CHAPTER 10

Tissue Culture of Fish Cell Lines

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I. Introduction

The purpose of this protocol is to provide fish health personnel with guidelines for effective poikilothermic cell culture techniques. Cell culture techniques are an important aspect of the quality control program for the National Wild Fish Health Survey. Healthy, sensitive and mycoplasma-free cells are essential for detection of fish viruses in free-ranging fish populations. This chapter outlines specific cell lines to utilize for various fish species, the steps necessary for optimal growth of cell lines, standardization among laboratories, and quality assurance procedures for tissue culture.

Redundancy is the cornerstone of a successful cell culture program. Every cell line cultured must be "backed up" by cells in frozen storage. Cell culture systems are biological entities with specific physiological needs, much like any other laboratory animals. They require ongoing care, adequate nutrition, a proper environment, and regular checkups. The fish health biologist must provide the cultures with an optimum environment for survival. If this environment is not provided, the cells can be unacceptable for viral testing of free-ranging fish populations.

II. Recommended Cell Lines for Various Fish Species

Refer to Table 1 in Chapter 11- Virology, for a listing of recommended cell lines and incubation temperatures to detect target viruses.

III. Maintenance of Stock Cell Lines & Passage of Cell Monolayers

A. MATERIALS

Fish cell line of choice
L-glutamine - 200 mM (100x)
Minimum Essential Medium (Eagle) with 10% Fetal bovine serum
Trypsin - EDTA
Culture flask (75 cm^2 or 25 cm^2)
Pipettes, sterile, cotton plugged, 1-mL, 5-mL, and 10-mL
70% isopropanol
Graduated cylinder, 100-mL
Glass bottle 100-mL
Beaker, 500-mL
Bleach

B. SELECTING AND EXAMINING CELL CULTURES

1. Work with only one cell line or one kind of primary culture at a time and decontaminate work surfaces between lines with 70% isopropanol. In this way, a single incident of contamination will not affect the entire stock.

2. Make certain that culture medium over cells is optically clear and free of evidence of

microbial or fungal contamination.

3. Only mycoplasma-negative cell lines should be used. The presence of infected lines in the laboratory provides an ongoing threat to the health of all cell lines.

4. Examine cell sheet with an inverted microscope and determine that cell morphology agrees with that of the description for that cell line. The cell culture must be confluent, have no foci or areas of necrosis or other factors that indicate culture may be infected or contaminated.

5. Use cells that are relatively young in the stationary phase. Cells allowed to remain too long in the stationary phase do not survive trypsinization and transfer.

C. PREPARING A STERILE WORK AREA

1. Thaw Trypsin-EDTA, this solution should be kept cool during use.

2. Wipe down all interior surfaces of the laminar flow hood with 70% isopropanol.

3. Disinfect all items introduced into the hood with 70% isopropanol, e.g., media containers and pipettors.

4. Load work area with appropriate pipettes, beaker, graduated cylinder, glass, bottles, and 75 cm^2 or 25 cm^2 culture flasks.

5. Wash hands and wrists thoroughly with an antibacterial soap prior to working with cell lines. Additional disinfection of the hands and wrists can be done with 70% isopropanol before and after each operation. Disposable latex gloves can also be worn to prevent the drying effects of alcohol to the skin.

D. PREPARING A CELL SUSPENSION

1. Aseptically decant medium from the flask of cells into a beaker containing diluted bleach or other disinfectant taking care to prevent backsplash of chlorine or disturbance of the cell layer.

2. Rinse cell monolayer with about 5 mL per 75 cm^2 flask or about 2 mL per 25 cm^2 flask of dispersing solution (trypsin-versene (TV) or trypsin – EDTA), wash all internal surfaces of the culture vessel, then decant solution into beaker containing bleach.

3. Add about 2 to 3 mL per 75 cm^2 flask or 1 to 2 mL per 25 cm^2 flask, of fresh dispersing solution using pipettor and make certain that it contacts the entire cell sheet. Rock the culture vessel during the next 3 to 5 minutes to ensure contact between dispersing solution and cell sheet and expedite cell separation.

Note: Cells will appear rounded when examined with an inverted light microscope and the monolayer will become opaque and grossly visible.

