Title: ISOThermal AMPLIFICATION METHOD FOR DETECTION OF NUCLEIC ACID MUTATION

Abstract: A method is provided for detection of a nucleic acid region in a biological sample, by use of an isothermal amplification, such as loop-mediated isothermal amplification (LAMP). In particular, the provided method is suitable for detection of the presence or absence of pathogenic organisms, such as the pathogenic JP clones of Aggregatibacter (Actinobacillus) actinomycetemcomitans. In addition, oligonucleotide primers are provided for performing the method. A kit is also provided for performing such methods. Moreover, a method for treatment of periodontitis is provided, wherein pathogenic organisms are detected by said isothermal amplification reaction followed by an antibiotic treatment.
Isothermal amplification method for detection of nucleic acid mutation

Field of invention
The present invention relates to a method for the detection of the presence or absence of a nucleic acid region within a nucleic acid target sequence. The method comprises performing an isothermal amplification reaction, wherein the amplification product comprises the nucleic acid region of interest. In particular, the method of the present invention comprises oligonucleotide primers for loop-mediated isothermal amplification of a promoter region of the leukotoxin gene in *Aggregatibacter (Actinobacillus) actinomycetemcomitans* for the detection of a specific 530 base pair deletion, which characterizes a hyper-virulent clone (JP2) of the species.

Background of invention
Deletion mutations of gene regions may be located in the coding region of the genes, and can upon transcription and translation result in the production of deleterious truncated polypeptides. Such deletions may also lead to frame shift mutations, wherein the translation frame is shifted due to the deletion. In addition, mutations in gene regions may also affect the further processing of the transcript, such as capping, splicing and 3'-processing, which either may lead to degradation of the transcript or to translational defects. Therefore, deletion mutations may be accompanied by structural or functional changes of the translated proteins. A deletion may also be located in a region outside the transcribed region of a gene, such as promoter or other regulatory regions. Deletions in these regions can affect the transcriptional regulation, which may have deleterious effects for the cell or its environment.

Identification of specific mutation may be used to identify specific subtypes of bacteria or viruses. For example, the specific JP2 clone of the gram-negative oral and systemic pathogen *Aggregatibacter actinomycetemcomitans* can be identified by a specific 530 base pairs deletion in the promoter region of its leukotoxin gene. The 530 base pairs deletion results in enhanced production of leukotoxin (1). *Aggregatibacter actinomycetemcomitans* has long been suspected as an etiological factor in periodontitis in juveniles (19, 21). A particular strong association has been reported between presence of the JP2 clone in the periodontal pockets and early-onset periodontitis (7).
Several methods exist for the identification of deletion mutations in nucleic acids, including well known techniques such as polymerase chain reaction (PCR), Southern blotting, Northern blotting, Western blotting, nucleic acid sequencing to name a few. The techniques differ with respect to efficiency, sensitivity and specificity, the PCR method probably being the most sensitive and specific method. Common to all the methods, however, is that they are relatively time and labour consuming, and they require rather advanced laboratory equipment. Neither of the techniques can be carried out in a single step, but require multiple handling and incubation steps, which makes it difficult to maintain the accuracy that is required. Therefore, these techniques are not suitable for detection of mutations or identification of pathogens for diagnosis of a disorder at the point of care.

Loop-mediated isothermal amplification (LAMP) is a relatively new amplification technique, which can be used for detection of nucleic acid mutations, and is particularly suited for detection of single nucleotide polymorphisms (Notomi, nucleic acids research, 2000, 28(12): e63; EP1020534; EP1231281). This technology employs specifically designed oligonucleotide primers, which synthesize and amplify target nucleotides under isothermal conditions. The LAMP method is reported to exceed PCR and other techniques in terms of sensitivity as well as specificity. Moreover, the LAMP method is extremely easily performed without advanced equipment and in a much shorter time frame than conventional amplification techniques and detection methods.

A limitation of the LAMP technology, however, is that the target DNA should be between 130 and 200 base pairs for efficient amplification. Moreover, the technique employs at least four oligonucleotide primers with six specific recognitions sites, which are required to participate in complex secondary loop structures. Therefore, the primers must be designed so that their melting temperatures fall within certain ranges. In addition, the melting temperature between the individual primers has to be balanced as well as the melting temperature between the different regions of the specific primer to insure that synthesis occur earlier from the inner primer set than from the outer primer set. All of these requirements strictly constrain the choice of sequences and lengths for optimal primer design. Therefore, LAMP is only applicable for detection of mutations which are located in regions that support the strict requirements for primer design.
Summary of invention

The present invention relates to a method for detection of the presence or absence of a nucleic acid region. Thus, the method can be used for detection of the presence or absence of a nucleic acid region within a nucleic acid target. This may for example be used for identification of a specific subtype or clone of a virus or bacteria or functionally important deletions/inserts in genes of prokaryotic or eukaryotic cells.

In one aspect, the present invention relates to a method for detecting the presence or absence of a nucleic acid region within a nucleic acid target comprising a providing a biological sample comprising a nucleic acid, b. performing an isothermal amplification reaction on said nucleic acid, employing oligonucleotide primers flanking said nucleic acid region, such that the amplification product comprises said nucleic acid region, and c. detecting said amplification product, wherein the presence of an amplification product is indicative of the absence of said nucleic acid region in said nucleic acid target, and the absence of amplification product is indicative of the presence of said nucleic acid region in said nucleic acid target or absence of said nucleic acid target in said biological sample.

In another aspect, the present invention relates to a method for determining the presence or absence of a toxic strain of Aggregatibacter actinomycetemcomitans comprising a. providing a biological sample comprising a nucleic acid, b. performing an isothermal amplification reaction on said nucleic acid specimen, wherein the amplification product comprises a nucleic acid region, and c. detecting said amplification product, wherein the presence of an amplification product is indicative of the presence of said toxic strain of Aggregatibacter actinomycetemcomitans in said biological sample, and the absence of amplification product is indicative of the absence of said toxic strain of Aggregatibacter actinomycetemcomitans in said biological sample.

In yet another aspect, the present invention pertains to an oligonucleotide primer comprising between 10 and 80 nucleotides for use in performing an isothermal amplification reaction on a nucleic acid target, wherein the amplification product comprises a nucleic acid region, and the subsequent detection of the presence of an amplification product is indicative of the absence of said nucleic acid region in said
nucleic acid target, and the absence of amplification product is indicative of the presence of said nucleic acid region in said nucleic acid target.

In a final aspect, the present invention relates to a kit for detection of presence or absence of a nucleic acid region within a nucleic acid target comprising i. a DNA polymerase catalyzing complementary strand synthesis which includes strand displacement, ii. a second primer, wherein the 3'-end of the second primer anneals to the 3'-side region of one of said target nucleotide sequence strands, and the 5'-side of the second primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin; iii. a first primer, wherein the 3'-end of the first primer anneals to the 3'-side region of the other said target nucleotide sequence strand, and the 5'-side of the first primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin; iv. nucleotide substrates; v. a third primer, wherein the third primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the first primer on the template functions as the origin; and vi. a fourth primer, wherein the fourth primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the second primer on the template functions as the origin.

Description of Drawings

Figure 1. LAMP primers used to detect Δ530 in the ltx promoter region of JP2 strains of A. actinomycetemcomitans. (a) Location of the primer sequences in the promoter region of the ltx gene operon. The ATG start codon of the ltxC gene (the first gene in the ltx operon) is shown in bold and underlined. The site of Δ530 and the BsmI restriction site are indicated by arrows. The numbers to the left indicate positions in the genome of A. actinomycetemcomitans strain HK1651 (http://www.genome.ou.edu/act.html). (b) The structure and sequence of the six primers used in the LAMP reaction.

Figure 2. Analysis of DNA fragments amplified by LAMP reactions performed on isolates and directly on plaque samples. (a) Visual detection of a white precipitate in positive samples. The figure shows a positive (strain JP2) and a negative (non-JP2
strain Y4) sample together with a negative control (neg.). (b) LAMP products were separated by 3% agarose gel electrophoresis and stained with EtBr. Lane 1. MW marker (GeneRuler 50 bp DNA ladder, Fermentas GMBH, Helsingborg, Sweden); lane 2. strain JP2; lane 3. strain HK1651 (JP2 clone strain); lane 4. strain HK975 (Y4, non-JP2 strain); lane 5. negative control; lane 6. clinical sample 362M (positive for JP2 clone and negative for non-JP2 types in PCR); lane 7. clinical sample 436l (positive for JP2 clone and non-JP2 types in PCR); lane 8. clinical sample 507l (negative for A. actinomycetemcomitans in PCR); lane 9. clinical sample 510l (negative for JP2 clone and positive for non-JP2 types in PCR); lanes 10 to 13, as lanes 2, 3, 6, and 7, respectively, digested with BsmI; lane 14. MW marker. The arrows to the right indicate the positions of fragments in bp in the MW marker. In lane 4 an excess of DNA was used as described in the text, and the genomic DNA is seen as a faint band in top of the gel. The low molecular smear in lanes 5, 8, and 9 is presumably due to primer-dimers, and it did not result in a visible white precipitate. In lanes 10 to 13 the two fragments of 106 and 116 bp migrate together in the gel. The band in lane 10 is weak because only a fraction of the sample was loaded onto the gel.

Figure 3. LAMP reaction (producing starting structure) in a JP2 strain and a non-JP2 strain of Aggregatibacter actinomycetemcomitans, respectively. The figure shows the basic principle of the LAMP reaction when the FIP or BIP primers are designed to contain an over 20 to 30 bp deletion within the "spacer region" between F1 and F2 or B1 and B2 is outlined in the figure. Using those primers, when the target includes the deletion, dumbbell-like starting structures are produced and the LAMP cycling continues. In contrast, when the target does not include the deletion, dumbbell-like starting structure is not produced and the LAMP cycling does not occur. Even if DNA synthesis proceeds in one step accidentally, the amplification reaction is either halted in other steps or is delayed since repetition of this reaction problem continually occurs at each cycling step of the DNA replication.

Detailed description of the invention
The object of the present invention is to provide a method for detection of the presence of a nucleic acid region within a specific target nucleic acid sequence. The method comprises amplification of the target nucleic acid region at isothermal conditions.
Definitions
To facilitate the understanding of the invention, a number of terms are defined below.

The term "mutation" as used herein, refers to a change in a nucleotide sequence of a nucleic acid compared with the wild type sequence as observed among other organisms (including viruses) of the same origin. Within a multicellular organism, differences observed among organs, cells, etc are also included as mutations. Mutations arise from point mutations, deletions, insertions, duplications, inversions, and translocations. Mutations may occur within the coding region of a gene, within intron sequences, or in regulatory regions, such as promoters, enhancers and 3'-processing signals. However, differences in nucleotide sequences found in other genomic sequences are also referred to as mutations. The present invention primarily relates to deletion mutations and insertion mutations. The term "deletion mutation" refers to a mutation, characterized by the absence of a nucleic acid region in a nucleic acid sequence, in which said region is observed in other organisms (including viruses). Conversely, the term "insertion mutation" refers to a mutation, wherein a nucleic acid region, has been inserted into a nucleic acid sequence.

The term "nucleic acid target" as used herein refers to a nucleic acid sequence, wherein the presence or absence of a nucleic acid region is detected by the methods and kits of the present invention. The nucleic acid target is targeted by oligonucleotide primers according to the present invention, and therefore, a nucleic acid sequence surrounded by annealing sites for the oligonucleotide primers of the present invention constitute the nucleic acid target of the present invention.

The term "nucleic acid region" as used herein refers to a nucleic acid sequence that may be comprised in the nucleic acid target. More specifically, the nucleic acid region may be inserted into the nucleic acid target, or the nucleic acid region may be deleted from the nucleic acid target. Importantly, the oligonucleotide primers according to the present invention hybridize to the 5'-side and the 3'-side of the nucleic acid target, i.e. on opposite sides of the nucleic acid region, or on opposite sides of the deletion site. The term "deletion site" as used herein refers to a site in a nucleic acid target, from which one or more nucleotides (the nucleic acid region) have been deleted. In the context of the present invention, the term "deletion site" relates to a site in the nucleic acid target from which a nucleic acid region is deleted in the nucleic acid target.
Consequently, the oligonucleotide primers according to the present invention do not hybridize to any parts of the nucleic acid region, nor does any of the oligonucleotide primers span the deletion site of the nucleic acid target.

Both of the expressions "5'-side" and "3'-side" refer to a direction in the strand which serves as the template. The nucleotide sequence of a nucleic acid is generally described from the 5'-side to the 3'-side of the sense strand.

The nucleotide sequence of the nucleic acid target includes both the sense strand and the nucleotide sequence of the complementary strand thereof, i.e. the antisense strand. Therefore, the nucleotide sequence of the nucleic acid target comprise either of the nucleotide sequence to be synthesized or the complementary strand thereof.

The terms "nucleic acid" and "polynucleotide(s)" are used interchangeably, and generally refers to any polynucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, nucleic acid as used herein refers to, among others, single-and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, nucleic acid as used herein can also refer to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. Specifically, the nucleic acid of the present invention may be derived from nucleic acid contained in said biological sample. For example, cDNA synthesized from mRNA, or nucleic acid amplified on the basis of nucleic acid derived from the biological sample, is a typical example of the nucleic acid of the present invention.

As used herein, the term "polynucleotide" includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or
modified bases, such as tritylated bases, to name just two examples, are
polynucleotides as the term is used herein.

The term polynucleotide in the present invention includes oligonucleotides. The term
"polynucleotide" is used in the case where the chain length is not limited, while the term
"oligonucleotide" is used to refer to a nucleotide polymer having a relatively short chain
length.

It will be appreciated that a great variety of modifications have been made to DNA and
RNA that serve many useful purposes known to those of skill in the art. The term
polynucleotide as it is employed herein embraces such chemically, enzymatically or
metabolically modified forms of polynucleotides, as well as the chemical forms of DNA
and RNA characteristic of viruses and cells, including simple and complex cells, inter
alia.

