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(54) Title: TEST KITS AND METHODS FOR RAPIDLY TESTING FOR CONTAMINATION BY MICROORGANISMS			
(57) Abstract The invention provides methods and kits for rapid detection of viable microorganisms including bacteria. The invention provides an enzyme detection system comprising synthetic substrates that are cleaved in the presence of an enzyme of a microorganism to release a tag which can be a fluorescent tag. The invention further provides a color developer that renders the tag visible in light other than ultraviolet light.			

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5 TEST KITS AND METHODS FOR
 RAPIDLY TESTING FOR CONTAMINATION BY MICROORGANISMS

 BACKGROUND OF THE INVENTION

1. Field of the Invention

 The invention is in the field of detection of viable
10 bacteria or microorganisms that may be present in food
 products, in liquids, in clinical samples such as blood or
 urine, on surfaces, on equipment, or any other suspect source.
 The basis of the invention is an enzyme detection system in
 which enzymes from a microorganism catalyze a chemical reaction
15 which, in the presence of appropriate substrates and
 procedures, results in a colored or fluorescent reaction
 product that can be easily assayed.

2. Description of the Background Art

20 The detection of viable bacteria and other
 microorganisms is important in the prevention of disease, and
 in diagnosis of infections when symptoms are present in an
 individual. In industrial settings, bacterial contamination of
 stock and products causes manufacturers and food service
25 personnel considerable loss of revenue. The detection of
 potential contamination is time-consuming and, therefore,
 costly. When missed, contamination by microorganisms during
 food manufacture, processing, and preparation results in the
 spread of food borne illnesses. In clinical settings, an
30 essential step in diagnosis and treatment of a patient
 presenting symptoms of infection is verification of the
 presence and identity of bacteria. The traditional method for
 detection of bacteria in both industrial and clinical settings
 requires culturing for 24-168 hours. At the end of this
35 culturing, or incubation, period, bacteria are determined to be
 present or absent and, if appropriate, identification of the
 type of bacteria may be made. The duration of these assays can
 lead to delays in production or treatment and is the reason
 more rapid methods have been sought. Methods now used for

determining low levels of bacteria are exemplified by various commercially available products.

One class of methods relies on sampling, growth in selective media, and counting of bacterial colonies. Bacterial growth usually takes at least twenty-four hours and more typically takes 48-72 hours. The identity of the bacteria, if any are present, is determined by the culture conditions that permitted colonies to form. Products based on this method include selective media and tools to facilitate collection or concentration of bacteria (e.g., Hach Co.-membrane filtration unit #22530-00; Millipore Corp. Samplers and swab kits; Hach Co. Paddle testers;) or visualization of colonies by a pH color indicator (e.g., Hach Co. Bromcresol Purple Acidity Method).

The "most probable number" or MPN method, relies on growth of bacteria (from contaminated samples) in liquid culture. A series of dilutions are made and a probable bacterial concentration of the starting material is determined by identifying those tubes in the dilution series that show bacterial growth after incubation.

Another method is specific to E. coli bacteria and relies on E. Coli glucuronidase, an enzyme that will metabolize 4-methylumbelliferyl- β -D-glucuronide (MUG) to product a fluorescent product. A positive reaction (bacteria present) typically requires 24 hours of incubation and a confirmed negative requires an additional 24 hours. The MUG test detects only E. Coli. (e.g., BioControl Systems, Inc. ColiComplete).

A method for determining total coliform bacteria involves growing bacteria on solid medium containing pH-sensitive chromogenic substrate. Bacterial colonies cause a local shift in pH that results in appearance of a colored product (e.g., BioControl Systems, Inc.; ColiComplete).

Each of these methods differs from the present invention, not only in the technology utilized, but in the amount of time required for the test. Products now available require 24 hours or more for culture enrichment, a delay that is costly and potentially dangerous. In fact, the advertising for each of the products described above emphasizes the rapidity of those methods relative to traditional methods. The

present invention is superior to existing methods because it is easy, sensitive, and allows the determination of viable bacteria and other microorganisms in two to four hours rather than 24-168 hours.

