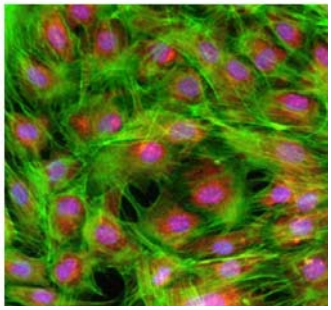
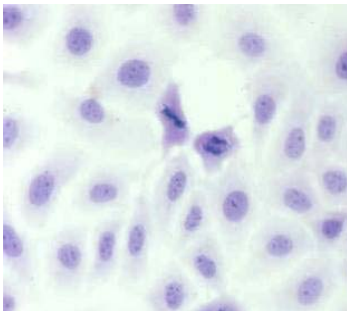
Mycoplasma Detection Using DNA Staining

Protocol





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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. When properly done (using control slides and cultures grown antibiotic-free for at least several passages), this testing method is over 95% effective.

Supplies

1. Positive and negative mycoplasma testing control slides (Bionique Testing Laboratories, Catalog # M-600; 518-891-2356).
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 5.5 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 800mL 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 ($C_6H_8O_7 \cdot H_2O$) in 800mL 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 0.45µm filter and store at 4°C.
4. **Stock DNA Stain**: To 100mL Hanks' balanced salt solution add 5mg of Hoechst stain #33258 (Sigma Catalog # B2883). Mix for 30 minutes while

protected from light. Filter sterilize and store at 4°C. Good for at least 1 year.

5. **Working DNA Stain**: After stirring for 30 minutes add 0.3mL of **stock DNA stain** concentrate to 100mL of **citrate-phosphate working buffer** and stir protected from light for an additional 30 minutes. Good for at least 1 day.
6. Plastic staining chambers (Fisher #12-569-35); polypropylene 50mL centrifuge tubes (Corning Catalog # 430828), glass Coplin jars or glass 100mm dishes (Corning Catalog # 3160-102) can also be used.
7. 24 x 60mm cover glasses (Corning Catalog # 2940-246)
8. Cells grown antibiotic-free on sterile glass slides or cover glasses in 100mm 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.

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.

Protocol

1. Remove the medium from your 100mm dish with slide and replace with 10ml **Carnoy's fixative** for 5 minutes. Remove fixative and replace with 10mL 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 prefixed control slide, 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 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 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

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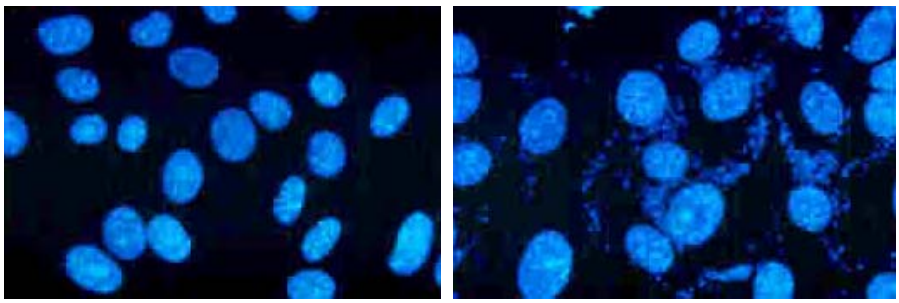
Both the **ATCC** (**www.atcc.org**) and **Bionique Testing Laboratories** (**www.bionique.com**) offer mycoplasma testing as a service. Contact these companies for additional information on these products.



Photomicrographs (1000x) of VERO cells stained with Hoechst 33258 dye. DNA-containing nuclei and mycoplasma stain brightly under ultraviolet light allowing the clean culture (left) to be easily distinguished from the infected culture (right). (Photomicrographs courtesy of Bionique Testing Laboratories, Inc.)

filtration package (365nm excitation, check with microscope manufacturer) using a high quality 40x or 100x lens. 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.

For additional product or technical information, please visit our web site at **www.corning.com/lifesciences** or call at 1-800-492-1110. International customers can call at 978-635-2200.

References

1. Chen, T .R. **In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain**. Exp. Cell Res. 104:255, 1977.
2. Lincoln, C.K., Gabridge, M.G**., Cell culture contamination: sources, consequences, prevention and elimination**. In Animal Cell Culture Methods, edited by J. P. Mather and D. Barnes, Methods in Cell Biology, Volume 57, pp. 49-65, Academic Press, San Diego, CA, 1998.
3. Freshney, R. I., **Culture of Animal Cells, A Manual of Basic Technique**, 3rd edition, Chapter 16, pp. 247-249, Alan R. Liss, Inc., N.Y., 1994.
4. Ryan, J., **Understanding and Managing Cell Culture Contamination,** Corning Technical Bulletin. (Available on the Corning Life Sciences web site at **www.corning.com/lifesciences**).

CORNING

Corning Incorporated
Life Sciences

Tower 2, 4th Floor
900 Chelmsford St.
Lowell, MA 01851
t 800.492.1110
t 978.442.2200
f 978.442.2476

www.corning.com/
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f (52-81) 8313-8589

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Corning Incorporated, One Riverfront Plaza, Corning, NY, 14831-0001