Title: IN SITU HYBRIDIZATION METHODS FOR REDUCING THE OCCURRENCE OF FALSE POSITIVES AND FOR TARGETING MULTIPLE MICROORGANISMS

Abstract

This invention provides novel methods for detecting microorganisms in biological samples. The methods utilize in situ hybridization techniques. In one preferred embodiment, the methods performing an in situ hybridization using two or more probes specific to the same target microorganism where the probes are each labeled with different and distinguishable label or are themselves different and distinguishable. When the probes co-localize to the same feature they are scored as positive for the target microorganism corresponding to those eprobes. Where only a single probe localizes to the target the result is scored as negative and/or, optionally subject to re-testing or other further analysis.
<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
<th>Code</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Albania</td>
<td>ES</td>
<td>Spain</td>
</tr>
<tr>
<td>AM</td>
<td>Armenia</td>
<td>FI</td>
<td>Finland</td>
</tr>
<tr>
<td>AT</td>
<td>Austria</td>
<td>FR</td>
<td>France</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
<td>GB</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>AZ</td>
<td>Azerbaijan</td>
<td>GE</td>
<td>Georgia</td>
</tr>
<tr>
<td>BA</td>
<td>Bosnia and Herzegovina</td>
<td>GH</td>
<td>Ghana</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>GN</td>
<td>Guinea</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>GR</td>
<td>Greece</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
<td>GR</td>
<td>Greece</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>HU</td>
<td>Hungary</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
<td>IE</td>
<td>Ireland</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>IL</td>
<td>Israel</td>
</tr>
<tr>
<td>BY</td>
<td>Belarus</td>
<td>IS</td>
<td>Iceland</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
<td>IT</td>
<td>Italy</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>JP</td>
<td>Japan</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>KE</td>
<td>Kenya</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>KG</td>
<td>Kyrgyzstan</td>
</tr>
<tr>
<td>CI</td>
<td>Côte d’Ivoire</td>
<td>KP</td>
<td>Democratic People’s</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>KR</td>
<td>Republic of Korea</td>
</tr>
<tr>
<td>CN</td>
<td>China</td>
<td>KZ</td>
<td>Kazakhstan</td>
</tr>
<tr>
<td>CU</td>
<td>Cuba</td>
<td>LC</td>
<td>Saint Lucia</td>
</tr>
<tr>
<td>CZ</td>
<td>Czech Republic</td>
<td>LI</td>
<td>Liechtenstein</td>
</tr>
<tr>
<td>DE</td>
<td>Germany</td>
<td>LK</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
<td>LR</td>
<td>Liberia</td>
</tr>
<tr>
<td>EE</td>
<td>Estonia</td>
<td>LS</td>
<td>Lesotho</td>
</tr>
<tr>
<td>LT</td>
<td>Lithuania</td>
<td>LU</td>
<td>Luxembourg</td>
</tr>
<tr>
<td>LV</td>
<td>Latvia</td>
<td>MC</td>
<td>Monaco</td>
</tr>
<tr>
<td>MD</td>
<td>Republic of Moldova</td>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>MK</td>
<td>The former Yugoslav</td>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td>MS</td>
<td>Republic of Macedonia</td>
<td>MN</td>
<td>Mongolia</td>
</tr>
<tr>
<td>MR</td>
<td>Mauritania</td>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td>MX</td>
<td>Mexico</td>
<td>NE</td>
<td>Niger</td>
</tr>
<tr>
<td>NL</td>
<td>Netherlands</td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
<td>PL</td>
<td>Poland</td>
</tr>
<tr>
<td>PT</td>
<td>Portugal</td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>RU</td>
<td>Russian Federation</td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>SE</td>
<td>Sweden</td>
<td>SG</td>
<td>Singapore</td>
</tr>
<tr>
<td>SI</td>
<td>Slovenia</td>
<td>SK</td>
<td>Slovakia</td>
</tr>
<tr>
<td>SN</td>
<td>Senegal</td>
<td>SZ</td>
<td>Swaziland</td>
</tr>
<tr>
<td>TD</td>
<td>Chad</td>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>TJ</td>
<td>Tajikistan</td>
<td>TM</td>
<td>Turkmenistan</td>
</tr>
<tr>
<td>TR</td>
<td>Turkey</td>
<td>TT</td>
<td>Trinidad and Tobago</td>
</tr>
<tr>
<td>UA</td>
<td>Ukraine</td>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>UG</td>
<td>Uganda</td>
<td>UZ</td>
<td>Uzbekistan</td>
</tr>
<tr>
<td>VN</td>
<td>Viet Nam</td>
<td>YU</td>
<td>Yugoslavia</td>
</tr>
<tr>
<td>ZW</td>
<td>Zimbabwe</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IN SITU HYBRIDIZATION METHODS FOR REDUCING THE OCCURRENCE OF FALSE POSITIVES AND FOR TARGETING MULTIPLE MICROORGANISMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to and benefit of Provisional Patent Application USSN 60/130,865, filed on April 22, 1999, under 35 U.S.C. §119 and/or 35 U.S.C. §120, as appropriate. Provisional Patent Application USSN 60/130,865 is incorporated herein by reference in its entirety for all purposes. This application is related to an application filed on 21 April 2000 entitled "BLOCKING NON-SPECIFIC BINDING OF GRANULOCYTES IN MICROORGANISM DETECTION" naming CURTIS T. THOMPSON, JOSEPH A. SPIDLE, DONNA G. ALBERTSON, and RICK SEGRAVES as inventors, which is also incorporated herein by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[ Not Applicable ]

FIELD OF THE INVENTION

This invention relates to the field of Fluorescent In Situ Hybridization (FISH). In particular, the invention pertains to improvements in FISH for the detection or quantification of microorganisms in biological samples.

BACKGROUND OF THE INVENTION

A methodology for rapid detection/quantification and identification of microorganisms has long been a concern to the medical, pharmaceutical, veterinary, and food processing fields, among others. Traditionally, microorganism identification/quantification has involved subsampling of the tissue, fluid, food product, etc. in question, and culturing of the subsample to grow colonies of bacteria that are present in that subsample.
A typical bacterial culture technique for screening for the presence of microorganisms/pathogens involves a series of media (nutrient fluid) transfers starting from a non-selective enrichment media (a "rich" media) and then transferring a portion of the primary enrichment medium to a selective media, or to a series of selective media. This process allows for the initiation of growth of potentially injured microorganisms in the non-selective primary enrichment media and, once the microorganisms have been revived, a small quantity of the non-selective enrichment medium is transferred into (or onto in the case of plates containing solidified media) the secondary (selective) media. This process, whose duration is often defined by human work patterns and the growth patterns of the microorganisms, can take several days to several weeks to complete.

One approach to detection of the presence of biologically active agents such as bacteria in a patient's body fluid, especially blood, involves bacterial culture in blood culture vials. A small quantity of blood is injected through an enclosing rubber septum into a sterile vial containing a "rich" culture medium, and the vial is then incubated at 37°C and monitored for microorganism growth over a period of hours or days. The presence of microorganisms is often detected visually. Generally, visual inspection involves monitoring the turbidity or eventual color changes of the liquid suspension of blood and culture medium. Alternatively, known instrumental methods detect changes in the carbon dioxide content (or pH value which is a function of CO₂ content) of the culture bottles, which is a metabolic by-product of the bacterial growth. These approaches, however, still impose significant delays for bacterial growth, are often subjective, and do not provide specific identification of the microorganism.

Such long screening times are clearly disadvantageous in commercial screening systems (e.g. screening food products for contamination, etc.), however, in the medical fields such delays can adversely effect a treatment regimen and may even be life threatening. Often in a medical or veterinary context, it is necessary to identify/characterize pathogenic microorganisms in a biological tissue or fluid sample in order to determine a particular treatment regimen. Antibiotics are often highly specific with respect to the organisms they target, and it is often necessary to identify the pathogenic organisms in order to select a particular antibiotic treatment. In addition, even within a particular species of pathogen (e.g. Mycobacterium tuberculosis) certain strains show greater degrees of drug
resistance than other strains, and it is important, and often critical, to identify a strain as drug resistant prior to treatment.

In recent years, nucleic acid amplification procedures (e.g. polymerase chain reaction (PCR)) have seen increasing use in identification of microorganisms. Such amplification methods, however, while faster, are highly labor intensive, expensive, and require separate reagents and reactions for each microorganism species or strain it is desired to identify.

Nucleic acid hybridization methods have also started to see some use for microorganism detection (see, e.g., U.S. Patent 5,055,394). This approach typically involves a "blotter hybridization" methodology (e.g. a Southern blot). Such procedures generally involve isolation of nucleic acids from the sample/microorganism, transfer of the samples to a filter or membrane, and hybridization of the transferred material with a nucleic acid probe. Such approaches involve considerable sample handling and so are labor intensive and risk the introduction of cross-contaminants. In addition, such methods are costly, often require radioisotope usage, and frequently lack relevant histological information that is useful to confirmation of diagnosis.

**SUMMARY OF THE INVENTION**

This invention provides novel methods of fluorescent in situ hybridization for the detection of microorganisms in a biological sample. The use of *in situ* hybridization methodologies preserves valuable histological information (e.g. tissue morphology, localization of the microorganism in or around the tissue, microorganism morphology, etc.) useful in diagnosis and confirmation of diagnosis. Moreover, the methods are generally compatible with histological methods already in use in pathology laboratories and therefore do not require significant reinstrumentation or re-training. In addition, the methods are relatively simple, highly accurate, rapid and provide effective discrimination between microorganism strains and/or species.

In one preferred embodiment, this invention provides methods of detecting a microorganism in a biological sample (e.g. a sample from a vertebrate), where the methods reduce the incidence of false positives. Such methods involve contacting the biological sample with a first nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of the microorganism and not (e.g. not substantially) to a nucleic acid of the
sample (e.g. a vertebrate); contacting the biological sample with a second nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not (e.g. not substantially) to a nucleic acid of the sample tissue (e.g. the vertebrate), where the first nucleic acid probe and the second nucleic acid probe are distinguishable. The method preferably further involves detecting hybridization of the first nucleic acid probe and the second nucleic acid probe to a target, where co-occurrence of the first nucleic acid probe and the second nucleic acid probe indicates the presence of the microorganism. Conversely, in preferred embodiments, hybridization of only the first nucleic acid probe or the second nucleic acid probe is interpreted as a false positive.

In some embodiments, the first probe may be contacted with the sample before the second probe, while in other embodiments, the first and second probes are contacted with each other before contacting the sample. In still other embodiments, the first probe, the second probe, and the sample are all contacted simultaneously.

The first and second probes are selected so as to specifically label (e.g. hybridize) to the same microorganism (e.g. species or strain). The first and second probes may be the same or different lengths and may specifically hybridize to the same nucleic acid sequence, to overlapping nucleic acid sequences or to different nucleic acid sequences. The first and/or second probe(s) may each be labeled with a single label or with multiple labels and certain preferred probes are molecular beacons and/or peptide nucleic acids (PNAs) and/or are synthetic oligonucleotides. The probes are often directly or indirectly labeled, e.g. with a fluorescent label, a colorimetric, a radioactive label, an enzymatic label, an electron dense label, or a magnetic label. Both probes may bear different kinds of labels (e.g. one probe fluorescently labeled and the other probe radioactively labeled) or the same kind of label (e.g. both probes fluorescently labeled). In one particularly preferred embodiment, the first nucleic acid probe and the second nucleic acid probe each are labeled with a fluorescent labels and the labels on each probe are distinguishable from each other.

