



(22) Date de dépôt/Filing Date: 2002/12/10

(41) Mise à la disp. pub./Open to Public Insp.: 2004/05/01

(30) Priorité/Priority: 2002/11/01 (60/422,877) US

(51) Cl.Int.⁷/Int.Cl.⁷ C12Q 1/68

(71) Demandeur/Applicant:
UNIVERSITY OF OTTAWA, CA

(72) Inventeurs/Inventors:
LEM, PAUL, CA;
SPIEGELMAN, JAMIE, CA

(74) Agent: OSLER, HOSKIN & HARCOURT LLP

(54) Titre : METHODE D'AMPLIFICATION SIMULTANEE DE PLUSIEURS GENES CIBLES

(54) Title: A METHOD FOR THE AMPLIFICATION OF MULTIPLE GENETIC TARGETS

(57) **Abrégé/Abstract:**

A method for simultaneous amplification and detection of multiple genetic targets is provided. Furthermore, a primer design protocol specific to the PCR reaction conditions of the present invention is also provided. The method of the present invention includes a PCR reaction mixture and primers specifically selected according to the reactions conditions provided. Multiple genetic targets are amplified simultaneously by this method, without requiring optimization of the reaction conditions.

ABSTRACT OF THE DISCLOSURE

A method for simultaneous amplification and detection of multiple genetic targets is provided. Furthermore, a primer design protocol specific to the PCR reaction conditions of the present invention is also provided. The method of the present invention includes a PCR reaction mixture and primers specifically selected according to the reactions conditions provided. Multiple genetic targets are amplified simultaneously by this method, without requiring optimization of the reaction conditions.

A METHOD FOR THE AMPLIFICATION OF MULTIPLE
GENETIC TARGETS

TECHNICAL FIELD

This invention relates to a method for the
5 amplification and detection of multiple genetic targets,
and components thereof. More specifically, the present
invention relates to the simultaneous amplification of
multiple genetic targets in a single reaction. In
particular, the present invention relates to an enhanced
10 Polymerase Chain Reaction (PCR) method and components
thereof.

BACKGROUND OF THE INVENTION

The Polymerase Chain Reaction (PCR) is a widely
used technique that employs a thermostable DNA polymerase
15 enzyme in conjunction with target-specific primers to
amplify target genetic sequences. The term "multiplex"
refers to the ability to amplify multiple genetic sequences
simultaneously, in a single reaction vessel, rather than
having to conduct each amplification reaction individually.
20 Thus, saving time and resources. Multiplex PCR generally
refers to a multiplex reaction using standard PCR reagents
and unmodified deoxynucleotide triphosphates (dNTPs) for
amplifying multiple genetic sequences simultaneously.
However, limitations exist in the number of target
25 sequences that are efficiently amplified with current
multiplex PCR systems. The products of such a multiplex
PCR reaction are routinely detected by a simple technique
like agarose gel electrophoresis and staining by a common
dye like ethidium-bromide. Other technologies "multiplex"
30 multiple genetic targets at one time, but then use
detection methods other than agarose gel electrophoresis.

For example, the Roche Light CyclerTM can multiplex more than one genetic target at a time, but detects amplified products using hybridization probes employing Fluorescent Resonance Energy Transfer (FRET) technology. Technologies
5 like the GenePrint PowerPlexTM system incorporate fluorescently-labeled nucleotides into the PCR products, for later fluorescent detection. Although these systems often enhance the detection capacity of multiplex PCR, they are technology-dependent, and expensive. There remains a
10 need for a simple multiplex PCR system that is reliable, efficient and cost-effective.

Many researchers have designed diagnostic assays based on multiplex PCR principles. Most multiplex PCR protocols are limited to the successful amplification of
15 just a few target genetic sequences in a single reaction. Several studies have been able to simultaneously amplify 3 to 5 multiplex PCR products (Maes et al., 2002; Paton and Paton, 2002). A study by Henegariu et al. (1997) reported visualization of 7 multiplex PCR products, but required
20 extensive optimization of reaction reagents and conditions in order to do so. Other studies use as many as seven primer pairs in the same reaction tube, but this is done with the expectation that the seven genetic targets will not all be present in the sample of interest (Markoulatos
25 et al., 2001; Elsayed et al., 2001; Hindiyeh et al., 2001).

Furthermore, multiplex PCR reaction conditions need to be empirically optimized depending on the primers used (Henegariu et al., 1997). Even then, there is no certainty that a given primer pair will work with others in a
30 multiplex PCR assay. Also, the addition or removal of a primer pair in an existing working assay may require the optimization process to begin all over again. It would be

useful to have a single set of multiplex PCR reaction conditions that do not require optimization each time a different primer is added or removed from the assay.

U.S. Patent Nos. 5,882,856 and 6,207,372 relate to
5 a universal primer sequence for multiplex DNA
amplification. In particular, these patents disclose
chimeric primers that serve as high stringency recognition
sequences in the amplification process and normalize the
degree of amplification of different targets. Although
10 these primers claim to have a uniformly high degree of
specificity on the annealing reactions that occur between
different primers present in the mixture and their cognate
target sequences in the DNA template without requiring the
need to adjust multiplex reaction conditions, the design of
15 these primers is both complex and time consuming.
Clearly, a technician could not readily optimize these
primers for use in a DNA amplification protocol, nor
replace a given set of primers in the midst of an
amplification assay.

20 U.S. Patent No. 6,333,179 relates to methods and
compositions for multiplex amplification of nucleic acids.
According to this patent, a predetermined ratio of primers
can be calculated according to a disclosed formula to
achieve approximate equi-molar yield of multiplex PCR
25 products. According to this formula, primer concentrations
are varied as a function of amplicon length. Such a method
is time-consuming, requiring individual calculations for
each primer pair. It would be desirable to have a method for
simultaneously amplifying multiple genetic targets that
30 could be repeatedly performed according to a set of common
directions without requiring optimization of the reaction
conditions.

In many cases it is desirable to be able to simultaneously amplify numerous genetic targets in a convenient and cost-effective manner. In the field of infectious disease, for example, often a practitioner is
5 interested in pinpointing a causative agent of infection from a large group of potential organisms. It would be beneficial to have a multiplex PCR system capable of simultaneously amplifying more than 5 target genetic sequences without the need for optimization steps. Thereby
10 providing the capability to quickly and conveniently detect a genetic target of interest.

In summary, there is a need for the ability to efficiently and economically amplify and detect multiple genetic targets in a single reaction vessel, without the
15 need for optimization of multiplex PCR reaction conditions.

In particular, in the case of genetic screening for diseases there is truly a need for a method for simultaneously amplifying multiple genetic targets without requiring optimization of reaction conditions.

20 **SUMMARY OF THE INVENTION**

An enhanced multiplex PCR method for simultaneous amplification of multiple genetic targets is provided. According to the present invention, multiple genetic targets can be quickly and easily detected without
25 requiring extensive optimization of the enhanced multiplex PCR method herein described. Furthermore, a primer design protocol specific to the PCR reaction conditions of the present invention is also provided. Amplification of at least ten genetic targets simultaneously in single reaction
30 vessel is provided in accordance with an embodiment of the present invention. In addition, the method of the present

invention is pre-optimized for amplification of multiple genetic targets and can be performed with primers designed according to a design protocol without the need for optimization of the multiplex PCR reaction conditions.

5 According to one aspect of the present invention, there is provided a method for simultaneously detecting multiple genetic targets, said method comprising: mixing a sample to be tested for the presence of said multiple genetic targets with pre-selected primer pairs specific to
10 said multiple genetic targets and a PCR reaction mixture; effecting a hot start initiation of amplification of said genetic targets; performing a series of PCR reaction steps to amplify each of said multiple genetic sequences in said sample; and detecting said multiple genetic targets present
15 in said sample; wherein said pre-selected primer pairs are provided to optimize amplification of said multiple genetic targets in said PCR reaction mixture in the absence of requiring optimization of reaction conditions of said method.

20 According to one aspect of the present invention, there is provided a method for simultaneously amplifying multiple genetic targets, said method comprising: mixing a sample to be tested for the presence of said multiple genetic targets with pre-selected primer pairs specific to
25 said multiple genetic targets and a PCR reaction mixture; effecting a hot start initiation of amplification of said genetic targets; performing a series of PCR reaction steps to amplify each of said multiple genetic sequences in said sample; wherein said pre-selected primer pairs are provided
30 to optimize amplification of said multiple genetic targets in said reaction mixture in the absence of requiring optimization of reaction conditions of said method.

