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(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.

Amplification Process

The present invention relates to processes for carrying out reactions in which nucleic acids are amplified, to means of 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 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, 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 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 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 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 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 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 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 "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 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, 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 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 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

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.

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

$$\text{DNA template} + \text{dNTP} \xrightarrow[\text{DNA polymerase}]{Mg^{++} +} \text{DNA template–dNMP} + \text{PPi}$$

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

$$(DNA)_{n \text{ residues}} + dNTP \leftrightarrow (DNA)_{n+1 \text{ residues}} + PPi$$

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 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.

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 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 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.

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 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 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 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 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 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 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.

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™ 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.

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 (*Tsp*NH), *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

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 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 sodium or potassium pyrophosphates including disodium pyrophosphate ($Na_2H_2P_2O_7$), anhydrous tetrasodium pyrophosphate ($Na_4P_2O_7$), tetrasodium pyrophosphate decahydrate ($Na_4P_2O_7.10H_2O$) and tetrapotassium pyrophosphate (anhydrous). Other soluble pyrophosphates which may be used include iron pyrophosphates such as ferric pyrophosphate ($Fe_4(P_2O_7)_3$), and soluble ammonium salts such as anhydrous tributylammonium pyrophosphate. Other soluble pyrophosphates are available from commercial sources.

A preferred inorganic pyrophosphate is tetrasodium pyrophosphate of formula $Na_4P_2O_7$.

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 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.

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.

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.

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 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. 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 *Sulfolbus acidicaldarius* pyrophosphatase, (Sac PPase - Meyer et al. Achives 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 *Aeropyrum pernix* inorganic pyrophosphatase obtainable from Genesys Limited, Farnborough UK.

Aeropyrum pernix K1, the first strictly aerobic hyperthermophilic archeaon, was isolated in 1993 from a coastal solfataric thermal vent at Kodaka ra-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 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

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 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 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.

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 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 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 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 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 fins 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.

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 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.

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.

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 interfere with the amplification reaction.

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 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 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 50μL PCR reaction mixture and more preferably from about 0.2-10 units per 50μL PCR reaction mixture. In this case, one unit is defined as the amount of enzyme catalysing the conversion of 1μmol pyrophosphate into 2μmol orthophosphate in one minute at 75°C under the following reaction conditions: 1mM $K_4P_2O_7$, 2mM $MgCl_2$, 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 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 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 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.

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 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 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.

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

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

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;

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

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;

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;

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;

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 pyrorphosphate, tetrasodium pyrophosphate decahydrate (PPi).

Reagent	**Volume**	**Final concn.**
10 x Reaction Buffer	5µl	1x
25mM $MgCl_2$	3µl	1.5mM
5mM dNTPs	2µl	200µM

5′ primer (10pm/µl)	5µl	1µM
3′ primer (10pm/µl)	5µl	1µM
Template	1ng	Lambda DNA
DNA polymerase (5u/µl)	0.25	1.25u
Water to Total volume	50.0µl	

Lambda 500bp Primer sequences

5′ Primer GAT GAG TTC GTG TCC GTA CAA CTG G (SEQ ID NO 14)

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

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,

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

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 2

Effect 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.

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

Lanes

1 + 2	1.5mM $MgCl_2$
3 + 4	5mM $MgCl_2$
5 + 6	7.5mM $MgCl_2$
7 + 8	10mM $MgCl_2$
9 + 10	1.5mM $MgCl_2$ + 3mM PPi
11 + 12	5mM $MgCl_2$ + 3mM PPi
13 + 14	7.5mM $MgCl_2$ + 3mM PPi
15	10mM $MgCl_2$ + 3mM PPi

The results show that the addition of Mg^{++} 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 3

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.

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

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

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 4

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.

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

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 non-HotStart DNA polymerase PCR generates a large number of non-specific amplification products.

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

Reagent	**Volume**	**Final concn.**
10x Reaction Buffer	5µl	1x
25mM $MgCl_2$	3µl	1.5mM
5mM dNTPs	2µl	200µM
5′ primer (100µm)	0.25	0.5/µM
3′ primer (100µm)	0.25	0.5/µM
Template 100ng/µl	50ng	Human xsomal DNA
5M Betaine	10.0	1M
DNA polymerase (5u/µl)	0.25	1.25u
Water to Total volume	**50.0µl**	

Angiotensin primer sequences

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:

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
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.

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

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

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.

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.

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 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 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 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 the specificity providing PPi and Sac PPase was used.

Example 6

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

The assay described in Example 4 was repeated alongside a 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 *Taq* polymerase PCR - without betaine
3 + 4	Standard *Taq* polymerase PCR - with betaine
5 + 6	Standard *Taq* polymerase PCR - without betaine but plus 3mM PPi and 0.2u Sac PPase
7 + 8	Standard *Taq* polymerase PCR - with betaine plus 3mM PPi and 0.2u Sac PPase
9 + 10	Standard *Taq* polymerase PCR - without betaine but plus 3mM PPi and 10u* *Thermococcus litoralis* PPase
11 + 12	Standard *Taq* polymerase PCR - with betaine plus 3mM PPi and 10u* *Thermococcus litoralis* PPase

* Units used in this case were as supplied by the manufacturer and are defined as the amount of enzyme that will generate 40nmoles of phosphate per minute under standard reaction conditions (10 minute reaction at 75°C in 50mM Tricine [pH 8.5], 1mM $MgCl_2$, 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.