Preferred biological samples include, but are not limited to, samples from vertebrates such as a human, a non-human primate, a bovine, an equine, a canine, a feline, a porcine, a murine, a hamster, a fish, a reptile, and an amphibian. The samples are typically obtained by a variety of standard methods well known to those of skill in the art, and include biopsies, body fluid samples, aspirates, swabs (e.g. buccal or nasal swabs). Preferred samples are fresh samples, frozen samples, or fixed samples. Typically biological tissue or
fluid samples include, but are not limited to pleural tissue samples, stomach or gut samples, epidermal samples, whole bloods, a blood fractions, oral fluid, feces, urine, cerebrospinal fluid, nasal fluid, and the like.

Preferred target microorganisms that are to be detected include, but are not limited to the microorganisms described herein. Particularly preferred microorganisms include, but are not limited to mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

In another embodiment, this invention provides a hybridization mix for detecting a microorganism in a biological sample (e.g. vertebrate tissue). A preferred hybridization mix includes a first nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of the microorganism and not to a nucleic acid of the sample tissue (e.g. a vertebrate); and a second nucleic acid probes that specifically hybridizes under stringent conditions to a nucleic acid of the microorganism and not to a nucleic acid of the sample (e.g. vertebrate tissue), where the first nucleic acid probe and said second nucleic acid probe are distinguishable. In some embodiments, the hybridization mix further comprises a hybridization buffer, and/or a detergent (e.g. SDS, more preferably 20% SDS). In one particularly preferred embodiment, the hybridization buffer, the detergent (e.g. SDS).

In preferred embodiments, the hybridization mix comprises blocking agents and/or probes (optionally labeled), for use with particular tissues and microorganisms as described herein.

In another embodiment, this invention provides a biological sample comprising a biological tissue from a vertebrate; a microorganism; and a hybridization complex comprising a first nucleic acid probe hybridized to a nucleic acid of said microorganism; and a second nucleic acid probe hybridized to a nucleic acid of the same microorganism, where the first nucleic acid probe and the second nucleic acid probe are distinguishable and the first nucleic acid probe and the second nucleic acid probe specifically hybridize, under stringent conditions, to a nucleic acid of said microorganism, but not to a nucleic acid in the biological tissue (e.g. vertebrate tissue). The sample is preferably affixed to a surface (e.g. a glass or plastic surface). The probes includes one or more of microorganism-specific probes (optionally labeled) as described herein.

Also provided is a kit detecting a microorganism in a biological sample (e.g. a vertebrate tissue sample). The kit preferably includes a first container containing a first
nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of the microorganism and not to a nucleic acid of the sample tissue (e.g. vertebrate tissue); and a second container containing a second nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of the sample tissue. The kit, optionally, further comprises instructional materials describing the use of the first and second probes for reducing the incidence of false positives in the identification of the microorganism. In some embodiments, the first container and the second container are the same container so the first probe and the second probe are combined. In various embodiments, the probes, labels, tissues, target microorganisms, and the like are as described herein.

In still another embodiment, this invention provides a device for preparing a biological sample for detection of a microorganism. The device preferably includes a first container containing a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of the "target" microorganism and not to a nucleic acid of the sample tissue (e.g. vertebrate tissue); a second container containing a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate; a third container containing a hybridization buffer; and a fourth container capable of containing one or more biological samples; where the first container, the second container, and the third container communicate with the fourth container such that the first nucleic acid probe, the second nucleic acid probe, and the hybridization buffer are delivered to said fourth container when desired.

In some embodiments, the first container and the second container are the same container. In other embodiments, the first container and the third container are the same container. In still other embodiments, the second container and the third container are the same container. The fourth container is, optionally, removable from the device. In certain embodiments, the fourth container is a microtiter plate or a slide holder. In various embodiments, any one of the containers may be in fluid communication with any of the other containers (e.g. through a valve). The device may also further comprise a heater.

In still another embodiment, this invention contemplates methods of detecting a plurality of different microorganisms in a biological sample from a vertebrate. The methods involve contacting the biological sample with a plurality of nucleic acid probes where each probe specifically hybridizes under stringent conditions to a nucleic acid of a
different microorganism and not to a nucleic acid of the vertebrate, and where the different probes comprising said plurality of probes are distinguishable; and detecting hybridization of the nucleic acid probes to targets, where hybridization of each probe indicates the presence of a different microorganism.

But for the fact that, in this embodiment, the probes are specific to different microorganism, preferred probes, labels, tissues, target microorganisms, and the like are as described above. Similarly this invention provides a hybridization mix comprising a plurality of different and distinguishable probes specific to different microorganisms, a biological sample comprising such probes hybridized to different microorganisms, kits comprising a plurality of different and distinguishable probes specific to different microorganisms, and a device for preparing a biological sample for detection of a plurality of microorganisms as described herein.

In another embodiment, this invention provides methods of detecting a microorganism in a biological sample (e.g. a sample from a vertebrate), where said biological sample comprises a granulocyte. The methods preferably involve contacting the biological sample with a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of the sample material (e.g., the vertebrate); and a blocking reagent (e.g., a non-coding nucleic acid, a negatively charged peptide, a negatively charged amino acid, a negatively charged carbohydrate, a negatively charged organic molecule having a molecular weight greater than about 150 MW, a negatively charged nucleoprotein, and a negatively charged lipid, whereby the blocking reagent inhibits binding/interaction of the nucleic acid probe with a granule in the granulocyte. Preferred methods further involve washing the biological sample to remove probe that is not hybridized to a nucleic acid of the target microorganism(s) and detecting remaining probe where the presence of the remaining probe indicates the presence of the corresponding target microorganism(s).

In some embodiments, the nucleic acid probe is contacted with the blocking agent before contacting the biological sample, while in other embodiments, the biological sample is contacted with the blocking agent before contacting the nucleic acid probe. In some embodiments the sample, the probe and the blocking agent are contacted simultaneously.
In certain embodiments, a particularly preferred blocking reagent comprises a non-coding nucleic acid (e.g. tRNA, cot-1 DNA, telomeric DNA, centrosomal DNA, etc.). The probe(s) may each be labeled with a single label or with multiple labels and certain preferred probes are molecular beacons and/or peptide nucleic acids (PNAs) and/or are synthetic oligonucleotides. The probes are often directly or indirectly labeled, e.g. with a fluorescent label, a colorimetric, a radioactive label, an enzymatic label, an electron dense label, a colorimetric label, or a magnetic label.

But for the fact that, in this embodiment, a (granulocyte) blocking agent is used, , preferred probes, labels, tissues, target microorganisms, and the like are as described herein. This invention thus, provides a hybridization mix comprising a (granulocyte) blocking reagent, , a biological sample comprising probes hybridized one or more microorganisms and comprising a (granulocyte) blocking agent, kits comprising a (granulocyte) blocking agent and appropriate probes, and a device for preparing a biological sample for detection of a microorganism in a sample comprising a granulocyte as described herein.

In certain embodiments, the methods described above are performed using intact cells and/or microorganisms. In other embodiments, the cells and/or the target microorganism(s) are disrupted or lysed thereby releasing their nucleic acids. In certain instances this may improve target detection. Methods of lysing cells are well known to those of skill in the art and include, but are not limited to hypotonic solutions, sonication, mechanical shear, digestive enzymes, and the like.

**DEFINITIONS**

The terms "patient" or "subject" are used herein to refer to human patients (e.g., hospital in patients, out-patients, patients in doctor's offices, clinic patients, etc.) and to refer to non-human patients. Thus, it is recognized that the methods of this invention are useful both in human medical context and in veterinary applications. "Patients" contemplated by this invention include, but are not limited to humans, non-human primates, bovines, ungulates, felines, equines, porcines, canines, lizards, amphibians, and the like. Particularly preferred veterinary patients include, but are not limited to, commercially important animal species, e.g. horses, cows, cattle, pigs, goats, llamas, dogs, cats, goats,
sheep, chickens, turkeys, and the like. The term "vertebrate" refers to a member of the class vertebrata.

A "granulocyte" is a type of white blood cell (leukocyte) typically filled with granules of compounds that digest microorganisms. Granulocytes are part of the innate immune system and have broad-based activity. They typically do not respond only to specific antigens as do B cells and T cells. Basophils, eosinophils, and neutrophils are all granulocytes.

The term "tissue" is used herein to refer to a sample of a biological material including biological samples comprising cells (e.g. samples of organs or solid tissues), and biological fluids (e.g. whole blood, blood fractions, cerebrospinal fluid, urine, peritoneal fluid, pleural fluid, etc.). Although not strictly a tissue, urine, fecal material, sputum, and the like, may be considered a tissue within certain contexts in this application.

The terms "fluorescent in situ hybridization" or "FISH", in accordance with common usage, are generally used to refer to in situ hybridization techniques using fluorescently labeled nucleic acid probes. In the context, of this invention, however, it is recognized that, while fluorescent probes are particularly preferred, "FISH" can be performed with a probe labeled with any label that provides an "optically detectable" signal. Thus, the probe may be labeled with a label that produces a colorimetric signal (e.g. colloidal gold, certain enzymes (e.g. β-galactosidase), etc.).

The terms "nucleic acid" or "oligonucleotide" or grammatical equivalents herein refer to at least two nucleotides (e.g., natural or modified) covalently linked together. A nucleic acid of the present invention is preferably single-stranded or double stranded and will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidite (Beaucage et al. (1993) Tetrahedron 49(10):1925) and references therein; Letsinger (1970) J. Org. Chem. 35:3800; Sprinzl et al. (1977) Eur. J. Biochem. 81: 579; Letsinger et al. (1986) Nucl. Acids Res. 14: 3487; Sawai et al. (1984) Chem. Lett. 805, Letsinger et al. (1988) J. Am. Chem. Soc. 110: 4470; and Pauwels et al. (1986) Chemica Scripta 26: 1419), phosphorothioate (Mag et al. (1991) Nucleic Acids Res. 19:1437; and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al. (1989) J. Am. Chem. Soc. 111:2321, O-methylphosphorothioamide linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and

A "probe" or a "nucleic acid probe", as used herein, refers to a collection of one or more nucleic acids whose hybridization to a target can be detected. The probe is labeled, e.g. as described below so that its binding to the target can be detected. The probe may be processed in some manner, for example, by blocking or removal of repetitive nucleic acids or enrichment with unique nucleic acids. Thus the word "probe" may be used herein to refer not only to the detectable nucleic acids, but to the detectable nucleic acids in the form in which they are applied to the target, for example, with blocking nucleic agents, etc. The blocking nucleic acid may also be referred to separately. What "probe" refers to specifically is clear from the context in which the word is used.

A "hybridization buffer" refers to a buffer used in performing a nucleic acid hybridization and/or washing unhybridized nucleic acids after a hybridization reaction.

Hybridization (or wash) buffers are well known to those of skill in the art and include, but are not limited to SSC, TE, TEN, SSPE, SSPE-T, and the like.
"Hybridizing" refers to the binding of two single stranded nucleic acids via complementary base pairing. A "hybridization complex" refers to a complex of two nucleic acids formed by a hybridization reaction and driven by complementary base pairing.

"Bind(s) substantially" or "binds specifically" or "binds selectively" or "hybridizing specifically to" refers to complementary hybridization between a nucleic acid (e.g. a nucleic acid probe) and a target sequence (e.g. a microbial nucleic acid) and embraces minor mismatches (in base pairing) that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence. These terms also refer to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes part I chapter 2 Overview of principles of hybridization and the strategy of nucleic acid probe assays, Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (see, Sambrook et al. (1989) Molecular Cloning - A Laboratory Manual (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY,
(Sambrook et al.) supra for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background, or unbound, probe signal. An example of medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

In one particularly preferred embodiment, stringent conditions are characterized by hybridization in 55% formamide, 2XSSC, pH 7.0 with one or more washes in 1XSSC for five minutes at 72°C.

The term "embedded" refers to a sample that has been infiltrated with a material to provide mechanical support and thereby reduce sample deformation during processes such as sectioning (preparing thin slices for viewing using a microscope). Embedding materials include waxes, such as paraffin wax, epoxies, gelatin, methacrylate, various nitrocellulose polymers and the like.

