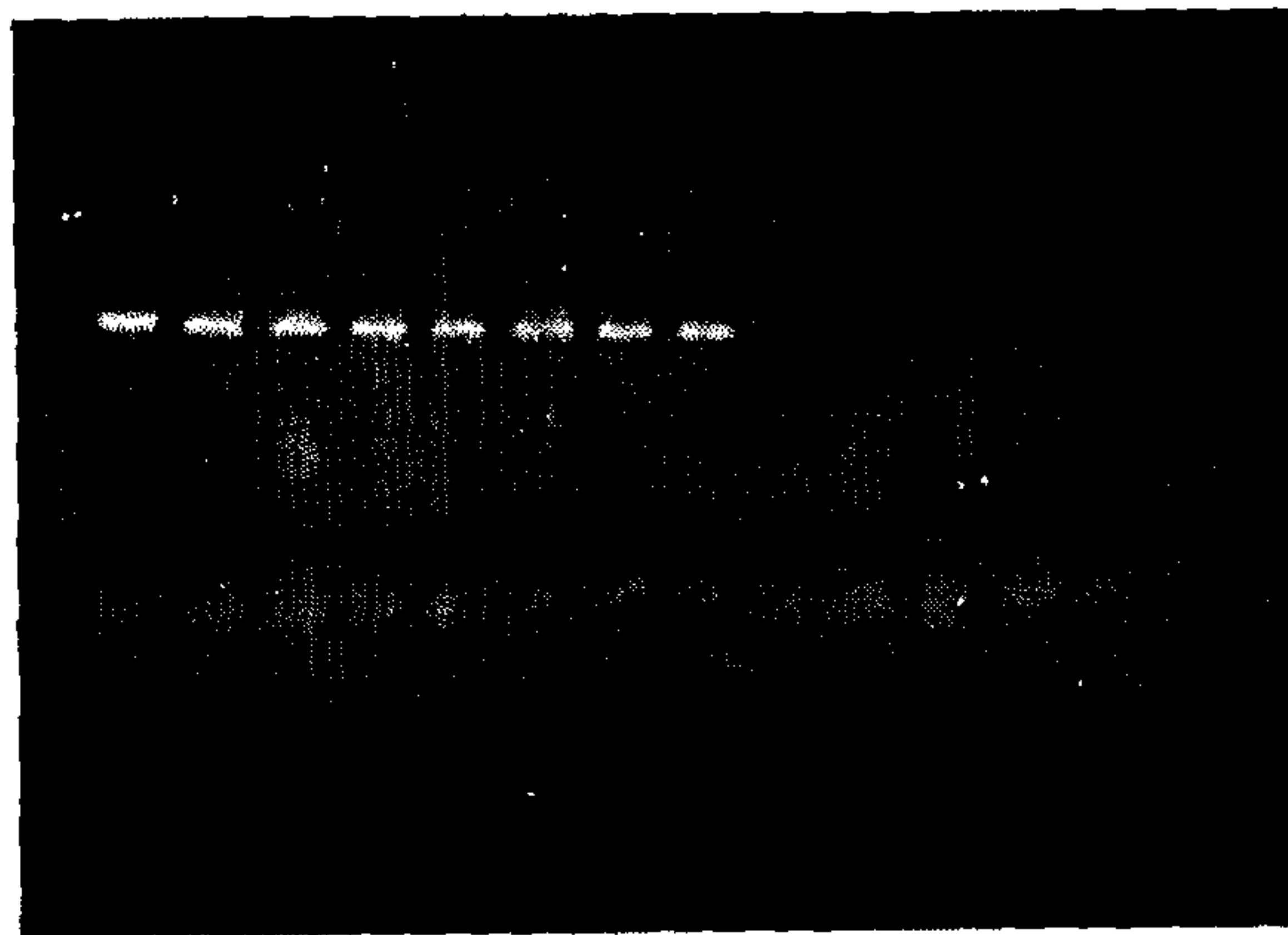


FIGURE 2

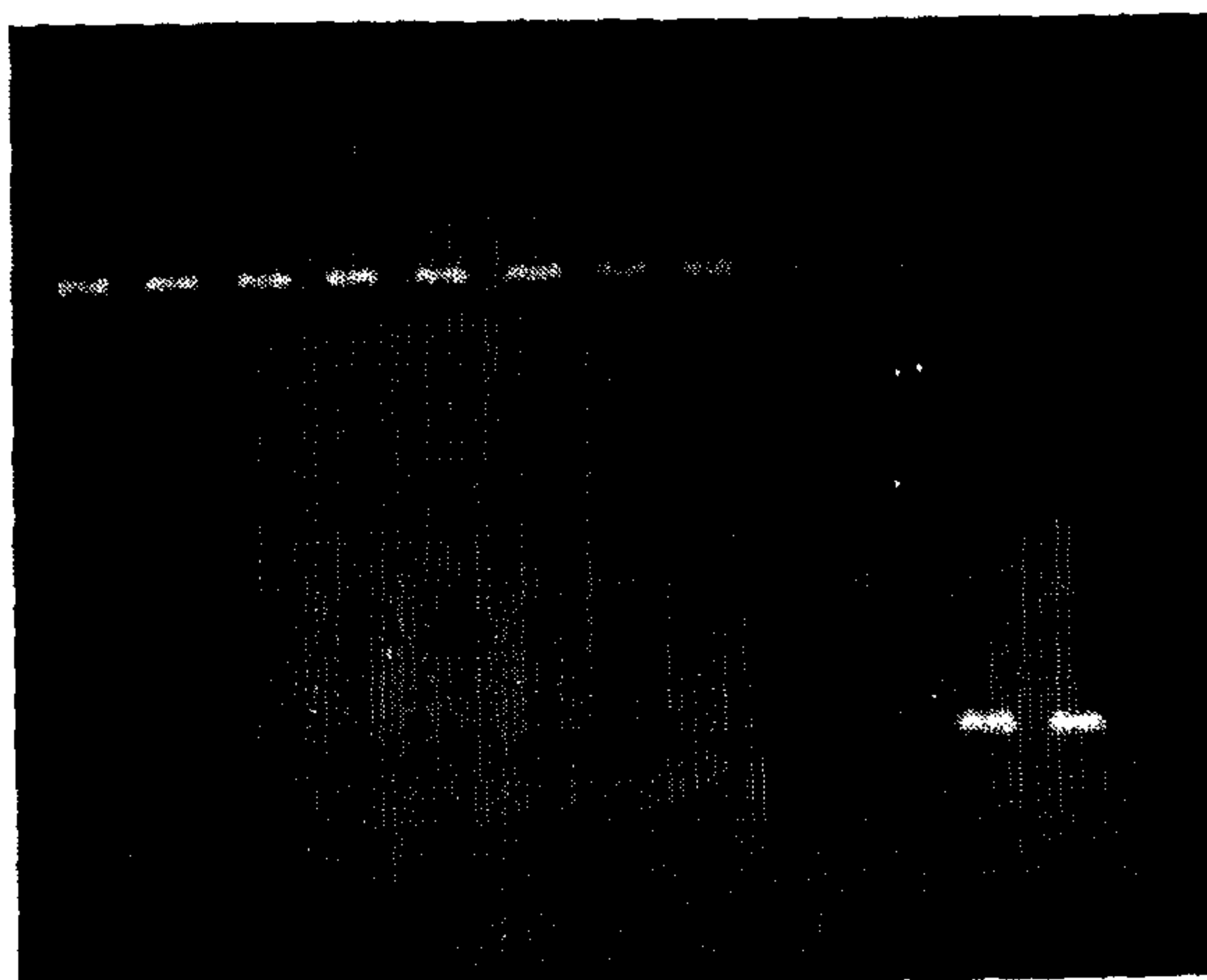


FIGURE 3

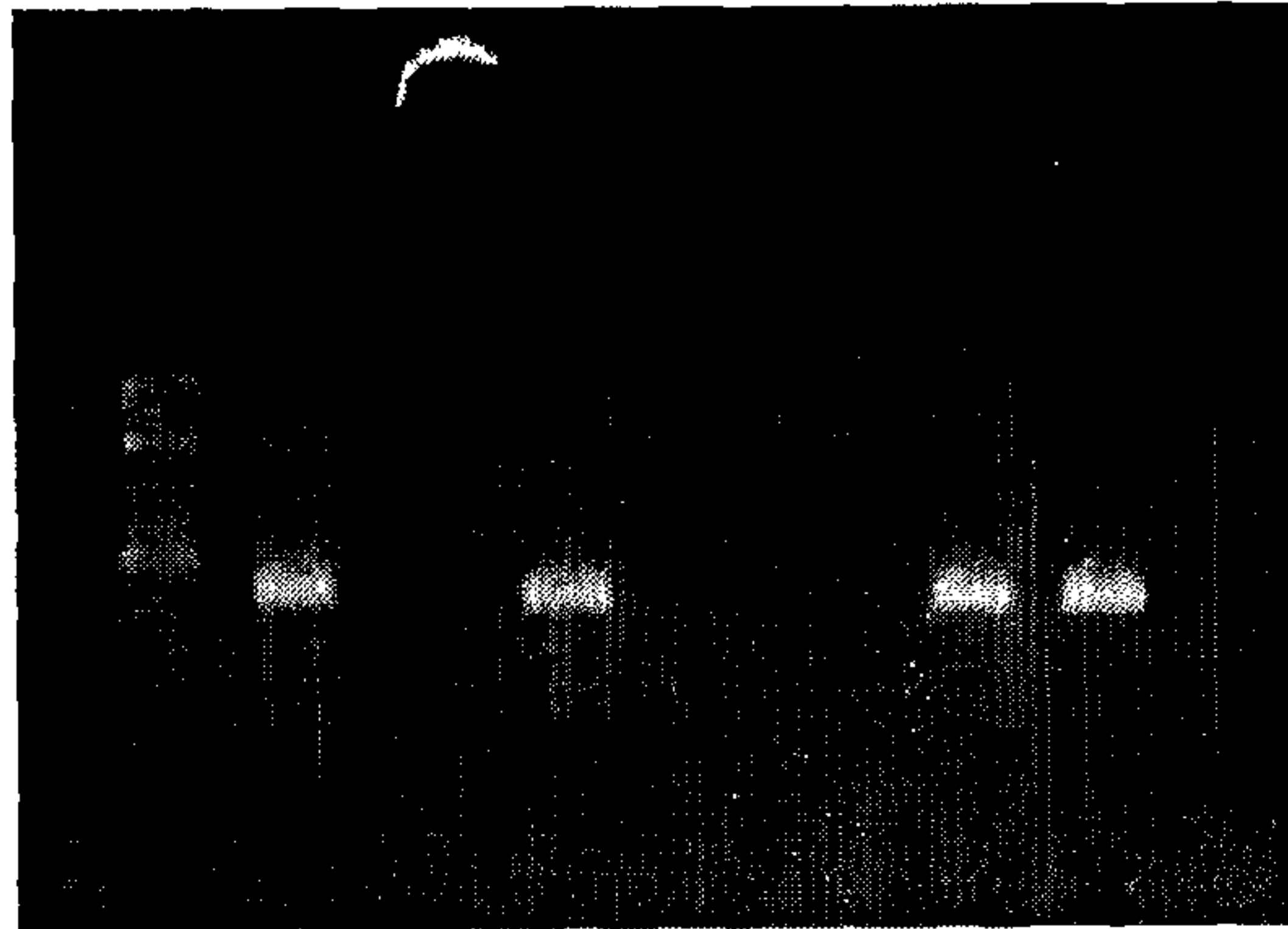


FIGURE 4

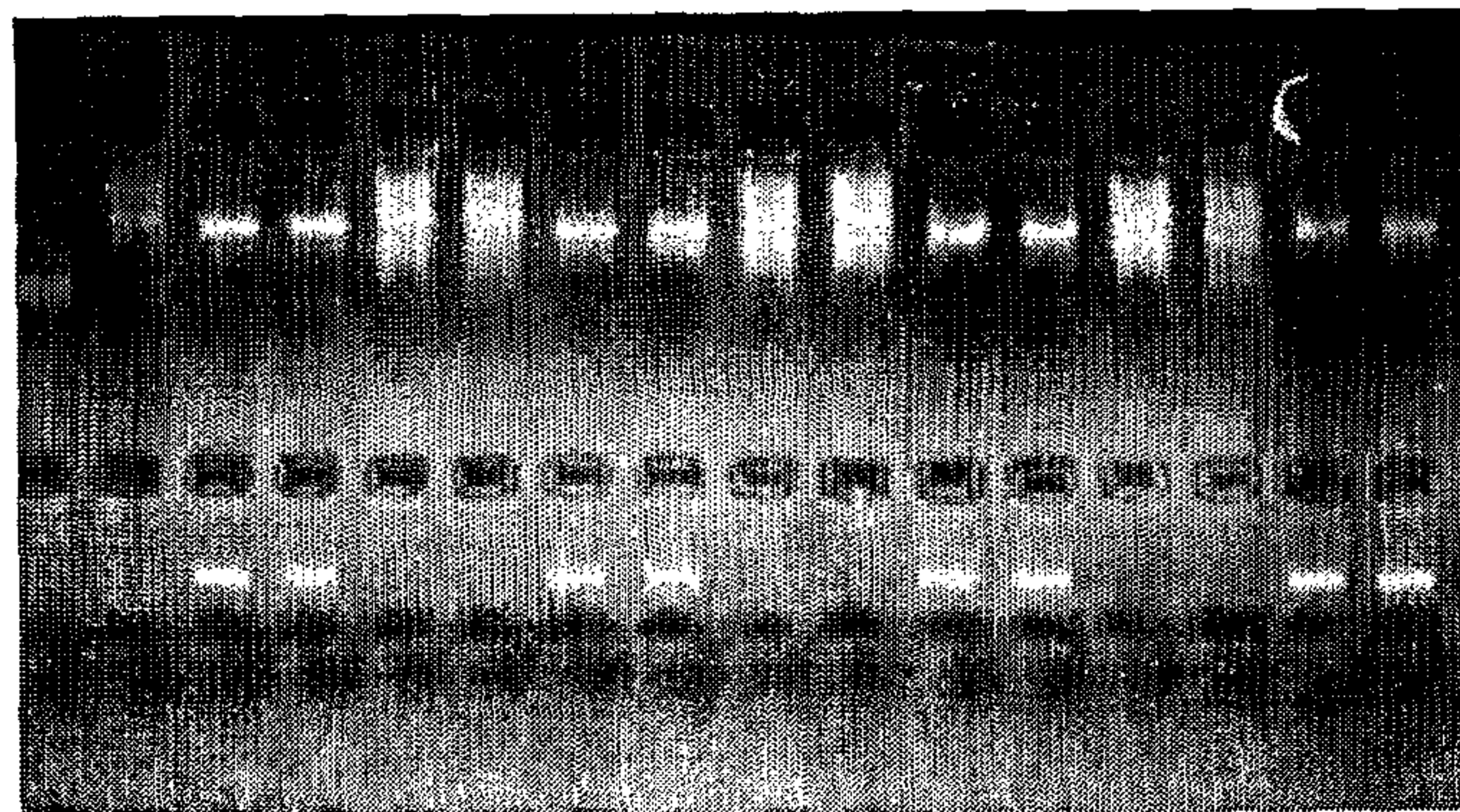


FIGURE 5

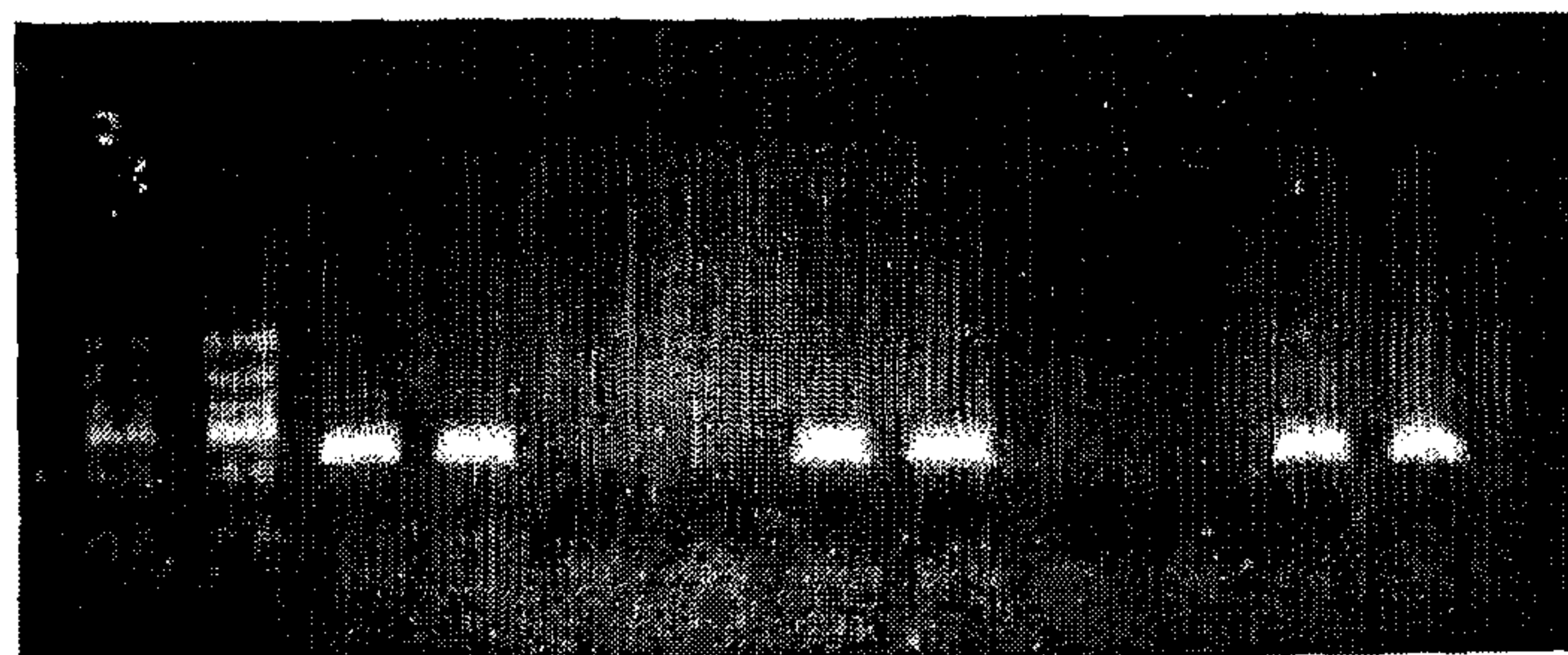


FIGURE 6

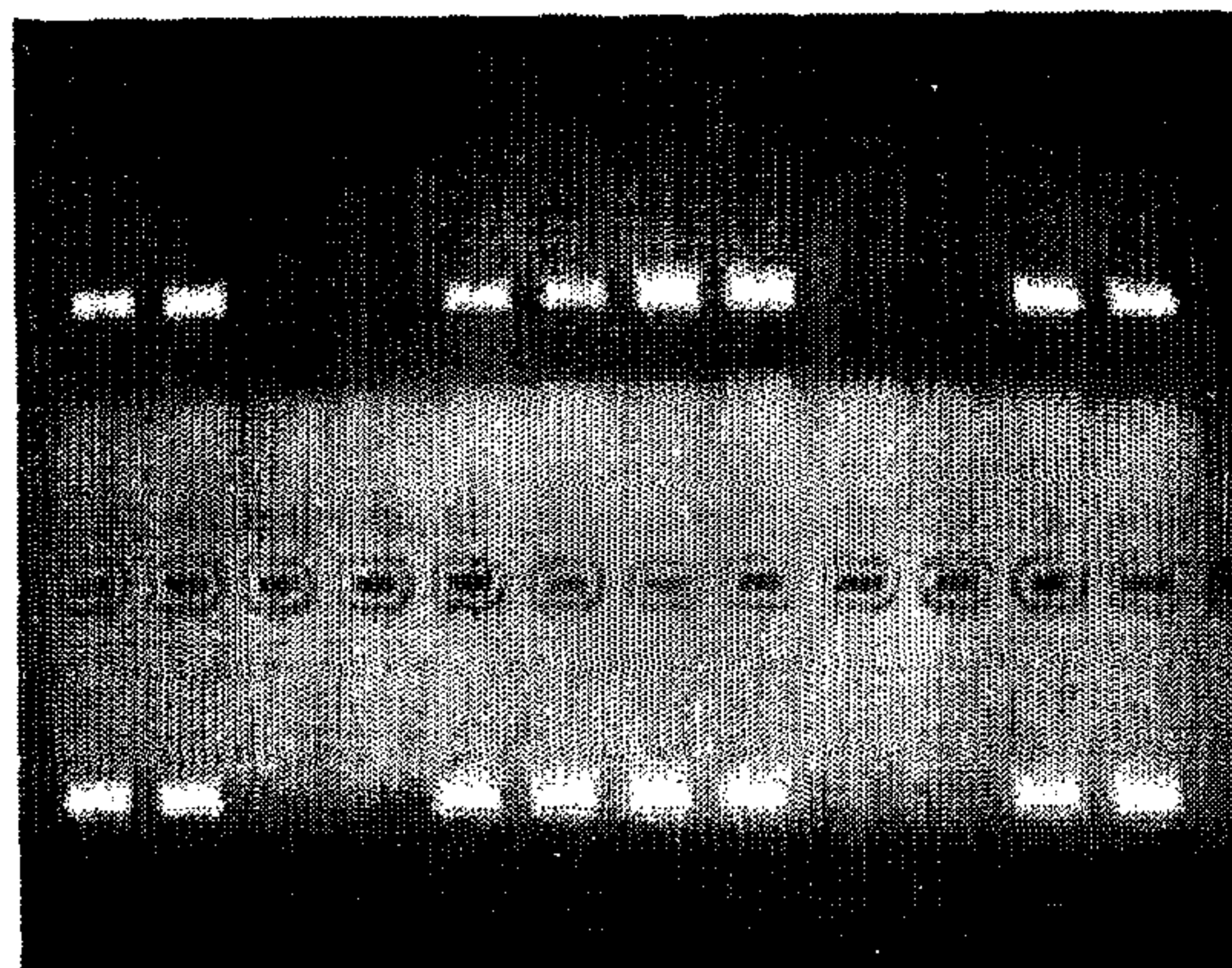


FIGURE 7a

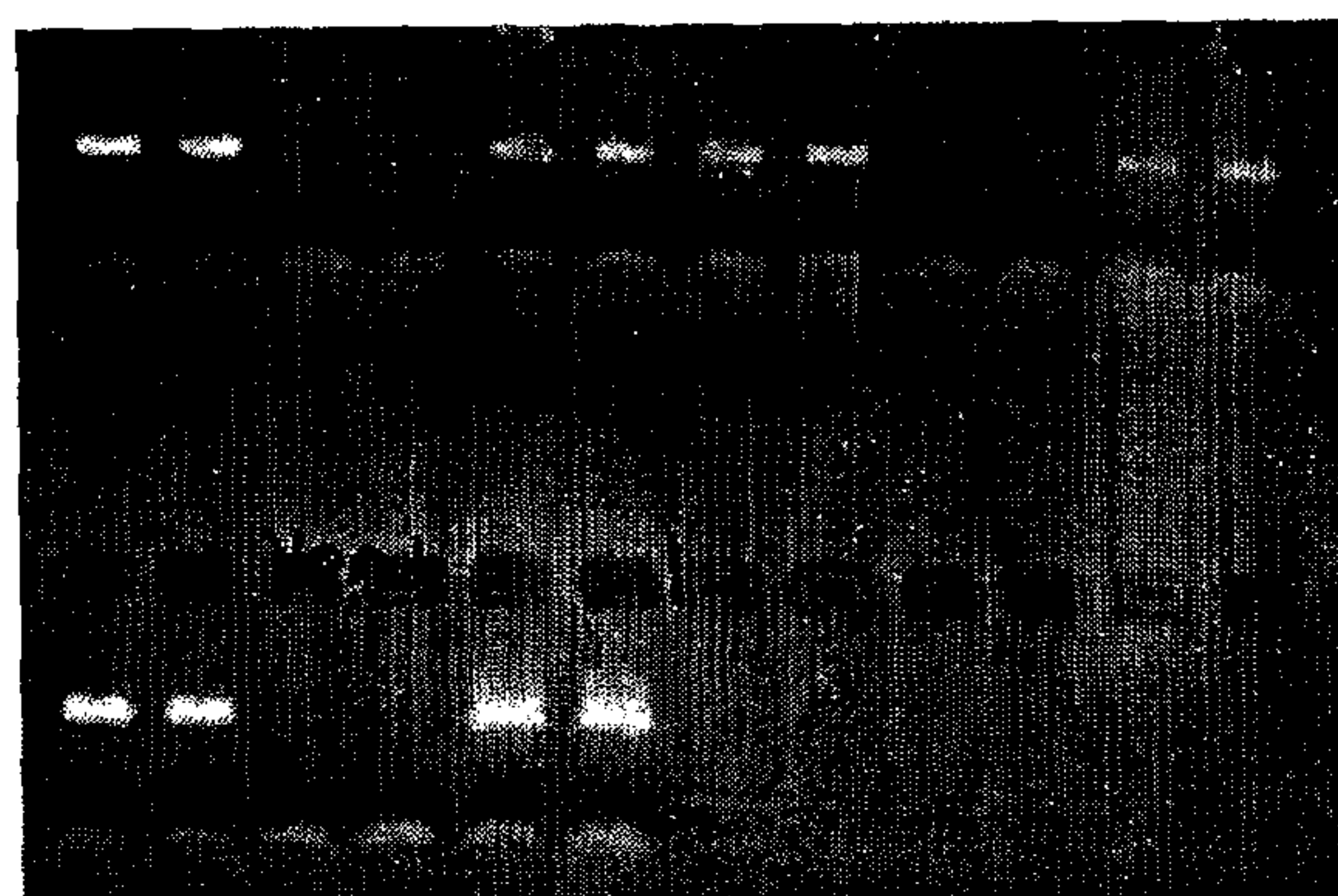


FIGURE 7b



FIGURE 8a

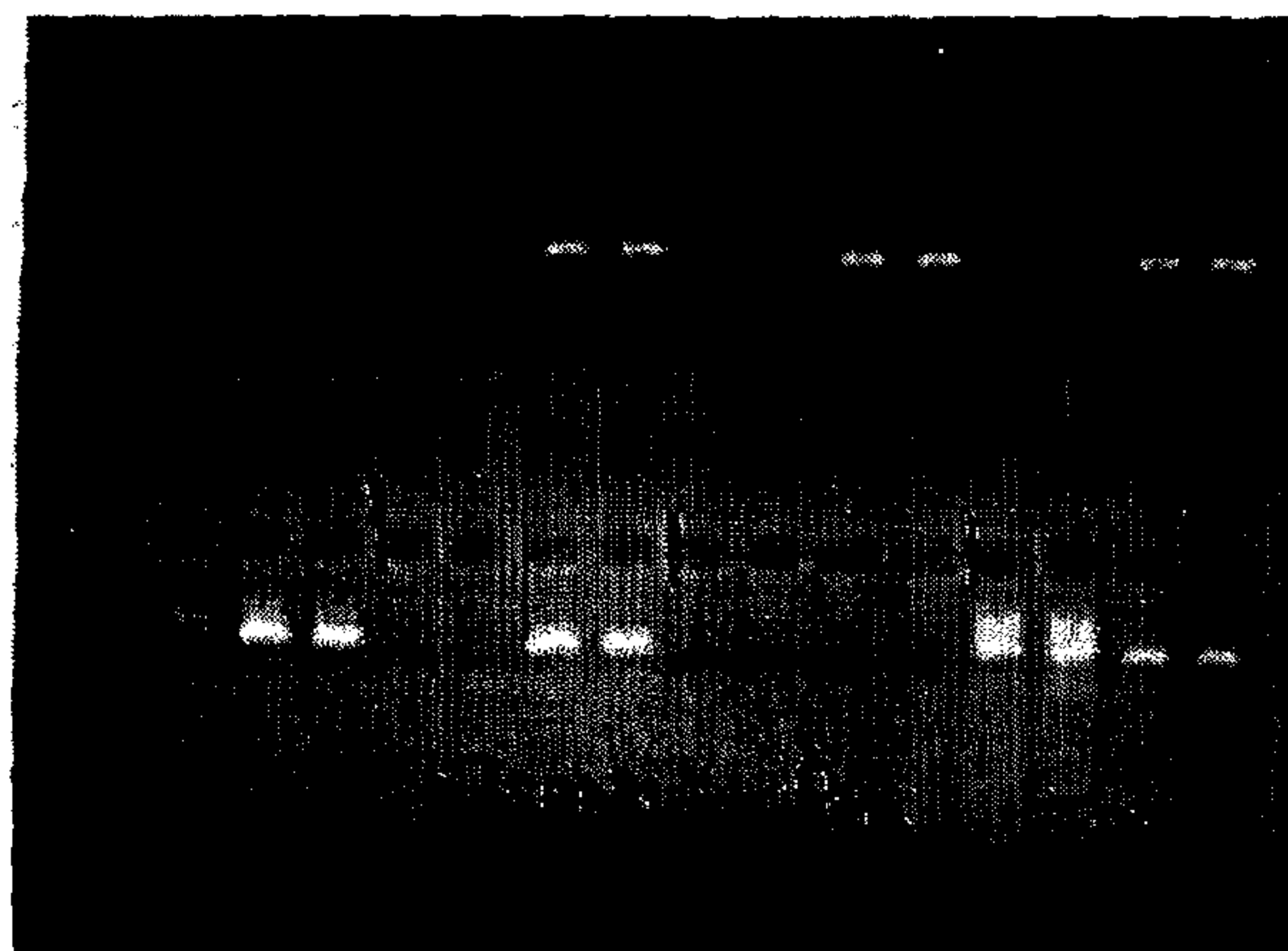


FIGURE 8b

