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(54) **Title**: SENSORS FOR MEASURING CONTAMINANTS

(57) **Abstract**: The present invention provides biosensors and methods of use for detecting the presence or absence of mycoplasma contamination through the detection of hydrolytic enzymes that are conserved among Mycoplasma species. Such hydrolytic enzymes include, but are not limited to, proteases, reductases and nucleases.

SENSORS FOR MEASURING CONTAMINANTS

BACKGROUND OF THE INVENTION

Mycoplasmas are very small microorganisms (Class Mollicutes) without cell walls that can cause infections in humans, animals, and plants. Mycoplasmas are also commonly found contaminating buffer solutions, and tissue culture media used in life science research. The *Mycoplasma* and *Acholeplasma* species, *Acholeplasma laidlawii, M. hyorhinis, M. orale, M. salivarium, M. arginini,* and *M. hominis,* account for about 98% of the tissue culture contaminants (McGarrity, G.J., & Carson, D.A., Adenosine phosphorylase-mediated nucleoside toxicity. Application towards the detection of mycoplasmal infection in mammalian cell cultures. Exp Cell Res. 1982 May; 139(1):199-205). As used herein, "mycoplasma" or "mycoplasmas" refers generally to members of the Class Mollicutes, including *Mycoplasma* and *Acholeplasma* species.

There is a clear unmet need for the real time detection of mycoplasmas for infection control monitoring in hospitals and for quality control of buffers and tissue culture media used in clinical laboratory testing and life science research.

SUMMARY OF THE INVENTION

The present invention provides biosensors and methods of use for detecting the presence or absence of mycoplasma contamination through the detection of hydrolytic enzymes that are conserved among *Mycoplasma* species. Such hydrolytic enzymes include, but are not limited to, proteases, reductases and nucleases

In preferred embodiments, the present invention provides a biosensor for detecting the presence or absence of Mycoplasma contamination comprising a support and a detectably labeled substrate for an enzyme produced and/or secreted by a mycoplasma, wherein the substrate is attached to the support. Typically, the enzyme is a Mycoplasma-specific hydrolytic enzyme selected from the group consisting of proteases, reductases and nucleases. In certain preferred embodiments, the enzyme is a Mycoplasma-specific protease selected from the group consisting of the gene product of *pepA1*(MCAP_0157), *pepA2* (MCAP_0195), *pepA* (leucyl aminopeptidase, such as MHP7448_0464), MCAP_0267 (metalloendopeptidase), *pepP* (Xaa-Pro endopeptidase, such as MCAP_0341 or MHP7448_0649),

MCAP_0509, mapP (methionine amino peptidase, MCAP_0675 or MHP7448_0173), mixtures thereof and homologous enzymes with at least 40% sequence identity. When the enzyme is a mycoplasma-specific protease, preferred substrates include leucine-(7-methoxycoumarin-4-yl)acetyl (leu-MCA), arginine-(7-methoxycoumarin-4-yl)acetyl (arg-MCA), methionine-(7-methoxycoumarin-4-yl)acetyl (met-MCA), an acetoxymethyl ester or maleimide derivative of blue dye number 1 coupled to a peptide substrate of the mycoplasma-specific protease.

In other preferred embodiments, the enzyme is a Mycoplasma-specific reductase selected from the group consisting of the gene product of *nrdE* (such as MCAP_0101), MCAP_0427, *trxB* (thioredoxin reductase, such as MCAP_0779 or MHP7448_0098), MCAP_0858 and mixtures thereof. When enzyme is a mycoplasma-specific reductase, suitable substrates include reactive black 5, 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), BODIPY®FL L-cystine, 2',7'-difluoro-4'-(2-(5-((dimethyl amino phenyl)azo) pyridyl)dithiopropionyl aminomethyl) fluorescein (DFDMAP-fluorescein),or an azo dye that is sensitive to decolorization by microbial reductases.

In yet other preferred embodiments, the enzyme is a mycoplasma-specific nuclease selected from the group consisting of the 5'-3' exonuclease encoded by MCAP_0047 or MHP7448_0581, the gene product of *nfo* (such as MCAP_0060 or MHP7448_0062), *vacB* (such as MCAP_0097 or MHP7448_0037), *uvrC* (such as MCAP_0252 or MHP7448_0066), *rnc* (ribonuclease III, such as MCAP_0492 or MHP7448_0398), MCAP_0768, *uvrB* (such as MCAP_0773 or MHP7448_0648), *uvrA* (such as MCAP_0774 or MHP7448_0091) and mixtures thereof. When the enzyme is a mycoplasma-specific nuclease, a preferred substrate is an acetoxymethyl ester or maleimide derivative of blue dye number 1 coupled to an aminoallyl-dNTP labeled nucleic acid substrate of the mycoplasma-specific nuclease. Typically the substrate is a reagent container, a culture medium container or a cell culture container.

In other aspects, the present invention provides a method of detecting mycoplasma contamination of a cell culture comprising the steps of providing a cell-permeable detectable label coupled to a cell-impermeant carrier in the culture medium wherein cleavage of the detectable label by a mycoplasma-specific enzyme

is followed by uptake of the detectable label into cells; and detecting labeled cells, thereby detecting mycoplasma contamination of the cell culture. In certain embodiments, the mycoplasma-specific enzyme is a protease and the detectable label is an acetoxymethyl ester of derivative of blue dye number 1 coupled to a peptide substrate of the mycoplasma-specific protease. Preferred proteases can be selected from the group consisting of the gene product of *pepA1*(MCAP_0157), *pepA2* (MCAP_0195), *pepA* (leucyl aminopeptidase, such as MHP7448_0464), MCAP_0267 (metalloendopeptidase), *pepP* (Xaa-Pro endopeptidase, such as MCAP_0341 or MHP7448_0649), MCAP_0509, mapP (methionine amino peptidase,MCAP_0675 or MHP7448_0173), and mixtures thereof. In other preferred embodiments, the mycoplasma-specific enzyme is a nuclease and the detectable label is an acetoxymethyl ester of derivative of blue dye number 1 coupled to a nucleic acid substrate of the mycoplasma-specific nuclease Preferred nucleases can be selected from the group consisting of the 5'-3' exonuclease encoded by MCAP_0047 or MHP7448_0581, the gene product of *nfo* (such as MCAP_0060 or MHP7448_0062), *vacB* (such as MCAP_0097 or MHP7448_0037), *uvrC* (such as MCAP_0252 or MHP7448_0066), *mc* (ribonuclease III, such as MCAP_0492 or MHP7448_0398), MCAP_0768, *uvrB* (such as MCAP_0773 or MHP7448_0648), *uvrA* (such as MCAP_0774 or MHP7448_0091) and mixtures thereof.

In other aspects, the present invention provides a method of determining the presence or absence of mycoplasma in a sample, comprising the steps of contacting the sample with a detectably labeled substrate for an enzyme produced and/or secreted by a mycoplasma under conditions that result in the modification of the substrate by the enzyme; and detecting the modification or the absence of the modification of the substrate wherein modification of the substrate indicates the presence of mycoplasma in the sample, and wherein the absence of modification of the substrate indicates the absence of mycoplasma in the sample. Preferably, the level of the detectable label is quantitatively related to the presence or amount of mycoplasma in the sample.

In preferred embodiments, the enzyme is a hydrolytic enzyme selected from a protease, a nuclease or a reductase. In certain embodiments, enzyme is a protease selected from group consisting of the gene product of *pepA1*(MCAP_0157), *pepA2* (MCAP_0195), *pepA* (leucyl aminopeptidase, such as MHP7448_0464),

MCAP_0267 (metalloendopeptidase), *pepP* (Xaa-Pro endopeptidase, such as MCAP_0341 or MHP7448_0649), MCAP_0509, mapP (methionine amino peptidase, such as MCAP_0675 or MHP7448_0173), and mixtures thereof. In other preferred embodiments, the enzyme is a reductase selected from the group consisting of the gene product of *nrdE* (such as MCAP_0101), MCAP_0427, *trxB* (thioredoxin reductase, such as MCAP_0779 or MHP7448_0098) , MCAP_0858 and mixtures thereof. In yet other preferred embodiments, the enzyme is a nuclease selected from the group consisting of the 5'-3' exonuclease encoded by MCAP_0047 or MHP7448_0581, the gene product of *nfo* (such as MCAP_0060 or MHP7448_0062), *vacB* (such as MCAP_0097 or MHP7448_0037), *uvrC* (such as MCAP_0252 or MHP7448_0066), *mc* (ribonuclease III, such as MCAP_0492 or MHP7448_0398), MCAP_0768, *uvrB* (such as MCAP_0773 or MHP7448_0648), *uvrA* (such as MCAP_0774 or MHP7448_0091) and mixtures thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

FIG. 1 is a photograph of the decolorization of an azo dye, reactive black 5, with supernatants of cultured bacteria. Each well was incubated with 10 μg of reactive black 5 plus 190 μl of culture supernatant from the following bacterium: *E. coli, E. faecalis, S. aureus, P. aeruginosa, S. pyogenes, and S. marcescens.* Such azo dyes were decolorized by most bacteria after incubation with the dye for about 18 hours. The decolorization is indicative of reductases produced by the bacteria.

FIG. 2A is a diagrammatic illustration of an embodiment of a contamination biosensor 200 placed on a container 100 for a reagent or culture medium. FIG. 2B a diagrammatic illustration of an embodiment of a contamination biosensor 210 placed on a container 110 for tissue culture.

FIG. 3 is a diagrammatic illustration of an embodiment of a mycoplasma contamination detection system for cell culture, showing in FIG. 3A a cell 300 in an

uncontaminated culture, and in FIG. 3B, a cell 300 in a contaminated culture containing a dye deposit 360 that is indicative of mycoplasma contamination.

FIG. 4A shows RNA samples used in RT-PCR. The lanes are a) 1kB DNA Ladder, b) BHK-21 cells infected with *Mycoplasma hyorhinis* as a monolayer, c) the
5 pellet of BHK-21 cells medium infected with *Mycoplasma hyorhinis*, and d) the pellet of *Mycoplasma hyorhinis* from mycoplasma enrichment broth (not from tissue culture cells).

FIG. 4B is a graphical representation of the expression of several *Mycoplasma hyorhinis* genes under conditions A-J: A) *lon*, 3T3 cells growing as a
10 monolayer, B) *lon*, BHK-21 cells growing in DMEM, C) *map*, 3T3 cells growing as a monolayer, D) *map*, BHK-21 cells growing in DMEM, E) *pepA*, 3T3 cells growing as a monolayer, F) *pepA*, BHK-21 cells growing in DMEM, G) *trxB*, 3T3 cells growing as a monolayer, H) *trxB*, BHK-21 cells growing in DMEM, I) *vacB*, 3T3 cells growing as a monolayer, J) *vacB*, BHK-21 cells growing in DMEM.

