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Product Information

Mycoplasma Stain Kit

Catalog Number **MYC1**
Storage Temperature 2–8 °C

Product Description

The Mycoplasma Stain Kit is designed for *in situ* detection of mycoplasma and other prokaryotic organisms in cell cultures. This procedure is based on the Hoechst Stain method cited by the Tissue Culture Association (TCA Procedure No. 75361).

Many methods are used to isolate and identify mycoplasma contaminants. These include direct growth on agar, broth or semi-solid media, enzymatic procedures, RNA labeling, autoradiography, and staining with DNA fluorochromes. These tests, with the exception of the DNA staining, require time, expertise, and/or a considerable amount of equipment and reagents.

DNA fluorochrome staining is one of the few known methods, which is sufficiently rapid and sensitive to allow frequent testing at each cell culture passage. With this method, a cell monolayer that is 50–80% confluent is fixed, stained with a DNA specific dye, and examined using fluorescent microscopy. Non-nuclear staining will be readily apparent and contaminants will show clearly against a black background. The nature of the contaminant may be determined by its morphology, size, and relationship to the cells. Several DNA fluorochromes such as DAPI, quinacrine mustard, and quinacrine dihydrochloride have been used in the same technique. However, none of these dyes performs as well as the Hoechst stain which shows minimal background fluorescence and slow quenching.

Components

Hanks' Balanced Salt Solution (HBSS) without phenol red and sodium bicarbonate (Catalog Number H5899)	3 × 35 ml
Hoechst Stain Solution 0.5 µg/ml (Catalog Number H6024)	1 × 10 ml
Mounting Medium (Catalog Number M1289)	1 × 10 ml
Mycoplasma Control Slides (Catalog Number M1414)	
Negative	1 × 10 each
Positive	1 × 10 each

Equipment and Reagents Required but Not Provided

- Growth medium and supplements for cell culture
- Incubator at 37 °C
- Refrigerator at 2–8 °C
- Various glassware
- Fluorescent microscope
- Carnoy's fixative (1 part of glacial acetic acid to 3 parts of methanol)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

It is recommended the entire procedure be reviewed before starting the assay.

Storage/Stability

The Hoechst Stain Solution (Catalog Number H6024) should be stored at 2–8 °C in the dark. The Hanks' Balanced Salt Solution (Catalog Number H5899) is stored at room temperature. The fixed Mycoplasma Control Slides (Catalog Number M1414) and Mounting Medium (Catalog Number M1289) are stored at 2–8 °C. See expiration date on the kit label.

A diluted, Working Hoechst Stain Solution should be stored at 2–8 °C in the dark and should not be kept more than 24 hours after preparation.

Procedure

Note: The use of an indicator cell is advisable in many instances. The procedure is exactly the same, except the cell line tested is aseptically added to a subconfluent culture of a clean (mycoplasma free) cell line such as 3T6, Vero, L929, or others, which are susceptible to mycoplasma infection. This cell line with test cells added is allowed to incubate for 2–5 days and is stained as described. The indicator cell line should be tested frequently and included as a negative control (i.e., an uninfected sample).

Growth of Cells

Cells should be grown in an appropriate container, e.g., slide, coverslip, Leighton tubes, or chamber slide.

The conditions for growth (temperature, atmosphere, etc.) should be followed as recommended for the cell line being grown. The cells should be grown to 50–80% confluency. Confluency greater than this makes interpretation of results difficult.

Notes: Suspension cells may be tested by direct inoculation onto a slide or coverslip. Allow to air dry before following the fixation and staining procedure.

Cell cultures should be passaged a minimum of three times without antibiotics prior to testing, as some antibiotics inhibit mycoplasma growth.

Some transformed cell lines will show some non-mycoplasmal cytoplasmic background fluorescence.

Staining Procedure

Note: Keep all solutions sterile. If any turbidity or precipitate is noted in the stain or diluent, discard the solution and start with fresh material.