According to one aspect of the present invention, there is provided a kit for simultaneously amplifying multiple genetic targets for detection, said kit comprising: a PCR reaction mixture having a final MgCl_2 concentration of 5 - 12.5mM; a set of pre-selected, target-specific primer pairs corresponding to each of said multiple genetic targets; and a set of instructions for using contents of said kit for simultaneously amplifying multiple genetic targets in a sample to be tested; wherein
10 said PCR reaction mixture is adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel without requiring optimization of pre-set amplification reaction conditions when said pre-selected, target-specific primer pairs are provided at a final
15 concentration of approximately $0.005\mu\text{M}$ - $0.05\mu\text{M}$ in the reaction mixture.

According to one aspect of the present invention, there is provided a PCR reaction mixture for use in simultaneously amplifying multiple genetic targets, said
20 mixture comprising: a PCR buffer reagent including 5mM - 10mM MgCl_2 ; wherein said PCR reaction mixture is adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel without requiring optimization of pre-set amplification reaction conditions.

25 It is an object of the present invention to provide an enhanced multiplex PCR method for simultaneously amplifying multiple genetic targets in a single reaction vessel.

It is a further object of the present invention to
30 provide an enhanced multiplex PCR method for simultaneously amplifying multiple genetic targets in a single reaction

vessel without requiring optimization of the reaction conditions.

It is another object of the present invention to provide a novel PCR reaction mixture adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel.

It is a further object of the present invention to provide primer pairs designed for use in an enhanced multiplex PCR method.

It is yet a further object of the present invention to provide a kit adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel.

BRIEF DESCRIPTION OF THE DRAWINGS

Further features and advantages of the present invention will become apparent from the following detailed description, taken in combination with the appended drawings, in which:

Fig. 1 is a flowchart exemplifying an enhanced multiplex PCR method in accordance with an aspect of the present invention;

Fig. 2 illustrates a primer selection criteria in accordance with an aspect of the present invention;

Fig. 3 is a flowchart of a series of PCR Cycling steps performed in accordance with an aspect of the present invention;

Fig. 4 illustrates the detection of ten different genes in methicillin-resistant *Staphylococcus aureus* in accordance with an aspect of the present invention;

Fig. 5 illustrates the detection sensitivity of ten different genetic targets of the present invention as displayed at varying initial concentrations of bacteria in accordance with an aspect of the present invention;

5 Fig. 6 illustrates the effect of varying magnesium chloride concentrations on the detection of ten different genetic targets in accordance with an aspect of the present invention; and

10 Fig. 7 illustrates the effects of varying primer concentrations on the detection of ten different genetic targets in accordance with an aspect of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

 An enhanced multiplex Polymerase Chain Reaction
15 (PCR) method for reliable and efficient amplification of multiple genetic targets in a single reaction vessel is provided by the present invention. The ability to simultaneously amplify and detect multiple genetic targets using a convenient and time-efficient method of enhanced
20 multiplex PCR as herein provided, provides a clear advantage over the prior art. The present invention provides a simple, efficient and economical method for achieving multiple target genetic sequence amplification in a single reaction. In particular, products of the enhanced
25 multiplex PCR method can be detected by routine and cost effective methods, such as agarose gel electrophoresis, for example.

 "Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the

concentration of a particular DNA sequence within a mixture of DNA sequences.

An "amplicon" is a product of the amplification of a target genetic sequence.

5 "Multiplex PCR" as used herein refers to the use of the polymerase chain reaction (PCR) for simultaneous amplification of multiple genetic targets in a single polymerase chain reaction (PCR) reaction. PCR as used herein may include touch-down PCR.

10 A "PCR reaction mixture" as used herein denotes a mixture adaptable for simultaneously amplifying multiple genetic targets under suitable conditions for PCR.

A "genetic target" as used herein denotes a genetic sequence capable of amplification by polymerase chain
15 reaction (PCR). A genetic target in accordance with the present invention includes any DNA sequence, including bacterial, viral, human, plant, and animal DNA, for example.

It is technically more challenging to design a
20 multiplex PCR system that can actually amplify multiple genetic targets. Different primer pairs have different amplification efficiencies, thus making it difficult to achieve adequate amplification of all primer pairs simultaneously. Due to the iterative nature of PCR cycles,
25 amplicons generated by more efficient primer pairs quickly become the dominant species in the mix. This dominant species then out-competes the less efficient primer pairs for PCR reagents, resulting in insufficient amplification of the less efficient species.

The present invention provides an enhanced multiplex PCR reaction method that includes pre-selected primer pairs for use in amplifying genetic targets. The enhanced multiplex PCR reaction method of the present invention further includes pre-set amplification reaction conditions. Pre-selected primer pairs of the present invention are target specific primer pairs designed according to a given primer design protocol. According to one embodiment of the present invention, when employed under pre-set amplification reaction conditions, a predetermined concentration of the primer pairs optimize the amplification efficiency of the genetic targets of interest. The unique reaction conditions of the present invention further serve to optimize the amplification efficiencies of the target specific primer pairs to effectively amplify multiple genetic targets simultaneously. According to a preferred embodiment of the invention 10 or more genetic targets can be simultaneously amplified in the same reaction vessel. The present invention can be employed with standard PCR equipment while achieving excellent sensitivity and specificity for the detection of multiple genetic targets.

Unique reaction conditions coupled with target-specific primer pairs and a series of thermal cycling conditions provide an enhanced multiplex PCR system that reliably and efficiently amplifies multiple target genetic sequences simultaneously, in accordance with one embodiment of the present invention. Primer pairs of the present invention are designed according to a primer design protocol or selected according to a primer selection criteria and employed in the enhanced multiplex PCR method in predetermined concentrations. The predetermined concentration of each primer pair in the reaction mixture

of the present invention is preferably the same. According to one embodiment of the present invention, the predetermined concentration of the primer pairs in the reaction mixture does not interfere with the pre-set optimization reaction conditions of the enhanced multiplex PCR method. Furthermore, target-specific primer pairs of the present invention can be added, removed or replaced in the reaction mixture of the present invention without requiring re-optimization of the reaction conditions.

10 According to this embodiment, the concentration of the primer pairs are predetermined with respect to the reaction conditions and allow for efficient amplification of all genetic targets. As provided in accordance with the present invention, the competing efficiency of the primer

15 pairs in the reaction mixture is reduced, and thus does not effect the optimization of the reaction conditions. This aspect of the invention is a significant improvement over the prior art. The present invention is easily tailored to amplify a preferred number of target genetic sequences

20 without requiring time-consuming optimization steps. For example, in the event that a sample is scheduled to be screened for 9 genetic targets, and it is subsequently determined that an additional genetic target is also of interest, the present invention may be adapted to

25 accommodate the screening of all 10 genetic targets, simultaneously. In doing so, the primer design protocol or primer selection criteria of the present invention would be used to obtain suitable primer pairs for the additional genetic targets of interest. In accordance with the method

30 of the present invention as herein disclosed, these additional primer pairs would be added to the reaction mixture to provide the predetermined concentration. Likewise, primer pairs can be removed from the reaction

mixture of the present invention without disturbing the optimization conditions of the reaction. In accordance with another embodiment of the present invention primer pairs can be replaced by other primer pairs as designed or
5 selected according to the primer design protocol or primer selection criteria respectively, as herein described.

In summary, the present invention provides the powerful new ability to test for multiple genetic targets simultaneously without the frustration of repeatedly
10 optimizing reaction conditions. The present invention can be readily incorporated into existing PCR products, or used to design a new generation of screening tests. In this manner a diagnostic screening assay of the present invention can be easily performed by a clinician and
15 results rapidly obtained. It is fully contemplated that the present invention includes a kit providing the materials for performing the enhanced multiplex PCR method herein described. The present invention has particular application in the diagnosis of infectious diseases where
20 many target organisms can be simultaneously screened in a timely and affordable fashion. The present invention has the potential to be applied to many other areas of DNA-based diagnostics.

MATERIALS AND METHODS

25 MATERIALS

Primer Design

Primers should be selected to have melting temperatures in the range of 55 to 65°C. For the purposes of the present invention the primers are preferably
30 selected to have a melting temperature within an approximate 2°C range. More preferably, the melting

temperatures of the primers are between 55°C to 57°C. Standardizing the melting temperature to a set range facilitates the uniformity of the primer hybridization kinetics.

5 **Amplicon Length**

The primers of the present invention may be designed to produce a PCR product or amplicon of virtually any size. In accordance with the present invention a PCR product or amplicon may include a target genetic sequence
10 as amplified and detected in accordance herewith. Typically amplicons of the present invention will range in size from 200 to 2000 base pairs (bp) or nucleotides (nt). According to a preferred embodiment of the present invention, primers are designed to produce a PCR product or
15 amplicon that is less than 900 base pairs (bp) or nucleotides (nt) in length. More preferably, amplicons will range in length from 212 to 823 bp. It is fully contemplated that the present invention is adaptable for the amplification of amplicons larger than 2000bp. In
20 accordance with this embodiment, consideration should be given to the type of enzyme employed in connection with the present invention as well as the means used for detecting the amplicon in question.