The term "non-embedded" refers to a sample that is not embedded, and was not previously embedded.

The term "organic solvent" includes aliphatic or aromatic hydrocarbon solvents including xylene, toluene, heptanes, octanes, benzene, acetone, acetonitrile and all isomers (e.g., ortho, meta, para) thereof.

The term "biological sample" refers to a biological material with which a microorganism, e.g. bacteria, may be associated. Frequently the sample will be a "clinical sample" which is a sample derived from a patient. Such samples include, but are not limited to, sputum, blood, blood fractions (e.g. serum), blood cells (e.g., white cells), tissue or fine needle biopsy samples, aspirates (e.g. fluids drained from abscesses), urine, oral fluid (e.g. fluid from the mouth, throat, etc.), nasal/sinus fluids, cerebrospinal fluid, peritoneal fluid, and pleural fluid, or cells therefrom. It will be recognized that the term "sample" may also include supernatant from cell cultures (which may contain free bacteria), cells from cell or
tissue culture and other media in which it may be desirable to detect microorganisms (e.g., food, water, nutritional supplements, etc.).

The terms "probe" or "nucleic acid probe" refers to a nucleic acid that specifically hybridizes with a specific sequence or subsequence of a "target" nucleic acid. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include, but are not limited to $^{32}$P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in an ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available.

A "labeled nucleic acid probe" is a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der waals or hydrogen "bonds" to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

A probe "labeled with a single label" refers to a nucleic acid probe that has affixed thereto only one species of label (e.g. a particular fluorophores).

A probe "labeled with a multiple labels" refers to a nucleic acid probe that has affixed thereto two or more species of label (e.g. a two different fluorophores).

A "molecular beacon" refers to a nucleic acid bearing two different fluorophores or a fluorophore and an absorber/quencher. When the molecular beacon is not hybridized to a target, it self hybridizes (e.g. forms a hairpin) juxtaposing the fluorophore with the "absorber" such that the fluorophore is quenched. When the molecular beacon hybridizes to a target nucleic acid, the hairpin is released, the fluorophore is moved away from the "absorber" and the fluorescence increases. More generally, the term "molecular beacon", as used herein refers to a molecule capable of participating in a specific binding reaction and whose fluorescence activity changes when the molecule participates in that
binding reaction. Molecular beacons are described at length by Tyagi and Kramer et al. (1996) Nature Biotechnology, 14: 303-308.

A "non-coding nucleic acid" refers to a nucleic acid that does not encode a protein. Non-coding nucleic acids include, but are not limited to highly repetitive DNA or RNA, G-C rich DNA or RNA, ribosomal RNA, DNA or RNA from exons, cot-1 (or other cot fractions) DNA or corresponding RNA, centrosomal DNA or RNA, tRNA, and the like. In certain embodiments, a non-coding nucleic acid also includes nucleic acids that may code for polypeptides, but that are not typically found in the sample cell(s), or the microorganism's, nucleic acids.

The term "SSC" refers to a citrate-saline solution of, typically, 0.15M sodium chloride and 20 mM sodium citrate. Solutions are often expressed as multiples or fractions of this concentration. For example, 6 X SSC refers to a solution having a sodium chloride and sodium citrate concentration of 6 times this amount or 0.9M sodium chloride and 120 mM sodium citrate. Similarly, 0.2 X SSC refers to a solution 0.2 times the SSC concentration or 0.03M sodium chloride and 4 mM sodium citrate. These solutions are controlled/buffered for a particular pH (e.g. pH 7).

A "microtiter plate" refers to a plurality of "wells" or "containers" mechanically affixed to each other so that they can be manipulated and/or transported as a single unit. The microtiter plate may have regular or irregular shaped wells having flat, conical, round, or other shaped bottoms. The well cross section can be circular, square, another regular polygon, or irregular. The microtiter plate may include filters, covers, or other removably affixed components. Particularly preferred microtiter plates are "standard format plates" (e.g. 96 well plates, etc.) such as are commonly used in the industry.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 illustrates a multi-station processing device for preparing in situ hybridized samples using the granulocyte blocking agent and/or multiple probe methods as described herein.

Figure 2 illustrates processing device for preparing in situ hybridized samples using the granulocyte blocking agent and/or multiple probe methods as described herein, where the reagents are pumped into a chamber housing the biological sample.
Figure 3 illustrates slides with granulocyte blocking reagent (tRNA) blocking
mast cell binding of the labeled probe.

Figure 4 illustrates slides without a granulocyte blocking reagent.

DETAILED DESCRIPTION

This invention provides novel methods of fluorescent in situ hybridization for
the detection of microorganisms in a biological sample. The use of in situ hybridization
methodologies preserves valuable histological information (e.g. tissue morphology,
localization of the microorganism in or around the tissue, microorganism morphology, etc.)
useful in diagnosis and confirmation of diagnosis. Moreover, the methods are generally
compatible with histological methods already in use in pathology laboratories and therefor
do not require significant reinstrumentation or re-training. In addition, the methods are
relatively simple, highly accurate, rapid and provide effective discrimination between
microorganism strains and/or species.

Prior to this invention, the use of hybridization methods to specifically
identify/quantify microorganisms in vertebrate tissue and/or fluid samples, has been
hampered by non-specific interaction of the hybridization probe with various components of
the sample. In particular, hybridization probes strongly interact with granulocytes (e.g.
vertebrate cells containing granulous bodies). This interaction between granulocytes (e.g.
eosinophils, neutrophils, mast cells) is particularly problematic, because a granulocyte-bound
probe typically displays a morphology that is extremely difficult to visually distinguish from
a microorganism of interest. In particular, without the methods of this invention parasites
(e.g. Trypanosomes, leishmania, malaria, Histoplasma capsulatum, and certain fungi) are
very difficult to morphologically distinguish from hybridization to a granule or to a
granulocyte.

It was a surprising discovery of this invention that interaction between a
nucleic acid probe and a granulocyte can be blocked by the use of a nucleic acid and thereby
reduce background signal and eliminate the frequency of false positives. This is particularly
surprising because the granules comprising granulocytes are not nucleic acids themselves.
Moreover, it was completely unexpected that nucleic acids (particularly non-coding nucleic
acids) would not only block non-specific binding of a nucleic acid probe to a granulocyte,
but that this would occur without interfering with hybridization of the nucleic acid probe to its target (the microorganism(s)).

Having discovered that nucleic acids (e.g. "non-coding" nucleic acids) could block nucleic acid probe/granulocyte interactions, it was then discovered that other agents, typically relatively large negatively charged molecules could also inhibit this interaction. Thus, other suitable blocking agents include, but are not limited to, negatively charged amino acids or peptide (e.g. ranging from about 1 amino acid up to about 100,000 amino acids), a, a negatively charged carbohydrate, a negatively charged organic molecule having a molecular weight (e.g. preferably having a molecular weight greater than about 150 MW), a negatively charged lipid, and the like.

Even with granulocyte blocking, (or without) it is also desirable to minimize the occurrence of false positives. In one embodiment this invention provides another method of minimizing false positives. This involves performing the hybridization with two or more probes specific to the same microorganism or even to the same nucleic acid sequence, where each of the two or more probes are labeled with different and distinguishable labels. Where two differently labeled probes co-localize on a target it indicates that the target is a real target microorganism. Where only a single labeled probe tags or identifies a target the result is treated as a false positive or, alternatively, other confirmatory approaches (e.g. visual microscopy, alternate staining, etc.) are taken.

In still another embodiment, it is desirable to screen for a plurality of different microorganisms at one time. In this approach, different nucleic acid probes are used that are specific to each target microorganism it is desired to detect. The different probes are labeled with distinguishable labels thereby permitting different microorganisms to be detected at the or identified same time.

These various approaches are not mutually exclusive. To the contrary, it is contemplated that any and/or all combination may be utilized. Thus, mast cell blocking can be used with multiple probes to different microorganisms and/or with multiply labeled probes to a single microorganism. One assay contemplates the use of two or more differently labeled probes to a single microorganism (to reduce false positives), while also utilizing probes for different microorganisms. Thus, for example, one experiment may utilize four differently labeled probes where two probes (probe A and probe B) are specific to microorganism X, and two probes (probe C and probe D) are specific to microorganism Y.
Co-localization of probes A and B indicate microorganism X is present, while co-localization of probes C and D indicate that microorganism Y is present. Both organism X and organism Y are detected and/or identified simultaneously. Moreover, microorganisms X and Y are distinguished from other (e.g. non-pathogenic) microorganisms (e.g. organism "Z") that might also be present.

The discoveries of this invention, e.g., granulocyte blocking, false positive reduction, and simultaneous screening of multiple microorganisms, while useful in a wide variety of hybridization formats are particularly useful for in situ hybridization formats, more particularly in fluorescent in situ hybridization (FISH) formats. The use of FISH for microorganism detection, the use of granulocyte blocking techniques, the use of multiple probe validation methods and the simultaneous detection of multiple microorganisms are described in detail below.

I. Fluorescent In Situ Hybridization for Microorganism detection.

Detection of microorganisms by in situ hybridization generally involves providing a biological sample comprising a nucleic acid to be tested, typically in a histological preparation comprising either intact or broken cells, hybridizing the nucleic acid sample with one or more probes that specifically bind to nucleic acid sequences found within the microorganism(s) it is desired to detect, and detecting or quantifying the hybridized probes, thereby identifying/quantifying the target microorganism(s). In preferred embodiments, the methods further involve blocking probe binding to granulocytes (e.g. mast cells) and/or multi-probe validation methods and/or simultaneous detection of multiple microorganisms. These methods are described in detail below.

A) Collection of a biological sample.

The biological sample is collected in accordance with standard methods well known to those of skill in the art. The term "biological sample", as used herein, refers to a sample obtained from an organism or from components (e.g., cells or cell extracts) of an organism. The sample may be of any biological tissue or fluid. Biological samples may also include organs or sections of tissues such as frozen sections taken for histological purposes. Biological samples include, but are not limited to whole blood or blood fractions (e.g. serum, buffy coat, etc.), cerebrospinal fluid, oral fluid (e.g. sputum), pleural
fluid, peritoneal fluid, nasal fluid, stomach fluid, urine, fecal matter, aspirates (e.g. from wounds and/or abscesses), and various tissue samples (e.g. cut tissue samples, needle biopsies, surgically removed tissues and/or organs, autopsy tissue samples), and the like, or any extract therefrom.

The biological sample may be a fresh sample or a sample that has been stored. Particularly preferred samples include fresh samples, frozen samples (e.g. cryogenically fixed samples), and dehydrated and/or fixed samples. The sample may be stored in a buffer and/or preservative and/or fixative as desired.

B) Nucleic acid probes for microorganisms.

i) Probe selection.

