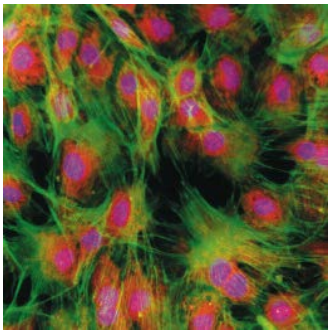
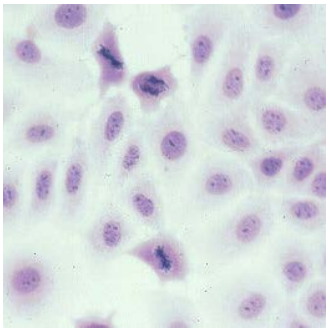
Mycoplasma Detection Using DNA Staining

Protocol

CORNING





Growing the cells on a mycoplasma-free indicator culture (such as 3T3 cells) for 7 to 10 days prior to staining can increase the sensitivity of this method.

Introduction

One of the most important, but frequently overlooked, cell culture procedures is testing cultures for microbial contamination, especially mycoplasma. It is critical for every cell culture laboratory to only use cell lines that have been carefully screened for mycoplasma. Fortunately, there is a simple fluorochrome DNA staining test that can detect both mycoplasma and virtually any other prokaryote contaminants. The testing method should be done using control slides and cultures grown antibiotic-free for at least several passages.

Supplies

1. Positive and negative mycoplasma testing control slides (Bionique Testing Laboratories Cat. No. M-600).
2. Citrate-Phosphate working buffer (for 1+ liter): While measuring pH, slowly add small amounts of Solution I to 1L of Solution II until a pH of 5 is reached. This can be dispensed and sterilized by filtration or autoclaving; store at 4°C.
   a) Solution I (for 1 liter): Dissolve 28.39g of dibasic sodium phosphate (Na_2HPO_4) in 800 mL of water. Then bring to final volume of 1L with water for a 0.2M solution.
   b) Solution II (for 1 liter): Dissolve 10.51g of citric acid monohydrate ($C_6H_8O_7H_2O$) in 800 mL of water. Once fully dissolved add 14.20g of dibasic sodium phosphate (Na_2HPO_4) and stir until dissolved. Then bring to a final volume of 1L with water.
3. Glycerin mounting medium: Add equal volumes of glycerin ($C_3H_8O_3$) and the citrate-phosphate working buffer, mix well, filter through a 0.45 µm filter and store at 4°C.
4. Stock DNA Stain: To 100 mL Hanks' Balanced Salt Solution add 5 mg of Hoechst stain 33258 (Sigma Cat. No. B2883). Mix for 30 minutes while protected from light. Filter sterilize and store at 4°C.
5. Working DNA Stain: After stirring for 30 minutes add 0.3 mL of stock DNA stain concentrate to 100 mL of citrate-phosphate working buffer, and stir protected from light for an additional 30 minutes. Working DNA stain is stable for at least 1 day stored in the dark.
6. Plastic staining chambers (Thermo Scientific Cat. No. 195); polypropylene 50 mL centrifuge tubes (Corning Cat. No. 430828), glass Coplin jars, or glass 100 mm dishes (Corning Cat. No. 3160-102) can also be used.
7. 24 x 60 mm cover glasses (Corning Cat. No. 2980-246).
8. Cells grown antibiotic-free on sterile glass slides or cover glasses in 100 mm dishes. Slides should be labeled with a diamond pen since the fixative may remove ink labeling.
9. Fresh Carnoy's fixative (3 parts methanol:1 part acetic acid).
10. Plastic gloves.
11. Fluorescent microscope with appropriate barrier and exciter filters.