4. When cells are sufficiently loosened by the dispersing solution, strike the flask lightly on the palm of your hand to dislodge them. Triturate with a sterile 5 mL pipette until cells are single or in aggregates of two or three cells.

5. Immediately add at least 10 mL of fresh growth media (MEM-10 w/ L-glutamine) to the flask and pipette several times to break up any aggregates of cells.

6. Considerations when working with either ASK or SHK-1 cell lines.
   a. Cell dispersion:
      i. Wash cells twice with sterile PBS without Ca^2 .
      ii. Add 1.5 mL trypsin solution to flask (75 cm^2).
      iii. Watch cells in microscope and pour off excess trypsin before the cells have completely rounded up. Knock carefully on the flask until the cells are loosened.

   b. Split ratio:
      i. 1:2 is adequate, but if cells are growing well, 1:3 will also work.
      ii. Split every 7-10 days at 20^{o}C.

   c. Use Leibovitz L-15 tissue culture medium (see Chapter 11, Appendix B).

E. DETERMINE THE APPROPRIATE MEDIUM VOLUME AND SPLIT RATIO

Determine the total volume of medium needed per flask to maintain the cell lines until the next propagation. Table 2 provides general guidelines of split ratios for various cell types. The culture flasks require minimum volumes as follows:

Medium volume	Flask size
5 mL	25 cm^2
20 mL	75 cm^2
50 mL	150 cm^2

Table 2 - Seeding Guidelines for Subculturing Fish Cell Lines

CELL LINE			Suggested	For	INCUBATION TEMP (°C)	
ATCC[a] Designation	Common[b] Name	Nominal split Ratio	Seeding Rate (per mL)	24-well PLATES	Suggested	Range
CRL-1681	CHSE-214	1:4 - 8	200,000	300,000	15 - 20	04 - 27
CCL-55	RTG-2	1:3 - 6	200,000	300,000	15 - 20	04 - 26
	EPC	1:4 - 8	500,000	500,000	20 - 25	15 - 30
CCL-42	FHM	1:4 - 6	500,000	500,000	25 - 30	0 - 36
CCL-59	BB	1:3 - 4	200,000	300,000	25 - 30	20 - 30
CCL-91	BF-2	1:2 - 3	200,000	300,000	25 - 30	20 - 30
	WSSK-1	1:4 - 8	300,000	300,000	15 - 25	15 - 30
	WSS-2	1:4 - 8	300,000	300,000	15 - 20	15 - 25
	CCO	1:2 - 4	200,000	300,000	25 - 30	20 - 30
CRL-2747	ASK	1:2 - 3	200,000	300,000	15 - 20	15 - 20
	SHK-1	1:2 - 3	200,000	300,000	15 – 20	15 - 20

[a]American Type Culture Collection, Rockville, MD designation for Certified Cell line.
[b]CHSE-214,Chinook Salmon Embryo; RTG-2, Rainbow Trout Gonad; EPC, Epithelioma Papulosum Cyprini; FHM, Fat Head Minnow; BB, Brown Bullhead; BF-2, Bluegill Fry; WSSK-1, White Sturgeon Skin; WSS-2, White Sturgeon Spleen; CCO, Channel Catfish Ovary; ASK, Atlantic Salmon Kidney; SHK-1, Salmon Head Kidney.

1. If cells are a confluent monolayer, were recently passed, but are older than 10 days, EPC and CHSE-214 cells can be split 1:8. Other cell lines can be split depending on prior performance and the flask size following the guidelines above. Cells should not be allowed to become too thick or old before splitting or they will clump and generally not perform as well as regularly passed cell cultures. Changing the medium in older flasks the day before splitting will help revive inactive cells and reduce clumping and cell debris.

2. If cell counts are done using a hemocytometer they should be approximately 2 x 10^5 to 5 x 10^6 cells/mL. Decant or pipette the correct aliquots of cell suspension into each flask; i.e., when diluting a 75 cm^2 flask of EPC cells to make 4 daughter flasks at a 1:8 split ratio. You must place 84 mL MEM-10/Hepes into a 100 mL glass bottle. After cell sheet is lifted withdraw 8 mL MEM-10 from glass bottle and place in flask of cells. This will give you the 1:8 split ratio. Triturate to break up clumps. Remove 4 mL of MEM-10 w/ cells from flask and place in 100 mL bottle containing 76 mL MEM-10. The 80 mL of cell suspension is then decanted equally into four 75 cm^2 flasks.