Nucleic acid probes for use in this invention are selected so that they specifically hybridize to (form a hybridization complex with) a nucleic acid in the microorganism(s) it is desired to detect and not with a nucleic acid in the vertebrate cell(s) or other microorganisms present in the sample being analyzed. Essentially any microorganism can be detected using the methods of this invention, however, pathogenic/parasitic microorganisms are typically of greatest interest. Such microorganisms include, but are not limited to mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes. Particularly preferred, medically relevant, bacterial microorganisms include, but are not limited to aerobic bacteria such as *Staphylococcus*, *Streptococcus*, *Neisseria*, *Branhamella*, *Moraxella*, *Acinetobacter*, *Bacillus*, *Listeria*, *Erysipelothrix*, *Propionibacterium*, *Streptobacillus*, *Calymmatobacterium*, etc., and anaerobes such as *Salmonella*, *Shigella*, *Campylobacter*, *Helicobacter*, *Vibrio* (e.g. *Vibrio cholerae*), *Escherichia* (e.g. *Escherichia coli* strains), *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Pseudomonas*, *Brucella*, *Pasteurella*, *Yersinia*, *Francisella*, *haemophilus*, *Bordetella*, *Corynebacterium*, *Mycobacteria*, *Nocardia*, *Actinomyces*, *Arachnia*, *Streptomyces*, *Leptospires*, *Borrelia*, *Spirillum*, *Treponema*, *Mycoplasmas*, *Rickettsiae*, *Chlamydia*, *Legionella*, etc. Other relevant pathogenic microorganisms, include, but are not limited to various fungi including yeasts and molds (e.g. *Ascomycetes*, *Basidimycetes*, in particulat *Histoplasma capsulatum*, etc.), protozoa (e.g. *Leishmania*, *Trypanosoma*, *Giardia*, *Trichomonas*, *Entamoeba*, *Dientamoeba*, *Naegleria*, *Acanthamoeba*, *Babesia*, *Plasmodium*, *Isospora*, *Sarcocystis*, *Sachomyces* (e.g. *S.*
*cerevisiae*, Cryptosporidium, Toxoplasma, Balantidium, Pneumocystis, etc.), amebas, hemoflagellates, malaria, toxoplasma, Pneumocystis, helminthes, schistosomes and other trematodes, cestodes, nematodes, and the like.

In certain embodiments, the nucleic acid probes are selected to specifically bind to a viral nucleic acid and thereby afford viral detection. Preferred virus targets include, but are not limited to *Herpesviridae* (*e.g.* Herpes simplex, Varicella zoster, Cytomegalovirus, *Herpes viruses* 6 and 7, etc.), Hepatitis viruses, Influenza, Parainfluenza, Respiratory syncytial viruses, adenoviruses, rhinoviruses, coronaviruses, Measles, Rubella, Mumps, Enteroviruses, Poxviruses, Alphaviruses, Flaviviruses, Bunyaviridae, Filoviruses, Rabies, Papillomaviruses, Paroviruses, Retroviruses (*e.g.* HIV), and the like.

Nucleic acid probe sequences particular to a given microorganism can be determined according to routine methods well known to those of skill in the art. Typically a suitable probe sequence can be initially identified by searching a nucleic acid database for a nucleic acid sequence common to the microorganism(s) it is desired to detect and rare or absent in other tissues/organisms. Bioinformatics tools (*e.g.* BLAST) may be used for searching nucleic acid databases and/or for identifying particular probes/consensus sequences, and these tools are well known to those of skill in the art. Moreover, such software tools are freely available on a number of internet web sites, available as freeware or shareware, or available in commercial bioinformatics packages (*e.g.* LaserGene™ by DNAStar, DNAassist™, etc.).

When one or more candidate probe sequences are identified, the relevant probes are made (*e.g.* as described below) and then tested in a relevant assay. Typically, this will be accomplished by performing hybridization (*e.g.*, an *in situ* hybridization) with the probe in a sample of interest containing the target microorganisms, and optionally in a sample lacking the target microorganisms. The degree of hybridization of the probe with the microorganism(s) of interest, as opposed to the sample tissue, is evaluated. Typically hybridization stringency will be increased to determine if a convenient stringency exists where the probe strongly hybridizes with the microorganism(s) of interest and not (substantially) with the host tissue.

Preferred probes will provide a signal when hybridized to the microorganism of interest that is distinguishable from "weak hybridization" or non-specific binding to the sample nucleic acid(s). More preferably the probe will provide a signal that is significantly
distinguishable (e.g. at the 90% or better, preferably at the 95% or better, more preferably at the 98% or better, and most preferably at the 99% or better confidence level) from interactions with the sample nucleic acid(s). In preferred embodiments the probes provide a signal at least 1.5 fold, preferably at least 2 fold, more preferably at least 5 fold, and most preferably at least 10 fold greater than the "background" signal provided by probes interacting with sample tissue nucleic acid(s).

A bioinformatics approach is not required to pre-select probes. Other approaches are known to those of skill in the art. Thus, for example, in one approach, the total nucleic acid (e.g. total DNA, total RNA, etc.) of the microorganism is used as a probe. In this instance, however, it will be recognized that such complex probes should be tested to verify that they are not unduly cross-reactive with other microorganisms and/or with the sample nucleic acid(s) before they are implemented as a regular diagnostic. Where it is desired to identify a probe that specifically distinguishes between two or more microorganism species and/or strains, a subtractive hybridization between the nucleic acids of the microorganisms of interest can be performed. The remaining nucleic acids not common to the microorganisms are good candidates for nucleic acids to distinguish the species or strains. Methods of performing subtractive hybridization are well known to those of skill in the art (see, e.g., Hampson, et al. (1992) Nucleic Acids Res., 20: 2899).

In addition, suitable probes for a wide number of microorganisms have already been identified. Thus, for example, probes for the detection of Legionella are described in U.S. Patent 5,055,394, probes for detection of Vibrio parahaemolyticus, toxigenic Escherchia coli, and Staphylococcus aureus are described in U.S. Patent 5,516,898, probes for the detection of pathogenic Campylobacter bacteria are described in U.S. Patent 5,494,795, probes for the detection of Salmonella are described in U.S. Patent 5,434,056, probes for the detection of Shigella are described in U.S. Patent 5,795,717, probes for the detection of various gram-negative bacteria are described in U.S. Patent 5,059,527, probes for the detection of Neisseria are described in U.S. Patent 4,245,038, probes for the detection of eubacterial organisms are described in U.S. Patent 6,025,132, probes for the detection of Chlamydia are described in U.S. Patent 5,814,490, and so forth. This list of probes is meant to be illustrative and not limiting. Numerous other probes for other microorganisms can be identified in the patent and scientific literature.
ii) Making and labeling probes.

Nucleic acid probes for use in the methods of this invention can be made according to a number of methods well known to those of skill in the art (see, e.g. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, or Ausubel et al. (1987) Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York)

In one embodiment, nucleic acids probes are prepared using methods of direct chemical synthesis by methods such as the phosphotriester method of Narang et al. (1979) Meth. Enzymol. 68: 90-99; the phosphodiester method of Brown et al. (1979) Meth. Enzymol. 68: 109-151; the diethylphosphoramidite method of Beaucage et al. (1981) Tetra. Lett., 22: 1859-1862, and the solid support method of U.S. Patent No. 4,458,066. Typical chemical syntheses routinely produce nucleic acids up to about 150 nucleotides in length, although careful syntheses can routinely produce nucleic acids up to 500 nucleotides in length. In addition, it is possible to ligate shorter sequences to produce very long nucleic acids.


Other probes can be created by isolating nucleic acids from representative target organisms and, optionally, cleaving the nucleic acids to a desired average length. The
nucleic acids are then labeled. Thus, the term nucleic acid probe includes single nucleotide sequences or combinations of different nucleotide sequences.

Preferred probes are sufficiently long that they will specifically hybridize to the nucleic acids of target microorganisms. Typically suitable probes are at least 10 nucleotides, preferably at least 15, more preferably at least 25, and most preferably at least 50 nucleotides in length. Certain preferred probes range in length from about 15 to about 100 nucleotides, preferably from about 20 to about 70 nucleotides and more preferably from about 30 or about 35 to about 50 nucleotides in length. In many instances longer probes are desired. Such longer probes are typically at least 100 nucleotides, preferably at least 200 nucleotides, more preferably at least 300 nucleotides in length, with such longer probes typically ranging in length from about 100 to about 400, more preferably from about 150 to about 300 nucleotides in length. Very long probes include probes longer than about 1000 nucleotides, and most preferably at least 2000 or even 3000 nucleotides in length. Preferred molecular beacons have a "hybridization" section ranging in length as indicated above, but may also have additional flanking nucleotides (e.g. 5 to 15 nucleotides at each end) to participate in hairpin formation.

Methods of labeling nucleic acids are well known to those of skill in the art. Preferred labels are those that are suitable for use in in situ hybridization. The nucleic acid probes may be labeled prior to the hybridization reaction. Alternatively, a detectable label which binds to the hybridization product may be used. Such detectable labels include any material having a detectable physical or chemical property and have been well-developed in the field of immunoassays.

As used herein, a "label" or a "detectable label" is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Useful labels in the present invention include radioactive labels (e.g. $^{32}$P, $^{125}$I, $^{14}$C, $^3$H, and $^{35}$S), fluorescent dyes (e.g. fluorescein, rhodamine, Texas Red, etc.), electron-dense reagents (e.g. gold), enzymes (as commonly used in an ELISA), colorimetric labels (e.g. colloidal gold), magnetic labels (e.g. Dynabeads™), and the like. Examples of labels which are not directly detected but are detected through the use of indirect labeling strategies include biotin and dioxigenin as well as haptens and proteins for which labeled antisera or monoclonal antibodies are available.
The particular label used is not critical to the present invention, so long as it
does not interfere with the in situ hybridization of the stain. However, stains directly labeled
with fluorescent labels (e.g. fluorescein-12-dUTP, Texas Red-5-dUTP, etc.) are preferred for hybridization.

A direct labeled probe, as used herein, is a probe to which a detectable label is
attached. Because the direct label is already attached to the probe, no subsequent steps are
required to associate the probe with the detectable label. In contrast, an indirect labeled
probe is one which bears a moiety to which a detectable label is subsequently bound,
typically after the probe is hybridized with the target nucleic acid.

Particularly preferred labels are detectible in as low copy number as possible
thereby maximizing the sensitivity of the assay and are detectible above any background
signal. Finally, a label is preferably chosen that provides a highly localized signal thereby
providing a high degree of spatial resolution. Particularly preferred fluorescent labels
include, but are not limited to fluorescein-12-dUTP and Texas Red-5-dUTP.

It will be recognized that fluorescent labels are not to be limited to single
species organic molecules, but include inorganic molecules, multi-molecular mixtures of
organic and/or inorganic molecules, crystals, heteropolymers, and the like. Thus, for
example, CdSe-CdS core-shell nanocrystals enclosed in a silica shell can be easily
have been covalently coupled to biomolecules for use in ultrasensitive biological detection

The labels may be coupled to the probes in a variety of means known to those
of skill in the art. In a preferred embodiment the nucleic acid probes will be labeled using
nick translation or random primer extension (Rigby, et al. (1977) J. Mol. Biol., 113: 237; or
Sambrook, et al., supra). A wide variety of labels appropriately derivatized for coupling to
nucleic acids are commercially available from a number of sources (see, e.g., Molecular
Probes, Eugene, OR).

One of skill in the art will appreciate that the probes of this invention need not
be absolutely specific for the microorganism. Rather, the probes are intended to produce
"staining contrast" permitting the microorganism to be distinguished against background.
Thus contaminating sequences which only hybridize to non-targeted sequences, for example,
impurities in a library, can be tolerated in the stain to the extent that the sequences do not reduce the staining contrast below useful levels.

C) Detection by in situ hybridization.

In situ hybridization (ISH) is a powerful and versatile tool for the detection and localization of nucleic acids (DNA and RNA) within cell or tissue preparations. Methods of in situ hybridization are well known to those of skill in the art. Several guides to the techniques are available such as Gall et al. Meth. Enzymol., 21:470-480 (1981) and Angerer et al. (1985) Pages 43-65 In: Genetic Engineering: Principles and Methods Setlow and Hollaender, eds. Vol 7, Plenum Press, New York.

Generally, in situ hybridization comprises the following major steps: (1) fixation of tissue or biological structure to analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA, and to reduce nonspecific binding; (3) hybridization of the mixture of nucleic acids to the nucleic acid in the biological structure or tissue; (4) posthybridization washes to remove nucleic acid fragments not bound in the hybridization and (5) detection of the hybridized nucleic acid fragments (e.g. via detection of a label). The reagent used in each of these steps and their conditions for use vary depending on the particular application.

