AURAMINE O STAIN

Preanalytical Considerations

I. PRINCIPLE

Acid-fast mycobacteria resist decolorization by acid-alcohol after primary staining owing to the high lipid (mycolic acid) content in their cell walls. The identification of mycobacteria with auramine O is due to the affinity of the mycolic acid in the cell walls for the fluorochromes. The dye will bind to the mycobacteria, which appear as bright yellow, luminous rods against a dark background. The potassium permanganate helps prevent non-specific fluorescence. All acid-fast organisms will be stained by auramine O, including some parasites. Slides stained with auramine O may be restained with Ziehl-Neelsen or Kinyoun stain directly, as long as the oil has been removed. This provides a convenient method of confirming and differentiating morphology of positive slides with the traditional stains. The fluorochromes stains are recommended for specimen examination because of their increased sensitivity and speed.

II. SPECIMENS

Clinical specimens or pure cultures suspected of harboring mycobacteria are stained for acid fastness. A direct smear (not concentrated) from a clinical specimen is discouraged because it lacks the sensitivity of a concentrated (centrifuged) smear. A negative result from a direct smear must be followed by a concentrated smear. Preparation of smear for staining is as follows:

A. In the case of a solid medium, an aqueous suspension is made. Take a small amount of material and suspend it in a drop of distilled water on a microscope slide. Do not make the smear too thick.

B. In the case of a liquid medium, a drop is used directly from the culture container.

C. Air dry the smear, then fix by passing the slide through a Bunsen burner flame two or three times. A better fixation method is to allow the slide to remain on an electric slide warmer at 65 to 75ºC for at least 2 h. Allow the slide to cool prior to staining.

III. MATERIALS

A. Reagents: Reagents may be purchased commercially or prepared in the laboratory.

a. Fluorochrome acid-fast stain
   i. Auramine O
   ii. 0.5% Acid-alcohol
   iii. Counterstain (potassium permanganate or acridine orange)

B. Supplies

a. Glass slides (25 by 75 mm), frosted ends desirable
b. Coverslips (22 by 22 mm; no. 1)
c. Sterile 50-ml conical polypropylene screw-cap tubes (aerosol free and graduated)

C. Equipment: Optional materials, depending on specimen source of laboratory protocol

a. Binocular microscope with 10X, 40X, and 100X objectives (or the approximate magnifications for low power, high dry power, and oil immersion examination).
b. Oculars should be 10X. Some workers prefer 5X; however, overall smaller magnification may make final organism identifications more difficult.
c. Centrifuge with aerosol-free sealed centrifuge cups
d. Vortex mixer

Analytical Considerations

IV. QUALITY CONTROL

A. A positive and negative control slide must be included whenever any acid-fast stain is performed and upon receipt of each new lot of materials, reagents, and media to verify the correct performance of the procedure. *Escherichia coli* is commonly used for the negative control (no fluorescence) and *M. tuberculosis* H37Ra ATCC 25177 is the positive control (yellow to orange fluorescence – color may vary with the filter system used).

B. The specimen is also checked for adherence to the slide (macroscopically).

C. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not recommended on a yearly basis.

D. Known positive microscope slides, Kodachrome 2 x 2 projection slides, and photographs (reference books) should be available at the work station.

E. Record all QC results; the laboratory should also have an action plan for ``out of control'' results.

V. PROCEDURE

A. Flood the slide with fluorochromes stain.
B. Stain for 15 min.
C. Rinse the slide with water; drain excess water from the slide.
D. Flood with 0.5% acid-alcohol
E. Decolorize for 30-60 s. (some protocols call for 2 min.)
F. Rinse the slide with water; drain excess water from the slide.
G. Flood the slide with potassium permanganate or acridine orange
H. Counterstain for 2 min; do not allow the slide to dry. NOTE: Timing is critical during the counterstaining step with potassium permanganate. Counterstaining for a longer time may quench the fluorescence of acid-fast organisms.
I. Rinse the slide with water; drain excess water from the slide.
J. Air dry; do not blot.
K. Examine the smear with a fluorescent microscope (K530 excitation filter and a BG 12 barrier filter or G365 excitation filter and an LP 420 barrier filter).
L. Examine smears using the high power objective (40X, total magnification, X400); verify using the oil immersion objective (100X, total magnification, X1,000). Some recommend screening with a 25X objective.