In accordance with another embodiment of the
25 present invention, amplicons are preferably of different lengths. A 20 bp difference in amplicon length is preferred when detection of the amplicons includes agarose gel electrophoresis and ethidium bromide staining. Alternatively, a single base pair difference in amplicon
30 length may also be detected in accordance with the present invention. In this instance, a detection system such as polyacrylamide gel electrophoresis may be employed. It

should be understood that the present invention may be employed to amplify genetic targets producing amplicons of any size. Furthermore, the present invention may be employed with a variety of detection means.

5 **GC Content**

Primers may be designed to have a GC content ranges from 20 to 80%. According to a preferred embodiment of the present invention, primer GC content ranges from 40 to 50%.

10 **PCR Reagents and Concentrations**

Magnesium Chloride (MgCl_2)

In accordance with the present invention a PCR reaction mixture includes a 5mM - 12.5mM final concentration of MgCl_2 . Preferably, a final MgCl_2
15 concentration is between 5 - 10 mM. More preferably, a final concentration of 7.5mM is provided.

Deoxynucleotidetriphosphates (dNTPs)

dNTP concentrations in the PCR reaction mixture of the present invention preferably ranges from 0.25mM to
20 1.25mM. More preferably, a final dNTP concentration of approximately 0.25 mM in a reaction mixture of the present invention is provided. In accordance with an embodiment of the present invention, a high concentration of dNTPs is provided to avoid the dNTP concentrations from becoming a
25 limiting reagent in the reaction.

Enzyme

PCR enzymes known in the art may be employed in accordance with the present invention, such as Taq polymerase, for example. According to a preferred
30 embodiment of the present invention, a hot start enzyme is employed, such as or Amplitaq Gold, for example.

Alternatively, in the absence of a hot start enzyme, a manual hot start step may be employed together with a standard PCR enzyme, as known in the art. It is fully contemplated that other enzymes capable of amplifying genetic sequences may be employed in accordance with the present invention.

Primer Concentrations

Primers of the present invention are provided to have a final concentration of $0.005\mu\text{M}$ to $0.05\mu\text{M}$. Preferably, the final primer concentrations of the present invention range from $0.005\mu\text{M}$ to $0.01\mu\text{M}$. More preferably, the final primer concentration of the PCR reaction mixture of the present invention is approximately $0.01\mu\text{M}$.

METHODS

Enhanced Multiplex PCR

Figs. 1 - 3 exemplify an enhanced multiplex PCR method according to a preferred embodiment of the present invention. Fig. 1 illustrates a step-wise method of the present invention. According to this embodiment of the invention, primer pairs are first selected or designed for specific genetic targets of interest in accordance with the criteria (A) outlined in Fig.2. A reaction mixture is subsequently prepared and the primer pairs and a target DNA sample added thereto. A hot start initiation of amplification is subsequently initiated. A series of PCR steps are carried out, as exemplified in B (Fig. 3). Once amplification and elongation are complete, detection of the genetic targets is performed. According to the embodiment exemplified in Fig. 3 initial denaturation is conducted at 95°C . This denaturation step may be carried out for 5 to 10 minutes. Preferably, denaturation is carried out for 10 minutes.

Denaturation is followed by amplification. According to an embodiment of the invention a hot-start initiation of amplification is provided, as is known in the art. Preferably, amplification includes a series of PCR
5 steps. PCR techniques applicable to the present invention include inter alia those described in PCR Primer: A Laboratory Manual, Dieffenback, C.W. and Dveksler, G.S., eds., Cold Spring Harbor Laboratory Press (1995); Enzymatic
10 amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N, Science (1985) Dec 20; 230(4732):1350-4. A first series of PCR steps preferably includes touchdown PCR. Touchdown or step-down PCR refers
15 to incremental decrease of the annealing temperature with each cycle. The objective is to increase the efficiency in each successive amplification step, while maintaining more rigorous primer specificity in the initial amplification steps. In accordance with a preferred embodiment of the
20 present invention, 15 to 20 cycles of touchdown PCR are performed. More preferably, 20 cycles of touchdown PCR are performed.

Touchdown PCR may be followed by a second series of PCR steps. A second series of PCR steps preferably
25 comprises of multiple cycles of regular PCR. A preferred range of regular PCR cycles is from 20 to 25 cycles. More preferably, 25 cycles of regular PCR are performed. It is fully contemplated that the PCR steps of the present invention may include steps of regular or touch-down PCR or
30 a combination thereof.

A final elongation step may be performed following the PCR steps. Typically, elongation is performed at 72°C

for approximately 7 minutes. Once elongation is complete, the reaction mixture may then be held at 6°C prior to detection.

As indicated above the amplicons of the present invention may be detected according to any detection system or method known in the art.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

Example 1

In this study, we used a multiplex PCR assay that amplified the *mecA*, *nuc*, and 16S rRNA genes in bacterial DNA obtained directly from the blood culture bottle. This allowed us to identify *S. aureus*, determine methicillin resistance, and monitor successful amplification using the 16S rRNA gene as an internal control.

Blood culture bottles that were flagged as positive by the Bact/Alert system (Organon Teknika Corp., Durham NC) were processed by the Microbiology laboratory using standard microbiologic methods (Kloos & Bannerman, 1999). *S. aureus* was identified by a positive tube coagulase test. Oxacillin susceptibility for *S. aureus* was determined using the oxacillin salt agar screen method (NCCLS M100-S10, M7) and for coagulase-negative Staphylococci (CoNS) using disk diffusion testing (NCCLS M100-S10, M2).

DNA for PCR was prepared from blood culture bottle contents by the benzyl alcohol-guanidine hydrochloride organic extraction method according to Fredericks and Relman (1998). The PCR reaction mixture consisted of PCR Buffer

(final concentration, 50 mM KCl, 9.0 mM MgCl₂, 10 mM Tris-HCl, pH 8.3), 0.25 mM each of dATP, dCTP, dTTP, and dGTP, 1.25 units of Taq DNA Polymerase (Roche Diagnostics), 2 μ L of target DNA, and each of the 6 primers at 0.017 μ M final
5 concentration.

Primers were designed (using gene sequences obtained from GenBank) for *mecA* (accession # Y00688), *nuc* (accession # V01281 J01785 M10924), and 16S rRNA (accession # AF076030). The primer sequences were as follows: *mecA*
10 forward primer (SEQ ID NO. 1), 5'- TGGTATGTGGAAGTTAGATTGG-3' and reverse primer (SEQ ID NO. 2), 5'- GGATCTGTACTGGGTTAATCAG3'; *nuc* forward primer (SEQ ID NO. 3), 5'-ATAGGGATGGCTATCAGTAATGT-3' and reverse primer (SEQ ID NO. 4), 5'-GACCTGAATCAGCGTTGTCTTC-3'; 16S rRNA forward
15 primer (SEQ ID NO. 5) 5-'TAGCCGACCTGAGAGGGTGAT-3' and reverse primer (SEQ ID NO. 6) 5'-GTAGTTAGCCGTGGCTTTCTG-3'. These primer pairs amplified a 1235 bp *mecA* fragment, 624 bp *nuc* fragment, and 228 bp 16S rRNA fragment, respectively.

20 PCR consisted of 40 cycles of amplification and was carried out in a GeneAmp PCR System 9600 thermal cycler. There was an initial heating step for 5 min at 95°C. The first 20 cycles of touchdown PCR consisted of 20s at 95°C, annealing for 45s starting at 63°C in the first cycle and
25 decreasing by 0.5°C for each of the subsequent 19 cycles, followed by extension for 45 s at 72°C. The last 20 cycles consisted of 20 s at 95°C, 45 s at 56°C, and 45 s at 72°C. Amplified products were detected by electrophoresis on 1.5% agarose gels that were stained with ethidium bromide and
30 visualized under UV light.

In cases where there were discrepancies between the *mecA* PCR result of the blood culture bottle contents and

the initial susceptibility result for the isolate recovered from the bottle, the isolate was re-tested for the *mecA* gene, oxacillin MIC was determined by agar dilution (NCCLS M100-S10, M7) and disk diffusion testing for oxacillin was
5 repeated (NCCLS M100-S10, M2).

One hundred and twelve bottles were tested in this study. This consisted of 81 bottles that grew staphylococci, 19 bottles that grew other organisms, and 12 bottles that showed no growth of bacteria (Table 1). There
10 were only 5 samples where the *mecA* PCR result of the bottle differed from the susceptibility result initially reported for the isolates recovered from the bottle. All 5 bottles contained CoNS and on retesting the isolates, the oxacillin disk diffusion and *mecA* PCR results agreed with the initial
15 *mecA* PCR result of the bottle (Table 2).

The direct PCR assay correctly identified all bottles with *S. aureus* and was more accurate than phenotypic testing for the determination of methicillin susceptibility of CoNS. In all 5 cases, where there was a
20 discrepancy, repeat disk diffusion testing and *mecA* PCR of the isolates obtained results consistent with the initial PCR results.