15 FIG. 5 shows the results of testing of primers with genomic DNA from *Mycoplasma hyorhinis*. The PCR products were run on a 1.5% agarose gel. We performed 35 cycles of hot start PCR with a initial melt of 95°C for 4 minutes, followed by a melt at 95°C for 45 seconds, an annealing at 50°C for 45 seconds, and a final extension step 72°C for 7 minutes. The lane order includes a 1 kB DNA ladder
20 (a), 5'-3' exonuclease (b &k), gcp (c & l), lon (d & m), map (e & n) , nfo (f & o), nox, (g&p), trxB (h &q), uvrA (i &r), p37 (j&s), all using 0.5μl of DNA template (B-J) or 1 μl of DNA diluted 1:10.

FIG. 6 shows the results of testing of further PCR primers with genomic DNA from *Mycoplasma hyorhinis*. Lanes include 1 kB DNA ladder (a), gcp (b), hrcA (c),
25 lgt (d), pepA (e), pepP (f), pth (g), rnc (h), uvrB (i), uvrC (j), vacB (k), 5'-3' (l), nox (m), p37 (n). The PCR product for pepF amplified the correct size product as well (not shown).

FIG. 7 shows the results of testing by RT-PCR of all primers that worked under 50°C AT. The RNA template used was the BHK-21 DMEM pellet that had
30 been previously treated with DNase. For each 2μl of a 1:10 dilution of template.

RT, 45°C for 10 minutes, 95°C for 15 minutes, amp. cycled 35 times 95°C 15s 50°C 45s, final extension at 72°C for 7 minutes. Lanes include 1 kB DNA ladder (a), 5'-3'(b), lgt (c), lon (d), map (e), nfo (f), nox (g), pepA (h), pepF (i), pth (j), rnc (k), trxB (l), uvrA (m), uvrB (n), vacB (o), p37 (p), no primer control.

5 FIG. 8 shows an agarose gel loaded with double stranded DNA (dsDNA, lanes b-i) or double stranded RNA (ribosomal RNA, lanes j-q) treated with *M. hyorhinis* extract from a cell culture infection or supernantants from infected or unifected cell cultures. The respective lanes contain: a) 1 kb DNA ladder, b) dsDNA exposed to an aliquot of *M. hyorhinis* extract for 30 minutes, c) dsDNA exposed to an 10 aliquot of the supernatant of an infected cell culture for 30 minutes, d) dsDNA exposed to an aliquot of the supernatant of an uninfected cell culture for 30 minutes, e) dsDNA exposed to H_2O for 30 minutes, f) dsDNA exposed to an aliquot of *M. hyorhinis* extract for 0 minutes, g) dsDNA exposed to an aliquot of the supernatant of an infected cell culture for 0 minutes, h) dsDNA exposed to an aliquot of the 15 supernatant of an uninfected cell culture for 0 minutes, i) dsDNA exposed to H_2O for 0 minutes, j) ribosomal RNA exposed to an aliquot of *M. hyorhinis* extract for 30 minutes, k) ribosomal RNA exposed to an aliquot of the supernatant of an uninfected cell culture for 30 minutes, l) ribosomal RNA exposed to an aliquot of the supernatant of an uninfected cell culture for 30 minutes, m) ribosomal RNA exposed 20 to exposed to H_2O 30 minutes, n) ribosomal RNA exposed to an aliquot of *M. hyorhinis* extract for 0 minutes, o) ribosomal RNA exposed to an aliquot of the supernatant of an infected cell culture for 0 minutes, p) ribosomal RNA RNA exposed to an aliquot of the supernatant of an un infected cell culture for 0 minutes, and q) ribosomal RNA exposed to an aliquot of H_2O for 0 minutes

25 FIG. 9 is a graph of the results of testing the thioredoxin reductase activities of *M. hyorhinis*, *E. coli* and *S. aureus*. There was no significant activity from 10^4-10^6 CFU/ml of *S. aureus* or *E. coli* using the DTNB substrate.

FIG. 10A and FIG. 10B are schematic diagrams of a substrate-linked enzymatic reporter reagent. In FIG. 10A, a bead 400 is covalently linked to 30 horseradish peroxidase 420 by a molecule DTSSP 410 that is a substrate for a reductase such as trxB. In FIG. 10B, a bead 400 is covalently linked to luciferase 440 by a molecule Leu 430 that is a substrate for a protease such as pepA.

FIG. 11 is a graph showing the effect of 1 mM DTT in enhancing the fluorescence of trxB substrates 2',7'-difluoro-4'-(2-(5-((dimethylaminophenyl) azo)pyridyl)dithiopropionyl aminomethyl) fluorescein (DFDMAP) and BODIPY®FL L-cystine.

5 FIG. 12 is a graph showing the effect of digitonin on the trxB assay.

FIG. 13 is a graph showing the effect of acid pH levels on the leu-MCA assay.

FIG. 14 is a graph showing the effect of basic pH levels on the leu-MCA assay.

10 FIG. 15 is a graph showing the sensitivity of the leu-MCA assay.

DETAILED DESCRIPTION OF THE INVENTION

Many of the genomes of the genus *Mycoplasma* have been sequenced. It is apparent that the microorganism has few biosynthetic genes, and the microorganism can only thrive in very rich growth mediums. Using a genomic approach in which we compared the genomes of 10 different mycoplasma (*M. gallisepticum, M. capricolum, M. genitalium, M. hyoppneumonia, M. mobile, M. mycoides, M penetrans, M. pneumonia, M. pulmonis, and M. synoviae*) at 40% sequence identity, we have identified 243 genes that are conserved in all *Mycoplasma* species studied to date. *Mycoplasma* species use an array of hydrolytic enzymes to uptake materials that compensates for having very few internal biosynthetic processes. The identified genes are listed in Table 1, below, using the *M. capricolum* notation.

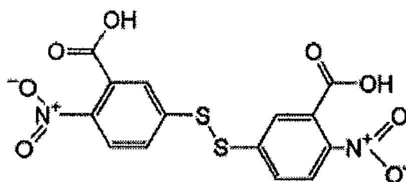
The common genes included a variety of enzymes that can be grouped into seven classes: synthetic enzymes, hydrolytic enzymes, chaperones, permeases, kinases, transcription factors, and ribosomal proteins. The presence of acetate kinase has been disclosed as an assay for the presence of Mycoplasma (U.S. published patent application No. 2004/0265942). However, this assay is an enzyme cascade assay requiring luciferase and is not amenable to a simple and direct method for measuring contamination in culture and *in vivo*.

The hydrolytic enzymes are interesting targets both for diagnosis and the treatment of a mycoplasma infection because they are secreted and likely involved in

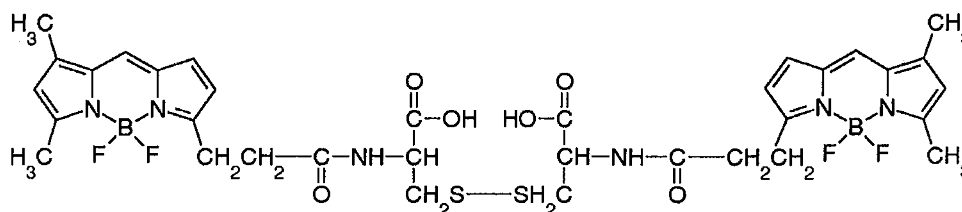
infection and virulence. The common hydrolytic enzymes of *Mycoplasma* species include: proteases, such as the gene products of MCAP_0157, MCAP_0195, MCAP_0267, MCAP_0341, MCAP_0509, MCAP_0675, nucleases, such as the gene products of MCAP_0047, MCAP_0060, MCAP_0097, MCAP_0252, MCAP_0492, MCAP_0768, MCAP_0773, MCAP_0774, and reductases, such as the gene products of MCAP_0101, MCAP_0427, MCAP_0779, and MCAP_0858.

Reductase activity can be measured through a Azo dye that gets decolorized by the release of reductases from many bacterial cells. An azo dye such as reactive black 5 or DABCYL (4-((4-(dimethylamino)phenyl)azo)benzoic acid) is completely decolorized by many bacterial cultured supernatant after just 18 hours on incubation. A sensor placed on the bottom of a culture dish, buffer container or even on a swab for measuring the presence of mycoplasma in human fluids can be used to ascertain bacterial contamination or infection. The benefit of a simple azo dye sensor is low cost although it may not specifically detect different bacteria. FIG. 1 is a photograph of a microtiter plate containing reactive black 5 decolorized by incubation with different pathogenic bacteria. Each well was incubated with 10 μg of reactive black 5 plus 190 μl of filtered culture supernatant from the following bacterium: *E. coli, E. faecalis, S. aureus, P. aeruginosa, S. pyogenes, and S. marcescens.* Such azo dyes are decolorized by most bacteria after incubation with the dye for about 18 hours. The decolorization is indicative of reductases produced by the bacteria. Mycoplasma reductases such as the gene products of MCAP_0101. MCAP_0427, MCAP_0779, and MCAP_0858 can also decolorize such substrates.

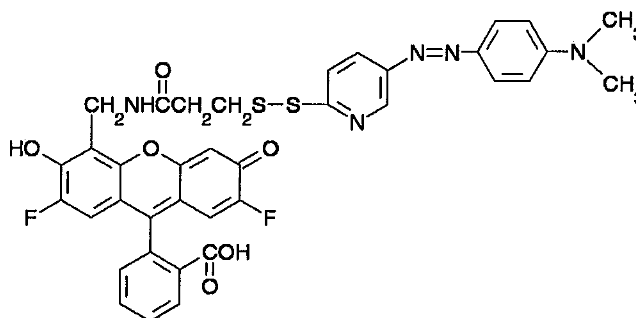
In other embodiments, substrates for reductases are reagents that produce a fluorescent signal. Suitable such reagents include DTNB (5,5'-Dithio-*bis*-(2-nitrobenzoic acid), also known as Ellman's reagent.



5 Two other suitable fluorogenic compounds are BODIPY®FL L-cystine,



and 2',7'-difluoro-4'-(2-(5-((dimethylaminophenyl)azo)pyridyl) dithiopropionyl aminomethyl) fluorescein,



Specific peptidase substrates can be used to identify a specific bacterium. Published patent applications disclosing both specific and broad-spectrum targets for detection of pathogens include WO 2005/042770, WO2005/012556 and WO20 04/087942, which are incorporated herein by reference. Mycoplasmas secrete a lysine-specific endopeptidase, an aminopeptidase and a carboxypeptidase that make it possible to specifically detect the presence of mycoplasma by using a substrate that is specific for these enzymes. Suitable aminopeptidases and carboxypeptidases have been purified by Watanabe and colleagues (Watanabe, T. (1988), Proteolytic activities of *Mycoplasma salivarium*, Adv Dent Res 2(2):297-300; Watanabe, T (1985) Proteolytic activity of mycoplasmas and ureaplasmas isolated freshly from human saliva, Medical Microbiology and Immunology 173(5): 251-255; Watanabe, T. et al., (1984) Aminopeptidase and caseinolytic activites of *Mycoplasma salivarium* Medical Microbiology and Immunology, 172 (4): 257-264). In a preferred embodiment, these purified or partially purified enzymes are used in a high-throughput screen to identify potential novel substrates.

