MycoProbe™

Mycoplasma Detection Kit

Catalog Number CUL001B

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
PRINCIPLE OF THE ASSAY	2
MYCOPLASMA SCREENING GUIDELINES	2
LIMITATIONS OF THE PROCEDURE	2
TECHNICAL HINTS	3
PRECAUTIONS	3
MATERIALS PROVIDED & STORAGE CONDITIONS	4
OTHER SUPPLIES REQUIRED	5
REAGENT PREPARATION	5
SAMPLE PREPARATION	6
ASSAY PROCEDURE	7
CALCULATION OF RESULTS	8
TYPICAL DATA	8
SENSITIVITY	8
CROSS-REACTIVITY	9
TROUBLESHOOTING GUIDE	10
REFERENCES	10

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Mycoplasma is a term used to denote any species included in the class *Mollicutes*. They are the smallest (0.2-2.0 µm in diameter) and simplest free-living parasitic organisms known (1). Mycoplasma lack a peptidoglycan cell wall and possess the smallest recorded genome in living cells (580-2,220 kilobases) (1, 2). Mycoplasma are parasites of many animal species and typically exhibit host and tissue specificity. In humans, *Mycoplasma (M.) pneumoniae* is the respiratory pathogen responsible for atypical pneumonia and mycoplasma are frequently isolated from patients with immunodeficiencies associated with disease states (1, 2).

Mycoplasma are common contaminants of eukaryotic cell cultures and are known to alter the phenotypic characteristics of host cell lines (1, 3, 4). The published incidence of mycoplasma infected cell cultures has ranged from 4-92% (3). Of the 18 most common species recognized as culture contaminants, *M. orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans*, and *Acholeplasma (A.) laidlawii* are the most frequently isolated, representing 80-90% of isolates (5). The small size of mycoplasma allows them to pass through 0.45 µm filters. Mycoplasma are resistant to common antibiotics such as penicillin and streptomycin. Mycoplasma contamination typically does not produce visible changes in cell culture medium despite the fact that they can reach titers of 10^8/mL. Sources of mycoplasma contamination include laboratory personnel, reagents, and mycoplasma-contaminated cell lines.

Mycoplasma contamination is detected by a number of methods. Microbiological culture is generally considered the most sensitive method for mycoplasma screening and is commonly used as the reference method for the evaluation of new mycoplasma detection techniques. However, culturing mycoplasma can take two to four weeks and cannot detect fastidious mycoplasma (6). Culturing mycoplasma requires special growth conditions and is generally restricted to specialized laboratories. Microscopic visualization of mycoplasma attached to host cells using fluorescent DNA staining displays extracellular fluorescence in addition to extranuclear fluorescence (7). This method is efficient for mycoplasma screening and particularly for detection of *M. hyorhinis* strains that cannot be cultivated on microbiological media. However, fluorescent staining cannot detect mycoplasma species that cyto-absorb poorly, and this method requires expertise for accurate interpretation of results. Enzyme-linked immunosorbent assays measuring mycoplasma-specific cell surface antigens have been described (8, 9) but typically lack sensitivity. A number of polymerase chain reaction (PCR)-based methods have been described for detection of mycoplasma (9-16) that achieve high sensitivity and may be amenable to species identification. Unfortunately, PCR-based detection of mycoplasma can be prone to false positive results due to amplicon contamination and false negative results due to use of excess sample. Mycoplasma screening methods utilizing selective biochemical activity have also been exploited (1, 17) but may give inconsistent results when comparing different cell lines.

The MycoProbe Mycoplasma Detection Assay is a 4.5 hour colorimetric microplate assay designed for routine screening of mycoplasma contamination in cultured cells. This assay detects Mycoplasma 16S ribosomal RNA (rRNA) using a colorimetric signal amplification system with sensitivity comparable to PCR without the susceptibility to common problems encountered with PCR-based mycoplasma detection kits. The MycoProbe Mycoplasma Detection Kit detects the most common mycoplasma cell culture contaminants, including *M. hyorhinis*, *M. arginini*, *M. fermentans*, *M. orale*, *M. Pirum*, *M. hominis*, *M. salivarium*, and *A. laidlawii*. These eight species account for approximately 95% of all mycoplasma contaminations. This kit can be used to detect mycoplasma contamination using either cell culture supernates or cultured cell pellets. Samples are prepared by dilution in the lysis buffer provided in the kit. Passage of cultured cells in antibiotic-free media is not required. The strip-well microplate format is amenable to a small number of samples as well as applications requiring high sample throughput. The positive control included in this kit is a synthetic DNA oligonucleotide.

