Status **Active** PolicyStat ID **16412194**

Beaumont

Origination	12/6/2022
Last Approved	8/20/2024
Effective	8/20/2024
Last Revised	8/20/2024
Next Review	8/20/2026

Document Contact	Sharon Scalise: Supv, Laboratory
Area	Laboratory-Histology
Applicability	Royal Oak

Histology Special Stain - Grocott Methenamine Silver - Royal Oak

Document Type: Procedure

I. PURPOSE AND OBJECTIVE:

The purpose of this document is to provide a procedure for the demonstration of fungus and Pneumocystis carinii. It will demonstrate the filaments (hypha) and the yeasts. It will also demonstrate Nocardia and Actinomyces.

II. PRINCIPLE:

The reaction is a silver precipitin reaction. Fungus walls contain polysaccharides. Chromic acid (chromium trioxide) is used to oxidize the polysaccharides to aldehydes. Chromic acid will also further oxidize these aldehydes to carboxylic acid, which will not react with silver. Only those areas in the tissue with a light content of polysaccharides, such as the fungus, glycogen, and mucin, will continue to stain. Areas with lesser amounts of polysaccharides, i.e. connective tissue, will not be stained. The sodium bisulfite is used to remove excess chromic acid from the tissue. Silver nitrate is the source of silver ions. Methenamine is used to reduce the silver. When heated, methenamine will breakdown into formaldehyde and ammonia. The formaldehyde reduces the silver ions to visible silver. The ammonia is an unwanted by-product, as it increases the pH, causing the sections to fall off the slides. Borax (sodium borate) is used to control the pH. Gold chloride tones the stain. Sodium thiosulfate removes unreduced silver. Light green is the counterstain.

III. SPECIMEN COLLECTION AND HANDLING:

A. Fixation

1. Any well-fixed tissue.

2. 10% neutral buffered formalin preferred.

B. Processing

1. Standard, overnight processing.

C. Section Thickness

1. Routine specimens-5µ.

D. Slide Drying

1. 30 minutes at 60°C.

E. Type of Slide

1. Plain slides.

IV. REAGENTS:

A. **10% Chromic Acid**

Chromic acid (chromium trioxide) 10.0 gm
Distilled water 100.0 mL

Dissolve together. Stable at room temperature for months; may be reused until weak.

B. **1% Sodium Bisulfite**

Sodium bisulfite 1.0 gm
Distilled water 100.0 mL

Dissolve together. Store at room temperature; stable for months.

C. **5% Sodium Borate (Borax)- use the vendor pre-made if available (stored at RT); if not available:**

Borax (sodium borate) 25.0 gm
Distilled water 500.0 mL

Dissolve together with the aid of gentle heat. Cool; stable at room temperature for months.

D. **5% Silver Nitrate**

Silver nitrate 5.0 gm
Distilled water 100.0 mL

Dissolve together in acid clean glassware. Store in refrigerator (3^0C.) in dark glass bottle; stable for 2-3 months.

E. **3% Methenamine- use vendor pre-made if available (stored at RT); if not available:**

Methenamine (hexamethylenetetramine) 3.0 gm
Distilled water 100.0 mL

Dissolve together. Store in refrigerator (3°C.); stable for months.

F. **Stock Methenamine-Silver Solution**

5% silver nitrate 5.0 mL
3% methenamine 100.0 mL

Mix together in acid clean glassware. A white precipitate forms, but will disappear with mixing. Store in refrigerator (3°C) in dark glass bottle; stable for 1-3 months.

G. **Working Methenamine-Silver Solution**

Stock methenamine-silver solution 25.0 mL

Distilled water	**25.0 mL**
5% borax	**2.0 mL**

JUST BEFORE USE, mix together in the order listed. Warm to room temperature before use; filter if cloudy. Discard after use.

H. **0.1% Gold Chloride- use vendor pre-made (store in refrigerator)**

I. **5% Sodium Thiosulfate**

Sodium thiosulfate	**5.0 gm**
Distilled water	**100.0 mL**

Dissolve together. Store at room temperature; stable for months.

J. **0.2% Light Green- use vendor pre-made if available (stored at RT); if not available:**

Light green SF Yellowish	**0.2 gm**
Distilled water	**100.0 mL**
Acetic acid	**0.2 mL**

Dissolve together. Store at room temperature; stable for months; may be reused until weak.

V. EQUIPMENT:

A. Balance
B. 60°C oven
C. Magnetic stirrer

VI. SUPPLIES:

A. Erlenmeyer flasks
B. Graduated cylinders
C. Funnel
D. Acid-cleaned coplin jars
E. Non-metal forceps
F. Filter paper

VII. QUALITY CONTROL (QC):

Tissue with fungus or Pneumocystis carinii.