The primary advantage of this multiplex assay is that it allows for the rapid identification of *S. aureus*
25 and detection of methicillin resistance in Staphylococci in positive blood culture bottles growing Gram-positive cocci in clusters. PCR was performed on bacterial DNA obtained directly from the blood culture bottle, without the need for subculture. This assay takes approximately 4 h starting
30 from the time a blood culture bottle is flagged positive to visualization of the PCR products on a gel. Conversely, it

would require > 24 h to obtain results using conventional phenotypic tests.

Example 2

Primer design

5 Primers were designed to give amplicons that ranged
in size between 200 to 900 bp. Where detection is by
agarose gel electrophoresis, a size difference of at least
20 bp between individual amplicons is preferred. Detection
by a more sensitive technique like capillary gel
10 electrophoresis may be employed in accordance with the
present invention to resolve a size difference of as little
as 1 bp.

When designing primers, the structural properties
of each primer set were selected according to the following
15 primer selection criteria:

Melting Temperature (T_m)	55 - 57°C
%GC	40 - 50%
optimal primer length	22 nt
Primer size (range)	18 - 27 nt

20 The above conditions were specified in a primer-
design program such as Oligo4.0 or Primer3 (freely
available at [http://www-genome.wi.mit.edu/cgi-](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)
[bin/primer/primer3_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)), for example. After generating
a possible candidate design, the new primer was checked for
25 internal stability and mispriming according to methods well
known in the art.

In cases where a primer pair did not work
immediately, a new primer pair was designed according to
the same principles, after reassessing the genomic
30 structure and/or stability at that priming site. The
reaction conditions were not adjusted in these cases.

PCR Reagents

	<u>Reagent</u>	<u>Working Concentration</u>
	AmpliTaq Gold PCR Buffer	10x
	(500mM KCl, 100mM Tris HCl;	
5	pH 8.3)	
	Magnesium chloride	25 mM
	dNTPs	1.25 μ M each
	<u>Enzyme</u>	
	AmpliTaq Gold	5 U/ μ L
10	<u>Primers</u>	
	Primer Master Mix	0.05 μ M each
	(primers + TE Buffer:	
	10mM Tris-HCl, pH 8.0 and 1mM EDTA)	

A hot start DNA polymerase enzyme was used in the PCR reaction steps, such as AmpliTaq Gold, for example. In this case, reactants were not wasted in the formation of unintended products and increased yields of the specific products were achieved. The final concentration of magnesium chloride in the reaction mix was 7.5 mM. Equal volumes of each primer were added together to make a mix containing all of the primers to be used in the multiplex assay. The concentration of each primer in this Primer Master Mix was 0.05 μ M. As a result, the final primer concentration in the PCR reaction mixture was approximately 0.01 μ M.

PCR reaction mixture

	<u>Reagent</u>	<u>Volume (uL)</u>
	Amplitaq Gold	3
	PCR Buffer	
5	MgCl ₂	9
	dNTPs	6
	DNA template	3
	H ₂ O	3
	AmpliTaQ Gold	0.5
10	Primer Master Mix	6
	Total:	30.5 uL

It is contemplated that fractions or multiples of these values can be used in accordance with the present invention if reagent volume ratios are preserved.

15 PCR thermal cycler program**Step 1: Initial Denaturation**

Time (min)	Temperature (°C)
10:00	95

Step 2: 20 cycles of Touchdown PCR

20	Time (min)	Temperature (°C)	Touchdown
	0:20	95	none
	1:00	63	decrease by 0.5°C each cycle
	1:00	72	none

25 Touchdown or step-down PCR refers to the incremental decrease of the annealing temperature with each cycle. Here, the annealing temperature was decreased by 0.5°C in each cycle. The objective was to increase the efficiency of each successive amplification step, while

maintaining more rigorous primer specificity in the initial amplification steps.

Step 3: 25 cycles of regular PCR

	Time (min)	Temperature (°C)
5	0:20	95
	0:45	56
	1:00	72

Step 4: Final elongation

	Time (min)	Temperature (°C)
10	7:00	72
	HOLD	6

Primer pairs designed in accordance with a primer design protocol of the present invention are herein provided as SEQ ID Nos. 7 - 26. Primer design, concentrations of PCR reagents and PCR reaction mixture were prepared as described above. A GeneAmp PCR System 9600 thermal cycler was programmed as detailed above. PCR products were detected by agarose gel electrophoresis, followed by staining with ethidium bromide. PCR products were identified based on size comparison to a standard DNA ladder. Figure 4 shows the results of this experiment.

Ten genes expressed in methicillin-resistant *Staphylococcus aureus* (MRSA) were identified from GenBank, as outlined hereinbelow. Detection of 10 genetic targets, corresponding to these ten different genes in methicillin-resistant *Staphylococcus aureus* was achieved on Agarose gel, in accordance with a method of the present invention (Fig. 4). As illustrated in Fig. 4 (Lane 1), from top to bottom, the ten genes are: (1) agr, 823 bp (ACCESSION M21854) (2) clumping factor, 726 bp (ACCESSION Z18852); (3)

16S rRNA, 653 bp (ACCESSION X68417); (4) hld, 554 bp (ACCESSION X17301); (5) femA, 419 bp (ACCESSION X17688 M23918); (6) rho, 376 bp (ACCESSION AF333962) (7) DNA polymerase III, 314 bp (ACCESSION Z48003 L39156); (8) 5 nuclease, 282 bp (ACCESSION V01281 J01785 M10924); (9) 23S rRNA, 244 bp, (ACCESSION X68425); and (10) hsp60, 212 bp (ACCESSION AF060189).

Example 3

Method

10 A 0.5 McFarland standard (1×10^8 CFU/mL) of MRSA bacteria was made up in sterile saline. Serial dilutions were made using sterile saline. The bacteria from 100 uL of each dilution were pelleted and resuspended in Lysis Buffer (50mM Tris-HCl (pH 8.0), 50mM NaCl, and 5 mM EDTA 15 (pH 8.0) for DNA extraction. Colony counts of bacteria on agar plates corroborated the accuracy of the McFarland standard. 2 uL from each DNA sample (100 uL each) was used for the method of the present invention as described herein above.

20 Results

The sensitivity of the method of the present invention is illustrated in Figure 5, with 10 amplicons visible in Lanes 1 and 2. Lanes 3 to 5 show progressive loss of amplified targets with a decrease in the initial 25 concentration (CFU/mL) of bacteria. A theoretical detection limit of approximately 500 CFU/mL (2 uL/PCR reaction * 500 CFU/1000 uL = 1 CFU / PCR reaction) was indicated in this example.

Example 4

30 The effect of varying magnesium chloride concentrations was investigated in accordance with an

embodiment of the present invention. Final MgCl_2 concentrations of 5.0mM to 10.0 mM were shown to allow detection of 10 target genes by agarose gel electrophoresis in accordance with the method described in Example 2 (Fig. 6 - Lanes 2,3 &4). No amplicons were detected when a final concentration of 2.5 mM MgCl_2 was provided in the PCR reaction mixture (Lane 1). Only 9 amplicons were visible (Lane 5) when the final concentration of MgCl_2 was increased to 12.5mM.

10 **Example 5**

The effects of varying primer concentrations were also studied (Fig. 7). Final primer concentrations were of 0.005 μm , 0.01 μm , 0.02 μm and 0.05 μm were tested.

At final primer concentrations 0.05 μm and 0.02 μm only 9 amplicons were visible (Fig. 7, Lanes 1 and 2). 10 amplicons were visible at final primer concentrations of 0.01 μm and 0.005 μm (Fig. 7, Lanes 3 and 4).

Conclusion

The present invention provides a single set of multiplex PCR conditions that will work with primers designed according to the primer design protocol as detailed herein. By employing this single set of multiplex PCR conditions, primer pairs designed in accordance with the primer design protocol of the present invention can be added or removed without having to change the reaction conditions of the enhanced multiplex PCR method.

The present invention amplifies all of the genetic targets if they are present. At least ten genetic targets can be amplified simultaneously, according to one embodiment of the present invention. More efficiently amplified primers do not overwhelm less efficiently

amplified primers in the reaction mix. A particular advantage of the present invention is that amplification is efficient enough to produce PCR products or amplicons that can be detected with inexpensive and simple methods like
5 agarose gel electrophoresis with ethidium-bromide staining.

As indicated in Examples 3, 4 and 5, a method of the present invention can detect at least 10 genetic targets. According to an embodiment of the present invention, detection can be accomplished by agarose gel
10 electrophoresis. A starting bacterial concentration (before DNA extraction) of 1×10^4 CFU/mL or greater is preferred. According to another embodiment of the present invention, a final $MgCl_2$ concentration of 7.5 - 10mM in the PCR reaction mixture is preferred for optimal amplification
15 and detection of at least 10 genetic targets. A final primer concentration between 0.005 and 0.05 μM is also preferred in order to amplify at least 10 genetic targets.