PRINCIPLE OF THE ASSAY

Cell culture supernate or cultured cell pellet samples are lysed. Samples are hybridized in a microplate with biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes that are targeted to the 16S ribosomal RNA (rRNA) of the eight most common mycoplasma contaminants. The hybridization solution is then transferred to a streptavidin-coated microplate and the rRNA/probe hybrid is captured. Following a wash to remove unbound material, an anti-digoxigenin alkaline phosphatase conjugate is added. After washing away unbound conjugate, a substrate solution is added. An amplifier solution is then added and color develops in proportion to the amount of mycoplasma rRNA in the original sample. Color development is stopped and the intensity of the color is measured using a standard colorimetric plate reader.

MYCOPLASMA SCREENING GUIDELINES

Routine screening of cultured cell lines for mycoplasma contamination is considered a prudent laboratory practice. It is recommended that screening for mycoplasma contamination be done at least quarterly, and more frequently if there is a history of mycoplasma infection or if a large number of cultures from outside laboratories are handled (4). Cell lines introduced to a cell culture facility, even from reputable sources, should be quarantined until tested for mycoplasma contamination. Recently thawed cell lines should be tested for mycoplasma contamination if not tested prior to freezing.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Use ribonuclease-free (RNase-free) reagents and supplies when running this assay.
- Do not mix or substitute reagents with those from other sources or lots.
- The kit should not be used beyond the expiration date on the kit label.
- Any variation in diluents, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can alter assay performance.
- This assay detects the following species: *M. hyorhinis*, *M. arginini*, *M. fermentans*, *M. orale*, *M. pirum*, *M. hominis*, *M. salivarium*, and *A. laidlawii*.
- A negative result does not indicate that other species of mycoplasma are absent. Additionally, mycoplasma may be present at levels below the detectable limits of this kit.
- Cell cultures that are visibly contaminated (*i.e.,* turbidity and yellow media) are probably due to *E. coli* or fungal infection and should not be tested. Visibly contaminated cell cultures should be discarded and fresh cultures should be started from frozen stock.
- This assay cannot be used for mycoplasma species identification.

TECHNICAL HINTS

- Avoid RNase contamination during reagent and sample preparation and while running the assay. The use of gloves, mask and a labcoat with tight-fitting cuffs is strongly recommended. RNase contamination will cause poor precision and/or depress the signal in sporadic wells.
- Careful washing of the microplate is essential to minimize non-specific binding of the conjugate. Non-specific binding may result in false positive results. When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay performance.
- Avoid contact of the conjugate with phosphate-based wash buffers and other sources of inorganic phosphate.
- Addition of the Substrate Solution or the Stop Solution will not result in a color change.
- Addition of the Amplifier Solution will result in a color change (colorless to shades of maroon).
- Amplifier Solution and Stop Solution should be added to the plate in the same order as the Substrate Solution.
- Warm reagents to room temperature and mix to homogeneity prior to pipetting.
- Avoid foaming when mixing reagents.
- To avoid cross-contamination, use a sterile pipet tip for each addition of control, sample or reagent. Also, use separate reservoirs for each reagent.
- Proper adhesion of plate sealers during incubation steps is necessary to prevent cross-contamination and/or sample evaporation.
- Cover plate with a new plate sealer before each incubation.

PRECAUTIONS

The Wash Buffer supplied in this kit contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution.