3. Label the flask(s) with cell line initials, passage number, date, splitting ratio, operator initials and lot #=s of medium used as follows:

   EPC Pass #99 08-14-97 1:8 TJO lot 105B ($NaHCO_3$)

4. Incubate cells at the optimum growth temperature (see Table 2) and allow them to form a cell monolayer without changing the medium.

   NOTE: After a salmonid cell layer is confluent, the flask can be transferred to a 15°C or 10°C incubator for holding until use. If they are to be held for extended periods of time (up to 3 months) the medium should be replaced with MEM-5 and the cells incubated at 4°C. With a change of growth medium and return to appropriate incubation temperatures the cells resume normal replication.

IV. Cell Counting Using the Hemocytometer

Rarely are cells counted during routine propagation of cell lines, however the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm^2 squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 mL, the cell concentration/mL is the average count per square x 10^4. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 min at 500 rpm) and resuspended in protein-free medium or Hanks salt solution prior to counting.

A. MATERIALS -
(see Chapter 11 -Appendix B: Media for Tissue Culture and Virology)

Hemocytometer chamber
75 cm^2 flask of cells
Trypan Blue (0.1% in PBS)
Microscope
Dilution tubes (12 x 75 mm)
Pasteur pipette
Hanks balanced salt solution, or MEM-0 (MEM w/o serum)
Trypsin - EDTA

Pipettes 1-mL, sterile, cotton plugged
22 x 22 mm cover-slips

B. PROCEDURE

1. Select a healthy (log phase) 75 cm^2 flask of cells and remove cells from flask surface following methods described in "Maintenance of stock cell lines and passage of cell monolayers".

2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20-50 cells/mm^2 (1 x 10^5 to 2 x 10^5 cells/mL). Dilutions vary depending on age of the cells, cell density and cell aggregation.

3. Aseptically transfer 0.5 mL of the cell suspension into a dilution tube.

4. Add 0.5 mL Trypan Blue stain (0.1%).

   Note: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

5. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipette to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Don't overfill or underfill the chambers.

6. Using a microscope with a 10x ocular and a 10x objective count 10 squares (5 from each chamber) as outlined above.

7. Calculate the # of cells/mL and the total # of cells as follows:

   Cells/mL = x (mean) count per square x 10^4 x Trypan Blue dilution factor

   Total cells in flask = cells/mL x total volume of cell suspension

   e.g., total # cells counted in 10 squares = 300 cells

   x count/square = 300 cells/10 squares = 30 cells

   cells/mL = 30 x 10^4 x 2 (dilution factor)
   cells/mL = 60 x 10^4 cells/mL
   cells/mL = 6.0 x 10^5 cells/mL

   Total cells = 6.0 10^5 cells/mL x 8 mL (original volume cell suspension)
   Total cells = 48.0 x 10^5 cells

Total cells = 4.80 x 10^6 cells

If the cells/mL calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

mL medium needed = (actual cells/mL) (vol. of cell suspension)/ desired cells/mL

e.g., actual count = 6 x 10^6 cells/mL
desired count = 1 x 10^6 cells/mL
volume of cell suspension = 8 mL
mL medium needed = x

x = mL medium needed = 6 x 10^6 cells/mL x 8 mL / 1 x 10^6 cells/mL

mL medium needed = 48 x 10^6 mL / 1 x 10^6

= 48 mL

Since you have 8 mL already in the flask, you would need to add 40 mL of medium to the flask before splitting to get the recommended seeding cell density for each new culture.

V. Cryopreservation of Fish Tissue Cell Lines

A. MATERIALS

Fish cell line of choice (75 cm^2 flask)
Trypsin-versene solution
Minimum Essential Medium (Eagle) with 10% FBS
Freezing medium (Origen)
Sterile (internal thread) cryovial tubes
Pipettes 5-mL, sterile, cotton plugged
12x75 mm test tube, sterile
75 cm^2 flasks

NOTE: Protective safety glasses and gloves are required when handling vials stored in liquid nitrogen.