The term "amino acid" and "amino acid sequence" refer to an oligopeptide, peptide,
polypeptide, or protein sequence, or a fragment of any of these, and to naturally
occurring or synthetic molecules. Where "amino acid sequence" is recited to refer to a
sequence of a naturally occurring protein molecule, "amino acid sequence" and like
terms are not meant to limit the amino acid sequence to the complete native amino
acid sequence associated with the recited protein molecule.

The term "anneal" as used herein refers to any formation of a double-stranded
structure of nucleic acid through base pairing based on the law of Watson-Crick.

Accordingly, even if a nucleic acid chain constituting base pairing is a single-stranded
chain, annealing occurs if intramolecular complementary nucleotide sequences are
base-paired. In the present invention, annealing and hybridization have the same
meaning in that the nucleic acid constitutes a double-stranded structure through base
pairing.

The term "template" as used herein refers to a nucleic acid serving as a template for
synthesis of a complementary nucleic acid strand. A complementary strand having a
nucleotide sequence complementary to a template thus has a meaning as a chain
corresponding to the template, but the relationship between the two is merely relative.
Thus, a nucleic acid strand synthesized as a complementary strand can function again as a template. That is, the complementary chain can become a template.

Isothermal amplification

Amplification according to the present invention is the process wherein a plurality of replicas of one or more starting molecule (template) is synthesised, without employing knowledge of the exact composition (e.g. nucleotide sequence) of the starting molecule. Hence, a template may be amplified even though the exact composition of said template is unknown. In one embodiment of the present invention amplification of a template comprises the process wherein a template is copied by a nucleic acid polymerase or polymerase homologue, for example a DNA polymerase or an RNA polymerase. The amplification process may include amplification of the nucleic acid template by reverse transcription.

Several nucleic acid amplification methods exist, including the widely used polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR), and strand displacement amplification SDA. These technologies employ different methods of reinitiating new rounds of transcription, such as heat denaturation of double stranded DNA products in PCR. 3SR and NASBA employ a set of transcription and reverse transcription reactions to amplify the target nucleic acid, whereas SDA uses restriction enzyme digestions and strand displacement DNA synthesis with modified nucleotides as substrate.

The present invention relates to a method, wherein a nucleic acid target is amplified by isothermal amplification. The hallmark of isothermal amplification is that the amplification reaction is performed at isothermal condition. Thus, in contrast to PCR amplification, a heat denaturing step is not required for amplification of a nucleic acid target. Isothermal amplification may be symmetric or asymmetric with respect to primer design. Examples of asymmetric isothermal amplification includes smart amplification process version 2 (SMAP 2), as described by Mitani et al. (Mitani et al. 2007. Nature Methods 4(3):257).

LAMP

The methods and kits of the present invention comprise amplifying a nucleic acid target by isothermal amplification. In one preferred embodiment of the present invention, the
isothermal amplification is loop-mediated isothermal amplification (LAMP), which amplifies nucleic acids with high specificity, efficiency and rapidity under isothermal conditions (Notomi et al., 2000. Nucleic acid research. Vol. 28(12)-e63). In short, LAMP employs a DNA polymerase and at least four oligonucleotide primers, which comprise sequences that recognize a total of six distinct sequences of the nucleic acid target. A first inner primer (forward inner primer (FIP)) comprising sequences of the sense and antisense strands of the target DNA initiates LAMP (see figure 3, left panel). After synthesis of a complementary strand primed by the first inner primer, strand displacement DNA synthesis primed by an outer primer (F3) releases a single-stranded DNA. This serves as template for DNA synthesis primed by second inner and outer primers (BIF and B3) that hybridize to the other end of the target, thereby producing a stem-loop DNA structure, see figure 3, left panel. In a subsequent cycling LAMP reaction, an inner primer (BIP or FIP) hybridizes to a loop on the nucleic acid product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA, wherein the stem is twice as long. The cycling reaction continues with accumulation of up to $10^5$ copies or more of the nucleic acid target. The nucleic acid amplification products are stem-loop polynucleotides with multiple inverted repeats of the nucleic acid target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the nucleic acid target in the same strand. Because LAMP recognizes the nucleic acid target by six individual sequences in the initial round of synthesis, and by four distinct sequences during the cycling LAMP reactions, the LAMP technology amplifies a nucleic acid target with high selectivity.

Detailed description of LAMP

In the present invention, the LAMP technique is utilized for detection of a nucleic acid region within a nucleic acid target. This method is based on the finding that LAMP only efficiently amplifies a nucleic acid target, with a limited number of nucleotides. Thus, in case the nucleic acid target comprises a nucleic acid region of a certain sequence length, the LAMP reaction will no longer amplify the nucleic acid target, thereby indicating the presence of said nucleic acid region within said nucleic acid target. Conversely, the detection of an amplification product in a LAMP reaction, wherein the distance between the primers exceeds the limits of functional LAMP cycling, indicates the absence of a nucleic acid region within the nucleic acid target of said LAMP reaction.
The LAMP reaction according to the present invention comprises the steps of:

(a) annealing a first primer to a nucleic acid target sequence to carry out complementary strand synthesis using the primer as the origin, wherein the 3'-end of the first primer anneals to a 3'-side region of one of the two target nucleotide sequence strands, and the 5'-side of the first primer includes a nucleotide sequence that comprises a region on the products of the complementary strand synthesis that uses this primer as the origin;

(b) putting the region, to be annealed with the second primer, in the elongation products of the first primer synthesized in the step (a) into a state allowing base pairing of the region to the second primer, annealing them to synthesize the complementary strand using the elongation product of the first primer as a template, wherein the 3'-end of the second primer anneals to the 3'-side region of the other said target nucleotide sequence strand, and the 5'-side of the second primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that comprises a region on the products of the complementary strand synthesis that uses the primer as the origin;

(c) carrying out complementary strand synthesis using the elongation product of the second primer itself as a template by annealing the 3'-end of the products with said predicted nucleotide sequence to the first primer in said elongation products;

(d) carrying out complementary strand synthesis using the complementary strand synthesized in step (c) itself as a template by annealing the 3'-end of the complementary strand synthesized in step (c) with said predicted nucleotide sequence to the second primer in said elongation products.

The LAMP reaction as described herein, for detecting the presence or absence of a nucleic acid region within a nucleic acid target according to the present invention, can be performed by incubating the components listed below under appropriate conditions.

More specifically, the present invention relates to a method for detecting the presence or absence of a nucleic acid region within a nucleic acid target comprising incubating the elements (i) to (vii) under conditions allowing complementary strand synthesis using the second and first primers as the origins:

i. a nucleic acid comprising a target nucleotide sequence;

ii. a DNA polymerase catalyzing complementary strand synthesis which includes strand displacement:
iii. a second primer, wherein the 3'-end of the second primer anneals to the 3'-side region of one of said target nucleotide sequence strands, and the 5'-side of the second primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin;

iv. a first primer, wherein the 3'-end of the first primer anneals to the 3'-side region of the other said target nucleotide sequence strand, and the 5'-side of the first primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin;

v. nucleotide substrates;

vi. a third primer, wherein the third primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the first primer on the template functions as the origin; and

vii. a fourth primer, wherein the fourth primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the second primer on the template functions as the origin.

A first or second primer, used in LAMP according to the present invention, comprises the following regions:

X2: a region containing a nucleotide sequence complementary to X2c, which region determines the 3'-side of the target nucleotide sequence; and X1c: a region located on the 5'-side of X2c mentioned above, and containing a nucleotide sequence complementary to the nucleotide sequence predicted in the region X1 which exists on the products elongated from the first primer. X1 is a sequence of the region on the elongation product of the first primer, said region being located between the 3'-end of the first primer and the specific nucleotide sequence. X1c also includes regions having substantially the same nucleotide sequence as that predicted for the region X1c.

The nucleic acid synthesized by loop-mediated isothermal amplification according to the present invention is composed substantially of mutually complementary chains linked via the loop-forming sequence. In general, a strand, which is not separated into 2 or more molecules upon dissociation of base pairing is called a single-stranded chain regardless of whether it partially involves base pairing or not. The complementary nucleotide sequence can form intramolecular base pairing in the same chain. An
intramolecular base-paired product, which can be obtained by permitting the nucleic acid having complementary nucleotide sequences linked alternately in a single-stranded chain according to LAMP as described herein to be base-paired in the same chain, gives a region constituting an apparently double-stranded chain and a loop not involving base pairing.

The nucleic acid comprising complementary nucleic acid sequences linked alternately in a single-stranded chain as described herein contains complementary nucleotide sequences capable of annealing in the same chain, and its annealed product can be defined as single-stranded nucleic acid constituting a loop not involving base pairing at a bent hinged portion. A nucleic acid comprising a nucleotide sequence complementary thereto can anneal to said loop. The loop-forming sequence is capable of base pairing so as to initiate the synthesis of a complementary chain for displacement, and is provided preferably with a sequence distinguishable from a nucleotide sequence located in the other region in order to achieve specific annealing. In a preferred embodiment, the loop-forming sequence contains substantially the same nucleotide sequence as a region F2c or R2c located at the 3'-side of a region (i.e. F1c or R1c) derived from nucleic acid as a template and annealed in the same chain.

In general, when a complementary chain synthesized with a certain sequence as a template anneals to a target nucleotide sequence to give the origin of synthesizing a complementary chain, this certain sequence is substantially the same as the target nucleotide sequence. For example, substantially the same sequence as F2 includes not only absolutely the same nucleotide sequence as F2 but also a nucleotide sequence capable of functioning as a template giving a nucleotide sequence capable of annealing to F2 and acting as the origin of synthesizing complementary chain.

Synthesis of the nucleic acid complementary nucleotide sequences linked alternately in a single-stranded chain can be initiated by use of a DNA polymerase having the strand displacement activity and nucleic acid which is provided at the 3'-terminal thereof with a region F1 capable of annealing to a part F1c in the same chain and which upon annealing of the region F1 to F1c, is capable of forming a loop containing a region F2c capable of base pairing. By use of this region as the origin of synthesis, a complementary chain previously synthesized with a sample sequence itself as a template is displaced. Then, a region B1c (arbitrary region) located at the 3'-terminal of
the displaced chain is in a state ready for base-pairing. A region having a complementary sequence to this B1c is annealed thereto, resulting in formation of a nucleic acid (2 molecules) having a nucleotide sequence extending from F1 to B1c and its complementary chain linked alternately via the loop-forming sequence. In the present invention, the arbitrary region such as B1c above can be selected arbitrarily provided that it can be annealed to a polynucleotide having a nucleotide sequence complementary to that region, and that a complementary chain synthesized with the polynucleotide as the origin of synthesis has necessary functions for the present invention.

The basic principle of the loop-mediated isothermal amplification reaction of a nucleic acid target by employing oligonucleotide primers and a DNA polymerase with strand displacement activity in the reaction according to the present invention is described by reference to Fig. 3. The oligonucleotide primer described elsewhere herein (FIP in Fig. 3) anneals at X2 (corresponding to F2) to nucleic acid as a template, to provide the origin of synthesis of complementary chain. In Fig. 3, a complementary chain synthesized from FIP as the origin of synthesis is displaced by synthesis of complementary chain from an outer primer (F3), as described elsewhere herein, to form a single-stranded chain (Fig. 3E). When synthesis of complementary chain to the resulting complementary chain is further conducted, the 3′-terminal of nucleic acid synthesized as complementary chain in Fig. 3F has a nucleotide sequence complementary to the oligonucleotide of the present invention. That is, because the 5′-terminal of the oligonucleotide of the present invention has the same sequence as a region X1c (corresponding to F1c), the 3′-terminal of the nucleic acid thus synthesized has a complementary sequence X1 (F1). Fig. 3 shows that the complementary chain synthesized from B1 (utilizing the BIP oligonucleotide primer) as the origin of synthesis is displaced by synthesis of complementary chain by primer B3 as the origin of synthesis, thereby allowing the displaced single stranded chain to form the characteristic hairpin loop starting structure, as shown in figure 3G, left panel. Once the 3′-terminal portion is made ready for base pairing by this displacement, X1 (F1) at the 3′-terminal anneals to X1c (F1c) in the same chain, and elongation reaction with itself as a template proceeds (Fig. 3G). Then, X2c (F2c) located at the 3′-terminal thereof is left as a loop not involving base pairing.
In a subsequent loop-mediated isothermal amplification cycling reaction, the X2 region (such as F2 in fig. 3) in the oligonucleotide according to the present invention anneals to the X2c (such as F2c) loop, and a complementary chain is synthesized with said oligonucleotide as the origin of synthesis. A product of complementary chain synthetic reaction with the previously synthesized product as a template is displaced by the strand displacement reaction so that it is made ready for base pairing.

By the use of one kind of oligonucleotide according to the present invention and an arbitrary reverse primer capable of conducting nucleic acid synthesis where a complementary chain synthesized with said oligonucleotide as a primer is used as a template, a plurality of nucleic acid synthetic products can be obtained. The desired nucleic acid product of the invention, however, has a complementary nucleotide sequence alternately linked in a single-stranded chain. Once converted into a single-stranded chain by treatment such as heat denaturation, the other product serves again as a template for forming the structure shown in fig. 3G, bottom. If the product as nucleic acid in the form of a double-stranded chain is converted into a single-stranded chain by heat denaturation, annealing occurs within the same chain at high probability without forming the original double-stranded chain. This is because a complementary chain having the same melting temperature (Tm) undergoes intramolecular reaction preferentially over intermolecular reaction. Each single-stranded chain derived therefrom annealed in the same chain is annealed in the same chain and returned to the state shown in fig. 3G, and each chain further gives one molecule of fig. 3E and 3G, respectively. Repetition of these steps allows the successive synthesis of nucleic acid having complementary nucleotide sequences linked alternately in a single-stranded chain. The template and the product formed in 1 cycle are increased exponentially, thereby making amplification very efficient.

For obtaining the state of Fig. 3F, the initially synthesized complementary chain should be ready for base pairing, at least in the region, which anneals to the reverse primer. This step can be achieved by an arbitrary method. To this end, an outer primer X3 (F3) is employed, which anneals to the first template at a region X3c (F3c) at the 3'-side of the region X2c (F2c) to which the oligonucleotide of the present invention anneals.