When handling cell culture samples, appropriate precautions should be taken to prevent exposure to mycoplasma and other hazardous biological agents.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Streptavidin Coated Microplate	890649	96 well polystyrene microplate (12 strips of 8 wells) coated with streptavidin.	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 3 months at 2-8 °C.*
Hybridization Microplate	895494	96 well polystyrene microplate.	May be stored for up to 3 months at 2-8 °C.*
Cell Lysis Diluent Concentrate	895540	2 vials (1.7 mL/vial) of a 10-fold concentrated solution.	
Sample Diluent	895284	2 vials (21 mL/vial) of a buffered protein solution with preservatives.	
Anti-Digoxigenin-AP Conjugate	890650	21 mL of a polyclonal antibody against digoxigenin conjugated to alkaline phosphatase with preservatives.	
Capture Probes	893000	1.1 mL of a six-fold concentrated stock solution.	
Detection Probes	893001	1.1 mL of a six-fold concentrated stock solution.	
Positive Control	892896	1.1 mL of a solution containing a synthetic DNA oligonucleotide.	
Wash Buffer Concentrate	895285	100 mL of a 10-fold concentrated solution with preservatives.	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Substrate	895884	1 vial of lyophilized NADPH with stabilizers.	Store in an upright position for up to 3 months at ≤ -80 °C.*
Substrate Diluent	895885	7 mL of a buffered solution with stabilizers.	
Amplifier	895886	1 vial of lyophilized amplifier enzymes with stabilizers.	
Amplifier Diluent	895887	7 mL of a buffered solution containing INT-violet with stabilizers.	
Float Collar	720045	Microplate float collar for water bath.	
Plate Sealers	N/A	12 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 490 nm with the correction wavelength set at 650 nm or 690 nm
- Pipettes and pipette tips
- Deionized water, RNase-free
- Squirt bottle or manifold dispenser
- 1000 mL graduated cylinder for preparation of Wash Buffer
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- 65 ± 1 °C water bath
- Vortex mixer
- Gloves and mask
- Tubes for sample preparation.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Cell Lysis Diluent (diluted 1:10) - Add 3.4 mL of Cell Lysis Diluent Concentrate to 30.6 mL of Sample Diluent to prepare 34 mL of Cell Lysis Diluent (diluted 1:10).

Probes (1X) - To assay an entire 96 well plate, add 1.0 mL of the Capture Probes and 1.0 mL of the Detection Probes to 4.0 mL of Sample Diluent. Adjust the volumes accordingly if assaying fewer than 96 wells. Make a fresh dilution of probes before running each assay.

Wash Buffer (1X) - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 100 mL of Wash Buffer Concentrate to 900 mL of RNase-free deionized water to prepare 1000 mL of Wash Buffer (1X).

Substrate Solution - Reconstitute the lyophilized Substrate with 6.0 mL of Substrate Diluent at least 10 minutes before use. Re-stopper and re-cap the vial and mix thoroughly.
Avoid contamination.

Amplifier Solution - Reconstitute the lyophilized Amplifier with 6.0 mL of Amplifier Diluent at least 10 minutes before use. Re-stopper and re-cap the vial and mix thoroughly.
Avoid contamination.

Positive Control - Ready to use; no preparation needed.

Negative Control - Use Sample Diluent as the negative control.

SAMPLE PREPARATION

Cell culture supernates (15 µL/well) or cell pellets (4,700 to 19,000 cells/well) are needed as samples in this assay. Passage of cultured cells in the absence of antibiotics (*i.e.,* penicillin and streptomycin) is not needed.

Cell cultures that are visibly contaminated (*i.e.,* turbidity and/or yellow media) are probably due to *E. coli* or fungal infection and should not be tested. Visibly contaminated cell cultures should be discarded and fresh cultures should be started from frozen stock. Mycoplasma contamination of cell cultures is typically not visible, even at high concentrations of mycoplasma (10^7-10^8 mycoplasma/mL).

CELL LYSATE SAMPLES

Cell pellet samples should be stored on ice until lysed with Cell Lysis Diluent (diluted 1:10) or stored at ≤ -20 °C for use at a later time. Prepare cell lysate samples using the following procedure:

1. Add 400 µL of Cell Lysis Diluent (diluted 1:10) to a cell pellet containing 5 x 10^5 cells, adherent or non-adherent, to obtain a final concentration of 1.25 x 10^6 cells/mL.
2. Pipet the cells up and down several times until they are resuspended.
3. Vortex for 15-20 seconds.
4. Dilute the cell lysate 10- to 40-fold with Cell Lysis Diluent (diluted 1:10) to obtain a final concentration of 3.125 x 10^4 to 1.25 x 10^5 cells/mL, respectively. For example, add 33 µL of cell lysate from Step 3 to 297 µL of Cell Lysis Diluent (diluted 1:10) to obtain a final concentration of 1.25 x 10^5 cells/mL. This sample dilution will provide sufficient volume to perform the assay in duplicate and provide excess for pipetting.
5. Assay immediately, cell lysates can be stored at ≤ -20 °C for use at a later time. Frozen cell lysate samples should be thawed on ice prior to use. Avoid multiple freeze-thaw cycles.

