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(54) **Title:** DETECTION AND IDENTIFICATION OF ORGANISMS VIA ANALYSIS OF NUCLEIC ACID SEQUENCES

(57) **Abstract:** The present invention relates to methods, compositions, and kits for identifying one or more organisms in a sample. The invention uses a combination of nucleic acid amplification, detectable labels, and restriction enzyme digestion of the amplified nucleic acid.

DETECTION AND IDENTIFICATION OF ORGANISMS VIA ANALYSIS OF NUCLEIC ACID SEQUENCES

[001] The present application claims priority to U.S. Provisional Serial No. 60/850,656, entitled “Detection and Identification of Organisms via Analysis of Nucleic Acid Sequences” filed on October 11, 2006, and which is incorporated herein by reference.

[002] The present invention, in various embodiments, relates to methods, compositions, and kits for detecting and identifying organisms in a sample by identifying single or multiple nucleotide variations in a nucleotide sequence of interest. The methods combine amplifying target nucleic acid sequences, digesting the nucleic acids with restriction enzymes and identifying the organism(s)/strain(s) in the sample based on the pattern of digestion predicted from the target nucleotide sequences. The kits and compositions provide the reagents needed to perform these methods. The present invention can be used on nucleic acids prepared from different types of organisms.

[003] Current methods for detecting and identifying organisms are often time-consuming. In some circumstances, the speed with which an organism can be identified is very important. For example, the faster a physician can diagnose an infection, the faster that physician can properly treat the infection and increase the likelihood of a successful outcome for the patient. Not only does the physician need to know what organism causes the infection, but he also needs to know whether the strain of organism is likely to be resistant to particular treatment regimes. Moreover, the faster public authorities can detect an organism in the environment, the more

quickly these authorities can make informed decisions on issues affecting public health. This is especially true for organisms used in bioterrorism such as anthrax and for organisms, such as the H5N1 strain of influenza also known as “bird flu,” that may one day cause a global pandemic. In addition, identifying specific strains in environmental samples will significantly reduce false positive results that could be generated from closely related agents. Rapid identification of strains will expedite the investigative process by security forces.

[004] In the case of bacteria, there are several detection and identification methods available including the use of selective bacterial media. This technique is limited in many ways. First, the length of time required to grow colonies on agar does not permit rapid detection, which is problematic as discussed above. Second, such methods are not particularly sensitive. If the bacteria are present in a sample in a number too small to form a colony, methods of detection that rely on colony growth would not detect bacteria in that sample. Moreover, selective media methods often require the use of several different media before an identification can be made. To identify a bacterium, clinicians often begin with a few selective media and based on results with those media proceed to plate the sample on additional types of selective media. Based on the pattern of growth on the media used, the clinician determines which bacteria are present in the sample. In this system, however, waiting for the results of growth on one selective medium before proceeding to the next medium based on that result can lead to long diagnosis times. Conversely, the clinician may try to minimize diagnosis time by using all available selective growth media for simultaneous testing. This strategy leads to a wasteful use of growth media. By using

a nucleic acid based method that identifies one or more organisms simultaneously, the invention can provide a higher sensitivity and a faster diagnosis time than media-based methods.

[005] Other current detection and identification methods for organisms include immunoassays using polyclonal or monoclonal antibodies. Immunoassays use polyclonal or monoclonal antibodies that specifically bind to a particular organism. Though these assays decrease the time needed to detect an infection, they nonetheless suffer from a lack of specificity. Antibodies raised to one organism can cross-react with other related organisms. Moreover, immunoassays require the production of specialized reagents for each particular organism to be detected. The present invention employs a minimal number of reagents to maximize the amount of information on organisms present in a sample. In the present invention, there is no need to prepare a reagent that is specifically designed to detect each organism.

[006] Finally, samples suspected of harboring an organism may also be tested for the ability to cause disease in animals. Although this technique improves the sensitivity of detecting organisms, it is costly and requires enough time for symptoms to develop in the animals. In addition, while animal tests may identify the microorganism generally, these tests do not provide information on the strain of organism present in the sample.

[007] Today, molecular biology techniques are also used to detect and identify organisms. These methods often rely on using reagents, such as PCR primers or probes for use in Southern or Northern blots, that bind to sequences that differ between the organisms to be identified. But these types of molecular assays can be

difficult to design when the genomes of the organisms to be identified are homologous to that of other species. For example, the *B. anthracis* genome is so similar to that of *B. cereus* and *B. thuringiensis* that these organisms were initially proposed to be the same species. Depending on the primer or probe length and composition, small differences in organism genome sequence may not prevent the probe or PCR primer from binding to a genomic sequence. The present invention solves this problem by using restriction enzymes to digest nucleic acid sequences amplified from an organism. Restriction enzymes digest nucleic acid on a sequence-specific basis and can detect a change of as little as one base pair at the enzyme's digestion site. This sequence sensitivity allows the invention to distinguish between very closely related organisms in a sample.

[008] Even if primer and probe binding were absolutely specific, these assays require a different primer set or probe for each organism to be detected. Thus, akin to antibody-based methods, a unique reagent would have to be made for each organism to be detected. In the case of PCR methods, each organism to be identified requires a set of primers that hybridize to a unique nucleic acid sequence present in each organism. Identifying these unique sequences for the purposes of identifying multiple organisms in a sample can be difficult and time-consuming. Moreover, PCR-based assays involve the time-consuming step of analyzing the products by gel electrophoresis to visualize the results. Similarly, blotting techniques also require time-consuming steps, including resolving DNA or RNA from an organism by gel electrophoresis and then detecting species-specific sequences with a labeled probe.

Although such methods may be used with the invention, the methods of the instant invention do not require analysis on a gel.

[009] In sum, prior methods that focus on the nucleic acid of an organism mainly depend on sequences that are specific to a particular organism. Primers that hybridize to such sequences are used to amplify or otherwise detect the sequences and determine the presence or absence of the organism. The utility of this approach is limited by the fact that it requires the identification of specific sequences that are unique to each organism of interest. Therefore, homologous sequences in closely related organisms can produce false positives. Furthermore, multiplexing to detect more than a single organism requires the design of several other primer pairs each of which are organism specific. For these reasons, such methods are not well suited to distinguishing between closely related organisms or between DNA sequences that have minimal variations.

[010] The instant invention facilitates the detection and identification of organisms in a sample by using a single set of reagents to identify a potentially large number of organisms. The invention circumvents the need to identify a unique nucleic acid sequence marker for each organism. In addition, the invention identifies an organism without the need for gel electrophoresis and allows for rapid results.

SUMMARY OF THE INVENTION

[011] The invention provides a nucleic acid-based technique that identifies one or more organisms in a sample. In embodiments where multiple organisms are identified, the organisms can be, for example, different strains of one organism, different species, or different genera. In some embodiments, the invention comprises

an amplification step using a pair of primers, one of which can be linked to a solid support and the other of which can be labeled with a detectable label. When a nucleic acid sequence of interest is present in the sample, amplification occurs. Following the amplification step, an aliquot of the amplicon(s) produced in the amplification reaction can be used in separate digestion reactions by exposing the amplicon(s) to a specific restriction enzyme in each reaction. If the appropriate digestion site is present in the amplicon(s), the restriction enzyme cuts the amplicon, thereby separating the detectable label from the rest of the amplicon, which can be attached to the solid support. Each organism gives rise to an amplicon with a nucleotide sequence that yields a unique restriction digestion pattern based on the set of restriction enzymes used. These unique digestion patterns for each organism can be used to specifically identify organisms in a sample.

[012] In some embodiments, the invention provides a method of identifying at least one organism in a sample comprising the steps of:

(a) forming a first composition comprising the sample, a first primer, and a second primer, wherein the first primer and the second primer can amplify one or more target nucleic acid sequences;

(b) incubating the first composition under conditions that permit at least one target nucleic acid sequence in the sample to be amplified to form a second composition comprising at least one amplicon;

(c) incubating all or a portion of the second composition with one or more enzymes that can cut DNA in a sequence-specific manner under conditions that allow the at least one enzyme to digest the at least one

amplicon if a recognition site for the enzyme is present in the at least one amplicon, wherein the at least one amplicon can be digested by at least one, but, if more than one enzyme is used, not all the enzymes, to generate an actual digestion pattern for each enzyme used;

(d) for each enzyme, determining whether or not digestion of the at least one amplicon occurred using at least one method other than gel electrophoresis;

(e) generating an actual digestion pattern of the at least one amplicon based on the determination in step (d) for each enzyme; and

(f) comparing the predicted digestion pattern for each enzyme to the actual digestion pattern of the at least one amplicon to identify at least one organism in the sample.

[013] In some embodiments, the presence of at least one amplicon in the sample can be detected before the at least one restriction enzyme is added. In certain embodiments, the presence of at least one amplicon in the sample can be determined at the same time the determination in step (d) is performed.

[014] Several techniques can be used to amplify a target nucleic acid sequence. In some embodiments, the target nucleic acid sequence can be amplified using polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), self-sustained sequence replication (SSSR), or Nucleic Acid Sequence-Based Amplification (NASBA®). In some embodiments, at least one target nucleic acid sequence can be amplified using a first primer and a second primer, wherein a portion of the at least one target nucleic acid sequence can

be substantially identical to (i) a portion of the first primer or its complement and (ii) a portion of the second primer or its complement. With respect to primers, the terms "first" and "second" are used for identification purposes only and do not indicate any particular order of the primers. In various embodiments, the first primer and the second primer can be used to amplify several target nucleic acid sequences in the sample. In some embodiments, the first primer can comprise a nucleic acid sequence that can be linked to a solid support. In some embodiments, the first primer can comprise a nucleic acid sequence and a solid support. In some embodiments, the second primer can comprise a detectable label. In some embodiments, the second primer can bind to a detectable label.

[015] In certain embodiments, a kit comprising one or more primers of the invention and at least one restriction enzyme can be used to practice the methods of the invention.

[016] Additional objects and advantages of the invention will be set forth in part in the description which follows or can be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[017] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[018] Figure 1 depicts one embodiment of the invention.

[019] Figure 2 shows a picture of amplification and digestion products derived from *K. oxytoca* (panel A) and *E. coli* (panel B) templates. Following amplification, aliquots of the amplicon were digested using BseMII, BbvI, SSPI, XmnI, Ase I and GsuI as indicated by the lane headers.

[020] Figure 3 shows bar graphs of the raw electrochemiluminescent measurement of the digested and undigested amplicons derived from *K. oxytoca* (A) and *E. coli* (B) chromosomal DNA. Following the amplification, aliquots of the amplicon were digested using BseMII, BbvI, SSPI, XmnI, Ase I and GsuI as indicated in the figure. The NC bar shows results without primer addition. The PC bar shows results without restriction enzyme addition.

[021] Figures 4A and 4B show bar graphs of measurements of the digested and undigested amplicons derived from 4 strains of *N. meningitidis*. Figure 4A shows raw electrochemiluminescent signals. Figure 4B normalizes these values as a percentage of the ratio of the difference of signal and the negative control to the difference of the positive and negative controls.

[022] Figure 5 shows a picture of amplification and digestion products derived from *N. meningitidis* templates. Following amplification, aliquots of the amplicon were digested using BseMII, BbvI, SSPI, XmnI, Ase I and GsuI as indicated by the panel headers.

DESCRIPTION OF THE INVENTION

I. Definitions

[023] It should be understood that the headings provided in this application are for organizational purposes only and in no way should be construed as limiting the description of the invention. The terms listed below are defined as follows:

[024] The term "**target nucleic acid**" refers to any nucleic acid that can be amplified to form an amplicon. Target nucleic acid can include, but is not limited to, chromosomal DNA, mitochondrial DNA, messenger RNA, ribosomal RNA, transfer RNA, and extrachromosomal DNA such as virulence plasmids.

[025] The term "**primer**" refers to a relatively short oligonucleotide that is complementary to a portion of the sequence of interest (the sequence of interest can be a fragment of a larger nucleic acid sequence). A primer can be the 5' terminus of one strand of the resulting extension product. A primer that is complementary at its 3' terminus to the target nucleic acid sequence on the template strand can be extended using a polymerase to synthesize a sequence complementary to the template. Modifications to the 3' end can affect the ability of an oligonucleotide to function as primer. An example of such a modification is the incorporation of a Locked Nucleic Acid (LNA) nucleotide, which can enhance the specificity of the primer (Latorra et al., *Hum. Mutat.* 22:79-85 2003). The length of the primer can be adjusted depending upon the particular application, but 15-30 nucleotides can be a common length. In some embodiments of the invention, the primer can be from about 6 to about 150 nucleotides in length. In some embodiments, the primer can be from about 10 to 40 nucleotides in length. In some embodiments, the primer can be from about 16 to 25

nucleotides in length. Primers can be used in pairs to amplify the nucleic acid sequence that falls between the two primer binding sites on the sequence of interest. In some embodiments, a primer can comprise at least 18 contiguous nucleotides from the sequence of interest. Primers can comprise nucleic acids, modified nucleic acids, and/or nucleic acid analogs.

[026] The term "**nucleic acid**" refers to a nucleobase sequence containing an oligomer, polymer, or polymer segment, having a backbone formed solely from naturally occurring nucleotides or unmodified nucleotides. Nucleic acids include, but are not limited to, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Non-limiting examples of naturally occurring nucleobases include: adenine, cytosine, guanine, thymine, and uracil.

[027] The term "**modified nucleic acid**" means an oligomer, polymer, or polymer segment comprising at least one modified nucleotide. Non-limiting examples of modified nucleotides include: 5-propynyl-uracil, 2-thio-5-propynyl-uracil, 5-methylcytosine, pseudoisocytosine, 2-thiouracil and 2-thiothymine, 2-aminopurine, N9-(2-amino-6-chloropurine), N9-(2,6-diaminopurine), hypoxanthine, N9-(7-deaza-guanine), N9-(7-deaza-8-aza-guanine) and N8-(7-deaza-8-aza-adenine). Other non-limiting examples of suitable modified nucleobases include those nucleobases illustrated in Figures 2(A) and 2(B) of Buchardt et al. (US 6,357,163).

[028] The term "**nucleic acid analog**" refers to synthetic molecules that can bind to a nucleic acid. For example, a nucleic acid analog primer can comprise peptide nucleic acids (PNAs), locked nucleic acids (LNAs), or any derivatized form of a nucleic acid. As used herein, "peptide nucleic acid" or "PNA" means any oligomer or

polymer comprising at least one or more PNA subunits (residues), including, but not limited to, any of the oligomer or polymer segments referred to or claimed as peptide nucleic acids in United States Patent Nos. 5,539,082; 5,527,675; 5,623,049; 5,714,331; 5,718,262; 5,736,336; 5,773,571; 5,766,855; 5,786,461; 5,837,459; 5,891,625; 5,972,610; 5,986,053; 6,107,470; 6,201,103; 6,228,982 and 6,357,163. The term PNA also applies to any oligomer or polymer segment comprising one or more subunits of those nucleic acid mimics described in the following publications: Lagriffoul et al., *Bioorg. Med. Chem. Lett.* 4: 1081-82 1994; Petersen et al., *Bioorg. Med. Chem. Lett.* 6:793-96 1996; Diderichsen et al., *Tett. Lett.* 37:475-78 1996; Fujii et al., *Bioorg. Med. Chem. Lett.* 7:637-40 1997; Jordan et al., *Bioorg. Med. Chem. Lett.* 7:687-90 1997; Krotz et al., *Tett. Lett.* 36:6941-44 1995; Lagriffoul et al., *Bioorg. Med. Chem. Lett.* 4:1081-82 1994; Diederichsen, U., *Bioorg. Med. Chem. Lett.* 7:1743-46 1997; Lowe et al., *J. Chem. Soc. Perkin Trans.* 11:539-46 1997; Lowe et al., *J. Chem. Soc. Perkin Trans.* 11:547-54 1997; Lowe et al., *J. Chem. Soc. Perkin Trans.* 11:555-60 1997; Howarth et al., *J. Org. Chem.* 62:5441-50 1997; Altmann, K-H et al., *Bioorg. Med. Chem. Lett.* 7:1119-22 1997; Diederichsen, U., *Bioorganic & Med. Chem. Lett.* 8:165-168 1998; Diederichsen et al., *Angew. Chem. Int. Ed.* 37:302-305 1998; Cantin et al., *Tett. Lett.* 38:4211-4214 1997; Ciapetti et al., *Tetrahedron* 53:1167-76 1997; Lagriffoule et al., *Chem. Eur. J.* 3:912-919 1997; Kumar et al., *Organic Letters* 3:1269-72 2001; and the Peptide-Based Nucleic Acid Mimics (PENAMs) of Shah et al. as disclosed in United States Patent No 5,705,333.

[029] The term "**locked nucleic acid**" or "**LNA**" refers to an oligomer or polymer comprising at least one or more LNA subunits. The term "**LNA subunit**"

means a ribonucleotide containing a methylene bridge that connects the 2'-oxygen of the ribose with the 4'-carbon. See generally, Kurreck, *Eur. J. Biochem*. 270:1628-44 2003.

[030] Bases in a primer can be joined by a linkage other than a phosphodiester bond, so long as it does not prevent hybridization. Thus, primers can have constituent bases joined by peptide bonds rather than phosphodiester linkages.

[031] A primer can bind to a target nucleic acid under certain conditions. In the context of two nucleic acid strands the term "**bind**" is synonymous with "**hybridize**" or "**anneal**". When two molecules hybridize, they can form a combination of the two molecules through one or more types of Watson-Crick or non-Watson-Crick base pairing. As used herein, the term "**complementary**" refers to nucleobases that can hybridize to each other. For example, adenine is complementary to thymine and cytosine is complementary to guanine.