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SUMMARY OF THE INVENTION

The present invention allows for rapid detection of low levels of bacteria or other microorganisms obtained from a variety of sources. The basis of the invention is a set of
10 enzyme detection systems, each consisting of a synthetic substrate that is cleaved in the presence of a bacterial enzyme. This cleavage reaction releases a reaction product (or products) that fluoresces under ultraviolet (UV) illumination, or, when treated with a developer (a Schiff base), appears
15 colored and can be detected visually in white light. Microcolonies of bacteria or other microorganisms can thus be detected on the basis of the fluorogenic or chromogenic reactions, or both. Not all microorganisms will cleave all substrates and not all substrates will be cleaved by all
20 microorganisms. Thus, microorganisms can be identified on the basis of their substrate specificities.

In some embodiments the invention involves culturing microorganisms that may be present. Culturing involves incubating the sample containing said microorganisms at a
25 temperature well suited for their growth, and, in, or on, a medium containing nutrients required for growth of the microorganisms. The preferred incubation temperature will be about the temperature at which the microorganisms grow most rapidly, which can be readily determined by reference to
30 standard microbiology textbooks (e.g., Stanier et al., 1976, The Microbial World, Prentice-Hall, Inc.) and which is well known in the art of microbiology. The optimal incubation temperature can vary for different organisms, but will most typically be about 37°C. The invention can be practiced at
35 temperatures other than the optimal temperature. The medium on which the microorganisms are cultured can likewise vary depending on the microorganism(s) being assayed for, but typically will include a support medium (e.g., agar, agarose,

or the like), nutrients (including, e.g., a carbon source; see Stainer et al. supra). Media and formulas for media for growth of microorganisms are varied, numerous, well known to the art, and readily available. In some embodiments the nutrient medium
5 can be a selective medium, or an enrichment medium; that is, the medium can contain nutrients (e.g. oxygen) that encourage growth of certain microorganisms and/or inhibit growth of other microorganisms. For example, addition of glucose (10 grams/liter [g/l]) and NH_4Cl (1 g/l) to basal nutrients (e.g.,
10 0.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g/l K_2HPO_4 , 0.05 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g/l CaCl_2 , 0.002 g/l $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.001 g/l $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$) and growth in a non-oxygenated atmosphere can enrich for enteric bacteria.

Because the substrates used in the present invention
15 can be used to give both fluorogenic and chromogenic products under appropriate conditions, the assay can be read using either ultraviolet or visible light. The advantage of the assay using UV illumination is greater sensitivity, and under UV illumination microcolonies can be reliably viewed after as
20 little as two hours incubation. The advantage of the assay using visible light is the convenience.

A method of detecting the presence of viable microorganisms is provided comprising the steps of:
(a) inoculating an aqueous buffer with the sample to be tested
25 by directly adding sample to the aqueous buffer to form an inoculated diluent or, alternatively, by placing a swab that has been in contact with the sample or surface to be tested into the aqueous buffer (b) mixing the inoculated diluent for about 15 seconds to disperse the sample (c) adding the
30 inoculated diluent, in such a manner as to cover the entire surface, to medium (e.g., an agar culture medium) containing (i) nutrients to support growth of the microorganisms to be tested for and, (ii) a synthetic substrate or substrates that may be cleaved by an enzyme of the microorganism to produce a
35 product (i.e., a tag or marker) that is fluorogenic, or, in the presence of an appropriate developer, chromogenic
(d) incubating the plate for about 4 hours at a temperature that supports rapid growth of the microorganism(s) and removing

the plate from the incubator (g) adding a color developing reagent to the surface of the media and (h) after about 10 seconds and less than 5 minutes, more usually after 30 seconds and no longer than 60 seconds, observing the presence of colored spots, indicating the presence of microcolonies and indicating a contaminated sample or observing the absence of such spots indicating no detectable contamination.

Another method of detecting the presence of viable microorganisms provided by the invention comprises the steps above and also including steps e and f following immediately after step d, said steps comprising: (e) illuminating the surface of the media with longwave (about 250-360 nm) ultraviolet light (f) observing the presence of pale spots over a darker background, indicating the presence of microcolonies and indicating a contaminated sample or observing the absence of such spots indicating no detectable contamination.