CELL CULTURE SUPERNATE SAMPLES

Cell culture supernate samples should be stored on ice prior to use or stored at ≤ -20 °C for use at a later time. Prepare cell culture supernate samples using the following procedure.

1. Dilute all cell culture supernate samples 10-fold in Cell Lysis Diluent (diluted 1:10). For example, add 33 µL of sample to 297 µL of Cell Lysis Diluent (diluted 1:10). This sample dilution will provide sufficient volume to perform the assay in duplicate and provide excess for pipetting.
2. Vortex for 15-20 seconds.
3. Assay immediately, or the diluted cell culture supernates can be stored at ≤ -20 °C for use at a later time. Frozen cell culture supernates should be thawed on ice prior to use. Avoid multiple freeze-thaw cycles.

ASSAY PROCEDURE

Wear gloves, mask, and a labcoat with tight-fitting cuffs during all assay steps. Bring all reagents to room temperature before use. Assaying all samples and controls in duplicate is recommended.

1. Prepare reagents and samples as instructed.
2. Wash the Hybridization Plate 2 times with Wash Buffer. Remove excess Wash Buffer by decanting or aspirating. Invert the plate and blot it against clean paper towels.
3. Add 50 µL of diluted Probes to the designated wells.
4. Add 150 µL of Positive Control, Sample Diluent (Negative Control), or sample to the designated wells. Cover with a plate sealer.
5. Apply the float collar to the Hybridization Plate and incubate the plate for 60 minutes in a 65 °C water bath.
6. Remove unused microplate strips from the Streptavidin Plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
7. Wash the Streptavidin Plate 2 times with Wash Buffer and remove excess Wash Buffer as described in Step 2.
8. Transfer 150 µL from each well of the Hybridization Plate to the washed Streptavidin Plate and apply a new plate sealer.
9. Incubate for 60 minutes at room temperature on a horizontal orbital shaker set at 500 ± 50 rpm.
10. Wash the Streptavidin Plate 4 times with Wash Buffer and remove excess Wash Buffer.
11. Add 200 µL of Anti-digoxigenin Conjugate to each well and cover with a new plate sealer.
12. Incubate for 60 minutes on the shaker at room temperature.
13. Wash the Streptavidin Plate 6 times with Wash Buffer and remove excess Wash Buffer.
14. Add 50 µL of Substrate Solution to each well and cover with a new plate sealer.
15. Incubate for 60 minutes on the shaker at room temperature. **Do not wash.**
16. Add 50 µL of Amplifier Solution to each well and cover with a new plate sealer.
17. Incubate for 30 minutes on the shaker at room temperature. **Do not wash.**
18. Add 50 µL of Stop Solution to each well.
19. Determine the optical density (OD) of each well within 30 minutes, using a microplate reader set to 490 nm. If wavelength correction is available, set to 650 nm or 690 nm. If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Determine the average of the duplicate optical density (O.D.) readings for each control and sample. Subtract the average negative control O.D. value from all average O.D. values. Negative results may have O.D. values below 0.0 after subtraction of the background.

The calculated positive control O.D. value should be ≥ 1.5. The results from calculated sample O.D. values can be obtained using the following table:

Calculated O.D. Values	Result	Interpretation
< 0.05	Negative	No mycoplasma detected.
0.05-0.10	Inconclusive	Sample is suspect for mycoplasma. Continue to culture for an additional 2-3 days and repeat the test. If sample gives a similar O.D., then no mycoplasma are detected.
> 0.10	Positive	Mycoplasma detected.

TYPICAL DATA

Cell Line	Sample Type	Dilution or Cells/mL	Calculated O.D. Values	Result
CTLL-2 mouse cytotoxic T cells	Supernate	1:10	1.025	Positive
BaF3 mouse pro-B cells	Supernate	1:10	0.183	Positive
HepG2 human hepatocellular carcinoma cells	Supernate	1:10	0.000	Negative
A431 human epithelial carcinoma cells	Cell Lysate	1.2×10^5	1.042	Positive
K562 human chronic myelogenous leukemia cells	Cell Lysate	1.2×10^5	1.912	Positive
K562 human chronic myelogenous leukemia cells	Cell Lysate	1.2×10^5	0.005	Negative