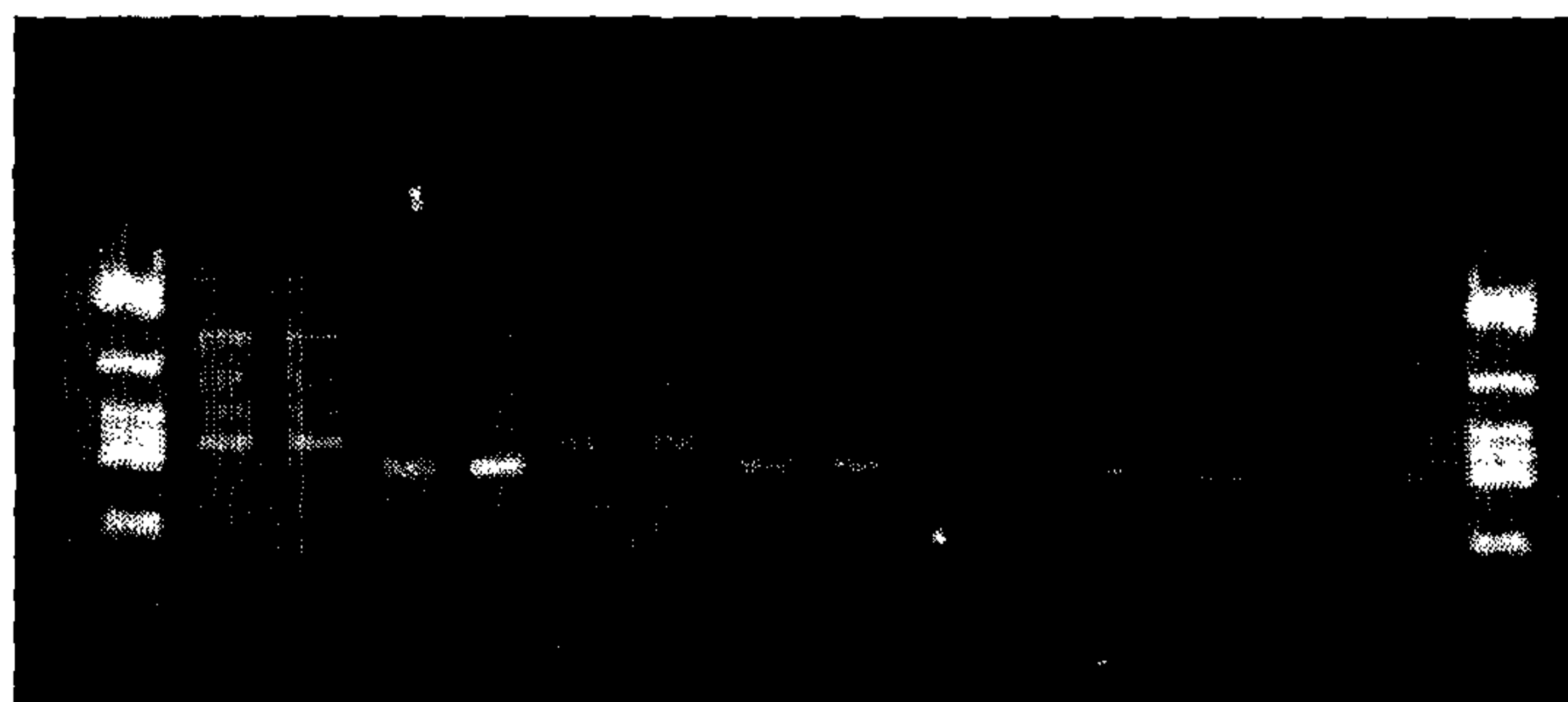


FIGURE 9

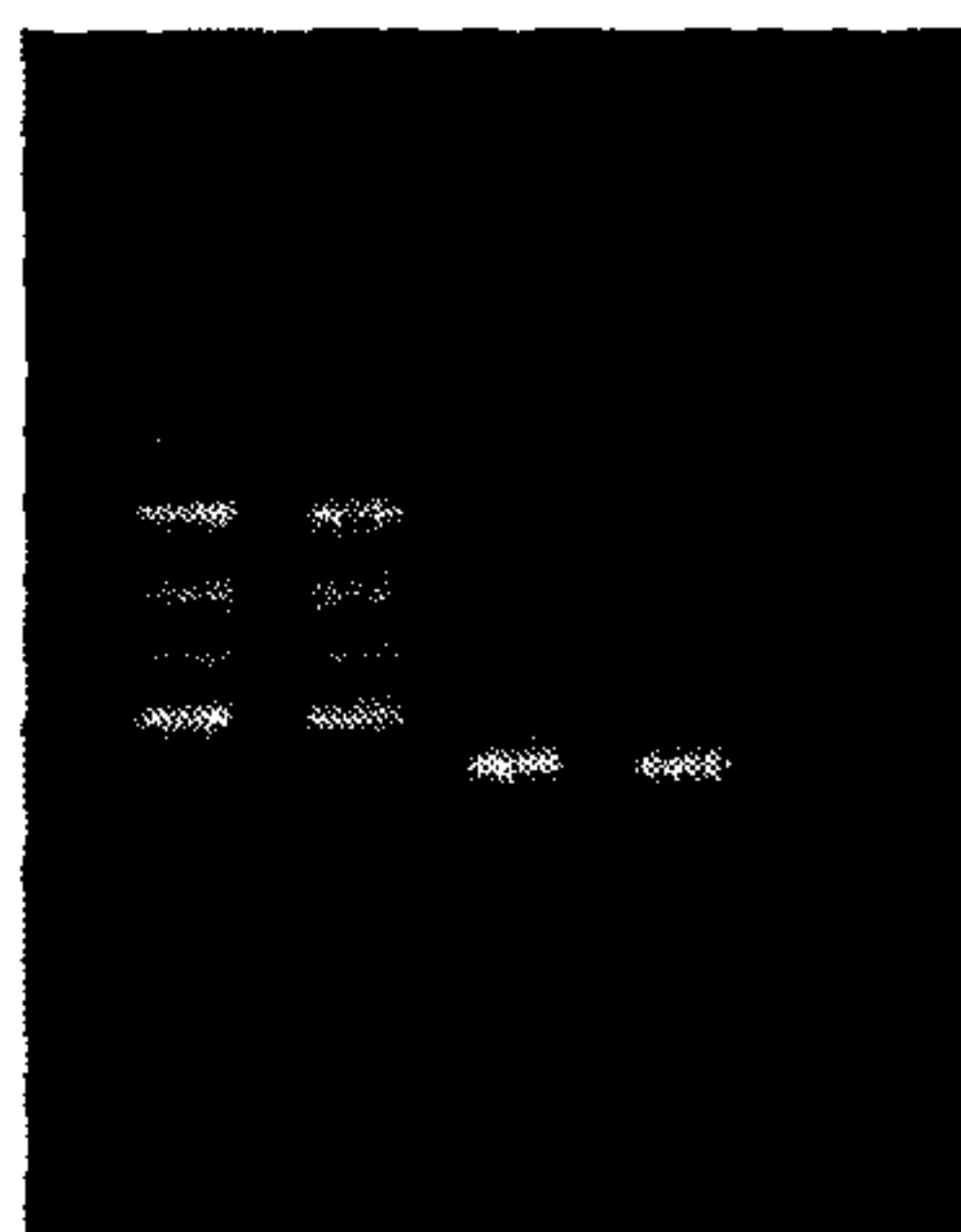


FIGURE 10

Figure 11

## Aeropyrum pernix sequence

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 (SEQ ID NO 2)

## SEQ ID NO 1

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## SEQ ID NO 26

```

atgacaggct gtctgaaaat tggtcctgga gatgaggctc cagatgttgt
gaatgtcgtt atagagatac ctatgaacag ttctgttaag tacgagttcg
acaaggaggc gtgtattgtt aagggtgata ggttccttta caccagcatg