B. FREEZING CELLS

1. Change medium in a 75 cm^2 flask of rapidly growing (log phase) cells. Incubate at appropriate temperature for cell line of choice for 24 hours.

2. Pour off medium and trypsinize the cell sheet 3x with trypsin-versene solution, let stand 2-5 minutes, rap the flask on palm of hand to dislodge cells and plate two 75 cm^2 flasks. Incubate overnight at appropriate temperature.

3. Remove cell monolayers from the flasks using method described in Section III. Maintenance of stock cell lines and passage of cell monolayers (page 2). The cells should be rapidly growing and do not need to be confluent.

4. Transfer the cell suspension to a sterile 12x75 mm test tube and centrifuge at 1000 rpm for 15 minutes to form a cell pellet.

5. After pelleting cells by centrifugation pour off supernatant and re-suspend pellet with 3 mL of Origen Freezing Medium, or other suitable solution such as 7% DMSO.

6. Aliquot 1.0 mL of cell suspension into each of 3 cryovials. Seal and label with cell line, passage number, and date.

Note: Before beginning the freezing process, the cells should equilibrate with the freeze medium for 5 min but no longer than 10 min, including the time it takes to resuspend and transfer the cells to the cryovials.

7. Place the cryovials in a freezing container containing isopropanol and freeze at -70°C for 24 hours.

8. After 24 hours, remove one cryovial to test for cell viability:
   - Rapidly defrost the contents of the cryovial in 30°C water bath.
   - Disinfect the outside of the cryovial with isopropanol.
   - Resuspend the cell pellet in 5 mL of MEM-10 and plate in a 25 cm^2 flask.
   - Incubate the cells for 24 hours at the proper temperature for the cell line.
   - After 24 hours observe flask for the attachment of cells and cell growth.

9. If the test culture has survived the freezing process, the remaining cryovials can be transferred into liquid nitrogen or -70°C ultra-low freezer for long term storage.

C. THAWING CRYOPRESERVED CELLS

1. Before thawing the cells, prepare and warm the MEM-10 to 15°C.

2. Pipette warmed growth medium (10 x the cell suspension) into a sterile 12x75 mm test tube. Repeat for the number of cryovials being thawed.

3. Thaw the cryovials of cells quickly in a 37°C water bath. As soon as the ice has melted, remove the cryovial from the water bath and decontaminate the outside of the cryovial with 70% isopropanol.

4. Aseptically transfer the cryovial contents to a sterile 25 cm^2 culture flask containing 5 mL of MEM-10. Incubate 24 hours at 20°C to determine cell viability. Exchange the growth medium after 24 hours.

VI. Quality Control in Tissue Culture

A. PRECEPTS OF CELL CULTURE

The following list provides guidelines in maintaining fish cell lines and avoiding tissue culture contaminants:

1. Maintain stock cultures separate from working cultures.
2. Employ a redundant approach for maintaining stock cultures.
3. Employ an annual testing program for mycoplasma for each year's serum supply.
4. Use high-purity or tissue culture quality water for all solutions.
5. Test the sterility of all locally prepared solutions before use. Discard any reagent of medium in which contamination is observed.
6. Avoid the use of any antibiotics in maintaining stock cultures.
7. Never mouth pipette samples, cell lines, or media.
8. Work with only one cell line at a time to prevent cross contamination by cells and/or potential contaminants.
9. Never handle all cultures of a cell line in one day. This prevents the risk of contaminating an entire active stock of a cell line if contamination is accidentally introduced.
10. Prepare and keep multiple daughter cultures.
11. Subculture stock cultures infrequently and keep salmonid cell lines at low temperature (4°C).
12. On an annual basis, discard working cultures of the previous year and replace with new working cultures from active or frozen stock cultures that have been tested for viral sensitivity and are mycoplasma free.
13. Test cell lines for mycoplasma contamination at 6 month intervals.
14. Keep detailed records of solution preparation and cell histories.
15. Have laminar flow hoods certified by a reputable company on an annual basis.