Example 7

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

A variety of thermostable DNA polymerases were employed in the 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:

Figure 7a – *Thermus* DNA polymerases

Lanes

Top Row

1 + 2	*Taq* polymerase 0mM PPi and no PPase
3 + 4	*Taq* polymerase 3mM PPi and no PPase
5 + 6	*Taq* polymerase 3mM PPi and 0.2u Sac PPase
7 + 8	*Tbr* polymerase 0mM PPi and no PPase
9 + 10	*Tbr* polymerase 3mM PPi and no PPase
11 + 12	*Tbr* polymerase 3mM PPi and 0.2u Sac PPase

Bottom Row

1 + 2	*Tth* polymerase 0mM PPi and no PPase
3 + 4	*Tth* polymerase 3mM PPi and no PPase
5 + 6	*Tth* polymerase 3mM PPi and 0.2u Sac PPase
7 + 8	*Tsp*NH polymerase 0mM PPi and no PPase
9 + 10	*Tsp*NH polymerase 3mM PPi and no PPase
11 + 12	*Tsp*NH polymerase 3mM PPi and 0.2u Sac PPase

Figure 7b - Archael Proof-reading DNA polymerases

Lanes

Top Row

1 + 2	*Pfu* polymerase 0mM PPi and no PPase
3 + 4	*Pfu* polymerase 3mM PPi and no PPase
5 + 6	*Pfu* polymerase 3mM PPi and 0.2u Sac PPase
7 + 8	9°N exo- polymerase 0mM PPi and no PPase
9 + 10	9°N exo- polymerase 3mM PPi and no PPase
11 + 12	9°N exo- 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	*Taq* polymerase without betaine
3 + 4	*Taq* polymerase with betaine
5 + 6	Accurase polymerase without betaine
7 + 8	Accurase polymerase with betaine
9 + 10	*Tbr* polymerase without betaine
11+ 12	*Tbr* polymerase with betaine
13 + 14	*Tth* polymerase without betaine
15 + 16	*Tth* polymerase with betaine

Fig 8b Angiotensin assay with PPi and Sac PPase (with and 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 *Tsp*NH polymerase without betaine

7 + 8 *Tsp*NH 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 8

Comparison of method of invention with conventional "Hotstart" methodologies

We have some initial results (Figure 9 and 10) that show that a 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.

Figure 9 Angiotensin assay

Lanes

1 + 2	*Taq* polymerase without betaine
3 + 4	*Taq* polymerase with betaine
5 + 6	Chemically modified *Taq* without betaine
7 + 8	Chemically modified *Taq* with betaine
9 + 10	Method of the invention (3mM PPi and 2u Sac PPase) without betiane
11 + 12	Method of the invention (3mM PPi and 2u Sac PPase) with betaine

It appears that under these circumstances, the chemically modified enzyme is inactive until it has a 10 min activation at 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.

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

Lanes

1 + 2	Anti-*Taq* antibody plus *Taq* polymerase without betaine
3 + 4	Anti-*Taq* antibody plus *Taq* polymerase with betaine

In an anti-*Taq* DNA polymerase antibody mediated HotStart, a 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.

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

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.

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 are shown below as 5′ to 3′ with the restriction sites shown in bold.

Upper primer, introducing the *Nde* I site: (SEQ ID NO 18)
TGCATG**CATATG**ACAGGCTGTCTGAAAATTG
Lower primer, introducing the *Hind* III site: (SEQ ID NO 19)
TAAGTGT**AAGCTT**GACTGTGGGGGCGGTGAAAG

Aligning the putative sequence from the genome with other 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 methionine (shown in bold in SEQ ID NO.1 in Figure 11).

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.

Initial hold of 3mins at 94°C

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

Final hold of 72°C 7 minutes

PCR conditions.

50pM Upper Primer (5'..TGCATG**CATATG**ACAGGCTGTCTGAAAATTG..3'-SEQ ID NO 18)

50pM Lower Primer (5'..TAAGTGT**AAGCTT**GACTGTGGGGGCGGTGAAAG..3' - SEQ ID NO 19)

1.5mM $MgCl_2$

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

75mM Tris, pH 8.8

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. 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 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 and re-suspended in 10mM Tris, 0.1mM EDTA.

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

vector (see Figure 16) in a total volume of 10µl, overnight at 16°C in 1x NEB ligation buffer using 200u of New England Biolabs T4 DNA ligase. The plasmid vector was pTTQ18NHK, 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.

20µl 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 5µl water.

0.5µl 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 1µl of 20mg/ml XGAL and 1µl 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 100µg/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.

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.

Following centrifugation at 10,000g for 10 minutes at room temperature, an aliquot from each lysed cells were analysed by SDS polyacrylamaide 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.

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_{600} 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.

Example 10

PCR 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 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). The standard Taq polymerase was substituted for the HotStart Taq polymerase provided with the kit.

PCR reaction mixture

1x Reaction Buffer
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)
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 $MgCl_2$
2mM NaPPi
Passive Reference
1 in 4 dilutions of Human genomic DNA starting with 7.5ng (2500 copies)

Cycling conditions

Initial denaturation of 94°C 3 minutes

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

The results are shown in Figure 19.

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 HotStart PCR but at a rapid rate with the additional benefit of increased specificity.