gtctacccct tcaactacgg gttcatacca ggcactctag aggaggacgg
agatcctgtt gacgtttctag ttattagccg ggagcccgtt gctcccggct
cgcttataga ggctgtgccc gtggccgtgt tagacatgga ggacgaggag
ggtccggaca gcaaggttgt tgccgtaccc aaggccaagc tggacccctt
attcgccagc tataaggacg ttggcgacat acctgatgcc ctgaaatcca
agataaagca cttcttcgag cactataagg agctggagcc tggaaagtgg
gttagagtga ctggatggag gcctgctgcc gatgcgaagg agattataag
gagggctata gagaggtata agggggcgtg a

```

Figure 12

## Alignment of PPase sequences with ClustalW

Aeropyrum = *Aeropyrum pernix* SEQ ID NO 2  
 Sulfolobus = *Sulfolobus solfataricus* SEQ ID NO 3  
 E.coli = *Escherichia coli* SEQ ID NO 4  
 Aquifex = *Aquifex aeolicus* SEQ ID NO 5  
 Pho = *Pyrococcus horikoshii* SEQ ID NO 6  
 Pab = *Pyrococcus abyssii* SEQ ID NO 7  
 Tli = *Thermococcus litoralis* SEQ ID NO 8  
 Themoplasma = *Themoplasma acidophilum* SEQ ID NO 9

CLUSTAL W (1.8) multiple sequence alignment

```

aeropyrum      MWTILPSKTGFVNSLSFITRLAKLSVRRVHAMTGCLKIGP-GDEAPDVVNVVIEIPM-NS
sulfolobus     -----MKLSP-GKNAPDVVNVVIEIPQ-GS
E.coli         -----MSLLNGPA-GKDLPEDIYVVIEIPANAD
aquifex        -----MGYDQLPP-GKNPPEDIYVVIEIPQ-GS
Pho            -----MNPFDHLEP-GPNVPEVVYALIEIPK-GS
Pab            -----MNPFDHLEP-GPNVPEVVYALIEIPK-GS
Tli            -----MNPFDHLEP-GPEVPEVVYALIEIPK-GS
thermoplasma   -----MESFYHSVPVGPKPPEEVYVIVEIPR-GS