B. STANDARDIZATION OF CELL LINES FOR THE WILD FISH SURVEY

Standardization of initial viral testing and corroborative methods is an important component of the Wild Fish Health Survey. All viral testing will utilize cell lines traceable to cell lines from the American Type Culture Collection (ATCC) when available. At the minimum, cell lines will be tested annually for mycoplasma infection and every three years for viral sensitivity. See Chapter 11, Table 1, for a list of primary and complimentary cell lines for the detection and isolation of specific viral pathogens of fish.

C. MYCOPLASMA SCREENING OF CONTINUOUS CELL LINES

Mycoplasma screening should be an integral part of the quality control program within all fish health diagnostic laboratories utilizing cell culture systems. Mycoplasmas are microbes that lack cell walls and are related to, but distinct from, bacteria. With a minimum size of 0.2 to 0.3 μm, mycoplasmas are the smallest free living organisms known.

Mycoplasmal contamination affects virtually every measurable cell culture parameter. Various enzymes, cytokines, and differentiated cellular functions can be suppressed and/or induced. Mycoplasmas may alter cell surface antigenic characteristics. Mycoplasmal contamination may induce chromosomal breakage, deplete nutrients from growth medium, and interfere with viral expression and/or viral isolation. Plaque and cellular morphology may be altered.

1. Asepsis

Mycoplasmas may be introduced by contaminated sera or reagents of a bovine or piscine origin (serum and trypsin), or contamination during handling of cell lines. However, the majority of contamination can be attributed to cross-contamination from other infected cell lines. Critical to preventing the majority of mycoplasma contamination is the establishment of effective aseptic techniques and routine testing. Effective aseptic techniques such as clean disinfected work surfaces, clean hands, and workers abstaining from talking during culture manipulations will prevent most sources of mycoplasma infection.

2. Routine Testing

a. Frequency

It is incumbent upon all fish health diagnostic laboratories to validate the absence of these covert microorganisms in their cell cultures. Routine testing should include annual screening to ensure stock cell lines, sera and trypsin are mycoplasma free. Mycoplasma testing of sera and trypsin should be done on a lot number basis.

All cell line stocks to be stored under cryogenic conditions should be tested prior to freezing. In addition, all cell lines received from outside sources, including commercial cell banks, should be quarantined and tested prior to use.

3. Screening Methods

There are a number of direct and indirect procedures for the detection of mycoplasma contaminants. In addition to direct methods such as staining with a DNA fluorochrome, there are an increasing number of ELISA and/or PCR mycoplasma test kits that also provide sensitive and rapid results. No single method may be entirely adequate, and each should be evaluated for overall sensitivity and performance.

a. Mycoplasma Test Kits

The following table lists several mycoplasma kits and their assay parameters as listed in the general information provided by the manufacturer. Follow manufacturer's instruction for mycoplasma testing of cell cultures and media.

Table 4 - Various Screening Methods for Mycoplasma in Cell Cultures

Mycoplasma Test Kits Available from Various Manufacturers[a]				
ASSAY PARAMETERS	Molecular Probes MycoFluor Kit Catalog # M7006	American Type Culture Collection (ATCC) Catalog # 90-1001K	Life Technologies (formerly Gibco) Catalog # 15672-017	Roche (formerly Boehringer Mannheim) Catalog # 1663-925
Sensitivity	Can Vary	.1-1.6 cfu/ 5 ul	Depends	>1000 cfu/mL
Detection Method	DNA Florachrome	Nested PCR	Visual	Standard PCR & ELISA
No of species detected	At least two	8 common	All common	All common
Time Required	Two hours	1 day	4-5 days	1-2 day
Number of Tests per Kit	100-200	50	50	96
Cost per Kit[b]	$161	$280	$172	$371
Complete Kit	No	Yes	No	Yes
Controls Provided	Reference "Morphs" similar to mycoplasm characteristics	Yes	Yes	Yes
Main Feature	Rapid	Sensitivity	Sensitivity	Sensitivity
Required Supplies and Equipment	Fluor microscope with DAPI filters	PCR Reagents Thermocycler Electrophoresis	ATCC-L96 cells Cell culture plates	ELISA Reader Thermocycler