1. Prepare a Working Hoechst Stain Solution from the Hoechst Stain Solution (Catalog Number H6024). A dilution of 1:10 (0.05 μg/ml) is suggested; however, the final concentration (0.05–0.5 μg/ml) for optimal fluorescence should be determined by titration. Dilute the Hoechst Stain Solution in the Hanks' Balanced Salt Solution (Catalog Number H5899) provided and mix well.
   Note: Caution should be exercised to avoid direct contact between the Hoechst stain solutions and skin. Handle the stain solutions carefully since the stain is a mutagen and binds well to DNA.
2. Remove the medium from the cell preparation, leaving just enough medium to cover the monolayer.
3. Add a sufficient volume of Carnoy's fixative (1 part glacial acetic acid to 3 parts absolute methanol) to cover the cell preparation, approximately equal to the volume of the remaining medium. Allow the slide/coverslip to fix for five minutes.
4. Remove the medium/fixative from the cell preparation and repeat the fixation procedure with Carnoy's fixative for 10 minutes.
5. Remove the fixative from the cell preparation and allow the slide/coverslip to air dry.
6. Add 1 ml of the Working Hoechst Stain Solution (step 1) to the slide/coverslip, and positive and negative control slides. Allow staining to proceed for 10–30 minutes at room temperature. Keep the preparation covered to prevent dehydration.
   Note: A positive and negative control slide should be incorporated with each staining procedure.
7. Remove the stain from the slide/coverslip.
8. Wash the slide/coverslip in deionized or distilled water.
9. Allow the slide/coverslip to air dry or alternatively gently blot dry.
10. Mount the slide/coverslip as follows:
    a. Slide - Apply a small amount of mounting medium on the upper cell sheet surface of the slide and cover with a coverslip.
    b. Coverslip - add 1 or 2 drops of the mounting medium to a glass slide. Put the coverslip on the mounting medium with cell side down.
11. Specimens should be examined by fluorescence microscopy (400–1,000×) under either oil or water immersion using appropriate filters:
    Filters - Hoechst 33258 complexed to DNA is excited at 360 nm and emits at 490–500 nm; therefore, barrier and exciter filters must be used which fall within these limits. An OSRAM® 50 W bulb or better is recommended as a light source. The use of epi-illumination is recommended.

Results

A negative culture will only show nuclear staining. Mitochondrial DNA will not stain by this technique. Occasionally micronuclei or other nuclear fragments from dead or disrupted cells will appear as spherical bodies. They may be distinguished from mycoplasma by their large size and brighter fluorescence.

A positive culture will show particulate or filamentous fluorescence around the cell nuclei. A heavy infection of mycoplasma will stain in the intercellular spaces. Bacteria, yeast, and other prokaryotes will show typical size, morphology, and growth characteristics (i.e., chains, budding, mycelia, etc).

On samples where absolute verification is needed, it is suggested direct growth on agar and in broth also be utilized.

Limitations of the Test

The references listed (1–4) should be consulted for further information. This test depends upon the isolation of a live agent. Extreme care must be taken in the handling of the initial specimens, and subsequent handling of the agent population during isolation and enrichment.

Failure to isolate an agent from material does not guarantee the suspected agent is absent. Factors such as improper time of collection of specimens, improper storage and transport of specimens, and poor laboratory technique can account for this failure.

References

1. Chen, T.R., *In Situ* detection of Mycoplasma Contamination in Cell Cultures by Fluorescent Hoechst 33258 stain. Experimental Cell Research, **104**, 255-262 (1977).
2. Chen, T.R., Microscopic Demonstration of Mycoplasma Contamination in Cell Culture and Cell Culture Media, Procedure 75361, Tissue Culture Manual, Vol 1, No. 4, 229-223 (1975).
3. Bordelon, M.R., Staining and Photograph for Chromosome Banding with Fluorescent Dyes, Quinacrine Mustard and Hoechst 33258, Procedure 61139, Tissue Culture Manual, Vol 3, No. 2, 587-592 (1977).
4. McGarrity G.J., Mycoplasma Infection of Cell Cultures - Microbiological Methods and Fluorescent Microscopy for the Direct Demonstration of Mycoplasma Infection of Cell Cultures, Del Guidice, R.A., and Hopps, H.E., (eds.), Plenum Press, (1978) pp. 57-69.

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