When a complementary chain is synthesized from said outer primer, F3, by a polymerase with strand displacement activity, the complementary chain synthesized
from the F2c as the origin of synthesis is displaced, allowing the region F1c to be annealed by F1 (Fig. 3E). The utilization of the strand displacement reaction allows the reaction up to now to proceed under isothermal conditions.

Importantly, complementary chain synthesis from the outer primer (F3) should be initiated after synthesis from F2c. The simplest way to obtain this is to provide a higher concentration of the inner primer than of the outer primer. Specifically, the primers are used at 2- to 50-fold, preferably 4- to 10-fold different concentrations, whereby allowing the reaction to proceed as expected. Moreover, the melting temperature (Tm) of the outer primer is set to be lower than the Tm of the X1-region (corresponding to F1 and R1) in the inner primer whereby the timing of synthesis can be controlled. The Tm relationship between the primer regions can be summarized as follows, where :: denotes base pairing between the indicated domains:

\[(F3::F3c) \leq (F2c::F2) \leq (F1c::F1), \text{ or} \]
\[(\text{outer primer::region at the 3'-side in the template}) \leq (X2c::X2) \leq (X1c::X1).\]

The fact that \((F2c::F2) \leq (F1c::F1)\), ensures the annealing between F1c::F1 prior to the annealing of F2 to the loop. The annealing between F1c::F1 is an intramolecular reaction and is therefore expected to proceed preferentially at high probability. Nevertheless, it is preferable to consider Tm in order to give more desired reaction conditions. Obviously, similar conditions should be considered even in the design of a reverse primer. Melting temperature (Tm) can be theoretically calculated by a combination of the length and composition of an annealing complementary chain.

Accordingly, those skilled in the art can derive preferable conditions on the basis of the disclosure of this specification. A suitable tool for primer design the computer program program PrimerExplorer V1, V2, V3 and/or V4 (V3 is available at https://primexplorer.jsp/lamp3.0.0/index.html).

Contiguous stacking is another methodology, which can be applied for the temporal control of annealing of the outer primer. Contiguous stacking is when an oligonucleotide not capable of annealing independently is made capable of annealing upon being contiguous to the part of a double-stranded chain (Chiara Borghesi-Nicoletti et al., Bio Techniques, 12, 474-477 (1992)). That is, the outer primer is designed so as to be contiguous to F2c (X2c) and not to be able to anneal.
independently. Thereby, the outer primer does not anneal until F2c (X2c) anneals, and thus the annealing of F2c (X2c) occurs predominantly to annealing of X3 (F3).

The nucleic acid target to be amplified by isothermal amplification according to the present invention, comprise DNA as well as RNA. If the template nucleic acid having F2c (X2c) is RNA, the reactions shown in fig. 3 can also be realized by a different method. For example, if this RNA chain is decomposed, R1c is made ready for base pairing. To this end, F2 is annealed to F2c in the RNA and a complementary DNA chain is synthesized by a reverse transcriptase. Then, the RNA serving as a template is decomposed by alkali denaturation or by enzymatic treatment with a ribonuclease acting on RNA in a double-stranded DNA/RNA hybrid, such as RNase H or some reverse transcriptases with ribonucleolytic activities, whereby the DNA synthesized from F2 is released as a single-stranded chain. In this way, the reverse primer can be annealed to R1c made capable of base pairing. Accordingly, the outer primer for rendering R1c ready for base pairing becomes unnecessary.

The strand displacement activity of reverse transcriptase can also be utilized for the strand displacement by an outer primer as described above. Thus, a reaction system can be constituted by a reverse transcriptase only. In this case, it is possible to synthesize a complementary chain from F2 annealing to F2c in the template, and to synthesize a complementary chain from the outer primer F3 as the origin of synthesis annealing to F3c located at the 3'-side of F2c and to simultaneously displace the previously synthesized complementary chain. When the reverse transcriptase performs the reaction of synthesizing a complementary chain with DNA as the template, all the reactions of synthesizing complementary chains proceed by the reverse transcriptase, including the synthesis of a complementary chain with R1c as the origin of synthesis annealing to R1c in the displaced complementary chain as the template, the synthesis of a complementary chain with B3 as the origin of synthesis annealing to B3c located at the 3'-side of R1c and the simultaneous displacement reaction. If the reverse transcriptase is not expected to exhibit the DNA/RNA strand displacement activity under specific reaction conditions, a DNA polymerase having the strand displacement activity described above may be used in combination. In a preferred embodiment of the present invention, a first single-stranded nucleic acid is obtained with RNA as a template as described above. Nevertheless, if a DNA polymerase such as Bca DNA polymerase, which has both strand displacement activity and reverse transcriptase
activity is used, not only synthesis of a first single-stranded nucleic acid from RNA but also subsequent reactions with DNA as a template can proceed similarly by the same enzyme.

The reaction system described above can proceed according to the present invention by utilization of a reverse primer with a specific structure that resembles the forward primer. In this context, a reverse primer serves as origin of complementary chain synthesis in the opposite direction as another primer. Herein, the reverse primer in relation to a forward primer is called a backward primer, and the reverse primer in relation to a backward primer is called a forward primer. Consequently, a reverse primer may be a forward primer or a backward primer depending on the context.

An oligonucleotide, serving as a reverse primer relative to a forward primer according to the present invention, comprise the arbitrary regions B2 and B1, which anneals to the B1c and B2c regions, respectively, of the complementary chain synthesized with F2 as a primer. By use of such a reverse primer, a series of reactions for forming a loop and for synthesizing and displacing a complementary chain from this loop occur in both the sense and antisense chains (forward side and backward side). see fig. 3. Consequently, the efficiency of synthesizing the nucleic acid with complementary nucleotide sequences linked alternately in a single-stranded chain according to the present invention is greatly improved, and a series of these reactions are feasible under isothermal conditions.

In the present invention, two kinds of oligonucleotide primers are employed: a forward inner primer (FIP) and a backward inner primer (BIP) with the following regions:

<table>
<thead>
<tr>
<th>Domains</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>X2</td>
<td>X1c</td>
<td></td>
</tr>
<tr>
<td>FIP</td>
<td>F2</td>
<td>F1c</td>
</tr>
<tr>
<td>BIP</td>
<td>B2</td>
<td>B1c</td>
</tr>
</tbody>
</table>

F2 is a complementary nucleotide sequence to a region F2c in a nucleic acid as the template, and B2 is a nucleotide sequence complementary to an arbitrary region R2c contained in a complementary chain synthesized with F2 as a primer. F1c and B1c are arbitrary nucleotide sequences located downstream of F2c and R2c respectively.
The F2 domain in FIP is first annealed to the F2c domain in a nucleic acid target as a template and used as the origin of synthesis of a complementary chain. The subsequent reaction steps are similar to those of the forward primer as explained above, see figure 3. The primer annealed as F3 is the outer primer, as described above. Still, a DNA polymerase with strand displacement activity is used for conducting the synthesis of a complementary chain with this primer as the origin of synthesis is used so that the complementary chain synthesized from the FIP is displaced and made ready for base pairing.

When B2c is made ready for base pairing, the B2 domain of the BIP as a reverse primer anneals thereto. Synthesis of a complementary chain with this site as the origin of synthesis proceeds until the chain reaches F1c at the 5'-terminal of FIP. Subsequently, the outer primer B3 anneals thereto to synthesize a complementary chain, during which strand displacement also proceeds so that the complementary chain synthesized from BIP as the origin of synthesis is displaced. In the complementary chain thus displaced, BIP is located at the 5'-side thereof and a sequence complementary to FIP is located at the 3'-terminal thereof. This complementary chain is shown in figure 3G, left panel, and designates as the starting structure.

The 3'-side of the displaced single-stranded nucleic acid, there is a sequence F1 complementary to F1c in the same chain. F1 rapidly forms intramolecular base-pairing to F1c in the same molecule to form the starting structure shown in fig. 3G, left panel, which supports the initiation of complementary chain synthesis. When the 3'-terminal (F1) anneals to F1c in the same chain, an F2c-containing loop is formed, which is ready for base pairing. The oligonucleotide FIP of the invention having a nucleotide sequence complementary to F2c anneals to the part of this loop and acts as the origin of complementary chain synthesis from the loop, proceeding while the reaction product in the previously initiated complementary chain synthesis from F1 is displaced.

Consequently, the complementary chain synthesized with itself as the template is made ready for base pairing again at the 3'-terminal. This 3'-terminal is provided with a region B1 capable of annealing to B1c in the same chain, and the two are annealed preferentially due to the rapid intramolecular reaction. The same reaction as the above-described reaction starting from the 3'-terminal synthesized with FIP as a template
proceeds in this region as well. Thereby, the nucleic acid having complementary nucleotide sequences linked alternately in the same single-stranded chain according to the present invention is continued to be extended from B1 as the starting point at the 3'-terminal by successive synthesis of a complementary chain and subsequent displacement thereof. Since B2c is always contained in the loop formed by intramolecular annealing of the 3'-terminal B1, the oligonucleotide (BIP) provided with B2 anneals to the loop at the 3'-terminal in the subsequent reaction.

The nucleic acid synthesized as complementary chain from the oligonucleotide annealing to the loop in the single-stranded nucleic acid elongated with itself as the template, supports the continued synthesis of the nucleic acid having complementary nucleotide sequences linked alternately in the same single-stranded chain according to the present invention. Synthesis of a complementary chain from the loop is completed when it reached BIP. Then, when the nucleic acid displaced by this nucleic acid synthesis initiates synthesis of complementary chain, the reaction reaches the loop which was once the origin of synthesis, and displacement is initiated again. Thus, the nucleic acid initiated by a primer annealing to the loop is also displaced, and as a result, the 3'-terminal B1 capable of annealing in the same chain is obtained. This 3'-terminal B1 anneals to B1c in the same chain to initiate synthesis of complementary chain. Accordingly, the starting structure shown in fig. 3G, left panel can function as a new nucleic acid which continues self-elongation and new nucleic acid formation.

The reaction of synthesizing nucleic acid, initiated from a primer annealing to the loop domain of the nucleic acid starting structure causes elongation from the 3'-terminal F1 as the origin of synthesis, as opposed to the reaction described above. So in the present invention, as one nucleic acid is elongated, the reaction of continuing to supply a new nucleic acid, from which elongation can be initiated, proceeds separately. Further, as the chain is elongated, a multitude of loop-forming sequences is produced not only at the terminal but also within the same chain. These loop-forming sequences are made ready for base pairing by the strand displacement synthetic reaction, thereby allowing an oligonucleotide to anneal thereto to serve as a base for the reaction of forming a new nucleic acid. Further efficient amplification is achieved by the synthetic reaction starting not only at the terminal but also within the chain. The newly formed nucleic acid is elongated itself and brings about subsequent formation of new nucleic acids. A series of these reactions continue to achieve very efficient amplification of
nucleic acid. Importantly, these reactions can be conducted under isothermal conditions.

The accumulated reaction products possess a structure comprising a nucleotide sequence between F1 and B1 and its complementary sequence linked alternately therein. However, both terminals of the repeating unit have a region consisting of the successive nucleotide sequences F2-F1 (or F2c-F1c) and B2-B1 (or B2c-B1c). This is due to the fact that the amplification according to the methods of the present invention is initiated from F2 (or B2) with an oligonucleotide as the origin of synthesis and then a complementary chain is elongated by the synthetic reaction from F1 (or B1) with the 3'-terminal as the origin of synthesis.

In a preferred embodiment of the present invention, the oligonucleotides FIP and BIP were used as oligonucleotides annealing to the part of a loop. However, even if these oligonucleotide primers with the composition described herein are not used, the isothermal amplification according to the present invention can be carried out by use of an oligonucleotide capable of initiating the synthesis of a complementary chain from the loop. That is, the elongating 3'-terminal, once displaced by a complementary chain synthesized from the loop, gives the part of a loop again. Since the nucleic acid comprising complementary nucleotide sequences linked alternately in a single-stranded chain is always used as a template in the complementary chain synthesis starting from the loop, it is evident that the amplification of the nucleic acid target can still be obtained. In this case, the synthesized nucleic acid allows synthesis of a complementary chain by forming a loop after displacement, but there is no 3'-terminal available for subsequent formation of a loop, and thus it cannot function as a new template. Accordingly, the product in this case, unlike nucleic acid initiated to be synthesized by FIP or BIP, is not exponentially amplified. Therefore, an oligonucleotide primer with the structure as described for FIP or BIP is preferred for highly efficient synthesis of nucleic acid based on the present invention.

In summary, a series of these reactions as described herein proceed by adding the following components to single-stranded nucleic acid as a template and then incubating the mixture at such a temperature that supports annealing of the domains in FIP and BIP with its complementary nucleotide sequence in a nucleic acid target, while maintaining the enzyme activity:
• At least 4 kinds of oligonucleotides (FIP, BIP, outer primer F3, and outer primer B3)
• DNA polymerase with strand displacement activity,
• An oligonucleotide serving as a substrate for DNA polymerase.

Polymerase
The present invention relates to methods and kits, wherein a nucleic acid target is amplified at isothermal condition. The isothermal amplification involves complementary strand synthesis catalyzed by a nucleic acid polymerase or polymerase homologue, for example a DNA polymerase or an RNA polymerase. In particular, the nucleic acid polymerase according to the present invention catalyzes complementary strand synthesis which includes strand displacement activity.

The DNA polymerase according to the present invention is selected from the group consisting of Bst DNA polymerase, Bca(exo-) DNA polymerase, Klenow fragment of DNA polymerase I, Vent DNA polymerase, Vent (Exo-) DNA polymerase (exonuclease activity-free Vent DNA polymerase), DeepVent DNA polymerase, DeepVent (Exo-) DNA polymerase (exonuclease activity-free DeepVent DNA polymerase), F29 phage DNA polymerase, MS-2 phage DNA polymerase, Z-Taq DNA polymerase (Takara Shuzo), KOD DNA-polymerase (TOYOBO), Taq MutS and Aac DNA polymerase. In a preferred embodiment, the polymerase is Bst DNA polymerase.

Moreover, mutants and/or functional homologs of the polymerases mentioned herein can be used in the present invention, to the extent that they are able to catalyze sequence-dependent complementary strand synthesis and the strand displacement activity. Such mutants include truncated enzymes having only the structures with the catalytic activity or mutant enzymes whose catalytic activity, stability, or thermal stability has been modified by amino acid mutations, and such.