[032] In certain embodiments, a primer can contain a mixture of nucleic acids, modified nucleic acids, or nucleic acid analogs as long as the primer specifically binds to its target sequence.

[033] In certain embodiments, a primer nucleotide sequence can be substantially identical to a portion of a polynucleotide sequence of interest, or its complement, such that the primer specifically binds to the portion of the organism's target nucleic acid sequence to be amplified. The term "**substantially identical**" means that two polynucleotides hybridize under high stringency conditions. The term "**high stringency**" generally refers to hybridization at 5 °C to 15 °C less than the temperature of dissociation or melting temperature (T_m). Those skilled in the art will

recognize that the T_m can be dependent upon, among other things, the polynucleotide's base pair composition, the length of the hybridized sequence, primer concentration, salt concentration, and on the solvent used. In the case of a PCR reaction, for example, high stringency conditions employ hybridization at 64 °C – 72 °C in a 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ solution.

[034] A primer need not be a perfect complement for successful hybridization and amplification to take place. For example, primers can contain some mismatched nucleotides in comparison to the organism's corresponding sequence and still specifically bind to the area of the organism's genome to be amplified. One skilled in the art can recognize that the optimum amount of identity between the primer and the target for successful specific amplification depends on a variety of readily controlled factors including the annealing temperature, the salt concentration, primer length, and the location of mismatches, if any. If the primer is an imperfect complement to the target, an extension product can result that incorporates the primer sequence, and during a later cycle, the complement to the primer sequence can be incorporated into the template sequence. In certain embodiments, a primer can incorporate any nucleic acid base, any modified nucleic acid, or any nucleic acid analog so that the primer extension product will incorporate these features to permit separation and detection of the primer extension product. While this amplification of imperfect complements is a detriment to prior art methods, in this invention specificity can be primarily achieved through the use of restriction enzymes. In some embodiments, imperfect complements can be expressly desired to be amplified, in order to reduce the number of unique primers.

[035] The terms "**restriction enzyme**" and "**restriction endonuclease**" are synonymous and refer to any of a group of enzymes that catalyze the sequence-specific cleavage of molecules that comprise nucleic acids, modified nucleic acids, and/or nucleic acid analogs.

[036] The term "**amplicon**" refers to a nucleotide sequence that can be amplified. In certain embodiments, an amplicon can be an amplified target nucleic acid sequence. In certain embodiments, the amplicon can be from about 50 base pairs to about 3000 base pairs in length or any length in between. In various embodiments, the amplicon can be from about 50 base pairs to about 1000 base pairs in length or any length in between. In some embodiments, the amplicon can be from about 50 base pairs to about 800 base pairs in length or any length in between. In various embodiments, the amplicon can be from about 50 base pairs to about 600 base pairs in length or any length in between. In certain embodiments, the amplicon can be from about 50 base pairs to about 400 base pairs in length or any length in between. In some embodiments, the amplicon can be from about 50 base pairs to about 200 base pairs in length or any length in between. In some embodiments, the amplicon can be from about 50 base pairs to about 100 base pairs in length or any length in between. In the case of primer-based methods of amplification, such as but not limited to PCR, an amplicon includes the sequences of the two primers and the sequence of the nucleic acid that lies between the two primer binding sites. Techniques for amplifying a DNA sequence are well known in the art. *See, e.g.*, Saiki R.K. et al., *Science* 230:1350-1354, 1985; Mullis et al. U.S. Pat. No. 4,683,195 and Mullis et al. U.S. Pat. No. 4,683,202.

[037] An amplicon nucleotide sequence can contain additions, deletions, or substitutions of nucleotide bases in comparison to the analogous sequence present in an organism's nucleic acid so long as the additions, deletions, or substitutions do not eliminate the recognition site for a restriction enzyme used in the methods of the invention. In some embodiments, an amplicon nucleotide sequence can contain from 0 to about 2, 0 to about 10, 0 to about 20, 0 to about 30, or 0 to about 40 nucleotide additions, deletions, or substitutions of nucleotide bases in comparison to the detectable portion of a polynucleotide sequence of interest.

[038] The term "**linked**" or "**linking**" refers to an association between two moieties. For example, hybridization can be a form of linking in that it involves the association of complementary oligonucleotides and/or polynucleotides.

[039] The term "**pair of binding partners**" or "**binding pair**" refers to a first entity that can bind (or become linked) to a second entity. In general, such complexes are characterized by a relatively high affinity and a relatively low to moderate capacity. Typically, specific interactions between the two members of a pair or binding partners can occur when the affinity constant Ka is higher than about 10^6 M^{-1}, or is higher than about 10^8 M^{-1}. A higher affinity constant indicates greater affinity, and thus greater specificity. For example, antibodies can bind antigens with an affinity constant in the range of 10^6 M^{-1} to 10^{12} M^{-1} or higher. If desired, nonspecific binding can be reduced without substantially affecting specific binding by varying the binding conditions using routine techniques known in the art. The conditions can be defined, for example, in terms of molecular concentration, ionic strength of the solution, temperature, time allowed for binding, or concentration of other molecules in a binding reaction.

[040] In some embodiments, a pair of binding partners can permit reversible linking between the primer and the solid support. In some embodiments, the interaction between the first member and the second member of a pair of binding partners can be (1) unstable at temperatures used for amplifying DNA, such that the first and second members will not stably bind to each other during the amplification reaction; and (2) stable at or below the temperature used for digesting DNA with one or more restriction enzymes. Once the DNA amplification reaction is complete, the reaction container can be cooled to a temperature at which the amplification products can bind to the solid support via the pair of binding partners.

[041] In some embodiments, a pair of binding partners can be nucleic acid sequences that are sufficiently complementary to each other but not complementary to a nucleic acid sequence in the amplicon. Sufficiently complementary nucleic acid sequences can hybridize as DNA/DNA hybrids, RNA/RNA hybrids, or DNA/RNA hybrids For example, in some embodiments, adenosine nucleotides in one DNA sequence can hybridize with thymidine nucleotides in another DNA sequence or uridine nucleotides in an RNA sequence. In some embodiments, guanine nucleotides in one DNA sequence can hybridize with cytosine nucleotides in another DNA sequence. In some embodiments, the nucleic acid sequences used for the pair of binding partners can be a mixture of nucleotides to provide, for example, the desired thermal stability and unique binding for measuring one or more target sequences. For example, binding between a small number of adenine and thymidine residues can be temperature sensitive. The temperature at which complementary nucleotide strands dissociate from each other is the melting temperature (T_m) of the strands. In general,

an approximate primer T_m can be calculated by adding 2 °C for each A or T in the primer and 4 °C for each G or C in the primer. Alternatively, T_m can be calculated using the following formulas. For sequences that are 13 or fewer nucleotides long: $T_m = (wA+xT) \times 2 + (yG+zC) \times 4$. For sequences that are 14 or more nucleotides long: $T_m = 64.9 + 41 \times (yG + zC - 16.4)/(wA + xT + yG + zC)$. In each formula, w, x, y, and z are the number of As, Ts, Gs, and Cs in the nucleotide sequence for which the T_m can be calculated. In certain embodiments, the T_m of a pair of binding partners can be lower than the lowest temperature used in an amplification reaction. When amplification is complete and the reaction tube cools to a temperature below the T_m of the binding partners, the pair of binding partners can bind, linking the amplicon to the solid support. In some embodiments, the solid support can be modified with a poly T tail and the primer can have a poly A tail, wherein the shorter of the two tails can be about 10, about 20, about 40, or about 50 residues long or any intermediate length. In various embodiments, the poly A tail can be linked to the solid support and the oligonucleotide primer can have a poly T tail at its 5'-end.

[042] In various embodiments, a pair of binding partners can be comprised of biotin and avidin or streptavidin. In some embodiments, the solid support can be derivatized with avidin and the primer can be biotinylated. In some embodiments, the solid support can be derivatized with streptavidin and the primer can be biotinylated. In some embodiments, the biotinylated primer can be linked to the solid support before the amplification reaction begins. In some embodiments, the primer can be covalently linked to the solid support via a crosslinker before the amplification reaction begins.

[043] The term "**solid support**" refers to a material that can be linked to a primer capable of hybridizing to a target nucleic acid sequence. Solid supports can be, but are not limited to, beads, membranes, synthetic organic polymers, microfuge tubes and inorganic oxides. Beads can be, but are not limited to, polystyrene beads. Beads can be magnetizable beads. The term "**magnetizable**" as used herein refers to a property of matter wherein the permeability of the matter differs from that of free space. Magnetizable beads include, but are not limited to, paramagnetic and superparamagnetic beads. Beads can also be metallic beads, including gold beads. In some embodiments, beads can have a diameter in the range of about 0.01 μm - about 100 μm, about 0.1 μm - about 50 μm, about 1 μm - about 20 μm, about 0.5 μm - about 10 μm, about 0.05 μm - about 5 μm, or about 1 μm - about 3 μm. Depending on the size of the bead, centrifugation techniques and/or filtration techniques can be used to isolate the amplicon from the amplification reaction.

[044] Membranes that can be used as solid supports in the invention comprise, for example, nitrocellulose, nylon, polyvinylidene fluoride (PVDF) or carboxylated polyvinylidene (U.S. Patent No.: 6,037,124). Membranes can be coated with various materials, including polyvinyl benzyl dimethyl hydroxyethyl ammonium chloride, polyvinyl benzyl benzoyl aminoethyl dimethyl ammonium chloride, polyvinyl benzyl tributyl ammonium chloride, copolymers of polyvinyl benzyl trihexyl ammonium chloride and polyvinyl benzyl tributyl ammonium chloride, copolymers of polyvinyl benzyl benzoyl dimethyl ammonium chloride and polyvinyl aminoethyl dimethyl ammonium chloride, and copolymers of polyvinyl benzyl phenyl ureidoethyl dimethyl

ammonium chloride or polyvinyl benzyl benzoyl dimethyl ammonium chloride (U.S. Patent No.: 5,336,596).

[045] The term "**antibody**," refers to an immunoglobulin or a part thereof, and encompasses any polypeptide (with or without further modification by sugar moieties (mono and polysaccharides)) comprising an antigen-binding site regardless of the source or method of production. The term includes, for example, polyclonal, monoclonal, monospecific, polyspecific, humanized, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, and CDR-grafted antibodies as well as fusion proteins. A part of an antibody can include any fragment which can bind antigen, including but not limited to Fab, Fab', $F(ab')_2$, Facb, Fv, ScFv, Fd, V_H, and V_L.

[046] The term "**specimen**" refers to any material taken from a biological or environmental source that may contain one or more target nucleic acid sequences from at least one organism of interest. Specimens can be drawn from any source upon which analysis is desired. For example, the specimen can arise from a host or biological fluids from a host, such as blood, plasma, serum, milk, semen, amniotic fluid, cerebral spinal fluid, sputum, bronchoalveolar lavage, nasopharyngeal wash, tears, urine, saliva, nasal swabs, throat swabs, or stool. Alternatively, the specimen can be a water sample obtained from a body of water, such as lake or river, or it may be from a source of drinking water, such as a tap, aquifer, reservoir, or water purification system. Specimens can be taken by a variety of means, including swabs of a surface. For example, a surface can be swabbed, and then a sample prepared by washing the swab with a liquid, thereby transferring an analyte from the surface into the liquid. Specimens can also be taken from the air.

[047] The term “**sample**” refers to the material actually tested. A sample comprises a specimen. Samples in most cases are in liquid form. The term “**liquid**,” as used herein comprises—in addition to the more traditional definition of liquid—colloids, suspensions, slurries, and dispersions of particles (including beads) in a liquid wherein the particles have a sedimentation rate due to earth’s gravity of less than or equal to about 1 mm/s. The sample can also be prepared by dissolving or suspending a specimen in a liquid, such as water or an aqueous buffer. For example, the air can be filtered, and the filter washed by a liquid, thereby transferring an analyte from the air into the liquid. Exemplary hosts include, but are not limited to, humans, animals, and plants.

[048] The term “**organism**” refers to living matter and viruses comprising nucleic acid that can be detected and identified by the methods of the invention. Organisms include, but are not limited to, bacteria, archaea, prokaryotes, eukaryotes, viruses, protozoa, mycoplasma, and fungi. Different organisms can be different strains, different varieties, different species, different genera, different families, different orders, different classes, different phyla, and/or different kingdoms.

[049] Further examples of organisms include bacterial pathogens such as: *Aeromonas hydrophila* and other species (spp.); *Bacillus anthracis*; *Bacillus cereus*; Botulinum neurotoxin producing species of *Clostridium*; *Brucella abortus*; *Brucella melitensis*; *Brucella suis*; *Burkholderia mallei* (formally *Pseudomonas mallei*); *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*); *Campylobacter jejuni*; *Chlamydia psittaci*; *Clostridium botulinum*; *Clostridium botulinum*; *Clostridium perfringens*; *Coccidioides immitis*; *Coccidioides posadasii*; *Cowdria ruminantium*

(Heartwater); *Coxiella burnetii*; Enterovirulent *Escherichia coli* group (EEC Group) such as *Escherichia coli* - enterotoxigenic (ETEC), *Escherichia coli* - enteropathogenic (EPEC), *Escherichia coli* - O157:H7 enterohemorrhagic (EHEC), and *Escherichia coli* - enteroinvasive (EIEC); *Ehrlichia* spp. such as *Ehrlichia chaffeensis*; *Francisella tularensis*; *Legionella pneumophilia*; *Liberobacter africanus*; *Liberobacter asiaticus*; *Listeria monocytogenes*; miscellaneous enterics such as *Klebsiella*, *Enterobacter*, *Proteus*, *Citrobacter*, *Aerobacter*, *Providencia*, and *Serratia*; *Mycobacterium bovis*; *Mycobacterium tuberculosis*; *Mycoplasma capricolum*; *Mycoplasma mycoides* ssp *mycoides*; *Peronosclerospora philippinensis*; *Phakopsora pachyrhizi*; *Plesiomonas shigelloides*; *Ralstonia solanacearum* race 3, biovar 2; *Rickettsia prowazekii*; *Rickettsia rickettsii*; *Salmonella spp.*; *Schlerophthora rayssiae var zeae*; *Shigella spp.*; *Staphylococcus aureus*; *Streptococcus*; *Synchytrium endobioticum*; *Vibrio cholerae* non-O1; *Vibrio cholerae* O1; *Vibrio parahaemolyticus* and other Vibrios; *Vibrio vulnificus*; *Xanthomonas oryzae*; *Xylella fastidiosa* (citrus variegated chlorosis strain); *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*; and *Yersinia pestis*.

[050] Further examples of organisms include viruses such as: African horse sickness virus; African swine fever virus; Akabane virus; Avian influenza virus (highly pathogenic); Bhanja virus; Blue tongue virus (Exotic); Camel pox virus; Cercopithecine herpesvirus 1; Chikungunya virus; Classical swine fever virus; Coronavirus (SARS); Crimean-Congo hemorrhagic fever virus; Dengue viruses; Dugbe virus; Ebola viruses; Encephalitic viruses such as Eastern equine encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis, and Venezuelan equine encephalitis virus; Equine morbillivirus; Flexal virus; Foot and mouth disease virus; Germiston virus; Goat pox

virus; Hantaan or other Hanta viruses; Hendra virus; Issyk-kul virus; Koutango virus; Lassa fever virus; Louping ill virus; Lumpy skin disease virus; Lymphocytic choriomeningitis virus; Malignant catarrhal fever virus (Exotic); Marburg virus; Mayaro virus; Menangle virus; Monkeypox virus; Mucambo virus; Newcastle disease virus (VVND); Nipah Virus; Norwalk virus group; Oropouche virus; Orungo virus; Peste Des Petits Ruminants virus; Piry virus; Plum Pox Potyvirus; Poliovirus; Potato virus; Powassan virus; Rift Valley fever virus; Rinderpest virus; Rotavirus; Semliki Forest virus; Sheep pox virus; South American hemorrhagic fever viruses such as Flexal, Guanarito, Junin, Machupo, and Sabia; Spondweni virus; Swine vesicular disease virus; Tick-borne encephalitis complex (flavi) viruses such as Central European tick-borne encephalitis, Far Eastern tick-borne encephalitis, Russian spring and summer encephalitis, Kyasanur forest disease, and Omsk hemorrhagic fever; Variola major virus (Smallpox virus); Variola minor virus (Alastrim); Vesicular stomatitis virus (Exotic); Wesselbron virus; West Nile virus; Yellow fever virus; and South American hemorrhagic fever viruses such as Junin, Machupo, Sabia, Flexal, and Guanarito.

[051] Further examples of organisms include parasitic protozoa and worms, such as: *Acanthamoeba* and other free-living amoebae; *Anisakis sp.* and other related worms *Ascaris lumbricoides* and *Trichuris trichiura*; *Cryptosporidium parvum*; *Cyclospora cayetanensis*; *Diphyllobothrium* spp.; *Entamoeba histolytica*; *Eustrongylides* sp.; Giardia lamblia; *Nanophyetus* spp.; *Shistosoma* spp.; *Toxoplasma gondii*; and *Trichinella*. Further examples of analytes include allergens such as plant pollen and wheat gluten.

[052] Further examples of organisms include fungi such as: *Aspergillus* spp.; *Blastomyces dermatitidis*; *Candida*; *Coccidioides immitis*; *Coccidioides posadasii*; *Cryptococcus neoformans*; *Histoplasma capsulatum*; Maize rust; Rice blast; Rice brown spot disease; Rye blast; *Sporothrix schenckii*; and wheat fungus.

[053] The phrase "**detectable label**" refers to an atom, moiety, functional group, molecule, or collection of molecules that can be attached to or incorporated in an oligomer or polymer to thereby render the oligomer or polymer detectable by an instrument or method. A detectable label can be, for example, a fluorophore, a chromophore, a spin label, a radioisotope, Quantum Dot, beads, aminohexyl, pyrene, biotin, an antigenic determinant detectable by an antibody, a chemiluminescence compound, or a electrochemiluminescent (ECL) moiety.