Typically, the nucleic acid sample is prepared by depositing cells, either as single cell suspensions or as tissue preparation, on solid supports such as glass slides and, optionally, fixed by choosing a fixative (e.g. formalin) which provides the best spatial resolution of the cells and the optimal hybridization efficiency (see, e.g., Coulton and Belleroche (1992) In Situ Hybridization: Medical Applications, Kluwer Academic Publishers, Boston; Eberwine et al. (1994) In Situ Hybridization: In Neurobiology; Advances in Methodology, Oxford University Press Inc., England; and Wilkinson (1992) In Situ Hybridization: A Practical Approach, Oxford University Press Inc., England).

In some applications it is necessary to block the hybridization capacity of repetitive sequences or to suppress non-specific interactions between the probe(s) and granulocytes present in the biological sample to reduce background noise and/or the instance of false positives (see discussion below).

Standard in situ hybridization techniques are used to probe a given sample. Hybridization protocols suitable for this invention are illustrated in the examples provided

Numerous non-isotopic systems have been developed to visualize labeled nucleic acid probes including, but not limited to a) fluorescence-based direct detection methods, b) the use of digoxigenin-and biotin-labeled nucleic acid probes coupled with fluorescence detection methods, and c) the use of digoxigenin-and biotin-labeled nucleic acid probes coupled with antibody-enzyme detection method, and the like. When fluorescence-labeled nucleic acid probes are hybridized to cellular DNA or RNA, the hybridized probes can be viewed directly using a fluorescence microscope. By using multiple nucleic acid probes with different fluorescence colors, simultaneous multicolored analysis (i.e., for identifying multiple microorganisms) can be performed in a single step on a single sample. Fluorochrome-directly-labeled nucleic acid probes eliminate the need for multi-layer detection procedures (e.g., antibody-based-systems), which allows fast processing and also reduces non-specific background signals.

In one preferred embodiment, scoring fluorescence signals using the FISH procedures described above generally requires a microscope with a filter, e.g. a triple bandpass filter to enhance signal detection. Such a microscopy system can be automated and under computer control to facilitate data collection. However, to date, human scanning typically proved faster and more accurate than computer controlled scanning.

In certain particularly preferred embodiments, the methods of this invention comprises the following: The DNA probe is precipitated, e.g. in a sodium acetate/ethanol mix, then pelleted and resuspended in a hybridization buffer solution containing a granulocyte blocking agent (e.g. tRNA). The solution preferably comprises hybridization buffer, a detergent, and a yeast tRNA, although the use of the detergent is not mandatory. In a particularly preferred embodiment, these components are 20% SDS and 100 mg/ml yeast tRNA, most preferably in the ratio 7:2:1 by weight. A preferred ratio of probe to blocking
agent (e.g. tRNA) is 1:50, preferably 1:500, and more preferably 1:5000, but may go higher e.g. 1:10,000 or greater. The probe is mixed and dissolved back into solution, and is then ready to use in the detection protocol. The detection protocol is preferably an in situ hybridization (ISH) protocol (e.g., FISH or other in situ hybridization technique). In ISH, the protocol preferably comprises a pretreatment of prepared samples, denaturation and hybridization of the samples, washing, and optionally counterstaining.

Such pretreatment preferably includes soaking in a series of washes, preferably xylene (or other organic solvent) if paraffin needs to be removed, followed by ethanol (or other alcohol), to remove any residue of fixative or solvents. In certain embodiments, a digestion is the preferred next step, disrupting the sample so as to allow better access to the targeted areas. In one embodiment, digestion is initiated using sodium thiocyanate, preferably 1M, at approximately 80°C for about 10 minutes. Slides are then washed in water, and placed in a digestion solution, preferably 5mg/ml pepsin, and preferably dissolved in an acid, such as 0.2M HCL. An additional washing step follows, preferably more than one wash in distilled water.

Slides/samples are then denatured to facilitate hybridization. Denaturation is accomplished by placing the slide/sample in a denaturation solution, preferably 70% formamide/2x SSC, and preferably by heating the sample, preferably to approximately 72°C. A pre-soaking step in denaturation solution at room temperature may, optionally, be added. Denaturation may take place at temperatures ranging from room temperature to approximately 100°C. Generally, the higher the temperature, the lower the formamide concentration needed.

Slides are then dehydrated, preferably in an ethanol series, while the probe is denatured by placing in an elevated temperature, e.g., 72°C. The probe is added to the specimen, and if a slide is used, it is then coverslipped and incubated, preferably in a humidified chamber at least overnight. The coverslip is then removed, and the slide/specimen is washed, e.g. in an SSC solution ranging from 0.4 X SSC to 1 X SSC for the first wash and 2 X SSC for a second wash. In certain embodiments, counterstain is added before viewing.

One or more probes can be utilized simultaneously in the protocol to target multiple microorganisms within the same sample. Various specimen types are amenable to this procedure as described above.
D) **Granulocyte (mast cell) blocking.**

As indicated above, it was a surprising discovery of this invention that interaction between a nucleic acid probe and a granulocyte can be blocked by the use of a nucleic acid. This is particularly surprising because the granules comprising granulocytes are not nucleic acids themselves. Moreover, it was completely unexpected that nucleic acids (particularly non-coding nucleic acids) would not only block non-specific binding of a nucleic acid probe to a granulocyte, but that this would occur without interfering with hybridization of the nucleic acid probe to its target (the microorganism(s)).

Having discovered that nucleic acids (e.g. "non-coding" nucleic acids) could block nucleic acid probe/granulocyte interactions, it was then discovered that other agents, typically relatively large negatively charged molecules could also inhibit this interaction. Thus, other suitable blocking agents include, but are not limited to a blocking reagent selected from the group consisting of a non-coding nucleic acid, a negatively charged amino acid, a negatively charged peptide comprising from about 2 to about 100,000 amino acids (preferably from about 2 to about 10,000 amino acids), more preferably from about 2 to about 1000 or 500 or 100 or 50 amino acids), a negatively charged carbohydrate, a negatively charged organic molecule having a molecular weight greater than about 150 MW (preferably greater than about 300 MW, more preferably greater than about 500 MW, also preferably less than about 50,000 MW, preferably less than about 10,000 MW, more preferably less than about 5,000 MW, and most preferably less than about 1000 MW), a negatively charged nucleoprotein, a negatively charged lipid, and the like.

As used herein, in a preferred embodiment, a non-coding nucleic acid refers to a nucleic acid that does not encoding a protein. Non-coding nucleic acids include, but are not limited to highly repetitive DNA or RNA, G-C rich DNA or RNA, DNA or RNA from exons, cot-1 (or other cot fractions) DNA or corresponding RNA, centrosomal DNA or RNA, tRNA, and the like. In certain embodiments., a non-coding nucleic acid also includes nucleic acids that may code for polypeptides, but that are not typically found in the sample cells, or the microorganism's, nucleic acids. Thus, where centrosomal or telomeric nucleic acids comprise sequences that encode polypeptides, they may still be regarded as "non-coding" for the purposes of this invention. Suitable non-coding nucleic acids can be identified using a bioinformatic approach as describe above. Alternatively such "non-coding" nucleic acid can be generated by performing a subtractive hybridization between the...
nucleic acids from the sample (including target microorganisms), a reference nucleic acid pool. The nucleic acids not subtracted out will be useful as "non-coding" nucleic acids in the present context.

The non-coding nucleic acids preferably range in length from individual nucleotides to nucleic acids ranging from about 5 nucleotides to about 10 nucleotides, preferably from about 5 nucleotides up to 50 or 100. In certain embodiments, the "non-coding" nucleic acid range in length from 100 to 1000, 50000, or even 10000 nucleotides or longer (e.g. 100,000 nucleotides). Conversely, in certain embodiments, preferred non-coding nucleic acids are no longer than 10,000, nucleotides, more preferably no longer than about 5,000 nucleotides, and most preferably no longer than 1,000 or 5000 nucleotides in length. In a particularly preferred embodiment, the blocking agent is a cot-1 DNA or a tRNA (e.g. a yeast tRNA).

While nucleic acids, as indicated above, are preferred granulocyte blocking agents, other negatively charged molecules are suitable. These include, but are not limited to negatively charged amino acids (e.g. Aspartic acid, Glutamic acid, etc.) negatively charged peptides and/or serum proteins (e.g. serum albumin, etc.), negatively charged carbohydrates, negatively charged organic molecules, e.g. having a molecular weight greater than about 150 MW, negatively charged nucleoproteins, negatively charged lipids (e.g. many phospholipids), and the like.

The blocking agent is used in a manner similar to blocking agents that are used to prevent non-specific binding of probes to nucleic acids. Typically the blocking agent is added to, or a component of, a hybridization mix. The blocking agent may be contacted with the probe(s) before they are contacted with a sample, or alternatively, the probe(s) and blocking agent(s) may be added simultaneously to the sample. In certain embodiments, the blocking agent is contacted with the sample before the probe. Preferred methods are illustrated in the examples.

E) Multiple labels for target validation.

In certain embodiments, this invention contemplates the use of multiple hybridization events to validate/confirm positive detection (to reduce false positives). In preferred embodiments, this is accomplished by using multiple probes to a single target microorganism where the probes are labeled with different and distinguishable labels. When
both probes hybridize to the same target microorganism (or same collection of microorganisms), they co-localize in the *in situ* hybridization. In other words, they label the same target(s). Such co-localization is interpreted as a valid (positive) target (microorganism) detection. Where only a single probe labels a putative target this is interpreted as an actual false positive or a potential false positive. Such a "hit" may be subject to further confirmatory analysis (*e.g.* microscopy under other illumination, the use of other stains, *etc.*).

In one embodiment, the different probes have identical nucleic acid sequences and differ only in the label. In other embodiments, the different probes are designed to hybridize to nucleic acid sequence(s) of the same microorganism. Such target nucleic acid sequences may be overlapping or distinct, and need not be of similar length.

The different and distinguishable labels may be of a different kind (*e.g.* a radioactive label and a fluorescent label), or the same kind (*e.g.* both fluorescent), but providing distinguishable signals (*e.g.*, red and green). In preferred embodiments, the labels are optically detectable (*e.g.* colorimetric, fluorescent, *etc.*) and the co-localization of the probes appears as a two different colors localized at the same "feature" or as a different color (the combination of the two or more probes) than the colors of each of the individual probes.

Thus, for example, in one embodiment, a first probe is labeled with fluorescein, while a second probe is labeled with Texas Red. The first probe alone appears green, while the second probe alone appears red. Both probes together (a confirmed positive) appear green and red on the same target.

**F) Multiple probes for multiple pathogens.**

In another embodiment, this invention contemplates the detection of multiple microorganisms simultaneously. This is accomplished by providing a plurality (*e.g.* two or more) probes each specific to a different microorganism. The probes are selected such that each probe targets a single species or strain of microorganism in a particular sample. Detection of each probe (*e.g.* scoring the probe as positive) indicates the presence and identity of the microorganism corresponding to that positive probe in the sample.
II. Scoring the assay.

The assays of this invention are scored according to standard methods well known to those of skill in the art. In one embodiment, the *in situ* hybridization results are scored by visual inspection (typically using fluorescence microscopy). Questionable tagging can be validated by altering filter sets and by reference to features visible under conventional optical or other (*e.g.* nemarsky) microscopy.

Typically a hybridization will be scored as positive when the label signal is clearly distinguishable above background. Where two confirmatory probes are used, as described above, the hybridization is scored as positive when both probes co-localize at the same feature(s). When only a single probe localizes at a feature, a re-test or further investigation is warranted. The labeled feature may, optionally be verified under alternate illumination. In preferred embodiments the probes provide a signal at least 1.5 fold, preferably at least 2 fold, more preferably at least 5 fold, and most preferably at least 10 fold greater than background as described above.