Mycoplasmas produce both secreted and membrane-bound nucleases that are involved in obtaining nucleotides for DNA synthesis. See Minion, C. J. D. Goguen (1986) Identification and Preliminary Characterization of External Membrane-Bound

Nuclease Activities in *Mycoplasma pulmonis,* Infection And Immunity, 51(1):352-354; Kannan, T. R.,& Baseman, J. B., (2006) ADP-ribosylating and vacuolating cytotoxin of *Mycoplasma pneumoniae* represents unique virulence determinant among bacterial pathogens. PNAS, 103:6724-6729; Bendjennat, M., et al., (1997) Purification and Characterization of *Mycoplasma penetrans* Ca2+/Mg2+-Dependent Endonuclease, Journal of Bacteriology 179:2210-2220; Minion, C. F., et al.,(1993) Membrane- Associated Nuclease Activities in Mycoplasmas. Journal of Bacteriology 175:7842-7847.

RNA or DNA sequences that are efficiently hydrolyzed by Mycoplasma nucleases that labeled with a detectable colorimetric or fluorescent dye can be used to detect the presence of mycoplasma contamination. A dye such as blue dye number 1 is not decolorized by microorganisms and would be a good choice for a colorimetric
5 reporter. The dye is labeled with a reactive aminoallyl-dUTP via a Klenow reaction using techniques known to one skilled in the art to covalently attach the dye to a nucleic acid. See Hasseman, J .J., et al., 2006 Microbial Genomic DNA Aminoallyl Labeling For Microarrays, The Institute For Genomic Research Standard Operating Procedure # M009. The aminoallyl groups on the nucleic acid would then be
10 available for labeling with a reactive fluorescent or chromogenic dye molecule. The dye-labeled nucleic acid can be attached to the surface of a sterile bottle. If the bottle after opening became contaminated with mycoplasmas, the spot of color on the inner surface of the bottle would be released, indicating that the bottle is contaminated. FIG. 2A is a diagrammatic illustration of a contamination biosensor 200 placed on a
15 container 100 for a reagent or culture medium. FIG. 2B a diagrammatic illustration of a contamination biosensor 210 placed on a container 110 for tissue culture.

Azo dyes such as reactive black 5 and DABCYL are decolorized by bacteria and can be used as a broad spectrum sensor for microbial contamination. Blue dye number 1, which is not decolorized by bacteria, can be used as a label of nucleic
20 acids or a peptide to give a specific probe for mycoplasmas or other contaminating microorganism. Fluorescent probes or the release of fluorescent micro-spheres can be used to indicate contamination. Contamination can be measured by eye, using a fluorimeter or colorimeter or on a microscope stage.

In another embodiment, a peptide or nucleic acid can be labeled with an acetoxymethyl ester of a dye, such as blue dye number 1, that upon hydrolytic cleavage would be taken up by cells in culture and thereby turn them blue to indicate the presence of mycoplasmas in the culture medium. FIG. 3 is a diagrammatic
5 illustration of an embodiment of such a mycoplasmas contamination detection system for cell culture, showing in FIG. 3A a cell 300 in an uncontaminated culture, and in FIG. 3B, a cell 300 in a contaminated culture containing a dye deposit 360 that is indicative of mycoplasmas contamination. An acetoxymethyl ester derivative of blue dye number 1 coupled to a peptide or nucleic acid carrier would be impermeable to
10 tissue culture cells until contamination with mycoplasmas. The proteases or nucleases from *Mycoplasma* spp. would cleave the carrier from the acetoxymethyl ester derivative of blue dye number 1, thereby allowing the acetoxymethyl ester derivative of blue dye number 1 to be taken up by the tissue culture cells. Tissue culture cells that become colored blue indicate that the culture was contaminated with
15 mycoplasmas. The colored cells can be observed with a light microscope. Alternatively a cell permeable fluorescent dye can be used and the fluorescing cells can be detected with a fluorescence microscope.

Table 1

Genes characterized by sequences common to *Mycoplasma* spp. (40% identity)

Gene	Symbol	Common Name
MCAP_0001	dnaA	chromosomal replication initiator protein DnaA
MCAP_0002	dnaN	DNA polymerase III, beta subunit
MCAP_0004	ksgA	dimethyladenosine transferase
MCAP_0008	dnaX	DNA polymerase III gamma-tau subunits
MCAP_0010	tmk	thymidylate kinase
MCAP_0011		DNA polymerase III, delta prime subunit
MCAP_0017	ftsH	ATP-dependent metalloprotease FtsH
MCAP_0022		acyl carrier protein phosphodiesterase, putative
MCAP_0026	rpsR	30S ribosomal protein S18
MCAP_0035	metG	methionyl-tRNA synthetase
MCAP_0038		ABC transporter, permease protein
MCAP_0039		ABC transporter, permease protein
MCAP_0040	gyrA	DNA gyrase, A subunit
MCAP_0041	gyrB	DNA gyrase, B subunit
MCAP_0045	secA	preprotein translocase, SecA subunit
MCAP_0047		5-3 exonuclease family protein
MCAP_0060		endonuclease IV
MCAP_0065	rplK	50S ribosomal protein L11
MCAP_0066	rplA	ribosomal protein L1
MCAP_0067	rplJ	50S ribosomal protein L10
MCAP_0068	rplL	50S ribosomal protein L7/L12
MCAP_0070	rpoB	DNA-directed RNA polymerase, beta subunit
MCAP_0071	rpoC	DNA-directed RNA polymerase beta subunit
MCAP_0074		ribose 5-phosphate isomerase B, putative
MCAP_0075	glyA	serine hydroxymethyltransferase
MCAP_0076	upp	uracil phosphoribosyltransferase
MCAP_0078	atpB	ATP synthase F0, subunit A
MCAP_0079	atpE	ATP synthase F0, subunit c
MCAP_0082	atpA1	ATP synthase F1, alpha subunit
MCAP_0083	atpG	ATP synthase F1, gamma subunit
MCAP_0084	atpD1	ATP synthase F1, beta subunit
MCAP_0094	ptsG	PTS system, glucose-specific IIABC component
MCAP_0096	smpB	SsrA-binding protein
MCAP_0097		Rnase R (VacB) and RNase II family 3-5 exoribonucleases
MCAP_0101	nrdE	ribonucleoside-diphosphate reductase 2, alpha subunit
MCAP_0104	prs	phosphoribosylpyrophosphate synthase
MCAP_0105	pth	peptidyl-tRNA hydrolase
MCAP_0107	dnaC	replicative DNA helicase
MCAP_0110	cysS	cysteinyl-tRNA synthetase
MCAP_0111		RNA methyltransferase, TrmH family, group 3
MCAP_0114	nusG	transcription antitermination protein NusG
MCAP_0119		oligopeptide ABC transporter, ATP-binding protein
MCAP_0120		oligopeptide ABC transporter, ATP-binding protein
MCAP_0124		hydrolase, TatD family
MCAP_0130	gltX	glutamyl-tRNA synthetase
MCAP_0136	fba	fructose-1,6-bisphosphate aldolase, class II
MCAP_0140	rpmE	ribosomal protein L31

Table 1 (continued)
Genes characterized by sequences common to *Mycoplasma* spp. (40% identity)

Gene	Symbol	Common Name
MCAP_0142		DHH phosphoesterase family protein, putative
MCAP_0143	tdk	thymidine kinase
MCAP_0144	prfA	peptide chain release factor 1
MCAP_0145		modification methylase, HemK family
MCAP_0151	rpsL	30S ribosomal protein S12
MCAP_0152	rpsG	30S ribosomal protein S7
MCAP_0153	fusA	translation elongation factor G
MCAP_0154	tuf	translation elongation factor Tu
MCAP_0157	pepA1	cytosol aminopeptidase
MCAP_0159	alaS	alanyl-tRNA synthetase
MCAP_0163		oligopeptide ABC transporter, ATP-binding protein
MCAP_0195	pepA2	cytosol aminopeptidase
MCAP_0200		spermidine/putrescine ABC transporter, permease protein and spermidine/putrescine-binding protein
MCAP_0201		spermidine/putrescine ABC transporter, permease protein
MCAP_0203	rplT	50S ribosomal protein L20
MCAP_0205	infC	translation initiation factor IF-3
MCAP_0208	gmk	guanylate kinase
MCAP_0213	eno	enolase4.2.1.11
MCAP_0216	hpt1	hypoxanthine phosphoribosyltransferase
MCAP_0220	pfkA	Phosphofructokinase
MCAP_0221	pyk	pyruvate kinase
MCAP_0222	thrS	threonyl-tRNA synthetase
MCAP_0223		NADH oxidase
MCAP_0224		lipoate-protein ligase
MCAP_0225	pdhA	pyruvate dehydrogenase complex, EI component, alpha subunit
MCAP_0226	pdhB	pyruvate dehydrogenase complex, E1 component, beta subunit
MCAP_0228	pdhD	dihydrolipoamide dehydrogenase
MCAP_0229	pta	phosphate acetyltransferase
MCAP_0230	ackA	acetate kinase
MCAP_0233	ptsI	phosphoenolpyruvate-protein phosphotransferase
MCAP_0234	crr	PTS system, glucose-specific IIA component
MCAP_0237	rpsD	30S ribosomal protein S4
MCAP_0245		GTP-binding conserved hypothetical protein
MCAP_0251	greA	transcription elongation factor GreA
MCAP_0252	uvrC	excinuclease ABC, C subunit
MCAP_0255		conserved hypothetical protein
MCAP_0258	valS	valyl-tRNA synthetase
MCAP_0260	rpe	ribulose-phosphate 3-epimerase
MCAP_0261	rsgA	ribosome small subunit-dependent GTPase A
MCAP_0267		metalloendopeptidase, putative
MCAP_0318	proS	prolyl-tRNA synthetase
MCAP_0321	lepA	GTP-binding protein LepA
MCAP_0323	aspS	aspartyl-tRNA synthetase
MCAP_0324	hisS	His-tRNA synthetase 6.1.1.21
MCAP_0330	rpsO	30S ribosomal protein S15
MCAP_0333	infB	translation initiation factor IF-2
MCAP_0336		transcription elongation protein nusA, putative
MCAP_0339	polC	DNA polymerase III, alpha subunit, Gram-positive type

Table 1 (continued)
Genes characterized by sequences common to *Mycoplasma* spp. (40% identity)