All references mentioned in the above specification are herein incorporated by reference. Other modifications of the present 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 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.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for conducting a nucleic acid amplification reaction, said method comprising forming a PCR reaction mixture by mixing together reagents necessary for carrying out an amplification reaction by the polymerase chain reaction, and a sufficient amount of a pyrophosphate salt to prevent primer extension, adding to said mixture at least 0.04 units per 50µL PCR reaction mixture of a thermostable pyrophosphatase enzyme (PPase), and subjecting said reaction mixture to conditions such that the said pyrophosphate salt is digested with a pyrophosphatase enzyme (PPase) whereupon an amplification reaction is carried out.

2. A method for conducting a nucleic acid amplification reaction, said method comprising

forming a PCR reaction mixture by mixing together reagents necessary for carrying out a PCR amplification reaction, and a sufficient amount of a pyrophosphate salt to prevent primer extension,

adding to said mixture a thermostable pyrophosphatase enzyme (PPase) which is obtainable from a hyperthermophilic archeaon, and

subjecting said reaction mixture to conditions such that the said pyrophosphate salt is digested with pyrophosphatase enzyme (PPase) whereupon an amplification reaction is carried out.

3. A method according to claim 1 or claim 2 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.

4. A method according to any one of claims 1 to 3 wherein the inorganic pyrophosphate is an alkali metal pyrophosphate.

5. A method according to claim 4 wherein the inorganic pyrophosphate is tetrasodium pyrophosphate of formula $Na_4P_2O_7$.

6. A method according to any one of claims 1 to 5 wherein the pyrophosphate is present in the reaction mixture at a concentration of at least 0.5mM.

7. A method according to claim 6 wherein the pyrophosphate is present at a concentration of from 1-10mM.

8. A method according to any one of claims 1 to 7 wherein the thermostable PPase is *Sulfolbus acidicaldarius* inorganic pyrophosphatase, (Sac PPase), *thermococcus litoralis* inorganic pyrophosphatase or *Aeropyrum pernix* inorganic pyrophosphatase.

9. A method according to any one of claims 1 to 8 wherein the thermostable PPase comprises the amino acid sequence shown in SEQ ID NO. 25 or a variant or fragment thereof.

10. A method according to claim 8 or claim 9 wherein the thermostable PPase is added to the reaction mixture on formation thereof.

11. A method according to claim 10 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.

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12. A method according to claim 2 wherein the PPase is added to the reaction mixture at a concentration of at least 0.04u per 50µL PCR reaction mixture.

13. A method according to any one of claims 1 to 12 wherein the PPase is added to the reaction mixture at a concentration of 0.08u per 50µL PCR reaction mixture.

14. A method according to claim 12 wherein the PPase is added to the reaction mixture at a concentration of from 0.2-10u per 50µL PCR reaction mixture.

15. A kit when used for conducting an amplification reaction according to any one of claims 1 to 14, said kit comprising a pyrophosphate salt, a thermostable pyrophosphatase enzyme, and optionally one or more reagents required for use in an amplification reaction.

16. A kit according to claim 15 which further comprises one or more primers necessary to carry out amplification of a particular target nucleic acid.

17. A kit according to claim 15 or claim 16 which further includes one or more fluorescently labelled reagents.

18. A kit according to claim 17 wherein the fluorescently labelled reagents are selected from one or more of an intercalating dye, a fluorescently labelled probe, a fluorescently labelled primer or a fluorescently labeled nucleotide.

19. A pyrophosphatase enzyme encoded by the polynucleotide

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sequence as shown in SEQ ID NO. 26 or a variant or fragment thereof.

20. A pyrophosphatase enzyme comprising the amino acid sequence as shown in SEQ ID NO. 25 or a variant or fragment thereof.

21. An isolated polynucleotide which encodes a pyrophosphatase enzyme according to claim 18 or claim 19.

22. A method for conducting an amplification reaction according to any one of claims 1 to 14, or a kit when used for conducting an amplification reaction according to any one of claims 15 to 18, or a pyrophosphatase enzyme according to any one of claims 19 to 20, or an isolated polynucleotide according to claim 21, substantially as herein before described with reference to the Figures and/or Examples.

