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(58) Field of Search:
INT CL **C12N, C12Q**
Other: **ONLINE: EPODOC, WPI, BIOSIS, MEDLINE**

(54) Title of the Invention: **Detecting viable microorganisms**
Abstract Title: **Detection of viable microorganisms by assaying for ligase and/or polymerase activity**

(57) A nucleic acid substrate for ligase and/or polymerase activity and its uses is claimed comprising:
a. a first strand b. a second strand c. a complementary strand that is complementary to the first and second strands such that the first and second strands hybridize with the complementary strand to produce a substantially double stranded structure wherein the first strand acts as a primer for polymerase activity and the complementary strand acts as a template strand enabling polymerase extension of the first strand, wherein the 5' end of the second strand is phosphorylated to permit ligation with the 3' end of the first strand in the presence of ligase activity and the second strand contains a mismatched region in the substantially double stranded structure wherein the complementary strand is sensitive to treatment to render it no longer a template strand, whereas the first and second strands are resistant to such treatment. Kits and alternatively blood culture bottles are claimed.

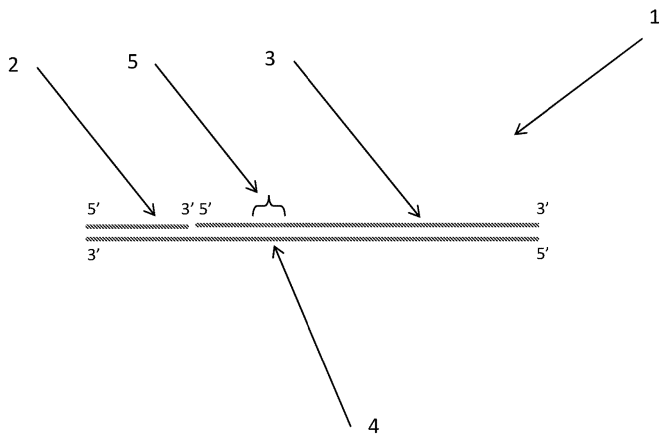

Fig. 1

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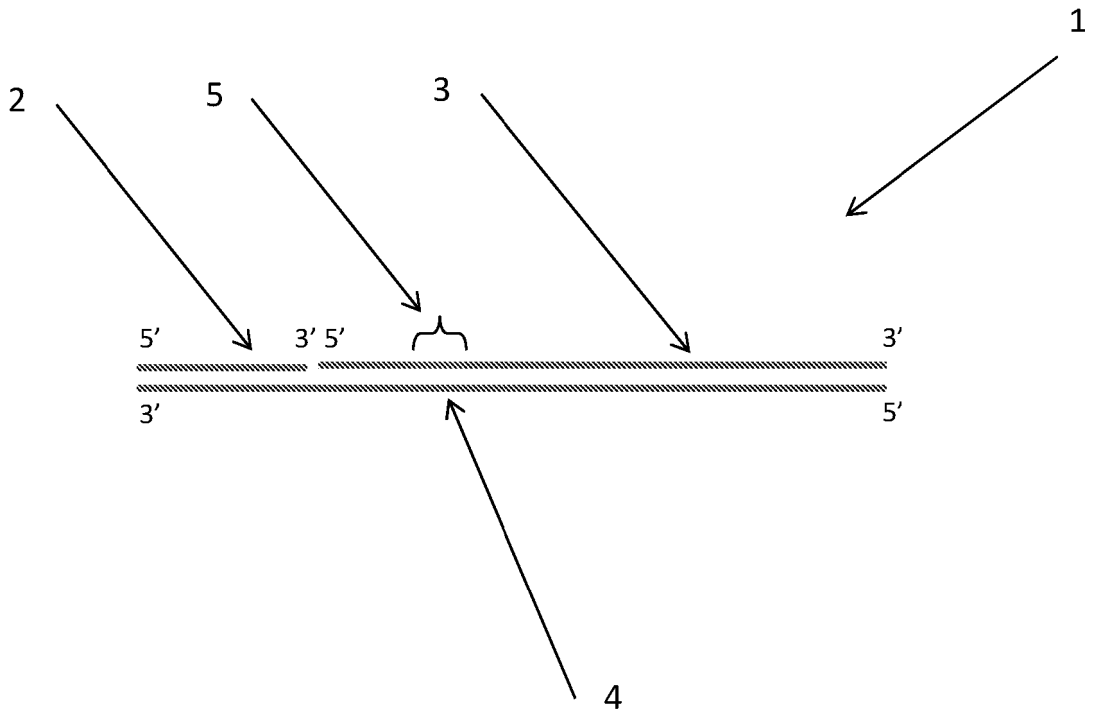

Fig. 1

DETECTING VIABLE MICROORGANISMS

FIELD OF THE INVENTION

The present invention relates generally to the field of detecting the absence or presence of microorganisms in a sample. The methods typically rely upon measuring microbial enzyme activity (if any) present in a sample and may relate to such methods which are capable of being performed using nucleic acid amplification techniques such as the polymerase chain reaction. The methods of the invention therefore enable determination of the absence and presence of microbial pathogens in samples such as un-purified blood, blood culture and other body fluids. This invention in particular relates to substrate nucleic acid molecules which permit ligase and polymerase activity to be determined and discriminated from one another. The invention also relates to improvements in work flow relating to blood culture sampling to assist with integration of the microorganism detection methods into laboratory testing protocols.

BACKGROUND TO THE INVENTION

Measuring the presence and levels of certain molecules which are associated with cell viability is important in a number of contexts. For example, measuring levels of ATP is useful in mammalian cells for growth analysis and toxicology purposes. Culture approaches can be used to detect small numbers of bacteria but such techniques require several days to complete, especially when attempting to detect small numbers of bacteria and also when detecting slower growing microorganisms.

Detection of adenylate kinase as an indicator of viability has also been proposed (Squirrell DJ, Murphy MJ, Leslie RL, Green JCD: A comparison of ATP and adenylate kinase as bacterial cell markers: correlation with agar plate counts. WO96/002665 describes a method for determining the presence and/or amount of microorganisms and/or their intracellular material present in a sample characterized in that the amount of adenylate kinase in the sample is estimated by mixing it with adenosine diphosphate (ADP), determining the amount of adenosine triphosphate (ATP) produced by the sample from this ADP, and relating the amount of ATP so produced to the presence/or amount of adenylate kinase and to microorganisms and/or their intracellular material, wherein the conversion of ADP to ATP is carried out in the presence of magnesium ions at a molar concentration sufficient to allow maximal conversion of ADP to ATP.

In WO2009/007719, ligases, in particular NAD- dependent ligases, are disclosed as a useful indicator of the presence of a (viable) microorganism in a sample. Ligases are enzymes which catalyze ligation of nucleic acid molecules. The ligation reaction requires either ATP or NAD+ as co-factor depending upon the ligase concerned. In this disclosure, the use of NAD-dependent ligase activity is utilized as an indicator of the presence of a (viable) microorganism in a sample.

WO2011/130584 describes a method for detection of viable microorganisms based on detection of DNA or RNA polymerases in which a sample is contacted with a nucleic acid substrate that acts as a substrate for microbial polymerase, incubated under conditions suitable for polymerase activity from intact microorganisms and any resulting nucleic acid product is determined using a nucleic acid amplification technique such as quantitative polymerase chain reaction. Such assays have been termed "ETGA assays", where ETGA stands for Enzymatic Template Generation and Amplification. A problem with ETGA assays for viable microorganisms in crude samples is the presence of contaminating polymerase activity outside the microorganisms arising from host (e.g. human) cells and dead microorganisms. The ETGA assay is unable to distinguish microorganism polymerase activity from that of the host or from dead microorganisms.

Applicant's co-pending application WO2010/119270 describes a method for removing enzyme activity (in this case, DNA ligase) outside intact microorganisms and this can be used also for removal of contamination nucleic acid polymerase activity.

DESCRIPTION OF THE INVENTION

The art has focused on detection of the presence of microorganisms rather than determining their absence. By "determining their absence" the applicants do not mean that the sample is necessarily sterile but may have an organism load that is sufficiently low as to be negative for practical purposes. For example, blood cultures are often taken from patients suspected of having bloodstream infections which can be associated with sepsis, a condition that can be rapidly fatal if left untreated. It is routine for clinical microbiology laboratories to incubate such specimens for at least five days before

reporting a negative result, during which time the patients are often kept on broad spectrum antibiotics. Typically up to 90% of such patients are negative, and so a large number of patients are left for 5 days on antibiotic therapy that is not necessary for their condition. A faster method for determining negative growth (relative to a five-day blood culture) would be of significant value in reducing the cost of unnecessary antibiotic therapy and provide health benefits in terms of reducing the risks of *C. difficile* infection, antibiotic toxicity and antimicrobial resistance. Identifying negative cultures requires the ability to detect both bacterial and fungal/yeast infections if present.