Another method of detecting the presence of viable microorganisms provided by the invention comprises steps (a) through (c) above followed by the following steps: (i) incubating the plate for at least 2 hours at a temperature that supports rapid growth of the microorganism(s) and removing the plate from the incubator (j) illuminating the surface of the media with longwave ultraviolet light and (k) observing the presence of pale spots over a darker background, indicating the presence of microcolonies and indicating a contaminated sample or observing the absence of such spots indicating no detectable contamination.

Another method of detecting the presence of viable microorganisms provided by the invention comprises steps (a) through (c) above followed by (i) though (j) above followed by the following steps: (k) returning the plate to the incubator and incubating the plate for about an additional 2 hours at a temperature that supports rapid growth of the microorganism(s) and (l) removing the plate from the incubator and (m) adding a color developing reagent to the surface of the media and (n) after about 10 seconds and less than 5 minutes, more usually after 30 seconds and no longer than 60 seconds, observing the presence of colored spots, indicating the

presence of microcolonies and indicating a contaminated sample or observing the absence of such spots indicating no detectable contamination.

Another method of detecting the presence of viable
5 microorganisms provided by the invention makes use of nutrient medium that contains, in addition to nutrients and a substrate, a color developer compound. The color developer compound can be added to the nutrient medium at any stage prior to addition of the inoculated diluent (e.g., either prior to, or following,
10 the pouring of agar nutrient medium into a culture plate.) This method comprises steps (a) through (b), above, followed by (c) adding the inoculated diluent, in such a manner as to cover the entire surface, to medium (e.g., an agar culture medium) containing (i) nutrients to support growth of the
15 microorganisms to be tested for, (ii) a synthetic substrate or substrates that may be cleaved by an enzyme of the microorganism to produce a product (i.e., a tag or marker) that is fluorogenic, or, in the presence of an appropriate developer, chromogenic, and (iii) a color developer compound;
20 followed by (p) incubating the plate for about 4 hours at a temperature that supports rapid growth of the microorganism(s) and removing the plate from the incubator, and (q) observing the presence of colored spots, indicating the presence of microcolonies and indicating a contaminated sample or observing
25 the absence of such spots indicating no detectable contamination.

Also provided are diagnostic kits for detecting the presence of viable microorganisms in a sample or on a surface comprising: (a) an aqueous buffer; (b) one or more sterile
30 petri dishes containing a culture medium (e.g., agar and nutrients appropriate for growth of the microorganism or microorganisms to be assayed) containing a substrate or substrates specific for the microorganism(s); (c) a color developer which reacts with the product produced in the
35 presence of viable microorganisms (i.e., the tag or marker released by cleavage of a substrate by an enzyme of the viable microorganisms) at about room temperature, and can be detected

by visual means; (d) quality control test strips for verifying reagent integrity; and (e) instructions.

Further provided are kits comprising a substrate or substrates, which can then be added to nutrient agar and used to prepare plates in a standard way well known to those skilled in the art.

The types of bacteria and microorganisms that can be detected by this invention include, but are not limited to: Salmonella, Listeria, Staph aureus, E. coli, Pseudomonas aeruginosa, and gram negative bacteria. This invention can be used to detect many types of microorganisms including bacteria, fungi, protists and others. Where the term "bacteria" is used in this specification, there is no intention to exclude other types of microorganisms.

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DESCRIPTION OF SPECIFIC EMBODIMENTS

In order to detect viable microorganisms in a test sample, or source material, a substrate is utilized which reacts with the microorganism or a by-product, such as an enzyme. A test sample can be any material (e.g., liquids, powders, particles) or surface that can contain or be a source of microorganisms. In some cases test samples can be added directly to an aqueous buffer; in other cases it will be necessary or convenient to use some means to transfer the test sample from a site to the aqueous buffer. Therefor, "test sample", as used herein, also refers to material or microorganisms on a swab or other transfer means that is used to transfer micororganisms from a potentially contaminated material or surface to an aqueous buffer. In some embodiments a color developer (usually a Schiff base) is also used. Usually an enzyme of a live or viable microorganism recognizes the substrate and cleaves the substrate to release a product, also called a tag or marker, or products which, in the preferred embodiment, will fluoresce under ultraviolet illumination. This tag or marker will appear colored in the presence of the color developer. A sufficient amount of the substrate and color developer must be used to react with the microorganisms and the components must interact for a

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sufficient length of time to detect viable organisms. The particular substrates used will depend upon the particular configuration of the test and its components and, more importantly, on the type(s) of microorganism or microorganisms being tested. Other ingredients or materials can be used in conjunction with the substrate, color developer, and the microorganism, such as buffers, solutions, and the like.

