

MycoFluor™ Mycoplasma Detection Kit (M-7006)

Quick Facts

Storage upon receipt:

These storage conditions apply only to the MycoFluor reagent and the mycoplasma MORFS suspension.

- 4°C
- Do not freeze
- Protect from light

Introduction

The MycoFluor™ Mycoplasma Detection Kit provides an ultrasensitive, rapid and simple fluorescence microscopic assay for the visual identification of mycoplasma infection in laboratory cell cultures. In order to detect mycoplasma, the fluorescent MycoFluor reagent is added directly to the culture medium, with or without cells, and the stained sample is then examined under a fluorescence microscope. The detection of mycoplasma in live-cell cultures or fixed-cell preparations takes about 15 minutes from when the reagent is added to the cells to when the results are obtained. The detection of mycoplasma in cell media requires about 30 minutes, depending on the amount of centrifugation required to concentrate potential contaminants.

Also provided with this kit are mycoplasma MORFS (Microscopic Optical Replicas for Fluorescence assayS), which serve as inert positive controls that mimic MycoFluor reagent-stained mycoplasma in size, shape and fluorescence intensity when viewed with a fluorescence microscope. The optical properties of the mycoplasma MORFS allow the researcher to discriminate between stained mycoplasma and other forms of background luminescence (including viruses, bacteria and cellular autofluorescence) without ever introducing infectious biological agents into the laboratory environment. No previous experience with mycoplasma testing is required.

Materials

Kit Contents

- **10 mL of 20X concentrated MycoFluor reagent**, sufficient for 100 tests of 2 mL samples. The MycoFluor reagent should be agitated vigorously or vortexed before using.
- **0.5 mL of mycoplasma MORFS stock suspension**, sufficient for generating 100 control slides. MORFS (Microscopic Optical Replicas for Fluorescence assayS), derived

from nonbiological material, are designed to mimic the size, shape, intensity and color of mycoplasma stained with the MycoFluor reagent according to the protocol as described in *Mycoplasma Detection Protocol*.

- **1 tube of coverslip sealant** used to rapidly mount the coverslip to the microscope slide
- **1 cotton swab** for application of coverslip sealant
- **4 reference photomicrographs**. The photomicrographs of mycoplasma-infected live and fixed cells are provided as examples of what one may see through the fluorescence microscope if mycoplasma contamination is detected.

Storage and Handling

Upon receipt and during prolonged storage, both the MycoFluor reagent and the mycoplasma MORFS stock suspension should be refrigerated and protected from light. All other kit contents are stable at room temperature. Protect the photomicrographs from condensation. **DO NOT FREEZE THE KIT.**

Principles

Mycoplasma infections are relatively common in laboratory cell cultures; it has been estimated that between 5% and 35% of all cell cultures are infected.¹ In countries that do not practice systematic detection and elimination, the rate of infection is much higher.² Mycoplasma infections, which are typically difficult to detect during routine cell culture work, can cause physiological and morphological distortions that affect experimental results.³ Furthermore, a mycoplasma infection in one cell culture has the potential of spreading to other uninfected laboratory cell cultures.

Several methods have been developed to detect mycoplasma, including direct culture in special growth media, enzyme-linked immunoassay, chromogenic substrates for mycoplasma-specific enzymes, radioisotopic detection of mycoplasma RNA and fluorescent nucleic acid stains.^{1,4} The MycoFluor Mycoplasma Detection Kit couples the use of a fluorescent nucleic acid stain with fluorescence microscopy to achieve a high level of sensitivity and reliability, equivalent to that of radioisotopic detection.¹ The minimum number of mycoplasma colony-forming units (CFU) required to detect an infection will depend on cell type, culture medium, mycoplasma species and other factors that affect the ability of the mycoplasma to establish a viable infection. In addition, the testing of live cells, fixed cells and concentrated culture media will have different detection sensitivities. Statistical significance should be established by repeat testing to monitor the proliferation of the mycoplasma in the infected culture (see *Systematic Monitoring of Cell Cultures*).

Mycoplasma Detection Protocol

Cell Preparation

1.1 During routine cell culture, seed one or more coverslips with each cell line to be tested for mycoplasma contamination.

1.2 Cultivate the cells in a petri dish according to the specific cell protocol and allow them to grow to medium density. At the time of testing, cells should be attached to the coverslip at less than 75% confluency.

1.3 Alternatively, select a coverslip of cells from a routine experimental lot. Dense or confluent cultures should be avoided because excessive cell debris or nuclear DNA may obscure results.