```

. . \* . \*: : .::\*\*\* .

```

aeropyrum      SVKYEFDKEACIVKVDRFLYTSMVYPFNYGFIPGTLEEDGDPVDVLVISREPVAPGSLIE
sulfolobus     NIKYEYDDEEGVIKVDRLYTSMNYPFNYGFIPGTLEEDGDPLDVLVITNYQLYPGSVIE
E.coli         PIKYEIDKESGALFVDRFMSTAMFYPCNYGYINHILSLDGPVDVLVPTPYPLQPGSVIR
aquifex        AVKYELDKDTGVIFVDRFLFTAMYYPFNYGFVPQTLADDGDPVDVLVISREPVVPGAVMR
Pho            RNKYELDKETGLLKLDRVLYTPFHYPVDYGIIPRTWYEDGDPFDIMVIMREPTYPLTIE
Pab            RNKYELDKKTGLLKLDRVLYSPFFYPVDYGIIPRTWYDDDDPFDIMVIMREPTYPLTIE
Tli            RNKYELDKKTGLIKLDRVLYSPFHYPVDYGIIPQTWYDDDDPFDIMVIMREPTYPGVLIE
thermoplasma   RVKYEIAKDFPGMLVDRVLYSSVYPVDYGLIPRTLYYDGDPMVMVLISQPTFFGAIMK

```

\*\*\* .. : :\*\*\*: :.. \*\* \*\*: : \* .\*\*\*.\*\*\*: \* :..

```

aeropyrum      AVPVAVLMEDEEGPDSKVVAVPKAKLDPLFASYKDVGDIPDALKSKIKHFFEHYKELEP
sulfolobus     VRPIGILYMKDEEGEDAKIVAVPKDTPSFSNIKDINDLPQATKNKIVHFFEHYKELEP
E.coli         CRPVGVLKMTDEAGEDAKLVAVPHSKLSKEYDHIKDVNDLPELLKAQIAHFFEHYKDLEK
aquifex        CRPIGMLEMRDEAGIDTKVIAVPHEKLDPSYSNIKTVDNLPEIVREKIKHFFEHYKELEP
Pho            ARPIGLFKMIDSGDKDYKVLAVPVE--DPYFKDWKDISDVPKAFLEIAHFFKRYKELE-
Pab            ARPIGLFKMIDSGDKDYKVLAVPVE--DPYFKDWKDIDDVPKAFLEIAHFFKRYKELQ-
Tli            ARPIGLFKMIDSGDKDYKVLAVPVE--DPYFNDWKDISDVPKAFLEIAHFFQRYKELQ-
thermoplasma   VRPIGMMKMVDQGETDNKILAVFDK--DPNVSYIKDLKDVNAHLLDEIANFFSTYKILE-

```

\*::: \* \*. \* \*::\*\* . \* : : : \* :\*\*\*. \*\* \*:

```

aeropyrum      GKWVRVTGWRPAADAKEIIRRAIERYKGA-----
sulfolobus     GKYVKISGWGSATEAKNRIQLAIKRVSGGQZ----
E.coli         GKWVKVEGWENAEAAKAEIVASFER-AKNKZ----
aquifex        GKWVKVENWKGLQDAIEEIKKGIENYKKNKEG---
Pho            GKEIIVEGWEGAEAAKREILRAIEMYKEKFGKKEZ
Pab            GKEIIVEGWEGAEAAKREILRAIELYKEKFGSKEZ
Tli            GKEIIVEGWENAEKAKQEILRAIELYKEKFKKZ--
thermoplasma   KKETKVLGWEGKEAALKEIEVSIKMYEEKYGKKNZ

```

\* : .\* \* \* .::

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Figure 13

686bp PCR product. (SEQ ID NO 10)

RE sites in bold, PPase gene in italics, primer sites underlined

TGCATGCATATGACAGGCTGTCTGAAAATTGGTCCTGGAGATGAGGCTCCAGATGTTGTGAATGTCGTT  
 ATAGAGATACCTATGAACAGTTCTGTTAAGTACGAGTTCGACAAGGAGGCGTGATTGTTAAGGTTGAT  
 AGGTTCCCTTTACACCAGCATGGTCTACCCCTTCAACTACGGGTTCATAACCAGGCACTCTAGAGGAGGAC  
 GGAGATCCTGTTGACGTTCTAGTTATTAGCCGGGAGCCCGTTGCTCCCGGCTCGCTTATAGAGGCTGTG  
 CCCGTGGCCGTGTTAGACATGGAGGACGAGGAGGGTCCGGACAGCAAGGTTGTTGCCGTACCCAAGGCC  
 AAGCTGGACCCCCCTATTCGCCAGCTATAAGGACGTTGGCGACATACCTGATGCCCTGAAATCCAAGATA  
 AAGCACTTCTTCGAGCACTATAAGGAGCTGGAGCCTGGAAAGTGGGTTAGAGTGA CTGGATGGAGGCCT  
 GCTGCCGATGCCAAGGAGATTATAAGGAGGGCTATAGAGAGGTATAAGGGGGCGTGATGAGGGCTTAAC  
 GGCTCACGTTTTCTGGGAGAGTGTGCGACCTTTGAGGGCGATCACCCCTCGCCAGCGTGCGTGTGCTTTT  
 GTCTATGATTATGGCTACAGTTCTTCTAGCCGCTTTACCCGCCCCCACAGT**CAAGCTT**TACACTTA

Figure 14

Modified polylinker sequence of pTTQ18NHK from initial ATG to the *Nde* I site and then the *Hind* III site (SEQ ID NO 11)

Met *Nde* I  
ATGACCACCACCACCACCATATGGGCATGCTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCG  
ACCTGCAGGCATGCAAGCTT  
*Hind* III