To this end, the foundation of the invention is methods of detecting the absence or presence of a micro-organism in a sample comprising:

(a) contacting the sample with a nucleic acid molecule which acts as a substrate for nucleic acid modifying activity of the micro-organism in the sample,

(b) incubating the thus contacted sample under conditions suitable for nucleic acid modifying activity; and

(c) specifically determining the absence or presence of a modified nucleic acid molecule resulting from the action of the nucleic acid modifying activity on the substrate nucleic acid molecule to indicate the absence or presence of the micro-organism.

Thus, the invention is based on developments in existing ETGA assays such as those assays relying upon detection of polymerase and/or ligase activity.

More specifically, the inventors have discovered that yeast, a potentially fatal source of bloodstream infection (if left untreated) display poor polymerase activity in ETGA assays. Accordingly, there is benefit in being able to perform assays in which both ligase and polymerase activity is detected. The inventors have devised a substrate nucleic acid molecule that permits both ligase and polymerase activity to be determined and, importantly, discriminated.

Accordingly, in a first aspect, the invention provides a nucleic acid substrate for ligase and/or polymerase activity comprising:

a. a first strand
b. a second strand

c. a complementary strand that is complementary to the first and second strands such that the first and second strands hybridize with the complementary strand to produce a substantially double stranded structure

wherein the first strand acts as a primer for polymerase activity and the complementary strand acts as a template strand to thus extend the first strand in the presence of polymerase activity

wherein the 5' end of the second strand is phosphorylated to permit ligation of the 3' end of the first strand and the 5' end of the second strand in the presence of ligase activity

wherein the second strand contains a sequence that does not base pair with the complementary strand sequence such that there is a mismatched region in the substantially double stranded structure

wherein the complementary strand is sensitive to treatment to render it no longer a template strand, whereas the first and second strands are resistant to such treatment.

A suitable structure is shown schematically in Fig. 1. As can be seen, the first and second strands when hybridized with the complementary strand form a largely continuous strand. The two are not joined however, the 3' end of the first strand is not (covalently) bonded to the 5' end of the second strand. This is important to permit ligation and/or extension reactions to take place if the appropriate enzyme activity is present in the sample.

The substrate may be extended by polymerase activity. All bacteria and fungi express DNA and RNA polymerase activity. The present invention does not rely upon discrimination of the source of polymerase activity. The first strand of the nucleic acid substrate acts as a primer for polymerase activity and the complementary strand acts as a template strand to thus extend the first strand in the presence of polymerase activity. This extension reaction effectively displaces the second strand which is downstream in the 5'-3' direction of the first strand.

The substrate may be ligated by bacterial or fungal ligase. Bacterial ligase is a DNA ligase which depends upon the nicotinamide adenine dinucleotide (NAD+) cofactor for activity. It is referred to hereinafter as "NAD-dependent ligase". In contrast, fungal ligases are ATP-dependent ligases which rely upon the cofactor adenosine triphosphate (ATP) for activity. They are referred to hereinafter as "ATP-dependent ligase". The

activity of both types of ligase is the formation of a phosphodiester bond between the 5' end of a nucleic acid molecule and the 3' end of a nucleic acid molecule. Thus, in the nucleic acid substrates of the invention, the 5' end of the second strand is phosphorylated to permit ligation of the 3' end of the first strand and the 5' end of the second strand in the presence of ligase activity.

As indicated, the complementary strand is sensitive to treatment to render it no longer a template strand, whereas the first and second strands are resistant to such treatment. This can be achieved by one of two broad approaches. Firstly, the complementary strand may be modified in a manner such that it is sensitive to treatment to render it no longer a template strand. The other parts of the nucleic acid substrate (i.e. the first and second strands) are not sensitive to the treatment. This is important to permit discrimination of polymerase and ligase activity in a downstream amplification process. This ensures that, during a subsequent amplification reaction (a step in ETGA assays), the polymerase used in the amplification reaction is unable to amplify the complementary strand using the first strand as a primer. Any suitable modification may be adopted. In some embodiments the complementary strand of the nucleic acid substrate comprises uracil bases/nucleotides such that upon treatment with uracil DNA glycosylase (UDG) enzyme the complementary strand is no longer able to serve as a template strand. The first and second strands do not contain uracil bases/nucleotides. Alternatively, the complementary strand may incorporate heat labile nucleotides such that prior to, or during, the subsequent amplification process, a heating step may be employed in order to (selectively) degrade the complementary strand. This prevents amplification of the complementary strand using the first strand as a primer in the amplification reaction. No heating step is employed in the initial detection method to permit extension and/or ligation.

In the second approach, the first and second strands may be modified such that they are resistant to a treatment applied to the complementary strand. For example, the first and second strands may be modified such that they are resistant to enzymatic, such as nuclease, degradation. Any suitable means may be employed in order to protect the first and second strands from nuclease activity. Non-limiting examples include incorporation of methylation into the nucleic acid molecule, end modification such as protection of the 3' and/or 5' ends and incorporation of synthetic nucleotides. In specific embodiments,

the synthetic nucleotides comprise phosphorothioate nucleotides and/or locked nucleic acid nucleotides. In certain embodiments, the synthetic nucleotides replace at least one up to all of the nucleotides in the nucleic acid molecule. In some embodiments, end modification is sufficient to protect from exonuclease activity. Thus, upon addition of an exonuclease to the sample, the complementary strand is preferentially degraded. This prevents that strand from being amplified in the subsequent detection step. However, the first and second strands, as part of a ligated or extended product, remain amplifiable as they are resistant to the exonuclease treatment. The modifications to the first and second strand should not preclude or inhibit the ligation or extension reactions.

The second strand contains a (portion of) sequence that does not base pair with the complementary strand sequence such that there is a mismatched region in the substantially double stranded structure. This is key to being able to subsequently discriminate those nucleic acid molecules that have been ligated versus those that have been extended by polymerase. In the presence of polymerase activity, the second strand is displaced and so a sequence complementary to the sequence of the complementary strand is produced. Thus, in the presence of polymerase activity the complementary (template) strand is copied thus displacing the second strand from the substantially double stranded structure. In the presence of ligase activity, the second strand is ligated to the first strand and so the region of mismatch is retained. As is discussed in greater detail herein, following an amplification reaction, the region of potential mismatch can be probed to determine the source of the enzyme activity in the sample (ligase and/or polymerase).

The mismatched region can be positioned anywhere within the second strand. However, in some embodiments, the mismatched region is not found within 1, 2, 3, 4, 5, 10, 15, 20, 25 or 30 nucleotides of one or both ends of the second strand, in particular the 5' end (to avoid any impact on ligation efficiency).

The mismatched region can be of any length provided that the mismatch is detectable and does not adversely affect the functioning of the assay (i.e. the extension or ligation reactions). Thus, the mismatch should not prevent overall hybridization between the first and second strand respectively and the complementary strand, even though the region of mismatch will not hybridize. Typically, the mismatch is a short region (relative to the

overall length of the molecule). In specific embodiments, the mismatched region is between (and including) 3-50, such as 5-25 or 6-12 nucleotides in length. The mismatched region may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or 25 nucleotides in length in some embodiments. Typically the mismatched region is flanked by significant regions of identity between the second strand and the complementary strand. The regions of identity may be at least 10, 15, 20, 30, 40, 50, 100 or more nucleotides in length.

The nucleic acid substrates may be provided in either orientation, according to standard nomenclature. Thus, in some embodiments, the first and second strands are sense strands and the complementary strand is an antisense strand. In other embodiments, the first and second strands are antisense strands and the complementary strand is a sense strand.

The nucleic acid substrates may be DNA based. They may incorporate synthetic nucleotide analogues as appropriate or may be RNA or PNA based for example, or mixtures thereof. Suitable modifications, for example, to protect the substrate from nuclease activity, may be included. Any suitable means may be employed in order to protect the nucleic acid molecule from nuclease activity. Non-limiting examples include incorporation of methylation into the nucleic acid molecule, end modification such as protection of the 3' and/or 5' ends and incorporation of synthetic nucleotides. In specific embodiments, the synthetic nucleotides comprise phosphorothioate nucleotides and/or locked nucleic acid nucleotides. In certain embodiments, the synthetic nucleotides replace at least one up to all of the nucleotides in the nucleic acid molecule. As is discussed above, modifications may preferentially be made to certain components of the substrate in specific embodiments.

The nucleic acid substrates may be labelled, such as using a fluorescent label, or FRET pair, in certain embodiments to facilitate detection. Suitable detection methods are described herein.

Thus, the substrate nucleic acid molecules include any natural nucleic acid and natural or synthetic analogues that are capable of being acted upon by nucleic acid modifying activity in order to generate the extended and/or ligated nucleic acid molecule. The substrate may be extended and/or ligated in specific embodiments.