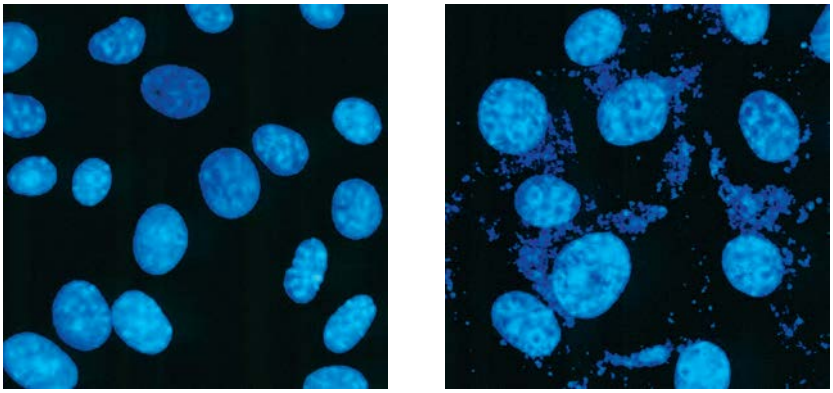
Protocol

1. Remove the medium from your 100 mm dish with the slide and replace with 10 mL Carnoy's fixative for 5 minutes. Remove fixative and replace with 10 mL fresh fixative for an additional 10 minutes. Then remove fixative and allow slide to completely air dry. Be careful not to scratch the cell monolayer. Slides can be stored dry for long periods (room temperature for at least a year) at this point.
2. Immerse your fixed and air-dried slide, along with the pre-fixed positive and negative control slides, into a staining chamber containing enough working DNA stain to fully cover the slide. Incubate at room temperature in the dark for 30 minutes. The DNA stain, Hoechst 33258, strongly binds to DNA and is considered a mutagen. Handle with care (wearing gloves is suggested) and avoid contact with the staining solution.
3. Rinse the stained slides in several changes of distilled water, and then add a drop of glycerin mounting medium to each slide (make sure you add it to the side containing the cells), and carefully cover (avoid air bubbles) with a large cover glass. Blot the slide to remove excess mounting medium. If desired, the cover glass can be sealed to the slide (using nail polish or hot wax) to make viewing easier.
4. View slides under a fluorescent microscope with an appropriate UV filtration package (365 nm excitation, check with microscope manufacturer) using a high quality 40X or 100X magnification. If staining is done correctly, all nuclei should fluoresce brightly. Mycoplasma will appear as very small bright extranuclear dots or rods; most other microbial contaminants will be larger. Compare the results on your test slide with your positive and negative control slides.

Caution: The DNA stain, Hoechst 33258, strongly binds to DNA and is considered a mutagen.

Handle with care (wearing gloves is suggested) and avoid contact with the staining solution.

Both the **ATCC** (**www.atcc.org**) and **Bionique Testing Laboratories** (**www.bionique.com**) offer mycoplasma testing as a service. Contact these companies for additional information on these products.



Photomicrographs of VERO cells stained with Hoechst 33258 dye. DNA-containing nuclei and mycoplasma stain brightly under UV light allowing the clean culture (left) to be easily distinguished from the infected culture (right). (Photomicrographs courtesy of Bionique Testing Laboratories.)

References

1. Chen TR. In Situ Detection of Mycoplasma Contamination in Cell Cultures by Fluorescent Hoechst 33258 stain. Exp. Cell Res. 104:255, 1977.
2. Lincoln CK, Gabridge MG. Cell Culture Contamination: Sources, Consequences, Prevention and Elimination. In Animal Cell Culture Methods, edited by JP Mather and D Barnes, Methods in Cell Biology, Volume 57:49-65, Academic Press, San Diego, CA, 1998.
3. Freshney RI. Culture of Animal Cells, a Manual of Basic Technique, 3rd edition, Chapter 16: 247-249, Alan R. Liss, Inc., NY, 1994.
4. A Guide to Understanding and Managing Cell Culture Contamination (Corning Guide CLS-AN-020) available at www.corning.com/lifesciences.

For additional product or technical information, visit **www.corning.com/lifesciences** or call 800.492.1110. Outside the United States, call +1.978.442.2200 or contact your local Corning sales office.

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