FIGURE 1

FIGURE 2

FIGURE 3

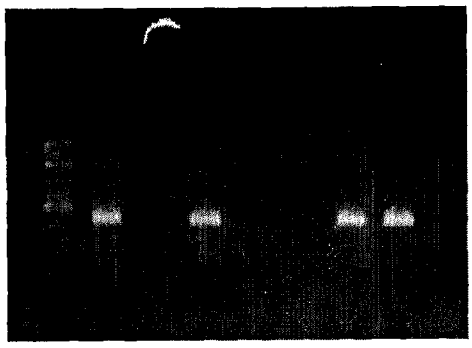

FIGURE 4

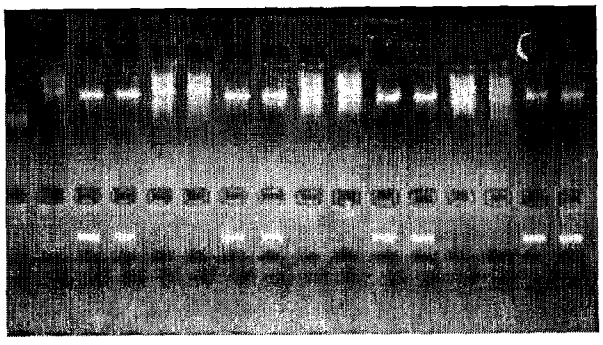

FIGURE 5

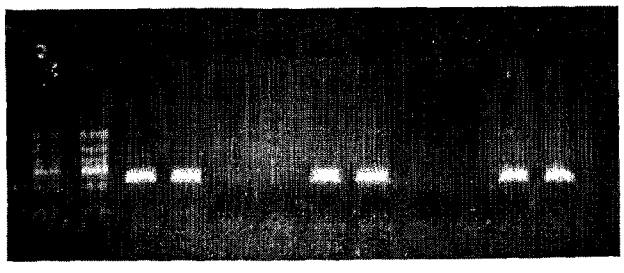

FIGURE 6

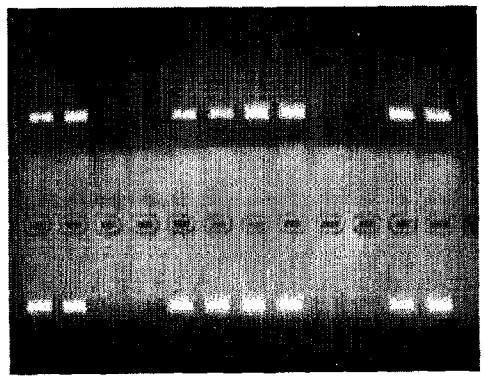

FIGURE 7a

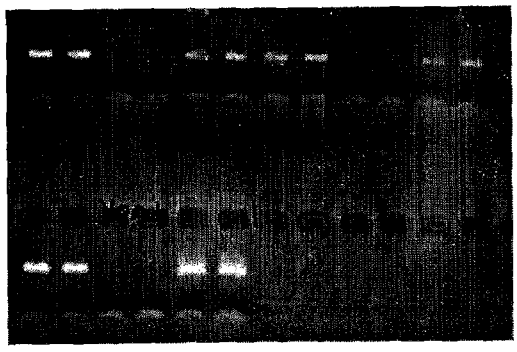

FIGURE 7b

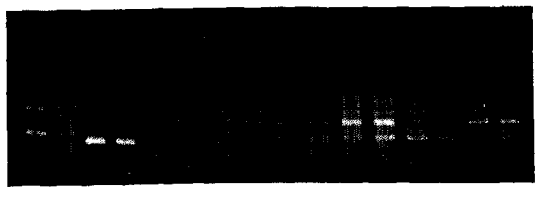

FIGURE 8a

FIGURE 8b

FIGURE 9

FIGURE 10

Figure 11

Aeropyrum pernix sequence

MWTILPSKTGFVNSLSFITRLAKLSVRRVHAMTGCLKIGPGDEAPDVVNVVIEIPMN
SSVKYEFDKEACIVKVDRFLYTSMVYPFNYGFIPGTLEEDGDPVDVLVISREPVAPG
SLIEAVPVAVLDMEDEEGPDSKVVAVPKAKLDPLFASYKDVGDIPDALKSKIKHFFE
HYKELEPGKWVRVTGWRPAADAKEIIRRAIERYKGA
(SEQ ID NO 2)

SEQ ID NO 1

```
1066801 taatcctaat tcgctttatg tggacgatcc ttcccagcaa aaccgggttt gttaacagcc
1066861 ttagctttat aactcgacta gccaaactat cggttagacg ggtgcatgca atgacaggct
1066921 gtctgaaaat tggtcctgga gatgaggctc cagatgttgt gaatgtcgtt atagagatac
1066981 ctatgaacag ttctgttaag tacgagttcg acaaggaggc gtgtattgtt aaggttgata
1067041 ggttccttta caccagcatg gtctacccct tcaactacgg gttcatacca ggcactctag
1067101 aggaggacgg agatcctgtt gacgttctag ttattagccg ggagcccgtt gctcccggct
1067161 cgcttataga ggctgtgccc gtggccgtgt tagacatgga ggacgaggag ggtccggaca
1067221 gcaaggttgt tgccgtaccc aaggccaagc tggacccccct attcgccagc tataaggacg
1067281 ttggcgacat acctgatgcc ctgaaatcca agataaagca cttcttcgag cactataagg
1067341 agctggagcc tggaaagtgg gttagagtga ctggatggag gcctgctgcc gatgcgaagg
1067401 agattataag gagggctata gagaggtata aggggggcgtg atgagggctt aacggctcac
1067461 gttttctggg agagtgtcgc acctttgagg gcgatcaccc tcgccagcgt gcgtgtgctt
1067521 ttgtctatga ttatggctac agttcttcta gccgctttca ccgcccccac agtcaataca
1067581 cttacaccta gaggttctgc gctgtatgct gtggatgtag ttgtagtaga cgccagcaca
1067641 ggatctgccc tggggttctc ccggtttgtc gtatccgcct acagaggggg ggtcggggat
1067701 gtgggtgtta tctactcttc gggggtctca gtatcagggt ctagtctgga aaggctgctg
```

MTGCLKIGPGDEAPDVVNVVIEIPMNSSVKYEFDKEACIVKVDRFLYTSMVYPFNYG
FIPGTLEEDGDPVDVLVISREPVAPGSLIEAVPVAVLDMEDEEGPDSKVVAVPKAKL
DPLFASYKDVGDIPDALKSKIKHFFEHYKELEPGKWVRVTGWRPAADAKEIIRRAIE
RYKGA (SEQ ID NO 25)

