

Catalog Number: 3030000

Mycoplasma Hoechst Stain Kit

1. Kit Contents

Item	Amount	Catalog Number
1. Hoechst Stain (0.5 µg/ml)	10 ml	3030145
2. 10X HBSS without Phenol Red and Sodium Bicarbonate	10 ml	3030245
3. Mounting Medium	10 ml	3030345
4. Fixed Control Slide	5/pk	3030400

NOTE:

1. Each kit contains stain sufficient for 100 tests.
2. Dilute the 10X HBSS reagent 1/10 for use with this kit.

2. Introduction

Many methodologies exist which are used to isolate and identify mycoplasma contaminants. Among these are direct growth on agar, broth or semisolid media, enzymatic procedures, RNA labeling, autoradiography, and staining with DNA fluorochromes. All of the above tests with the exception of the DNA staining require time, expertise and a substantial amount of equipment and reagents.

The DNA fluorochrome staining is the only known method which is sufficiently rapid and sensitive to allow frequent testing at each passage. A cell sheet between 50-80% confluent is fixed and stained with the DNA specific dye and examined under fluorescent microscopy. Non-nuclear staining will be readily apparent and contaminants will stand out boldly 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 but none of the above fluorochromes perform as well as the Hoechst stain with respect to fluorescent effect, slow quenching and minimum background fluorescence.

3. Components

A. Hoechst Stain (3030145)

Hoechst Compound #33258 0.5 µg/ml

Hoechst stain contains Thimerosal at 0.1%

Dilute 1:10 into 1X Hanks Balanced Salt Solution to stain cells. It may be necessary to titrate the stain to obtain the optimal fluorescence. See notes below in Staining section.

B. Mounting Medium (3030345)

Citric Acid Monohydrate 4662 mg/L

NaHPO₄ 8247 mg/L

Glycerol 500 ml

Water to 1000 ml

C. 10X Hanks Balanced Salt Solution (HBSS) Without Phenol Red and Sodium Bicarbonate (3030245)

CaCl₂·2H₂O 1855 mg/L

Glucose 10000 mg/L

KCl 4000 mg/L

KH₂PO₄ 600 mg/L

MgSO₄·7H₂O 2000 mg/L

NaCl 80000 mg/L

NaH₂PO₄ 475 mg/L

Water to 1000 ml

(Caution: Dilute HBSS 1:10 for use with the Hoechst Stain Kit)

D. Fixed Control Slide Kit (3030400) contains 5 slides, each with:

1. Vero Cells (negative)
2. Vero Cell infected with *Mycoplasma hyorhinis*
3. Mouse-mouse hybridoma (negative)
4. Mouse-mouse hybridoma cells infected with *Acholeplasma laidlawii*

4. Potency

The Hoechst Stain should give maximum fluorescent intensity when diluted 1:10 with Hanks Balanced Salt Solution, pH 7.0, achieving a final concentration at 0.05 ug/ml. A lesser or greater dilution may be necessary depending on the microscope being utilized. It is suggested that the investigators determine their own optimum working dilution by testing various dilutions with the known positive specimens included in the kit.

5. Specificity

This stain is highly specific for DNA. It is thought to occupy one of the grooves of the DNA double helix, binding by intercalation. The stain will cause cellular nuclei to fluoresce; however, the other major DNA cytoplasmic components - mitochondria - will not. All prokaryotic organisms (Mycoplasmas, bacteria, yeast, fungi) will give off fluorescence. The identification of the contaminant must be made by noting size, morphology and relationship to the cell.

Control Slides

Zone 1

Vero cell line, a continuous monkey kidney cell line. Zone shows typical results for genetically stable adherent cells demonstrating uniform nuclear staining with little extranuclear staining.

Zone 2

Vero cell line co-cultivated with *Mycoplasma hyorhinis* represents the ideal picture of a cytoadsorbing pleomorphic mycoplasma contaminating a stable continuous cell line.

Zone 3

Mouse-mouse hybridoma, an example of a genetically unstable nonadherent cell line, demonstrates the pleomorphism of nuclear structure and extranuclear staining often observed with hybridomas, tumor-derived cell lines and/or cells growing under non-optimal conditions.

Zone 4

Mouse-mouse hybridoma co-cultivated with *Acholeplasma laidlawii* represents the more frequently encountered situation of having to discern normal nuclear and extranuclear staining from contamination with a pleomorphic, non-cytoadsorbing mycoplasmal species.

One of the main benefits of this assay procedure is that the stain is not specific only to mycoplasmas, but is a rapid screening procedure for any contaminant. So far all mycoplasma species and strains tested were easily demonstrated by this method.

6. Warning

This product is intended for *in vitro* diagnostic use, but is not limited to this use. The Hoechst-compound is known to bind to DNA by intercalation and is not easily removed. Therefore, care must be taken to avoid direct contact or ingestion of the stain.

7. Storage

Concentrated Hoechst stain, Hanks Balanced Salt Solution, slides and mounting medium may be stored at 2°C to 8°C or below.

8. Stability

See expiration date on vial for the stain and on slide box for the slides. Dilute stain (1:10 dilution) should be stored at 2°C to 8°C and must not be kept longer than three weeks.