In certain embodiments, the assay is scored using automated data acquisition methods. This can be accomplished by using a microscope configured with an image acquisition system. Alternatively, in certain embodiments, the slide (or other hybridization surface) is juxtaposed directly to an image acquisition device (*e.g.* a CCD camera).

The image is scanned, and software analyzes the image and scores positive hits. Software to optimize level of detection, noise rejection, trace and/or identify and/or count features is well known to those of skill in the art. Such image analysis software is commercially available from a number of suppliers (*e.g.* MediaCybernetics, TOOLBOX Visilog, from Noesis Vision Inc., OPTIMAS, from Optimas Corp. of Bothell, WA, SigmaScan from Jandell, San Rafael, CA; GLOBAL LAB from Data Translation of Marlboro, MA; Oncor-Image from Oncor, Gaithersburg, MD; CellScan from Scanalytics/CSPI; and the like). Such packages offer a wide variety of features such as the ability to perform computation-intensive deconvolution algorithms to improve image definition. Such software packages are designed to be readily adapted to a wide variety of uses, particularly the analysis of microscope images.
III. Devices for automated FISH.

In another embodiment, this invention contemplates devices for automated sample preparation for the detection of microorganisms. Automated devices for histological preparation are well known to those of skill in the art. Generally the devices fall into two basic types. In the first type, the sample(s) are sequentially moved through a series of baths or "stations", each bath containing a particular sample preparation reagent or each station performing a particular physical or chemical operation. One such device is illustrated by U.S. Patent 5,601,650 from which Figure 1 is obtained. As illustrated in Figure 1, the device preferably comprises a housing 42 with several processing stations 16. The processing stations 16 have vats 48 with reagents for preprocessing, dyeing, hybridization, or washing, and a drying chamber 64. Typically, one vat 48 will comprise one or more nucleic acid probes, as described herein. In certain embodiments, the same vat, or a different vat, will comprise a hybridization mix, and/or a granulocyte blocking reagent as described herein. Three are optionally vats for serial dehydration of the sample, for optional sample digestion, for nucleic acid denaturation, and for essentially any step in the preparation and in situ hybridization of the tissue. Particular vats will be maintained at optimum pH, salinity, and temperature to facilitate nucleic acid denaturation and/or re-annealing of the probe(s) to their target nucleic acids, and/or for washing off unbound probe (e.g. at high stringency). Certain vats are optionally configured with heaters to allow optimum temperature conditions to be maintained or systematically varied.

In one embodiment, the processing stations 16 form in the housing 42 two parallel rows; and between these rows there is a drip area 36, which is, optionally, designed as a chamber through which water passes. A conveyor 18 is arranged above the processing stations 16. Furthermore, a feed station 38 and a removal station 40, which includes a horizontally moveable drawer 44, are provided in the front region of the housing 42.

The conveyor 18 enables transport movements in three coordinate axes. Thus, the processing stations 16 can be approached in any arbitrary order. A first transport carriage 120 can be moved in the cross direction. It rests on two housing-sided rails and is equipped with a parallel drive, which ensures a synchronous movement on both tracks.

A second transport carriage 122 for a movement in the longitudinal direction is arranged on the first transport carriage 120. Since the span width of this second transport carriage 122 is smaller, it can make do without a parallel drive. The second transport
carriage 122 in turn carries a lifting and lowering device 124. The two transport carriages 120 and 122 and the lifting and lowering device 124 are driven by actuators which are designed as stepping motors, or regulated direct current motors as servomotors. The position reached is detected by means of sensors, which are not shown here for reasons owing to a better overview. This design enables a very exact, repeatable positioning.

The actuators are a component of a controller, which also includes a computer and a memory. The conveyor 18 is operated with the controller. A program line, which consists of transport times, processing times, processing temperatures and processing stations 16 to be approached, is stored in the controller or computer memory. Depending on the selected dyeing method a specific program line, which causes the computer 28 to control the movement sequence by way of the actuators is selected in the memory. When several different dyeing and/or hybridization methods run simultaneously, the program start of different dyeing methods is controlled with the aid of the stored program lines in the sense of a non-overlapping parallel run of the program lines. Thus, if, for example, the dyeing process is initiated for a first object slide holder, then the program determines with the aid of the program line of the dyeing method for the on-going dyeing method and the program lines for other dyeing methods the start time at which the next dyeing method can begin. As soon as this start time is reached, the next object slide holder is included in the processing sequence.

To prepare a dyeing and/or hybridization method, preparations, e.g. on microscope slides are inserted into object slide holders which are attached to coupling element 20. The slide holders are automatically taken in succession by the conveyor 18 from this feed station 38 and conveyed to the processing stations 16 provided by the program. Upon completing the staining and/or hybridization process the object slide holders are placed into a removal station 40.

Each slide may be encoded (e.g. with a bar code) that is read by the device and that offers sample identification information and/or that selects a particular processing protocol. The encoded information in certain embodiments, simply selects a previously programmed processing protocol, or, alternatively, the encoded information provides all the necessary protocol information and, in effect, programs the device. This approach allows for non-sequential processing and/or positive identification of the sample.
In the second type of sample processing device, the sample is held in a single chamber, while reagents are sequentially introduced and removed. A representative type of this device is illustrated in U.S. Patent 4,141,342 from which Figure 2 is obtained. A device of this sort can be readily adapted to perform in situ hybridizations.

Thus, in one embodiment, the tissue processor according to the invention processes histological tissue specimens through fixing, dehydrating, clearing and embedding agents automatically, and prehybridization and/or hybridization and/or wash protocols automatically. Referring to the schematic diagram of Figure 2, tissue capsules 10 are placed in a perforated tissue basket 12 and the basket is positioned in a tissue container 14. The container is closed and sealed by lid 16. A simple "O" ring seal may be used to insure a vacuum tight fit.

The tissue container 14 is plumbed to a vacuum pump 18, a vacuum gauge 20 and to a vacuum exhaust valve 22. A heater, such as band type heater 24 is provided for the container 14. An inlet and drain port 26 located in the bottom of the container 14 is in fluid communication via valve block 28 which includes solenoid operated valves 30, 32 and 34 with a first paraffin bath 36, a plurality of solvent baths 38a, 38b, 38c, . . . etc. and a second paraffin bath 40. It will be appreciated that while the device of Figure 2 is illustrated with respect to paraffin baths, the paraffin baths can be substituted with hybridization mix and/or hybridization probes and/or blocking agent(s) as described herein. The heater can then govern the hybridization and wash temperatures.

Each solvent or hybridization bath is in fluid communication with valve 32 by means of a rotary valve 42. Valve 42 is a multiple port valve having a common port 43 sequentially coupled to, say twelve, selectable ports (44a, 44b, 44c, . . . etc.). Preferably, the selectable ports are radially extending in a fixed valve body and connectable with the common port by a rotating slide. Ten of the ports, for example, are plumbed to ten solvent/hybridization reagent baths, and a remaining port is plumbed to a fitting (not shown) for purging or flush purpose. When the valve 32 is open, the tissue container 14 and the tissue capsules 10 contained therein are connected to one of the ten baths selected by the rotary valve 42. Similarly, when valves 30 and 34 are open, the tissue container 14 and the tissue capsules contained therein are connected, in turn, to the baths 36 and 40.

The tissue processor is, in the preferred embodiment, controlled by a programming module 100, which determines the sequence and timing of all processing
operations. Generally speaking, the operation of the tissue processor according to the invention may be summarized as follows: Tissue specimens to be processed are placed in a container optionally sealed against atmosphere. The specimens are selectively subjected to treating agents or fluids introduced into the container. In a preferred embodiment of the invention, the rotary valve rotates to a first selected solvent bath, usually a fixation bath. Valve 32 opens and vacuum pump 18 is energized causing the solvent (treating agent) to enter the container until all of it is transferred from the solvent bath, at which time the solvent valve 32 closes and the pump 18 is de-energized. The solvent is optionally stirred gently by a stirrer 46 magnetically driven from outside the container, thus moving it among the tissue capsules. When the first bath cycle is completed, the solenoid operated vacuum exhaust valve 22 is opened to atmosphere and the solvent valve 32 reopens permitting the solvent to drain via gravity into the solvent bath. The same cycle is repeated through each of the solvent baths selected by the rotary valve 42. For the hybridization bath cycles, heated reagents flow from baths 36, 40 through valves 30 or 34, respectively, and into the tissue container 14 which is also heated to a preset temperature.

If desired, vacuums may be applied to the tissue container 14 during all bath cycles or during the hybridization bath cycles only. If desired, a low level, preset temperature may be applied to the tissue container 14 during all the bath treatments. In any event, the container 14 and valve block 28 are preheated and maintained at an appropriate temperature prior to and during the hybridization.

It will be appreciated that these tissue hybridization devices are simply illustrative. Numerous variations of such devices, or other work stations (e.g. Biomek, Beckman Instruments, Inc.) incorporating the methods, hybridization probes and blocking reagents described herein will be apparent to those of skill in the art using the teachings provided herein.

IV. Hybridization mix and kits for detecting microorganisms.

In still another embodiment, this invention provides a hybridization mix for use in the hybridization protocols described herein. In one embodiment the hybridization mix comprises a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of a microorganism as described herein and a granulocyte blocking reagent as described herein. The hybridization mix provides a convenient pre-formulated reagent
system that is optionally provided in a kit and is useful for detecting one or more microorganisms in a biological sample. The hybridization mix optionally comprises a hybridization buffer (e.g. SSC) and/or a detergent (e.g. Tween, SDS, etc.).

Particular hybridization mixes can be optimized to detect microorganisms characteristic of a particular tissue type. Thus, for example, a hybridization mix optimized for pleural effusions, sputum, or lung biopsies may comprise one or more probes for mycobacterial strains.

The hybridization mix can be provided as a fluid, in a frozen form or as a dried (e.g. lyophilized) powder. The powder form can be reconstituted in alcohol or, more preferably in water.

In another embodiment, this invention provides kits for practice of the methods of this invention. In a preferred embodiment, the kits comprise a container containing one or more nucleic acid probes as described herein. One kit includes one or more nucleic acid probes and, optionally, a granulocyte blocking reagent as described herein. The probes may, optionally, constitute a multiple probe system with multiple probes to the same target microorganism for target validation as described herein. Alternatively, or in addition, the probe(s) may comprise multiple probes to simultaneously detect a plurality of microorganisms.

The kits, may optionally include one or more reagents, and/or devices to facilitate practice of the methods described herein. Such reagents may include, but are not limited to various buffers, labels, sampling devices, filter sets (e.g. for fluorescence microscopy), reference slides, and the like.

In addition, the kits optionally include labeling and/or instructional materials providing directions (i.e., protocols) for the practice of the methods of this invention. Preferred instructional materials teach the use granulocyte (e.g. mast cell) blockers, and/or multiple probes for target validation and/or multiple probes for simultaneous detection of a plurality of microorganisms in vertebrate tissue samples.

While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips),
optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

V. Reference preparations.

In certain embodiments, this invention provides biological samples subjected to *in situ* hybridization in accordance with the methods described herein. In preferred embodiments, the samples thus comprise a biological sample from a vertebrate and the target microorganism(s) labeled with labeled probe(s) hybridized to their respective target and thereby forming a hybridization complex. The preparation optionally includes a granulocyte blocking agent as described herein. In certain embodiments, the preparation comprises two differently labeled probes hybridized to nucleic acids of the same microorganism and/or a plurality of different probes hybridized to different microorganisms. In certain embodiments, the sample is immobilized on a surface (e.g. a glass microscope slide).

The samples thus prepared are useful in a number of contexts. Such prepared samples are useful for example, as reference materials for training pathologists. Using well-prepared samples in accordance with this invention, pathologists can learn to rapidly scan slides and distinguish various labeled target microorganism in particular tissue samples.