Preferably, the nucleic acid substrate is present in excess, and in particular in large molar excess, over the nucleic acid modifying activity (provided by the microorganisms) in the sample. This is an important technical distinction over prior art methods. Because a novel extended and/or ligated nucleic acid molecule is detected, only the presence of this molecule in the sample is essential for the detection methods to work effectively. Thus, it is not detrimental to the methods of the invention if other nucleic acid molecules are present in the sample such as from the microorganisms to be detected or from mammalian or other sources which may be found in the sample to be tested for example.

In some embodiments, the substrate and/or primers may incorporate complementary non-naturally occurring molecules which can base pair with each other, to avoid non-specific detection of genomic DNA. As an example, pyDAD and puADA may be incorporated into primers and substrate molecules as appropriate (Sismour et al., Nucleic Acids Research, 2004, Vol. 32, No. 2: 728-735).

The invention provides for use of a nucleic acid substrate as described herein for detecting polymerase and/or ligase activity in a sample. Specific methods are described herein.

The invention further provides a kit for detecting the presence or absence of polymerase and/or ligase activity in a sample comprising:

a. a nucleic acid substrate as defined herein
b. a probe which hybridizes to a target sequence comprising the mismatched region contained within the second strand; and/or
c. a probe which hybridizes to a target sequence comprising the sequence corresponding to the mismatched region in the complementary strand; and/or
d. a first primer which hybridizes to the product of ligation between the first and second strands and/or to the product of polymerization from the first strand acting as primer and the complementary strand acting as template and directs amplification of the product of ligation/polymerization; and/or
e. a second primer which hybridizes to the amplification product resulting from use of the first primer and directs further amplification of that amplification product; and/or

f. uracil DNA glycosylase and/or a nuclease enzyme such as an exonuclease enzyme (depending upon the form of substrate used).

Thus, in addition to the nucleic acid substrate, the kit may include a probe which hybridizes to a target sequence comprising at least a portion of the mismatched region contained within the second strand. This enables ligated molecules, which may subsequently have been amplified, to be detected.

Similarly, in addition to the nucleic acid substrate, the kit may include or further include a probe which hybridizes to a target sequence comprising the sequence corresponding to at least a portion of the mismatched region in the complementary strand. This enables extended molecules, produced as a consequence of polymerase activity (in which the second strand is displaced) to be detected (optionally following an amplification step). Generally, the kit may include both probe types.

Either one or both probes may be labelled to facilitate detection. Typical labels include chromophores, fluorophores and electrochemically active species. Where both probe types are included in the kits each probe may be differently labelled. By "differently labelled" is meant a detectably different label thus permitting the probes to be easily distinguished from one another (e.g. using one detection methodology). The skilled person would be readily able to select suitable different labels. For example the labels may have different wavelengths of maximal emission. Suitable pairs of fluorophores which may be utilised include Cy3 and FAM or Texas Red and FAM. Other fluorophores which may be employed include Alexa dyes, JOE, TET, HEX etc.

The kit may additionally or alternatively incorporate reagents for performing a subsequent amplification step (once the ligated and/or extended nucleic acid substrate has been generated). Such reagents include a first primer which hybridizes to the product of ligation between the first and second strands and/or to the product of polymerization from the first strand acting as primer and the complementary strand acting as template and directs amplification of the product of ligation/polymerization. Generally, the primer hybridizes outside of the mismatch region and thus will amplify extension or ligation products. However, in some embodiments, the first primer may incorporate the probe function such that the primer binds to either extension or ligation

products but not both. In such embodiments, two separate first primers may be required to allow amplification of both product types. The discussion of probes above therefore applies mutatis mutandis to these embodiments. For example, primers may be labelled and differently labelled if they perform the probe function in relation to identifying the origin of the mismatch sequence.

Similarly, the kit may incorporate a second primer which hybridizes to the amplification product resulting from use of the first primer and directs further amplification of that amplification product. Suitable primer pairs can be designed by one skilled in the art against any defined nucleic acid substrate molecule. In certain embodiments, the second primer (but typically not both the first and second primer although this may be possible if the mismatch region is large enough) may incorporate the probe function such that the primer binds to either extension or ligation products but not both. In such embodiments, two separate second primers may be required to allow amplification of both product types. The discussion of probes above therefore applies mutatis mutandis to these embodiments. For example, primers may be labelled and differently labelled if they perform the probe function in relation to identifying the origin of the mismatch sequence.

As already discussed, the complementary strand of the nucleic acid substrate is sensitive to treatment to render it no longer a template strand, whereas the first and second strands are resistant to such treatment. In some embodiments, the complementary strand comprises modification such that it is sensitive to treatment to render it no longer a template strand, such as incorporation of uracil bases such that upon treatment with uracil DNA glycosylase (UDG) enzyme the complementary strand is no longer a template strand. Alternatively, the first and second strands may be modified to protect them from a treatment, such as (exo)nuclease treatment as discussed herein. This ensures that, during a subsequent amplification reaction (a step in ETGA assays), the polymerase used in the amplification reaction is unable to amplify the complementary strand using the first strand as a primer. Accordingly, in some embodiments, the kit incorporates uracil DNA glycosylase. UDG is available from various sources. UDG may be termed UNG and this is the approved gene symbol (according to the HUGO Gene Nomenclature Committee). The enzyme may be recombinantly produced in a suitable expression system (e.g. in E. coli). UDG may be from any origin, e.g. bacterial or

human. The human gene reference sequence is NM_080911 and the Genbank accession number is A64377. Homologs exist in a number of species including mice and rats. Suppliers include New England Biolabs and Thermo Scientific. The enzyme is typically applied to control carry-over contamination in PCR. In other embodiments, the kit contains modified first and second strands and a nuclease, such as an exonuclease.

The substrates and kits of the invention may usefully be applied to discriminate bacteria from fungi in a (clinical) sample. Suitable methods are discussed in further detail herein. Generally, the invention may require deactivation of (bacterial) NAD-dependent ligase activity in the sample. Thus, the kit of the invention may include reagents for deactivating NAD-dependent ligase activity. Any suitable reagents may be employed. For example, the reagents may be affinity based reagents that permit removal of NAD-dependent ligase activity from the sample or inactivate the NAD-dependent ligase activity within the sample. The reagents may specifically bind to NAD-dependent ligase. Suitable reagents may comprise antibodies, aptamers or peptides. They may be immobilised on a solid support to facilitate rapid removal from the sample. For example they may be immobilised on beads, such as magnetic beads that can be readily separated from the sample. In the context of a kit the solid support may be provided separately optionally together with means for immobilising the specific binding agent on the solid support. The antibody may be of monoclonal or polyclonal origin. Typically, the antibody is an IgG immunoglobulin isotype. Antigen-binding fragments and antibody derivatives may also be utilised, to include without limitation Fab fragments, ScFv, single domain antibodies, nanoantibodies, heavy chain antibodies, chimeric antibody fusions etc. which retain antigen-specific binding function and these are included in the definition of “antibody”. Methods for generating specific antibodies are known to those skilled in the art. Antibodies may be of human or non-human origin (e.g. rodent, such as rat or mouse) and be humanized etc. according to known techniques (Jones *et al.*, Nature (1986) May 29-Jun. 4;321(6069):522-5; Roguska *et al.*, Protein Engineering, 1996, 9(10):895-904; and Studnicka *et al.,* Humanizing Mouse Antibody Frameworks While Preserving 3–D Structure. Protein Engineering, 1994, Vol.7, pg 805).

The aptamer may be a nucleic acid molecule, to include natural nucleotides and derivatives/analogues and non-natural nucleotides, or a peptide molecule, to include natural amino acids and derivatives/analogues and non-natural amino acids bonded with

standard or non-standard peptide bonds, or one or more operably connected nucleic acid molecules and/or peptide molecules. In specific embodiments the aptamer is a DNA, RNA or XNA aptamer. Nucleic acid aptamer selection can be made using methods known to those skilled in the art, for example using in vitro selection or SELEX. In certain embodiments the aptamer is a peptide aptamer comprising a variable peptide domain, attached at both ends to a protein scaffold. The variable peptide domain may be up to 30 amino acids long, preferably 5 to 25 amino acids long, more preferably 10 to 20 amino acids long. The scaffold may be any protein which has appropriate solubility and compacity properties, for example the bacterial protein Thioredoxin-A, Peptide aptamer selection can be made using methods known to those skilled in the art, for example using the yeast two-hybrid system.

By "specifically bind" it is meant that an antibody binds to an epitope via its antigen binding domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. The term is well known in the art. Accordingly, an antibody specifically binds to an epitope when it binds to that epitope, via its antigen binding domain more readily than it would bind to a random, unrelated epitope. Likewise, a specific binding agent such as an aptamer, protein or peptide specifically binds to a target molecule when it binds to that molecule more readily than it would to a random, unrelated molecule. In the present invention specificity of binding ensures that NAD-dependent ligase is inactivated/removed but ATP-dependent ligase activity in the sample is not inactivated/removed.