DISCLAIMER

a - This information is provided as a guideline only. The assay parameters described were obtained from general advertising and/or catalog descriptions for each product. Check with the manufacturer for updated and more specific information.

b - The prices listed are approximate list prices as of Nov 2003. Please check with the manufacturer or scientific supply company for current pricing information.

b. DNA Fluorochrome Staining

DNA fluorochrome procedure has been shown to be 96-98% effective. It is a good, reliable indirect assay, however it requires some expertise in diagnosing low-level contamination.

i. MATERIALS

Tissue culture cells grown in antibiotic-free media for at least 3 passages.
Trypsin-EDTA solution
Petri dish (use sterile non-toxic, "tissue culture clean")
Sterile coverslips 22x22 mm (heat or flamed sterilized)
MEM-10
Pipettes 5-mL, 10-mL, sterile, cotton plugged
20-mL cold , fresh fixative
Forceps
Bisbenzimide staining solution
Microscope slides
Mounting medium
Pasteur pipettes
Positive and Negative controls
Coverslips

ii. REAGENTS

Refer to MSDS and use all personal protective equipment when working with Bisbenzamide – strong mutagen.

Bisbenzamide Fluorochrome Stain - Stock Concentrate

Bisbenzamide fluorochrome stain	5.0 mg
HBSS 1x, w/o $NaHCO_3$ and phenol red	100 mL
Thimersol (final conc., 1:10,000)	0.01gm

Mix thoroughly at 22-25^{0}C w/ magnetic stirrer for 30 min. and wrap bottle in aluminum foil and store in dark at 2-8^{0}C.

Working Stain Dilution (100 mL)

1. Add 0.1 mL of stock concentrate to 100 mL of HBSS for final concentration of 0.05 ug/mL (Optimal fluorescence may range from 0.05 to 0.5 ug/l).
2. Store in a dark bottle wrapped in aluminum foil at 2-8^{0}C.
3. Before use, mix thoroughly with magnetic stirrer at 22-25^{0}C for 30 min.
4. Examine periodically for microbial contamination. Discard when contaminated. Millipore filtration diminishes fluorescence.

Fixative

Glacial Acetic Acid - Methanol (1:3)

iii. PROCEDURE

1. Plating cells

a. Examine the cell sheet under inverted microscope.

b. Decant growth medium from flask.

c. Remove cell monolayer from flask using method described in "Maintenance of stock cell lines: passage of confluent cell monolayers".

d. Place 10 mL MEM-10 in petri dish containing sterile coverslips.

e. Using the same pipette, add 4 to 5 mL MEM-10 to the flask of cells and triturate the cell suspension to disrupt clumps.

f. Place 5 mL of the cell suspension into the petri dish and discard the remainder.

g. Label the petri dish with your initials, and date. Incubate at the same temperature used for propagation of the cell culture being tested.

2. Staining cells

a. Carefully examine coverslips in petri dish using inverted microscope.

b. If the cells are healthy and confluent, draw off the medium with a 10 mL pipette.

c. Immediately add 10 mL cold, fresh fixative to the petri dish. Fix for 5 to 10 min.

d. Pipette off the fixative and repeat the fixative step.

e. Remove the second fixative application and thoroughly drain the coverslips.

f. Flood the coverslips with several drops of bisbenzimide stain, and allow them to incubate for 30 minutes at ambient temperature. Remember to mix stain prior to use.

g. Remove the stain with a Pasteur pipette.

h. Rinse the coverslips 3 times with sterile distilled water.

i. Drain one coverslip on a paper towel.

3. Mounting and Examining Cells

a. Label a slide with the position of the coverslips; i.e., (+), (-), and test.

b. Place 3 drops of mounting medium on the slide.

c. Mount positive and negative controls and test coverslips **cell side up** on the microscope slide.

d. Add an additional drop of mounting medium to the top surface of each coverslip.

e. Place a 22x50 mm coverslip over all.

f. Examine the slide using oil immersion with a fluorescence microscope for the presence of yellow-green fluorescent mycoplasma on the surface of individual cells.

g. Compare the test samples to the negative control to assess the amount of non-specific background staining or fluorochrome debris that may occur.

h. Compare any suspect samples to the positive control slide for size, location and staining intensity of any suspect bacteria.

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