Gene	Symbol	Common Name
MCAP_0340	cdsA	phosphatidate cytidylyltransferase
5 MCAP_0341		Xaa-Pro peptidase
MCAP_0342	trpS	tryptophanyl-tRNA synthetase
MCAP_0358	atpA2	ATP synthase F1, alpha subunit
MCAP_0359	atpD2	ATP synthase F1, beta subunit
MCAP_0364		RNA methyltransferase, TrmH family
10 MCAP_0365		hydrolase of the HAD superfamily, putative
MCAP_0367	hrcA	heat-inducible transcription repressor HrcA
MCAP_0369	dnaK	Chaperone protein dnaK (Heat shock protein 70) (Heat shock 70 kDaprotein) (HSP70)
MCAP_0371	rpsB	30S ribosomal protein S2
15 MCAP_0372	tsf	translation elongation factor Ts
MCAP_0374	pyrH	uridylate kinase
MCAP_0375	frr	ribosome recycling factor
MCAP_0376	argS	arginyl-tRNA synthetase
MCAP_0383	pheS	phenylalanyl-tRNA synthetase, alpha subunit
20 MCAP_0384	pheT	phenylalanyl-tRNA synthetase, beta subunit
MCAP_0388	mraW	S-adenosyl-methyltransferase MraW 2.1.1.- 479149
MCAP_0393	ileS	isoleucyl-tRNA synthetase
MCAP_0395		ribosomal large subunit pseudouridine synthase, RluA family
MCAP_0410		conserved hypothetical protein, TIGR00096
25 MCAP_0412	rplU	50 ribosomal protein L21
MCAP_0414	rpmA	50S ribosomal protein L27
MCAP_0423		ImpB/MucB/SamB family protein
MCAP_0427		pyridine nucleotide-disulphide oxidoreductase
MCAP_0439	ldh	L-lactate/malate dehydrogenase
30 MCAP_0445		triacylglycerol lipase
MCAP_0446		triacylglycerol lipase, putative
MCAP_0449		lipoate-protein ligase
MCAP_0454	gtsA	glycerol ABC transporter, ATP-binding protein
MCAP_0456	parC	DNA topoisomerase IV, A subunit
35 MCAP_0457	parE	DNA topoisomerase IV, B subunit
MCAP_0462		RNA methyltransferase, TrmH family
MCAP_0465	pgi	glucose-6-phosphate isomerase
MCAP_0469		aminotransferase, class V
MCAP_0472		HIT family protein
40 MCAP_0474	ung	uracil-DNA glycosylase
MCAP_0476	gid	glucose inhibited division protein
MCAP_0478	metK	S-adenosylmethionine synthetase
MCAP_0479		conserved hypothetical protein TIGR00282
MCAP_0481	ftsY	signal recognition particle-docking protein FtsY
45 MCAP_0488	rpmB	50S ribosomal protein L28
MCAP_0492		ribonuclease III
MCAP_0495		structural maintenance of chromosomes (SMC) superfamily protein
MCAP_0497	apt	adenine phosphoribosyltransferase
50 MCAP_0503	rpoD	RNA polymerase sigma factor RpoD
MCAP_0504	dnaG	DNA primase
MCAP_0505	glyS	glycyl-tRNA synthetase
MCAP_0507	era	GTP-binding protein Era

Table 1 (continued)
Genes characterized by sequences common to *Mycoplasma* spp. (40% identity)

Gene	Symbol	Common Name
MCAP_0509		Peptidase C39 family protein
MCAP_0510		DJ-1 family protein
MCAP_0516	lon	ATP-dependent protease La
MCAP_0517	tig	trigger factor
MCAP_0519	efp	translation elongation factor P
MCAP_0521		conserved hypothetical protein
MCAP_0523	trmU	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase
MCAP_0529		nicotinate (nicotinamide) nucleotide adenylyltransferase /conserved hypothetical domain
MCAP_0532		Spo0B-associated GTP-binding protein, putative
MCAP_0544	rplS	50S ribosomal protein L19
MCAP_0545	trmD	tRNA (guanine-N1)-methyltransferase
MCAP_0547	rpsP	30S ribosomal protein S16
MCAP_0549	ffh	signal recognition particle protein
MCAP_0551	recA	recA protein
MCAP_0577	engA	GTP-binding protein engA
MCAP_0578	cmk	cytidylate kinase
MCAP_0581	ppa	inorganic pyrophosphatase
MCAP_0589		ribulose-phosphate 3-epimerase, putative
MCAP_0601	scpB	chromosomal segregation and condensation protein B
MCAP_0606		hydrolase, alpha/beta fold family
MCAP_0609		Uncharacterised membrane protein, UPF0154 family
MCAP_0610	tkt	transketolase
MCAP_0613		glucose-inhibited division protein, putative
MCAP_0616		fructose/tagatose bisphosphate aldolase, class II
MCAP_0623		metallo-beta-lactamase superfamily protein
MCAP_0631	pgk	phosphoglycerate kinase
MCAP_0632	gap	glyceraldehyde-3-phosphate dehydrogenase
MCAP_0635	mutM	formamidopyrimidine-DNA glycosylase
MCAP_0636	polA	DNA polymerase I
MCAP_0637	dnaE	DNA-directed DNA polymerase III (polc)
MCAP_0639	tyrS	tyrosyl-tRNA synthetase
MCAP_0659	leuS	leucyl-tRNA synthetase
MCAP_0662	rpsI	30S ribosomal protein S9
MCAP_0663	rplM	50S ribosomal protein L13
MCAP_0666		cobalt ABC transporter, permease protein
MCAP_0667		cobalt ABC transporter, ATP-binding protein, putative
MCAP_0668		cobalt ABC transporter, ATP-binding protein, putative
MCAP_0669	rplQ	50S ribosomal protein L17
MCAP_0670	rpoA	DNA-directed RNA polymerase, alpha chain
MCAP_0671	rpsK	30S ribosomal protein S11
MCAP_0672	rpsM	30S ribosomal protein S13
MCAP_0675	map	methionine aminopeptidase, type I
MCAP_0676	adk	adenylate kinase
MCAP_0677	secY	preprotein translocase, SecY subunit
MCAP_0678	rplO	50S ribosomal protein L15
MCAP_0679	rpsE	30S ribosomal protein S5
MCAP_0680	rplR	50S ribosomal protein L18
MCAP_0681	rplF	50S ribosomal protein L6
MCAP_0682	rpsH	ribosomal protein S8
MCAP_0684	rplE	ribosomal protein L5

Table 1 (continued)

Genes characterized by sequences common to *Mycoplasma* spp. (40% identity)

Gene	Symbol	Common Name
MCAP_0686	rplN	ribosomal protein L14
MCAP_0687	rpsQ	ribosomal protein S17
MCAP_0689	rplP	ribosomal protein L16
MCAP_0690	rpsC	ribosomal protein S3
MCAP_0691	rplV	ribosomal protein L22
MCAP_0692	rpsS	30S ribosomal protein S19
MCAP_0693	rplB	50S ribosomal protein L2
MCAP_0694	rplW	50S ribosomal protein L23
MCAP_0695	rplD	50S ribosomal protein L4
MCAP_0696	rplC	50 ribosomal protein L3
MCAP_0697	rpsJ	30S ribosomal protein S10
MCAP_0707		potassium uptake protein, TrkH family, putative
MCAP_0708		potassium uptake protein, TrkA family, putative
MCAP_0709	gatB	glutamyl-tRNA(Gln) amidotransferase, B subunit
MCAP_0710	gatA	glutamyl-tRNA(Gln) amidotransferase, A subunit
MCAP_0712	ligA	DNA ligase, NAD-dependent
MCAP_0714		ribosomal large subunit pseudouridine synthase, RluA family
MCAP_0716	ptsH	phosphocarrier protein hpr
MCAP_0750	tpiA	triosephosphate isomerase
MCAP_0751		HAD-superfamily hydrolase subfamily IIB, protein
MCAP_0752	gpmI	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
MCAP_0755	deoC	deoxyribose-phosphate aldolase
MCAP_0757	deoA	pyrimidine-nucleoside phosphorylase
MCAP_0761	trmB	tRNA (guanine-N(7)-)-methyltransferase
MCAP_0765	hpt2	hypoxanthine phosphoribosyltransferase
MCAP_0768		deoxyribonuclease, TatD family
MCAP_0773	uvrB	excinuclease ABC, B subunit
MCAP_0774	uvrA	excinuclease ABC, A subunit
MCAP_0778	lgt1	prolipoprotein diacylglyceryl transferase
MCAP_0779	trxB	thioredoxin reductase
MCAP_0780	lgt2	prolipoprotein diacylglyceryl transferase
MCAP_0781		conserved hypothetical protein
MCAP_0792	topA	DNA topoisomerase I
MCAP_0805	engD	GTP-dependent nucleic acid-binding protein engD
MCAP_0807	gidB	methyltransferase gidB
MCAP_0808	pgsA	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
MCAP_0814		transporter protein, putative
MCAP_0818	rpsT	30S ribosomal protein S20
MCAP_0819	trmE	tRNA modification GTPase TrmE
MCAP_0821		glycoprotease family protein
MCAP_0824	asnS	asparaginyl-tRNA synthetase
MCAP_0834		hydrolase, haloacid dehalogenase-like family, putative
MCAP_0836	lysS	lysyl-tRNA synthetase
MCAP_0839	serS	seryl-tRNA synthetase
MCAP_0844		PTS system glucose-specific IIBC component
MCAP_0849	deoD	purine nucleoside phosphorylase
MCAP_0856	gidA	glucose inhibited division protein A
MCAP_0858		pyridine nucleotide-disulphide oxidoreductase
MCAP_0860		membrane protein, putative
MCAP_0870	rpmH	50S ribosomal protein L34

Example 1
Mycoplasma hyorhinis Enzymes

The subset of *Mycoplasma* genes from the *Mycoplasma hyorhinis* genome that were selected for further study are listed in Table 2, below. DNA PCR primers
5 were made for each of these genes and the PCR are shown in FIG. 5 and FIG. 6.

Table 2
Hydrolytic enzymes in common among *Mycoplasma ssp.*

	Gene	Symbol	Common Name
1	MHP7448_0010	hrcA	heat-inducible transcription repressor
2	MHP7448_0037	vacB	VACB-like ribonuclease II
3	MHP7448_0062	nfo	endonuclease IV
4	MHP7448_0066	uvrC	excinuclease ABC subunit C
5	MHP7448_0082	nox	NADH oxidase
6	MHP7448_0091	uvrA	excinuclease ABC subunit A
7	MHP7448_0097	lgt	prolipoprotein diacylglyceryl transferase
8	MHP7448_0098	trxB	thioredoxin reductase
9	MHP7448_0173	map	methionine aminopeptidase
10	MPH7448_0204	pth	prptidyl-tRNA hydrolase
11	MHP7448_0398	rnc	ribonuclease III
12	MHP7448_0464	pepA	leucyl aminopeptidase
13	MHP7448_0521	pepF	oligoendopeptidase F
14	MHP7448_0524	lon	heat shock ATP-dependent protease
15	MHP7448_0581		5'-3' exonuclease
16	MHP7448_0635	gcp	O-sialoglycoprotein endopeptidase
17	MHP7448_0648	uvrB	excinuclease ABC subunit B
18	MHP7448_0659	pepP	XAA-PRO aminopeptidase

Purification of Total RNA

Total RNA was isolated from *Mycoplasma hyorhinis* grown in BHK-21 and Swiss 3T3 tissue culture cells (FIG. 4A). RNA was purified both from the infected
10 tissue culture media and culture cells (BHK-21 & Swiss 3T3) from the infected dish. RNA was purified using either the Invitrogen Triazol® Max bacterial RNA isolation kit or an acid phenol-guanidium thiocyanate and chloroform extraction procedure. Although the acid phenol-guanidium thiocyanate and chloroform extraction procedure had better quality RNA as judged by gel electrophoresis in a 1.5% agarose
15 gel, the RNA from the Triazol® Max bacterial RNA kit had more mycoplasma RNA as judged by PCR of the p37 control gene. As a positive control for the RT-PCR reactions, we amplified the p37 gene from the total infected BHK-21 and 3T3 cell media. As a negative control, we also isolated total RNA from uninfected tissue

culture media and uninfected BHK-21 or 3T3 cells and then performed RT-PCR using the p37 primer set. RT PCR was performed in a iCycler iQ PCR Detection System (Bio-Rad) using the SYBR Green One-Step Quantitative RT-PCR kit. Alternatively, for the preliminary studies RT PCR was performed with the Ambion
5 Ag-Path kit. FIG. 4A shows an exemplary result of agarose gel electrophoresis of the RNA samples used in RT-PCR. The lanes are a) 1kB DNA Ladder, b) BHK-21 cells infected with *Mycoplasma hyorhinis* as a monolayer, c) the pellet of BHK-21 cells medium infected with *Mycoplasma hyorhinis*, and d) the pellet of *Mycoplasma hyorhinis* from mycoplasma enrichment broth (not from tissue culture cells).