In effect, the present invention creates a common platform that researchers and clinicians can use to develop
20 new multiplex PCR tests. If a new genetic target is discovered, the method of the present invention can be adapted to include this target in the reaction mixture for amplification with the existing targets without requiring any changes to reaction conditions. Conversely, if a
25 certain genetic target is no longer desirable, its primer pair can be removed from the existing assay, without requiring any changes to the reaction conditions.

To summarize, the present invention includes the advantages of (1) significant savings in reagent costs and
30 technologist time and labour due to the elimination of optimization procedures; (2) rapid product development: different PCR primer pairs will work together right away

without competitive inhibition; and (3) flexibility: new PCR primers can be added or subtracted from existing assays without adjusting reaction conditions.

By selecting target-specific primers, the present invention can be used to develop diagnostic assays in a variety of fields. For example, the present invention can be used to provide high-throughput, sensitive and specific diagnostic tests for infectious diseases, screening panels for genetic diseases, or for the detection of disease-causing genes. In particular, the present invention can be tailored to provide a screening method and kit to detect potential causative organisms of human encephalitis in cerebrospinal (CSF) samples (e.g. herpes simplex virus, human herpes virus 6, *Cryptococcus*, *Listeria*, *H. influenzae* type B, *S. pneumoniae*, *E. coli*, etc.) The present invention also finds application in the fields of blood product screening and genetic testing such as prenatal screening for genetic diseases and detection of cancer-causing genes in pathology samples, for example.

In the case of diseases such as meningitis/encephalitis, there are at least 10 different bacteria and viruses that can cause the classic symptoms of fever, stiff neck, and altered mental status. Unfortunately, it is prohibitively expensive to test for all 10 organisms individually. As a result doctors are usually forced to treat patients empirically, without adequate knowledge of the true cause of disease. Empirical treatment is expensive, promotes antibiotic resistance, and puts patients at risk of adverse drug reactions. It may not even cover the rarer causes of disease. The present invention allows for the simultaneous detection of multiple genetic targets, such as genetic disease markers of a

disease of interest, thereby allowing a physician to quickly, reliably and economically arrive at an accurate diagnosis.

The present invention may be provided as a
5 diagnostic kit. A kit of the present invention may be tailored for the diagnosis of a variety of diseases and/or conditions such as meningitis/encephalitis, STDs, respiratory infections, etc. According to one embodiment of the present invention, a kit will contain PCR reaction
10 tubes that are pre-loaded with a PCR reaction mixture. Such a kit may also include multiple target-specific primer pairs for the amplification and detection of genetic targets of interest. According to one embodiment of the invention, each tube will be ready for immediate use, and
15 the laboratory technologist will simply add sample DNA and then run it using standard PCR equipment. Alternatively, a kit of the present invention may include some or all of the ingredients necessary to establish the suitable reaction conditions of the invention, such as a PCR reaction
20 mixture, enzymes, and target-specific primers for example. A kit of the present invention may include predefined measures of the components for performing multiple repeats of the enhanced multiplex PCR method. Test controls may also be provided as a component of the kit of the present
25 invention to confirm the reaction conditions of a given amplification reaction. For example, positive and negative controls may be provided. In the case of a bacterial target, highly conserved targets like 16s rRNA and 23s rRNA genes may be employed. In the case of human molecular
30 testing, human GAPDH or beta-globin genes may be used as positive test controls. These are highly conserved genes that can be used to confirm the presence of human DNA in a sample. Similarly, negative test controls may also be

employed to provide indicators of the reaction conditions. A kit of the present invention preferably includes a set of instructions for practicing the method thereof. The kits of the present invention may be designed to be compatible
5 with the major brands of PCR machines. Alternatively, the kits of the present invention may be tailored for a fully automated system specific to the present invention with integrated DNA extraction, and PCR amplification and detection functions.

10 The embodiment(s) of the invention described above is(are) intended to be exemplary only. The scope of the invention is therefore intended to be limited solely by the scope of the appended claims.

Table 1. PCR analysis of Bact/Alert blood cultures for mecA, nuc and 16S rRNA genes

Organism initially reported from bottles	mecA Result			nuc Result		
	No. of bottles tested	No. of bottles positive for 16S rRNA	No. of mecA positive bottles	No. of mecA negative bottles	No. of nuc positive bottles	No. of nuc negative bottles
^a MS-S. aureus	20	20	0	20	20	0
^b MR-S. aureus	2	2	2	0	2	0
MS-S. aureus and MR-CoNS	1	1	1	0	1	0
MR-CoNS	27	26 ^c	25	1	0	26
MS-CoNS	28	28	4	24	0	28
MR-CoNS and MS-CoNS	3	3	3	0	0	3
Non-staphylococcal isolates ^e	19	18 ^d	0	18	0	18

a MS, methicillin susceptible.

b MR, methicillin resistant.

c One bottle was PCR negative for 16S rRNA, mecA and nuc genes, indicating PCR inhibition.

d This isolate did not grow for susceptibility testing at time blood culture results was reported. Subsequent testing of the organism indicated an oxacillin MIC of 0.25 ntg/I, disk diffusion zone diameter of 25.5 mm (S), and negative PCR for mecA.

e These bottles contained *Enterococcus* spp. (5 bottles), *Streptococcus* spp. (9 bottles), Corynebacteria (1 bottle), and Gram-negative bacilli (4 bottles).

Table 2. Analysis of isolates with oxacillin susceptibility results that were discrepant with the blood culture bottle result.

Bottle Number	Isolate from this bottle initially reported as	Isolate retest results			
		mecA PCR of bottle	Oxacillin susceptibility by DD	Oxacillin MIC	mecA PCR
10	^a MR-CoNS	-	S	0.5	-
8	^b MS-CoNS	+	R	2.0	+
92	MS-CoNS	+	R	ND ^c	+
93	MS-CoNS	+	R	ND	+
94	MS-CoNS	+	R	ND	+

a MR, methicillin resistant

b MS, methicillin susceptible

c ND: Not done.

- 32 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

University of Ottawa
Room 3042
800 King Edward Avenue
Ottawa, Ontario
K1N 6N5, Canada

(ii) TITLE: "A METHOD FOR THE AMPLIFICATION OF MULTIPLE GENETIC TARGETS"

(iii) NUMBER OF SEQUENCES: 26

(iv) CORRESPONDENCE ADDRESS:

Suite 1500
50 O'Connor Street
Ottawa, Ontario
K1P 6L2, Canada

Telephone: (613) 235-7234
Facsimile: (613) 235-2867

(v) COMPUTER READABLE FORM:

COMPUTER: IBM Compatible
OPERATING SYSTEM: Windows 2000
SOFTWARE: PatentIn version 3.1

(vi) CURRENT APPLICATION DATA:

APPLICATION NUMBER: 2,410,795
FILING DATE: December 10, 2002
CLASSIFICATION: C12Q-1/68

(vii) PRIOR APPLICATION DATA:

APPLICATION NUMBER: US 60/422,877
FILING DATE: November 1, 2002
CLASSIFICATION:

(viii) PATENT AGENT INFORMATION:

NAME: Osler, Hoskin & Harcourt, LLP
REFERENCE NUMBER: 16029

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 33 -

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus mecA gene forward primer

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

tggtatgtgg aagttagatt gg

22

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus mecA gene reverse primer

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

ggatctgtac tgggttaatc ag

22

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus nuc gene forward primer

- 34 -

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

atagggatgg ctatcagtaa tgt

23

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus nuc gene reverse primer

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

gacctgaatc agcgttgtct tc

22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus 16S rRNA gene forward primer

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

tagccgacct gagagggtga t

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- 35 -

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus 16S rRNA gene reverse primer

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

gtagttagcc gtggctttct g

21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus agr gene left primer

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

gccataagga tgtgaatgta tg

22

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 36 -

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus agr gene right primer

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

cagctatata gtgcatttgc ta

22

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus clumping factor gene left primer

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

ggctactggc ataggtagta ca

22

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus clumping factor gene right primer

- 37 -

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

gctgaatctg aaccactatc tg

22

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus 16S rRNA gene left primer

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

ggattagata ccctggtagt cc

22

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus 16S rRNA gene right primer

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

cttcgggtgt tacaaactct c

21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- 38 -

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus hld gene left primer

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

attagggatg caggtcttag c

21

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus hld gene right primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ctataagctg cgatgttacc aa

22

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 38a -

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus femA gene left primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

taccgcttta aacgtggatt

20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus femA gene right primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

gatatcacac acttgcaaac ac

22

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus rho terminaton factor gene left
primer