Alternatively, chemical reagents for deactivating NAD-dependent ligase activity in the sample may be employed and included in the kits of the invention. Examples include:

- Chloroquine,
- hydroxychloroquine,
- quinacrine.
- 2-Amino-5-diethylaminopentane,
- spermidine,
- putrescine,
- DDPP(2,4-diamino-7-dimethylamino-pyrimido[4,5-d]pyrimidine),
- diaminopyrimidine,
- triaminopyridimine,

- diaminoquinazoline,
- 6-Amino-2-(dimethylamino)-4-pyrimidinol
- N(2),N(2)-dimethyl-2,4,6-pyrimidinetriamine,
- N(7)-propylpyrimido[4,5-d]pyrimidine-2,4,7-triamine
- trimethoprim,
- methotrexate
- Reducing conditions (to reduce NAD and thus prevent it from activating NAD dependent ligase).

These reagents may preferentially affect NAD-dependent ligase activity and not significantly affect, or affect to a lesser degree, ATP-dependent ligase activity in the sample.

These reagents may inhibit NAD-dependent ligase activity where the ligase is not already "charged" with NAD. By "charged" is meant that an AMP group derived from NAD is covalently attached to the NAD-dependent ligase, typically via a conserved lysine residue within the KXDG motif of the NAD-dependent ligase. In order to exhaust already charged ligase, a dummy or false substrate may be included in the kits (for addition to the sample). This false substrate can be acted upon by the ligase but is not detected and does not impair subsequent detection of the nucleic acid substrate molecule of the invention. Thus, typically the false substrate does not include the primer and/or probe binding sites found in the nucleic acid substrate molecules of the invention. Thus, the kits may incorporate both a chemical reagent to inhibit NAD-dependent ligase activity and a false substrate molecule.

As already mentioned, the extended and or ligated nucleic acid substrate is typically detected by virtue of an amplification reaction. Accordingly, the kit may incorporate suitable reagents for amplification, such as PCR in particular quantitative PCR (qPCR). Thus, in some embodiments, the kit comprises or further comprises one or more of a polymerase, dinucleotide triphosphates, $MgCl_2$ and buffer. Any suitable polymerase may be utilised. Examples include thermostable polymerases such as Taq or Pfu polymerase and the various derivatives of those enzymes. Suitable buffers are also well known and commercially available and may be included in a PCR mastermix that includes the majority of the components required for PCR amplification.

The kits of the invention are useful for detecting polymerase and/or ligase activity in a sample. The invention provides corresponding methods using the substrate nucleic acid molecules described herein. Thus, in a further aspect there is provided a method for detecting polymerase and/or ligase activity in a sample, comprising:

a. adding a nucleic acid substrate as defined herein to the sample
b. incubating the sample and substrate under conditions permissive of polymerase and/or ligase activity (if present in the sample)
c. treating the sample, for example by adding UDG to the sample or heating the sample if a substrate is used with a heat-labile complementary strand or adding a nuclease if a protected first and second strand is utilised, in order to render the complementary strand no longer a template strand
d. performing a nucleic acid amplification step in order to amplify any ligated first and second strand in the sample (due to ligase activity) and/or extended first strand in the sample (due to polymerase activity)
e. detecting the amplification products to detect polymerase and/or ligase activity in the sample.

In specific embodiments, step e comprises as part of the detection, discriminating amplification products arising from ligase or polymerase activity in the sample. This may be achieved using suitable primers and/or probes which bind to the mismatched region or the sequence corresponding to the mismatched region in the complementary strand. Thus, in some embodiments step e comprises the addition of a first probe which hybridizes to a target sequence comprising the mismatched region contained within the second strand together with a second probe which hybridizes to a target sequence comprising the sequence corresponding to the mismatched region in the complementary strand. In such embodiments, discriminating amplification products arising from ligase or polymerase activity relies upon detecting hybridization of the probes to their respective target sequences. In such embodiments, the probes may be differently labelled for convenience of detection, as discussed in further detail herein. Alternatively, depending upon the amplification technology employed, the primers may incorporate the probe functionalities (e.g. if using hairpin primers or hairpin probe containing primers, such as Scorpion or Amplifluor primers) and thus a separate probe is not necessarily required.

As discussed above, when attempting to discriminate bacteria from fungi in the sample, there may be a requirement to remove or deactivate any NAD dependent ligase in the sample. Thus, in some embodiments, the method comprises, prior to step a, removing from the sample or deactivating in the sample any NAD-dependent ligase activity. This can be achieved in any suitable manner as discussed herein (which discussion applies mutatis mutandis), such as using an affinity agent or a chemical reagent (to include reducing conditions). As also discussed above, in order to exhaust already charged ligase, a dummy or false substrate may be utilised in the methods prior to step a. This false substrate can be acted upon by the ligase but is not detected and does not impair subsequent detection of the nucleic acid substrate molecule of the invention. Thus, typically the false substrate does not include the primer and/or probe binding sites found in the nucleic acid substrate molecules of the invention. Thus, the methods may require use of both a chemical reagent to inhibit NAD-dependent ligase activity and a false substrate molecule in some embodiments.

These methods are useful to determine the absence or presence of microorganisms in a sample. Thus, the invention provides a method of detecting the absence or presence of a microorganism in a sample comprising performing a method as described herein and using the detection of polymerase and/or ligase activity in the sample as an indicator of the absence or presence of a microorganism in the sample. The microorganism may be a bacterium or a fungus (e.g. a yeast). The methods may involve further characterising the nature of the microorganism once detected in the sample.

The invention further provides a method for detecting and discriminating bacteria and/or fungi in a sample, comprising:

a. removing from the sample or deactivating in the sample any NAD-dependent ligase activity
b. adding a nucleic acid substrate as defined herein to the sample
c. incubating the sample and substrate under conditions permissive of polymerase and/or ligase activity (if present in the sample)
d. treating the sample, for example by adding UDG to the sample or heating the sample if a substrate is used with a heat-labile complementary strand or adding a nuclease if a protected first and second strand is utilised, in order to render the complementary strand no longer a template strand

e. performing a nucleic acid amplification step in order to amplify any ligated first and second strand in the sample (due to ligase activity) and/or extended first strand in the sample (due to polymerase activity)

f. detecting and discriminating the amplification products to detect polymerase and/or ligase activity in a sample, wherein the presence of polymerase activity in the sample but no ligase activity indicates the presence of bacteria in the sample but not fungi and/or wherein the presence of ligase activity in the sample indicates the presence of fungi and/or wherein the presence of both polymerase and ligase activity in the sample indicates the presence of fungi and possibly also bacteria.

This method may require, as a preliminary step (depending upon the sample type utilised), removal of non-microorganism (such as mammalian) sources of nucleic acid modifying activity from the sample. This may be achieved for example by lysing non-microorganism cells in the sample as a preliminary step, followed by inactivation and/or removal of the nucleic acid modifying activity provided by the non-microorganism cells. The fact that the microorganisms remain intact protects them from inactivation or removal of their own nucleic acid modifying activity during this step. This may then be followed by a second lysis step to release the microorganism source of nucleic acid modifying activity to be detected.

In some embodiments (relating to step a of the method), the lysis of microorganisms may be separated into a lysis step for fungi and a subsequent lysis step for bacteria. The subsequent lysis step may be performed in the presence of inhibitors of NAD-dependent ligase (as described herein). This permits ligase activity detected according to the methods to be attributed to fungi only, because (ATP-dependent) ligase activity from the fungi is not impaired by the inhibitors until the bacteria have been lysed and the inhibitors added. By lysing the fungal cells initially and incubating for a suitable period of time (under suitable conditions), the fungal ligase (if present in the sample) will already have acted on the nucleic acid substrate before the bacterial cells are lysed and the inhibitors added (which may then impact on further activity of the fungal ligases).

Alternatively, in other embodiments, the lysis of microorganisms may be separated into a lysis step for bacteria followed by removal of the bacterial lysed cell material and a

subsequent lysis step for fungi. This may avoid the need to utilise NAD-dependent ligase inhibitors. Optionally, however, the bacterial lysis step may be performed in the presence of inhibitors of NAD-dependent ligase (as described herein). This permits ligase activity detected according to the methods to be attributed to fungi only, because (ATP-dependent) ligase activity from the fungi is not impaired by the inhibitors since they are removed with the bacterial lysed cell mixture. The presence of NAD-dependent ligase inhibitors may reduce the risk of carry over contamination into the fungal lysis step (which could potentially give a false positive signal of fungal ligase activity if active NAD-dependent ligase was carried over).