[054] In some embodiments, a detectable label can be directly attached to a primer. In various embodiments, a detectable label can be indirectly attached to a primer. In some embodiments, a detectable label can be attached to a primer using a linker. In various embodiments, a hapten can be attached to a primer and can be recognized by a detectably labeled antibody. For example, a primer attached to a hapten can be used in a PCR reaction to produce an amplicon. After the PCR reaction, a labeled antibody that recognizes the hapten binds to the amplicon, thereby labeling the amplicon. Haptens can be, but are not limited to, dinitrophenyl (DNP), fluorescein isothiocyanate (FITC), 5(6)-carboxyfluorescein, 2,4-dinitrophenyl, rhodamine, bromodeoxy uridine, acetylaminofluorene, mercury trinitrophenol, estradiol, and biotin.

[055] Detectable labels can also be fluorophores. Fluorophores that can be used in the method of the present invention include, but are not limited to, infrared (IR) dyes, Dyomics dyes, phycoerythrine, cascade blue, Oregon green 488, pacific blue, rhodamine derivatives such as rhodamine green, 5(6)-carboxyfluorescein, cyanine dyes (i.e., Cy2, Cy3, Cy 3.5, Cy5, Cy5.5, Cy 7) (diethyl-amino)coumarin, fluorescein (i.e., FITC), tetramethylrhodamine, lissamine, Texas Red, AMCA, TRITC, bodipy dyes, and Alexa dyes. Fluorophores with large Stoke's shifts can be used in the present invention, for example, by utilizing at least two fluorophores in a fluorescent resonant energy transfer (FRET) arrangement. For example, the fluorophores can be located in the same bead (see, e.g., U.S. Patent No. 5,326,692), or they can be covalently coupled (e.g., Tandem dyes, U.S. Patent Nos. 5,783,673; 5,272,257; and 5,171,843 such as Alexa Fluor® APC-Alexa Fluor 750 (Molecular Probes; Carlsbad, CA, USA)).

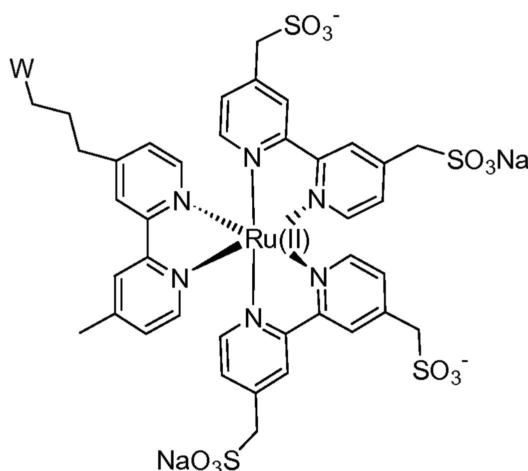
[056] Detectable labels can also be beads or other containers of detectable labels. For example, U.S. Patent Application Publication No. 2006/0078912 discloses containers of ECL moieties comprising more than 10^9 moieties that can be linked to a binding partner, and U.S. Patent No. 5,326,692 discloses fluorescently labeled microparticles that incorporate multiple labels to increase both the signal generated and its Stoke's shift. See, for example, TransFluoSpheres® (Molecular Probes; Eugene, OR, USA).

[057] In certain embodiments of the invention, the detectable label can be a molecular beacon (i.e., a conformation-sensitive label attached to a hairpin loop-containing oligonucleotide) as described, for example, in Kostrikis, L. et al., *Science* 279:1228-29 1998 and in Tyagi, S. et al., *Nat. Biotechnol.* 16:49-52 1998. This type

of label can be used, for example, as a probe rather than a primer. In some embodiments, the probe spans across a restriction enzyme site so the molecular beacon's conformational shape is dependent on the presence of the restriction enzyme site and the proper restriction enzyme.

[058] Detectable labels can also be radioisotopes and chemiluminescent compounds. Radioisotopes that can be used in the present invention include, but are not limited to ^{32}P and ^{35}S. Chemiluminescent compounds that can be used in the present invention include, but are not limited to adamantyl 1,2-dioxetane arylphosphate, other 1,2-dioxetanes, acridinium esters, and acridinium sulphonamides.

[059] ECL moieties that can be used as detectable labels in the present invention can comprise a metal. In some embodiments, the metal can be selected from ruthenium, rhenium, osmium, lanthanum, and europium. Representative ECL moieties are described in U.S. Patent Nos. 5,221,605; 5,591,581; 5,858,676; and 6,808,939. In some embodiments, the ECL moiety can be ruthenium(II) tris-bipyridyl ($[Ru(bpy)_3]^{2+}$), also known as BV-TAG™. In certain embodiments, the ECL moiety can be $[Ru(sulfo\text{-}bpy)_2bpy]^{2+}$ (also known as BV-TAG *Plus*) whose structure is provided by:



wherein W is a functional group attached to the ECL moiety capable of reacting with a biological material, binding reagent, enzyme substrate or other assay reagent, thereby forming a covalent linkage. The covalent linkage may be chosen from a NHS ester, an activated carboxyl, an amino group, a hydroxyl group, a carboxyl group, a hydrazide, a maleimide, and a phosphoramidite.

[060] Methods for preparing primers comprising ECL labels are well known in the art, as described, for example, in U.S. Patent 6,174,709.

[061] In various embodiments, ECL labels can be used with coreactants. The tem "**coreactant**," refers to a chemical compound that either by itself or via its electrochemical reduction oxidation product(s), participates in the ECL reaction sequence.

[062] In some embodiments, coreactants can be chemical compounds that, upon electrochemical oxidation / reduction, can yield, either directly or upon further reaction, strong oxidizing or reducing species in solution. For example, a coreactant can be peroxodisulfate (i.e., $S_2O_8^{2-}$, persulfate), which can be irreversibly electro-reduced to form oxidizing $SO_4^{\bullet -}$ ions. For example, the coreactant can also be oxalate (i.e., $C_2O_4^{2-}$), which can be irreversibly electro-oxidized to form reducing $CO_2^{\bullet -}$ ions. A class of coreactants that can act as reducing agents is amines or compounds containing amine groups, including, for example, tri-n-propylamine (i.e., $N(CH_2CH_2CH_2)_3$, TPA). In some embodiments, tertiary amines can be better coreactants than secondary amines. In some embodiments, secondary amines can be better coreactants than primary amines.

[063] Coreactants include, but are not limited to, lincomycin; clindamycin-2-phosphate; erythromycin; 1-methylpyrrolidone; diphenidol; atropine; trazodone; hydroflumethiazide; hydrochlorothiazide; clindamycin; tetracycline; streptomycin; gentamicin; reserpine; trimethylamine; tri-n-butylphosphine; piperidine; N,N-dimethylaniline; pheniramine; bromopheniramine; chloropheniramine; diphenylhydramine; 2-dimethylaminopyridine; pyrilamine; 2-benzylaminopyridine; leucine; valine; glutamic acid; phenylalanine; alanine; arginine; histidine; cysteine; tryptophan; tyrosine; hydroxyproline; asparagine; methionine; threonine; serine; cyclothiazide; trichlormethiazide; 1,3-diaminopropane; piperazine, chlorothiazide; hydrazinothalazine; barbituric acid; persulfate; penicillin; 1-piperidinyl ethanol; 1,4-diaminobutane; 1,5-diaminopentane; 1,6-diaminohexane; ethylenediamine; benzenesulfonamide; tetramethylsulfone; ethylamine; di-ethylamine; tri-ethylamine; tri-iso-propylamine; di-n-propylamine; di-iso-propylamine; di-n-butylamine; tri-n-butylamine; tri-iso-butylamine; bi-iso-butylamine; s-butylamine; t-butylamine; di-n-pentylamine; tri-n-pentylamine; n-hexylamine; hydrazine sulfate; glucose; n-methylacetamide; phosphonoacetic acid; and/or salts thereof.

[064] ECL coreactants include, but are not limited to, 1-ethylpiperidine; 2,2-Bis(hydroxymethyl)-2,2',2"-nitrilotriethanol (BIS-TRIS); 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-Tris propane) (BIS-TRIS propane); 2-Morpholinoethanesulfonic acid (MES); 3-(N-Morpholino)propanesulfonic acid (MOPS); 3-Morpholino-2-hydroxypropanesulfonic acid (MOPSO); 4-(2-Hydroxyethyl)piperazine-1-(2-hydroxypropanesulfonic acid) (HEPPSO); 4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid (EPPS); 4-(N-Morpholino)butanesulfonic acid (MOBS);N,N-Bis(2-

hydroxyethyl)glycine (BICINE); DAB-AM-16, Polypropylenimine hexadecaamine Dendrimer (DAB-AM-16); DAB-AM-32, Polypropylenimine dotriacontaamine Dendrimer (DAB-AM-32); DAB-AM-4, Polypropylenimine tetraamine Dendrimer (DAB-AM-4); DAB-AM-64, Polypropylenimine tetrahexacontaamine Dendrimer; DAB-AM-8, Polypropylenimine octaamine Dendrimer (DAB-AM-8); di-ethylamine; dihydronicotinamide adenine dinucleotide (NADH); di-iso-butylamine; di-iso-propylamine; di-n-butylamine; di-n-pentylamine; di-n-propylamine; di-n-propylamine; ethylenediamine tetraacetic acid (EDTA); Glycyl-glycine (Gly-Gly); N-(2-Acetamido)iminodiacetic acid (ADA); N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES); N-(2-Hydroxyethyl)piperazine-N'-(4-butanesulfonic acid) (HEPBS); N,N-Bis(2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (DIPSO); N,N-Bis(2-hydroxyethyl)taurine (BES); N-ethylmorpholine; oxalic acid; Piperazine-1,4-bis(2-hydroxypropanesulfonic acid) (POPSO); s-butylamine; sparteine; t-butylamine; triethanolamine; tri-ethylamine; tri-iso-butylamine; tri-iso-propylamine; tri-n-butylamine; tri-n-butylamine; tri-n-pentylamine; N,N,N',N'-Tetrapropyl-1,3-diaminopropane; oxalate; peroxodisulfate; piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES); tri-n-propylamine; 3-dimethylamino-1-propanol; 3-dimethylamino-2-propanol; 1,3-Bis(dimethylamino)-2-propanol; 1,3-Bis(diethylamino)-2-propanol; 1,3-Bis(dipropylamino)-2-propanol; N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES); piperazine-N,N'-bis-3-propanesulfonic acid (PIPPS); piperazine-N,N'-bis-4-butanesulfonic acid (PIPBS); 1,6-diaminohexane-N,N,N',N'-tetraacetic acid; 4-(di-n-propylamino)-butanesulfonic acid; 4-[bis-(2-hydroxyethane)-amino]-butanesulfonic acid; azepane-N-(3-propanesulfonic acid); N,N-bis propyl-N-4-aminobutanesulfonic

acid; piperazine-N,N'-bis-3-methylpropanoate; piperazine-N-2-hydroxyethane-N'-3-methylpropanoate; piperidine-N-(3-propanesulfonic acid); piperidine-N-(3-propionic acid) (PPA); 3-(di-n-propylamino)-propanesulfonic acid; and/or salts thereof.

II. Assay methods

[065] In some embodiments, the invention provides a method for detecting and identifying one or more organisms in a sample by combining a nucleic acid amplification reaction, a detectable label, and the use of restriction enzymes. In certain embodiments, the invention provides a method of identifying at least one organism in a sample comprising the steps of:

(a) forming a first composition comprising the sample, a first primer, and a second primer, wherein the first primer and the second primer can amplify one or more target nucleic acid sequences;

(b) incubating the first composition under conditions that permit at least one target nucleic acid sequence in the sample to be amplified to form a second composition comprising at least one amplicon;

(c) incubating all or a portion of the second composition with one or more enzymes that can cut DNA in a sequence-specific manner under conditions that allow the at least one enzyme to digest the at least one amplicon if a recognition site for the enzyme is present in the at least one amplicon, wherein the at least one amplicon can be digested by at least one, but, if more than one enzyme is used, not all the enzymes, to generate an actual digestion pattern for each enzyme used;

(d) for each enzyme, determining whether or not digestion of the at least one amplicon occurred using at least one method other than gel electrophoresis;

(e) generating an actual digestion pattern of the at least one amplicon based on the determination in step (d) for each enzyme; and

(f) comparing the predicted digestion pattern for each enzyme to the actual digestion pattern of the at least one amplicon to identify at least one organism in the sample.

[066] In some embodiments, the invention provides the method of paragraph [065] wherein the target nucleic acid sequence can be amplified by a method selected from PCR, LCR, SDA, and SSSR. In certain embodiments, a preliminary step of amplifying a target nucleic acid sequence with NASBA can be performed.

[067] In various embodiments, the invention provides the method of paragraph [065], wherein amplification of the target nucleic acid sequence can be achieved using (1) a first primer comprising a nucleic acid sequence that can be linked to a solid support and (2) a labeled second primer, wherein (i) a first portion of the at least one target nucleic acid sequence can be substantially identical to at least 10 consecutive nucleotides, preferably at one end, of the first primer or its complement, and (ii) a second portion of the at least one target nucleic acid sequence can be substantially identical to at least 10 consecutive nucleotides, preferably at one end, of the second primer or its complement.

[068] In certain embodiments, the invention provides the method of paragraph [065], wherein the actual restriction enzyme digestion pattern can be determined by measuring a decrease in the label signal.

A. PRIMER AND RESTRICTION ENZYME SELECTION

[069] In some embodiments, the methods of the invention can be used to distinguish organisms at different levels of resolution. In some embodiments, the invention can distinguish between organisms in a sample that are in different genera. In certain embodiments, the invention can distinguish between organisms in a single genus that are of different species. In various embodiments, the invention can distinguish between organisms that are different strains of a single genus and species. In certain embodiments, the invention can distinguish between strains of an organism that are resistant to various forms of treatment such as drug treatment. In some embodiments, the invention can distinguish between homozygotes and heterozygotes.

[070] At the broader resolutions of organism distinction (e.g., species or genera distinction), this invention provides advantages over gel-based methods because this invention detects enzyme digestion more directly. Gel-based methods measure band positions that can be related to the length of the amplicon or digestion products. Thus, organisms that have different length amplicons or digestion products appear differently. In contrast, this invention is more robust to length changes so long as those changes do not affect the primer sites, restriction enzyme sites, or labeling sites. This can even be an advantage for strain-level determinations, if naturally (or otherwise) occurring deletions and mutations significantly affect amplicon or digestion product length.

[071] In some embodiments, the methods of the invention entail amplification and labeling of a target nucleic acid sequence from one or more organisms in a sample. In some embodiments, a single pair of primers can amplify target nucleic acid sequences from more than one organism. Each possible labeled amplicon can be different from the others by one or more base pairs. In some embodiments, the method of the invention can identify one organism in a sample, by using a pair of primers to amplify one target nucleic acid sequence in the sample. In various embodiments, the method of the invention can identify two organisms in a sample, by using a pair of primers to amplify two target nucleic acid sequences in the sample. In various embodiments, the method of the invention can identify three organisms in a sample, by using a pair of primers to amplify three target nucleic acid sequences in the sample. In various embodiments, the method of the invention can identify several organisms in a sample, by using a pair of primers to amplify several target nucleic acid sequences in the sample. To distinguish between possible target nucleic acid sequences from each organism following amplification, the invention uses restriction enzymes, which can detect differences between nucleic acid sequences of as little as one base pair. The set of restriction enzymes used can be chosen so that the predicted target nucleic acid sequence of each possible organism shows a unique restriction pattern. In some embodiments, a target nucleic acid sequence can be predicted based on the known nucleic acid sequences present in the organisms that may be in the sample. An organism can be identified based on which restriction enzymes do or do not cut the amplified target nucleic acid sequence.

[072] In certain embodiments, a genomic nucleic acid sequence for amplification, a sequence of interest, can be identified by aligning all or a portion of the genomic nucleic acid sequences derived from an organism with sequences available in a nucleic acid sequence database such as Genbank or EMBL. The genomic sequence can be compared with sequences of other organisms in the database in order to find homologous sequences. The set of aligned sequences can be used to design a common primer set that can be used to amplify a sequence of interest derived from each of the organisms, with a minimal level of primer-dimer or non-specific by-product formation. The sequence of each amplicon that can be produced using the selected primer set can be analyzed to develop a restriction map for each amplicon. The unique combination of restriction enzyme sites in each amplicon that are used gives rise to a “predicted restriction enzyme digestion pattern.”

[073] The target nucleic acid sequence of interest can be chosen to produce a unique set of restriction enzyme recognition sites to allow identification of each amplicon sequence from other sequences amplified by the same pair of primers. In embodiments where organisms of different genera are identified, the target amplicon sequence can be highly conserved in all species of one genus while the corresponding sequence in the other genus/genera consistently differs from that highly conserved sequence. In embodiments where organisms of different species are identified, the target sequence can be highly conserved in all members of one species but differ in members of other species in the same genus. In embodiments where different strains are identified, the target nucleic acid sequence for amplifying can be conserved within a particular strain but differ among other strains in the same genus and species so as

to produce a different digestion pattern, depending on which restriction enzymes are used.