Figure 15

## pTTQ18NHK sequence (SEQ ID NO 12)

>pTTQ18NHK Sequence

|        |                                                          |      |
|--------|----------------------------------------------------------|------|
| GAAC   | TGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA | 60   |
| ATGA   | TGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGG | 120  |
| CAAG   | AGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCA | 180  |
| GTCAC  | AGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATA  | 240  |
| ACCAT  | GAGTGATAACACTGCGGCCAATTACTTCTGACAACGATCGGAGGACCGAAGGAG   | 300  |
| CTAAC  | CGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCG  | 360  |
| GAGCT  | GAAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCA | 420  |
| ACAAC  | CGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTA | 480  |
| ATAGAC | TGGATGGAGGCGGATAAAGTTGCAGGACCATTCTGCGCTCGGCCCTTCCGGCT    | 540  |
| GGCTG  | GTTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCA | 600  |
| GCACT  | GGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAG  | 660  |
| GCAACT | TATGGATGAACGAAATAGACAGATCGTCGATAGGTGCCTCACTGATTAAGCAT    | 720  |
| TGGTA  | AACTGTGACACCAAGTTTACTCATATATACTTTAGATTGATTTAAACTTCATTTT  | 780  |
| TAATTT | AAAAAGGATCTAGGTGAAGATCCTTTTTTGATAATCTCATGACCAAATCCCTTAA  | 840  |
| CGTGAG | TTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGA  | 900  |
| GATCCT | TTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCACCGTACCAGCG   | 960  |
| GTGGTT | TGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAGTGGCTTCAGC   | 1020 |
| AGAGCG | CAGATACCAAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG    | 1080 |
| AACTCT | GTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAAGTGGCTGCTGCC  | 1140 |
| AGTGGC | GATAAGTCGTGCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG    | 1200 |
| CAGCGT | CGGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTAC     | 1260 |
| ACCGAA | CTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGA   | 1320 |
| AAGGCG | GACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTT   | 1380 |
| CCAGGG | GAAACGCCTGGTATCTTTATAGTCCTGTGGGTTCGCCACCTCTGACTTGAG      | 1440 |
| CGTCGA | TTTTTGATGCTCGTCAGGGGGCGGAGCCTATGGAAAACGCCAGCAACGCG       | 1500 |
| GCCTTT | TACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTCTCTGCGTTA    | 1560 |
| TCCCCT | GATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGC   | 1620 |
| AGCCGA | ACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGC   | 1680 |
| AAACGC | CTCTCCCCGCGCGTTGGCCGATTTCATTAATGCAGAATTAATTCTCATGTTTGA   | 1740 |
| CAGCTT | ATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGC   | 1800 |
| TGTGGT | TATGGCTGTGCAGGTGTAATCACTGCATAATTCGTGTGCTCAAGGCGCACTC     | 1860 |
| CCGTTT | CTGGATAATGTTTTTTCGCCGACATCATAACGGTTCCTGGCAAATATCTGAAAT   | 1920 |
| GAGCTG | TGTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAAT  | 1980 |
| TTACAC | AGGAAACACATATATGCACCACCACCACCACCATATGGGCATGCTGAATTCGA    | 2040 |
| GCTCGG | TACCCGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCG    | 2100 |
| TCGTTT | TACAACGTCGTGACTGGGAAAACCTGGCGTTACCCAACCTTAATCGCCTTGCG    | 2160 |
| CACATC | CCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCAGATCGCCCTTCCC | 2220 |
| AACAGT | TGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATC   | 2280 |
| TGTGCG | GTATTTTACACCCGCATAAATTCCTGTTTTGGCGGATGAGAGAAGATTTTCAGC   | 2340 |
| CTGATA | CAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCCTGGCGGC  | 2400 |
| AGTAGC | CGCGGTGGTCCCACCTGACCCCATGCCGAACCTCAGAAGTGAAACGCCGTAGCGCC | 2460 |
| GATGGT | AGTGTGGGTCTCCCCATGCGAGAGTAGGGAACCTGCCAGGCATCAAATAAAACG   | 2520 |
| AAAGGC | TCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTTGTGTCGGTGAACGCTCT   | 2580 |
| CCTGAG | TAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGG   | 2640 |
| GTGGCG | GGCAGGACGCCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCT  | 2700 |
| GACGGT | ATGGCCTTTTTCGCTTCTACAAACTCTTCTGTCGTCATATCTACAAGCCATCC    | 2760 |
| CCCCAC | AGATAACGGTAACTAGCCTCGTTTTTGTCATCAGGAAAGCAGGGAATTTATGGTG  | 2820 |
| CACTCT | CAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAAC   | 2880 |
| ACCCGT | CTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGT  | 2940 |
| GACCGT | CTCCGGGAGCTGCATGTGTGAGAGGTTTTCACCGTCATCACCGAAACGCGCGAG   | 3000 |
| ACGAA  | AGGGCCTGATTAGAAAACTCATCGAGCATCAAATGAACTGCAATTTATTCATA    | 3060 |
| TCAGGA | TATCAATACCATAATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCA   | 3120 |
| CCGAGG | CAGTTCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCA    | 3180 |
| ACATCA | ATAACCTATTAATTTCCCTCGTCAAAAAATAAGGTTATCAAGTGAGAAATCA     | 3240 |
| CCATGA | GTGACGACTGAATCCGGTGAGAATGGCAAAAGNTTATGCATTTCTTTCCAGACT   | 3300 |
| TGTTCA | ACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTA   | 3360 |
| TTTCA  | TTCGTTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTA    | 3420 |
| CAAAC  | AGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCA  | 3480 |
| CCTGA  | ATCAGGATATTCTTCTAATACCTGGAATGCTGTTGATGGTCGGAAGAGGCATAAAT | 3540 |
| AGTA   | ACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAAT | 3600 |
| TCCGT  | CAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTG  | 3660 |
| CCATG  | TTTCAGAAACAACCTCTGGCGCATCGGGCTTCCCATATAAATCAGCATCCATGTTG | 3720 |
| CCTGA  | TGCCCCGACATTATCGCGAGCCATTTATACCCATATAAATCAGCATCCATGTTG   | 3780 |
| GAATTT | AATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTT   | 3840 |
| GTATT  | ACTGTTTATGTAAGCAGACAGTTTATGTTTCATGATGATATATTTTATCTTGT    | 3900 |
| GCAAT  | GTAACATCAGGGCCTCGTGATACGCCTATTTTATAGGTTAATGTCATGATAATA   | 3960 |

Figure 15 cont'd...

```

ATGGTTTCTTAGACGTGAGGTTCTGTACCCGACACCATCGAATGGTGCAAAACCTTTTCGC 4020
GGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTA 4080
ACGTTATACGATGTGCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTG 4140
AACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAG 4200
CTGAATTACATTCCCAACCGCGTGGCACAACAACCTGGCGGGCAAACAGTCGTTGCTGATT 4260
GGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAA 4320
TCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTGATGGTAGAACGAAGCGGCGTC 4380
GAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATT 4440
AACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCG 4500
GCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAA 4560
GACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCAATTGGGTCACCAGCAAATCGCGCTG 4620
TTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATAT 4680
CTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCC 4740
GGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTT 4800
GCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTT 4860
GGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCG 4920
CCGTAAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACAGCGTGGACCGCTTG 4980
CTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTG 5040
AAAAGAAAAACCACCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGAT 5100
TCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC 5160
AATTAATGTAAGTTAGCTCACTCATTAGGCACCCAGGCTTTACACTTTATGCTTCCGAC 5220
CTGCAAGAACCTCACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTG 5280
TTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAAT 5340
GCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTAT 5400
TCCCTTTTTTTCGCGCATTTTGCCTTCCTGTTTTTGTCTACCCAGAAACGCTGGTGAAAGT 5460
AAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATC 5503

```

Figure 16

pTTQ18NHK sequence containing PPase (bold) and remainder of PCR product cloned (italics) (SEQ ID NO 13)

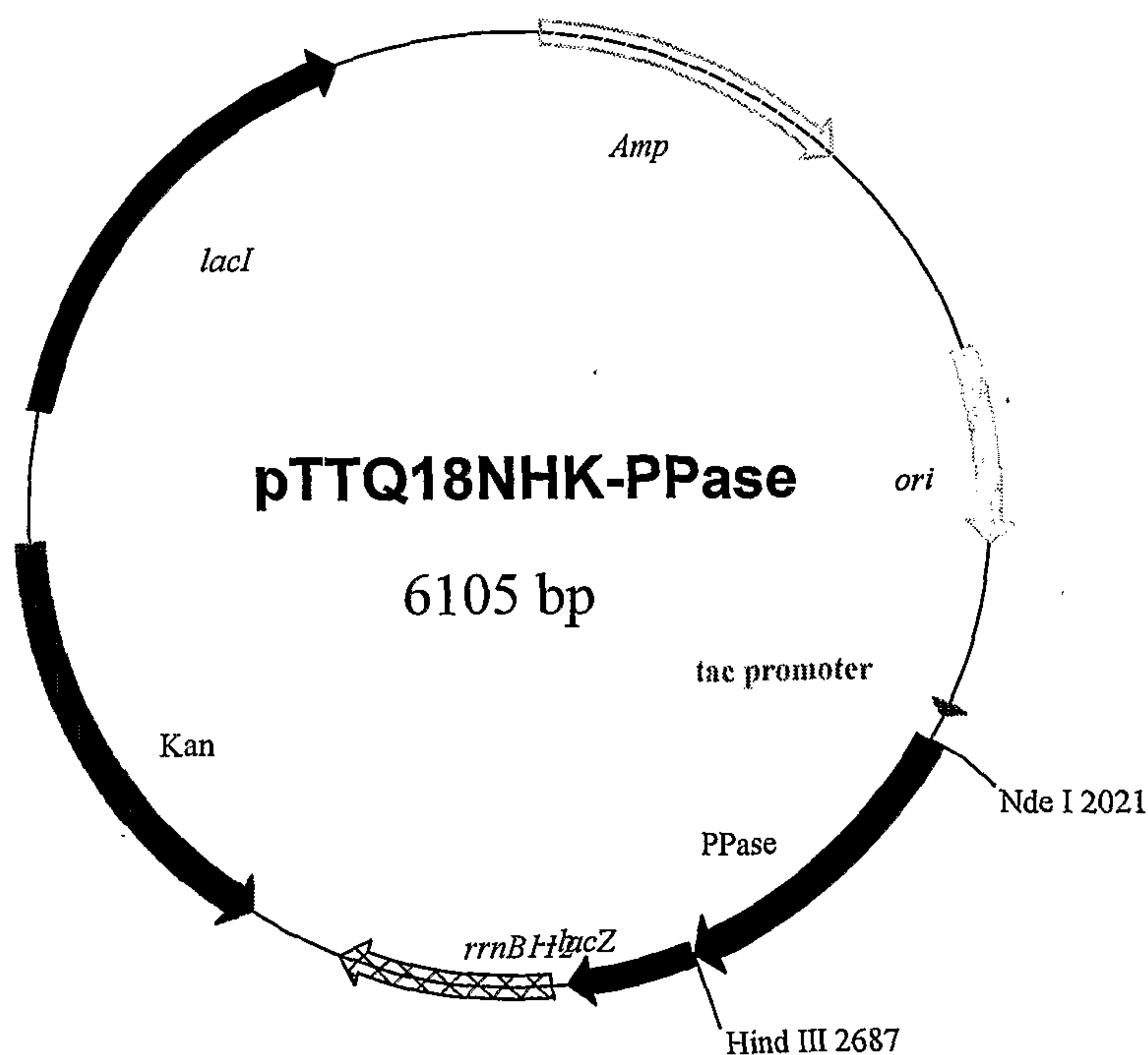


Figure 16 cont'd...