The substrate can be any hydrolytic substance or mixture of substances acted upon by an enzyme or microorganism by-product of a microorganism or bacteria, indicating viability of the microorganism. The interaction of the substrate and the microorganism results in a modification of the substrate to release the tag or marker, which can be a luminescent, fluorescent, fluorogenic, colored, chromogenic or radioactive material. The substrate tag further reacts with the color developer to give the resulting color in the assay.

Substrates used in this invention can be represented by the formula "B-T" where "B" is a base compound conjugated to a tag (or marker) "T". Substrates used in this invention include a mixture of one or more of L-arginine-T, L-phenylalanine-T, L-leucine-T, L- γ -glutamine-T, L-alanine-T, L-pyroglutamic acid, ABZ-alanine-glycine-leucine-alanine-T, and L-tosyl-glycyl-L-prolyl-L-arginyl-T where the T group is the tag or marker and is one or more of: 7-amino-4-methylcoumarin; 4-methoxy-2-naphthylamine; 7-amino-4-trifluoromethylcoumarin; 7-amino-4-trifluoromethylquinoline; 6-aminoquinoline; β -naphthylamide; para-nitroanilide; and the like. ("ABZ" is aminobenzoic acid.)

The substrate can also be phosphate conjugated to either of 7-hydroxy-4-methylcoumarin or 7-hydroxy-4-trifluoromethylcoumarin. In addition, the substrate can be 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt.

Typically, microorganisms will be assayed using substrates comprising particular base compounds (B) which are cleaved by the type of microorganism being assayed for and not, generally, by other types of microorganisms likely to be present in a sample. For example, to assay Salmonella bacteria, B will typically be L- γ -glutamine; for Listeria

bacteria, B will typically be one of carbobenzoxy-phenylalanine-argine, succinyl-alanine-alanine-phenylalanine, or methoxysuccinyl-alanine-alanine-proline-alanine, with methoxysuccinyl-alanine-alanine-proline-alanine most preferred; for Staph aureus bacteria, B will typically be L-tosyl-glycyl-L-prolyl-L-arginyl; for Pseudomonas aeruginosa bacteria, B will typically be ABZ-alanine-glycine-leucine-alanine; for E. coli bacteria, B will typically be β -D-glucuronide conjugated to one of 7-hydroxy-4-methylcoumarin or 7-hydroxy-4-trifluoromethyl-coumarin. In order to assay more than one type of microorganism, combinations of base compounds can be used, or base compounds that are cleaved by several classes, or types, of microorganisms. For example, to assay all gram negative bacteria, B will typically be L-alanine, and to assay all types of bacteria present, B will typically be a mixture comprising L-arginine, L-leucine, L-phenylalanine, L-alanine, and L-pyroglutamic acid.

Useful color developers include a color developer compound in solution, with an aqueous solution preferred. Useful color developer compounds include: para-dimethylaminocinnamaldehyde, 5-nitrosalicylaldehyde (5-NSA), benzaldehyde, p-nitrobenzaldehyde, and the like, with paradimethylaminocinnamaldehyde being preferred. The color developer will most typically contain the color developer compound at a concentration of about 2 to 15 grams per liter.

Quality control test strips can be used to verify the integrity of reagents. The quality control test strip will be a filter paper or other carrier having positive control and a negative control areas marked. The positive control area can be impregnated with any one of the tags listed above, namely: 7-amino-4-methylcoumarin; 4-methoxy-2-naphthylamine; 7-amino-4-trifluoromethylcoumarin; 7-amino-4-trifluoromethylquinolone; 6-aminoquinolone; β -naphthylamide; para-nitroanilide; and the like. The negative control area can be impregnated with an inert material or can be left blank.