[074] The number of primer / restriction enzyme combinations depends in part on the desired results. For example, to choose 1 organism out of O candidate organisms in a sample, there are O possible outcomes (plus a “no organism present” outcome and optionally plus a “data are inconsistent with any one of the candidate organisms” outcome). For example, to choose 1 or 2 organisms out of O candidate organisms in a sample, there are $(O^2 + O)/2$ possible outcomes (plus a “no organism present” outcome and optionally plus a “data are inconsistent with any one or two of the candidate organisms” outcome). As a second example, to choose 1 or 2 or 3 organisms out of O candidate organisms in a sample, there are $(O^3 + 5 O)/6$ possible outcomes (plus a “no organism present” outcome and optionally plus a “data are inconsistent with any one, two, or three of the candidate organisms” outcome). More generally, to choose any of 1 – p organisms out of O candidate organisms in a sample ($p \le O$), there are $\sum_{i=1}^{p} \frac{O!}{(O-i)!\,i!}$ possible outcomes (plus a “no organism present” outcome and optionally plus a “data are inconsistent with any combination of p or fewer of the candidate organisms” outcome). Exemplary values for O (the number of candidate organism) are 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, 500, 1000, as well as other integers between those listed as well as integers greater than 1000. Exemplary values for p (the maximum number of different organisms in the sample) are all values less than or equal to O, including but not limited to 1 and where applicable 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, and 50.

[075] Once the desired results are known, (i.e., what is the value of "p", and what is the collection of candidate organisms), the primer / restriction enzyme combinations can be selected to uniquely identify all the possible outcomes. In some embodiments, the primer / restriction enzyme combinations can be selected to maximize the smallest difference between the predicted restriction enzyme digestion patterns for any two of the possible outcomes. In some embodiments, the system can be made robust to noise by making the Hamming distance between the predicted restriction enzyme digestion patterns for any two of the possible outcomes be larger than the Hamming distance arising from errors for those two outcomes. For example, 4 restriction enzymes (R1, R2, R3, and R4) can be used to determine two species, species A or B. Species A could have a digestion pattern of + + + - for R1-R4 respectively, with a + indicating enzyme digestion and a – indicating no enzyme digestion. Species B could have a digestion pattern of + - + - or - - - +. The second digestion pattern for species B (- - - +) has a greater (and the greatest) Hamming distance, while the first digestion pattern for species B (+ - + -) has the smallest non-zero Hamming distance.

[076] In various embodiments, the amplicon can be digested with one or more restriction enzymes that can cut DNA in a sequence-specific manner. In some embodiments, the restriction enzymes can be selected with at least one of the following criteria in mind: 1) an enzyme that digests each of the predicted amplicons, i.e., that can act, for example, as a positive control for digestion; 2) an enzyme that can be specific to the nucleic acid sequence of each amplicon produced; 3) commercial availability of the restriction enzyme; 4) the specificity and enzymatic

activity of the enzyme; and 5) the optimal temperature of the digestion reaction. In embodiments where the invention distinguishes among different species of a genus, a restriction enzyme can be chosen based upon a restriction enzyme recognition site that is common to all the strains of a given species sequence (to, for example, avoid false results due to mutational variation with a given species) and not present in at least one of the other genera to be distinguished. The term "restriction enzyme recognition site" refers to the DNA nucleotide sequence position/region at which the restriction enzyme binds to and cleaves the DNA molecule. Restriction enzymes that can be used with the invention include, but are not limited to, AcyI, BbvI, NarI, TfiI, TseI, BseMII, BsrDI, HaeII, KpnI, TauI, HindII, Hin4I, BfiI, and BsrI. Additional restriction enzymes are well known in the art and can be found in, for example, the New England Biolabs catalog and its REBASE website. See also, Roberts R. J. et al., *Nucleic Acid Research*, Vol 33, p. D230 – D232, 2005. The instant invention need not be limited to the use of restriction enzymes. Any reagent that cleaves a nucleic acid in a sequence-specific manner can be used with the invention, for example, integrase, recombinase, transposase, topoisomerase, etc.

[077] High-temperature tolerant restriction enzymes can also be used, for example, AclI, ApaLI, ApeKI, AvrII, BamHI, BclI, BglII, BlpI, BsaXI, BsoBI, BsrFI BstBi, BstEII, BstNI, BstUI, BstZ17I, BtsCI, CspCI, HpaI, KpnI, MwoI, Nb.BsrDI, NciI, PaeR7I, PhoI, PI-PspI, PpuMI, PsPGI, PvuII, SfiI, SmlI, TfiI, TliI, TseI, Tsp45I, Tsp509I, TspMI, TspRI, and Tth111. High-temperature tolerant restriction enzymes can be useful, for example, because they can survive the high temperatures associated with PCR or other amplification techniques. In some embodiments, restriction enzymes are either

in the amplification mixture during amplification or located sufficiently close to the amplification mixture that they are exposed to high temperatures (e.g., ≥ 60 °C, ≥ 70 °C, ≥ 80 °C, ≥ 90 °C) during amplification.

[078] In some embodiments, one restriction enzyme can be used. In some embodiments, more than one restriction enzyme can be used.

B. **AMPLICON LABELING**

[079] An amplicon can be detected by a variety of means well known to persons skilled in the art. In some embodiments, a detectable label can be incorporated into the amplified DNA during synthesis by using labeled nucleotides. In certain embodiments, a detectable label can be associated with one of the oligonucleotide primers. Exemplary techniques for analyzing, staining, and labeling nucleic acids are well known in the art and can be found, for example, in Biren, B., Green, E.D., Klapholz, S., Myers, R.M., and Roskams, J., 1997, Analyzing DNA, Cold Spring Harbor Press; and in Kricka, L, (editor), 1995, Nonisotopic Probing, Blotting, and Sequencing, Academic Press.

[080] In certain embodiments, at least one ECL label can be incorporated into one of the PCR primers. For example, a primer can be labeled with an amino group introduced during synthesis using tag NHS and tag phosphoramidite, respectively, where the “tag” can be an ECL moiety.

[081] In certain embodiments, at least one fluorescent or chemiluminescent label can be incorporated into one of the PCR primers. For example, a primer can be labeled with an amino group introduced during synthesis using tag NHS and tag phosphoramidite, respectively, where the “tag” can be a fluorophore or chemiluminescent moiety.

[082] In some embodiments, a labeled amplicon can be rendered detectable by incorporating a binding moiety into a primer that is not labeled or, if labeled nucleotides are used, into either primer. Binding moieties that can be used in the present invention include, but are not limited to, biotin and digoxigenin.

[083] In some embodiments, a digoxigenin label can be detected through a variety of reactions including, but not limited to, fluorescent reactions, chemiluminescent reactions, and ECL reactions. In some embodiments, a biotin label can be detected through at least one of a labeled form of streptavidin (i.e., alkaline phosphatase streptavidin or FITC-streptavidin) or a labeled anti-biotin antibody.

[084] In some embodiments of the invention, an amplicon can be detected by incorporating a primer comprising a detectable label and then measuring the labeled amplification product. In certain embodiments, a labeled amplicon can be separated from unincorporated primer comprising the detectable label by physical means such as centrifugation, dialysis, column purification, or exposure to magnetic or electric fields. For example, amplicons can be attached to a solid support. Magnetizable beads can be the solid support and labeled amplicons can be separated from unincorporated labeled primers by exposure to a magnet.

[085] Amplicons can comprise one or more labels. In some embodiments using amplicons linked to a solid support, at least one candidate restriction enzyme site can be located between at least one label and the solid support. Thus, enzymatic cutting of the amplicon by the restriction enzyme changes the amount of label linked to the solid support thereby making the presence of the restriction enzyme site measurable.

[086] Amplicons can also be labeled with probes that span a candidate restriction enzyme site. Upon heating and cooling the amplicon, the probe can preferentially bind to uncut amplicons. The probe-amplicon complex can be measured through a solid support system as described above or with a homogeneous method such as a molecular beacon. In some embodiments, amplicons are not labeled with probes that span a restriction enzyme site.

C. **AMPLIFICATION**

[087] A target nucleic acid sequence can be amplified by several methods. Methods of nucleotide sequence amplification include, but are not limited to, the polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA; U.S. Pat No 5,409,818), ligase chain reaction (LCR; Wu, D.Y. et al., *Genomics* 4:560-569 1989), strand displacement amplification (SDA; Walker et al, *Nucleic Acids Res.* 20:1691-96 1992), and self-sustained sequence replication (SSSR; Guatelli, J.C. et al., *Proc. Natl. Acad. Sci. USA* 87:1874-78 1990).

[088] In some embodiments, one target nucleic acid sequence can be amplified from the sample. In some embodiments, two target nucleic acid sequences can be amplified from the sample. In various embodiments, three target nucleic acid sequences can be amplified from the sample. In various embodiments, several target nucleic acid sequences can be amplified from the sample (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 50, 75, 100, 200, 300, 400, 500, 600, 1000, 10000, 20000, 50000, 100000, 1000000 or more target sequences or any number in between).

[089] In some embodiments, only one set of primers is used. In other embodiments, multiple sets of primers are used (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 50, 75, 100, 200, 500, 1000 or more primer sets or any number in between).

When multiple sets of primers are used, they can target the same or different organisms.

[090] In some embodiments, the method of the invention comprises extracting at least one target nucleic acid from a sample, wherein the nucleic acid can be DNA or RNA. In some embodiments, the two primers can be mixed with DNA extracted from a sample and with a DNA polymerase, deoxyribonucleotide triphosphates, buffer, and salts and placed in a thermal cycler instrument in a typical PCR reaction. In certain embodiments, RNA can be extracted from a sample and converted to DNA using reverse transcriptase before mixing the resulting DNA with a DNA polymerase, deoxyribonucleotide triphosphates, buffer, and salts for amplification. In some embodiments, if the test substance contains an organism of interest, thousands to millions of copies of the amplicon can be synthesized.

[091] In some embodiments, amplification is not performed; rather, only an extension is performed. Extension-only methods require the detection of fewer labeled amplicons, but have the advantage of not having to amply the nucleic acid. In these embodiments, the resulting products are extension products. These extension products can be used in an analogous manner to labeled amplicons for determining the presence of an organism. Extension-only methods require one or more primers. After the primer and a target nucleic acid hybridize, a polymerase is used to build a double-stranded nucleic acid that, depending on the target nucleic acid sequence, may contain one or more restriction enzyme sites that can be used to identify the organism.

[092] Methods for extracting nucleic acids from a sample are well known in the art. Common methods include treatment of samples with proteolytic enzymes followed

by extraction with organic solvents such as phenol and chloroform (see e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989)) and binding to silica in the presence of chaotropic agents (see e.g., Boom, et al. U.S. Pat. No. 5,234,809). Methods for handling blood specimens and nasal swabs are described, for example, in Rantakokko-Jalava K. and Viljanen, M.K., *Clin. Microbiol. Infect.* 9:1051-56 2003.

D. DETERMINATION OF THE ACTUAL RESTRICTION ENZYME DIGESTION PATTERN

[093] In various embodiments, an amplicon can be linked to a solid support. This linking serves at least two purposes: (1) to facilitate separation of the labeled amplicon from labels that are not incorporated into amplicons; and (2) to allow removal of labels separated from amplicons via restriction enzyme digestion. In some embodiments, covalent bonds can link the amplicon to a solid support. In some embodiments, an amplicon can be linked to a solid support through a pair of binding partners. In some embodiments, the pair of binding partners can permit reversible linking between the solid support and the amplicon, for example, the pair of binding partners can comprise nucleic acids, modified nucleic acids, and/or nucleic acid analogs in such a configuration that have a melting temperature above room temperature (e.g., ≥ 30 °C, ≥ 40 °C, ≥ 45 °C, ≥ 50 °C, and/or ≥ 60 °C) and less than amplification and/or extension temperatures (e.g., ≤ 50 °C, ≤ 60 °C, ≤ 70 °C, 80 °C, ≤ 90 °C, and/or ≤ 98 °C).

[094] In various embodiments, a primer can be linked to a solid support. This linking serves at least two purposes: (1) to facilitate separation of the labeled amplicon from labels that are not incorporated into amplicons; and (2) to allow removal of labels

separated from amplicons via restriction enzyme digestion. In some embodiments, covalent bonds can link the primer to a solid support. In some embodiments, the primer can be linked to a solid support through a pair of binding partners. In some embodiments, the pair of binding partners can permit reversible linking between the solid support and the primer, for example, the pair of binding partners can comprise nucleic acids, modified nucleic acids, and/or nucleic acid analogs in such a configuration that have a melting temperature above room temperature (e.g., ≥ 30 °C, ≥ 40 °C, ≥ 45 °C, ≥ 50 °C, and/or ≥ 60 °C) and less than amplification and/or extension temperatures (e.g., ≤ 50 °C, ≤ 60 °C, ≤ 70 °C, 80 °C, ≤ 90 °C, and/or ≤ 98 °C).

[095] Amplicons can be attached to a solid support via a primer used to make the amplicon in a variety of ways. In some embodiments, a primer can be covalently linked to a solid support. In some embodiments, a primer can be covalently linked to the solid support, for example, by synthesizing a primer with a primary amine at the 5' end and by reacting the primer with a carboxylated solid support. In various embodiments, a primer can be covalently linked to the surface of the solid support by a variety of well-known chemistries, such as carbodiimide coupling (see, e.g., Katz et. al., 1994, *J. Electroanal. Chem.* 367:59; Narvaez et al., 1997, *J. Electroanal. Chem.* 430:227) or maleimide reactions (see, e.g., Marty et al. 2004, *CMLS Cellular and Molecular Life Sci.* 61:1785). In some embodiments, the primer can be covalently linked to a solid support before beginning the amplification reaction. In some embodiments, the primer, contained in an amplicon, can be covalently linked to a solid support after the amplification reaction using, for example, amino linkers such as aminohexyl.

[096] In some embodiments, the primer and the solid support can be chemically modified with moieties that allow the primer to be cross-linked to the solid support. Such moieties can be, for example, photoactivatable crosslinkers such as phenyl azide, nitrophenyl azide, psoralen, azido-methylcoumarin, and any other crosslinkers known to the art. In some embodiments, the cross-linker can be [1-ethyl-3-(dimethylaminopropyl)carbodiimide] hydrochloride. In some embodiments, the primer can be cross-linked to the solid support after the amplification reaction.

[097] In certain embodiments, the primer can be linked to a magnetizable bead. An amplicon comprising the primer linked to the magnetizable bead can be isolated by subjecting the beads to a magnetic field.

[098] For each primer / restriction enzyme combination that is used in the analysis, a separate determination of whether or not the recognition sequence for the restriction enzyme was found in an amplicon is made. These determinations along with the determination of whether an amplicon was generated result in an “actual restriction enzyme digestion pattern.” The actual restriction enzyme digestion pattern identifies the organism(s) present in the sample when these patterns match the predicted restriction enzyme digestion pattern of an organism.

[099] Figure 1 illustrates embodiments of the invention in which a sample tube containing one or more organisms’ target nucleic acid can be amplified using one primer that can be labeled and another primer that can be linked to a magnetizable bead. If a target nucleic acid sequence of interest is present in the sample, amplification occurs to form amplicons. Aliquots of the amplicon preparation are added to tubes containing different restriction enzymes and one or more tubes lacking

restriction enzymes. After permitting the restriction enzymes to cut amplicons having their respective recognition sequences, a magnetic field is used to separate labels that are linked to the beads through an intact amplicon from other labels. The linked labels are measured to determine (a) if an amplicon was formed (from the tubes lacking restriction enzymes) and (b) which restriction enzymes cut the amplicons. Thus, the process described in this paragraph generates an actual restriction enzyme digestion pattern.

[0100] In various embodiments, restriction enzyme digestion of an amplicon can be detected by measuring a loss of signal from the support-bound amplicon. For example, when a labeled primer is used, digestion of the amplicon by a restriction enzyme can result in cleavage of the amplicon, separating the label from the rest of the amplicon, which is still attached to a solid support. The labeled portion of the amplicon can be removed from the sample containing the support-bound amplicon and the sample can then be analyzed for a loss of signal from the label. In some embodiments, the detached segments can be removed and the amount of detectable label remaining can be measured and compared with the label signal of an amplicon that is not digested with a restriction enzyme. In certain embodiments, restriction enzyme digestion of an amplicon can be detected by measuring a partial decrease in the signal from the detectable label as opposed to a total loss of signal if the label were completely removed by restriction enzyme digestion. For example, labeled nucleotides can be used during amplification, and the resulting labeled amplicon digested with one or more restriction enzymes. If an amplicon contains a restriction enzyme recognition site, the signal from the detectable label decreases in proportion

to the portion of amplicon removed from the solid support due to enzymatic cleavage. The cleaved portion of the amplicon can be removed from the sample and the sample then analyzed for a reduction in the signal from the label. This reduction of signal can be detected by comparing the signal of the digested sample to the signal of an undigested amplicon preparation.

[0101] In some embodiments, the sample is split before amplification. For example, if there are P sets of primers and RP combinations of primers & restriction enzymes to be used, the sample can be split into at least RP containers (e.g., P+RP or more than P+RP containers if replicates are desired). In some embodiments, RP containers can be used if there is a separate method to determine if amplicons were formed (e.g., incorporation of radioactive nucleotides into acid-precipitable material); otherwise, P+RP is a more practical lower limit. A potential advantage of splitting the sample before amplification is a reduction in the number of times additions have to be made to the reaction mixtures: the restriction enzymes may be added simultaneously with the amplification reagents.

[0102] In some embodiments, the sample is split after amplification. After the amplicons are generated, the amplified sample can be split into least RP containers (e.g., P+RP or more than P+RP containers if replicates are desired). In some embodiments, RP containers can be used if there is a separate method to determine if amplicons were formed (e.g., incorporation of radioactive nucleotides into acid-precipitable material); otherwise, P+RP is a more practical lower limit Potential advantages of splitting the sample after amplification can be (a) a reduction in counting (Poisson) variability if the copy number of the target nucleic acid is small, (b) a

reduction in variability in amplicon concentration due to having fewer (e.g., one) mixture undergoing amplification.