10 The results of preliminary studies indicate that the following genes *vacB, trxB, map, pepA, lon,* and *uvrB* are transcribed at a high level. FIG. 4B is a graphical representation of the expression of several *Mycoplasma hyorhinis* genes under conditions A-J: A) *lon*, 3T3 cells growing as a monolayer, B) *lon*, BHK-21 cells growing in DMEM, C) *map*, 3T3 cells growing as a monolayer, D) *map*, BHK-21
15 cells growing in DMEM, E) *pepA*, 3T3 cells growing as a monolayer, F) *pepA*, BHK-21 cells growing in DMEM, G) *trxB*, 3T3 cells growing as a monolayer, H) *trxB*, BHK-21 cells growing in DMEM, I) *vacB*, 3T3 cells growing as a monolayer, J) *vacB*, BHK-21 cells growing in DMEM. The level of expression from strongest to weakest for these abundant mRNA is *trxB>pepA>map>vacB>lon>uvrB*.
20 Substrates for the hydrolytic enzymes corresponding to these putative abundant mRNAs from *Mycoplasma* were identified using both literature and patent searches. Certain selected examples are provided in Table 3, below.

Table 3
Mycoplasma Hydrolase Substrates

Gene Symbol	Substrates	References
vacB	ds RNAse activity	J. Cell. Physiol. 143(3) 416-419
trxB	DTNB	Cayman Chemical Company
Map	Rhodamine based fluorogenic substrates	J. Biomol. Screening 7(6) 531-540, 2002
pepA	Leucine at N-terminal of a peptide	Curr. Microbiol. 48(1)32-38, 2004
lon	Glutaryl-Ala-Ala-Phe-methoxynaphthylamine + ATP	J. Biol. Chem. 260(22) 1 1985
uvrB	(UvrA)2(UvrB)1 com	Proc. Natl Soc. USA 86(14) 5237-5241, 1985.

The genes that were determined by quantitative RT-PCR results to be highly
25 expressed in *Mycoplasma hyorhinis* when infecting 3T3 cells or BHK-21 cells: *trxB*, *pepA*, *lon*, *vacB*, *map*, and *uvrB*. The substrates for the enzymes produced by these

gene products is reported in Table 4, below.

Table 4 *Mycoplasma hyorhinis* **Gene Encoding Enzymes and Enzyme Substrates**		
Gene	**Enzyme**	**Substrate**
trxB	Thioredoxin reductase	DTNB
pepA	Leucine aminopeptidase	leu-MCA
lon	ATP-dependent protease	glt-ala-ala-phe-MCA
map	Methionine aminopeptidase	met-MCA
vacB	exoribonuclease	dsRNA
uvrB	excinuclease	dsDNA

We examined the nuclease activities of VacB and UvrB by challenging extracts from *M. hyorhinis* isolated from a cell culture infection or medium from
5 *Mycoplasma*-infected or uninfected cell culture with either double stranded (ds) RNA or dsDNA. The reaction was incubated for 30 minutes at 37°C and the products were then analyzed by agarose gel electrophoresis, as shown in FIG. 8.

FIG. 8 shows an agarose gel loaded with double stranded DNA (dsDNA, lanes b-i) or double stranded RNA (ribosomal RNA, lanes j-q) treated with *M.*
10 *hyorhinis* extract from a cell culture infection or supernantants from infected or unifected cell cultures. The respective lanes contain: a) 1 kb DNA ladder, b) dsDNA exposed to an aliquot of *M. hyorhinis* extract for 30 minutes, c) dsDNA exposed to an aliquot of the supernatant of an infected cell culture for 30 minutes, d) dsDNA exposed to an aliquot of the supernatant of an uninfected cell culture for 30 minutes,
15 e) dsDNA exposed to H_2O for 30 minutes, f) dsDNA exposed to an aliquot of *M. hyorhinis* extract for 0 minutes, g) dsDNA exposed to an aliquot of the supernatant of an infected cell culture for 0 minutes, h) dsDNA exposed to an aliquot of the supernatant of an uninfected cell culture for 0 minutes, i) dsDNA exposed to H_2O for 0 minutes, j) ribosomal RNA exposed to an aliquot of *M. hyorhinis* extract for 30
20 minutes, k) ribosomal RNA exposed to an aliquot of the supernatant of an uninfected cell culture for 30 minutes, l) ribosomal RNA exposed to an aliquot of the supernatant of an uninfected cell culture for 30 minutes, m) ribosomal RNA exposed to exposed to H_2O 30 minutes, n) ribosomal RNA exposed to an aliquot of *M. hyorhinis* extract for 0 minutes, o) ribosomal RNA exposed to an aliquot of the
25 supernatant of an infected cell culture for 0 minutes, p) ribosomal RNA RNA

exposed to an aliquot of the supernatant of an un infected cell culture for 0 minutes, and q) ribosomal RNA exposed to an aliquot of H_2O for 0 minutes.

There was no detectable dsDNAse activity (lanes b & c) in the *M. hyorhinis* extract or *M. hyorhinis*-infected medium suggesting that there is insufficient urvB
5 activity for this enzyme to serve as a suitable basis for a diagnostic test for Mycoplasma contamination. Although dsRNAse activity was observed in the *M. hyorhinis* extract and *M. hyorhinis*-infected medium (lanes j and k), the uninfected tissue culture media control also had appreciable dsRNAse activity. This finding indicates that vacB activity would not be a suitable basis for a *Mycoplasma*
10 diagnostic test due to cross-reactivity from ribonucleases present in the culture medium of uninfected cells.

Table 5
Mycoplasma hyorhinis
Assays for Proteolytic Activity

Gene	Enzyme	Substrate	Condition	Vmax
lon	ATP-dependent protease	glt-ala-ala-phe-MCA	Buffer	55.8
			Uninfected medium	1460
			Infected medium	1802
			Mycoplasma positive control	3354
pepA	Leucine aminopeptidase	leu-MCA	Buffer[A]	2874
			Uninfected medium	454
			Infected medium	906
			Mycoplasma positive control	522
[A]This reading is probably artefactually high, and may indicate a bubble in the well. Subsequent experiments showed that Vmax in buffer was essentially zero.				
map	Methionine aminopeptidase	met-MCA	Buffer	19.4
			Uninfected medium	430
			Infected medium	627
			Mycoplasma positive control	733

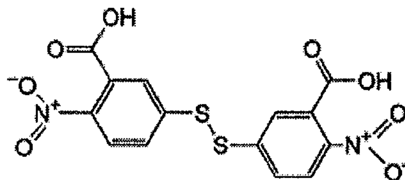
Further studies examined the suitability of the aminopeptidases map or lon or the protease pepA for use in a diagnostic test. These studies used several fluorogenic substrates consisting of small chain amino acids coupled to a methoxy coumarin
15 fluorescent probe (MCA, (7-methoxycoumarin-4-yl)acetyl). The results of the initial studies are provided in Table 5, above. The test conditions were "buffer," phosphate-buffered saline (PBS), "uninfected medium," medium from cell cultures not infected with *Mycoplasma*, "infected medium," medium from cell cultures infected with *Mycoplasma,* and a *Mycoplasma* positive control derived from a *Mycoplasma*

culture. In each case the uninfected media control had substantial background proteolytic activity, with pepA being the most candidate with a signal-to-noise ratio (S/N) of about 2, where S/N= (Vmax infected medium)/ (Vmax uninfected medium).

Example 2

5 **Detection of *Mycoplasma* Using trxB**

In contrast to the protease and the double stranded nuclease markers, thioredoxin reductase (trxB) had significant activity specific to tissue culture cells co-infected with *Mycoplasma hyorhinis*. In earlier studies, we demonstrated that Mycoplasma thioredoxin reductase activity was measured in infected culture medium 10 using the substrate DTNB (5,5'-Dithio-*bis*-(2-nitrobenzoic acid), also known as Ellman's reagent.



Other suitable fluorogenic thioredoxin reductase substrates have been reported in the literature.

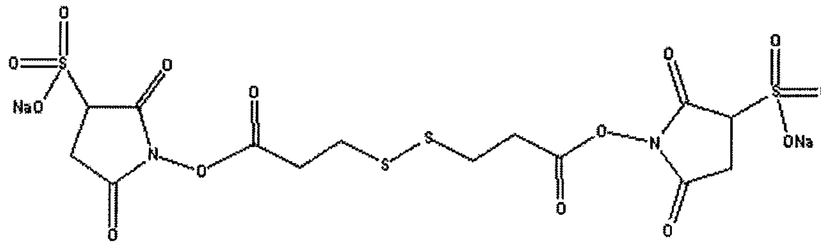
15 Since thioredoxin reductases are widely distributed in eukaryotes and prokaryotic cells, there is a possibility that thioredoxin reductases from other microbes may cross-react with this assay to give a false positive result. One possible approach would be to use gentle lysis buffers that disrupt *Mycoplasma* cells, which do not have a cell wall, but do not appreciably lyse other bacteria that possess a cell 20 wall. Studies demonstrated that there was no appreciable hydrolysis of DTNB by up to 10^6 CFU/ml *E. coli* or *S. aureus* (FIG. 9). This result indicates that the thioredoxin reductases of other gram-positive and gram-negative bacteria, represented by *E. coli* and *S. aureus*, are either minor enzymatic components or have a much lower specific activity than that of *M. hyorhinis*. This finding is consistent with the previous 25 demonstration that Mollicutes such as *Mycoplasma* have a very highly active thioredoxin reductase system (NTS) (0.09-0.25 SA units) in the presence of NADPH. This high NTS activity is presumed to be useful for *Mycoplasma* for the

detoxification of reactive oxygen compounds, since the Mollicutes have simple genomes that lack the genes encoding enzymes such as catalase, peroxidase and oxygen dismutase that function to remove H_2O_2 and other oxygen radicals in other bacteria (Gibson, D.G., et al., Complete chemical synthesis, assembly, and cloning of
5 a *Mycoplasma genitalium* genome. Science. 319(5867):1215-1220 (2008)).