Of the polymerases mentioned herein, Bst DNA polymerase and Bca(exo-) DNA polymerase are particularly preferred. These enzymes display high catalytic activity, and are thermally stable. The thermal stability and catalytic activity of the polymerase at a desired temperature is important, since the primer annealing to a double-stranded nucleic acid and subsequent complementary strand synthesis are conducted under the
same thermal condition. Since primer annealing often requires a temperature slightly higher than the optimal temperature for normal polymerase activity, a thermostable polymerase is generally preferred. Nevertheless, the reaction can be achieved under a wide variety of conditions by thermostable enzymes.

In one example, Vent (Exo-) DNA polymerase is a highly thermostable enzyme and has strand replacing activity. Furthermore, addition of a single strand-binding protein accelerates the reaction of complementary strand synthesis by DNA polymerase which comprises strand displacement (Paul M. Lizardi et al., Nature Genetics 19, 225-232, July, 1998). This method may be applied to the present invention, and therefore, acceleration of complementary strand synthesis is expected by the addition of single strand-binding protein. When Vent (Exo-) DNA polymerase is used, T4 gene 32 may be used as the single strand-binding protein.

Amplification temperature
Temperature cycling such as in PCR is not necessary for nucleic acid amplification according to the present invention. The amplification is performed at a temperature, which supports stable base pairing of at least a part of an oligonucleotide primer, which serves as the origin of complementary chain synthesis. For example, a desired condition for promoting stable base pairing is to set the amplification temperature lower than melting temperature (Tm) of the region of a oligonucleotide primer, which anneals to the nucleic acid target. In general, melting temperature (Tm) is regarded as the temperature at which 50 % of nucleic acids having mutually complementary nucleotide sequences are base-paired. Incubation of the reaction mixture at melting temperature (Tm) or less is not an essential requirement in the present invention, but is one of the reaction conditions to be considered for attaining high efficiency of synthesis. If nucleic acid to be used as a template is a double-stranded chain, the nucleic acid should, in at least a region to which the oligonucleotide anneals, be made ready for base pairing. For this, heat denaturation is generally conducted, and this may be conducted only once as pre-treatment before the reaction is initiated. This initial heat denaturation is conducted by incubating the nucleic acid target at at least 50 degrees Celsius, such as at least 60, at least 70, at least 75, at least 80, at least 85, at least 90, preferably at least 95 or at least 98 degrees Celsius.
Nucleic acid amplification according to the present invention is performed at isothermal conditions. The optimal temperature for amplification varies and may for example depend on primer characteristics, such as sequence length, melting temperature as described elsewhere herein, and choice of polymerase. In one embodiment of the present invention, the amplification temperature is at least 40 degrees Celsius, such as at least 45, for example at least 50, such as at least 52, such as at least 54, for example at least 56, such as at least 58, such as at least 60 such as at least 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 78 or 80 degrees Celsius. In a preferred embodiment, the amplification temperature is at least 59, at least 60, at least 61, at least 62, or at least 63 degrees Celsius.

The amplification reaction may be terminated by increasing the temperature to at least 70, 75, 80, 85, 90, or at least 95 degrees Celsius. The temperature increase leads to inactivation of the polymerase, and thus terminates the amplification reaction.

Buffer composition

Isothermal amplification according to the present invention is conducted in the presence of a buffer, which maintain a suitable pH for optimal enzyme activity, comprising salts required for annealing or for supporting the catalytic activity of the enzyme, possibly a protective agent for the enzyme, and when necessary a regulator for melting temperature (Tm). As the buffer, e.g. Tris-HCl having a buffering action in a neutral to weakly alkaline range is used. The pH is adjusted depending on the DNA polymerase used. As the salts, KCl, NaCl, (NH₄)₂SO₄ etc. are suitably added to maintain the activity of the enzyme and to modulate the melting temperature (Tm) of the nucleic acids and oligonucleotides. The protective agent for the enzyme may include bovine serum albumin or sugars. Further, dimethyl sulfoxide (DMSO) or formamide is used as a standard regulator of melting temperature (Tm). By use of the regulator for melting temperature (Tm), annealing of the oligonucleotide can be regulated under limited temperature conditions. Further, betaine (N,N,N-trimethylglycine) or a tetraalkyl ammonium salt is also effective for improving the efficiency of strand displacement by virtue of its isostabilization. Betaine is provided in an amount of 0.2 to 3.0 M, preferably 0.5 to 1.5 M to the reaction solution. These different regulators for melting temperature act for lowering melting temperature, and consequently, those conditions giving suitable stringency and reactivity are empirically determined in consideration of the concentration of salts, reaction temperature etc.
Oligonucleotide primers

In one aspect the present invention relates to oligonucleotide primers, which can be used in the methods and kits according to the present invention. An important feature of the present invention is that the oligonucleotide primers of the present invention hybridize to the 5'-side and the 3'-side of the nucleic acid target, respectively. Specifically, the primers hybridize to sequences in the nucleic acid target on opposite sides of the nucleic acid region, or on opposite sides of the deletion site. Consequently, the oligonucleotide primers according to the present invention do not hybridize to any parts of the nucleic acid region, nor does any of the oligonucleotide primers span the deletion site of the nucleic acid target.

The method and kits according to the present invention involve an isothermal amplification step, which employs at least four oligonucleotide primers.

The important nucleic acid intermediate structure of the isothermal amplification according to the present invention can be obtained in various methods. This nucleic acid intermediate structure is characterized by 3'-terminal with a region F1 capable of annealing to a part F1c in the same chain and which upon annealing of the region F1 to F1c is capable of forming a loop comprising a region F2c capable of base pairing. In a preferred embodiment, said nucleic acid structure is provided by employing the following oligonucleotide primers.

Oligonucleotide primer suitable as a first primer or second primer according to the present invention consists of at least two regions X2 and X1c, wherein X1c is attached to the 5'-side of X2. X2 is a region with a nucleic acid sequence complementary to a region X2c in nucleic acid with a specific nucleotide sequence. X1c is a region having substantially the same nucleotide sequence as a region X1c located at the 5'-side of the region X2c in nucleic acid having a specific nucleotide sequence. In the terminology of the present invention, the region X can be either F or B, representing a region in the 5'-side or the 3'-side of the nucleic acid target, respectively (see fig. 3, left panel).

The regions X2 and X1c constituting an oligonucleotide primer of the present invention for the nucleic acid having a specific nucleotide sequence are typically located contiguously. However, if there is a common part in both nucleotide sequences, the two
can be partially overlapping. Because X2-region should function as a primer, it should always be located in the 3'-terminus of the oligonucleotide. Conversely, X1c should be arranged at the 5'-terminal, thereby being able to give the function of a primer as described below to the 3'-terminal of a complementary chain synthesized with the nucleic acid as a template. The complementary chain obtained with this oligonucleotide as the origin of synthesis serves as a template for synthesis of complementary chain in the reverse direction in the next step, and finally the part of the oligonucleotide of the present invention is copied as a template into a complementary chain. The 3'-terminal generated by copying has the nucleotide sequence X1, which anneals to X1c in the same chain to allow loop formation.

The oligonucleotides according to the present invention are able to form complementary base pairing and give an -OH group serving as the origin of synthesis of complementary chain at the 3'-terminal. In one embodiment, the backbone of the oligonucleotide comprise phosphodiester linkages, however, the oligonucleotides are not necessarily limited to such backbones. For example, it may comprise a phosphothioate derivative having S in place of P as a backbone or a peptide nucleic acid based on peptide linkages. Typically, the bases of the oligonucleotides of the present invention may be those capable of complementary base pairing. There are 5 natural bases, namely A, C, T, G and U, but the base can also be an analogue such as bromodeoxyuridine. The oligonucleotide used in the present invention functions preferably not only as the origin of synthesis but also as a template for synthesis of complementary chain.

The oligonucleotide according to the present invention has such a chain length as to be capable of base pairing with a complementary chain and to maintain necessary specificity under the given environment in the various reactions of synthesizing nucleic acid described below. Specifically, it is composed of 5 to 200 base pairs, such as 10 to 80 nucleotides, and more preferably 10 to 50 nucleotides, such as at least 15, such as at least 20, for example at least 25, such as at least 30, such as at least 35, for example at least 40, such as at least 45, or for example at least 50 nucleotides.

Additionally, the chain length of the annealing part of the oligonucleotides of the present invention is at least 5 nucleotides. However, a length of 10 bases or more is desired statistically in order to expect specificity as the nucleotide sequence. Still, the
chemical synthesis of a longer nucleotide sequence is difficult and more errors occur
the longer the chain length is, and thus the chain lengths described above is
exemplified as a desired range. The chain length exemplified here refers to the chain
length of a part annealing to a complementary chain. As described below, the
oligonucleotide according to the present invention can anneal finally to at least 2
regions individually. Accordingly, it should be understood that the chain length
exemplified here is the chain length of each region constituting the oligonucleotide.

The oligonucleotides of the present invention may be labelled with a known labelling
substance, including binding ligands such as digoxin and biotin, enzymes, fluorescent
substances and luminescent substances, and radioisotopes. The techniques of
replacing a base constituting an oligonucleotide by a fluorescent analogue are known
by those of skill within the art. Oligonucleotides according to the present invention can
also be bound to a solid phase. Alternatively, an arbitrary part of the oligonucleotide
may be labelled with a binding ligand such as biotin, and it can be immobilized
indirectly via a binding partner such as immobilized avidin. When the immobilized
oligonucleotide is used as the origin of synthesis, nucleic acid as the synthetic reaction
product is captured by the solid phase, thus facilitating its separation. The separated
product can be detected by a nucleic acid-specific indicator or by hybridization with a
labelled probe.

The oligonucleotides of the present invention is not limited to the 2 regions described
above, and can contain an additional region. While X2 and X1c are arranged at the 3'-
and 5'-terminals respectively, an arbitrary sequence can be inserted in-between. For
example, the inserted sequence can be a restriction enzyme recognition site, a
promoter recognized by RNA polymerase, or DNA encoding a ribozyme. By using it as
a restriction enzyme recognition sequence, the nucleic acid having a complementary
sequence alternately linked in a single-stranded chain as the synthetic product of the
present invention can be cleaved into double-stranded nucleic acids of the same
length. Arrangement of a promoter sequence recognized by RNA polymerase, allows
the synthetic product of the present invention to serve as a template to permit further
transcription into RNA. By further arranging DNA coding for ribozyme, a system where
the transcriptional product is sequences are those functioning after formed into a
double-stranded chain. Accordingly, when the single- stranded nucleic acid according
to the present invention has formed a loop, these sequences do not function. They do
not function until the nucleic acid is elongated and annealed in the absence of a loop to a chain having a complementary nucleotide sequence.

Loop-mediated isothermal amplification according to the present invention employs at least four oligonucleotide primers, designated FIP, BIP, F3 and B3, as defined in Table 1 below.

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotide primers</th>
<th>Aliases</th>
<th>Nucleic acid domains</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>First primer</td>
<td>Forward inner primer, FIP</td>
<td>F1c + F2</td>
<td>SEQ ID NO: 1 and 10</td>
</tr>
<tr>
<td>Second primer</td>
<td>Backwards inner primer, BIP</td>
<td>B1c + B2</td>
<td>SEQ ID NO: 2 and 11</td>
</tr>
<tr>
<td>Third primer</td>
<td>Forward primer, F3</td>
<td>F3</td>
<td>SEQ ID NO: 3 and 12</td>
</tr>
<tr>
<td>Fourth primer</td>
<td>Forward inner primer, B3</td>
<td>B3</td>
<td>SEQ ID NO: 4 and 13</td>
</tr>
<tr>
<td>First loop primer</td>
<td>Loop forward, LF</td>
<td>LF</td>
<td>SEQ ID NO: 5</td>
</tr>
<tr>
<td>Second loop primer</td>
<td>Loop backward, LB</td>
<td>LB</td>
<td>SEQ ID NO: 6 and 14</td>
</tr>
</tbody>
</table>

In addition to the four oligonucleotide primers (FIP, BIP, F3 and B3) mentioned above, the isothermal amplification may employ at least two additional oligonucleotide primers, designated “loop primers”. According to the present invention, a loop primer can be used to improve the amplification efficiency of loop-mediated isothermal amplification. The loop primer is an oligonucleotide providing an origin for complementary strand synthesis between a region derived from FIP in the elongation products of the FIP and the arbitrary region corresponding to FIP (the region comprising the nucleotide sequence complementary to the 5’-end of FIP). Similarly for BIP, a loop primer can be used to provide an origin for complementary strand synthesis between a region derived from the BIP in the elongation products of the above-mentioned BIP and the arbitrary region corresponding to BIP (the region comprising the nucleotide sequence complementary to the 5’-end of RA). Herein, the loop primer corresponding to FIP is referred to as the first loop primer and the loop primer corresponding to BIP is referred to as second loop primer. The loop primer was designed based on the fact that the
amplification products of LAMP often comprise loops to which neither BIP nor FIP can anneal. The efficiency of LAMP is considerably improved by the combined use of at least one loop primer with the LAMP method. When loop primers are used, LAMP is initiated after adding a first loop primer and/or a second loop primer together with BIP, FIP, and the outer primers.

Specifically, the present invention relates to an oligonucleotide primer comprising between 10 and 80 nucleotides for use in performing an isothermal amplification reaction on a nucleic acid target, wherein the amplification product comprises a nucleic acid region, and the subsequent detection of the presence of an amplification product is indicative of the absence of said nucleic acid region in said nucleic acid target, and the absence of amplification product is indicative of the presence of said nucleic acid region in said nucleic acid target. Specifically, the oligonucleotide according to the present invention is selected from the group consisting of SEQ ID NO: 1 to 6 and SEQ ID NO: 10 to 14. In one embodiment, the first primer is SEQ ID NO: 1 or 10, the second primer is SEQ ID NO: 2 or 12, the third primer is SEQ ID NO: 3 or 13, and the fourth primer is SEQ ID NO: 4 or 14. In a preferred embodiment, the first primer is SEQ ID NO: 1. In another preferred embodiment, the second primer is SEQ ID NO: 2. In yet another preferred embodiment of the present invention, third primer is SEQ ID NO: 3. In another preferred embodiment, the fourth primer is SEQ ID NO: 4.