Well-prepared samples produced in accordance with this invention can provide effective positive controls for evaluating hybridization protocols in particular microscopy systems. Also, well characterized samples can be used to evaluate the efficacy of automated slide scanning software and hardware. Other uses of such prepared samples will be readily apparent to those of skill in the art.

VI. Other commercial applications.

It has long been recognized that the potential for bacterial contamination exists in products which are designed for human consumption or use. Manufacturers and/or processors of these products destined for human consumption generally test these products to ensure the quality and safety of the products for human consumption. Such products can include raw meat, prepared food items, food preparation equipment, drinking water, nutritive supplements (for humans or animals, bathing water, and other vectors in which microorganisms can reside which allows their contact or transfer to humans or animals.
Typically, organisms such as enteric bacteria, *i.e.*, *Enterobacteriaceae*, such as *Salmonella* and *Escherichia coli*, and Gram positive organisms, such as *Staphylococcus* and *Enterococcus*, are the organisms tested for in products destined for human and animal consumption or use. These microorganisms are generally present in the colon, intestines, or fecal matter of humans or animals. When food products such as poultry, red meat, seafood, eggs, or any foods which contain these products come into contact with fecal matter during handling or processing, the potential exists for contamination and subsequent transfer of these organisms to the end users or consumers, *i.e.*, humans.

Not only can the presence of sufficient numbers of microorganisms cause the deterioration of a food product such as by causing spoilage, additionally, if consumed by a human or animal, it can also cause disease. In the United States alone, the number of cases of food poisoning associated with the consumption of contaminated food products is conservatively estimated to be in the multi-millions per year. While most human cases of bacterial food poisoning only result in acute symptomatic disease which includes nausea, vomiting, diarrhea, chills, fever, and exhaustion; for those individuals such as infants, the elderly, pregnant women, neonates, and those with immunocompromised systems, death can occur.

The total economic loss attributable to bacterial food poisoning has been estimated to reach into the hundreds of millions of dollars each year due to lost productivity, increased use of the medical insurance system, and increased use of the medical provider system.

In order to prevent the transmission of foodborne bacterial pathogens, the manufacturers and/or processors of food products routinely test samples of their products in order to identify contaminated products before the product is placed into the stream of commerce leading to human consumption. Because pathogenic microorganisms can be present in very small numbers in a food product which may contain a large number or variety of other pathogenic and/or non-pathogenic microorganisms, methods for the detection of small quantities of pathogens and the ability to distinguish them from non-pathogenic microorganisms are desired.

*The in situ* hybridization methodologies described herein are well suited to rapid and efficient screening of such food products, agricultural materials, or other commercial materials subject to parasitic, bacterial, viral, or other, contamination.
EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

A preferred embodiment of the granulocyte (e.g., mast cell) blocking protocol of the invention was performed as follows: The microbial probe (a *Leishmania donovani* probe targeting the entire genome) was precipitated with 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 X volume -4°C 100% ethanol. The solution was mixed thoroughly, and then centrifuged for 30 minutes at 10,000 rpm. Supernatant was carefully removed without disturbing the pellet, and residual supernatant was evaporated. The pellet containing the probe was then resuspended in hybridization buffer, 20% SDS and 100 mg/ml yeast tRNA (Gibco BRL, Grand Island, NY) in the ratio of 7:2:1. The resulting solution was mixed thoroughly, and the pellet allowed to dissolve completely.

Example 2

The following experiment was conducted on specimens (a mixture of cultured tryptomastigotes from *Leishmania donovani* and *Trypanosoma cruzi*) containing leishmania or trypanosoma organisms.

Slides having the organism were placed in a xylene wash for five minutes at room temperature and then transferred to a fresh xylene wash for another five minutes. Excess xylene was drained. The slides were then washed in 100% ethanol for five minutes at room temperature, and in fresh ethanol for another five minutes. Excess ethanol was drained, and the slides were placed on a hot plate to dry. Digestion processing began by placing slides in 1M NaSCN at 80°C for ten minutes, then washing twice in fresh distilled water for five minutes at room temperature. Excess water was wicked off. Slides were placed in 5 mg/ml Pepsin dissolved in 0.2 M HCl (Sigma, St. Louis, MO) for 20-30 minutes (depending on level of digestion) at 37°C, then washed twice in fresh distilled water for five minutes at room temperature. Excess water was wicked off of slides.

Slides were placed in denaturation solution (70% formamide (Gibco, Grand Island, NY), 2X SSC) for 5-8 minutes at 72°C. After denaturing, slides were dehydrated in a 70/80/95% ethanol series for two minutes at -4°C. During this time, probe was denatured.
(20 μl/slide) at 72°C for five minutes. Slides were dried after last ethanol wash, and 20 μl of
denatured probe was added. The slide was coverslipped and sealed with rubber cement, then
incubated in a humidified chamber for 24-48 hours at 37°C.

Coverslips were removed carefully, and the slides were washed in 0.4X
SSC/0.3% NP-40 for two minutes at 72°C. They were then drained and transferred to
2X SSC/0.1% NP-40 at room temperature for five seconds, and air-dried in darkness. 15 μl
of counterstain was added before coverslipping and sealing with clear fingernail polish.

**Example 3**

The following experiment was conducted on formalin-fixed, paraffin-
embedded lymph node tissue from a biopsy (4micron sections from the block) containing
mycobacterium.

Slides were fixed at least 30 minutes in 10% buffered formaldehyde at room
temperature. Slides were then washed twice in a 70/85/100% ethanol series for two minutes
each at room temperature, then washed in a xylene wash for five minutes at room
temperature, and then an additional fresh xylene wash for another five minutes. Excess
xylene was drained. Slides were placed in a 100% ethanol wash for five minutes at room
temperature, and the wash was repeated with fresh ethanol for another five minutes.

A digestion process was initiated by placing slides in 1M NaSCN at 80°C for
ten minutes. Slides were then washed twice in fresh distilled water for five minutes at room
temperature, and excess water wicked off of sample. Slides were placed in 5 mg/ml Pepsin
(dissolved in 0.2M HCl) (Sigma, St. Louis, MO) solution for five minutes at 4°C, then
placed in 5 mg/ml Pepsin solution for 20-30 minutes at 37°C. The slides were washed twice
in fresh distilled water for five minutes at room temperature, and excess water was wicked
off of slides.

Slides were placed in denaturation solution (70% formamide (Gibco, Grand
Island, NY), 2X SSC) for 5-8 minutes at 72°C. After denaturing, slides were dehydrated in a
70/80/95% ethanol series for two minutes at -4°C. During this time, the probe was denatured
at 72°C for five minutes. Slides were dried after last ethanol wash, and 20 μl of denatured
probe was added. The slide was coverslipped and sealed with rubber cement, then incubated
in a humidified chamber for 24-48 hours at 37°C.
Coverslips were carefully removed and slides were washed in 0.4X SSC/0.3% NP-40 for two minutes at 72°C, then drained and transferred to 2X SSC/0.1% NP-40 at room temperature for five seconds. The slides were then dried in darkness. 15 µl of counterstain was added before coverslipping and sealing with clear fingernail polish.

5 Example 4

The following experiment was conducted on specimens containing mycobacterium. Pretreatment was as above in Example 3 up to the point of digestion.

Digestion was initiated by placing the slides in 1M NaSCN at 80°C for ten minutes. Slides were then washed twice in fresh distilled water for five minutes at room temperature, heat-dried on 60-70°C, then placed on ice to cool. Slides were then placed in 4 mg/ml Pepsin solution for five minutes at 4°C, then placed in 4 mg/ml Pepsin solution for five minutes at 37°C.

Slides were placed in a denaturation solution of 70% formamide/2X SSC/0.1% Tween 20 for one hour at room temperature, then drained, warmed, and then denatured for five minutes at 72°C. The slides were then cooled to 37°C and blotted dry. Denatured probe was added to the slide, which was then coverslipped, sealed and left overnight.

Subsequently, the coverslips were floated off in 1X SSC at room temperature, and the slide was washed in a series of washes: 1X SSC for five minutes at 72°C, 2X SSC for five minutes at 37°C, 2X SSC for five minutes at room temperature, fresh distilled water for five minutes at room temperature, and an ethanol series of 70/85/100%, two minutes each at room temperature. The slides were then air-dried at 37°C. Antifade was added, and the slides were coverslipped and sealed.

Example 5

The following experiment was conducted on samples of of formalin-fixed, paraffin-embedded human skin biopsy material.

Slides having the specimen were placed in a xylene wash for five minutes at room temperature, then transferred to a fresh xylene wash for an additional five minutes. Excess xylene was drained, and the slides were then washed in 100% ethanol for five minutes, and again in fresh ethanol for an additional five minutes. Excess ethanol was
drained, and the slides were placed on a hot plate to dry. Digestion was initiated by placing the slides in 1M sodium thiocyanate at 80°C for ten minutes. The slides were washed twice in fresh distilled water for five minutes at room temperature, and the excess water was wicked off. Slides were then placed in 5 mg/ml Pepsin (Sigma, St. Louis) dissolved in 0.2M hydrochloric acid for 20-30 minutes at 37°C. Slides were then removed from this solution, and washed twice for five minutes each in fresh distilled water at room temperature.

Hybridization was conducted by placing the slides in a denaturation solution comprising 70% formamide (Gibco BRL, Grand Island, NY) and 2X SSC for 5-8 minutes at 72°C. After denaturing, slides were dehydrated in a 70/80/95% ethanol series for two minutes at -4°C. While the slides were in ethanol, the probe was denatured at 72°C for five minutes. Slides were then dried, and 20 μl of probe (centromeric probe for chromosome 17 purchased from Vysis) was added to each. Coverslips were added and sealed with rubber cement, after which the slides were incubated in a humidified chamber for 24-48 hours at 37°C.

After the allotted time, the slides were removed from the chamber, and the coverslips were removed. The slides were then washed in 0.5X SSC for five minutes at 72°C. After draining, the slides were transferred to 1X PBD at room temperature for two minutes, and dried in a 70/85/95% ethanol series for one minute each at room temperature.

Then, 15 μl of counterstain was added to each slide. The slide was coverslipped and sealed with clear fingernail polish. Figure 3 and Figure 4 show the marked difference obtained with the use of tRNA. As shown in Figure 3, the slides probed with the tRNA/probe mixture lack the non-specific and additional fluorescence shown in Figure 4. This additional fluorescence is due to the binding of the probe by the mast cells in the sample.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of
this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.
CLAIMS

What is claimed is:

1. A method of detecting a microorganism in a biological sample from a vertebrate, said method comprising:
   i) contacting said biological sample with a first nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate;
   ii) contacting said biological sample with a second nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate, where said first nucleic acid probe and said second nucleic acid probe are distinguishable;
   iii) detecting hybridization of said first nucleic acid probe and said second nucleic acid probe to a target, where co-occurrence of said first nucleic acid probe and said second nucleic acid probe indicates the presence of said microorganism.

2. The method of claim 1, wherein hybridization of only said first nucleic acid probe or said second nucleic acid probe is interpreted as a false positive.

3. The method of claim 1, wherein said first nucleic acid probe is labeled with a single label.

4. The method of claim 1, wherein said first nucleic acid probe is a molecular beacon.

5. The method of claim 1, wherein said first nucleic acid probe and said second nucleic acid probe are peptide nucleic acids.

6. The method of claim 1, wherein said first nucleic acid probe and said second nucleic acid probe are synthetic oligonucleotides.

7. The method of claim 1, wherein said first nucleic acid probe and said second nucleic acid probe have the same nucleotide sequence.
8. The method of claim 1, wherein said first nucleic acid probe and said second nucleic acid probe are labeled with a labels selected from the group consisting of a fluorescent label, a radioactive label, an enzymatic label, an electron dense label, a colorimetric label, and a magnetic label.