After collection, the sample being tested for the presence of microorganisms is resuspended in an aqueous buffer solution, such as a tris(hydroxymethyl)-aminomethane

hydrochloride ("Tris-HCl") solution, and the like. The buffer solution can also contain a non-ionic detergent, such as Triton X-100[®], and/or a reducing agent such as DL-dithioerythritol (DTE), DL-dithiothreitol (DTT), thioglycolic acid, and the like. The buffer solution can also contain a surfactant, such as polysorbate 80, typically (when present) at a concentration of 0.25-2%. These buffer solutions must be compatible with the substrates and the microorganisms, and must not affect their interaction with the substrate.

The types of bacteria and microorganisms that can be detected by this invention include, but are not limited to: Salmonella, Listeria, Staph aureus, E. coli, Pseudomonas aeruginosa, and gram negative bacteria. The invention can also be used to detect the total count of bacteria (i.e., detect all types of bacteria present rather than a specific type). Yeasts and molds and other fungi can also be detected by the invention as well as other microorganisms such as alga, protists and others.

20 EXAMPLES

The present invention is illustrated by the following examples. These examples are not intended to limit the scope of the invention.

25 Materials:

The following materials are prepared for use:

Aqueous Buffer:

Tris(hydroxymethyl)-aminomethane hydrochloride	0.10M (Molar)
30 Thioglycolic Acid	0.01M
Non-ionic detergent (e.g., Triton X-100 [®])	0.04M

The buffer components are dissolved in deionized water and the pH is adjusted to about 8.3 +/- 0.2 with 1 M sodium hydroxide.

Color Developer:

Para-dimethylaminocinnamaldehyde	6.0 gm (grams)
Hydrochloric acid (37%)	100.0 ml (milliliter)
Deionized water	900.0 ml

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The color developer components are mixed and the resulting pH is about 2.5 +/- 0.5.

Positive Control:

10 7-amino-4-methylcoumarin	0.025 gm
Methanol	25.000 ml
Buffer	100.000 ml

The components of the control are mixed together.

15

Substrate Solution:

The substrate solution is prepared at a concentration of about 0.5 mg/ml (milligrams per milliliter) using:

20 N,N-Dimethyl-formamide	5.000 ml
Methanol	200.000 ml
Substrate	0.100 gm

The substrates for the examples are: L-γ-glutamine conjugated to 7-amino-4-methylcoumarin; β-D-glucurononide conjugated to 7-hydroxy-4-methylcoumarin; L-γ-glutamine conjugated to para-nitroanilide; L-tosyl-glycyl-L-prolyl-L-arginyl conjugated to p-nitroanilide; ABZ-alanine-glycine-leucine-alanine conjugated to para-nitroanilide, L-alanine-6-aminoquinoline, L-γ-glutamine-7-amino-4-methylcoumarin, and a pool of: L-arginine, L-alanine, L-leucine, L-phenylalanine, and L-pyroglutamic acid - all tagged with 7-amino-4-methylcoumarin.

Example 1

35 Sites at a food processing plant were tested for bacterial contamination. Eight pieces of processing equipment were treated for contamination by swabbing a minimum of 40 square inches per piece of equipment. The swabs were placed

into 1.0 ml 0.1 M Tris-HCl, pH 8.3, and vortexed briefly to form an inoculated diluent. 0.5 ml of each sample in diluent was placed on a separate test plate containing the substrate L-alanine-6-aminoquinoline, to test for the presence of gram negative bacteria. The plates were incubated for 4.5 hours at 37°C. The plates were read under long wave UV light and colonies counted (results in Table I). Ten drops of a color developer containing p-dimethylaminocinnamaldehyde were added to each plate and colonies that turned purple were counted (results in Table I).

TABLE I

Test Site	Colonies under UV	Purple colonies
Meat slicer A	35	35
Meat slicer B	17	17
Conveyer belt 371	>100	>100
Conveyer belt 938	>100	>100
Storage rack in poultry section	>100	>100
Storage rack in beef section	>100	>100
Shrink wrap machine in beef section	63	63
Poultry storage bin 3452	40	40

These results demonstrate the rapid detection of fewer than 20 viable bacteria from a test sample, and demonstrate the correspondence between the number of colonies apparent under UV illumination and the number visible after addition of color developer to the system.