In a specific embodiment, the oligonucleotide primers suitable for the methods and kits according to the present invention are a specific combination of primers. Table 2 shows different specific combinations of primers for use in an isothermal amplification reaction according to the present invention. Each of the combination 1 through 12 listed below represents a specific embodiment of the present invention, and can accordingly be claimed individually.

Table 2

<table>
<thead>
<tr>
<th>Combinations</th>
<th>SEQ ID NOs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Aa2- FIP</td>
<td></td>
</tr>
<tr>
<td>Aa2- BIP</td>
<td></td>
</tr>
<tr>
<td>Aa2- F3</td>
<td></td>
</tr>
<tr>
<td>Aa2- B3</td>
<td></td>
</tr>
<tr>
<td>Aa2- LF</td>
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<tr>
<td>Aa2- LB</td>
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<tr>
<td>Aa1- FIP</td>
<td></td>
</tr>
<tr>
<td>Aa1- BIP</td>
<td></td>
</tr>
<tr>
<td>Aa1- F3</td>
<td></td>
</tr>
<tr>
<td>Aa1- B3</td>
<td></td>
</tr>
<tr>
<td>Aa1- LB</td>
<td></td>
</tr>
</tbody>
</table>

| 1   | X  | X  | X  | X  |
| 2   | X  | X  | X  | X  |
In a preferred embodiment of the present invention, the combination of primers is as defined in combination no. 2. In another preferred embodiment of the present invention, the combination of primers is as defined in combination no. 3. In yet another preferred embodiment of the present invention, the combination of primers are as defined in combination no. 4. In another preferred embodiment of the present invention, the combination of primers is as defined in combination no. 5. In another preferred embodiment of the present invention, the combination of primers is as defined in combination no. 6. In another preferred embodiment of the present invention, the combination of primers is as defined in combination no. 7. In a particularly preferred embodiment of the present invention, the combination of primers is as defined in combination no. 8.

It is understood that the oligonucleotides disclosed herein, also comprise oligonucleotides, which are at least 70% identical, such as at least 75% identical, for example at least 80% identical, such as at least 85% identical, such as at least 90% identical, such as at least 95% identical, for example at least 98% identical, such as at least 99% identical.

Distance between F2 and B2 domains
A central aspect of the present invention is the fact that isothermal amplification under the conditions disclosed herein, will only efficiently amplify a nucleic acid target when the distance between the F2 (or F2c) and Bc2 (or B2) domains in the nucleic acid target is below a certain threshold. Consequently, an isothermal amplification reaction, in which the 5'-side and 3'-side oligonucleotide primers are located far apart, will only
yield an amplification product if a nucleic acid region between the primers of the 5'-side and the 3'-side of the nucleic acid target has been deleted, thereby bringing the primers in close enough proximity to allow efficient amplification.

Therefore, the distance between the F2 (or F2c) and Bc2 (or B2) domains comprised in the oligonucleotide primers for detecting the absence of a nucleic acid region in a nucleic acid target is at least 90 nucleotides, such as at least 95, such as at least 100, for example at least 105, such as at least 110, such as at least 120, such as at least 130, for example at least 140, for example at least 150, such as at least 160, such as at least 170, such as at least 180, such as at least 190, such as at least 200, for example at least 220, such as at least 240, for example at least 260, such as at least 280, such as at least 300, for example at least 350, such as at least 400, such as at least 450, for example at least 500, such as at least 550, such as at least 600, such as at least 650, such as at least 700, such as at least 750, for example at least 800, such as at least 850, such as at least 900, such as at least 950, such as at least 1000, such as at least 1200, such as at least 1400, such as at least 1600, such as at least 1800, such as at least 2000, such as at least 4000, for example at least 6000, such as at least 8000, such as at least 10000, such as at least 20000, such as at least 30000, such as at least 40000, such as at least 50000, such as at least 60000, such as at least 80000, such as at least 100000 nucleotides. However, there is no upper limit for the distance between oligonucleotide primers hybridizing to the 5'-side and the 3'-side of the nucleic acid target. In a preferred embodiment, the distance is between 200 and 1000 nucleotides.

However, the optimal distance between the F2 (or F2c) and Bc2 (or B2) domains of the nucleic acid target according to the present invention depends on the length of the nucleic acid region, the absence of which is detected by the methods and kits of the present invention. In those mutants, wherein said nucleic acid region is deleted, the distance between the between the F2 (or F2c) and Bc2 (or B2) domains is less than 1000 nucleotides, such as less than 900, such as less than 800, such as less than 700, for example less than 600, such as less than 500, such as less than 400, for example less than 350, such as less than 300, such as less than 250, for example less than 200, for example less than 180, such as less than 160, such as less than 150, such as less than 140, for example less than 130, such as less than 120, for example less than 110, such as less than 100, for example less than 90, for example less than 80, such
as less than 70, such as less than 60, such as less than 50, for example less than 40, such as less than 30, such as less than 20, such as less than 10 nucleotides.

In a preferred embodiment, the distance between the F2 (or F2c) and Bc2 (or B2) domains in the nucleic acid target in those mutants, wherein said nucleic acid region is absent, is between 90 and 150 nucleotides, such as between 90 and 100 nucleotides, between 100 and 110 nucleotides, between 110 and 120 nucleotides, between 120 and 130 nucleotides, between 130 and 140 or between 140 and 150 nucleotides.

The present invention also relates to methods and kits for detecting the presence of a nucleic acid region within a nucleic acid target. The presence of a nucleic acid region within the nucleic acid target is detected according to the present invention by the lack of amplification product after providing conditions for isothermal amplification of said nucleic acid target. Isothermal amplification as disclosed herein is non-functional when the distance between F2 (or F2c) and Bc2 (or B2) domains in the nucleic acid target exceeds a certain threshold. Therefore, the presence of a nucleic acid region in the nucleic acid target between the 5'-side and 3'-side oligonucleotide primers impedes isothermal amplification as disclosed herein. Presence of a nucleic acid region in a specific nucleic acid target may for example be a result of an insertion mutation.

For detecting the presence of a nucleic acid region in a nucleic acid target according to the present invention, the distance between the F2 (or F2c) and Bc2 (or B2) domains in the nucleic acid target is less than 500, such as less than 400, for example less than 300, such as less than 200, such as less than 180, such as less than 160, such as less than 150, 140, 130, 120, 110, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55 50, 45, 40, 35, 30, 25, 20, 15, 10 or 5 nucleotides. In a preferred embodiment, the distance is between 90 and 150 nucleotides, such as at least 100 nucleotides.

In a preferred embodiment, the distance between the F2 (or F2c) and Bc2 (or B2) domains in the nucleic acid target, in which said nucleic acid region is not present is between 90 and 150 nucleotides, such as between 90 and 100 nucleotides, between 100 and 110 nucleotides, between 110 and 120 nucleotides, between 120 and 130 nucleotides, between 130 and 140 or between 140 and 150 nucleotides.

Detection
The nucleic acid synthesized in the present invention is a single-stranded chain but is composed of partial complementary sequences. Thus, the majority of the nucleic acid
sequences are base-paired. Consequently, the amplified nucleic acid target of the present invention, can be detected by means of a fluorescent agent, such as a double-stranded chain-specific intercalating agent, such as ethidium bromide, SYBR Green I, Pico Green, Loopamp Fluorescent Detection Reagent (Eiken Chemical Co.), or cationic polymers (Yasuyoshi Mori, Tsuyoshi Hirano, Tsugunori Notomi. BMC Biotechnology, Vol.6, No.3, 2006), wherein the increased fluorescence correlates with increased levels of double stranded nucleic acid product. In one embodiment, it is possible to monitor the nucleic acid amplification in real time, by continuously measuring the fluorescence in a closed system. Different apparatuses support such real time monitoring of fluorescence. In one example, a Lightcycler can be used. Similar to use of a double-stranded chain-specific intercalating agent, transfer of fluorescent energy can also be utilized for detection of nucleic acid products. Any intercalating agents and dyes suitable for detection of nucleic acid amplification product, may be applied before and/or after the amplification step of the present invention.

As the isothermal amplification reaction according to the present invention progresses, the reaction by-product pyrophosphate ions bind to magnesium ions and form a white precipitate of magnesium pyrophosphate, the amount of which reflects the amount of nucleic acid produced by amplification reaction. Therefore, in a specific embodiment, the amplification of nucleic acid target according to the present invention is detected by measuring the amount of Mg-Pyrophosphate precipitate as described by Mori et al (Mori et al., 2004. J. Biochem. Biophys. Methods 59:145-157). The amount of Mg-Pyrophosphate precipitate may be detected by optical methods or simply evaluated quantitatively or qualitatively by the naked eye.

According to a preferred nucleic acid amplification method of the present invention, a large number of loops capable of base pairing are produced in a single strand of nucleic acid. This means that a large number of probes can be hybridized with one molecule of nucleic acid to permit highly sensitive detection. It is thus possible to realize not only the improvement of sensitivity but also a method of detecting nucleic acid based on a special reaction principle such as aggregation. A probe may be immobilized onto fine particles such as polystyrene latex and added to the amplification product of the present invention, whereupon the aggregation of latex particles is observed as the hybridization of the product with the probe proceeds. Highly sensitive and quantitative observation is feasible by optically measuring the strength of the
aggregation. Because the aggregation can also be observed with the naked eyes, a reaction system not using an optical measuring device can also be constituted.

The amplification product of the present invention may also be detected by chromatographic methods, utilizing a visually detectable label permitting many labels to be bound to each nucleic acid molecule. In one embodiment, an immunoassay (immunochromatography) is used. In this method an analyte is sandwiched between an antibody immobilized on a chromatographic medium and a labelled antibody, while unbound labelled component is washed away. In this method, a labelled probe toward a part of a loop is prepared and immobilized onto a chromatographic medium to prepare a capturing probe for trapping thus permitting analysis chromatography. A sequence complementary to a part of the loop can be utilized as the capturing probe. Since the reaction product of the present invention has a large number of loops, the product binds to a large number of labelled probes to give a visually recognizable signal.

The presence in amplification products of the present invention of loop regions capable of base pairing enables a wide variety of other detection systems. In one embodiment, a detection system utilizing surface plasmon resonance using an immobilized probe for this loop portion is applied. Moreover, if a probe for the loop portion is labelled with a double-stranded chain-specific intercalating agent, even more sensitive fluorescent analysis is feasible.

In another embodiment, the ability of the nucleic acid amplification product of the present invention to form a loop capable of base pairing at both the 3'- and 5'-sides is utilized. For example, one loop is designed to have a common nucleotide sequence between a normal type and an abnormal type, while the other loop is designed to generate a difference therebetween. It is possible to constitute a characteristic analytic system in which the presence of a gene is confirmed by the probe for the common portion while the presence of an abnormality is confirmed in the other region. Because the nucleic acid amplification according to the present invention is performed at isothermal conditions, an important advantage is that real-time analysis can be conducted by a general fluorescent photometer.
In a specific embodiment, the large number of loops comprised in the amplification product of the present invention can itself be used as probes. In a DNA chip, probes should be accumulated at high density in a limited area. By use of the amplification product of the present invention, a large number of probes capable of annealing can be immobilized at high density. That is, the reaction product according to the present invention may be immobilized as probes on a DNA chip. After amplification, the reaction product may be immobilized by any techniques known in the art, or the immobilized oligonucleotide is utilized as the oligonucleotide in the amplification reaction of the present invention, resulting in generating the immobilized reaction product. By use of such immobilized probes, large numbers of sample DNAs can be hybridized in a limited area.

Nucleic acid target
The present invention relates to methods and kits for detecting the presence or absence of a nucleic acid region within a nucleic acid target. The nucleic acid target according to the present invention is targeted by at least four oligonucleotide primers as described elsewhere herein. The nucleic acid target is a nucleic acid sequence, which may comprise an insertion mutation or a deletion mutation.

The methods and kits of the present invention are suitable for detecting the presence or absence of a nucleic acid region in any nucleic acid target. In one embodiment, the nucleic acid target is the leukotoxin gene. In a specific embodiment, the nucleic acid target is the Aggregatibacter actinomycetemcomitans leukotoxin gene or a fragment thereof. The Aggregatibacter actinomycetemcomitans leukotoxin is expressed from an operon comprising four genes designated ItxC, ItxA, ItxB, and ItxD. In a preferred embodiment, the nucleic acid target is promoter region of an Aggregatibacter actinomycetemcomitans leukotoxin gene or a fragment thereof.

A sequence of the promoter region of an Aggregatibacter actinomycetemcomitans leukotoxin gene is disclosed in SEQ ID NO: 7 (a non-JP2 strain) and SEQ ID NO: 8 (a JP2 strain).

In another embodiment of the present invention, the nucleic acid target is the CCR-5 chemokine receptor gene (Nature. 1996 Aug 22:382(6593):722-5, and 1996 Aug
Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene.

In yet another embodiment of the present invention, the nucleic acid target comprise markers for the genetic characterization of a non- spoilage variant isolated from beer-spoilage Lactobacillus brevis ABBC45 (J Appl Microbiol. 2004;96(5):946-53).

The amount of nucleic acid required for isothermal amplification of the target nucleic acid according to the method of the present invention is in the range of femtograms to micrograms. It is appreciated by the person skilled in the art that in practical terms no upper limit for the amount of nucleic acid to be analysed exists. The problem that the skilled person encounters is that the amount of sample to be analysed is limited. Therefore, it is beneficial that the method of the present invention can be performed on a small amount of sample and thus a limited amount of nucleic acid in said sample.