9. The method of claim 1, wherein said first nucleic acid probe and said second nucleic acid probe each are labeled with a fluorescent labels and the labels on each probe are distinguishable from each other.

10. The method of claim 1, wherein said biological sample is a sample from a vertebrate selected from the group consisting of a human, a non-human primate, a bovine, an equine, a canine, a feline, a porcine, a murine, a fish, a hamster, a lizard, and an amphibian.

11. The method of claim 1, wherein said biological sample is selected from the group consisting of a biopsy, a body fluid sample, and an aspirate.

12. The method of claim 1, wherein said sample is selected from the group consisting of a fresh sample, a frozen sample, and a fixed sample.

13. The method of claim 1, wherein said sample is a biological tissue or fluid selected from the group consisting of lung tissue, stomach tissue, epidermal tissue, gut tissue, , whole blood, a blood fraction, oral fluid, feces, urine, cerebrospinal fluid, and nasal fluid.

14. The method of claim 13, wherein said microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

15. A hybridization mix for detecting a microorganism in a biological sample from a vertebrate, said hybridization mix comprising:

   a first nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate;
and a second nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate, where said first nucleic acid probe and said second nucleic acid probe are distinguishable.

16. The hybridization mix of claim 15, further comprising a hybridization buffer.

17. The hybridization mix of claim 15, further comprising a detergent.

18. The hybridization mix of claim 17, wherein said detergent comprises 20% SDS.

19. The hybridization mix of claim 15, wherein said probe is labeled with a label selected from the group consisting of a fluorescent label, a radioactive label, an enzymatic label, an electron dense label, a colorimetric label, and a magnetic label.

20. The hybridization mix of claim 19, wherein said first nucleic acid probe is labeled with a fluorescent label.

21. The hybridization mix of claim 15, wherein said first nucleic acid probe is labeled with a single label.

22. The hybridization mix of claim 15, wherein said first nucleic acid probe is a molecular beacon.

23. The hybridization mix of claim 15, wherein said first nucleic acid probe and said second nucleic acid probe are selected from the group consisting of a peptide nucleic acid, and a synthetic oligonucleotide.

24. The hybridization mix of claim 15, wherein said probe specifically hybridizes to a nucleic acid of a microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

25. A biological sample comprising a biological tissue from a vertebrate;
a microorganism;
a hybridization complex comprising a first nucleic acid probe
hybridized to a nucleic acid of said microorganism; and
a second nucleic acid probe hybridized to a nucleic acid of the
same microorganism, wherein said first nucleic acid probe and said second nucleic
acid probe are distinguishable and said first nucleic acid probe and said second
nucleic acid probe specifically hybridize, under stringent conditions, to a nucleic acid
of said microorganism, but not to a nucleic acid in said biological tissue.

26. The biological sample of claim 25, wherein said sample is affixed to a
glass surface.

27. The biological sample of claim 25, wherein said probe is labeled with
a label selected from the group consisting of a fluorescent label, a radioactive label, an
enzymatic label, an electron dense label, a colorimetric label, and a magnetic label.

28. The biological sample of claim 27, wherein said first nucleic acid
probe is labeled with a fluorescent label.

29. The biological sample of claim 25, wherein said first nucleic acid
probe is labeled with a single label.

30. The biological sample of claim 25, wherein said first nucleic acid
probe is a molecular beacon.

31. The biological sample of claim 25, wherein said first nucleic acid
probe and said second nucleic acid probe are independently selected from the group
consisting of a peptide nucleic acid, and a synthetic oligonucleotide.

32. The biological sample of claim 25, wherein said first nucleic acid
probe and said second nucleic acid probe both specifically hybridize to a nucleic acid of a
microorganism is selected from the group consisting of mycobacteria, leishmania,
trypanosoma, protozoa, mycoplasma, and spirochetes.
33. A kit for detecting a microorganism in a biological sample from a vertebrate, said kit comprising:
   a first container containing a first nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate; and
   a second container containing a second nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate.

34. The kit of claim 26, further comprising instructional materials describing the use of the first and second probes for reducing the incidence of false positives in the identification of the microorganism.

35. The kit of claim 26, wherein said first container and said second container are the same container.

36. The kit of claim 26, wherein said microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

37. A device for preparing a biological sample for detection of a microorganism, said device comprising:
   a first container containing a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate; and
   a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate;
   a third container containing a hybridization buffer;
   a fourth container capable of containing one or more biological samples;
   wherein said first container, said second container, and said third container communicate with said fourth container such that said first nucleic acid probe, said second nucleic acid probe, and said hybridization buffer are delivered to said fourth container when desired.
38. The device of claim 37, wherein said first container and said second container are the same container.

39. The device of claim 37, wherein said first container and said third container are the same container.

40. The device of claim 37, wherein said second container and said third container are the same container.

41. The device of claim 37, wherein said first container and said second and said third container are the same container.

42. The device of claim 37, wherein said fourth container is removable from said device.

43. The device of claim 37, wherein said fourth container is a microtiter plate.

44. The device of claim 37, wherein said device further comprises a heater.

45. The device of claim 37, wherein any one of said first, said second, said third, or said fourth containers is fluid communication to any other of said first, said second, said third, or said fourth containers.

46. The device of claim 47, wherein here said fluid communication is though a valve.

47. A method of detecting a plurality of different microorganisms in a biological sample from a vertebrate, said method comprising:
   i) contacting said biological sample with a plurality of nucleic acid probes where each probe specifically hybridizes under stringent conditions to a nucleic acid of a different microorganism and not to a nucleic acid of said vertebrate, and where the different probes comprising said plurality of probes are distinguishable;
   iii) detecting hybridization of said nucleic acid probes to targets, where hybridization of each probe indicates the presence of a different microorganism.
48. The method of claim 47, wherein said plurality of nucleic acid probes comprises a probe labeled with a single label.

49. The method of claim 47, wherein said plurality of nucleic acid probes comprises a molecular beacon.

50. The method of claim 47, wherein said plurality of nucleic acid probes comprises a peptide nucleic acid.

51. The method of claim 47, wherein said plurality of nucleic acid probes comprises a synthetic oligonucleotide.

52. The method of claim 47, wherein said plurality of nucleic acid probes comprises a probe labeled with a label selected from the group consisting of a fluorescent label, a radioactive label, an enzymatic label, an electron dense label, a colorimetric label, and a magnetic label.

53. The method of claim 47, wherein said plurality of nucleic acid probes are labeled with fluorescent labels and the labels on each probe are distinguishable from each other.

54. The method of claim 47, wherein said biological sample is a sample from a vertebrate selected from the group consisting of a human, a non-human primate, a bovine, an equine, a canine, a feline, a porcine, a murine, a fish, a hamster, a lizard, and an amphibian.

55. The method of claim 47, wherein said biological sample is selected from the group consisting of a biopsy, a body fluid sample, and an aspirate.

56. The method of claim 47, wherein said sample is selected from the group consisting of a fresh sample, a frozen sample, and a fixed sample.

57. The method of claim 47, wherein said sample is a biological tissue or fluid selected from the group consisting of lung tissue, stomach tissue, epidermal tissue, gut tissue, whole blood, a blood fraction, oral fluid, feces, urine, cerebrospinal fluid, and nasal fluid.
58. The method of claim 47, wherein said microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

59. A hybridization mix for detecting a plurality of microorganisms in a biological sample from a vertebrate, said hybridization mix comprising:
   a plurality of nucleic acid probes where each probe specifically hybridizes under stringent conditions to a nucleic acid of said a different microorganism and not to a nucleic acid of said vertebrate, and where said the different probes comprising said plurality of probes are distinguishable.

60. The hybridization mix of claim 59, further comprising a hybridization buffer.

61. The hybridization mix of claim 59, further comprising a detergent.

62. The hybridization mix of claim 59, wherein said detergent comprises 20% SDS.

63. The hybridization mix of claim 15, wherein said probe is labeled with a label selected from the group consisting of a fluorescent label, a radioactive label, an enzymatic label, an electron dense label, a colorimetric label, and a magnetic label.

64. The hybridization mix of claim 19, wherein said first nucleic acid probe is labeled with a fluorescent label.

65. The hybridization mix of claim 15, wherein said first nucleic acid probe is labeled with a single label.

66. The hybridization mix of claim 15, wherein said first nucleic acid probe is a molecular beacon.

67. The hybridization mix of claim 15, wherein said first nucleic acid probe and said second nucleic acid probe are selected from the group consisting of a peptide nucleic acid, and a synthetic oligonucleotide.
68. The hybridization mix of claim 15, wherein said probe specifically hybridizes to a nucleic acid of a microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

69. A biological sample comprising
   a biological tissue from a vertebrate;
   a microorganism;
   a hybridization complex comprising a first nucleic acid probe hybridized to a nucleic acid of said microorganism; and
   a second nucleic acid probe hybridized to a nucleic acid of the same microorganism, wherein said first nucleic acid probe and said second nucleic acid probe are distinguishable and said first nucleic acid probe and said second nucleic acid probe specifically hybridize, under stringent conditions, to a nucleic acid of said microorganism, but not to a nucleic acid in said biological tissue.

70. The biological sample of claim 25, wherein said sample is affixed to a glass surface.

71. The biological sample of claim 25, wherein said first nucleic acid probe is labeled with a label selected from the group consisting of a fluorescent label, a radioactive label, an enzymatic label, an electron dense label, a colorimetric label, and a magnetic label.

72. The biological sample of claim 27, wherein said first nucleic acid probe is labeled with a fluorescent label.

73. The biological sample of claim 25, wherein said first nucleic acid probe is labeled with a single label.

74. The biological sample of claim 25, wherein said first nucleic acid probe is a molecular beacon.

75. The biological sample of claim 25, wherein said first nucleic acid probe and said second nucleic acid probe are independently selected from the group consisting of a peptide nucleic acid, and a synthetic oligonucleotide.
76. The biological sample of claim 25, wherein said first nucleic acid probe and said second nucleic acid probe both specifically hybridize to a nucleic acid of a microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

77. A kit for detecting a microorganism in a biological sample from a vertebrate, said kit comprising:

- a first container containing a first nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate; and
- a second container containing a second nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of said microorganism and not to a nucleic acid of said vertebrate.

78. The kit of claim 26, wherein said first container and said second container are the same container.

79. The kit of claim 26, wherein said microorganism is selected from the group consisting of mycobacteria, leishmania, trypanosoma, protozoa, mycoplasma, and spirochetes.

80. A device for preparing a biological sample for detection of a plurality of microorganisms, said device comprising:

- a first container containing a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of a first microorganism and not to a nucleic acid of said vertebrate; and
- second container a nucleic acid probe that specifically hybridizes under stringent conditions to a nucleic acid of a second microorganism and not to a nucleic acid of said vertebrate;
- a third container containing a hybridization buffer;
- a fourth container capable of containing one or more biological samples;
wherein said first container, said second container, and said third container communicate with said fourth container such that said first nucleic acid probe, said second nucleic acid probe, and said hybridization buffer are delivered to said fourth container when desired.

81. The device of claim 80, wherein said first container and said second container are the same container.

82. The device of claim 80, wherein said first container and said third container are the same container.

83. The device of claim 80, wherein said second container and said third container are the same container.

84. The device of claim 80, wherein said first container and said second and said third container are the same container.

85. The device of claim 80, wherein said fourth container is removable from said device.

86. The device of claim 80, wherein said fourth container is a microtiter plate.

87. The device of claim 80, wherein said device further comprises a heater.

88. The device of claim 80, wherein any one of said first, said second, said third, or said fourth containers is fluid communication to any other of said first, said second, said third, or said fourth containers.

89. The device of claim 88, wherein said fluid communication is though a valve.