Although initial attempts to detect trxB with DTNB were successful and the DTNB did not cross react with *Staphylococcus aureus* or *Escherichia coli* (FIG. 9), the sensitivity of the assay was unacceptable (>10^6 CFU/ml). Other fluorescent probes such as BODIPY® FL L-cystine and the 2',7'-difluoro-4'-(2-(5-((dimethyl
10 amino phenyl)azo) pyridyl)dithiopropionyl aminomethyl) fluorescein (abbreviated as DFDMAP-fluorescein) were studied to improve the sensitivity and the signal-to-noise ratio of the assay. Assays were performed with 40 mM Tris pH 7.2, 100 mM NaCl ± detergent.

A detergent lysis buffer procedure that would hydrolyze the simple
15 *Mycoplasma* cell membranes but not lyse the tissue culture cells was needed. Methyl-6-O-(N-heptylcarbamoyl)-α-D-glucopyranoside (HECAMEG) is a preferred detergent. We have found that 0.5% HECAMEG was sufficient to lyse *Mycoplasma* cells while not disrupting the membranes of the cells grown in the culture medium. In contrast, 0.25% Triton X-100®, 0.4% BriJ 35®, and digitonin resulted in either
20 significant increase in the background or loss in the true level of trxB activity. The activity of the fluorescent substrates is low compared to reduction with DTT suggesting that they may not be ideal or specific for trxB (FIG. 11). As expected, 0.1 mM NADPH enhanced the activity of trxB (FIG. 12). As a substrate for trxB, Bodipy ® FL L-cystine provided a signal that was 7500 times that of the buffer
25 control, however this signal was an artifact of the digitonin. DFDMAP fluorescein had a moderately improved detection level of 10,000-20,000 RFU at 10^7-10^8 CFU/ml.

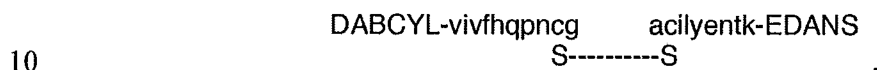
An alternative substrate to produce the thioredoxin reductase signal was to measure the release of horseradish peroxidase (HRP) from a chromatography bead
30 tethered with the heterobifunctional crosslinking reagent 3,3′-dithiobis [sulfosuccinimidylpropionate] (DTSSP).



The HRP-DTSSP-BEAD conjugate is shown schematically in FIG. 10A.

It was expected that frxB would be able to reduce the disulfide bridge of DTSSP, thereby releasing the HRP to react with its substrate tetramethylbenzidine (TMB), and in the presence of Mycoplasma, produce a blue color. However, it was found that the HRP-DTSSP-BEAD conjugate also cross-reacted to the tissue culture uninfected medium sample.

Other alternative substrates can use fluorescence energy transfer (FRET) with a disulfide bridge between EDANS and DABSYL, as shown below:



Alternatively, DFDMAP and BODIPY® FL L-cysteine moieties could be coupled to the thioredoxin peptide or the central Gly-Ala residues to enhance the specificity of these fluorescent probes. Initial studies of these approaches have not shown improved sensitivity or reduction of background of the uninfected media control.

Example 3
Detection of *Mycoplasma* Using Proteases

The proteases pepA, lon, and map were evaluated for use in the detection of *Mycoplasma*. pepA and lon were found to be expressed at a higher level than map based on RT-qPCR results (FIG. 5 and FIG. 6). Arginine amino peptidase activity has also been reported in *Mycoplasma* species.

A presently preferred substrate is leu-MCA that had significant activity above the uninfected media control under the gentle conditions used to lyse the *Mycoplasma* (0.05 % HECAMEG, 1 mM $MgCl_2$, 100mM NaCl, 40 mM Tris buffer,

pH 8.5). The MCA-Leu substrate produces a signal level of 500 mOD in 30 minutes with *M. hyorhinis*, while *M. hyorhinis* has weak activity for arg-MCA. Results are provided in Table 6, below.

Table 6
Vmax Measured Using MCA Labeled Substrates Under Different Conditions

	Vmax				
Condition	**Arg-MCA**	**Leu-MCA**	**Met-MCA**	**Leu/Met**	**All 3**
Buffer	0	236	125	0	193
Medium	676	2118	1731	2024	1443
Uninfected Medium	29034	17422	19004	14937	25770
Infected Medium	60903	415190	302082	373456	271170
Signal/Noise	2.10	23.83	15.90	25.00	10.52

5 In further studies, we determined that the background of the uninfected cells could be reduced even further by adjusting the pH, with an optimum at pH 8.5. FIG. 13 is a graph showing the effect of acid pH levels on the leu-MCA assay. FIG. 14 is a graph showing the effect of basic pH levels on the leu-MCA assay.

Detergent lysis of *M. hyorhinis* with HECAMEG gives better signal than
10 sonication. Manganese or magnesium also improves the signal to noise ratio. In the studies on the effects of divalent cations, 1 mM $MgCl_2$ of the standard mixture was replaced by 1 mM $MnCl_2$, 1 mM $MgSO_4$ or 1 mM EDTA, as indicated in Table 7, below.

Table 7
Effect of Divalent Cations
Vmax, MCA-Leu Substrate

Condition	**EDTA**	**$MnCl_2$**	**$MgSO_4$**	**$MnCl_2$**
Buffer	0	0	483	84
Medium	1710	1703	-	-
Uninfected	3873	3819	5125	3032
Infected	45241	89467	90365	95120
Signal/Noise	11.68	23.43	17.63	31.37

15 Using the leu-MCA substrate, a sensitivity of 10^5 CFU/ ml can be achieved (FIG. 15). Further increases in sensitivity may be obtained using a bis-leu rhodamine 110 labeled substrate, or using luciferase-leucine-bead complex, as shown schematically in FIG. 10B.

The sensitivity of the present assay using the leu-MCA substrate was
20 compared to two commercially available *Mycoplasma* detection tests: a *Mycoplasma*

PCR ELISA test (Roche cat # 11 663 925 910), and the MycoAlert Sample Kit, (Lonza cat# LT37-618). The results are presented in Table 8, below.

Table 8
Test Sensitivity Comparison

	Present leu-MCA Assay	**PCR ELISA test**	**MycoAlert Sample Kit**
***M. hyorhinis* (CFU/ml)**	10^5	80	10^6
Time Required For Test	20 minutes	2 Days	20 minutes

The cross reactivity of the present assay using the leu-MCA substrate was evaluated with the following microorganisms: two bacteria (*S. aureus* , *E. coli*) and three species of fungus(*Candida albicans, Aspergillis niger, Saccharomyces cerevisiae*) Only in the case of a completely turbid cultures was there weak low cross reactivity with the present assay using the leu-MCA substrate.

CLAIMS

What is claimed:

1. A biosensor for detecting the presence or absence of mycoplasma contamination comprising a support and a detectably labeled substrate for an hydrolytic enzyme produced and/or secreted by a mycoplasma, wherein the substrate is attached to a support.

2. The biosensor of claim 1 wherein the enzyme is selected from the group consisting of proteases, reductases and nucleases.

3. The biosensor of claim 1 wherein the enzyme is selected from the group consisting of the gene product of *pepA1*, MCAP_0195, MCAP_0267, MCAP_0341, MCAP_0509, MCAP_0675 and mixtures thereof.

4. The biosensor of claim 1 wherein the enzyme is a reductase and the substrate is reactive black 5, 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), BODIPY®FL L-cystine, or 2',7'-difluoro-4'-(2-(5-((dimethyl amino phenyl)azo) pyridyl)dithiopropionyl aminomethyl) fluorescein (DFDMAP-fluorescein).

5. The biosensor of claim 4 wherein the reductase is selected from the group consisting of the gene product of *nrdE* (such as MCAP_0101), MCAP_0427, *trxB* (thioredoxin reductase, such as MCAP_0779 or MHP7448_0098) , MCAP_0858 and mixtures thereof.

6. The biosensor of claim 1 wherein the mycoplasma-specific enzyme is a nuclease and the detectable label is an acetoxymethyl ester or maleimide derivative of blue dye number 1 coupled to an aminollyl-dNTP labeled nucleic acid substrate of the mycoplasma-specific nuclease.

7. The biosensor of claim 6 wherein the nuclease is selected from the group consisting of the gene product of the 5'-3' exonuclease encoded by MCAP_0047 or MHP7448_0581, the gene product of *nfo* (such as MCAP_0060 or MHP7448_0062), *vacB* (such as MCAP_0097 or MHP7448_0037), *uvrC* (such as MCAP_0252 or MHP7448_0066), *mc* (ribonuclease III, such as MCAP_0492 or MHP7448_0398), MCAP_0768, *uvrB* (such as MCAP_0773 or MHP7448_0648), *uvrA* (such as MCAP_0774 or MHP7448_0091) and mixtures thereof.

8. The biosensor of claim 1 wherein the support is a reagent container, a bead, a polymer film, a culture medium container or a cell culture container.

9. A method of detecting mycoplasma contamination of a cell culture

comprising the steps of providing a cell-permeable detectable label coupled to a cell-impermeant carrier in the culture medium wherein cleavage of the detectable label by a mycoplasma-specific enzyme is followed by uptake of the detectable label into cells; and detecting labeled cells, thereby detecting mycoplasma contamination of the
5 cell culture.

10. The method of claim 9 wherein the mycoplasma-specific enzyme is a protease and the detectable label is leucine-(7-methoxycoumarin-4-yl)acetyl (leu-MCA), arginine-(7-methoxycoumarin-4-yl)acetyl (arg-MCA), methionine-(7-methoxycoumarin-4-yl)acetyl (met-MCA), or an acetoxymethyl ester.

10 11. The method of claim 10 wherein the protease is selected from the group consisting of *pepA1*(MCAP_0157), *pepA2* (MCAP_0195), *pepA* (leucyl aminopeptidase, such as MHP7448_0464), MCAP_0267 (metalloendopeptidase), *pepP* (Xaa-Pro endopeptidase, such as MCAP_0341 or MHP7448_0649), MCAP_0509, mapP (methionine amino peptidase, MCAP_0675 or
15 MHP7448_0173), and mixtures thereof.

12. The method of claim 9 wherein the mycoplasma-specific enzyme is a nuclease and the detectable label is an acetoxymethyl ester of derivative of blue dye number 1 coupled to a nucleic acid substrate of the mycoplasma-specific nuclease

13. The method of claim 12 wherein the nuclease is selected from the group
20 consisting of the 5'-3' exonuclease encoded by MCAP_0047 or MHP7448_0581, the gene product of *nfo* (such as MCAP_0060 or MHP7448_0062), *vacB* (such as MCAP_0097 or MHP7448_0037), *uvrC* (such as MCAP_0252 or MHP7448_0066), *rnc* (ribonuclease III, such as MCAP_0492 or MHP7448_0398), MCAP_0768, *uvrB* (such as MCAP_0773 or MHP7448_0648), *uvrA* (such as MCAP_0774 or
25 MHP7448_0091) and mixtures thereof.