The amount of the nucleic acid to be analysed is thus at least 1 fg, such as at least 2 fg, for example at least 5 fg, such as at least 10 fg, for example at least 25 fg, such as at least 50 fg, for example at least 75 fg, such as at least 100 fg, for example at least 125 fg, such as at least 150 fg, for example at least 200 fg, such as at least 225 fg, for example at least 250 fg, such as at least 275 fg, for example at least 300 fg, 400 fg, for example at least 500 fg, such as at least 600 fg, for example at least 700 fg, such as at least 800 fg, for example at least 900 fg, such as at least 1 pg, such as at least 10 pg, for example at least 25 pg, such as at least 50 pg, for example at least 75 pg, such as at least 100 pg, for example at least 125 pg, such as at least 150 pg, for example at least 200 pg, such as at least 225 pg, for example at least 250 pg, such as at least 275 pg, for example at least 300 pg, 400 pg, for example at least 500 pg, such as at least 600 pg, for example at least 700 pg, such as at least 800 pg, for example at least 900 pg or such as at least 1 ng, such as at least 10 ng, for example at least 25 ng, such as at least 50 ng, for example at least 75 ng, such as at least 100 ng, for example at least 125 ng, such as at least 150 ng, for example at least 200 ng, such as at least 225 ng, for example at least 250 ng, such as at least 275 ng, for example at least 300 ng, 400 ng, for example at least 500 ng, such as at least 600 ng, for example at least 700 ng, such as at least 800 ng, for example at least 900 ng or such as at least 1 μg.

In one preferred embodiment the amount of nucleic acid as the starting material for the method of the present invention is in the range of 25 fg to 500 pg, such as at least 1 pg.
The amount of the target nucleic acid used for isothermal amplification according to the method of the present invention may also be measured in copy numbers of the target nucleic acid locus. Still, it is appreciated by the person skilled in the art that in practical terms no upper limit for the amount of nucleic acid to be analysed exists. The amount of the nucleic acid to be analysed is at least 1 copy, such as at least 2 copies, such as at least 3 copies, for example at least 4 copies, such as at least 5 copies, for example at least 6 copies, such as at least 7 copies, such as at least 8 copies, such as at least 9 copies, for example at least 10 copies, such as at least 15 copies, for example at least 20 copies, such as at least 30 copies, such as at least 40 copies, such as at least 50 copies, for example at least 60 copies, such as at least 70 copies, for example at least 80 copies, such as at least 90 copies, such as at least 100 copies, such as at least 120 copies, for example at least 140 copies, such as at least 160 copies, for example at least 180 copies, such as at least 200 copies, such as at least 250 copies, for example at least 300 copies, such as at least 350 copies, for example at least 400 copies, such as at least 450 copies, such as at least 500 copies, such as at least 550 copies, for example at least 600 copies, such as at least 650 copies, for example at least 700 copies, such as at least 750 copies, such as at least 800 copies, such as at least 850 copies, for example at least 900 copies, such as at least 950 copies, for example at least 1000 copies, such as at least 1100 copies, such as at least 1200 copies, such as at least 1300 copies, for example at least 1400 copies, such as at least 1500 copies, for example at least 1600 copies, such as at least 1700 copies, such as at least 1800 copies, such as at least 1900 copies, for example at least 2000 copies, such as at least 2500 copies, for example at least 3000 copies, such as at least 3500 copies, such as at least 4000 copies, such as at least 4500 copies, for example at least 5000 copies, such as at least 5500 copies, for example at least 6000 copies, such as at least 7000 copies, such as at least 8000 copies, such as at least 9000 copies, for example at least 10000 copies, such as at least 12000 copies, for example at least 14000 copies, such as at least 16000 copies, such as at least 18000 copies, such as at least 20000 copies, for example at least 30000 copies, such as at least 40000 copies, for example at least 50000 copies or such as at least 60000 copies.

In one preferred embodiment the copy number of the nucleic acid target as the starting material for the method of the present invention is at least 250 copies. In another
preferred embodiment the copy number is at least 500 copies. In yet another preferred embodiment the copy number is at least 1000 copies.

Disorders

The present invention relates to methods, oligonucleotides and kits for detecting the presence or absence of a nucleic acid region in a specific nucleic acid target. In some cases, the presence of a nucleic acid region in a nucleic acid target is a result of an insertion mutation. Conversely, the absence of a nucleic acid region in a nucleic acid target may be a result of a deletion mutation within the nucleic acid target. Insertion mutations and/or deletion mutations are known to trigger a wide range of disorders, or increase the risk of developing such disorders.

Thus, the methods and kits of the present invention can be used for the diagnosis of a disorder. In addition, the methods and kits according to the present invention can be used for predicting the risk of developing a disorder. In particular, the present invention relates in an in vitro method for the diagnosis of a disorder, wherein the presence or absence of a nucleic acid region within a nucleic acid target is indicative of said disorder or indicative of elevated risk of developing said disorder. In one embodiment, the presence or absence of said nucleic acid region is indicative of an elevated risk of developing said disorder of at least 5%. In another embodiment, the presence or absence of said nucleic acid region is indicative of an elevated risk of developing said disorder of at least 10%, such as at least 20%, such as at least 30%, such as at least 40%, such as at least 50%, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 100%, such as at least 120%, such as at least 140%, such as at least 160%, such as at least 180%, such as at least 200%, such as at least 250%, such as at least 300%, such as at least 350%, such as at least 400%, such as at least 450%, such as at least 500%, such as at least 550%, such as at least 600%, such as at least 650%, such as at least 700%, such as at least 750%, such as at least 800%, such as at least 850%, such as at least 900%, such as at least 950%, such as at least 1000%, such as at least 1100%, such as at least 1200%, such as at least 1300%, such as at least 1400%, such as at least 1500%, such as at least 1600%, such as at least 1700%, such as at least 1800%, such as at least 1900%, such as at least 2000%, such as at least 3000%, such as at least 4000%, such as at least 5000%, such as at least 6000%, such as at least 7000%, such as at least 8000%, such as at least 9000%, such as at least 10000%. 

Specifically, the methods and kits of the present invention can be used for the diagnosis of a disorder or prediction of the risk of developing a disorder, wherein said disorder is selected from the group consisting of cancer, oral cancer, oral disorders, periodontitis, adult periodontitis and early onset periodontitis.

The methods and kits of the present invention can be used for detecting microorganisms, including specific clones or strains of microorganisms, as described elsewhere herein. Specifically, such microorganisms may be involved in inducing disorders in animals and human beings.

In a specifically preferred embodiment, the methods and kits of the present invention can be used for detecting a toxic strain of *Aggregatibacter actinomycetemcomitans*, such as a JP2 clone. *Aggregatibacter actinomycetemcomitans* is a gram negative bacterium that is associated with a various infectious disease processes such as endocarditis, subcutaneous abscesses, brain abscesses, actinomycotic processes, and several forms of periodontal disorders (Block et al., 1973, A. J. Med. Sci. 276:387-392; Page et al., 1966, N. Engl. J. Med. 275:181-186; Zambon, 1985, J. Clin. Periodontal, 12:1-20; Zambon et al., 1983, Infect. Immun. 41:19-27). An example of a periodontal disorder is early onset periodontitis, which is an oral disease afflicting adolescents and pre-teens and is caused by *Aggregatibacter actinomycetemcomitans*. Symptoms of early onset periodontitis include inflammation of the gingiva and destruction of the tissues supporting the teeth. *Aggregatibacter actinomycetemcomitans* produces several potential virulence factors including a proteinaceous leukotoxin (Kraig et al., 1990, Infect. Immun. 58:920-929; Lally et al., 1991, Microb. Pathog. 111-121) which is a member of the RTX (repeats-in-toxin) family of bacterial cytolsins (Welch, 1991, Mol. Microbiol.5:521-528). The *Aggregatibacter actinomycetemcomitans* leukotoxin exhibits a unique cytolytic specificity destroying human polymorphonuclear leukocytes and macrophages, but without lysing other types of cells, such as epithelial and endothelial cells, fibroblasts, erythrocytes and platelets (Baehni et al., 1981, Arch. Oral Biol. 27:671-676; Taichman et al., 1980, Infect. Immun. 28:258-278; Taichman et al., 1984, J. Period. Res. 19:133-145; Taichman et al., 1987, Oral Microbiol. Immun. 2:97-104).
Thus in a preferred embodiment the methods and kits of the present invention can be used for the diagnosis of or prediction of the risk of developing oral disorders. In a particularly preferred embodiment, said disorder is periodontitis. In another particularly preferred embodiment, said disorder is early onset periodontitis.

Samples
According to the present invention, the presence or absence of a nucleic acid region within a nucleic acid target can be detected in vitro by providing any biological sample comprising nucleic acid. Thus, detection of the presence or absence of a nucleic acid region may be performed on nucleic acid comprised in plaque, dental plaque, bio film, cells, human cells, bacteria, tissue, saliva, tumor tissue, yeast, nucleic acids, nucleic acid vectors, or body fluids, such as sputum, urine, blood and sweat. The biological sample according to the present invention may be selected from the group consisting of plaque, dental plaque, bio film, cells, human cells, bacteria, tissue, saliva, tumor tissue, yeast, nucleic acids, nucleic acid vectors, body fluids, sputum, urine, blood and sweat. In a preferred embodiment the sample is selected from the group consisting of plaque, dental plaque and bio film. In another preferred embodiment the sample is dental plaque.

The sample may be obtained by any method known by those of skill within the art. In one embodiment the sample is obtained from the gingival pocket.

A specific aspect of the present invention relates to detection of a toxic strain of *Aggregatibacter actinomycetemcomitans*. Therefore, the samples according to the present invention include any biological sample comprising *Aggregatibacter actinomycetemcomitans*. In a specific embodiment, the biological sample comprises a toxic strain, such as a JP2 strain of *Aggregatibacter actinomycetemcomitans*.

The nucleic acid to be analysed according to the present invention may be extracted from the samples by a variety of techniques such as that described by Haubek et al., 1997, J Clin Microbiol 35: 3037-3042. However, the sample may be used directly.

Any nucleic acid, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it contains, or is suspected of containing, the specific nucleic acid sequence containing the target site.
Therefore, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded may be used as target material. Where RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized as known to a person skilled in the art. A DNA-RNA hybrid which contains one strand of each may also be utilized. A mixture of nucleic acids may also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers may be so utilized.

The specific nucleic acid sequence which is to be amplified may be a part of a larger molecule or is present initially as a discrete molecule. The nucleic acid sequence to be amplified need not to be present in a pure form, it may for example be a fraction of a complex mixture of other DNA molecules, and/or RNA.

The sample may originate from any sample, including a human being. For example the sample may be from mouse, hamster, rat, rabbit, cow, dog, pig, cat, sheep, goat, monkey, ape or humans. In a preferred embodiment the sample is from a human.

The methods and kits of the present invention can be used for detecting microorganisms, including specific clones or strains of microorganisms. Such microorganisms may be involved in inducing disorders in animals and human beings. Furthermore, the microorganisms may be involved in contamination of water tanks, food, air, cultures and environmental samples.

Method of treatment

In a specific aspect, the present invention relates to a method for treatment of periodontitis in a human being comprising a. detecting the presence of a toxic strain of Aggregatibacter actinomycetemcomitans by i. providing a biological sample comprising a nucleic acid, ii. performing an isothermal amplification reaction on said nucleic acid specimen, wherein the amplification product comprises a nucleic acid region, and iii. detecting said amplification product, wherein the presence of an amplification product is indicative of the presence of said toxic strain of Aggregatibacter actinomycetemcomitans in said biological sample, b. treating the human being with antibiotics.
Treatment with antibiotics
When a toxic strain of *Aggregatibacter actinomycetemcomitans* (such as a JP2 clone) has been detected in a patient by the method of the present invention, the patient is preferably treated with antibiotics, such as amoxicillin combined with metronidazole or other antimicrobials to which said bacteria are susceptible.

Kit
According to the present invention, a kit can be provided as a set of pre-combined components of the isothermal amplification reaction of the present invention. Thus, in one aspect, the present invention relates to a kit for detection of presence or absence of a nucleic acid region within a nucleic acid target. Said kit comprises: a DNA polymerase catalyzing complementary strand synthesis which includes strand displacement; a second primer, wherein the 3'-end of the second primer anneals to the 3'-side region of one of said target nucleotide sequence strands, and the 5'-side of the second primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin; a first primer, wherein the 3'-end of the first primer anneals to the 3'-side region of the other said target nucleotide sequence strand, and the 5'-side of the first primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin; nucleotide substrates; a third primer, wherein the third primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the first primer on the template functions as the origin; and a fourth primer, wherein the fourth primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the second primer on the template functions as the origin.; and amplification buffer

Further, the kit may also contain loop primers to improve the reaction efficiency as described elsewhere herein. Moreover, standard samples as positive or negative controls, instruction for use and such, may be attached to the kit. Also, the kit may contain an indicator component for detecting amplification products. Such indicators are described elsewhere herein, and include, for example, fluorescent dyes such as
EtBr, CYBR green, probes generating signals when recognizing the nucleotide sequence of amplification products; and so on.

**Examples**

**Example 1**
A Novel Loop-Mediated Isothermal Amplification (LAMP) Method for Detection of the JP2 Clone of *Aggregatibacter actinomycetemcomitans* in Subgingival Plaque

A JP2 clone of *Aggregatibacter actinomycetemcomitans* constitutes a particular pathogenic subpopulation of the species that causes aggressive periodontitis in juveniles of West and North-West African descent. A characteristic of a JP2 clone is a 530 bp deletion in the promoter of the leukotoxin gene operon. A novel LAMP method as disclosed herein was developed for detection of a JP2 clone of *Aggregatibacter actinomycetemcomitans* in subgingival plaque samples. This method differentiates a JP2 clone from other strains of *Aggregatibacter actinomycetemcomitans* based on the characteristic deletion. Reduced distance between primer regions increases the amplification efficiency and results in an amplification product only from bacteria of the JP2 clone. The method is highly specific and the detection limit is 10 genome copies. It does not require special equipment and may be relevant to clinical settings without laboratory access, e.g., in Africa, where the clone is endemically occurring.

**Bacterial strains:**
A total of 27 *A. actinomycetemcomitans* strains were used in this study, including 11 JP2 strains and 16 non-JP2 strains for which presence versus absence of the Δ530 deletion was determined by PCR with primers ltx3 and ltx4 as described (17). The non-JP2 strains included 4, 6, 2, 2, and 2 strains of the serotypes a, b, c, d, and e, respectively, and were selected to represent the different evolutionary subpopulations of the species (18). In addition, we tested reference strains of three closely related species including *Aggregatibacter (Haemophilus) aphrophilus* HK1147, *Aggregatibacter (Haemophilus) segnis* HK307, and *Mannheimia haemolytica* CCUG12392. Bacteria were grown on chocolate agar plates (Statens Serum Institut, Copenhagen, Denmark) in air supplemented with 5% CO2 at 37 °C.