1.4 If cells are nonadherent, a cell adhesion reagent can be used to attach a sample of cells to a coverslip. This adhesion reagent may contain collagen, fibronectin, gelatin, laminin, polylysine, polyphenolic proteins or other compounds.⁵⁻⁷

Testing of Live Cells

2.1 Melt the coverslip sealant by placing the tube in a small beaker of water and heating the water to 80–90°C on a hot plate or in a microwave oven. Do not allow the water to evaporate or to mix with the sealant. The sealant should never be heated above 100°C because it may produce flammable vapors. Keep the sealant melted until completion of the test. Place the cotton swab in the tube of melted sealant in preparation for step 2.7.

2.2 Equilibrate the 20X concentrated MycoFluor reagent to room temperature and agitate vigorously or vortex before using. If the reagent has been refrigerated for several weeks, it may contain a precipitate that will dissolve upon vortexing.

2.3 If the MycoFluor mycoplasma test is to be conducted on fixed cells, skip to the protocol for testing of fixed cells as described in *Testing of Fixed Cells*. If the MycoFluor mycoplasma test is to be conducted on cell-free growth medium, skip to the protocol for testing of culture media as described in *Testing of Culture Media*. For testing of live cells, continue to step 2.4.

Note: Positive controls may be generated by spiking stained test preparations (live cells, fixed cells or media) with the mycoplasma MORFS supplied with the kit (see *Control Slides with Mycoplasma MORFS*).

2.4 Add 1 volume of 20X concentrated MycoFluor reagent to 19 volumes of culture medium. For optimum results, prepare this dilution accurately. For example, add 100 µL of 20X concentrated MycoFluor reagent to 1.9 mL of growth medium in a petri dish. New growth medium can be used, but we recommend using the original growth medium in which the cells were cultured, because it will be equilibrated to the cells and may contain mycoplasma in suspension.

2.5 Incubate the cells with the MycoFluor reagent for 10 minutes at room temperature or at 37°C.

2.6 Remove the coverslip with tweezers, hold it vertically, and gently blot the excess medium that accumulates on its edge. **DO NOT ALLOW THE COVERSLIP TO DRY.** Place the coverslip, cell side down, onto a clean microscope slide.

2.7 Seal the coverslip quickly by painting the sealant around the edges of the coverslip with the cotton swab. To avoid moving the coverslip during this procedure, first secure the corners of the coverslip with the sealant. Note that the sealant will harden in seconds so it may be helpful to dip the swab in the tube of melted sealant between application to each coverslip edge. First time users should practice sealing wetted blank coverslips.

2.8 After the sealant is dry (about 10 seconds), remove residual medium and MycoFluor reagent from the top surface of the coverslip by placing a drop of distilled water on the coverslip. Gently shake off the drop and blot dry. **DO NOT APPLY PRESSURE TO THE COVERSLIP.**

2.9 Proceed to the microscopy protocol described in *Microscopy*.

Testing of Fixed Cells

3.1 Prepare the fixative by mixing a 3:1 (v/v) solution of 100% methanol:glacial acetic acid.

3.2 Gently add fixative directly to the medium containing the coverslip until the indicator-containing medium changes color (requires approximately 1 volume of fixative per volume of medium, though the amount of fixative used is not critical).

3.3 After about 5 minutes, remove the solution of fixative and medium and add fresh fixative, using a volume sufficient to cover the cells.

3.4 After an additional 10 minutes, remove the fixative and gently wash twice with deionized water.

3.5 Bring the 35 mm culture dish to 1.9 mL with deionized water for subsequent staining.

3.6 Add 100 µL MycoFluor reagent to 1.9 mL of fixed-cell preparation to achieve proper dilution of the 20X concentrated MycoFluor reagent. For optimum results, prepare this dilution accurately. Proceed to step 2.6 above.

Testing of Culture Media

4.1 Before testing for mycoplasma contamination with the MycoFluor reagent, the cell culture medium should be processed to remove cell debris and to enhance the likelihood that mycoplasma in suspension at very low concentrations will be detected. Proliferation in media may be enhanced by anaerobic incubation in 95% N₂ and 5% CO₂.¹

4.2 Sample several mL of the medium directly from the culture dishes in which the cells have been growing. Centrifuge the sample at 1300 × g for 10 minutes to pellet any cells and debris and carefully transfer the supernatant into microfuge tubes.

4.3 Centrifuge the microfuge tubes at 12,500 × g for 15 minutes.

4.4 Carefully remove and discard the supernatant, leaving about 0.5 mL of medium in the tube. Resuspend any pellet that may have formed using this 0.5 mL of medium.

4.5 Add 26 µL of 20X concentrated MycoFluor reagent to 0.5 mL of the medium.

4.6 Pipet 10 μL of the stained medium onto a clean microscope slide and cover with a clean coverslip. Proceed to step 2.7 above.