14. A method of determining the presence or absence of mycoplasma in a sample, comprising the steps of:

contacting the sample with a detectably labeled substrate for an enzyme produced and/or secreted by a mycoplasma under conditions that result in the
30 modification of the substrate by the enzyme; and

detecting the modification or the absence of the modification of the substrate wherein modification of the substrate indicates the presence of mycoplasma in the sample, and wherein the absence of modification of the substrate indicates the

absence of mycoplasma in the sample.

15. The method of claim 14 wherein the level of the detectable label is quantitatively related to the presence or amount of mycoplasma in the sample.

16. The method of claim 14 wherein the enzyme is a hydrolytic enzyme
5 selected from a protease, a nuclease or a reductase.

17. The method of claim 14 wherein the enzyme is selected from the group consisting of the gene product of *pepA1*(MCAP_0157), *pepA2* (MCAP_0195), *pepA* (leucyl aminopeptidase, such as MHP7448_0464), MCAP_0267 (metalloendopeptidase), *pepP* (Xaa-Pro endopeptidase, such as MCAP_0341 or
10 MHP7448_0649), MCAP_0509, mapP (methionine amino peptidase, MCAP_0675 or MHP7448_0173), and mixtures thereof.

18. The method of claim 14 wherein the enzyme is selected from the group consisting of the 5'-3' exonuclease encoded by MCAP_0047 or MHP7448_0581, the gene product of *nfo* (such as MCAP_0060 or MHP7448_0062), *vacB* (such as
15 MCAP_0097 or MHP7448_0037), *uvrC* (such as MCAP_0252 or MHP7448_0066), *mc* (ribonuclease III, such as MCAP_0492 or MHP7448_0398), MCAP_0768, *uvrB* (such as MCAP_0773 or MHP7448_0648), *uvrA* (such as MCAP_0774 or MHP7448_0091) and mixtures thereof.

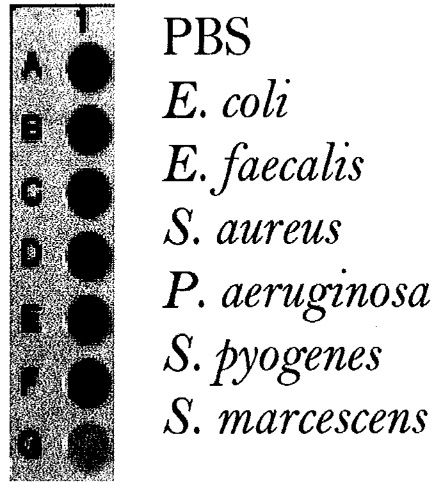


Fig. 1

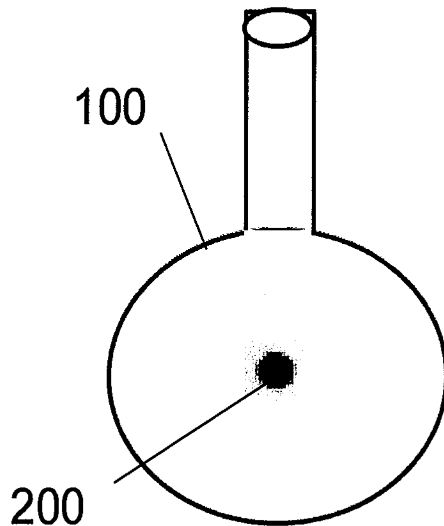


Fig. 2A

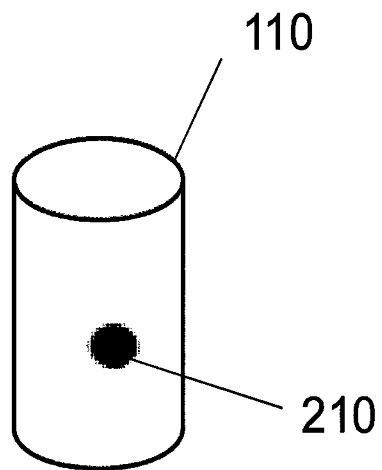


Fig. 2B

Fig. 3A

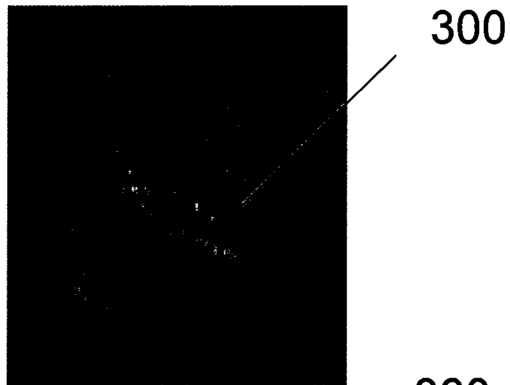
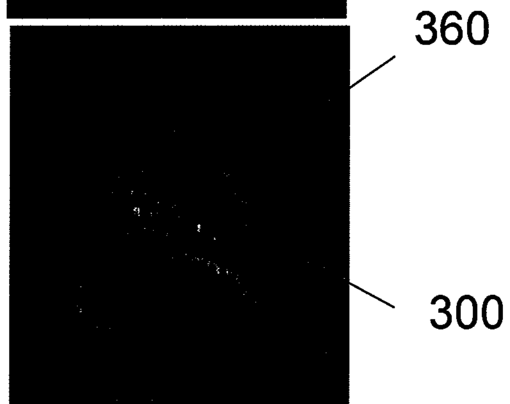