**Subgingival samples:**
Subgingival plaque samples on paper points were collected from adolescents in Morocco and send to Denmark in 1 ml 0.9 % NaCl as described (6). After vortexing the bacteria were collected by centrifugation, resuspended in 100 µl water and boiled for 5 min as described previously (17). The samples were stored at -20 °C.

Preparation of bacterial DNA:
To determine the specificity of the method bacteria were propagated and DNA extracted by proteinase K treatment followed by phenol-chloroform extraction and precipitation as described (5). For the sensitivity test DNA was purified using DNeasy Tissue Kit as recommended by the supplier (Qiagen, Hilden, Germany) and its quality assayed by agarose gel electrophoresis. The absorbencies A260 and A280 were used to spectrophotometrically measure the quantity of the genomic DNA. The number of genome copies was calculated on the basis of a genome size of 2.1 Mb.

LAMP reaction:
The LAMP reaction was performed in a 25 µl reaction volume containing 1.6 µM each of primers FIP and BIP, 0.2 µM each of primers F3 and B3, 0.4 µM each of primers LF and LB, 8 U of the Bst DNA polymerase large fragment (New England Biolabs, Beverly, MA), 1.4 mM each dNTP (Roche Diagnostics A/S, Hvidovre, Denmark), 0.8 M betaine (Sigma-Aldrich Denmark, Vallensbaek Strand, Denmark), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 8 mM MgSO4, 0.1% Tween-20, and template DNA (in a volume up to 5 µl). The primers were purchased from DNA Technology (Aarhus, Denmark) in HPLC-purified form and dissolved in TE buffer. The mixture was incubated at 63 °C for 60 min, and then heated at 80 °C for 2 min to terminate the reaction.

Presence of a precipitate was scored visually. For experimental confirmation of the visually recorded results, the amplification products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. Prior to digestion with the restriction enzyme BsmI at 65 °C as recommended by the manufacturer (Roche Diagnostics A/S) the amplification products were purified using DNeasy Tissue Kit. To avoid contamination all reactions were set up in a sterile bench in a room separate from the one used for electrophoretic analysis using pipettes dedicated to this and filter tips.

DNA sequencing:
To confirm their identity, the amplification products were purified using DNeasy Tissue Kit and sequenced using the Thermo Sequenase dye terminator cycle sequencing kit (GE Healthcare, Buckinghamshire, UK) and an Applied Biosystems PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA).

Results and discussion
Among bacteria of the species *A. actinomycetemcomitans* the Δ530 deletion is unique to members of the JP2 clone. However, the low percentage of G + C in the sequence around Δ530 in the ltx promoter region makes it impossible to design appropriate LAMP primers in this region including a primer spanning the site of deletion. As an alternative, we developed a new approach to differentiate between the JP2 clone and other genotypes of *A. actinomycetemcomitans* based on the difference in distance between primer regions ("spacer regions"). The LAMP primers shown in Fig. 1 were designed from the genome sequence of *A. actinomycetemcomitans* strain HK1651 belonging to the JP2 clone (available at http://www.genome.ou.edu/act.html) using the program PrimerExplorer V3 (available at https://primerexplorer.jp/lamp3.0.0/index.html). According to this software the recommended distance between the 5' ends of F2 and B2 is 120-180 bp for optimal amplification and according to Notomi and coworkers DNA of more than 500 bp amplifies very poorly (13). Using the primers shown in Fig. 1 this distance is 167 bp in members of the JP2 clone and 697 bp in non-JP2 clones of *A. actinomycetemcomitans*. In agreement with our hypothesis an amplification product was obtained when whole-cell DNA from *A. actinomycetemcomitans* strain HK921 (JP2) was the template whereas DNA from non-JP2 strain HK975 (Y4) did not result in a product as revealed both by visual inspection and by agarose gel electrophoresis. Throughout this study evaluation of the LAMP reactions showed complete agreement between a white precipitate recorded by visual inspection and an amplification product detectable by agarose gel electrophoresis. The optimal temperature for the LAMP reaction was experimentally determined to be 63 °C.

Specificity of the LAMP reaction. To evaluate the specificity of the LAMP reaction, we tested 27 *A. actinomycetemcomitans* strains, including 11 JP2 strains and 16 non-JP2 strains, and members of the three closely related species *Aggregatibacter (Haemophilus) aphrophilus*, *Aggregatibacter (Haemophilus) segnis*, and *Mannheimia haemolytica*, the latter of which produces an RTX leukotoxin homologous to that of *A.*
**actinomycetemcomitans.** Using approximately 1 ng whole-cell DNA in the LAMP reaction, amplification products were observed from all the JP2 strains, whereas none of the non-JP2 strains of *A. actinomycetemcomitans* and none of the representatives from the three other species resulted in any amplification product. Addition of excess DNA (25 ng) from non-JP2 strain HK975 (Y4) did not result in a product (Fig. 2). To ensure that the amplification products had sequences corresponding to the selected target we took advantage of the fact that strain JP2 has a BsmI recognition site between sequences F1c and B1c (Fig. 1). In full agreement, the products after digestion with BsmI were the predicted sizes of 106 and 116 bp (Fig. 2). Their identity was further confirmed by sequencing using primers F2 and B2. Although the amplicons and thereby also the sequences were a mixture of different products, the itx promoter region of strain JP2 containing Δ530 was unequivocally identified within the obtained sequences. Thus, the LAMP reaction is highly specific for the designated target and, thus, for strains of the JP2 clone.

Sensitivity of the LAMP reaction. Serial 10-fold dilutions of genomic DNA from *A. actinomycetemcomitans* strain HK921 (JP2) were tested in the LAMP reaction, and the results were compared with those obtained by conventional PCR performed as described (17). The detection limits for the LAMP assay and the PCR were 10 and 100 genome copies, respectively, in 5 μl sample. The detection limit for the LAMP when incubating for 30 min instead of 60 min was 100 genome copies, indicating that the reaction was very rapid.

LAMP applied to analysis of clinical specimens. A total of 72 subgingival plaque samples were tested for presence of the JP2 clone of *A. actinomycetemcomitans* by both the LAMP reaction and by the PCR (Table 3). In the PCR targeting the itx promoter region and performed as described (17) 23 μl of subgingival plaque sample were used in a 25 μl reaction. In the LAMP test 5 μl were used as described above. The two tests agreed on presence of the JP2 clone in 42 samples and on absence of the clone in 24 samples. In the remaining six samples (8.3 %) the LAMP test demonstrated the presence of the JP2 clone while the PCR gave a negative result. Digestion of the LAMP products from the latter six samples with BsmI resulted in the two characteristic fragments and thus confirmed that they represented the selected target. The higher sensitivity of the LAMP test conceivably contributed to the higher detection rate. In addition, previous studies demonstrated that the LAMP reaction is
more tolerant to biological substances perturbing the reaction than the PCR methodology (9).

In contrast to the LAMP test, the PCR test concurrently is able to detect non-JP2 members of *A. actinomycetemcomitans*. Among the 42 samples positive for the JP2 clone in both PCR and LAMP tests six were also positive for non-JP2 types of *A. actinomycetemcomitans* in the PCR. Non-JP2 types were also found by PCR in one of the six samples in which only the LAMP detected the JP2 clone, and in 12 of the 24 samples that were negative for the JP2 clone in both PCR and LAMP. Thus, the presence of non-JP2 *A. actinomycetemcomitans* seems not to influence the detection of the JP2 clone by LAMP.

In conclusion, this specific isothermal amplification method can be used for detection of a JP2 clone of *A. actinomycetemcomitans*. Based on differences in spacer regions resulting from a characteristic deletion in the JP2 genome the LAMP method detects the JP2 clone in plaque samples with a specificity equivalent to and a sensitivity exceeding that of previously described PCR methods. Specific detection of infection with a JP2 clone of *A. actinomycetemcomitans* has significant implications for prevention and treatment of aggressive periodontitis in susceptible human populations.

Because the LAMP reaction is easy to set up and does not require special equipment it can be a useful tool in clinical settings with limited access to laboratory technology.

Table 3. Detection of *A. actinomycetemcomitans* with and without the Δ530 deletion by PCR with primers ltx3 and ltx4 (17) compared to the LAMP described here on 72 plaque samples.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Positive</td>
<td>5</td>
<td>1</td>
<td>36</td>
<td>6</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>13</td>
<td>36</td>
<td>6</td>
<td>72</td>
</tr>
</tbody>
</table>

*a A. actinomycetemcomitans*
Negative: no product
Positive: amplification of a product
The LAMP reaction is unable to detect non-JP2 types of *A. actinomycetemcomitans*
and a positive reaction is due to simultaneous presence of both the JP2 clone (missed
by the PCR) and non-JP2 types of the bacterium.
Example 2
The temperatures 60 °C, 63 °C, and 65 °C was tested, and it was found that the reaction performed best at 63 °C.

This example illustrates a time course experiment, where the efficiency of detecting a JP2 clone is tested at different incubation intervals. Detection appears to reach a plateau after incubation for at least 50 minutes, where only 10 copies of JP2 genomic clones can be detected. see table 4.

Table 4. Detection sensitivities of the LAMP assay for JP2 clone.

<table>
<thead>
<tr>
<th>reaction (min)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>10²</th>
<th>10³</th>
<th>10⁴</th>
<th>10⁵</th>
<th>10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>20</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*a, amplification occurred; -, amplification did not occur.*
References


Sequences

SEQ ID NO: 1
First oligonucleotide primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa2-FIP)
ACGATTGCGCTAATCTATTACGGTTACTCTATGAATACTGGAAACTTGT

SEQ ID NO: 2
Second oligonucleotide primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa2-BIP)
TCTCGCGGAAAAACTATTGGGATTATGAATAAGATAACCAAAACCAC

SEQ ID NO: 3
Third oligonucleotide primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa2-F3)
CCGTTTTATTCAGTTCCAAG

SEQ ID NO: 4
Fourth oligonucleotide primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa2-B3)
TCTCCATATTAATCTCTTGTT

SEQ ID NO: 5
First loop primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa2-LF)
ACTAACCCTTTGTACAAAATCTGA

SEQ ID NO: 6
Second loop primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa2-LB)
CCAAAGTACTTTTAATGATGGCA

SEQ ID NO: 7
Nucleic acid sequence of the promoter region of the leukotoxin gene operon in *Aggregatibacter actinomycetemcomitans* strain 652 (a non-JP2 strain)
ttttgagtattaataatcacaaataacggaattgctatagataaagagtattattcataaatcataaatatttttagataaatattataataaatcattatatttctatgtgactattaaagaatcgggtagtaaattagttctctcaaaataataaacaacatagtgaatatcttctcgcgtaaagttatctttaagattaaacccttgaagaagggaaagcagagatgtcgcggctacgaagatagttctcttttatttcgatttattccgccaatcattgataaatgttcgtcgttcgcagatgataatatttattgatcgcgcttcatccctctccctctctcaagtgtgctttatcgcagcagatataatgtaatatatattatcatcttaattatcggcttattctctattatcatcttttttttcttttatttcatctttattatcatctattttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
caactacgaatcactcatatttaatatggtattattgcaaaaaataataaagcttgtaatatattcctgttaataataaggttaaataagtttatattttatcatgtgttaacaataataatataatcatagtctatatttgattcgtataatgagttggcattttcagtctcatgcgatcgtgtaagttatttsgta

SEQ ID NO: 10
First oligonucleotide primer (FIP) for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa1-FIP)
ACAAGTTTCCAGTATTTGAGTAGTCTCCCAAGTAAATGAAGTT

SEQ ID NO: 11
Second oligonucleotide primer (BIP) for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (Aa1-BIP)
TTGGCATTTCGCGAAAAACATAAACCAAACTCAATCCAG

SEQ ID NO: 12
Third oligonucleotide primer (F3) for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (Aa1-F3)
GCTCCATTTTATCTCCGTT

SEQ ID NO: 13
Fourth oligonucleotide primer (B3) for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (Aa1-B3)
CTCCATATTAATCTCCTTGT

SEQ ID NO: 14
Second loop primer for LAMP of *Aggregatibacter actinomycetemcomitans* leukotoxin promoter (aa1-LB)
ACCAAAGTACTTTTATGATGGCA
Claims

1. A method for detecting the presence or absence of a nucleic acid region within a nucleic acid target comprising
   a. providing a biological sample comprising a nucleic acid,
   b. performing an isothermal amplification reaction on said nucleic acid, employing oligonucleotide primers flanking said nucleic acid region, such that the amplification product comprises said nucleic acid region, and
   c. detecting said amplification product,

wherein the presence of an amplification product is indicative of the absence of said nucleic acid region in said nucleic acid target, and the absence of amplification product is indicative of the presence of said nucleic acid region in said nucleic acid target or absence of said nucleic acid target in said biological sample.

2. The method according to claim 1, further comprising
   a. performing an additional isothermal amplification reaction on said nucleic acid, wherein the amplification product does not comprise said nucleic acid region
   b. detecting said amplification product of said additional isothermal amplification reaction,

wherein the presence of an amplification product of said additional isothermal amplification reaction is indicative of the presence of said nucleic acid target in said nucleic acid specimen, and the absence of an amplification product of said additional isothermal amplification reaction is indicative of the absence of said nucleic acid target in said biological sample.

3. The method according to any of the preceding claims, wherein said isothermal amplification reaction is symmetric or asymmetric isothermal amplification.

4. The method according to any of the preceding claims, wherein said isothermal amplification reaction is loop-mediated isothermal amplification (LAMP), NASBA, 3SR, SDA, or smart amplification process version 2 (SMA P 2).

5. The method according to any of the preceding claims, wherein said isothermal amplification reaction is loop-mediated isothermal amplification (LAMP).
6. The method according to any of the preceding claims, wherein said isothermal amplification reaction comprises at least four primers.