VIII. SPECIAL SAFETY PRECAUTIONS:

A. Chromic Acid (Chromium Trioxide)
    1. Is an oxidant.
    2. Store separately from other material.
    3. Explosive when mixed with combustible material.
    4. May cause cancer.

5. May be fatal if inhaled.
6. Toxic in contact with skin and if swallowed.
7. May cause sensitization by inhalation and skin contact.
8. Danger of serious damage to health by prolonged exposure.
9. May cause heritable genetic damage.
10. Possible risk of impaired fertility.

B. Sodium Bisulfite
   1. Has low hazard for recommended handling.

C. Sodium Borate (Borax)
   1. Has low hazard for recommended handling.

D. Methenamine (Hexamethylenetetramine)
   1. Is an irritant to eyes, skin, and respiratory system.

E. Gold Chloride
   1. Causes skin and eye irritation.

F. Sodium Thiosulfate
   1. Is an irritant.

G. Light Green SF Yellowish
   1. Toxicological properties have not been investigated.

H. Acetic Acid
   1. Is an acid.
   2. Add drop by drop to solution.
   3. May cause skin / eye burns.

IX. PROCEDURE:

Step	Action	Time	Notes
1	Deparaffinize and hydrate sections through graded alcohol to distilled water.		
2	Place in 10% chromic acid at room temperature.	10 minutes	**If chromic acid is dark orange to brown, throw out; it is too weak.**
3	Wash well in running water at room temperature.	1 minute	
4	Decolorize in 1% sodium bisulfite.	1 minute	
5	Rinse in running water.	1 minute	

6	Rinse in distilled water, 2-3 changes.	5-10 seconds	
7	Place in WORKING silver-methenamine in 60°C oven or water bath. (**Or heat in microwave on high 30 seconds; pour off, pour on. Repeat this step then losley cover and let sit on counter until correct color. Check every 20 seconds as this reaction can happen quickly and become too dark if not stopped in time**)	22-45 minutes	Make up working solution JUST BEFORE USE. Leave in solution until section is golden brown and fungus is brown. Pneumocystis carinii require longer staining time than fungi.
8	Rinse in distilled water.	1 minute	
9	Tone in 0.1% gold chloride until background is clear and fungus are black.	10-60 seconds	Check with microscope. The fungus should be stained gray on the inside, with a black delineation on the outside and the septa.
10	Rinse in distilled water, 2-3 changes.	10 seconds	
11	Place in 5% sodium thiosulfate.	1 minute	
12	Rinse in running water.	1 minute	
13	Counterstain in 0.2% light green SF Yellowish.	10-30 seconds	The counterstain should NOT obscure the staining of the fungus.
14	Rinse in running tap water, 2-3 changes until background is a pale green.	5-10 seconds	
15	Dehydrate through graded alcohols, clear with xylene.		
16	Coverslip.		

X. LIMITATIONS:

A. The following may influence the validity of test results:

1. Overheating the silver-methenamine will cause the methenamine to breakdown quickly. This will increase the amount of silver ions, causing the silver to stain the background, slide, and coplin jar. An increased amount of ammonia will also be formed. This will increase the pH of the solution, causing the sections to fall off the slides.
2. If connective tissue is staining (elastin, collagen), either the slides were left in the methenamine silver too long, the methenamine-silver was overheated, or the chromic acid is too weak, so did not over-oxidize the connective tissue.
3. Use acid-cleaned coplin jars and non-metal forceps.

XI. RESULTS:

A. Fungus (hyphae or yeast) - **gray-black**
B. Pneumocystis carinii - **gray-black**
C. Actinomyces, Norcardia - **gray-black**
D. Glycogen, mucin - **gray-black**
E. Cellulose, chitin - **gray-black**
F. Reducing substances (calcium, lipids, melanin, etc.) - **gray-black**
G. Background - **green**

XII. REFERENCES:

A. Bancroft JD and Steven A: Theory and Practice of Histological Techniques, 3rd edition, New York, NY. Churchill-Livingstone, 1990.
B. Carson FL: Histotechnology: A Self-Instructional Text. Chicago, IL, ASCP Press, 1990.
C. Sheehan DC, Hrapchak BB: Theory and Practice of Histotechnology, 2nd edition. Columbus, Ohio, Battelle Press, 1980.

Approval Signatures

Step Description	Approver	Date
Medical Director	Laura Walters: OUWB Clinical Faculty	8/20/2024
Policy and Forms Steering Committee (if needed)	Sharon Scalise: Supv, Laboratory	8/15/2024
	Sharon Scalise: Supv, Laboratory	8/15/2024
	Jamie Pert: Mgr, Division Laboratory	8/15/2024
	Sharon Scalise: Supv, Laboratory	8/14/2024

Applicability

Royal Oak