Microscopy

Prior to conducting mycoplasma tests, we highly recommend preparing a control slide containing the mycoplasma MORFS (see *Control Slides with Mycoplasma MORFS*) and viewing the slide under a fluorescence microscope to establish a visual reference for identifying potential mycoplasma contamination. The mycoplasma MORFS suspension contains two sizes of particles with diameters between 0.1 μm and 0.3 μm , spanning the approximate size range of various mycoplasma species. Alternatively, “positive” control slides can be prepared by adding a sample of the mycoplasma MORFS to experimental cell samples.

5.1 Prepare the microscope with a near ultraviolet fluorescence filter (e.g., excitation at about 365 nm and either a bandpass (450 \pm 30 nm) or longpass (>400 nm) emission filter). When a bandpass filter is used, the mycoplasma MORFS and the stained mycoplasma will appear violet to blue. When a longpass filter is used, the stained mycoplasma will appear whitish blue.

5.2 For optimum results, a 100X oil immersion objective is suggested, but a 60X oil immersion objective will also suffice.

5.3 First examine a control slide containing the mycoplasma MORFS under the optical conditions specified. Then examine the test specimen for any extranuclear blue fluorescence. It will help to focus up and down through the sample and scan several fields on the slide. Bright-field phase microscopy may be useful for locating the cells prior to viewing fluorescence, especially in the case of live-cell specimens where fluorescence may be almost nonexistent.

5.4 Look for objects that appear similar in size, shape, color and intensity to the mycoplasma MORFS. The reference photomicrographs (see *Reference Photomicrographs* below) provide a guide to the expected visual appearance of the MycoFluor reagent on live and fixed cells and on mycoplasma in suspension. **Caution: Fluorescence resulting from excitation at wavelengths other than 365 \pm 30 nm may be associated with objects other than the stained mycoplasma.**

Reference Photomicrographs

These reference photomicrographs are only limited views of real mycoplasma contaminations and actual infections may have a variety of appearances, including sparse individual entities, chains and dense clusters of small spherical objects; filamentous forms may also occur. Specific appearances may depend on cell type, mycoplasma species, degree of infection, presence of additional biological contaminants and optics used in the observation.

Photomicrograph #1

Fluorescence of Mycoplasma MORFS

The mycoplasma MORFS were suspended in a solid mounting medium and then excited at 365 nm and detected with a 450 \pm 30 nm bandpass filter and a 100/1.3 Plan Neofluar objective lens. The particle diameters, between 0.1 μm and 0.3 μm ,

span the approximate size of individual mycoplasma from a variety of species. The emission spectra of the MORFS are designed to have a homogeneous intensity that closely matches that of mycoplasma stained according to the MycoFluor mycoplasma detection protocol. Comparison of the appearance of the highly standardized MORFS in a freshly prepared control slide (see *Control Slides with Mycoplasma MORFS*) with photomicrograph #1 can be used to gauge equipment-dependent differences in image quality.

Photomicrograph #2

Fluorescence of *Mycoplasma arginini* in Live Cultured Cells Stained with the MycoFluor Reagent

The fluorescence from *Mycoplasma arginini* stained with the MycoFluor reagent has been superimposed on the attenuated bright-field image of nonfluorescent infected cultured cells. The imaging conditions are the same as in photomicrograph #1. The conditions specified in the MycoFluor mycoplasma detection protocol have been optimized so that the MycoFluor reagent readily stains mycoplasma associated with the outside of the cells but does not produce significant intracellular fluorescence. The animal cells were located initially by bright-field phase microscopy and subsequently examined for fluorescence.

Photomicrograph #3

Fluorescence of *Mycoplasma hyorhinis* in Fixed Cultured Cells Stained with the MycoFluor Reagent

In fixed cultured cells, the MycoFluor reagent has access to the nuclei of the infected cells, as well as to the mycoplasma. The imaging conditions are the same as those in photomicrograph #1. The mycoplasma that are separated from the bright nuclei are readily visible, but mycoplasma on or near the nuclei will be obscured by the relatively intense nuclear fluorescence. The optical properties of the stained mycoplasma in live cells are similar to those in fixed cells.

Photomicrograph #4

Fluorescence of Mycoplasma-Free Fixed Cultured Cells Stained with the MycoFluor Reagent

As in photomicrograph #3, the nuclei of fixed cultured cells are intensely stained with the MycoFluor reagent; however, in this mycoplasma-free preparation, fluorescent extranuclear objects are not detected. Also note that mitochondria do not appear to stain with the MycoFluor reagent using this protocol. The imaging conditions are the same as those in photomicrograph #1. The optical properties of stained nuclei in cultured cells varies and often contains bright punctate objects; thus, fluorescent objects that appear on or immediately adjacent to the nuclei of fixed cells should not be interpreted as infections without further testing.