Example 2

To test two pieces of meat (numbered 1 and 2) for suspected Salmonella contamination, swabs were run across the meat. The swabs were placed into 1.0 ml 0.1 M Tris-HCl, pH 8.3, and vortexed briefly to form an inoculated diluent. 0.5 ml of each sample in diluent was placed on a separate test plate containing the substrate L-γ-glutamine-7-amino-4-

methylecoumarin, with the sample from piece #1 being plated on plate #1, and the sample from piece #2 being plated on plate #2. The plates were incubated for 4 hours at 37°C. The plates were read under long wave UV light and colonies counted (results in Table II). Ten drops of a color developer containing p-dimethylaminocinnamaldehyde were added to each plate and colonies that turned purple were counted (results in Table II).

TABLE II

Plate No.	Colonies under UV	Purple colonies
1	0	0
2	39	39

These results demonstrated that meat piece #2 was contaminated with Salmonella, but that piece #1 was not contaminated.

Example 3

The surfaces and equipment of a restaurant were tested for general sanitation (i.e., the total bacteria count) by touching each of six sites in the restaurant with swabs (one swab per site). The swabs were placed into 1.0 ml 0.1 M Tris-HCl, pH 8.3 and vortexed briefly to form an inoculated diluent. 0.5 ml of each sample in diluent was placed on a separate test plate. Each plate contained a pool of: L-arginine, L-alanine, L-leucine, L-phenylalanine, and L-pyroglutamic acid - all tagged with 7-amino-4-methylecoumarin. The plates were incubated for 4 hours at 37°C. The plates were read under long wave UV light and colonies counted (results in Table III). Ten drops of a color developer containing p-dimethylaminocinnamaldehyde were added to each plate and colonies that turned purple were counted (results in Table III).

TABLE III

Plate No.	Colonies under UV	Purple colonies
1	49	49
2	21	21
3	0	0
4	>100	>100
5	53	53
6	79	79

These results demonstrate the use of the invention in assaying for general sanitation and shows the ability of the test to identify sanitary sites (i.e., the sample of plate #3) as well as sites of contamination.

Example 4

This example illustrates the use of the invention in an industrial setting in which the ultraviolet illumination step is used to obtain the most rapid possible result.

To verify the integrity of the kit reagents, two drops of color developer are added to the positive and negative sections of a quality control test strip containing 7-amino-4-methylcoumarin in the positive area only. The positive test area of the test strip rapidly turns purple, indicating that the color developer is working. A 0.1 milliliter sample of tomato soup concentrate is added to a tube containing 0.5 ml of diluent. The tube is briefly (15 seconds) vortexed to mix the contents. The inoculated diluent is then poured over the surface of agar medium contained in a 60 mm petri plate. The agar medium consists of agar, nutrient media, and in addition, L- γ -glutamine conjugated to 7-amino-4-methylcoumarin. The plate is incubated at about 37°C for about two hours, removed from the incubator and illuminated in a dark area with long-range ultraviolet light. Under the UV illumination yellow/white spots appear, indicating Salmonella microcolonies. The plate is returned to the incubator for an additional two hours (for a total of about four hours at about 37°C) and then removed a second time. Six drops of color developer are

dropped onto the surface of the agar medium. After about 30 seconds purple spots appear on the blue background, again indicating Salmonella microcolonies. The purple spots are rapidly counted and the concentration of bacteria in the original sample is calculated.

Example 5

This example illustrates the use of the invention in an industrial setting in which, for the sake of convenience, the UV step is omitted.

The end of a sterile swab is used to wipe the end of the apparently clean blade of a meat-cutting machine. The end of the swab is then immersed into a tube containing 0.5 mls of diluent. The tube is briefly (about 15 seconds) vortexed to mix the contents after which the swab is discarded. The inoculated diluent is then poured over the surface of agar medium contained in a 60 mm petri plate. The agar medium consists of agar, nutrient media, and in addition, β -D-glucurononide conjugated to 7-hydroxy-4-methylcoumarin. The plate is incubated at about 37°C for about four hours and removed from the incubator. Six drops of color developer are dropped onto the surface of the agar medium. After about 30 seconds purple spots appear on the blue background, indicating E. coli microcolonies. The purple spots are rapidly counted and found to be outside of acceptable limits for bacterial contamination.