- 38b -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

aacaatctgg tttaggtcgt g

21

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

primer Staphylococcus aureus rho termination factor gene right

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

tggaatgatt catactgagg ag

22

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

 Staphylococcus aureus DNA polymerase III gene left primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

gtagaattaa cgcaacatca cc

22

(2) INFORMATION FOR SEQ ID NO: 20:

- 38c -

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus DNA polymerase III gene right
primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

cacgctgtac ctaccaataa tc

22

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus nuclease (nuc) gene left primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

gtcctgaagc aagtgcattt ac

22

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

- 38d -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus nuclease (nuc) gene right primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

gacctgaatc agcggtgtct tc

22

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus 23S rRNA gene left primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

atttgagagg agctgtcctt ag

22

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

- 38e -

Staphylococcus aureus 23S rRNA gene right primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

attagtattc gtcagctcca ca

22

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus heat shock protein 60 kDA (hsp60)

gene left primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

gacaaagcag ttaaagttgc tg

22

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE: Artificial

(ix) FEATURE:

(D) OTHER INFORMATION:

Staphylococcus aureus heat shock protein 60 kDA (hsp60)

gene right primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ccttcaacca cttctagttc ag

22

I/WE CLAIM:

1. A method for simultaneously amplifying multiple genetic targets, said method comprising:

selecting primer pairs specific to said multiple genetic targets according to a primer selection criteria;

effecting a hot start initiation of amplification of said multiple genetic targets in a single reaction vessel together with said primer pairs and a PCR reaction mixture;

performing a series of PCR reaction steps to amplify each of said multiple genetic sequences in said sample;

wherein said PCR reaction mixture is adaptable for simultaneously amplifying multiple genetic targets in said single reaction vessel without requiring optimization of pre-set amplification reaction conditions of said method when said primer pairs are provided at a final concentration of approximately $0.005\mu\text{M}$ - $0.05\mu\text{M}$ in the reaction mixture.
2. The method of claim 1 wherein each of said primer pairs are provided to have a final concentration of approximately $0.01\mu\text{M}$ in said reaction mixture.
3. The method of claim 1 wherein said PCR reaction mixture includes at least 5.0 mM MgCl_2 .

4. The method of claim 1 wherein said primer selection criteria includes selecting primer pairs having a melting temperature in the range of 55 to 65°C.
5. The method of claim 4 wherein said melting temperatures of said pre-selected primer pairs are within a 2°C range of variation.
6. The method of claim 5 wherein the melting temperatures of said pre-selected primer pairs are 55 - 57°C.
7. The method of claim 1 wherein said primer selection criteria includes selecting primer pairs having a GC content of 40-50%.
8. The method of claim 1 wherein said primer selection criteria includes selecting primer pairs that are 18 - 27 nucleotides in length.
9. The method of claim 1 wherein said series of PCR reaction steps includes a first and second PCR step.
10. The method of claim 1 wherein said series of PCR reaction steps includes a step of touchdown PCR.
11. The method of claim 10 wherein said step of touchdown PCR is performed in a first set of PCR steps.
12. The method claim 10 wherein said first set of PCR steps includes 20 cycles of touchdown PCR.
13. The method of claim 9 wherein said second set of reaction steps includes at least 20 cycles of PCR.

14. The method of claim 13 wherein said second set of reaction steps includes 25 cycles of PCR.
15. A method for preparing a PCR reaction mixture for simultaneously multiplexing multiple genetic targets, said method comprising:
 - adjusting the final MgCl_2 concentration of a PCR buffer suitable for a PCR reaction to at least 5.0mM; and
 - adding a hot start initiation means to said buffer;
 - wherein said PCR reaction mixture is adaptable for simultaneously multiplexing multiple genetic targets in the presence of pre-selected primer pairs having a final concentration of 0.005 - 0.05 μM when added to said mixture.
16. The method of claim 15 wherein each of said pre-selected primer pairs are provided to have a final concentration of approximately 0.01 μM in said reaction mixture.
17. The method of claim 15 wherein the final concentration of MgCl_2 in said PCR reaction mixture is approximately 7.5 mM.
18. The method of claim 15 wherein said pre-selected primer pairs are selected to be and 18 - 27 nucleotides in length; have a melting temperature in the range of 55 to 65°C; and a GC content of 40-50%.

19. The method of claim 18 wherein said melting temperatures of said pre-selected primer pairs are within a 2°C range of variation.
20. The method of claim 19 wherein the melting temperatures of said pre-selected primer pairs are 55 - 57°C.
21. A method for simultaneously amplifying multiple genetic targets, said method comprising:
- mixing a sample to be tested for the presence of
said multiple genetic targets with pre-selected primer pairs specific to said multiple genetic targets and a PCR reaction mixture;
 - effecting a hot start initiation of amplification of said genetic targets;
 - performing a series of PCR reaction steps to amplify each of said multiple genetic sequences in said sample;
 - wherein said pre-selected primer pairs are provided to optimize amplification of said multiple genetic targets in said reaction mixture in the absence of requiring optimization of reaction conditions of said method.
22. The method of claim 21 wherein each of said primer pairs are provided to have a final concentration in said reaction mixture of 0.005 - 0.05µM.

23. The method of claim 22 wherein each of said primer pairs are provided to have a final concentration of approximately 0.01 μ M in said reaction mixture.
24. The method of claim 21 wherein said PCR reaction mixture includes at least 5.0 mM MgCl_2 .
25. The method of claim 21 wherein said PCR reaction mixture includes approximately 7.5 mM MgCl_2 .
26. The method of claim 21 wherein said pre-selected primer pairs are selected to be 18 - 27 nucleotides in length; have a melting temperature in the range of 55 to 65°C; and have a GC content of 40-50%.
27. The method of claim 26 wherein said melting temperatures of said pre-selected primer pairs are within a 2°C range of variation.
28. The method of claim 27 wherein the melting temperatures of said pre-selected primer pairs are 55 - 57°C.
29. The method of claim 21 wherein said series of PCR reaction steps includes a first and second PCR step.
30. The method of claim 21 wherein said series of PCR reaction steps includes a step of touchdown PCR;
31. The method of claim 30 wherein said step of touchdown PCR is performed in a first set of PCR steps.
32. The method claim 29 wherein said first set of PCR steps includes 20 cycles of touchdown PCR.

33. The method of claim 29 wherein said second set of reaction steps includes at least 20 cycles of PCR.
34. The method of claim 33 wherein said second set of reaction steps includes 25 cycles of PCR.
35. A method for simultaneously detecting multiple genetic targets, said method comprising:
- mixing a sample to be tested for the presence of
 - said multiple genetic targets with pre-selected primer pairs specific to said multiple genetic targets and a PCR reaction mixture;
 - effecting a hot start initiation of amplification of said genetic targets;
 - performing a series of PCR reaction steps to amplify each of said multiple genetic sequences in said sample; and
 - detecting said multiple genetic targets present in said sample;
 - wherein said pre-selected primer pairs are provided to optimize amplification of said multiple genetic targets in said PCR reaction mixture in the absence of requiring optimization of reaction conditions of said method.
36. The method of claim 35 wherein gel electrophoresis is employed in detecting said multiple genetic targets.

37. The method of claim 35 wherein each of said primer pairs are provided to have a final concentration in said reaction mixture of 0.005 - 0.05 μ M.
38. The method of claim 35 wherein each of said primer pairs are provided to have a final concentration of approximately 0.01 μ M in said reaction mixture.
39. The method of claim 35 wherein said PCR reaction mixture includes at least 5.0 mM MgCl₂.
40. The method of claim 35 wherein said PCR reaction mixture includes approximately 7.5 mM MgCl₂.
41. The method of claim 35 wherein said pre-selected primer pairs are selected to be 18 - 27 nucleotides in length; have a melting temperature in the range of 55 to 65°C; and have a GC content of 40-50%.
42. The method of claim 41 wherein said melting temperatures of said pre-selected primer pairs are within a 2°C range of variation.
43. The method of claim 42 wherein the melting temperatures of said pre-selected primer pairs are 55 - 57°C.
44. The method of claim 35 wherein said series of PCR reaction steps includes a first and second PCR step.
45. The method of claim 35 wherein said series of PCR reaction steps includes a step of touchdown PCR;

- 46 -

46. The method of claim 45 wherein said step of touchdown PCR is performed in a first set of PCR steps.
47. The method claim 44 wherein said first set of PCR steps includes 20 cycles of touchdown PCR.
48. The method of claim 44 wherein said second set of reaction steps includes at least 20 cycles of PCR.
49. The method of claim 48 wherein said second set of reaction steps includes 25 cycles of PCR.
50. The method of claim 44 wherein said first series of PCR reaction steps comprises 20 cycles of touchdown PCR including 20s at 95°C, 1 min at 63°C - decreased by 0.5 each cycle, and 1 min at 72°C.
51. The method of claim 44 wherein said second series of PCR reaction steps comprises 25 cycles of PCR, including 20s at 95°C, 45s at 56°C and 1 min at 72°C.
52. The method of claim 51 including 7 min at 72°C.
53. The method of claim 1 wherein said multiple genetic targets are DNA sequences.
54. The method of claim 53 wherein DNA sequences are selected from the group consisting of bacterial DNA, viral DNA, plant DNA, animal DNA or human DNA.
55. The method of claim 9 wherein said first series of PCR reaction steps comprises 20 cycles of touchdown PCR including 20s at 95°C, 1 min at 63°C - decreased by 0.5 each cycle, and 1 min at 72°C.