[0103] In some embodiments, the sample is split after partial amplification. For example, if real-time PCR (RT-PCR) is used with labeled-probes spanning the restriction enzyme sites, the sample can be split after e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 cycles. These first cycles can reduce the counting (Poisson) variability, while maintaining the ability of RT-PCR to estimate concentrations in part by determining the cycle numbers needed to reach a certain signal level.

[0104] Aliquots of amplicons can be split into multiple containers by pipetting into and out of individual containers (e.g., tubes). Alternatively, a single cartridge can have multiple containers fluidically connected so that amplicons are not exposed to the exterior of the cartridge when the amplicons are split into separate containers having restriction enzymes.

[0105] In various embodiments, digested or undigested amplicons can be quantified, and the quantitative results can be used to help determine the presence of multiple organisms in the sample.

[0106] The artisan can contemplate additional specific embodiments of the invention based on the general description above. Some exemplary embodiments are described below, but the artisan can easily discern additional examples. For example, in various embodiments, different target nucleic acid sequences present in the organisms of a sample can be amplified via PCR with one primer labeled with a detectable label and another primer linked to a bead. Following the amplification reaction, the amplicon can be isolated via centrifugation, filtration, or column

purification. In a second reaction, aliquots of the amplicon preparation can be added to several tubes, each tube containing a different restriction enzyme, chosen by the criteria described above. The restriction digests can then be incubated under conditions that permit the restriction enzymes to digest the amplicon at the enzyme recognition sites. The nucleic acids that remain linked to the solid support after enzyme digestion can be separated from the labeled nucleic acids via centrifugation, filtration, or column purification. The samples can then be analyzed to determine whether the detectable label remains associated with the solid support via the amplicon (i.e., a recognition site for the restriction enzyme was not present in the amplicon) or whether the detectable label has been removed due to digestion by the restriction enzyme. The organisms that the target nucleic acid was derived from in the sample can be identified by comparing the actual pattern of enzymatic digestion obtained by analyzing the samples to a predicted pattern of enzymatic digestion based on the nucleotide sequence of the amplicon from each possible target organism. In some embodiments, the label can be an ECL label and the samples can be analyzed on an M1M analyzer.

[0107] For example, in some embodiments, different target nucleic acid sequence(s) of organism(s) in a sample can be amplified with one primer comprising a detectable label and the other primer coupled to one member of a pair of binding partners, wherein the other member of the pair of binding partners can be linked to a magnetizable bead. A separate amplification reaction can be prepared, one amplification reaction for each restriction enzyme to be used in the second reaction. The amplicon(s) can be isolated from each amplification reaction by exposing the

amplification reaction to a magnet. The captured amplicon(s) can then be digested with a set of restriction enzymes, chosen by the criteria described above. A different enzyme can be added to each of the isolated amplification reactions. The nucleic acids that remain linked to the solid support after enzyme digestion can be cleaved from the labeled nucleic acids by exposure to a magnet. The samples can then be analyzed to determine whether the detectable label remains associated with the solid support via the amplicon or whether the detectable label has been removed due to digestion by the restriction enzyme. If an amplicon contains a recognition site that can be cleaved by a specific restriction enzyme, the detectable label is removed from the bead, causing the amplicon to lose its detectable signal. The organisms in the sample can be identified by comparing the actual pattern of enzymatic digestion obtained by analyzing the samples to the pattern of enzymatic digestion predicted from the nucleotide sequences of the target amplicons.

[0108] In some embodiments, target nucleic acid sequence(s) of organism(s) in a sample can be amplified with one chemically modified primer capable of crosslinking to a bead, and a labeled second primer. After the amplification reaction(s) are complete, the amplicons can be captured onto chemically modified beads to crosslink the amplicon with the bead via the modified primer. In some embodiments, the beads are magnetizable beads in which case the amplicons can be isolated by exposure to a magnet. In a second reaction, aliquots of the amplicon preparation can be added to several tubes, each tube containing a different restriction enzyme, chosen by the criteria described above. The restriction digests can then be incubated under conditions that permit the restriction enzymes to digest the amplicon(s) at the enzyme

recognition sites. The nucleic acids that remain linked to the solid support after enzyme digestion can be cleaved from the labeled nucleic acids by exposure to a magnet. The samples can then be analyzed to determine whether the detectable label remains associated with the magnetizable beads or whether the detectable label has been removed due to digestion of the amplicon by the restriction enzyme. For example, whether an amplicon has been digested can be determined by comparing the signal from an undigested sample to the signal from a sample incubated with a restriction enzyme. The organisms in the sample can be identified by comparing the actual pattern of enzymatic digestion obtained by analyzing the samples to the pattern of enzymatic digestion predicted from the nucleotide sequences of the target amplicons. In some embodiments, the label can be an ECL label. In some embodiments, the samples can be analyzed on an M1M analyzer.

[0109] For example, in some embodiments, different target nucleic acid sequences of organisms in a sample can be amplified with one chemically modified primer capable of crosslinking to a bead, labeled deoxyribonucleotides, and a second primer. After the amplification reaction is complete, the amplicons can be captured onto chemically modified beads to crosslink the amplicon with the bead via the modified primer. In some embodiments, the beads are magnetizable beads, in which case the amplicons can be isolated by exposure to a magnet. In a second reaction, aliquots of the amplicon preparation can be added to several tubes, each tube containing a different restriction enzyme, chosen by the criteria described above. The restriction digests can then be incubated under conditions that permit the restriction enzymes to digest the amplicon at the enzyme recognition sites. The nucleic acids

that remain linked to the solid support after enzyme digestion can be cleaved from the nucleic acids that have been cleaved by exposure to a magnet. The samples can then be analyzed to determine whether a decrease in the signal from the detectable label associated with the magnetizable beads has occurred. For example, a decrease in signal can be determined by comparing the signal from an undigested sample to the signal from a sample incubated with a restriction enzyme. The organisms in the sample can be identified by comparing the actual pattern of enzymatic digestion obtained by analyzing the samples to the pattern of enzymatic digestion predicted from the nucleotide sequences of the target amplicons. In some embodiments, the label can be an ECL label. In some embodiments, the samples can be analyzed on an M1M analyzer.

[0110] In various embodiments, a kit comprising one or more primers of the invention and at least one restriction enzyme can be used to practice the methods of the invention. In certain embodiments, the kit comprises at least one pair of primers, each primer pair comprising at least 12 contiguous nucleotides of a target nucleic acid sequence of interest or at least 12 contiguous nucleotides complementary to a target nucleic acid sequence of interest, wherein the primers specifically amplify the target nucleic acid sequence from at least one organism in the sample. In some embodiments, the kit further comprises reagents for carrying out one or more amplification reactions and one or more restriction enzyme digestions. In some embodiments, the kit further comprises a means for nucleic acid amplification and is portable. A timed and temperature controlled flow design having multiple incubation chambers can be used. Following incubation in one chamber (extension reaction), the

sample then flows into several chambers containing specific restriction enzymes. Following incubation with the restriction enzyme(s), the sample then flows into a detection chamber. In embodiments where a thermostable restriction enzyme is used, a single chamber can be used for the extension/digestion reactions. The sample can then flow into a detection chamber.

III. Analysis methods

[0111] Each organism of interest and each primer / restriction enzyme pair, can be analyzed to determine if (a) an amplicon will be created, and (b) if the restriction enzyme will or will not cut the amplicon. This analysis can use sequence information from the organism available, for example, from databases such as Genbank or EMBL. The collection of analyses for all the primer / restriction enzymes pairs for each organism gives rise to a “predicted enzyme digestion pattern.” Optionally, the predicted enzyme digestion pattern can be refined by determining the actual enzyme digestion pattern with a known nucleic acid sequence derived from a specific organism/strain. This refinement may be useful, for example, if one restriction enzyme used is not as efficient as the other restriction enzymes. In this case, not all of the amplicon that have restriction sites for that restriction enzyme may be cleaved; consequently, the signal change for the presence and absence of a particular restriction site may differ among the restriction enzyme digests.

[0112] In some embodiments, each datum in the predicted enzyme digestion pattern comprises binary values for measurements that result from restriction enzymes, one value indicates that the restriction enzyme made a cut, and one value indicates that the restriction enzyme did not make a cut. For measurements that did

not have restriction enzymes and had a primer, one value indicates that an amplicon was created and one value indicates that an amplicon was not created.

[0113] In some embodiments, each datum in the predicted enzyme digestion pattern can be more than 2 values (e.g., greater than or equal to 3, 4, 10, 100, $2^7 - 1$, $2^8 - 1$ $2^{11} - 1$, $2^{12} - 1$, $2^{14} - 1$, $2^{15} - 1$, $2^{16} - 1$, 10^5, 10^6, $2^{23} - 1$, or $2^{24} - 1$ values). For measurements that result from restriction enzymes, each datum is the expected numerical value for that primer / restriction enzyme / organism combination. For measurements that did not have restriction enzymes and had a primer, each datum is the expected numerical value for that primer/organism combination.

[0114] For either the binary or the multi-value predicted enzyme digestion pattern, each datum can be the result of simple or complex analysis steps. For example, each datum can be simply the average value of a photodetector from a measurement technique that uses a luminescent label. In some embodiments, multiple measurements can be combined for each datum. For example, a background measurement can be taken (e.g., in the absence of sample, or in the absence of primer), and each datum is taken to be the ratio or difference between the measurement and the background. As a another example, a measurement using primers and no restriction enzyme (called a positive) can be used with primer/restriction enzyme pair data (and optionally a background measurement) to produce a datum that is (a) the ratio or difference of the positive to the primer/restriction enzyme pair data or (b) the ratio of the difference between the positive and the background to the difference between the primer/restriction enzyme pair data and the background.

A. SINGLE ORGANISM DETERMINATION

[0115] For detecting a single-organism (i.e., p=1), the goal is to determine which one organism, among a possibly large number of candidates, is found in a sample. In some embodiments, the analysis can also result in the conclusion that none of the candidate organisms are in the sample. In some embodiments, the analysis can also result in the conclusion that an unidentified organism or multiple types of organisms are present. The analysis uses the actual enzyme digestion pattern and the collection of all of the predicted enzyme digestion patterns from all the candidate organisms. As described above, to choose 1 organism out of O candidate organisms in a sample, there are O possible outcomes (plus a “no organism present” outcome and optionally plus a “data are inconsistent with any one of the candidate organisms” outcome).

[0116] In general terms, the analysis for a single organism compares the actual enzyme digestion pattern to each of the collection of all of the predicted enzyme digestion patterns for all the possible outcomes and chooses the best match. In one embodiment, the results can be analyzed by first choosing the predicted enzyme digestion pattern that minimizes the sum of the squared differences (SSD) between a particular predicted and the actual enzyme digestion patterns. Next, this minimum SSD can be compared to a threshold value. If the SSD is smaller than or equal to the threshold, report the organism that produced the minimum SSD; if the SSD is greater than the threshold, report that the data are inconsistent with any one of the candidate organisms. This last reporting can be modified by examining whether or not any amplicon was created to determine whether or not organisms having a nucleic acid sufficiently complementary to the primers was present

[0117] Analysis methods other than SSD are also contemplated. For example, the data can first be normalized to reduce the variations in SSD based on signal level changes. As another example, the data can first be weighted in such a way that the expected standard deviation is constant for each of the values in the predicted enzyme digestion pattern (a coefficient of variation of 1% yields a much bigger standard deviation for means of 100,000 than for means of 100). For 2-level values, the SSD method can be used. For example, if positive is equated with a value of 1 and negative is equated with a value of 0; then the SSD method is equivalent to the sum of the exclusive or's (XOR) of the elements of the predicted and actual enzyme digestion pattern. In other embodiments, norms other than the square can be used on the differences: the sum of the absolute differences, the sum of the cube of the absolute differences, and the maximum of the absolute differences are a few more exemplary embodiments. In some embodiments, ratios rather than differences can be used.

B. MULTIPLE ORGANISM DETERMINATION

[0118] For multi-organism determination (i.e., p>1), the goal is to determine which combination of any of p or fewer organisms, among a possibly large number of candidates, is found in a sample. Of course, the single organism determination can be considered a special case of this more general analysis. The analysis can also result in the conclusion that none of the candidate organisms are in the sample. In some embodiments, the analysis can also result in the conclusion that an unidentified organism or more than p types of organisms are present. The analysis uses the actual enzyme digestion pattern and the collection of all of the predicted enzyme digestion patterns from all the candidate organisms. As described above, to choose at most p

organisms out of O candidate organisms in a sample, there are $\sum_{i=1}^{p} \frac{O!}{(O-i)!\,i!}$ possible outcomes (plus a "no organism present" outcome and optionally plus a "data are inconsistent with any combination of p or fewer of the candidate organisms" outcome).

[0119] One potential issue with multi-organism detection can be a variation in the concentration of the organisms in the original sample. Thus, there can be a scaling factor for each of the predicted enzyme digestion patterns relating to the amplicon concentration for each organism. The range of required scaling factors can be minimized or eliminated by the assay methods. For example, the number of PCR amplicons can be ultimately limited by the number of primers available. As the number of PCR cycles is increased (e.g., 20, 25, 30, 35, 40, 45, 50, 55, 60 or more or any number of cycles in between), the amplification efficiency can first decrease for nucleic acids that were originally high in concentration. Thus, the range of amplicon concentration can be less that the range of the nucleic acid concentration.

[0120] For each of the possible outcomes, one can first compute scaling factors and the resulting residual error (e.g., mean squared error). If a scaling factor is negative or zero, that outcome can be dismissed: the negative case is non-physical and the zero case would be duplicative of another outcome with fewer organisms. In some embodiments, outcomes that have a small positive scaling factor can also be dismissed. For example, the outcomes that produce a ratio of the largest to smallest scaling factors greater than the maximum possible corresponding amplicon concentration (e.g., 10x, 100x, 1000x, 10000x) can be dismissed. The residual error

can also be weighted by the number of organisms with non-zero scaling factors to compensate for the general numerical rule that greater degrees of freedom results in better fits, even if those fits are non-physical. If the (possibly weighted) residual error of the possible outcome with the smallest residual error is sufficiently small, that outcome can be reported as the organism or organisms present in the sample. Otherwise, the analysis can result in reporting a “data are inconsistent with any combination of p or fewer of the candidate organisms” outcome.

[0121] To compute the scaling factors and the residual error, the following procedure is one of many that can be employed. Let n represent the number of measurements in each enzyme digestion pattern. If each measurement can take on v statistically different values, then n has to be greater than or equal to $\log_2$ $\sum_{i=1}^{p} \frac{O!}{(O-i)!\,i!}$ divided by $\log_2(v)$ in order to uniquely identify the p or fewer organisms. For example, n can be greater than or equal to $\log_2$ $\sum_{i=1}^{p} \frac{O!}{(O-i)!\,i!}$. The predicted enzyme digestion pattern for an organism can be considered as an n-dimensional vector. Suppose that the possible outcome currently under consideration has P organisms ($1 \leq P \leq p$). Then a predicted matrix M can be constructed of the P n-dimensional vectors of the relevant predicted enzyme digestion patterns. Let α represent the n-dimensional vector of the actual enzyme digestion pattern. The problem can be reduced to solving the matrix equation $M f = \alpha$, where f is the P dimensional vector of scaling factors. The least-squares solution (see, e.g., Linear Algebra and Its Applications, Gilbert Strang, 3rd Edition, 1988), is $f = (M^T M)^{-1} M^T \alpha$,

where M^T is the transpose of M, and $(M^TM)^{-1}$ is the matrix inverse of (M^TM). If the inverse does not exist, a pseudo inverse can be used. The residual mean-square error is $\alpha^T\alpha - \alpha^TMf$.

IV. Examples

[0122] The following examples are provided for illustrative purposes only and are not intended to limit or restrict the scope of the invention.

Example 1: Identification of *K. oxytoca* or *E. coli*

[0123] In this example, a sample was analyzed for the presence of either *Klebsiella oxytoca* (Accession # AJ133197) or *Escherichia coli* (AY065817.1). In this single-organism determination (i.e., p=1), the number of possible outcomes is 4: (1) neither *K. oxytoca* nor *E. coli* is present, (2) *K. oxytoca* and not *E. coli* is present, (3) *E. coli* and not *K. oxytoca* is present, and (4) something is present, but it is not only *K. oxytoca* nor is it only *E. coli*.

Primer and restriction enzyme selection

[0124] A partial gene sequence for a topoisomerase derived from *Klebsiella oxytoca* (Accession # AJ133197) was used to find homologous DNA sequences in the NCBI databank using the BLAST algorithm (www.ncbi.nih.gov/BLAST/), for divergent sequences/discontiguous megablast. Several genes from different strains of *K. oxytoca* and sequences derived from other bacteria were found to have significant homology to the sequence, as shown in Table 1. As shown in Table 1, many organisms and strains share significant homology to the topoisomerase gene from *Klebsiella oxytoca* (Accession # AJ133197). Thus, one primer pair is likely all that is needed to amplify most of these organisms/strains. With proper restriction enzyme

selection, these closely related organisms can be distinguished. Rather than attempting to distinguish among all of these organisms/strains, this example focuses on distinguishing between a *K. oxytoca* strain and an *E. coli* strain.