Example 6

This example illustrates the use of the invention in a clinical setting.

A blood sample is obtained from a patient with an apparent bacterial infection. 0.1 ml of blood is added to each of four tubes, each tube containing 0.5 ml of diluent. The samples are mixed and the contents of each tube is poured over the surface of a different plate, marked a, b, c, or d. Each plate contains agar. In addition to agar, plate a, used to assay for E. coli, contains nutrient media suitable for growth of E. coli and β -D Glucuronide conjugated to 7-hydroxy-4-

methylecoumarin. Plate b, used to assay for Salmonella contains, in addition to agar, nutrient media suitable for growth of Salmonella, and L- γ -glutamine conjugated to para-nitroanilide. Plate c, used to assay for Staph aureus,
5 contains agar, nutrient media suitable for growth of Staph aureus, and L-tosyl-glycyl-L-prolyl-L-arginyl conjugated to para-nitroanilide. Plate d, used to assay for Pseudomonas aeruginosa, contains agar, nutrient media suitable for growth of Pseudomonas aeruginosa, and ABZ-alanine-glycine-leucine-
10 alanine conjugated to para-nitroanilide. .

The plates are incubated at about 37°C for about four hours and removed from the incubator. Six drops of color developer are dropped onto each of the plates. On plate b, but not on plates a, c, or d, purple spots rapidly appear. This
15 result indicates that the patient's blood contains Salmonella bacteria but not E. coli, Staph aureus, or Pseudomonas aeruginosa bacteria. The Salmonella microcolonies (purple spots) are counted and the number of bacteria per milliliter blood is calculated.

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It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those skilled in the art upon
25 reviewing the above description. The scope of the invention, therefore, should be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

Although the foregoing invention has been described
30 in some detail by way of illustration and example, for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

All publications and other references cited herein
35 are expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of detecting the presence of viable microorganisms from any test sample comprising the steps of:

5 (a) inoculating an aqueous buffer with test sample to form an inoculated diluent;

(b) adding the inoculated diluent to a culture medium containing a substrate of an enzyme of a microorganism or microorganisms, wherein said substrate can be cleaved by
10 said enzyme to produce a product or products capable of fluorescence under ultraviolet light;

(c) incubating said culture medium for less than twelve (12) hours at a temperature well suited for growth of said microorganisms;

15 (d) illuminating the surface of said culture medium with ultraviolet light and observing by their fluorescence said product or products; and

(e) correlating the presence of said fluorescence with the presence of viable microorganisms.

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2. A method of detecting the presence of viable microorganisms from any test sample comprising the steps of:

(a) inoculating an aqueous buffer with test sample to form an inoculated diluent;

25 (b) adding the inoculated diluent to a culture medium containing a substrate of an enzyme of a microorganism or microorganisms, wherein said substrate can be cleaved by said enzyme to produce a product or products capable of fluorescence under ultraviolet light;

30 (c) incubating said culture medium for less than twelve (12) hours at a temperature well suited for growth of said microorganisms;

(d) illuminating the surface of said culture medium with ultraviolet light and observing by their fluorescence said
35 product or products;

(e) correlating the presence of said fluorescence with the presence of viable microorganisms;

(f) adding a color developer which is specific for said product or products and causes said product or products to appear colored; and

5 (g) correlating the appearance of color with the presence of viable microorganisms.

3. A method of detecting the presence of viable microorganisms from any test sample comprising the steps of:

10 (a) inoculating an aqueous buffer with test sample;

(b) adding the inoculated diluent to a culture medium containing a substrate of an enzyme of a microorganism or microorganisms, said substrate being capable of being cleaved by said enzyme to produce a product or products;

15 (c) incubating said culture medium for less than twelve (12) hours at a temperature well suited for growth of said microorganisms;

(d) adding a color developer which is specific for said product or products and causes said product or products to appear colored; and

20 (e) correlating the appearance of color with the presence of viable microorganisms.