Separate lysis steps may be achieved by use of appropriate selective lysis reagents. For example commercially available reagents include B-Per (Thermo Scientific) for lysis of bacterial cells and Y-Per (Thermo Scientific) for lysis of yeast/fungal cells.

Step a may require applying to the sample reagents for deactivating NAD-dependent ligase activity. Any suitable reagents may be employed, as discussed in detail in respect of the kits of the invention. For example, the reagents may be affinity based reagents that permit removal of NAD-dependent ligase activity from the sample or inactivate the activity within the sample. The reagents may specifically bind to NAD-dependent ligase. Suitable reagents may comprise antibodies, aptamers or peptides. They may be immobilised on a solid support to facilitate rapid removal from the sample. For example they may be immobilised on beads, such as magnetic beads that can be readily separated from the sample. The antibody may be of monoclonal or polyclonal origin. Typically, the antibody is an IgG immunoglobulin isotype. Antigen-binding fragments and antibody derivatives may also be utilised, to include without limitation Fab fragments, ScFv, single domain antibodies, nanoantibodies, heavy chain antibodies, chimeric antibody fusions etc. which retain antigen-specific binding function and these are included in the definition of “antibody”. The aptamer may be a nucleic acid molecule, to include natural nucleotides and derivatives/analogues and non-natural nucleotides, or a peptide molecule, to include natural amino acids and derivatives/analogues and non-natural amino acids bonded with standard or non-standard peptide bonds, or one or more operably connected nucleic acid molecules and/or peptide molecules. In specific embodiments the aptamer is a DNA, RNA or XNA aptamer. In certain embodiments the aptamer is a peptide aptamer comprising a variable peptide domain, attached at both

ends to a protein scaffold. The variable peptide domain may be up to 30 amino acids long, preferably 5 to 25 amino acids long, more preferably 10 to 20 amino acids long. The scaffold may be any protein which has appropriate solubility and compacity properties, for example the bacterial protein Thioredoxin-A.

Alternatively, chemical reagents for deactivating NAD-dependent ligase activity in the sample (but not ATP-dependent ligase activity) may be employed. Examples include:

- Chloroquine,
- hydroxychloroquine,
- quinacrine.
- 2-Amino-5-diethylaminopentane,
- spermidine,
- putrescine,
- DDPP(2,4-diamino-7-dimethylamino-pyrimido[4,5-d]pyrimidine),
- diaminopyrimidine,
- triaminopyridimine,
- diaminoquinazoline,
- 6-Amino-2-(dimethylamino)-4-pyrimidinol
- N(2),N(2)-dimethyl-2,4,6-pyrimidinetriamine,
- N(7)-propylpyrimido[4,5-d]pyrimidine-2,4,7-triamine
- trimethoprim,
- methotrexate
- Reducing conditions (to reduce NAD and thus prevent it from activating NAD dependent ligase).

These reagents may preferentially affect NAD-dependent ligase activity and not significantly affect, or affect to a lesser degree, ATP-dependent ligase activity in the sample.

These reagents may inhibit NAD-dependent ligase activity where the ligase is not already “charged” with NAD. By “charged” is meant that an AMP group derived from NAD is covalently attached to the NAD-dependent ligase, typically via a conserved lysine residue within the KXDG motif of the NAD-dependent ligase. In order to exhaust already charged ligase, a dummy or false substrate may also be employed in the methods of the

invention. This false substrate can be acted upon by the ligase but is not detected and does not impair subsequent detection of the nucleic acid substrate molecule of the invention. Thus, typically the false substrate does not include the primer and/or probe binding sites found in the nucleic acid substrate molecules of the invention. Thus, the methods may incorporate both a chemical reagent to inhibit NAD-dependent ligase activity and a false substrate molecule.

The addition of the nucleic acid substrate is after the NAD-dependent ligase has been inactivated or removed to prevent the NAD-dependent ligase from acting on the substrate.

The sample is then incubated under conditions suitable for nucleic acid modifying activity. This may involve incubation at an optimum temperature for nucleic acid modifying activity. For example, the sample may be incubated at a temperature between around 15 and 40 degrees Celsius, such as around 37 degrees Celsius. This may be for any suitable period of time, for example between 5 and 60 minutes, such as around 5, 10, 15, 20, 25 or 30 minutes. Following this (i.e. at some point prior to step e of the method, which may be after step c or step d), the nucleic acid modifying activity may be inactivated prior to the modified nucleic acid molecule detection step. This may be achieved by elevating the temperature, for example to a temperature above 60 degrees Celsius, such as 95 degrees Celsius for a suitable time period. This may be a relatively short time period such as 1, 2, 3, 4, 5, 10, 15 or more minutes.

Step d requires treating the sample, for example by adding UDG to the sample or heating the sample if a substrate is used which incorporates a heat-labile complementary strand or adding a nuclease if a protected first and second strand is utilised in order to render the complementary strand no longer a template strand. The complementary strand of the nucleic acid substrate may comprise uracil bases such that upon treatment with uracil DNA glycosylase (UDG) enzyme the complementary strand is no longer a template strand. This ensures that, during the subsequent detection steps, typically a subsequent amplification reaction (a step in ETGA assays), the polymerase used in the amplification reaction is unable to amplify the complementary strand using the first strand as a primer.

The method then requires performing a nucleic acid amplification step in order to amplify any ligated first and second strand in the sample (due to ligase activity in the sample, typically ATP-dependent ligase activity because the NAD-dependent ligase activity has already been removed or deactivated) and/or extended first strand in the sample (due to polymerase activity in the sample). Amplification techniques are discussed in greater detail herein and include PCR, specifically quantitative PCR.

The final step of the method relies upon detecting and discriminating the amplification products to detect polymerase and/or ligase activity in a sample. The presence of polymerase activity in the sample but no ligase activity indicates the presence of bacteria in the sample but not fungi. This is because the bacterial NAD-dependent ligase activity has already been removed/inactivated. If fungi were present in the sample, ATP-dependent ligase activity would be detected.

The presence of ligase activity in the sample indicates the presence of fungi. Bacterial NAD-dependent ligase activity has already been removed/inactivated in the method and so the detection and/or quantification of ligated nucleic acid substrate indicates a fungal source of (ATP-dependent) ligase activity.

The presence of both polymerase and ligase activity in the sample indicates the presence of fungi. As already indicated, bacterial NAD-dependent ligase activity has already been removed/inactivated in the method and so the detection and/or quantification of ligated nucleic acid substrate indicates a fungal source of (ATP-dependent) ligase activity. The presence of polymerase activity in addition to the ligase activity could be provided either by fungi or bacteria.

It is also possible that the relative levels of nucleic acid modifying activity will assist in discriminating the type of microorganism in the sample. The inventors have observed that fungal ligase activity may be significantly faster than polymerase activity. Thus, relative levels (of the extended and ligated products) may permit discrimination of fungi from bacteria. For example a sample containing predominantly ligase activity but also limited polymerase activity is likely to contain only fungi, whereas a sample containing more similar levels of ligase and polymerase activity is likely to be a mixed sample containing both fungi and bacteria. Relative levels can be determined with reference to

levels of amplification products, for example by a simple comparison of cycle threshold (Ct) value. Absolute quantification, using suitable standard curves, may also be possible if required.

In order to discriminate the ligation and extension products, step f of the method may comprise the addition of a first probe which hybridizes to a target sequence comprising the mismatched region contained within the second strand together with a second probe which hybridizes to a target sequence comprising the sequence corresponding to the mismatched region in the complementary strand. Discriminating amplification products from ligase or polymerase activity relies upon detecting hybridization of the probes to their respective target sequences. As discussed herein in further detail, which discussion applies mutatis mutandis, the probes may be differently labelled for example with detectably distinct fluorophores. The probes may be incorporated into amplification primers in some embodiments.

More generally, the invention provides a method of detecting the absence or presence of a micro-organism in a sample comprising:

a. adding a nucleic acid substrate as defined herein to the sample,
b. incubating the sample and substrate under conditions permissive of polymerase and/or ligase activity (if present in the sample); and
c. specifically determining the absence or presence of an extended or ligated nucleic acid substrate resulting from the action of the polymerase and/or ligase activity to indicate the absence or presence of the micro-organism.

A “sample” in the context of the present invention is defined to include any sample in which it is desirable to test for the presence of a microorganism, such as a fungus (e.g. a yeast) or a bacterium, expressing nucleic acid modifying activity. Thus the sample may comprise, consist essentially of or consist of a clinical sample, such as a blood sample. The methods of the invention are particularly applicable to the rapid determination of negative blood cultures. Thus, the sample may comprise a blood culture sample from a patient suspected of suffering from, or being screened for, a bloodstream infection. The sample may be any suitable volume such as 1 to 10ml, preferably a 1ml blood culture sample.