Table 1: Sequences Producing Significant Alignments Based on *Klebsiella oxytoca*[1]

Accession Number	Organism	Homologous length	BLAST homology score
gi|11761961|gb|AF303643.1|AF303643	*Klebsiella oxytoca*	614	7e-173
gi|11761955|gb|AF303640.1|AF303640	*Klebsiella oxytoca*	587	9e-165
gi|11761971|gb|AF303648.1|AF303648	*Klebsiella oxytoca*	585	3e-164
gi|11761973|gb|AF303649.1|AF303649	*Klebsiella oxytoca*	562	3e-157
gi|11761969|gb|AF303647.1|AF303647	*Klebsiella oxytoca*	531	5e-148
gi|11761947|gb|AF303636.1|AF303636	*Klebsiella oxytoca*	519	2e-144
gi|11761923|gb|AF303624.1|AF303624	*Klebsiella oxytoca*	514	9e-143
gi|11761985|gb|AF303655.1|AF303655	*Klebsiella planticola*	433	2e-118
gi|11761979|gb|AF303652.1|AF303652	*Klebsiella ornithinolytica*	433	2e-118
gi|11761975|gb|AF303650.1|AF303650	*Klebsiella planticola*	433	2e-118
gi|11761941|gb|AF303633.1|AF303633	*Klebsiella pneumoniae*	415	3e-113
gi|14289140|gb|AY034616.1|	*Klebsiella pneumoniae*	410	2e-111
gi|11761981|gb|AF303653.1|AF303653	*Klebsiella pneumoniae*	404	9e-110
gi|11761963|gb|AF303644.1|AF303644	*Klebsiella pneumoniae*	398	5e-108
gi|11761977|gb|AF303651.1|AF303651	*Klebsiella terrigena*	392	3e-106
gi|61654906|gb|AY776325.1|	*Shigella dysenteriae*	362	5e-97
gi|61654898|gb|AY776321.1|	*Shigella dysenteriae*	362	5e-97

Accession Number	Organism	Homologous length	BLAST homology score
gi|81244029|gb|CP000036.1|	*Shigella boydii*	362	5e-97
gi|73854091|gb|CP000038.1|	*Shigella sonnei*	362	5e-97
gi|18476192|gb|AY049061.1|	*Enterobacter aerogenes*	358	7e-96
gi|24080789|gb|AE005674.1|	*Shigella flexneri*	356	3e-95
gi|61654904|gb|AY776324.1|	*Shigella dysenteriae*	356	3e-95
gi|61654900|gb|AY776322.1|	*Shigella dysenteriae*	356	3e-95
gi|26111730|gb|AE014075.1|	*Escherichia coli*	356	3e-95
gi|30043918|gb|AE014073.1|	*Shigella flexneri*	356	3e-95
gi|85674274|dbj|AP009048.1|	*Escherichia coli*	350	1e-93
gi|48994873|gb|U00096.2|	*Escherichia coli*	350	1e-93
gi|18389177|gb|AY065817.1|	*Escherichia coli*	350	1e-93
gi|18389175|gb|AY065816.1|	*Escherichia coli*	350	1e-93
gi|47118301|dbj|BA000007.2|	*Escherichia coli*	350	1e-93
gi|882431|gb|U28377.1|ECU28377	*Escherichia coli*	350	1e-93
gi|147105|gb|M58408.1|ECOPARC	*Escherichia coli*	350	1e-93
gi|56126533|gb|CP000026.1|	*Salmonella enterica*	327	1e-86
gi|9623017|gb|AF227958.1|AF227958	*Serratia marcescens*	323	2e-85
gi|71057502|emb|AM050347.1|	*Salmonella paratyphi*	321	7e-85
gi|62126203|gb|AE017220.1|	*Salmonella enterica*	321	7e-85
gi|4007019|emb|Y18300.1|CJE18300	*Campylobacter jejuni*	317	1e-83
gi|49609491|emb|BX950851.1|	*Erwinia carotovora*	310	2e-81

Accession Number	Organism	Homologous length	BLAST homology score
gi|16421729|gb|AE008846.1|	*Salmonella typhimurium*	298	6e-78
gi|16504049|emb|AL627277.1|	*Salmonella enterica*	298	6e-78
gi|154234|gb|M68936.1|STYPARCF	*Salmonella typhimurium*	298	6e-78
gi|45437513|gb|AE017138.1|	*Yersinia pestis*	294	9e-77
gi|51587641|emb|BX936398.1|	*Yersinia pseudotuber culosis*	294	9e-77
gi|15978734|emb|AJ414144.1|	*Yersinia pestis*	294	9e-77
gi|3046559|gb|AF056286.1|AF056286	*Citrobacter freundii*	287	2e-74
gi|18175499|gb|AY064399.1|	*Yersinia enterocolitica*	283	3e-73
gi|3046561|gb|AF056287.1|AF056287	*Enterobacter sakazakii*	281	1e-72

[1] The identical sequence in the database for a specific strain has been deleted. If the alignment provided identical sequences (100%) homology or significant homology between organisms/or strains that will not provide unique restriction enzyme patterns, these sequences were removed.

[0125] Sequences for *K. oxytoca* and *Escherichia coli* (AY065817.1) were used to identify a target region of approximately 165 bp and to design a primer set. The PCR primers used to amplify both the *K. oxytoca* and *E. coli* amplicon were:

Forward Primer: 5'-TTCTCCTACCGCTATCCGCTGGT-3' (SEQ ID NO. 1)

Reverse Primer: 5'-GAAGTTTGGTACCCAGTCAACGGT-3' (SEQ ID NO. 2)

[0126] The predicted nucleic acid sequence of the amplicon for detecting and identifying *K. oxytoca* was as follows, with the position of each primer identified for the forward primer (bold) and the reverse primer (underline):

5'-**TTCTCCTACCGCTATCCGCTGGT**TGACGGTCAGGGAAACTGGGGGCG

CCGGACGATCCTAAATCCTTCGCCGCAATGCGTTATACCGAATCCCGTTTGTCGA

AGTATGCTGAACTGCTGCTGAGCGAACTGGGGCAAGG CACCGTTGACTGG GTACCAAACTTC-3' (SEQ ID NO. 3).

[0127] The predicted nucleic acid sequence of the amplicon for detecting and identifying *E. coli* was as follows, with the position of each primer identified for the forward primer (bold) and the reverse primer (underline):

5'-**TTCTCTTACCGTTATCCGCTGGT**TGATGGTCAGGGGAACTGGGGCGCGCC GGACGATCCGAAATCGTTCGCGGCAATGCGTTACACCGAATCCCGGTTGT CGAAATATTCCGAGCTGCTATTGAGCGAGCTGGGGCAGGGGACGGCTGAC TGGGTGCCAA ACTTCGACG-3' (SEQ ID NO. 4).

[0128] These amplicons (SEQ ID NOs. 3 and 4) were analyzed to identify possible digestion sites using an online restriction mapping program available at www.restrictionmapper.org/. The program was set up to look for recognition sites that were at least 5 nucleotides long without providing a limit on the number of times the amplicon could be cut by a restriction enzyme. Table 2 provides a restriction map for the *K. oxytoca* amplicon and Table 3 provides a restriction map for the *E. coli* amplicon.

Table 2: Restriction Map for the *K. oxytoca* Amplicon

Enzyme Name	Sequence	Site Length	Overhang	Number of Cuts	Cut Positions
AcyI	GRCGYC	6	five prime	1	46
BbvI	GCAGC	5	five prime	1	104
NarI	GGCGCC	6	five prime	1	46
TfiI	GAWTC	5	five prime	1	88
TseI	GCWGC	5	five prime	1	117
BseMII	CTCAG	5	three prime	1	112
BsrDI	GCAATG	6	three prime	1	80
HaeII	RGCGCY	6	three prime	1	49
KpnI	GGTACC	6	three prime	1	157
TauI	GCSGC	5	three prime	1	73

Enzyme Name	Sequence	Site Length	Overhang	Number of Cuts	Cut Positions
HindII	GTYRAC	6	blunt	2	24, 147
Hin4I	GAYNNNNNVTC	6	three prime	2	46, 78
BfiI	ACTGGG	6	three prime	3	48, 139, 159
BsrI	ACTGG	5	three prime	3	43, 134, 154

Table 3: Restriction Map for the *E. coli* Amplicon

Enzyme Name	Sequence	Site Length	Overhang	Number of Cuts	Cut Positions
SspI	AATATT	6	blunt	1	106
XmnI	GAANNNNTTC	6	blunt	1	64
AscI	GGCGCGCC	8	five prime	1	44
BbvI	GCAGC	5	five prime	1	101
BccI	CCATC	5	five prime	1	19
BsePI	GCGCGC	6	five prime	1	44
BseYI	CCCAGC	6	five prime	1	129
TfiI	GAWTC	5	five prime	1	88
TseI	GCWGC	5	five prime	1	114
BsrDI	GCAATG	6	three prime	1	80
TauI	GCSGC	5	three prime	1	73
BfiI	ACTGGG	6	three prime	2	48, 159
BsrI	ACTGG	5	three prime	2	43, 154
Hin4I	GAYNNNNNVTC	6	three prime	2	46, 78

The unique and common restriction enzymes for each amplicon were selected based on one or more of the following criteria: 1) common to each organism; 2) specific to each amplicon; 3) common to all the strains of a given species sequence; 4) commercial availability of the restriction enzyme; 5) the specificity and enzymatic activity of the enzyme; and 6) the optimal temperature of the digestion reaction. As for the specificity, Tables 2 and 3 have 8 restriction enzymes (AcyI, HaeII, Hin4I, HindII, TauI, TfiI, TseI, and XmnI) that recognize more than 1 sequence: sequence code N

refers to A or C or G or T; R means A or G; S means C or G; V means A or C or G; W means A or T; and Y means C or T. Based on this analysis, the following restriction enzymes were chosen to differentially identify *K. oxytoca* and *E. coli*: BseM II, Bbv I, Ssp I, Xmn I, Ase I, and Gsu I. The enzymes BbvI and GsuI are common to both organisms, and therefore establish some level of certainty that an actual digestion pattern is the result of one of these two organisms. The enzymes BseMII, SSPI, XmnI, and AseI provide specificity to distinguish between the two organisms. In this example, only 1 strain of K. *oxytoca* and E. *coli* were desired to be distinguished, thus, the criteria of selecting restriction enzymes common to all the strains of a given species sequence was unnecessary. All the restriction enzymes were commercially available. All the restriction enzymes had exquisite specificity and had comparable enzymatic activities on the unit enzyme scale. For this proof of concept, differences in required digestion temperatures among the restriction enzymes was not a criterion. The predicted digestion pattern of each amplicon with each of these enzymes is shown below in Table 4. A plus sign indicates cleavage while a minus sign indicates that the given restriction enzyme does not cleave the amplicon sequence.

Table 4: Positions and Specific Sequences that the Restriction Enzymes Cleave

Restriction Enzyme	Specific DNA sequence	*K. oxytoca* (Amplicon position)	*E. coli* (Amplicon position)
BseMII	CTCAG	+ (112)	-
BbvI	GCAGC	+ (201)	+ (229)
SSPI	AATATT	-	+ (234)
XmnI	GAANNNNTTC	-	+ (192)
AseI	GGCGCGCC	-	+ (172)
GsuI	CTGGAG	-	-

Amplicon Labeling

[0129] Two primer sets (SEQ ID NOs. 1 and 2), purchased from Biosource, were used in this example. In primer set A, the forward and reverse primers were unlabeled. In primer set B, the forward primer was labeled with an electrochemiluminescent label BV-TAG *Plus* (BioVeris Corp., Gaithersburg, MD) at the 5'-prime position and the reverse primer was labeled with biotin at the 5'-prime position. PCR was used to incorporate the labeled primers into amplicons to form labeled amplicons.

Amplification

[0130] PCR was used to create the labeled amplicons.

Determination of the predicted restriction enzyme digestion pattern

[0131] For each candidate organism, ECL signal is to be generated by coupling a magnetizable bead to the labeled amplicon and using a magnetic field to separate ECL label linked to the magnetizable bead from ECL label not linked (e.g., free in solution). If a labeled amplicon has a restriction enzyme site for a restriction enzyme present in solution, the ECL label is separated from the magnetizable bead, resulting in a reduction in ECL signal. The predicted restriction enzyme digestion pattern also includes a negative control where no primers (or primer set A) are used that can generate labeled amplicons. The predicted restriction enzyme digestion pattern also includes a positive control for the amplification reaction where no restriction enzymes are used.

[0132] As can be seen in Table 5, the predicted restriction enzyme digestion patterns for the 2 species differ, which is the end-goal of the primer / restriction

enzyme selection process. This binary-value predicted restriction enzyme digestion pattern can be improved with experimental data. For example, the experimental data in the next section can be used to make these patterns more quantitative by using more than 2 values.

Table 5: Predicted restriction enzyme digestion pattern

		Predicted ECL signal level	
Primer	Restriction Enzyme	*K. oxytoca*	*E. coli*
Set B	BseMII	low	high
Set B	BbvI	low	low
Set B	SSPI	high	low
Set B	XmnI	high	low
Set B	AseI	high	low
Set B	GsuI	high	high
Set B	none	high	high
none or Set A	none	low	low

Determination of the actual restriction enzyme digestion pattern

[0133] Chromosomal DNA was prepared from 50 µL each of a *K. oxytoca* and an *E. coli* culture that was incubated at 37 °C overnight. To extract the DNA from the cells, 20 µL of bacterial culture was added to 180 µL TE buffer (1 mM Tris, 0.01 mM EDTA, pH 8.0). The mixture was incubated at 95 °C for 10 min, and centrifuged in a microcentrifuge at 13,000 rpm for 5 minutes. The supernatant was transferred to a microfuge tube. Nucleic acid was precipitated using DNA co-precipitating reagents from Bioline Inc., (Randolph, MA) per the manufacturer's instructions. Each chromosomal DNA preparation was reconstituted in TE buffer and stored at -20 °C.

[0134] PCR reactions were performed using both primer set A and primer set B. All PCR reagents were from Roche Molecular (Catalog # 12032937001; Pleasanton, CA), except for the dNTPs which were from Bioline (Catalog # B10-

39045, Boston, MA). Five microliters of each DNA preparation was added to a reaction mix containing 2.5 mM $MgCl_2$, each primer at a concentration of 200 nM, 200 µM dATP, 200 µM dGTP, 200 µM dCTP, and 200 µM dTTP in 1X PCR buffer. Taq DNA polymerase was then added to a concentration of 2.5 unit/50 µL PCR reaction immediately before placing the reaction tubes in PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA). The final volume of each PCR reaction was 50 µL. The reactions were incubated for 35 cycles of 15 seconds at 95 ^{o}C followed by 30 seconds at 55 ^{o}C and then 30 seconds at 72 ^{o}C. Prior to initiating the first cycle, the reaction mix was incubated at 95 ^{o}C for 1 min to denature the double stranded template. Following PCR, the amplicons were purified using a PCR preparation kit from Qiagen (Cat. No. 28104), and each DNA preparation was reconstituted with 30 µL TE

[0135] Restriction enzymes were used as directed by the manufacturer's (New England Biolabs) instructions. Digestions were carried out using 1 unit of enzyme at 37 ^{o}C for 1 hour. Either 3.0 or 1.5 µL of each amplicon preparation was used for each digestion reaction. Following the digestion reaction, the amplification and digestion samples from the amplification reaction performed with unlabeled primer were detected using ethidium bromide staining to detect and identify fragments derived from the amplification of *K. oxytoca* (Fig. 2A) and *E. coli* (Fig. 2B) DNA. Each digestion reaction was loaded on a 2% agarose pre-cast gel (Invitrogen Corp.) and the bands were visualized on a UV transilluminator (UltraViolet Products). The low molecular weight ladder was purchased from Invitrogen. .

[0136] Amplification and digestion products from the amplification reaction performed with the ECL-labeled primer were detected using

electrochemiluminescence as shown in Figure 3. Following the amplification, amplification products were digested using BseMII, BbvI, SSPI, XmnI, Ase I and GsuI as indicated in the figure. Each restriction enzyme digest was mixed with 80 μL of 0.5 mg/mL streptavidin-coated M-280 magnetic beads (Dynal BioTech, part number 110029). If digestion with a particular enzyme occurred, the BV-TAG™ *Plus* and biotin labels were separated, removing the ECL label from the amplicon bound to the bead. In this case, the presence of a restriction site in the amplicon resulted in a decrease in ECL signal as compared to an undigested sample. The negative control sample defined as NC, was a PCR reaction without the template, and was treated under the same conditions as the positive control sample. The positive control sample, defined as PC, was a PCR reaction that was not treated with any restriction enzyme.

[0137] The data in Figure 3 match the predictions in Table 5. Thus, if either of these samples were unknown, the predicted restriction enzyme patterns could be used to identify the organism.

Example 2: Identification of *N. meningitidis* strains

[0138] In this example, a sample was analyzed for the presence of one of four strains of *N. meningitidis* (Accession # AJ133197) or *Escherichia coli* (AY065817.1). In this single-organism determination (i.e., p=1), the number of possible outcomes is 6: (1) none of the strains are present, (2-5) one of the strains is present, and (6) something is present, but it is not just one strain.

Primer and restriction enzyme selection

[0139] The DNA sequence for *N. meningitides* aspartate b-semialdehyde dehydrogenase gene (partial coding sequence) from GenBank with the accession number of AF205574.1 was used. Two primer sequences were designed based on the published gene sequence and were used to amplify genomic DNA preparations derived from the four *N. meningitides* strains that are commercially available from ATCC with the following accession numbers: 53415D, 53416D, 53417D, and 53418D.

[0140] The gene sequence (AF205574.1) for the *N. meningitides* gene is given below. Also given is a primer pair that can be used to amplify the sequence.