7. The method according to any of the preceding claims, wherein said isothermal amplification reaction comprises the steps of

5

a. incubating the elements (i) to (vii) under conditions that allow complementary strand synthesis wherein second and first primers are used as origins:

i. a nucleic acid comprising a target nucleotide sequence;

ii. a DNA polymerase catalyzing complementary strand synthesis which includes strand displacement;

iii. a second primer, wherein the 3'-end of the second primer anneals to the 3'-side region of one of said target nucleotide sequence strands, and the 5'-side of the second primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin;

iv. a first primer, wherein the 3'-end of the first primer anneals to the 3'-side region of the other said target nucleotide sequence strand, and the 5'-side of the first primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin;

v. nucleotide substrates;

vi. a third primer, wherein the third primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the first primer on the template functions as the origin; and

vii. a fourth primer, wherein the fourth primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the second primer on the template functions as the origin.
8. The method according to claim 7, wherein the nucleotide sequences arranged on both 5'-ends of the second primer and the first primer, contain a nucleotide sequence that is complementary to the predicted nucleotide sequence of said specific region or the complementary strand thereof.

9. The method according to claim 7, further comprising
   a. a first loop primer, which provides an origin for complementary strand synthesis between a region originating from the first primer of the amplification product of the first primer and an arbitrary region for the first primer; and/or
   b. a second loop primer, which provides an origin for complementary strand synthesis between a region originating from the second primer of the amplification product of the second primer and an arbitrary region for the second primer.

10. The method according to claim 7, wherein the DNA polymerase is selected from the group consisting of Bst DNA polymerase, Bca(exo-) DNA polymerase, Klenow fragment of DNA polymerase I, Vent DNA polymerase, Vent (Exo-) DNA polymerase (exonuclease activity-free Vent DNA polymerase), DeepVent DNA polymerase, DeepVent (Exo-) DNA polymerase (exonuclease activity-free DeepVent DNA polymerase), F29 phage DNA polymerase, MS-2 phage DNA polymerase, Z-Taq DNA polymerase (Takara Shuzo), KOD DNA-polymerase (TOYOBO), Taq MutS and Aac DNA polymerase, or fragments and/or functional homologues thereof.

11. The method according to any of the preceding claims, wherein said biological sample is plaque, dental plaque, bio film, cells, human cells, bacteria, tissue, saliva, tumor tissue, yeast, nucleic acids, nucleic acid vectors, blood, or cerebrospinal fluid.

12. The method according to claim 11, wherein said biological sample is dental plaque.

13. The method according to claim 11, wherein said biological sample originates from the gingival pocket.

14. The method according to any of the preceding claims, wherein said biological sample comprises Aggregatibacter actinomycetemcomitans.
15. The method according to any of the preceding claims, wherein said biological sample comprises a JP2 clone of *Aggregatibacter actinomycetemcomitans*.

16. The method according to any of the preceding claims, wherein the presence of amplification product is indicative of presence of a clone of *Aggregatibacter actinomycetemcomitans*.

17. The method according to claim 16, wherein said clone is a JP2 clone of *Aggregatibacter actinomycetemcomitans*.

18. The method according to any of the preceding claims for diagnosis of a disorder.

19. The method according to claim 18, wherein the presence or absence of said nucleic acid region is indicative of said disorder or indicative of elevated risk of developing said disorder.

20. The method according to claim 19, wherein the presence or absence of said nucleic acid region is indicative of an elevated risk of at least 50% of developing said disorder.

21. The method according to any of the preceding claims, wherein said disorder is selected from the group consisting of cancer, oral cancer, oral disorders, periodontitis, early onset periodontitis, sepsis, endocarditis, and HIV.

22. The method according to any of the preceding claims, wherein said disorder is oral disorders.

23. The method according to any of the preceding claims, wherein said disorder is periodontitis.

24. The method according to any of the preceding claims, wherein said disorder is early onset periodontitis.

25. method according to any of the preceding claims, wherein said amplification product is detected by gel electrophoresis, southern blotting or polymerase chain reaction (PCR).

26. The method according to any of the preceding claims, wherein said amplification product is detected by any intercalating nucleic acid agent.
27. The method according to any of the preceding claims, wherein said agent is ethidium bromide, SYBR Green I, Pico Green, Loopamp Fluorescent Detection Reagent (Eiken Chemical Co.), or cationic polymers.

28. The method according to any of the preceding claims, wherein the amplification product is detected by quantifying the amount of magnesium pyrophosphate precipitate, wherein the amount of magnesium pyrophosphate precipitate reflects the amount of amplification product.

29. The method according to any of the preceding claims, wherein said amplification product is detected during the isothermal amplification reaction.

30. The method according to any of the preceding claims, wherein said amplification product is detected after the isothermal amplification reaction.

31. The method according to any of claims 25 and 30, wherein said amplification product is detected photometrically.

32. The method according to any of claims 26 to 31, wherein said amplification product is detected by visual inspection (with the naked eye).

33. The method according to any of the preceding claims, wherein said nucleic acid target is selected from the group consisting of the leukotoxin gene \( \text{(Aggregatibacter actinomycetemcomitans)} \) or the CCR5 chemokine receptor gene.

34. The method according to any of the preceding claims, wherein said nucleic acid target is the leukotoxin promoter of \( \text{Aggregatibacter actinomycetemcomitans} \).

35. The method according to any of the preceding claims, wherein said nucleic acid target comprises a nucleic acid sequence as defined by SEQ ID NO: 7, or part thereof or SEQ ID NO: 8, or part thereof.

36. The method according to any of the preceding claims, wherein said nucleic acid region is at least 50 nucleotides.

37. The method according to any of the preceding claims, wherein said nucleic acid region is at least 200 nucleotides.

38. The method according to any of the preceding claims, wherein said nucleic acid region is at least 500 nucleotides.
39. The method according to any of the preceding claims, wherein said nucleic acid region comprises the promoter region of the leukotoxin gene of *Aggregatibacter actinomycetemcomitans*, as defined by SEQ ID NO: 7, or part thereof.

40. The method according to any of the preceding claims, wherein said nucleic acid region comprises a nucleic acid sequence as defined by SEQ ID NO: 9, or part thereof.

41. The method according to any of the preceding claims, wherein the presence of JP2 is indicative of elevated risk of developing periodontitis.

42. A method for determining the presence or absence of a toxic strain of *Aggregatibacter actinomycetemcomitans* comprising

a. providing a biological sample comprising a nucleic acid,

b. performing an isothermal amplification reaction on said nucleic acid specimen, wherein the amplification product comprises a nucleic acid region, and

c. detecting said amplification product.

wherein the presence of an amplification product is indicative of the presence of said toxic strain of *Aggregatibacter actinomycetemcomitans* in said biological sample, and the absence of amplification product is indicative of the absence of said toxic strain of *Aggregatibacter actinomycetemcomitans* in said biological sample.

43. The method according to claim 42, as defined in any of claims 1 to 40.

44. The method according to any of claims 42 and 43, wherein said toxic strain of *Aggregatibacter actinomycetemcomitans* is a JP2 clone.

45. The method according to any of claims 43 and 44, wherein said first primer is SEQ ID NO: 1.

46. The method according to any of claims 43 and 44, wherein said second primer is SEQ ID NO: 2.

47. The method according to any of claims 43 and 44, wherein said third primer is SEQ ID NO: 3.

48. The method according to any of claims 43 and 44, wherein said fourth primer is SEQ ID NO: 4.
49. The method according to any of claims 43 and 44, wherein said first loop primer is SEQ ID NO: 5.

50. The method according to any of claims 43 and 44, wherein said second loop primer is SEQ ID NO: 6.

51. The method according to any of claims 43 and 44, wherein said first primer is SEQ ID NO: 1, said second primers is SEQ ID NO: 2, said third primer is SEQ ID NO: 3, and said fourth primer is SEQ ID NO: 4, and said first loop primer is SEQ ID NO: 5, and said second loop primer is SEQ ID NO: 6.

52. An oligonucleotide primer comprising between 10 and 80 nucleotides for use in performing an isothermal amplification reaction on a nucleic acid target, wherein the amplification product comprises a nucleic acid region, and the subsequent detection of the presence of an amplification product is indicative of the absence of said nucleic acid region in said nucleic acid target, and the absence of amplification product is indicative of the presence of said nucleic acid region in said nucleic acid target.

53. The oligonucleotide according to claim 52 for detection of the JP2 clone of Aggregatibacter actinomycetemcomitans.

54. The oligonucleotide according to claim 53, wherein said oligonucleotide is at least 70 % identical to SEQ ID NO: 1.

55. The oligonucleotide according to claim 53, wherein said oligonucleotide is at least 70 % identical to SEQ ID NO: 2.

56. The oligonucleotide according to claim 53, wherein said oligonucleotide is at least 70 % identical to SEQ ID NO: 3.

57. The oligonucleotide according to claim 53, wherein said oligonucleotide is at least 70 % identical to SEQ ID NO: 4.

58. The oligonucleotide according to claim 53, wherein said oligonucleotide is at least 70 % identical to SEQ ID NO: 5.

59. The oligonucleotide according to claim 53, wherein said oligonucleotide is at least 70 % identical to SEQ ID NO: 6.

60. The oligonucleotide primer according to claim 52, wherein said primer is selected from the group consisting of SEQ ID NOs: 10 to 14.
61. A kit for detection of presence or absence of a nucleic acid region within a nucleic acid target comprising

i. a DNA polymerase catalyzing complementary strand synthesis which includes strand displacement

ii. a second primer, wherein the 3'-end of the second primer anneals to the 3'-side region of one of said target nucleotide sequence strands, and the 5'-side of the second primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin;

iii. a first primer, wherein the 3'-end of the first primer anneals to the 3'-side region of the other said target nucleotide sequence strand, and the 5'-side of the first primer includes a nucleotide sequence complementary to the predicted nucleotide sequence that constitutes a region on the products of the complementary strand synthesis reaction that uses the primer as the origin;

iv. nucleotide substrates;

v. a third primer, wherein the third primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the first primer on the template functions as the origin;

vi. a fourth primer, wherein the fourth primer serves as the origin of the complementary strand synthesis reaction, further wherein the 3'-side of the annealing region of the second primer on the template functions as the origin, and

62. The kit according to claim 61, further comprising amplification buffer.

63. The kit according to claim 61, wherein said oligonucleotide primers support loop-mediated isothermal amplification of a nucleic acid target.

64. The kit according to any of claims 61 and 63, wherein said nucleic acid target is a leukotoxin gene.
65. The kit according to any of claims 61 and 64, wherein said nucleic acid target comprises a nucleic acid sequence as defined by SEQ ID NO: 7, or part thereof or SEQ ID NO: 8, or part thereof.

66. The kit according to any of claims 61 to 65, wherein at least one oligonucleotide primer is selected from the group consisting of SEQ ID NO: 1 to 6.

67. The kit according to any of claims 61 to 66 for diagnosis of a disorder.

68. The kit according to any of claims 61 to 67 for diagnosis of periodontitis.

69. The kit according to claim 68, wherein periodontitis is early onset periodontitis.

70. The kit according to any of claims 61 to 66 for detection of a toxic clone of \textit{Aggregatibacter actinomycetemcomitans}.

71. The kit according to any of claims 61 to 66 for detection of a JP2 clone of \textit{Aggregatibacter actinomycetemcomitans}.

72. A method for treatment of periodontitis in a human being comprising

a. detecting the presence of a toxic strain of \textit{Aggregatibacter actinomycetemcomitans} by

i. providing a biological sample comprising a nucleic acid,

ii. performing an isothermal amplification reaction on said nucleic acid specimen, wherein the amplification product comprises a nucleic acid region, and

iii. detecting said amplification product, wherein the presence of an amplification product is indicative of the presence of said toxic strain of \textit{Aggregatibacter actinomycetemcomitans} in said biological sample.

b. treating the human being with antibiotics.

73. The method according to claim 72, as defined in any of claims 1 to 51.
a

1977975  TCTCGTTTATTCAGTTCACAAAAAGTAAATGAAGTTTACTCTATGAAATACACTGGAAACTTGT
        F3        F2
1977915  TCAGAATTTTGTAACAAAAAGTTAGAATAACCGTAGGATAGGACAAAAATCGTTGGCAT
        LF                F1c
1977855  TCTCGGCAGAAAAAATCTATTTGGAAATACCCAAGATCTTTTTAATGATGGCATGCTGTGGATAT
        B1c               LB  
1977795  TGGGTGGTTACATCTTATCCAAAATAAATTTAATACACTACAGGAGATTTAAATTACGGAAAC
        B2                B3

b

F3    5’ CCGTTTTATTCAGTTCACAAAG 3’
B3    5’ TCTCCATATTAATCTCTTTGT 3’
F1P   5’ ACGATTTGTCATATCTTACGTTACTCTATGAAATACCTGGAAACTTGTT 3’ (F1c+F2)
B1P   5’ TCTCGGCAGAAAAACTATTTGGAAATTAAGGAGAAACACCAC 3’ (B1c+B2)
LF    5’ ACTAACCCTTTTGACTACAAAAATTCCTGA 3’
LB    5’ CCAAGTACTTTTTATGATGGCA 3’

Fig. 1
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>paragraph [0025]; claims 1,3,13; figure 7; examples 1-7</td>
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<td>paragraphs [0035], [0062], [0072], [0094], [0095], [0099], [0100], [0108]; claims 26,30; figure 1; example 5</td>
<td>12-17, 22-24, 34-51, 53-60, 72,73</td>
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* Further documents are listed in the continuation of Box C.

See patent family annex.

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or of other special reasons (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search
9 January 2009

Date of mailing of the international search report
23/01/2009

Name and mailing address of the ISA
European Patent Office, P. B. 5818 Patentlaan 2 NL - 2280 HNrijswijk Tel. (+31-70) 340-2040, Fax (+31-70) 340-3016

Authorized officer

Nurmi, Jussi
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<td>ROMANO JOSEPH W ET AL: &quot;Genotyping of the CCR5 chemokine receptor by isothermal NASBA amplification and differential probe hybridization&quot; CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 6, no. 6, November 1999 (1999-11), pages 959-965, XP002509538 ISSN: 1071-412X abstract; figures 1,3 page 960, column 1, paragraph 2 page 959, column 1 page 960, column 1, paragraph 3</td>
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<td>abstract column 10, line 53 - line 60; claims 1,2</td>
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