56. The method of claim 9 wherein said second series of PCR reaction steps comprises 25 cycles of PCR, including 20s at 95°C, 45s at 56°C and 1 min at 72°C.
57. The method of claim 56 including 7 min at 72°C.
58. The method of claim 9 wherein said multiple genetic targets are DNA sequences.
59. The method of claim 58 wherein DNA sequences are selected from the group consisting of bacterial DNA, viral DNA, plant DNA, animal DNA or human DNA.
60. The method of claim 29 wherein said first series of PCR reaction steps comprises 20 cycles of touchdown PCR including 20s at 95°C, 1 min at 63°C - decreased by 0.5 each cycle, and 1 min at 72°C.
61. The method of claim 29 wherein said second series of PCR reaction steps comprises 25 cycles of PCR, including 20s at 95°C, 45s at 56°C and 1 min at 72°C.
62. The method of claim 61 including 7 min at 72°C.
63. The method of claim 29 wherein said multiple genetic targets are DNA sequences.
64. The method of claim 63 wherein DNA sequences are selected from the group consisting of bacterial DNA, viral DNA, plant DNA, animal DNA or human DNA.
65. A PCR reaction mixture for use in simultaneously amplifying multiple genetic targets, said mixture comprising:

a PCR buffer reagent including 5mM - 10mM MgCl₂;

wherein said PCR reaction mixture is adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel without requiring optimization of pre-set amplification reaction conditions.

66. The mixture of claim 65 wherein said PCR buffer reagent includes dNTPs having a final concentration of approximately 0.25mM each;

67. A PCR reaction mixture for use in simultaneously amplifying multiple genetic targets in an amplification reaction, said mixture comprising:

a PCR buffer reagent including 5mM - 10mM MgCl₂;

dNTPs having a final concentration of approximately 0.25mM each; and

pre-selected primer pairs corresponding to target genetic sequences to be amplified; each of said primers having a final concentration of approximately 0.005 μ M - 0.05 μ M in the reaction mixture;

wherein said PCR reaction mixture is adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel without requiring optimization of pre-set amplification reaction conditions.

68. A kit for simultaneously amplifying multiple genetic targets for detection, said kit comprising:

a PCR reaction mixture having a final MgCl₂ concentration of 5 - 12.5mM;

a set of pre-selected, target-specific primer pairs corresponding to each of said multiple genetic targets; and

a set of instructions for using contents of said kit for simultaneously amplifying multiple genetic targets in a sample to be tested;

wherein said PCR reaction mixture is adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel without requiring optimization of pre-set amplification reaction conditions when said pre-selected, target-specific primer pairs are provided at a final concentration of approximately $0.005\mu\text{M}$ - $0.05\mu\text{M}$ in the reaction mixture.

69. The kit of claim 68 wherein said PCR reaction mixture is pre-loaded in at least one reaction vessel.

70. The kit of claim 69 wherein said at least one reaction vessel further includes said set of pre-selected, target-specific primer pairs.

71. The kit of claim 68 further comprising, a DNA polymerase enzyme.

72. The kit of claim 71 wherein said DNA polymerase enzyme is a hot start enzyme.

73. A method for simultaneously amplifying multiple genetic targets, said method comprising:

mixing a sample to be tested for the presence of said multiple genetic targets with pre-

- 50 -

selected primer pairs specific to said multiple genetic targets and a PCR reaction mixture including a final MgCl_2 concentration of at least 5.0mM;

effecting means for a hot start initiation of amplification of said genetic targets; and performing a series of PCR reaction steps including a step of touchdown PCR;

wherein said amplification is optimized when said pre-selected primer pairs are provided to have a final concentration of 0.005 - 0.05 μM in said reaction mixture.

74. The method of claim 73 wherein each of said pre-selected primer pairs are provided to have a final concentration of approximately 0.01 μM .
75. The method of claim 73 wherein said pre-selected primer pairs are selected to have a melting temperature in the range of 55 to 65°C.
76. The method of claim 75 wherein said melting temperatures of said pre-selected primer pairs are within a 2°C range of variation.
77. The method of claim 76 wherein the melting temperatures of said pre-selected primer pairs are 55 - 57°C.
78. The method of claim 73 wherein each of said pre-selected primer pairs include a GC content of 40-50%.

79. The method of claim 73 wherein each of said pre-selected primer pairs is 18 - 27 nucleotides in length.
80. The method of claim 79 wherein each of said pre-selected primer pairs is 22 nucleotides in length.
81. The method of claim 73 wherein the final concentration of MgCl_2 in said PCR reaction mixture is 5 to 12.5mM.
82. The method of claim 81 wherein said PCR reaction mixture includes more than 6mM of MgCl_2 .
83. The method of claim 82 wherein said PCR reaction mixture includes 7.5 mM of MgCl_2 .
84. The method of claim 73 wherein ten or more genetic targets are simultaneously detected.
85. The method of claim 73 wherein said means for effecting a hot start initiation is a hot start enzyme.
86. The method of claim 85 wherein said hot start enzyme is a Taq Polymerase enzyme.
87. The method of claim 86 wherein said enzyme is Amplitaq GoldTM.
88. The method of claim 73 wherein said means for effecting a hot start initiation includes a DNA polymerase enzyme and a heating step.

89. A method for simultaneously detecting multiple genetic targets in a sample to be tested, said method comprising:
- selecting primer pairs corresponding to said multiple genetic targets according to a pre-defined primer selection criteria;
 - mixing said primer pairs with said sample to be tested and a PCR reaction mixture; said PCR reaction mixture including a final concentration of at least 5.0 mM MgCl₂;
 - effecting means for a hot start initiation of amplification of said genetic targets;
 - performing a series of PCR reaction steps including a step of touchdown PCR; and
 - detecting for the presence of said multiple genetic targets in said sample;
- wherein when said primer pairs are provided in said reaction mixture to have a final concentration of 0.005 - 0.05 μ M amplification of said multiple genetic targets is optimized in the absence of requiring optimization of reaction conditions of said method.
90. The method of claim 89 wherein each of said pre-selected primer pairs are provided to have a final concentration of approximately 0.01 μ M.
91. The method of claim 89 wherein said pre-selected primer pairs are selected to have a melting temperature in the range of 55 to 65°C.

92. The method of claim 91 wherein said melting temperatures of said pre-selected primer pairs are within a 2°C range of variation.
93. The method of claim 92 wherein the melting temperatures of said pre-selected primer pairs are 55 - 57°C.
94. The method of claim 89 wherein each of said pre-selected primer pairs include a GC content of 40-50%.
95. The method of claim 89 wherein each of said pre-selected primer pairs is 18 - 27 nucleotides in length.
96. The method of claim 95 wherein each of said pre-selected primer pairs is 22 nucleotides in length.
97. The method of claim 89 wherein said PCR reaction mixture includes 5 to 12.5mM of MgCl₂.
98. The method of claim 97 wherein said PCR reaction mixture includes 5 to 10 mM of MgCl₂.
99. The method of claim 98 wherein said PCR reaction mixture includes more than 6mM of MgCl₂.
100. The method of claim 99 wherein said PCR reaction mixture includes 7.5 mM of MgCl₂.
101. The method of claim 89 wherein ten or more genetic targets are simultaneously detected.

102. The method of claim 89 wherein said means for effecting a hot start initiation is a hot start enzyme.
103. The method of claim 102 wherein said hot start enzyme is a Taq Polymerase enzyme.
104. The method of claim 103 wherein said enzyme is Amplitaq GoldTM.
105. The method of claim 89 wherein said means for effecting a hot start initiation includes a DNA polymerase enzyme and a heating step.
106. The method of claim 89 wherein said series of PCR reaction steps includes 20 cycles of touchdown PCR including 20s at 95°C, 1 min at 63°C - decreased by 0.5 each cycle, and 1 min at 72°C.
107. The method of claim 106 wherein said series of PCR reaction steps further includes 25 cycles of PCR, including 20s at 95°C, 45s at 56°C and 1 min at 72°C.
108. The method of claim 107 further including 7 min at 72°C.
109. The method of claim 89 wherein said genetic targets are DNA sequences.
110. The method of claim 109 wherein DNA sequences are selected from the group consisting of bacterial DNA, viral DNA, plant DNA, animal DNA or human DNA.