In some embodiments the sample may be or comprise an *in vitro* assay system for example. Samples may comprise, consist essentially of or consist of beverage or food samples or preparations thereof, or pharmaceutical or cosmetic products such as personal care products including shampoos, conditioners, and moisturisers etc., all of which are tested for microbial contamination as a matter of routine. The sample may comprise, consist essentially of or consist of tissue or cells and may comprise, consist essentially of or consist of a sputum or a blood sample or a platelet sample for example. In addition, the methods and kits of the invention may be used to monitor contamination of surfaces, such as for example in locations where food is being prepared. Contamination is indicated by the presence of microbial nucleic acid modifying activity. The contamination may be from any microbial source, in particular bacterial or fungal (e.g yeast) contamination. Furthermore, the invention is also useful in monitoring environmental conditions such as water supplies, wastewater, marine environments etc. The invention is also useful in monitoring bacterial growth in fermentation procedures and in air sampling where bacteria or spore content can be assessed in hospital, industrial facilities or in biodefence applications.

In certain embodiments, the sample contains a non-microorganism source of polymerase and/or ligase activity and the method comprises deactivating the non-micro-organism source of polymerase and/or ligase activity prior to adding the nucleic acid substrate to the sample. The source may comprise mammalian cells, such as blood cells in some embodiments.

In specific embodiments, deactivating the non-microorganism source of polymerase and/or ligase activity prior to adding the nucleic acid substrate to the sample comprises treating the sample under high pH conditions in a manner such that micro-organism polymerase and/or ligase activity is not significantly affected. The duration of the high pH conditions is typically less than 20 minutes and may be not more than 10, 9, 8, 7, 6 or 5 minutes and may be around 5, 6, 7, 8, 9 or 10 minutes. In yet further embodiments, the treatment is carried out for between around 2 and 15 minutes, such as around 5 minutes. By “around” is meant plus or minus 30 seconds.

Any suitable reagent may be added to the sample in order to provide high pH conditions. In particular embodiments, the high pH conditions comprise contacting the sample with

an alkali. In particular embodiments, NaOH or Na2CO3 is used. In specific embodiments, the concentration of the NaOH or Na2CO3 is around 5mM or greater.

The high pH conditions typically inhibit the activity of nucleic acid modifying enzymes including ATP-dependent ligase and polymerases from non-microorganism sources such as mammalian cells, but do not inhibit the activity of the microbial ligases or polymerases. This may be due to the greater resistance of microbial enzymes to these conditions and/or to differential lysis conditions employed in the methods to ensure that only the non-microorganism enzymes are exposed to the high pH conditions. High pH is generally a pH of at least around 10, such as around 10, 11, 12, 13 or 14. Low pH is generally a pH of less than or equal to around 4, such as around 4, 3, 2, or 1. By "around" is meant 0.5 of a pH unit either side of the stated value. Altering the pH of the sample may be achieved using any suitable means, as would be readily appreciated by one skilled in the art. Microbial enzymes such as polymerases and ligases may be resistant to extremes of pH, whereas mammalian ligases may be inactivated under the same pH conditions. This permits selective detection of microbial ligases in a sample containing both mammalian cells and microbial cells. In specific embodiments, the conditions that inhibit the activity of non-microorganism nucleic acid modifying activity, such as ATP-dependent ligase, from mammalian cells but which do not inhibit the activity of the microorganism source of nucleic acid modifying activity, such as microbial ligases, comprise treating the sample with sodium hydroxide (NaOH) or sodium carbonate (Na2CO3). Such agents can readily be used, as shown herein, to increase the pH of the sample to high pH thus inactivating mammalian ligase activity whilst leaving the microbial (fungal and bacterial) ligases active. Suitable concentrations and volumes of the appropriate agent can be applied by a skilled person. In certain embodiments, however, the NaOH is at least around 5mM NaOH.

In further embodiments, the pH is around 12 to inactivate mammalian nucleic acid modifying activity (such as polymerase and/or ATP-dependent ligase activity), but not microbial nucleic acid modifying activity (such as polymerase and/or ligase activity). In specific embodiments, pH conditions may be increased to at least around 11, or at least 11.2. This treatment may result in lysis of micro-organisms in the sample and thus lead to nucleic acid modifying activity (e.g. polymerase and/or ligase) release into the sample. This permits detection of nucleic acid modifying activity (e.g. polymerases and/or ligases)

in the sample, originating from the micro-organism, without the need for a separate cell lysis step. Under these conditions, mammalian ligases (such as blood ATP-dependent ligases) are inactivated. However, typically the methods include a separate step for lysing microorganisms in the sample, as discussed in greater detail herein below.

In some embodiments, the treatment under high pH conditions is stopped by adding a reagent to lower the pH. Suitable reagents include a buffer and/or an acid. In specific embodiments, the buffer comprises a Tris-HCl buffer (e.g. pH 7.2 or 8). Other suitable agents for lowering the pH include acids such as hydrochloric acid (HCl) and sulphuric acid (H_2SO_4). These (and other) acids may be incorporated into a buffer as would be readily appreciated by one skilled in the art.

In all methods of the invention specifically determining the absence or presence of the extended and/or ligated nucleic acid molecule may comprise, consist essentially of or consist of a nucleic acid amplification step. This serves to make the methods of the invention maximally sensitive. Such amplification techniques are well known in the art, and include methods such as PCR (in particular qPCR), NASBA (Compton, 1991), 3SR (Fahy et al., 1991), Rolling circle replication, Transcription Mediated Amplification (TMA), strand displacement amplification (SDA) Clinical Chemistry 45: 777-784, 1999, the DNA oligomer self-assembly processes described in US6261846 (incorporated herein by reference), ligase chain reaction (LCR) (Barringer et al., 1990), selective amplification of target polynucleotide sequences (US 6410276), arbitrarily primed PCR (WO 90/06995), consensus sequence primed PCR (US 4437975), invader technology, strand displacement technology and nick displacement amplification (WO 2004/067726). The list above is not intended to be exhaustive. Any nucleic acid amplification technique may be used provided the appropriate nucleic acid product is specifically amplified. Similarly, sequencing based methodologies may be employed in some embodiments to include any of the range of next generation sequencing platforms.

Amplification is achieved with the use of amplification primers specific for the sequence of the modified nucleic acid molecule which is to be detected. In order to provide specificity for the nucleic acid molecules primer binding sites corresponding to a suitable region of the sequence may be selected. The skilled reader will appreciate that the nucleic acid molecules may also include sequences other than primer binding sites

which are required for detection of the novel nucleic acid molecule produced by the modifying activity in the sample, for example RNA Polymerase binding sites or promoter sequences may be required for isothermal amplification technologies, such as NASBA, 3SR and TMA.

One or more primer binding sites may bridge the boundary of the mismatch region of the substrate nucleic acid molecule such that an amplification product is only generated if one or other of ligation/extension has occurred, for example. Thus, two separate primers may be needed to detected both the ligation and extension products. Alternatively, primers may bind either side of the mismatch region and direct amplification of this region with probe based detection of the nature of the mismatch. Primers and the substrate nucleic acid molecule(s) may be designed to avoid non-specific amplification (e.g. of genomic DNA in the sample).

The primers may incorporate synthetic nucleotide analogues as appropriate or may be RNA or PNA based for example, or mixtures thereof. The primers may be labelled, such as with fluorescent labels and/or FRET pairs, depending upon the mode of detection employed. Probes may be utilised, again which may be labelled, as desired.

Thus, in certain aspects, the methods of the invention are carried out using nucleic acid amplification techniques in order to detect the extended or ligated nucleic acid molecule produced as a direct result of the action of nucleic acid-modifying activity on the substrate nucleic acid molecule which indicates the presence of a microorganism in the sample. In certain embodiments the technique used is selected from PCR (including qPCR), NASBA, 3SR, TMA, SDA and DNA oligomer self-assembly.

Detection of the amplification products may be by routine methods, such as, for example, gel electrophoresis but in some embodiments is carried out using real-time or end-point detection methods. Preferably quantitative methods are employed.

A number of techniques for real-time or end-point detection of the products of an amplification reaction are known in the art. These include use of intercalating fluorescent dyes such as SYBR Green I (Sambrook and Russell, Molecular Cloning - A Laboratory Manual, Third edition), which allows the yield of amplified DNA to be estimated based

upon the amount of fluorescence produced. Many of the real-time detection methods produce a fluorescent read-out that may be continuously monitored; specific examples including molecular beacons and fluorescent resonance energy transfer probes. Real-time and end-point techniques are advantageous because they keep the reaction in a "single tube". This means there is no need for downstream analysis in order to obtain results, leading to more rapidly obtained results. Furthermore keeping the reaction in a "single tube" environment reduces the risk of cross contamination and allows a quantitative output from the methods of the invention. This may be particularly important in the context of the present invention where health and safety concerns may be of paramount importance (such as in detecting potential microbial infection in a patient samples for example).