Fig. 3B



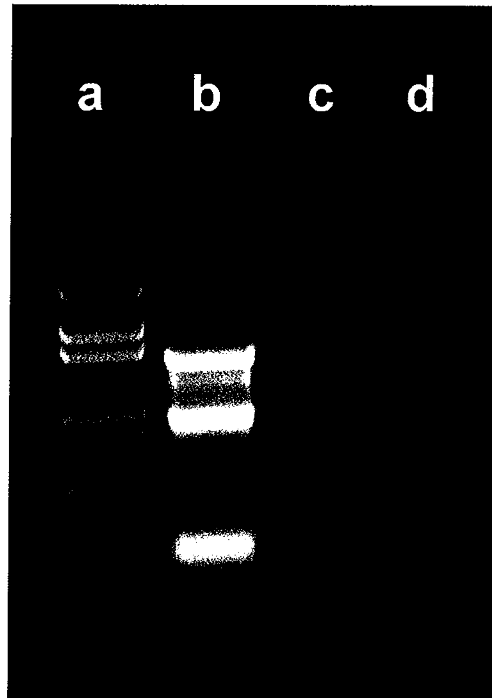


Fig. 4A

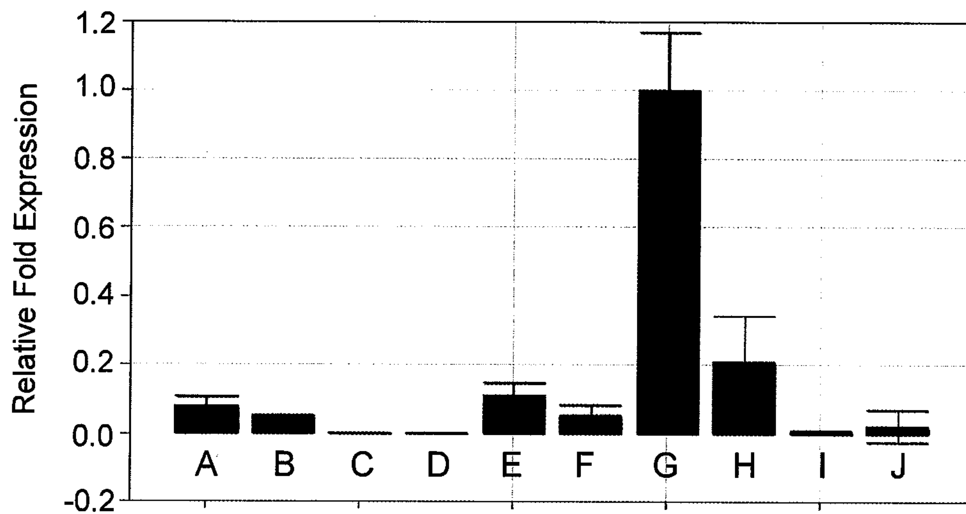


Fig. 4B

Fig. 5

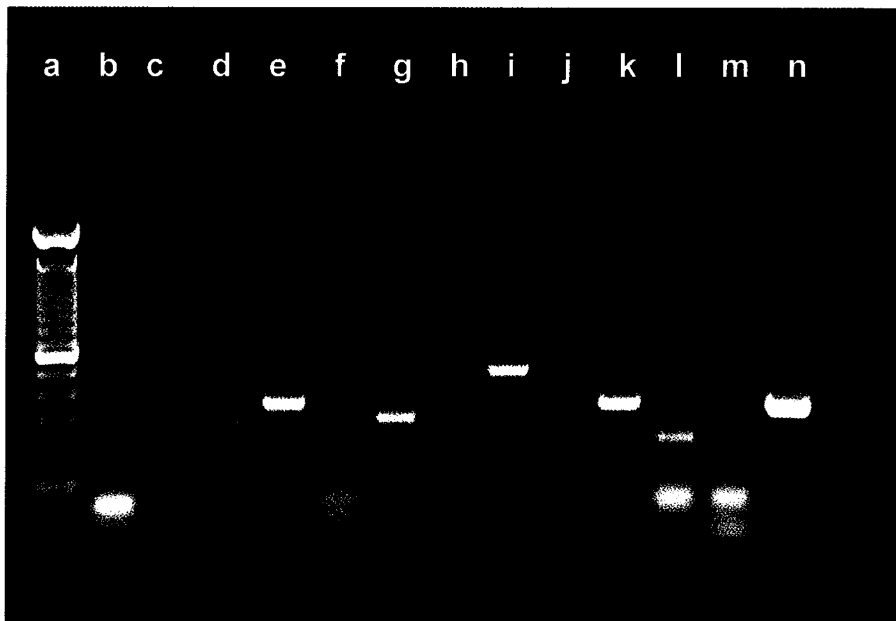


Fig. 6

Fig. 7

Fig. 8

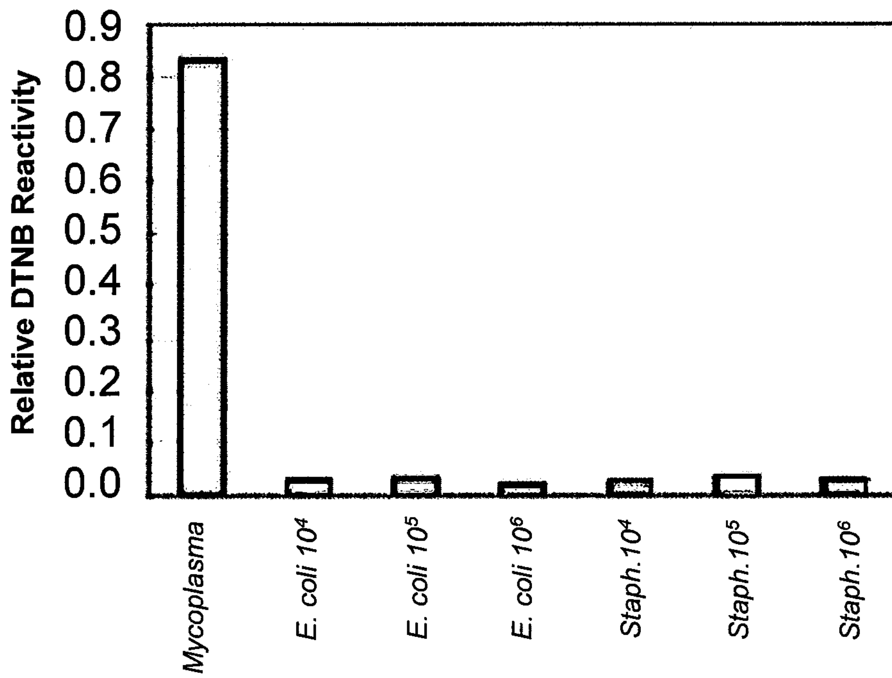


Fig. 9

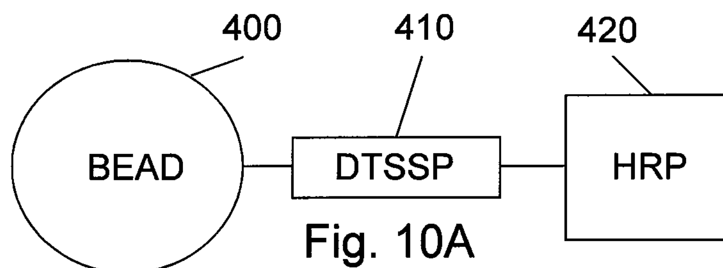


Fig. 10A

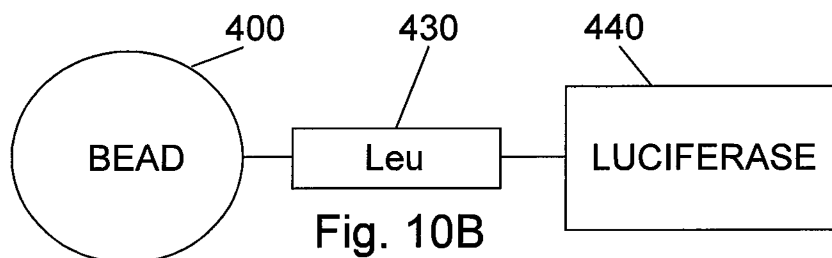


Fig. 10B

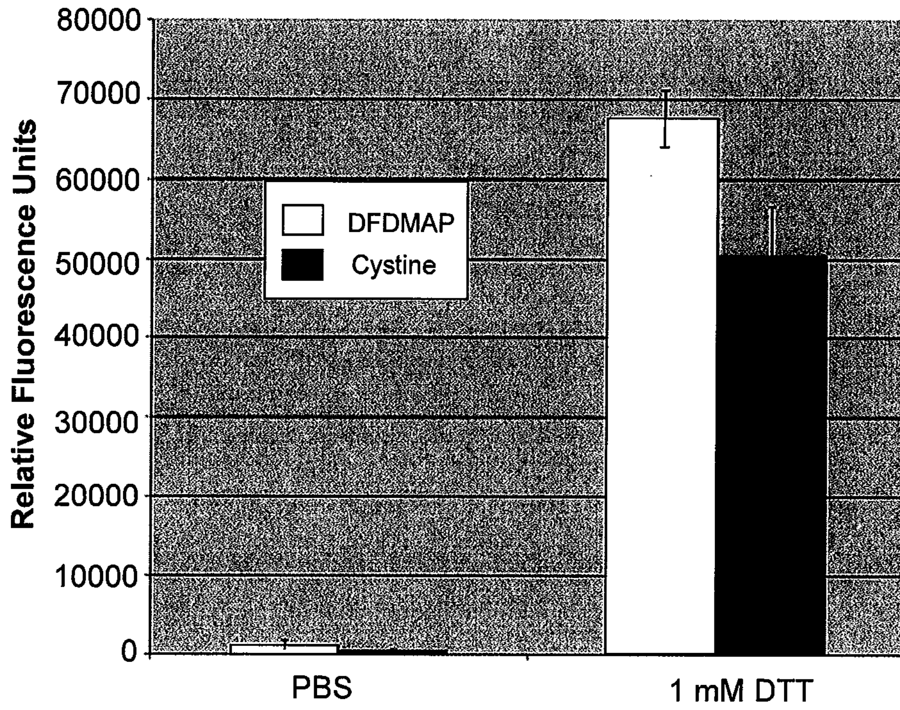


Fig. 11

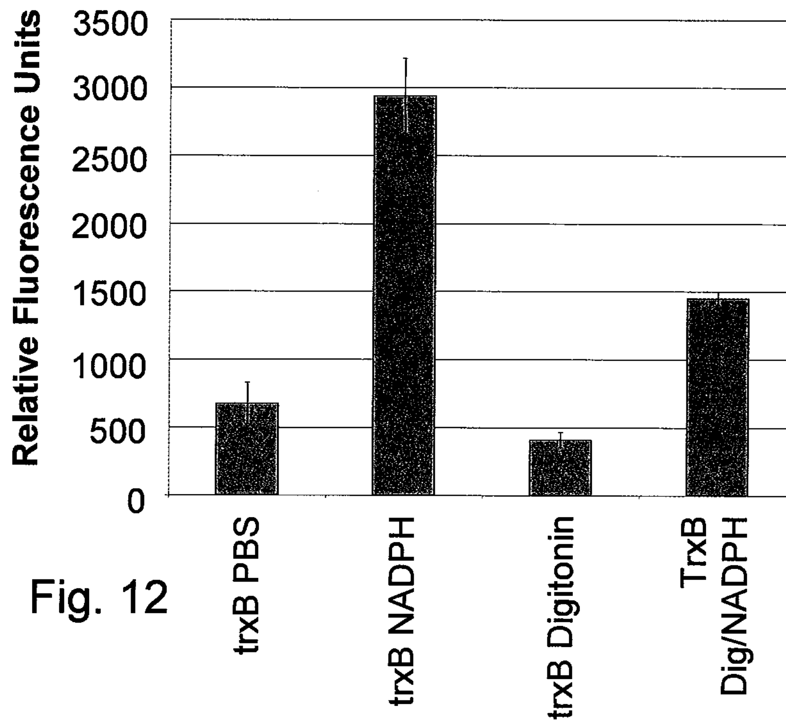


Fig. 12

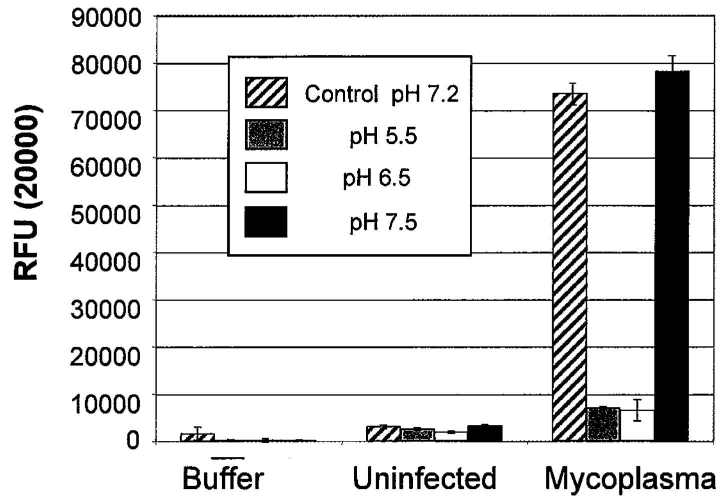


Fig. 13

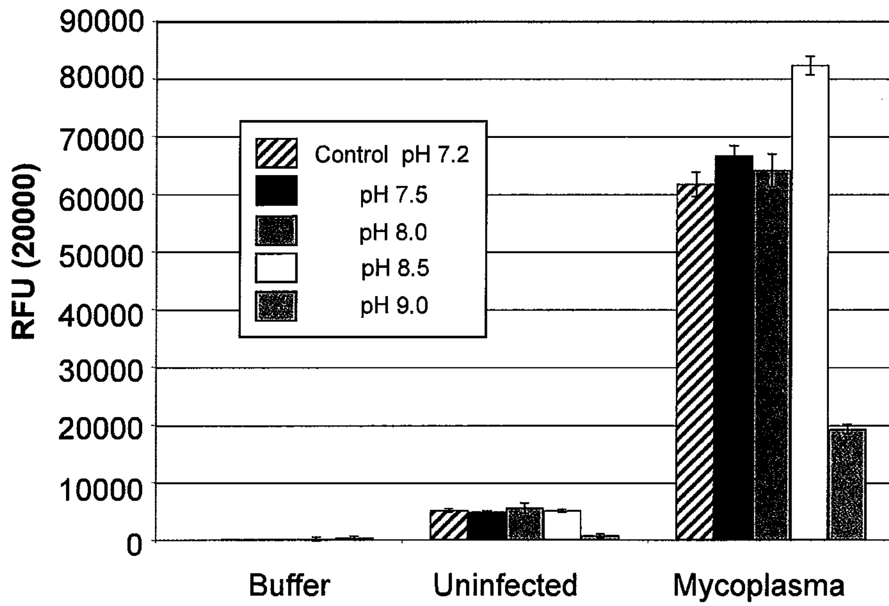


Fig. 14

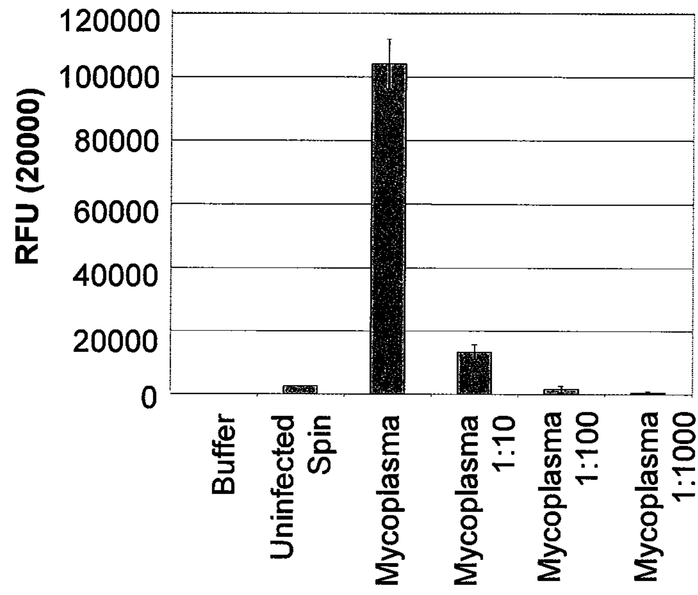


Fig. 15