4. The method of claim 2 or claim 3, wherein the microorganisms are bacteria.

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5. The method of claim 2 or claim 3, wherein the substrate has the formula B-T, and:

B is selected from the group consisting of:

30 L-arginine, L-phenylalanine, L-leucine, L-alanine, L- γ -glutamine, L-alanine, L-tosylglycyl-L-prolyl-L-arginyl, L-pyroglutamic acid, ABZ-alanine-glycine-leucine-alanine, carbobenzoxy-phenylalanine-argine, succinyl-
35 alanine-alanine-phenylalanine, and methoxysuccinyl-alanine-alanine-proline-alanine; and

T is selected from the group consisting of:
7-amino-4-methylcoumarin, 4-methoxy-2-naphthylamine, 7-amino-4-trifluoromethylcoumarin, 7-amino-4-trifluoromethylquinolone, 6-aminoquinalone, β -naphthylamide, and para-nitroanilide.

6. The method of claim 2 or claim 3, wherein the substrate has the formula Phosphate-T and T is 7-hydroxy-4-methylcoumarin or 7-hydroxy-4-trifluoromethylcoumarin.

7. The method of claim 2 or claim 3 wherein the substrate is 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine salt.

8. The method of claim 2 or claim 3 wherein the color developer is selected from the group consisting of: paradimethylaminocinnamaldehyde, 5-nitrosalicylaldehyde, benzaldehyde, and p-nitrobenzaldehyde.

9. The method of claim 2 or claim 3 wherein more than one microorganism is detected.

10. The method of claim 5 wherein the microorganism is Salmonella bacteria and B is L- γ -glutamine.

11. The method of claim 5 wherein the microorganism is Listeria bacteria and B is carbobenzoxy-phenylalanine-argine, succinyl-alanine-alanine-phenylalanine, or methoxysuccinyl-alanine-alanine-proline-alanine.

12. The method of claim 5 wherein the microorganism is a gram negative bacteria and B is L-alanine.

13. The method of claim 5 wherein the microorganism is Staph aureus bacteria and B is L-tosyl-glycyl-L-prolyl-L-arginyl.

14. The method of claim 5 wherein the microorganism is Pseudomonas aeruginosa bacteria and B is ABZ-alanine-glycine-leucine-alanine.

5 15. The method of claim 2 or claim 3 wherein the microorganism is E. coli bacteria and the substrate is β -D-glucuronide conjugated to 7-hydroxy-4-methylcoumarin or 7-hydroxy-4-trifluoromethylcoumarin.

10 16. The method of claim 5 wherein the microorganisms are bacteria and B is a mixture comprising L-arginine, L-leucine, L-phenylalanine, L-alanine, and L-pyroglutamic acid.

15 17. A diagnostic kit for rapid detection of microorganisms comprising:

 (a) a culture medium comprising a substrate, wherein said substrate is cleaved by an enzyme of a viable microorganism to produce a product; and

20 (b) a color developer, wherein the color developer reacts with said product, wherein said product can be detected in light, other than ultraviolet light, by visual means.

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US94/03207

A. CLASSIFICATION OF SUBJECT MATTER IPC(5) :C12Q 1/34 1/37, 1/26, 1/02, 1/00, 1/04; G01N 33/566, 33/537. US CL :435/18, 34, 29, 25, 24, 23, 7.8, 4; 436/501, 536, 538, 805, 824. According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/18, 34, 29, 25, 24, 23, 7.8, 4; 436/501, 536, 538, 805, 824. Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,603,108 (BASCOMB) 29 July 1986, see entire document.	1-17
Y	US, A, 5,173,434 (MORRIS ET AL) 22 December 1992, see entire document.	1-17
A	FR, 2,654,113 A (DEUBEL VINCENT) 10 May 1991, see entire document.	1-17
A	WO,A,90/04641 (RELMAN ET AL) 03 May 1990, see entire document.	1-17
A	WO,A,92/03458-A (ZEBEDEE ET AL) 05 March 1992, see entire document.	1-17
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
A	document defining the general state of the art which is not considered to be part of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E	earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O	document referring to an oral disclosure, use, exhibition or other means	*G* document member of the same patent family
P	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 06 JUNE 1994		Date of mailing of the international search report 17 JUN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer LOUISE N. LEARY <i>L. Leary</i> Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US94/03207

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS: microorganism, fluorescence, enzyme, amino acid, coumarin, bacteria

CAS: microorganism, fluorescence, enzyme, amino acid, coumarin, bacteria