>PTTQ18NHK-PPASE SEQUENCE

|                                                                |      |
|----------------------------------------------------------------|------|
| GAAC TGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTTCCA | 60   |
| ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGG   | 120  |
| CAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCA   | 180  |
| GTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATA   | 240  |
| ACCATGAGTGATAAACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG    | 300  |
| CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCG   | 360  |
| GAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCA   | 420  |
| ACAACGTTGCGCAAACCTATTAAC TGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTA | 480  |
| ATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCT   | 540  |
| GGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCA   | 600  |
| GCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAG   | 660  |
| GCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCAT   | 720  |
| TGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAACTTCATTTT    | 780  |
| TAATTTAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAATCCCTTAA     | 840  |
| CGTGAGTTTTCTGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGA  | 900  |
| GATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACACCGCTACCAGCG     | 960  |
| GTGGTTTGTGTTGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAC TGGCTTCAGC  | 1020 |
| AGAGCGCAGATACCAAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG    | 1080 |
| AACTCTGTAGCACCCGCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC   | 1140 |
| AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG   | 1200 |
| CAGCGGTGCGGCTGAACGGGGGGTTCTGTCACACAGCCCAGCTTGGAGCGAACGACCTAC   | 1260 |
| ACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGA   | 1320 |
| AAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTT   | 1380 |
| CCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTGGGTTTCGCCACCTCTGACTTGAG    | 1440 |
| CGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAACGCCAGCAACGCG    | 1500 |
| GCCTTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTA  | 1560 |
| TCCCCTGATTCTGTGGATAAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGC  | 1620 |
| AGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGC   | 1680 |
| AAACCGCCTCTCCCCGCGCGTTGGCCGATTCAATTAATGCAGAATTAATTCTCATGTTGA   | 1740 |
| CAGCTTATCATCGACTGCACGGTGACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGC    | 1800 |
| TGTGGTATGGCTGTGCAGGTGTAATCACTGCATAAATCGTGTGCTCAAGGCGCACTC      | 1860 |
| CCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAAT   | 1920 |
| GAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAAT   | 1980 |
| TTACACAGGAAACACATATATGCACCACCACCACCACCATATGACAGGCTGTCTGAAAA    | 2040 |
| TTGGTCTCTGGAGATGAGGCTCCAGATGTTGTGAATGTCGTTATAGAGATACCTATGAACA  | 2100 |
| GTTCTGTTAAGTACGAGTTTCGACAAGGAGGCGTGTATTGTTAAGGTTGATAGGTTCCTTT  | 2160 |
| ACACCAGCATGGTCTACCCCTTCAACTACGGGTTTACATACCAGGCACTCTAGAGGAGGACG | 2220 |
| GAGATCCTGTTGACGTTCTAGTTATTAGCCGGGAGCCCGTTGCTCCCGGCTCGCTTATAG   | 2280 |
| AGGCTGTGCCCGTGGCCGTGTTAGACATGGAGGACGAGGAGGGTCCGGACAGCAAGGTTG   | 2340 |
| TTGCCGTACCCAAGGCCAAGCTGGACCCCTATTTCGCCAGCTATAAGGACGTTGGCGACA   | 2400 |
| TACCTGATGCCCTGAAATCCAAGATAAAGCACTTCTTCGAGCACTATAAGGAGCTGGAGC   | 2460 |
| CTGGAAAGTGGGTTAGAGTGACTGGATGGAGGCCTGCTGCCGATGCGAAGGAGATTATAA   | 2520 |
| GGAGGGCTATAGAGAGGTATAAGGGGGCGTGATGAGGGGCTTAACGGCTCACGTTTTCTGG  | 2580 |
| GAGAGTGTCGCACCTTTGAGGGCGATCACCTTCGCCAGCGTGCGTGTGCTTTTGTCTATG   | 2640 |
| ATTATGGCTACAGTTCTTCTAGCCGCTTTTACCGCCCCCACAGTCAAGCTTGGCACTGGC   | 2700 |
| CGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACCTAATCGCCTTGC   | 2760 |
| AGCACATCCCCCTTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTC  | 2820 |
| CCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCA   | 2880 |
| TCTGTGCGGTATTTACACCGCATAAATTCCCTGTTTTGGCGGATGAGAGAAGATTTTCA    | 2940 |
| GCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCG   | 3000 |
| GCAGTAGCGCGGTGGTCCACCTGACCCCATGCCGAACCTCAGAAGTGAAACGCCGTAGCG   | 3060 |
| CCGATGGTAGTGTGGGGTCTCCCATGCGAGAGTAGGGAACCTGCCAGGCATCAAATAAAA   | 3120 |
| CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCTGTTTTATCTGTTGTTTGTGCGGTGAACGCT | 3180 |
| CTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGA   | 3240 |
| GGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATC   | 3300 |
| CTGACGGATGGCCTTTTTGCGTTTTCTACAACTCTTCCTGTCGTATATCTACAAGCCAT    | 3360 |
| CCCCCACAGATACGGTAAACTAGCCTCGTTTTTGCATCAGGAAAGCAGGGAATTTATGG    | 3420 |
| TGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCA   | 3480 |
| ACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCT   | 3540 |
| GTGACCGTCTCCGGGAGCTGCATGTGTGAGAGGTTTTACCGTCATCACCGAAACGCGCG    | 3600 |
| AGACGAAAGGGCCTGATTAGAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCA    | 3660 |

Figure 16 cont'd...

TATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACCT 3720  
CACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTC 3780  
CAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAAT 3840  
CACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGNTTATGCATTTCTTTCCAGA 3900  
CTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGT 3960  
TATTCATTCTGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAAT 4020  
TACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTT 4080  
CACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCNNGGGATCGCAGTGG 4140  
TGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAA 4200  
ATTCGCTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTT 4260  
TGCCATGTTTCAGAAACAACCTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCG 4320  
CACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGT 4380  
TGGAATTTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCC 4440  
TTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTTCATGATGATATATTTTTATCTT 4500  
GTGCAATGTAACATCAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA 4560  
TAATGGTTTCTTAGACGTGAGGTTCTGTACCCGACACCATCGAATGGTGCAAAACCTTTC 4620  
GCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAG 4680  
TAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGG 4740  
TGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGG 4800  
AGCTGAATTACATTCCCAACCGCGTGGCACAACAACCTGGCGGGCAAACAGTCGTTGCTGA 4860  
TTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTGCGAAATTGTGCGGCGGATTA 4920  
AATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTGATGGTAGAACGAAGCGGCG 4980  
TCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCA 5040  
TTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTT 5100  
CGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTCTCCCATG 5160  
AAGACGGTACGCGACTGGGCGTGGAGCATCTGGTTCGATTGGGTCAACAGCAAATCGCGC 5220  
TGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAAT 5280  
ATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGT 5340  
CCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGG 5400  
TTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCG 5460  
TTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCC 5520  
CGCCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACAGCGTGGACCGCT 5580  
TGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCCGTCTCACTGG 5640  
TGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCG 5700  
ATTCATTAATGCAGCTGGCACGACAGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAAC 5760  
GCAATTAATGTAAGTTAGCTCACTCATTAGGCACCCAGGCTTTACACTTTATGCTTCCG 5820  
ACCTGCAAGAACCTCACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATT 5880  
TGTTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAA 5940  
ATGCTTCAATAATATTGAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTT 6000  
ATTCCTTTTTTTCGGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAA 6060  
GTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATC 6105

Figure 17

Pyrostart B-Actin Taqman on human genomic