1 GCTGGCGCGG TATGGTCGGT TCGGTTTTGA TGCAGCGTATGAAAGAAGAAAACGACTTCG
61 CCCACATTCC TGAAGCGTTT TTCTTTACCA CTTCCAACGT CGGCGGCGCA GCCCCTGATT
121 TCGGTCAGGC AGCCAAAACA TTGTTGGATG CCAACGATGT TGCCGAATTG GCAAAAATGG
181 ACATCATCGT TACCTGTCAG GGCGGCGATT ACACCAAATC CGTCTTCCAA CCCCTGCGCG
241 ACAGCGGCTG GAACGGCTAC TGGGTTGACG CGGCGTCATC CCTGCGCATG AAAGACGATG
301 CGATTATCGT CCTCGACCCG GTCAACCGCA ACGTCATCGA CAACGGCCTC AAAA (SEQ ID NO. 5)

Forward Primer: 5'-GCTGGCGCGGTATGGTCGGT (SEQ ID NO. 6)
Reverse Primer: 5'-GAGGCCGTTGTCGATGACGT (SEQ ID NO. 7)

[0141] Initially, each of the four genomic DNAs was used in PCR in order to verify that amplification of a similar length amplicon could be produced from each strain's genomic template. Following the verification that the above primer set targets each DNA sequence derived from the four strains, aliquots of the four genomic templates and the primer set was sent to Lofstrand Inc. (Gaithersburg, MD) for sequence information. Below see the sequence of each amplicon derived from the four strains. The sequences that are in bold and underlined indicate the forward and reverse primer positions, respectively. For any sequence variation, addition or deletion at each the primer position, the position is indicated with a small case letter.

The sequences that are in italics indicate restriction enzyme digest positions. A letter k represents an unknown base from the sequencing data.

Neisseria meningitides (53415D) serogroup B
PCR amplicon
Primers: Neisseria For-1 and Rev-2

Contig Length: 353 bases

CT**GCTGGCGCGGTATGGTCGGT**TCGGTTTTGAT*GCAGC*GTATGAAAGAAGAAAA
CGACTTCGCCCACATTC*CTGAAG*CGTTTTTCTTTACCACTTCCAACGTCGGCGGC
GCAGCCCCTGATTTCGGTCAGGCAGCCAAAACATTGTTGGATGCCAACGATGTTG
CCGAATTGGCAAAAATGGACATCATCGTT*ACCTGCCAAGG*CGGCGATTACACCAA
ATCCGTCTTCCAACCCCTGCGCGACAGCGGCTGGAACGGCTACTGGGTTGACGC
GGCGTCATCCCTGCGCATGAAAGACGATGCGATTATCGTCCTCGACCCGGTCAA
CCGCAACGTCATCGACAACGGCCTCA (SEQ ID NO. 8)

Neisseria meningitides (53416D) serogroup C
PCR amplicon
Primers: Neisseria For-1 and Rev-2

Contig Length: 354 bases

T**GtCTGGCGCGGTATGGTCGGT**TCGGTTTTGAT*GCAGC*GTATGCAAGAAGAAAAC
GACTTCGCCCACATTCCCGAAGCGTTTTTCTTTACCACTTCCAACGTCGGCGGCG
CAGCCCCTGATTTCGGTCAGGCAGCCAAAACATTGTTGGATGCCAACGATGTTGC
CGAATTGGCAAAAATGGACATCATCGTTACCTGTCAGGGCGGCGATTACACCAAA
TCCGTGTTCCAAGCCCTGCGCGACAGCGGCTGGAACGGCTACTGGGTTGACGC
GGCATCTTCTCTGCGCATGAAAGACGACGCGATTATCGTCCTCGACCCTGTCAAC
CGCAACGTCATCGACAACGGCCTCAA (SEQ ID NO. 9)

Neisseria meningitides (53417D) serogroup A
PCR amplicon
Primers: Neisseria For-1 and Rev-2

Contig Length: 356 bases

CCT**GCTGGCGCGGTATGGTCkGT**TCGGTTTTGAT*GCAGC*GTATGAAAGAAGAAAA
CGACTTCGCCCACATT*CCTGAA*GCGTTTTTCTTTACCACTTCCAACGTCGGCGGC
GCAGCCCCTGATTTCGGTCAGGCAGCCAAAACATTGTTGGATGCCAACGATGTTG
CCGAATTGGCAAAAATGGACATCATCGTTACCTGTCAGGGCGGCGATTACACCAA
ATCCGTCTTCCAACCCCTGCGCGACAGCGGCTGGAACGGCTACTGGGTTGACGC
GGCGTCATCCCTGCGCATGAAAGACGATGCGATTATCGTCCTCGACCCGGTCAA
CCGCAACaGTCATCGACAACGGCCCTCA (SEQ ID NO. 10)

Neisseria meningitides (53418D) serogroup B
PCR amplicon
Primers: Nseisseria For-1 and Rev-2

Contig Length: 355 bases

T**GCTGGCGCGGTATGGTCGGT**TCGGTTTTGAT*GCAGC*GTATGAAAGAAGAAAAC
GACTTCGCCCACATTCCCGAAGCGTTTTTCTTTACCACTTCCAACGTCGGCGGCG
CAGCCCCTGATTTCGGTCAGGCGGCTAAAACATTATTGGACGCGAAC*AACGTT*GC
CGAGCTGGCAAAAATGGACATCATCGTT*ACCTGCCAAGG*CGGCGACTACACCAA
ATCCGTCTTCCAAGCCCTGCGCGACAGCGGCTGGAACGGCTACTGGATTGACGC
GGCATCCTCGCTGCGTATGAAAGACGACGCGATTATCGTCCTCGACCCCGTCAA
CCGCAA<u>caGTCATCGACAAACGGCCTC</u>AA (SEQ ID NO. 11)

[0142] Two primer sets (C and D), both using SEQ ID NO. 6 and 7, purchased from Biosource, and were used in this example. For analysis by agarose gel electrophoresis, the forward and reverse primers were unlabeled (primer set C). In primer set D, the forward and reverse primers were labeled with a BV-TAG^{TM} *Plus* and biotin at the 5'-postions, respectively. The predicted PCR product for each strain of *N. meningitides* is shown in the sequence data above.

[0143] Each of the four sequences was analyzed using "restrictionmapper" to identify and select restriction enzymes that provide a unique digestion pattern for each of the four amplicons. The restriction enzymes in Table 6 were selected for these examples. For Figure 4, in the bar graph, NC (no signal) indicates a negative control (all components were present except template in the PCR mix and sample was treated under the same condition without actually adding restriction enzyme). Samples with restriction enzymes Acl I, BbvI, BspMI, StyI and Eco571 are also shown in the figures. Finally, PC in the figures is a positive signal where no restriction enzyme was added to the PCR reaction. The vertical axis in Figure 4A is ECL signal. The vertical axis in

Figure 4B is the ECL signal, after it has be normalized to a percentage of the positive control signal.

Table 6: Specific Sequences that the Restriction Enzymes Cleave

Restriction Enzyme	Sequence Target	ATCC# 53415D (Digestion site)	ATCC# 53416D (Digestion site)	ATCC# 53417D (Digestion site)	ATCC# 53418D (Digestion site)
AclI	AACGTT	-	-	-	+
BbvI	GCAGC$(N)_8$	+	+	+	+
BspMI	ACCTGC$(N)_4$	+	-	-	+
StyI	CCWWGG	+	-	-	+
Eco571	CTGAAG$(N)_{16}$	+	-	+	-

Determination of the predicted restriction enzyme digestion pattern

[0144] For each candidate organism, ECL signal was generated by coupling a magnetizable bead to the labeled amplicon and using a magnetic field to separate ECL label linked to the magnetizable bead from ECL label not linked (e.g., free in solution). If a labeled amplicon has a restriction enzyme site for a restriction enzyme present in solution, the ECL label is separated from the magnetizable bead, resulting in a reduction in ECL signal. The predicted restriction enzyme digestion pattern also included a negative control where no primers (or primer set C) were used that can generate labeled amplicons. The predicted restriction enzyme digestion pattern also included a positive control for the amplification reaction where no restriction enzymes are used.

[0145] As shown in Table 7, the predicted restriction enzyme digestion patterns for the 4 strains differ, which is the end-goal of the primer / restriction enzyme selection process. This binary-value predicted restriction enzyme digestion pattern can be improved with experimental data. For example, the experimental data in the

next section can be used to make these patterns more quantitative by using more than 2 values.

Table 7: Predicted restriction enzyme digestion pattern

		Predicted ECL signal level			
Primer	Restriction Enzyme	53415D	53416D	53417D	53418D
Set D	AclI	high	high	high	low
Set D	BbvI	low	low	low	low
Set D	BspMI	low	high	high	low
Set D	StyI	low	high	high	low
Set D	Eco571	low	high	low	high
Set D	none	high	high	high	high
none or Set C	none	low	low	low	low

Determination of the actual restriction enzyme digestion pattern

[0146] Chromosomal DNA from each of the four strains was purchased from ATCC. The same procedure was used as that in Example 1.

[0147] The results shown in Figure 4 match the predictions in Table 7. Figure 4B shows an exemplary alternate analysis method. If binary valued data are desired, a threshold of 50% of the positive control signal can differentiate between "high" and "low" signals to compare with Table 7. Thus, if either of these samples were unknown, the predicted restriction enzyme patterns could be used to identify the organism.

[0148] The gel results are shown in Figure 5. The lanes that had the Eco57I enzyme show a common problem with gels that is eliminated using the principals of this invention. In these gel lanes, no clean bands are visible. Instead, there is a band much larger than the amplicon with a corresponding long smear that suggests an artifact due to "gel-shift" –the binding of the restriction enzyme to the amplicon and thereby reducing the mobility of the amplicon through the gel matrix. This makes the amplicon band appear larger and creates a smear as the DNA fragments dissociate

from the protein throughout the duration of the electrophoresis . This type of artifact makes it difficult to confidently analyze the results under circumstances that requires a quick and unambiguous decision.

[0149] Additional embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only. All references either discussed or referred to herein are incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of identifying an organism or group of organisms in a sample comprising the steps of:

(a) forming a first composition comprising the sample, a first primer, and a second primer, wherein the first primer and the second primer can amplify one or more target nucleic acid sequences;

(b) incubating the first composition under conditions that permit at least one target nucleic acid sequence in the sample to be amplified to form a second composition comprising at least one amplicon;

(c) incubating all or a portion of the second composition with one or more enzymes that can cut DNA in a sequence-specific manner under conditions that allow the at least one enzyme to digest the at least one amplicon if a recognition site for the enzyme is present in the at least one amplicon, wherein the at least one amplicon can be digested by at least one, but, if more than one enzyme is used, not all the enzymes, to generate an actual digestion pattern for each enzyme used;

(d) for each enzyme, determining whether or not digestion of the at least one amplicon occurred using at least one method other than gel electrophoresis;

(e) generating an actual digestion pattern of the at least one amplicon based on the determination in step (d) for each enzyme; and

(f) comparing the predicted digestion pattern for each enzyme to the actual digestion pattern of the at least one amplicon to identify at least one organism in the sample.

2. The method of claim 1, wherein at least one of the one or more enzymes is a restriction endonuclease.

3. The method of claim 1, wherein the method distinguishes among organisms in different genera.

4. The method of claim 1, wherein the method distinguishes among different species in a single genus.

5. The method of claim 1, wherein the method distinguishes among different strains in a single species.

6. The method of claim 1, wherein the method can determine which one organism is present in the sample among a number of candidate organisms, wherein the number of candidate organisms is greater than or equal to 500.

7. The method of claim 1, wherein the method distinguishes which one organism is present in the sample among a number of candidate organisms, wherein the number of candidate organisms is less than 500.

8. The method of claim 1, wherein the method can determine which combination of any of p or fewer organisms among O candidate organisms, wherein p is less than or equal to O, and O is greater than or equal to 500.

9. The method of claim 1, wherein the method can determine which combination of any of p or fewer organisms among O candidate organisms, wherein p is less than or equal to 50, p is less than equal to O, and O is less than 500.

10. The method of claim 9, wherein p is less than 11, p is less than or equal to O, and O is less than 50.

11. The method of claim 9, wherein p is less than or equal to 3 and p is less than or equal to O.

12. The method of any of claims 1 – 11, wherein the at least one nucleic acid sequence is amplified using a method selected from polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), self-sustained sequence replication (SSSR) and Nucleic Acid Sequence-Based Amplification (NASBA).

13. The method of claim 1, wherein the at least one target nucleic acid sequence is amplified using a first primer and a second primer.

14. The method of claim 13, wherein a portion of each of the at least one target nucleic acid sequences is substantially identical to (i) the first primer or its complement and (ii) the second primer or its complement.

15. The method of claim 13, wherein the first primer comprises a detectable label.

16. The method of claim 15, wherein the detectable label is a radioactive label, a fluorescent label, an enzyme, a chemiluminescent moiety, or an electrochemiluminescent (ECL) moiety.

17. The method of claim 16, wherein the ECL moiety comprises a metal.

18. The method of claim 17, wherein the metal is ruthenium, rhenium, or osmium.

19. The method of claim 18, wherein the ECL moiety is ruthenium(II) tris-bipyridyl ($[Ru(bpy)_3]^{2+}$) or $[Ru(sulfo\text{-}bpy)_2bpy]^{2+}$.

20. The method claim 13, wherein the at least one target nucleic acid sequence is amplified using at least two primers.

21. The method of claim 13 or 20, wherein each primer comprises at least 12 contiguous nucleotides.

22. The method of claim 1 wherein the at least one target nucleic acid sequence is a DNA sequence.

23. The method of claim 1 wherein the at least one target nucleic acid sequence is an RNA sequence.

24. The method of claim 13, wherein the second primer is linked to a solid support.

25. The method of claim 24, wherein the second primer comprises biotin or digoxigenin.

26. The method of claim 24, wherein the second primer is linked to the solid support before the amplification reaction.

27. The method of claim 24, wherein the second primer is linked to the solid support after the amplification reaction.

28. The method of claim 24, wherein the second primer comprises a nucleic acid sequence that is complementary to a nucleic acid sequence attached to a solid support and wherein the primer’s complementary sequence is not complementary to a nucleotide sequence in the amplicon.

29. The method of claim 24, wherein the solid support is a bead.

30. The method of claim 29, wherein the bead is a magnetizable bead.

31. The method of claim 1, wherein

(a) the at least once amplicon contains at least one enzyme recognition site; and

(b) the actual enzyme digestion pattern is determined by measuring the label.

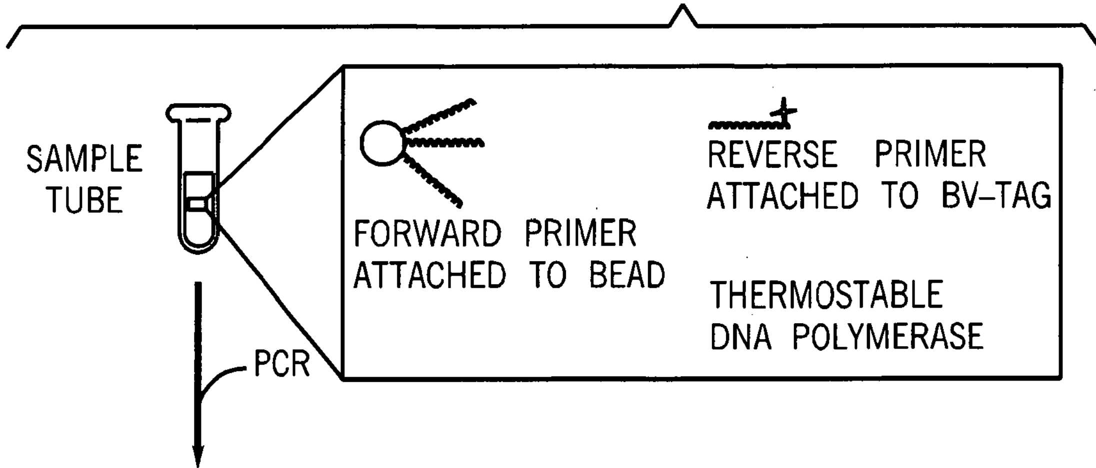
32. The method of any of claims 1 – 11 and 13 – 31, wherein the sample is split before the amplification step into at least one amplification tube, one amplification tube for each enzyme to be used.

33. The method of claim 32, wherein a restriction enzyme is added to at least one amplification tube before amplification.

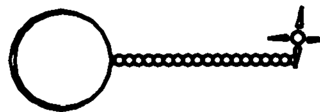
34. The method of any of claims 1 – 11 and 13 - 31, wherein the sample is split after the amplification step into at least one tube for each enzyme.

35. The method of any of claims 1 – 11 and 13 - 31, wherein one or more enzymes are added after the amplification step.

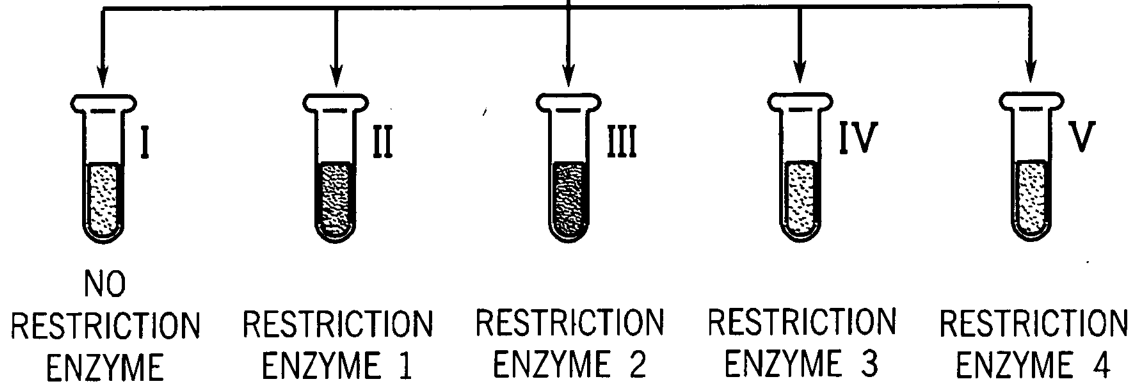
FIG. 1



IF SAMPLE CONTAINS ORGANISM OF INTEREST AMPLIFICATION WILL OCCUR



ADD TO ANALYSIS TUBES CONTAINING RESTRICTION ENZYMES



IF SEQUENCE IS PRESENT FOR THE SPECIFIC CLEAVAGE RESTRICTIONS DIGESTION OCCURS. ASSUME 1 & 4 CLEAVE.