SEQ ID NO 26

```
atgacaggct gtctgaaaat tggtcctgga gatgaggctc cagatgttgt
gaatgtcgtt atagagatac ctatgaacag ttctgttaag tacgagttcg
acaaggaggc gtgtattgtt aaggttgata ggttccttta caccagcatg
gtctacccct tcaactacgg gttcatacca ggcactctag aggaggacgg
agatcctgtt gacgttctag ttattagccg ggagcccgtt gctcccggct
cgcttataga ggctgtgccc gtggccgtgt tagacatgga ggacgaggag
ggtccggaca gcaaggttgt tgccgtaccc aaggccaagc tggacccccct
attcgccagc tataaggacg ttggcgacat acctgatgcc ctgaaatcca
agataaagca cttcttcgag cactataagg agctggagcc tggaaagtgg
gttagagtga ctggatggag gcctgctgcc gatgcgaagg agattataag
gagggctata gagaggtata aggggggcgtg 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 abysii*	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-GKNAPDVVNVLVEIPQ-GS
E.coli          ---------------------------------MSLLNGPA-GKDLPEDIYVVIEIPANAD
aquifex         ---------------------------------MGYDQLPP-GKNPPEDIYVVIEIPQ-GS
Pho             ----------------------------------MNPFHDLEP-GPNVPEVVYALIEIPK-GS
Pab             ----------------------------------MNPFHDLEP-GPNVPEVVYALIEIPK-GS
Tli             ----------------------------------MNPFHDLEP-GPEVPEVVYALIEIPK-GS
thermoplasma    ----------------------------------MESFYHSVPVGPKPPEEVYVIVEIPR-GS
                                                      .  . * . *: : .::***   .

aeropyrum       SVKYEFDKEACIVKVDRFLYTSMVYPFNYGFIPGTLEEDGDPVDVLVISREPVAPGSLIE
sulfolobus      NIKYEYDDEEGVIKVDRVLYTSMNYPFNYGFIPGTLEEDGDPLDVLVITNYQLYPGSVIE
E.coli          PIKYEIDKESGALFVDRFMSTAMFYPCNYGYINHILSLDGDPVDVLVPTPYPLQPGSVIR
aquifex         AVKYELDKDTGVIFVDRFLFTAMYYPFNYGFVPQTLADDGDPVDVLVISREPVVPGAVMR
Pho             RNKYELDKETGLLKLDRVLYTPFHYPVDYGIIPRTWYEDGDPFDIMVIMREPTYPLTIIE
Pab             RNKYELDKKTGLLKLDRVLYSPFTYPVDYGIIPRTWYDDDDPFDIMVIMREPTYPLTIIE
Tli             RNKYELDKKTGLIKLDRVLYSPFHYPVDYGIIPQTWYDDDDPFDIMVIMREPTYPGVLIE
thermoplasma    RVKYEIAKDFPGMLVDRVLYSSVVYPVDYGLIPRTLYYDGDPMDVMVLISQPTFPGAIMK
                  ***  ..   : :**.: :.. ** :** :      *.**.*::*       *  ::.

aeropyrum       AVPVAVLDMEDEEGPDSKVVAVPKAKLDPLFASYKDVGDIPDALKSKIKHFFEHYKELEP
sulfolobus      VRPIGILYMKDEEGEDAKIVAVPKDKTDPSFSNIKDINDLPQATKNKIVHFFEHYKELEP
E.coli          CRPVGVLKMTDEAGEDAKLVAVPHSKLSKEYDHIKDVNDLPELLKAQIAHFFEHYKDLEK
aquifex         CRPIGMLEMRDEAGIDTKVIAVPHEKLDPSYSNIKTVDNLPEIVREKIKHFFEHYKELEP
Pho             ARPIGLFKMIDSGDKDYKVLAVPVE--DPYFKDWKDISDVPKAFLDEIAHFFKRYKELE-
Pab             ARPIGLFKMIDSGDKDYKVLAVPVE--DPYFKDWKDIDDVPKAFLDEIAHFFKRYKELQ-
Tli             ARPIGLFKMIDSGDKDYKVLAVPVE--DPYFNDWKDISDVPKAFLDEIAHFFQRYKELQ-
thermoplasma    VRPIGMMKMVDQGETDNKILAVFDK--DPNVSYIKDLKDVNAHLLDEIANFFSTYKILE-
                  *:.:: * *.   * *::**      .       * : ::       :* :**. ** *:

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

Figure 13

686bp PCR product. (SEQ ID NO 10)

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

TGCATG**CATATG***ACAGGCTGTCTCAAAATTGGTCCTGGAGATGAGGCTCCAGATGTTGTGAATGTCGTT*
ATAGAGATACCTATGAACAGTTCTGTTAAGTACGAGTTCGACAAGGAGGCGTGTATTGTTAAGGTTGAT
AGGTTCCTTTACACCAGCATGGTCTACCCCTTCAACTACGGGTTCATACCAGGCACTCTAGAGGAGGAC
GGAGATCCTGTTGACGTTCTAGTTATTAGCCGGGAGCCCGTTGCTCCCGGCTCGCTTATAGAGGCTGTG
CCCGTGGCCGTGTTAGACATCGAGGACGACGAGGGTCCGGACAGCAAGGTTGTTGCCGTACCCAAGGCC
AAGCTGGACCCCCTATTCGCCAGCTATAAGGACGTTGGCGACATACCTGATGCCCTGAAATCCAAGATA
AAGCACTTCTTCGAGCACTATAAGGAGCTGGAGCCTGGAAAGTGGGTTAGAGTGACTGGATGGAGGCCT
GCTGCCGATGCGAAGGAGATTATAAGGAGGGCTATAGAGAGGTATAAGGGGGCGTGATGAGGGCTTAAC
GGCTCACGTTTTCTGGGAGAGTGTCGCACCTTTGAGGGCGATCACCCTCGCCAGCGTGCGTGTGCTTTT
GTCTATGATTATGGCTACAGTTCTTCTAGCCGCTTTCACCGCCCCCACAGTC**AAGCTT**ACACTTA

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**
ATGCACCACCACCACCACCATATGGGCATGCTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCG

ACCTGCAGGCATGCAAGCTT
***Hind* III**

Figure 15

pTTQ18NHK sequence (SEQ ID NO 12)