111. The method of claim 73 wherein said series of PCR reaction steps includes 20 cycles of touchdown PCR including 20s at 95°C, 1 min at 63°C - decreased by 0.5 each cycle, and 1 min at 72°C.
112. The method of claim 111 wherein said series of PCR reaction steps further includes 25 cycles of PCR, including 20s at 95°C, 45s at 56°C and 1 min at 72°C.
113. The method of claim 112 further including 7 min at 72°C.
114. The method of claim 73 wherein said genetic targets are DNA sequences.
115. The method of claim 114 wherein DNA sequences are selected from the group consisting of bacterial DNA, viral DNA, plant DNA, animal DNA or human DNA.

Application number numéro de demande: 2410795

Figures: 4-5-6-7

Pages: _____

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
10^{ème} étage)

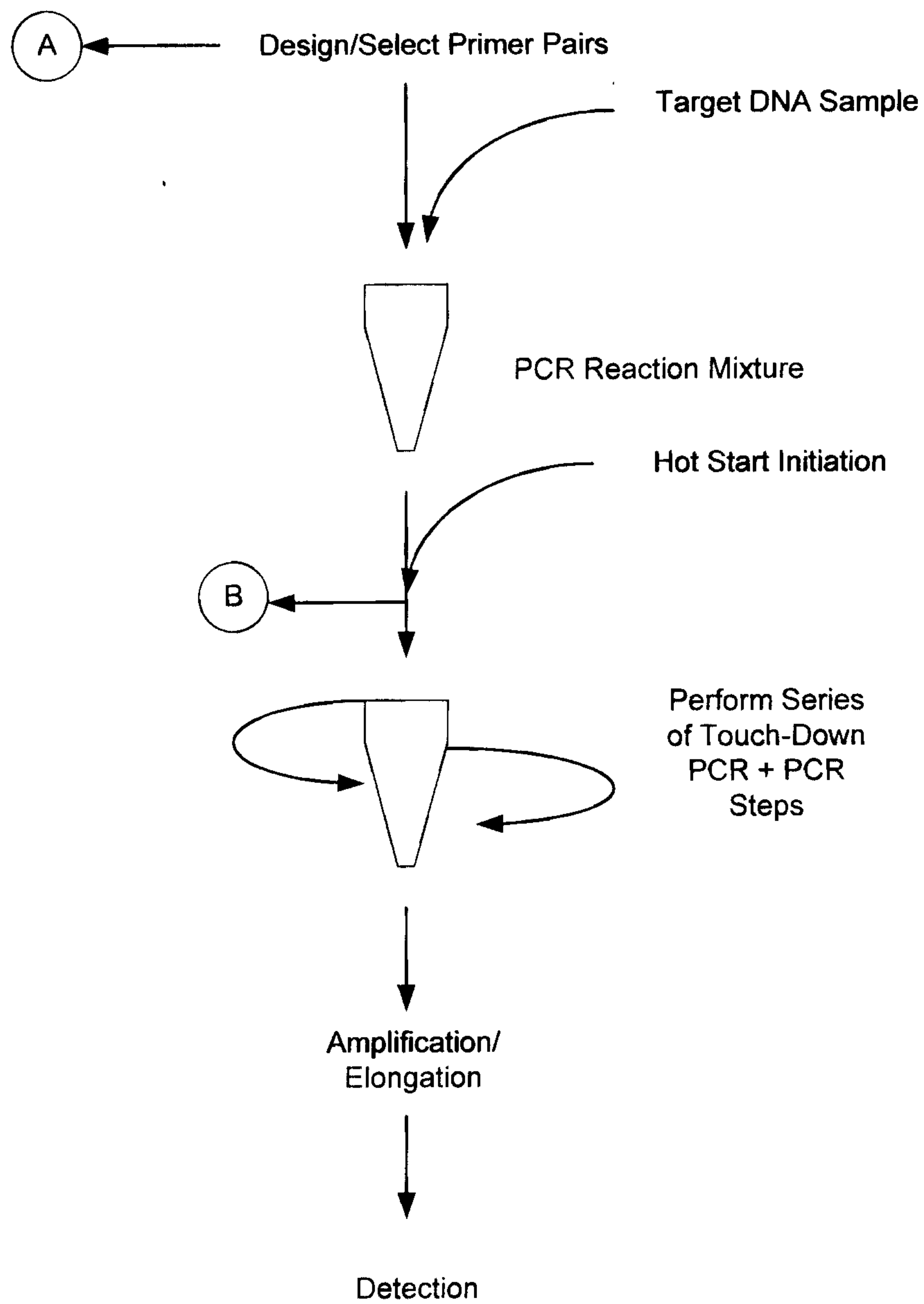


Figure 1

Figure 2. Primer Selection Protocol

Length	18 – 27nt (22 nt- preferred)
GC Content	20 – 80% (40 - 50% preferred)
Melting Temperature (T _m)	55- 65 °C (57 °C preferred)

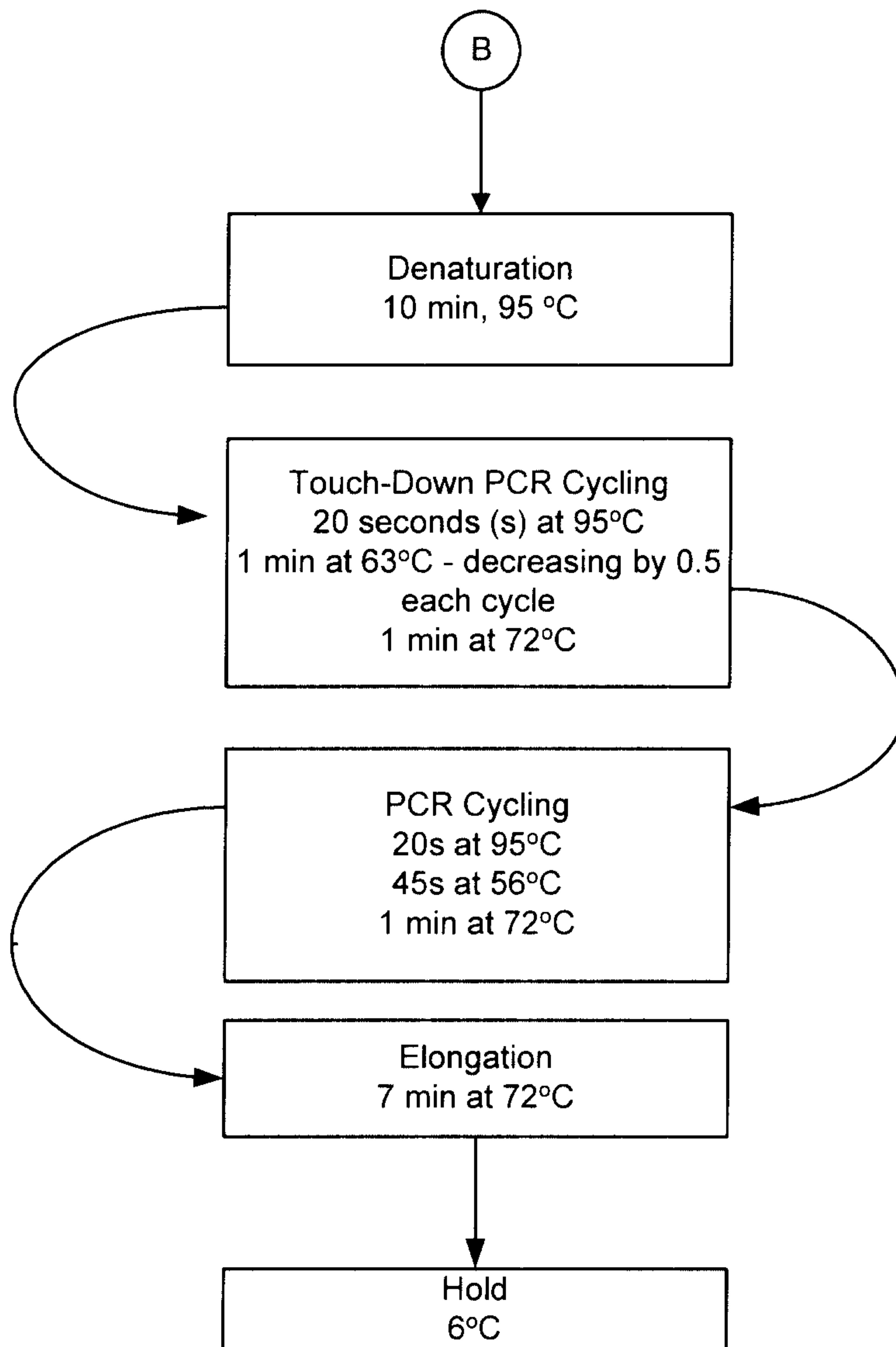


Figure 3