CLEAVAGE	NO	YES	NO	NO	YES
ECL	+	–	+	+	–

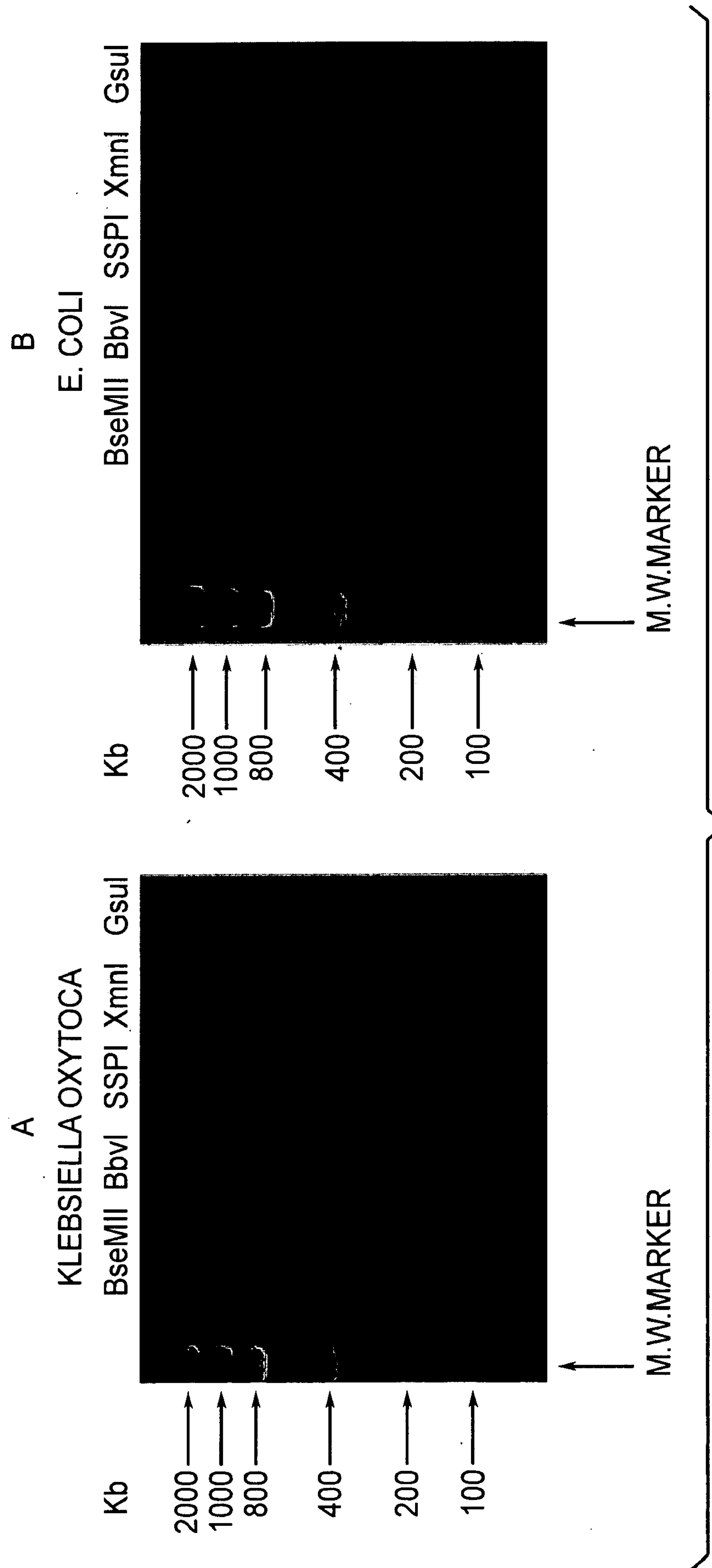


FIG. 2

FIG. 3A

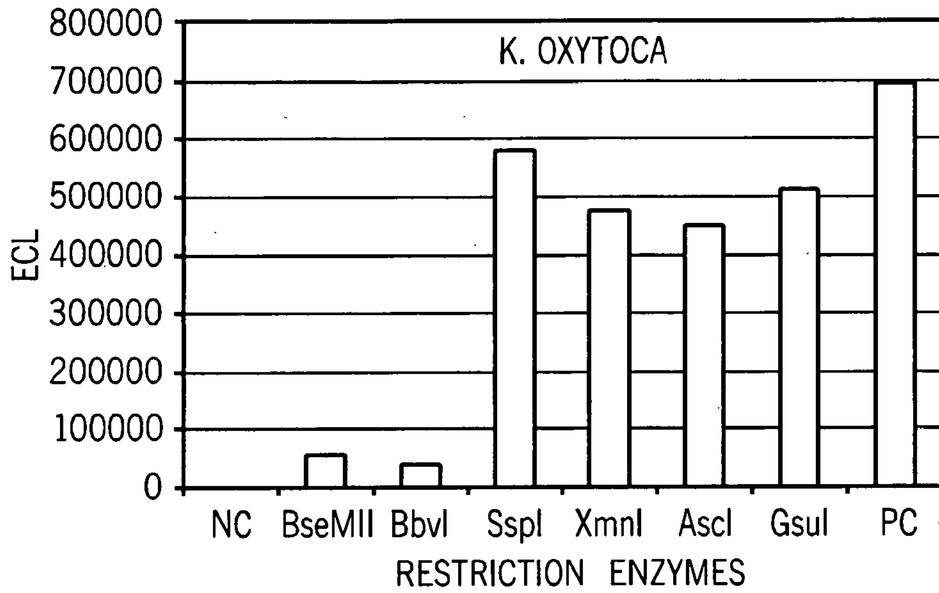
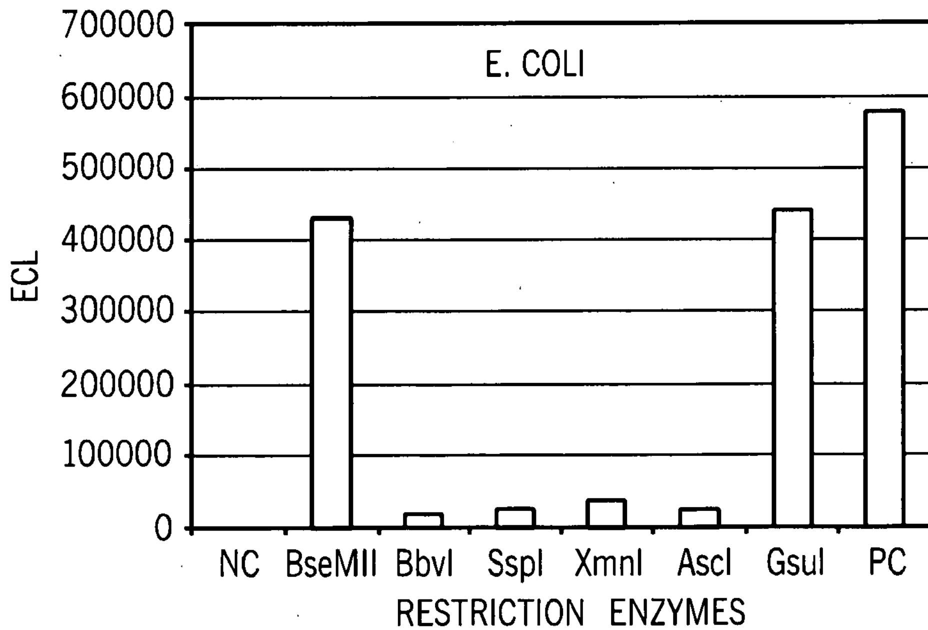


FIG. 3B



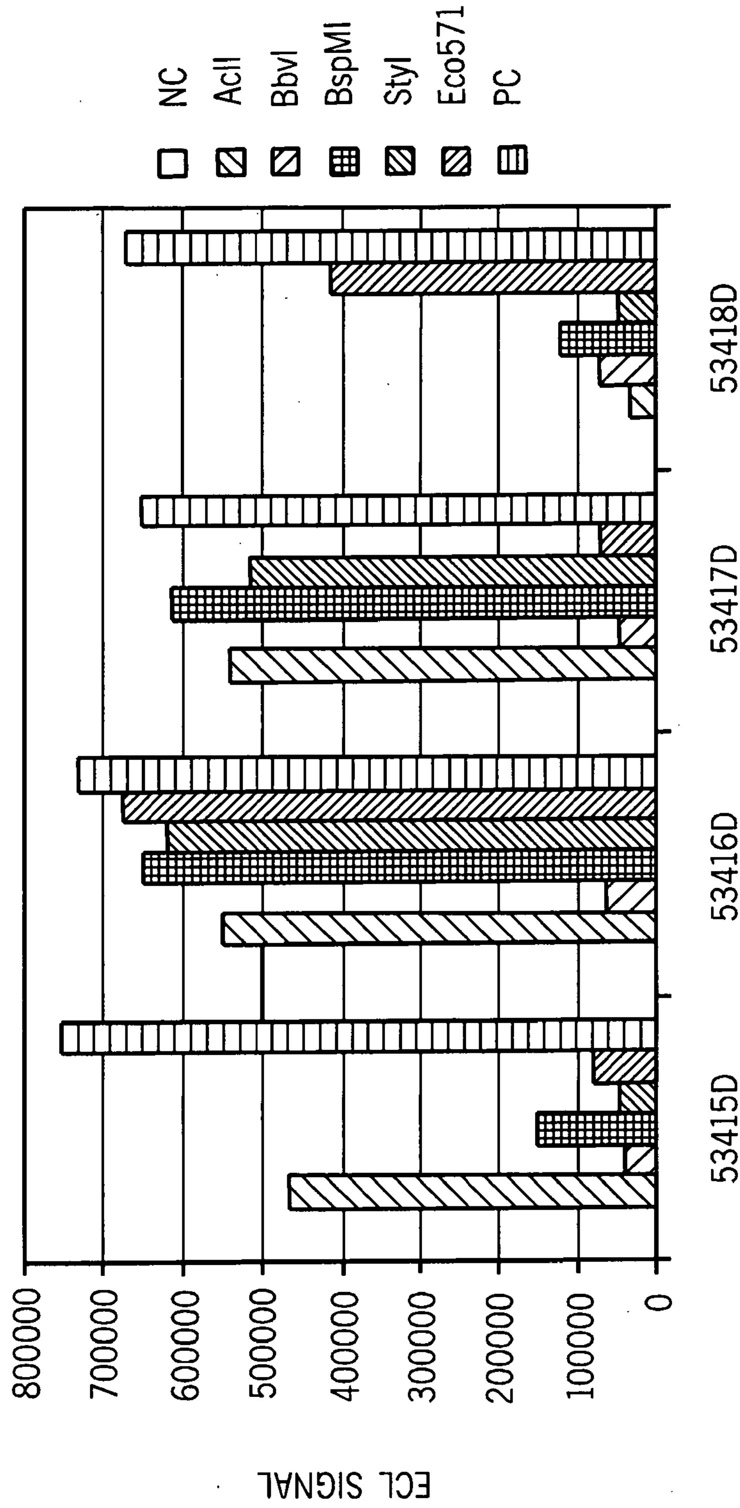


FIG. 4A

FIG. 4B

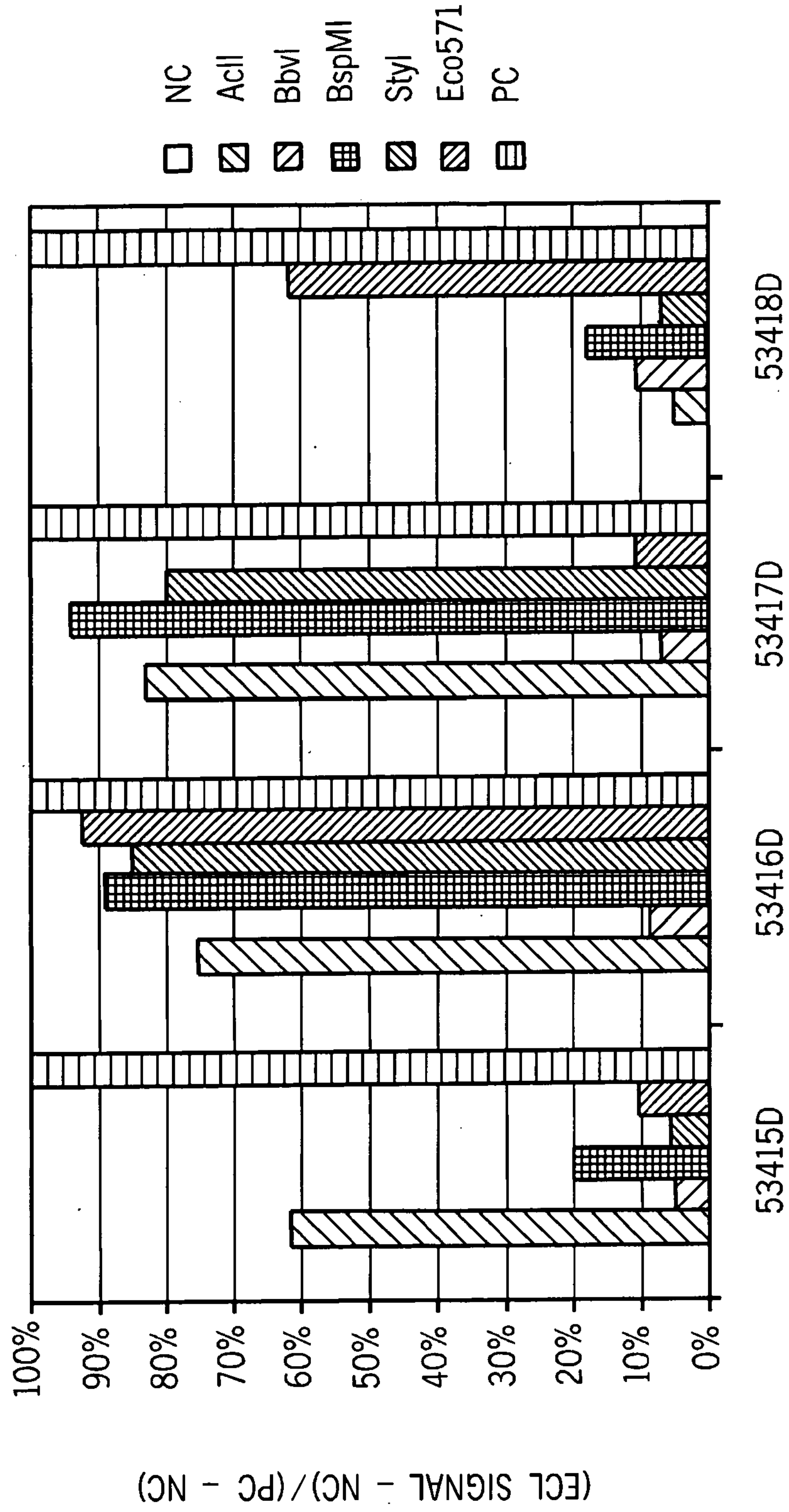
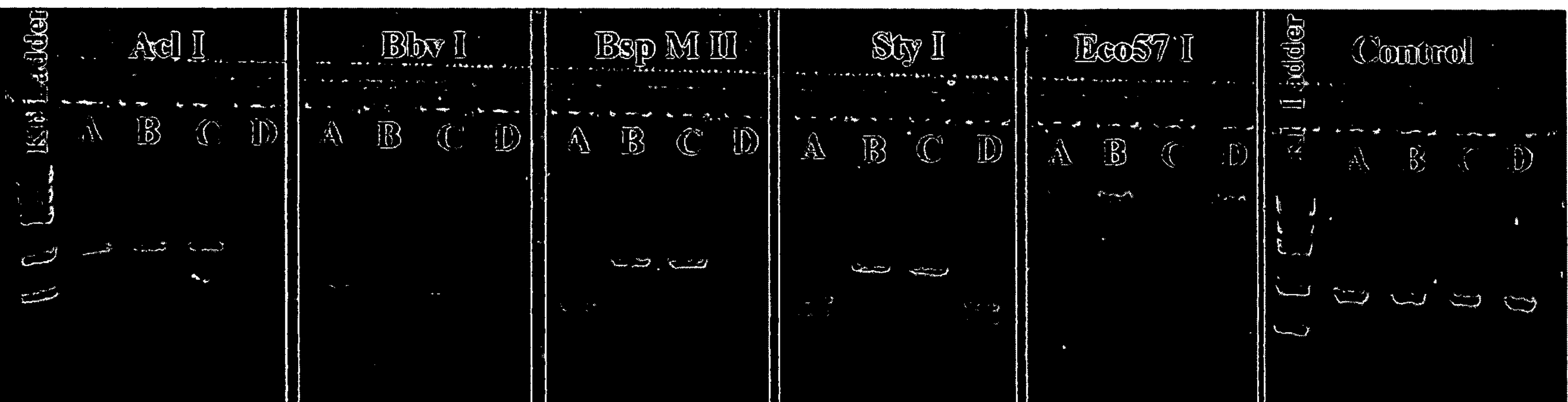


FIG. 5

RESTRICTION DIGESTION OF AMPLICONS DERIVED FROM FOUR NEISSERIA meningitidis STRAINS



A-N. meningitidis ATCC # 53415D
B-N. meningitidis ATCC # 53416D
C-N. meningitidis ATCC # 53417D
D-N. meningitidis ATCC # 53418D