```
>pTTQ18NHK Sequence
GAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA  60
ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGG  120
CAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCA  180
GTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATA  240
ACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG  300
CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCG  360
GAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCA  420
ACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTA  480
ATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCT  540
GGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCA  600
GCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAG  660
GCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCAT  720
TGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTT  780
TAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAA  840
CGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGA  900
GATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG  960
GTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGC  1020
AGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG  1080
AACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC  1140
AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG  1200
CAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTAC  1260
ACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGA  1320
AAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTT  1380
CCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAG  1440
CGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCG  1500
GCCTTTTTACGGTTCCTGGCCTTTTGCTGCCCTTTTGCTCACATGTTCTTTCCTGCGTTA  1560
TCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGC  1620
AGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGC  1680
AAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGAATTAATTCTCATGTTTGA  1740
CAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGC  1800
TGTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTC  1860
CCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAAT  1920
GAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAAT  1980
TTCACACAGGAAACACATATATGCACCACCACCACCATATGGGCATGCTGAATTCGA  2040
GCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCG  2100
TCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAG  2160
CACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCC  2220
AACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATC  2280
TGTGCGGTATTTCACACCGCATAAATTCCCTGTTTTGGCGGATGAGAGAAGATTTTCAGC  2340
CTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGC  2400
AGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCC  2460
GATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACG  2520
AAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCT  2580
CCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGG  2640
GTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCT  2700
GACGGATGGCCTTTTTGCGTTTCTACAAACTCTTCCTGTCGTCATATCTACAAGCCATCC  2760
CCCCACAGATACGGTAAACTAGCCTCGTTTTTGCATCAGGAAAGCAGGGAATTTATGGTG  2820
CACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAAC  2880
ACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGT  2940
GACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAG  3000
ACGAAAGGGCCTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATA  3060
TCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCA  3120
CCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCA  3180
ACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCA  3240
CCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGNTTATGCATTTCTTTCCAGACT  3300
TGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTA  3360
TTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTA  3420
CAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCA  3480
CCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCNGGGATCGCAGTGGTG  3540
AGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAAT  3600
TCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTG  3660
CCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCA  3720
CCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTG  3780
GAATTTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTT  3840
GTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATCTTGT  3900
GCAATGTAACATCAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATA  3960
```

Figure 15 cont'd...

```
ATGGTTTCTTAGACGTGAGGTTCTGTACCCGACACCATCGAATGGTGCAAAACCTTTCGC  4020
GGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTA  4080
ACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTG  4140
AACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAG  4200
CTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGATT  4260
GGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAA  4320
TCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTC  4380
GAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATT  4440
AACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCG  4500
GCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAA  4560
GACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTG  4620
TTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATAT  4680
CTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCC  4740
GGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTT  4800
GCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTT  4860
GGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCG  4920
CCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTG  4980
CTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTG  5040
AAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGAT  5100
TCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGC  5160
AATTAATGTAAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGAC  5220
CTGCAAGAACCTCACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTG  5280
TTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAAT  5340
GCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTAT  5400
TCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGT  5460
AAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATC  5503
```

Figure 16

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

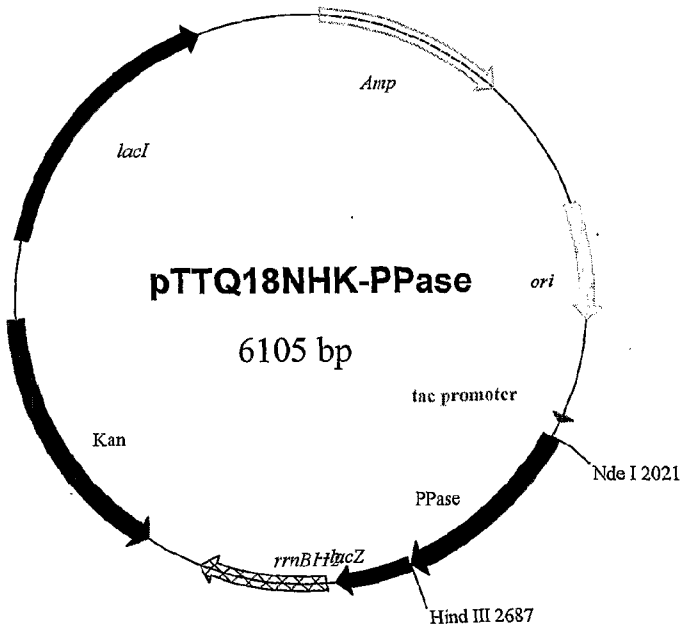

Figure 16 cont'd...

>PTTQ18NHK-PPASE SEQUENCE