VI. RESULTS

A. Mycobacteria are approximately 1 to 10 µm long and typically appear as slender rods. However, they may also appear curved or bent, coccobacillary, or even filamentous. Some may be beaded or banded.
B. Reports should provide results based on organisms counted; see table below.
C. The results from this staining procedure should be reported only if the positive control smears are acceptable.

Postanalytical Considerations

VII. REPORTING RESULTS

A. Report the organism and stage (do not use abbreviations
Examples (Negative) No acid-fast bacilli seen
Example (Positive): Data listed below are numbers of AFB per indicated number of microscopic fields (F's). This takes into account the larger number of fields covered with the lower magnification examined under the fluorescent microscope.

B. Quantitate as indicated below:

Fluorochrome			Report
250X	**450X**	**1,000X***	
0	**0**	**0**	**No AFB seen**
1-2/30F	**2/70F**	**2/300F**	**Doubtful, repeat**
1-9/10F	**2-18/50F**	**1-9/100F**	**1+**
1-9/F	**4-36/10F**	**1-9/10F**	**2+**
10-90/F	**4-36/F**	**1-9/F**	**3+**
>90/F	**>36/F**	**>9/F**	**4+**

***Carbolfuchsin stain only**

VIII. PROCEDURE NOTES

A. Overheating (burning) during fixation can be avoided by just touching the back of the slide to the back of the hand each time the slide has been passed through the flame. Remember, the heating block is recommended for fixation.

B. Do not stain smears, which have only been air-dried; they must also be fixed prior to staining.

C. Smears should not be too thick. After air-drying, examine under a microscope. If there are no areas of bacteria separation, more water should be added to dilute the smear.

D. After staining, it is essential that the back surface of the slide is wiped clean.

E. If washing with distilled water is not done adequately, crystallization of the stain may appear on the slide.

IX. LIMITATIONS OF THE PROCEDURE

A. The acid-fast stain is nonspecific. Slow-growing mycobacteria (not just *M. tuberculosis*) are consistently acid-fast. Microorganisms other than mycobacteria that demonstrate various degrees of acid-fastness include *Nocardia, Rhodococcus, Legionella micdadei*, cysts of *Cryptosporidium* spp., and *Cyclospora* spp.

B. The acid-fast smear is insensitive. Sensitivities ranging from 22 to 81% have been reported. A negative smear does not rule out tuberculosis because levels are only 5,000 to 10,000 bacilli per ml of sputum.

C. Since heat fixing and staining may not kill all the mycobacteria, discard slides in a sharps receptacle and wear gloves.

SUPPLEMENTAL READING

1. **Ebersole, L.L.** 1992. Acid-fast stain procedures, *In* H.D. Isenberg (ed), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
2. **Isenberg, H.D.** 1998. *Essential Procedures for Clinical Microbiology*. American Society for Microbiology, Washington, D.C.
3. **Salfinger, M., and H.E. Pfyffer.** 1994. The new diagnostic Mycobacteriology laboratory. *Eur. J. Clin. Microbiol. Infect. Dis*. **13**:961-979.

APPENDIX

Auramine O stain reagents available from Medical Chemical Corporation are as follows:

REAGENT	CATALOG NUMBER	SIZE AND CATALOG NUMBER
Auramine O	418D	418D-8oz 8oz 418D-1gl 1 gal
Fluorescent Decolorizer	AAD	AAD-8oz 8 oz AAD-1gl 1 gal
Potassium Permanganate	PCC	PCC-8oz 8 oz PCC-1gl 1 gal