Real-time and end-point quantitation of PCR reactions may be accomplished using the TaqMan® system (Applied Biosystems), see Holland et al; Detection of specific polymerase chain reaction product by utilising the 5'-3' exonuclease activity of Thermus aquaticus DNA polymerase; Proc. Natl. Acad. Sci. USA 88, 7276-7280 (1991), Gelmini et al. Quantitative polymerase chain reaction-based homogeneous assay with flurogenic probes to measure C-Erb-2 oncogene amplification. Clin. Chem. 43, 752-758 (1997) and Livak et al. Towards fully automated genome wide polymorphism screening. Nat. Genet. 9, 341-342 (19995) (incorporated herein by reference). This type of probe may be generically referred to as a hydrolytic probe. Suitable hydrolytic/Taqman probes for use in real time or end point detection are also provided. The probe may be suitably labelled, for example using the labels detailed herein.

In the Molecular Beacon system, see Tyagi & Kramer. Molecular beacons - probes that fluoresce upon hybridization. Nat. Biotechnol. 14, 303-308 (1996) and Tyagi et al. Multicolor molecular beacons for allele discrimination. Nat. Biotechnol. 16, 49-53 (1998) (incorporated herein by reference), the beacons are hairpin-shaped probes with an internally quenched fluorophore whose fluorescence is restored when bound to its target. These probes may be referred to as hairpin probes.

A further real-time fluorescence based system which may be incorporated in the methods of the invention is the Scorpion system, see Detection of PCR products using self-probing amplicons and fluorescence by Whitcombe et al. Nature Biotechnology 17,

804 - 807 (01 Aug 1999). Additional real-time or end-point detection techniques which are well known to those skilled in the art and which are commercially available include Lightcycler® technology, Amplifluour® primer technology, DzyNA primers (Todd et al., Clinical Chemistry 46:5, 625-630 (2000)), or the Plexor™ qPCR and qRT-PCR Systems.

Thus, in further aspects of the invention the products of nucleic acid amplification are detected using real-time or end point techniques. In specific embodiments of the invention the real-time technique consists of using any one of hydrolytic probes (the Taqman® system), FRET probes (Lightcycler® system), hairpin primers (Amplifluour® system), hairpin probes (the Molecular beacons system), hairpin probes incorporated into a primer (the Scorpion® probe system), primers incorporating the complementary sequence of a DNAzyme and a cleavable fluorescent DNAzyme substrate (DzYNA), Plexor qPCR and oligonucleotide blocking systems.

Amplification products may be quantified to give an approximation of the microbial nucleic acid modifying activity in the sample and thus the level of microorganisms in the sample. Thus, “absence or presence” is intended to encompass quantification of the levels of microorganisms in the sample.

In certain embodiments, the reaction mixture will contain all of; the sample under test, the substrate nucleic acid molecule(s), reagents, buffers and enzymes required for amplification of the modified nucleic acid molecule optionally in addition to the reagents required to allow real time or end-point detection of amplification products. Thus the entire detection method for the nucleic acid modifying activity (from the one or more bacterial cells or micro-organisms of interest) may occur in a single reaction, with a quantitative output, and without the need for any intermediate washing steps. Use of a “single tube” reaction is advantageous because there is no need for downstream analysis in order to obtain results, leading to more rapidly obtained results. Furthermore keeping the reaction in a “single tube” environment reduces the risk of cross contamination and allows a quantitative output from the methods of the invention. Also, single tube reactions are more amenable to automation, for example in a high throughput context.

Alternatively, the methods of the invention may be carried out in step-wise fashion. Thus, in a first step it may first be necessary to prepare the sample in a form suitable for use in the method of the invention. For example, as discussed herein, selective cell lysis or increasing cellular permeability may be required. Capture of specific nucleic acid modifying activity, such as polymerase or ligase, may also be desirable again as described herein. Other (sources of) nucleic acid modifying activity, such as nuclease activity, may be inhibited etc.

The methods of the invention are intended to assist in determining the absence or presence of viable microorganisms in a sample rapidly and economically. The methods are much quicker than conventional blood culture testing and may benefit from an indication of when the blood culture can be sampled in order to perform the ETGA methods. To this end, the invention further provides a blood culture bottle for micro-organism culture comprising:

a. an opening to which is added culture medium and a sample that may contain a micro-organism
b. a visual indicator of time elapsed since the sample was added to the culture medium, wherein the visual indicator indicates a pre-determined period of time at the end of which the sample is ready for further testing.

Any suitable visible indicator may be employed. In specific embodiments, the visible indicator comprises a light source such as a light emitting diode (LED). In certain embodiments, the visual indicator changes colour after the pre-determined period of time is reached. For example, the colour change may be from red to green. The pre-determined period of time may be set according to requirements. In specific embodiments, the period of time is set between (and including) around 4 to 16 hours, such as 6 to 12 hours, or 4, 5, 6, 7, 8, 9, 10, 11 or 12 hours. By “around” is meant plus or minus 30 minutes.

The methods of the invention may include culturing the sample in a blood culture bottle as described herein prior to performing the method.

DESCRIPTION OF THE FIGURE

Figure 1 is a schematic representation of a "multi-detection" nucleic acid substrate permitting detection and identification of ligase and polymerase activity.

DETAILED DESCRIPTION

Figure 1 shows a nucleic acid substrate (1) of the invention. The multi-detection substrate consist of three oligonucleotides; a first single strand (2), a second single strand downstream of the first strand (3) and a strand complementary to the first and second strands (4). The first strand (2) is complementary to the 3' end region of the complementary strand (4). The second strand (3) is also complementary to the complementary strand (4) apart from a short internal region, typically of 6-12 bases, that is not complementary ((5) and shown in red); the 5' and 3' ends of the second strand (3) therefore still base pair strongly with the complementary strand (4). The second strand (3) also carries a 5' phosphate modification (allowing ligation). The complementary strand (4) contains uracil bases that can be removed using UDG enzyme.

When exposed to a mixture containing a ligase, the first strand (2) will be ligated to the second strand (3) creating a template containing the mismatched sequence; this is the ligated substrate.

When exposed to a sample containing polymerase, the second strand (3) will be displaced (or proof-read and corrected) by the polymerase extending the DNA strand from the first strand (2) using the complementary strand (4) as the template; this is the polymerase extended substrate.

When added to a PCR amplification reaction containing UDG enzyme and different fluorescent probes specific for the ligated substrate (eg. Cy3 labelled) and the polymerase extended substrate (e.g. FAM labelled), it will be possible to identify whether the template was generated by polymerase or ligase. UDG enzyme must be used prior to the PCR amplification steps to prevent Taq polymerase from extending the DNA strand from the first strand (2) to create an amplifiable template.

Discrimination of bacteria and fungi

In a preparation reaction that disables the activity of NAD-dependent ligase (only produced by bacteria), but allows the activity of ATP-dependent ligase (only produced by eukaryotes, including fungi) it would be possible to use the result of the multi-detection substrate to determine whether the ligase or polymerase reaction has occurred, and hence the presence of bacteria (polymerase activity only) or fungi (a mixture of polymerase and ligase activity, or just ligase activity).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Moreover, all embodiments described herein are considered to be broadly applicable and combinable with any and all other consistent embodiments, as appropriate.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

1. A nucleic acid substrate for ligase and/or polymerase activity comprising:
   a. a first strand
   b. a second strand (downstream in the 5'-3' direction of the first strand)
   c. a complementary strand that is complementary to the first and second strands such that the first and second strands hybridize with the complementary strand to produce a substantially double stranded structure

wherein the first strand acts as a primer for polymerase activity and the complementary strand acts as a template strand to thus extend the first strand in the presence of polymerase activity

wherein the 5' end of the second strand is phosphorylated to permit ligation of the 3' end of the first strand and the 5' end of the second strand in the presence of ligase activity

wherein the second strand contains a sequence that does not base pair with the complementary strand sequence such that there is a (short) mismatched region in the substantially double stranded structure

wherein the complementary strand is sensitive to treatment to render it no longer a template strand, whereas the first and second strands are resistant to such treatment.

2. The substrate of claim 1 wherein the complementary strand comprises uracil bases such that upon treatment with uracil DNA glycosylase (UDG) enzyme the complementary strand is no longer a template strand.

3. The substrate of claim 1 or 2 wherein the mismatched region:
   a. is not found within 20 nucleotides of the ends of the second strand; and/or
   b. is between (and including) 6-12 nucleotides in length.

4. The substrate of any one of claims 1 to 3 wherein in the presence of polymerase activity the complementary (template) strand is copied thus displacing the second strand from the substantially double stranded structure.

5. The substrate of any one of claims 1 to 4 wherein the first and second strands are sense strands and the complementary strand is an antisense strand.