```
GAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA  60
ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGG  120
CAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCA  180
GTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATA  240
ACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG  300
CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCG  360
GAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCA  420
ACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTA  480
ATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCT  540
GGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCA  600
GCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAG  660
GCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCAT  720
TGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTT  780
TAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAA  840
CGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGA  900
GATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG  960
GTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGC  1020
AGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG  1080
AACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC  1140
AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG  1200
CAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTAC  1260
ACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGA  1320
AAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTT  1380
CCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAG  1440
CGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCG  1500
GCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTA  1560
TCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGC  1620
AGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGC  1680
AAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGAATTAATTCTCATGTTTGA  1740
CAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGC  1800
TGTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTC  1860
CCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAAT  1920
CAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAAT  1980
TTCACACAGGAAACACATATATGCACCACCACCACCACCATATGACAGGCTGTCTGAAAA  2040
TTGGTCCTGGAGATGAGGCTCCAGATGTTGTGAATGTCGTTATAGAGATACCTATGAACA  2100
GTTCTGTTAAGTACGAGTTCGACAAGGAGGCGTGTATTGTTAAGGTTGATAGGTTCCTTT  2160
ACACCAGCATGGTCTACCCCTTCAACTACGGGTTCATACCAGGCACTCTAGAGGAGGACG  2220
GAGATCCTGTTGACGTTCTAGTTATTAGCCGGGAGCCCGTTGCTCCCGGCTCGCTTATAG  2280
AGGCTGTGCCCGTGGCCGTGTTAGACATGGAGGACGAGGAGGGTCCGGACAGCAAGGTTG  2340
TTGCCGTACCCAAGGCCAAGCTGGACCCCCTATTCGCCAGCTATAAGGACGTTGGCGACA  2400
TACCTGATGCCCTGAAATCCAAGATAAAGCACTTCTTCGAGCACTATAAGGAGCTGGAGC  2460
CTGGAAAGTGGGTTAGAGTGACTGGATGGAGGCCTGCTGCCGATGCGAAGGAGATTATAA  2520
GGAGGGCTATAGAGAGGTATAAGGGGGCGTGATGAGGGCTTAACGGCTCACGTTTTCTGG  2580
GAGAGTGTCGCACCTTTGAGGGCGATCACCCTCGCCAGCGTGCGTGTGCTTTTGTCTATG  2640
ATTATGGCTACAGTTCTTCTAGCCGCTTTCACCGCCCCCACAGTCAAGCTTGGCACTGGC  2700
CGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGC  2760
AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTC  2820
CCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCA  2880
TCTGTGCGGTATTTCACACCGCATAAATTCCCTGTTTTGGCGGATGAGAGAAGATTTTCA  2940
GCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCG  3000
GCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCG  3060
CCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAA  3120
CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCT  3180
CTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGA  3240
GGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATC  3300
CTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTCCTGTCGTCATATCTACAAGCCAT  3360
CCCCCCACAGATACGGTAAACTAGCCTCGTTTTTGCATCAGGAAAGCAGGGAATTTATGG  3420
TGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCA  3480
ACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCT  3540
GTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCG  3600
AGACGAAAGGGCCTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCA  3660
```

Figure 16 cont'd...

TATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACT 3720
CACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTC 3780
CAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAAT 3840
CACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGNTTATGCATTTCTTTCCAGA 3900
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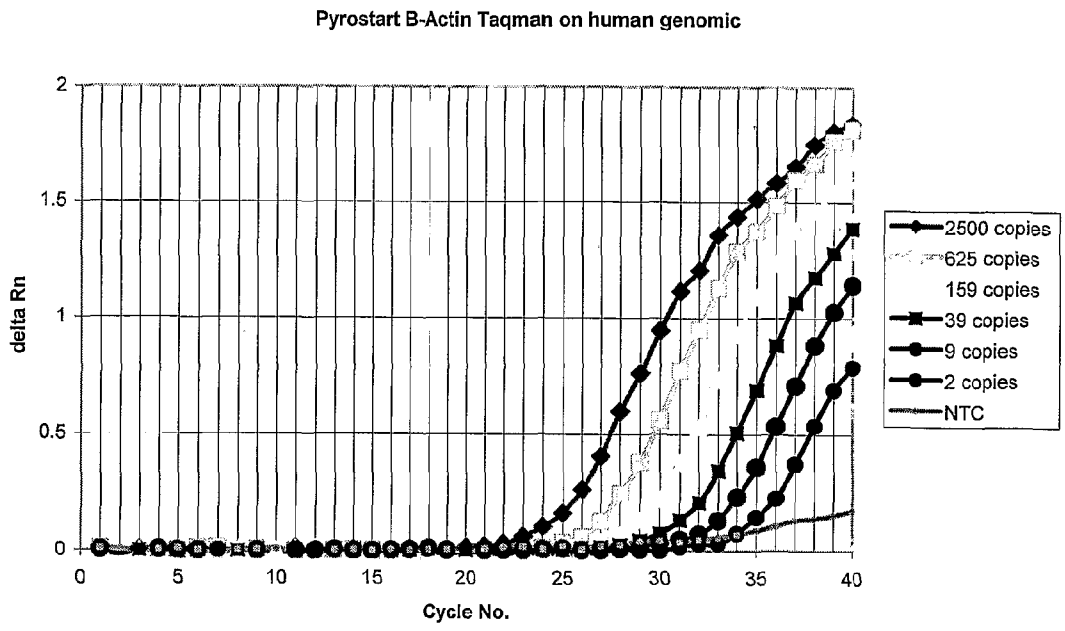
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Pro Gly Ser Leu Ile Glu Ala Val Pro Val Ala Val Leu Asp Met Glu
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Pro Phe Asn Tyr Gly Phe Ile Pro Gly Thr Leu Glu Glu Asp Gly Asp
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Asn Lys Ile Val His Phe Phe Glu His Tyr Lys Glu Leu Glu Pro Gly
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Ala Met Phe Tyr Pro Cys Asn Tyr Gly Tyr Ile Asn His Ile Leu Ser
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5

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Thr Asp Glu Ala Gly Glu Asp Ala Lys Leu Val Ala Val Pro His Ser
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Lys Leu Ser Lys Glu Tyr Asp His Ile Lys Asp Val Asn Asp Leu Pro