9. Materials Required but not Provided

1. Sterile distilled water for dilution of 10X HBSS.
2. Incubator (37°C)
3. Refrigerator
4. Various glassware
5. Fluorescent microscope
6. Carnoy's fixative or 1 part glacial acetic acid to 3 parts absolute methanol.

10. Procedure

A. Growth of Cells

Cells should be grown on slides or coverslips in petri dishes or Leighton tubes as desired. The conditions for growth (media, temperature, atmosphere, etc.) should be followed as indicated by the cell line. The cells should be allowed to grow to 50-80% confluency (It is difficult to interpret results in confluent monolayers). It is an excellent idea to plant and stain a slide or coverslip culture with each split of the cell line, thus maintaining an ongoing evaluation of the cell line for mycoplasma, bacteria, yeast and other prokaryotes.

B. Staining

Note 1: The control slides (#3030400) are already fixed, but unstained, so you may skip over steps 2-5 below. Also please see #4 under Precautions section.

Note 2: It is recommended that the stain be used at a dilution of 1:10 of the stock solution (0.05 ug/ml) but can be titrated to determine the optimal fluorescence which may range from 0.05 to 0.5 ug/ml.

Note 3: The Hank's Balanced Salt Solution is provided in a 10X concentrate. It must first be diluted with sterile distilled water to a 1X solution for use in step #1.

1. Add the prepared 1x HBSS to the Hoechst Stain solution, to prepare the proper stain dilutions as determined in Note #2. Mix together thoroughly and allow to warm to room temperature.
2. To prepare your cells, first aspirate or remove by pipette almost all of the medium from the petri dish or Leighton tube, leaving only enough medium to barely cover the coverslip.
3. Add a sufficient amount of Carnoy's Fixative (1 part glacial acetic acid to 3 parts absolute methanol) to cover the slide sufficiently (approximately a volume equal to the amount of medium left in the petri dish or Leighton tube) and allow the slide/coverslip to fix for 5 minutes.
4. Remove the medium/fixative and repeat fixation procedure with Carnoy's Fixative for an additional 10 minutes.
5. Air dry the slide/coverslip for 30 minutes.
6. Add 1.0 ml of the working dilution of stain (prepared in step #1) to the slide/coverslip and allow staining to continue for 10-30 minutes at room temperature. (Keep the slide/coverslip covered to prevent dehydration).
7. Remove the stain.
8. Wash the slide/coverslip three times in deionized or distilled water at room temperature allowing 1 minute soaking time per wash. (Do not use salt solutions).
9. Allow the slide/coverslip to air dry.

C. Mounting

1. If cells are grown on a SLIDE - Apply a small amount of mounting medium (product #3030345) on the upper-cell sheet surface of the slide and cover with a coverslip.
2. If cells are grown on a COVERSLIP - Add 1 or 2 drops of the mounting medium to a glass slide, put the coverslip on the mounting medium surface side up. Add 1 or 2 drops of additional mounting medium to the upper (cell surface) side of the coverslip and cover with a clean coverslip.

11. Interpretation of Results

Specimens should be observed by fluorescent microscopy at between 400-1000X with oil immersion as described below:

FILTERS - the choice of a fluorescent microscope is usually determined by budget or what is available. Hoechst Stain #33258 complexed to DNA is excited at 360 nm and emits at 490-500 nm; therefore, barrier exciter filters must be used which fall within these limits.

A negative culture will show only nuclear staining (not that mitochondrial DNA will not be demonstrated by this staining 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 cell culture will show the cellular nuclei surrounded by small pinpoint dots of fluorescence, either aggregated in clusters or rows. They will have a uniform size ranging from 0.1 - 0.3 μm in diameter and may be pleomorphic. The morphology may range from spherical bodies to filamentous-like forms. Bacteria, yeast and other prokaryotes will show typical size, morphology and growth characteristics (i.e., chains, budding, mycelia, etc.).

12. Limitation of the Test

The references listed should be consulted for further information. The test depends upon the isolation of a live agent; therefore, extreme care must be taken in the handling of the specimens and subsequent handling during isolation and enrichment of the agent population.

Failure to isolate an agent from material does not indicate that the suspected agent is absent. There are many factors which can account for this failure, such as improper time of collection of specimens, improper storage and transport of specimens or the use of insensitive procedures on the part of the laboratory.

13. Precautions

1. Handle the stain carefully since it is a mutagen and does bind well to DNA.
2. Keep all solutions as sterile as possible. If any turbidity or precipitation is noted in the stain or diluent, discard the solution and begin with fresh material.
3. Some transformed cell lines will show non-mycoplasmal cytoplasmic background fluorescence.
4. A control slide should be incorporated with each staining procedure to provide the necessary controls.

14. References

1. Chen, T. , "In situ detection of Mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain." *Exp. Cell Res.*, v. **104**, 255-262, (1977)
2. Chen, T. "Microscopic demonstration of Mycoplasma contamination in cell culture media. Procedure 75361", *Tissue Culture Manual*.
3. Bordelon, "Staining and photography for chromosome banding with fluorescent dyes, quinacrine mustard and Hoechst 33258. Procedure 61138", *Tissue Culture Manual*.
4. McGarrity, G.J., "Mycoplasma infection of cell cultures", in *Microbiological methods and fluorescent microscopy for the direct demonstration of Mycoplasma infection in cell cultures.*, Del Guidice, R.A. and Hopps, H.E., Eds., Plenum Press, 1978.