SENSITIVITY

This assay detects the following mycoplasma species at the levels shown below. To determine the minimal amount detectable, each species was grown in pure culture, serially diluted, and tested. The first five species listed account for 80-85% of mycoplasma contamination in cultured animal cells. CFU=Colony Forming Units

Mycoplasma Species	Sensitivity (CFU/well)
M. arginini	15
M. orale	65
M. fermentans	75
A. laidlawii	240
M. hyorhinis	560
M. pirum	30
M. hominis	225
M. salivarium	2500

CROSS-REACTIVITY

Assay cross-reactivity was tested using 1 x 10^4 CFU/well of microbes and 1.2 x 10^5 cells/well of the mammalian cell lines listed below. This assay recognizes the eight mycoplasma species listed in the sensitivity table and two closely related prokaryotic species (based on 16S rRNA homology), *Ureaplasma urealyticum* and *Lactobacillus casei*. *U. urealyticum* is a mycoplasma associated with human urogenital diseases and is not found as a cell culture contaminant. *U. urealyticum* was detectable using as few as 2.7 x 10^3 CFU/well. *L. casei* is a lactic acid fermenting bacteria that is not found as a cell culture contaminant. No significant cross-reactivity was observed for other microbes in the panel. Mammalian cells did not show cross-reactivity or interference when tested using the recommended concentration range.

Organism	Classification	Result
Ureaplasma urealyticum	Mollicute (mycoplasma)	Positive
Lactobacillus casei	Gram Positive Bacteria	Positive
Bacillus subtilis	Gram Positive Bacteria	Negative
Escherichia coli	Gram Negative Bacteria	Negative
Torulopsis candida	Yeast	Negative
Cryptococcus albidus	Yeast	Negative
Geotrichum sp.	Mold	Negative
Penicillium sp.	Mold	Negative
Cladosporium sp.	Mold	Negative
Human	K562	Negative
Mouse	EL-4	Negative
Rat	NR-8383	Negative

TROUBLESHOOTING GUIDE

OBSERVATION	PROBLEM	CORRECTIVE ACTION
High background level	Insufficient washing	Wash per protocol being sure to remove all Wash Buffer from wells before addition of next component.
	Contamination with alkaline phosphatase	Keep work area clean and free of alkaline phosphatase. It may be necessary to wear a mask when preparing the reagents and performing the assay since alkaline phosphatase is detectable in saliva.
Poor precision	Plate not washed before use	Wash per protocol.
	RNase contamination	Use RNase-free technique.
	Pipetting error	Use a new pipette tip for each pipetting step and use proper technique.
No signal for positive control	Component or step omitted	Read protocol thoroughly before repeating the assay.

REFERENCES

1. Hay, R.J. *et al.* (1989) Nature **339**:487.
2. Razin, S. *et al.* (1998) Microbiol. Mol. Biol. Rev. **62**:1094.
3. McGarrity, G.J. *et al.* (1984) In Vitro **20**:1.
4. McGarrity, G.J. and H. Kotani (1985) in *The Mycoplasma: Cell Culture Mycoplasmas*, Volume IV, Razin, S. and M.F. Barile eds., Academic Press, New York, pp. 353-390.
5. McGarrity, G.J. *et al.* (1979) In Vitro **15**:73.
6. Masover, G.K. and F.A. Becker (1998) Methods Mol. Biol. **104**:207.
7. Masover, G.K. and F.A. Becker (1998) Methods Mol. Biol. **104**:217.
8. Gabridge, M.G. *et al.* (1986) In Vitro Cell Dev. Biol. **22**:491.
9. Garner, C.M. *et al.* (2000) Br. J. Biomed. Sci. **57**:295.
10. Wirth, M. *et al.* (1994) Cytotechnology **16**:67.
11. van Kuppeveld, F.J. *et al.* (1994) Appl. Environ. Microbiol. **60**:149.
12. Uphoff, C.C. and H.G. Drexler (1999) Hum. Cell **12**:229.
13. Uphoff, C.C. and H.G. Drexler (2002) In Vitro Cell Dev. Biol. Anim. **38**:79.
14. Razin, S. (1994) Mol. Cell Probes **8**:497.
15. Hopert, A. *et al.* (1993) In Vitro Cell Dev. Biol. Anim. **29A**:819.
16. Blanchard, A. *et al.* (1991) FEMS Microbiol. Lett. **65**:37.
17. Hatanaka, M. *et al.* (1975) Proc. Natl. Acad. Sci. USA **72**:1401.