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Glu Leu Leu Lys Ala Gln Ile Ala His Phe Phe Glu His Tyr Lys Asp
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Asp Gly Asp Pro Val Asp Val Leu Val Ile Ser Arg Glu Pro Val Val
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Pro Gly Ala Val Met Arg Cys Arg Pro Ile Gly Met Leu Glu Met Arg
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Asp Glu Ala Gly Ile Asp Thr Lys Val Ile Ala Val Pro His Glu Lys
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Leu Asp Pro Ser Tyr Ser Asn Ile Lys Thr Val Asp Asn Leu Pro Glu
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Ile Val Arg Glu Lys Ile Lys His Phe Phe Glu His Tyr Lys Glu Leu
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Pro Phe His Tyr Pro Val Asp Tyr Gly Ile Ile Pro Arg Thr Trp Tyr
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Glu Asp Gly Asp Pro Phe Asp Ile Met Val Ile Met Arg Glu Pro Thr
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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
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Phe Leu Asp Glu Ile Ala His Phe Phe Lys Arg Tyr Lys Glu Leu Glu
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Lys Glu

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Pro Phe Phe Tyr Pro Val Asp Tyr Gly Ile Ile Pro Arg Thr Trp Tyr
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Tyr Pro Leu Thr Ile Ile Glu Ala Arg Pro Ile Gly Leu Phe Lys Met
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Ile Asp Ser Gly Asp Lys Asp Tyr Lys Val Leu Ala Val Pro Val Glu
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Asp Pro Tyr Phe Lys Asp Trp Lys Asp Ile Asp Asp Val Pro Lys Ala
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Phe Leu Asp Glu Ile Ala His Phe Phe Lys Arg Tyr Lys Glu Leu Gln
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Gly Lys Glu Ile Ile Val Glu Gly Trp Glu Gly Ala Glu Ala Ala Lys
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Arg Glu Ile Leu Arg Ala Ile Glu Leu Tyr Lys Glu Lys Phe Gly Ser
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Lys Glu

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<213> Thermococcus litoralis

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Pro Phe His Tyr Pro Val Asp Tyr Gly Ile Ile Pro Gln Thr Trp Tyr
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Asp Asp Asp Asp Pro Phe Asp Ile Met Val Ile Met Arg Glu Pro Thr
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Tyr Pro Gly Val Leu Ile Glu Ala Arg Pro Ile Gly Leu Phe Lys Met
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Ile Asp Ser Gly Asp Lys Asp Tyr Lys Val Leu Ala Val Pro Val Glu
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Asp Pro Tyr Phe Asn Asp Trp Lys Asp Ile Ser Asp Val Pro Lys Ala
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Phe Leu Asp Glu Ile Ala His Phe Phe Gln Arg Tyr Lys Glu Leu Gln
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Gly Lys Glu Ile Ile Val Glu Gly Trp Glu Asn Ala Glu Lys Ala Lys
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Ser Ser Val Val Tyr Pro Val Asp Tyr Gly Leu Ile Pro Arg Thr Leu
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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
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Met Val Asp Gln Gly Glu Thr Asp Asn Lys Ile Leu Ala Val Phe Asp
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Lys Asp Pro Asn Val Ser Tyr Ile Lys Asp Leu Lys Asp Val Asn Ala
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His Leu Leu Asp Glu Ile Ala Asn Phe Phe Ser Thr Tyr Lys Ile Leu
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Glu Lys Lys Glu Thr Lys Val Leu Gly Trp Glu Gly Lys Glu Ala Ala
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Lys Lys Asn

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: pTTQ18NHK
vector including the PPase sequence

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<220>
<223> Description of Artificial Sequence: Primer

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<220>
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<220>
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<210> 17
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<220>
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<400> 19
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<210> 20

<400> 20
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<210> 22
<211> 23
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<220>
<223> Description of Artificial Sequence: Primer

<400> 22
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<210> 23
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 23
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<210> 24
<211> 27
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<220>
<223> Description of Artificial Sequence: Probe

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<210> 25
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<213> Aeropyrum pernix

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Ala	Pro	Gly	Ser	Leu	Ile	Glu	Ala	Val	Pro	Val	Ala	Val	Leu	Asp	Met
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