6. The substrate of any one of claims 1 to 4 wherein the first and second strands are antisense strands and the complementary strand is a sense strand.

7. Use of a substrate of any one of claims 1 to 6 for detecting polymerase and/or ligase activity in a sample.

8. A kit for detecting the presence or absence of polymerase and/or ligase activity in a sample comprising:

a. a nucleic acid substrate according to any one of claims 1 to 6
b. a probe which hybridizes to a target sequence comprising the mismatched region contained within the second strand; and/or
c. a probe which hybridizes to a target sequence comprising the sequence corresponding to the mismatched region in the complementary strand; and/or
d. a first primer which hybridizes to the product of ligation between the first and second strands and/or to the product of polymerization from the first strand acting as primer and the complementary strand acting as template and directs amplification of the product of ligation/polymerization; and/or
e. a second primer which hybridizes to the amplification product resulting from use of the first primer and directs further amplification of that amplification product; and/or
f. uracil DNA glycosylase and/or a nuclease.

9. The kit of claim 8 wherein the probe b and/or probe c is labelled.

10. The kit of claim 8 or 9 which contains both probe b and probe c and in which each probe is differently labelled.

11. The kit of any one of claims 8 to 10 further comprising reagents for deactivating NAD-dependent ligase activity.

12. The kit of any one of claims 8 to 11 further comprising one or more of a polymerase, dinucleotide triphosphates, $MgCl_2$ and buffer.

13. Use of a kit of any one of claims 8 to 12 for detecting polymerase and/or ligase activity in a sample.

14. A method for detecting polymerase and/or ligase activity in a sample, comprising:

a. adding a nucleic acid substrate as defined in any one of claims 1 to 6 to the sample
b. incubating the sample and substrate under conditions permissive of polymerase and/or ligase activity (if present in the sample)
c. treating the sample in order to render the complementary strand no longer a template strand
d. performing a nucleic acid amplification step in order to amplify any ligated first and second strand in the sample (due to ligase activity) and/or extended first strand in the sample (due to polymerase activity)
e. detecting the amplification products to detect polymerase and/or ligase activity in the sample.

15. The method of claim 14 wherein step c comprises adding UDG to the sample in order to render the complementary strand no longer a template strand.

16. The method of claim 14 or 15 wherein step e comprises:

a. as part of the detection, discriminating amplification products from ligase or polymerase activity; and/or
b. the addition of a first probe which hybridizes to a target sequence comprising the mismatched region contained within the second strand together with a second probe which hybridizes to a target sequence comprising the sequence corresponding to the mismatched region in the complementary strand and wherein discriminating amplification products from ligase or polymerase activity relies upon detecting hybridization of the probes to their respective target sequences.

17. The method of claim 16 wherein the probes are differently labelled.

18. The method of anyone of claims 14 to 17 which comprises prior to step a removing from the sample or deactivating in the sample any NAD-dependent ligase activity.

19. A method of detecting the absence or presence of a micro-organism in a sample comprising performing a method of any one of claims 14 to 18 and using the detection of polymerase and/or ligase activity in the sample as an indicator of the absence or presence of a micro-organism in the sample.

20. The method of claim 19 wherein the micro-organism is a bacterium or a fungus.

21. A method for detecting and discriminating bacteria and/or fungi in a sample, comprising:

a. removing from the sample or deactivating in the sample any NAD-dependent ligase activity
b. adding a nucleic acid substrate as defined in any one of claims 1 to 6 to the sample
c. incubating the sample and substrate under conditions permissive of polymerase and/or ligase activity (if present in the sample)
d. treating the sample in order to render the complementary strand no longer a template strand
e. performing a nucleic acid amplification step in order to amplify any ligated first and second strand in the sample (due to ligase activity) and/or extended first strand in the sample (due to polymerase activity)
f. detecting and discriminating the amplification products to detect polymerase and/or ligase activity in a sample, wherein the presence of polymerase activity in the sample but no ligase activity indicates the presence of bacteria in the sample but not fungi and/or wherein the presence of ligase activity in the sample indicates the presence of fungi and/or wherein the presence of both polymerase and ligase activity in the sample indicates the presence of fungi and possibly also bacteria.

22. The method of claim 21 wherein step d comprises adding UDG to the sample.

23. The method of claim 21 or 22 wherein step f comprises the addition of a first probe which hybridizes to a target sequence comprising the mismatched region contained within the second strand together with a second probe which hybridizes to a target sequence comprising the sequence corresponding to the mismatched region in the complementary strand and wherein discriminating amplification products from ligase or polymerase activity relies upon detecting hybridization of the probes to their respective target sequences, optionally wherein the probes are differently labelled.

24. A method of detecting the absence or presence of a micro-organism in a sample comprising:

a. adding a nucleic acid substrate as defined in any one of claims 1 to 6 to the sample,
b. incubating the sample and substrate under conditions permissive of polymerase and/or ligase activity (if present in the sample); and
d. specifically determining the absence or presence of an extended or ligated nucleic acid substrate resulting from the action of the polymerase and/or ligase activity to indicate the absence or presence of the micro-organism.

25. The method of any one of claims 14 to 24 wherein the sample is a clinical sample.

26. The method according to any one of claims 14 to 25 wherein the sample contains a non-micro-organism source of polymerase and/or ligase activity and the method comprises deactivating the non-micro-organism source of polymerase and/or ligase activity prior to adding the nucleic acid substrate to the sample.

27. The method according to claim 26 wherein deactivating the non-micro-organism source of polymerase and/or ligase activity prior to adding the nucleic acid substrate to the sample comprises treating the sample under high pH conditions in a manner such that micro-organism polymerase and/or ligase activity is not significantly affected.

28. The method of claim 27 wherein the sample is treated under high pH conditions for no more than 5 minutes.

29. A blood culture bottle for micro-organism culture comprising:

a. an opening to which is added culture medium and a sample that may contain a micro-organism

b. a visual indicator of time elapsed since the sample was added to the culture medium, wherein the visual indicator indicates a pre-determined period of time at the end of which the sample is ready for further testing.

30. The blood culture bottle of claim 29 wherein the visual indicator comprises an LED.

31. The blood culture bottle of claim 29 or 30 wherein the visual indicator changes colour after the pre-determined period of time is reached.

32. The blood culture bottle of claim 31 wherein the colour change is from red to green.

33. The blood culture bottle of anyone of claims 29 to 32 wherein the pre-determined period of time is set between (and including) 4 to 16 hours.

34. The method of any one of claims 14 to 28 wherein the clinical sample is cultured in a blood culture bottle of any one of claims 29 to 33 prior to performing the method.

35. A nucleic acid substrate as defined herein with reference to the accompanying drawing.

36. A kit as defined herein with reference to the accompanying drawing.

37. A method as defined herein with reference to the accompanying drawing.

38. A blood culture bottle as defined herein with reference to the accompanying drawing.

Intellectual Property Office

Application No:	GB1412310.3	**Examiner:**	Dr Patrick Purcell
Claims searched:	1-28, 34-37	**Date of search:**	2 April 2015

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	14, 21, 24-26	WO2009/007719 A (ISEAO TECHNOLOGIES LIMITED) see whole document, esp. page 3, line 6-page 7, line 3, page 15, line 22-page 17, line 16, page 18, lines 5-25, page 24, line 21-page 26, line 6
X	14, 21, 24-26	Nucleic Acids Research, Vol 40, 2012, DR Zweitzig et al, "Characterization of a novel DNA polymerase activity assay sensitive, quantitative and universal detection of viable microbes", e109 see whole document, esp. pages 2-4, Materials and Methods, page 5, column 2 & Fig 1, available online at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3413125/pdf/gks316.pdf
A		Transfusion, Vol 54, 2014, DR Zweitzig et al, "A novel approach for rapid detection of bacterially contaminated platelet concentrates via sensitive measurement of microbial DNA polymerase activity", 1642-51. Epub 2013 Nov 5. available online at http://onlinelibrary.wiley.com/doi/10.1111/trf.12487/abstract
A		WO2012/094343 A (TRILINK BIOTECHNOLOGIES)
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A		US5976806 A (MAHAJAN ET AL)
A		WO01/29211 A (ENCHIRA BIOTECHNOLOGY CORP.)
A		BMC Microbiology, Vol 2, 2002, E Busti et al, "Bacterial discrimination by means of a universal array approach mediated by LDR (ligase detection reaction)", 27 Available online at http://www.biomedcentral.com/content/pdf/1471-2180-2-27.pdf

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X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

C12N; C12Q

The following online and other databases have been used in the preparation of this search report

ONLINE: EPODOC, WPI, BIOSIS, MEDLINE

International Classification:

Subclass	Subgroup	Valid From
C12Q	0001/68	01/01/2006
C12N	0015/11	01/01/2006
C12Q	0001/04	01/01/2006