Analysis and Troubleshooting

Optical Filter Specifications for Mycoplasma Detection

The filter specifications for mycoplasma detection with the MycoFluor reagent are important for interpretation of the results. Some cells may contain fluorescent inclusions that emit light at wavelengths different from those specified in the MycoFluor mycoplasma detection protocol. Such observations should not be taken as evidence of mycoplasma infection. Cellular fluorescence that may occur upon excitation at wavelengths signifi-

cantly different from 360 nm or with emission wavelengths greater than 500 nm has not been correlated with mycoplasma.

Systematic Monitoring of Cell Cultures

Systematic monitoring of cell cultures for mycoplasma infection is highly recommended. The presence of only a few mycoplasma-like particles should not be taken as evidence of an infection. A significant number of the cells (greater than 25%) on a slide should contain at least a few fluorescent particles resembling the mycoplasma MORFS. If an infection is suspected, repetitive testing of the cell culture with the MycoFluor reagent should indicate an increase in the number of the mycoplasma over time. Proliferating mycoplasma on cells or in suspension may consist of individual entities, chains or dense clusters. Please note that the mycoplasma may appear smaller in fixed preparations than in physiological samples. Statistical significance should be established by repeat testing to monitor proliferation of mycoplasma in an infected culture. In the unlikely event that all test samples appear to be infected, indicator cell lines guaranteed to be free of mycoplasma, bacteria and fungi can be obtained from ATCC, including the 3T6-Swiss albino (ATCC CCL 96) and Vero (ATCC CCL 81) cell lines.⁸ Samples of all new cell stocks should be tested to determine that they are free of mycoplasma and preserved in liquid N₂ to serve as negative controls for subsequent culture testing.

Control Slides with Mycoplasma MORFS

A control slide can be made from the mycoplasma MORFS suspension. Simply agitate the MORFS stock suspension and pipet 5 µL onto a clean microscope slide, apply a coverslip and seal, as in step 2.7. In addition, "positive" control samples can be generated from stained preparations of live cells, fixed cells or culture media by adding the mycoplasma MORFS. Agitate the MORFS stock suspension and pipet 5 µL or less onto a clean microscope slide before applying a coverslip containing stained live or fixed cells to the slide, as in step 2.6. Likewise, when

generating positive controls for the testing of culture media, pipet 5 µL or less of the mycoplasma MORFS stock suspension on a clean microscope slide, add 5 µL of stained medium to the slide and cover with a coverslip as in step 4.6. These positive controls should be observed under a fluorescence microscope and compared with stained samples that have not been spiked with MORFS. Some of the MORFS will move around by Brownian motion, while others will associate with cells or glass. A significant dilution of the MORFS stock may be required to mimic a sparse infection. Dense clusters and filamentous forms will be absent.

Negative Results

Negative results should not be interpreted as the absolute absence of mycoplasma. As previously mentioned, the sensitivity of detection will depend on many factors that influence mycoplasma proliferation. Testing for mycoplasma infection with the MycoFluor reagent is both sensitive and simple; thus, it is the ideal method for routinely checking cell cultures and media for contamination. Direct culture of mycoplasma on agar and in broth over several days provides the most definitive indication of infection.⁴

Elimination of Mycoplasma

Positive identification of an infection will necessitate the elimination of all sources of contamination. Generally, infected cells and media are discarded, and all cell culture materials, including incubators, are thoroughly disinfected. Standard disinfectants include bleach, phenols, quaternary ammonium and iodine compounds.⁹ Filter membranes that effectively remove mycoplasma from solutions are available.² The use of antibiotics may yield varying degrees of success and should be considered only after other alternatives have been explored.¹

Extra caution should be exercised when handling any cell culture materials suspected of containing mycoplasma or other infectious agents.

References

1. Nature 339, 487 (1989); 2. BioPharm 22 (April 1992); 3. In Vitro 20, 1 (1984); 4. Methods Enzymol 58, 18 (1979); 5. Science 212, 1038 (1981); 6. J Biol Chem 258, 2911 (1983); 7. Exp Neurol 95, 652 (1987); 8. Methods Mycoplasma 2, 173 (1983); 9. Microbiology, M.J. Pelczar and R.D. Reid, Eds., McGraw-Hill (1972) pp. 441–461.

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

| Cat # | Product Name | Unit Size |
|--------------|---|------------------|
| M-7006 | MycoFluor™ Mycoplasma Detection Kit | 1 kit |

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