CLPS

Current Laboratory Practice Series

USE OF FLUOROCHROME STAINING FOR DETECTING ACID-FAST MYCOBACTERIA

December 2000

DEPARTMENT OF HEALTH AND HUMAN SERVICES
CENTERS FOR DISEASE CONTROL AND PREVENTION
ATLANTA, GA 30333

CURRENT LABORATORY PRACTICE SERIES

CLPS

USE OF FLUOROCHROME STAINING FOR DETECTING ACID-FAST MYCOBACTERIA

Part I - Fluorochrome Staining Procedure–36 slides
Part II - Examining and Reporting Fluorochrome-Stained Smears–13 slides
Audio Cassette tape: Part I - 23 minutes, **Part II** - 14 minutes

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INTRODUCTION

Target Audience

This program is designed for public health and clinical laboratory personnel with basic to intermediate levels of experience in mycobacteriology, who will prepare, stain and examine smears for acid-fast bacilli.

Program Description

The program is a two-part sound slide presentation. Part I provides basic information on preparation and fixing smears, fluorochrome staining and reading acid-fast smears. Part II contains photomicrographs and descriptions of smears stained with various fluorochrome staining procedures. The viewer will examine and participate in practice exercises for one method of quantitative reporting of acid-fast smear results. A relatively dark room should be used to ensure optimal visibility of acid-fast organisms in the photomicrographs.

An explanation of terms, tables, staining procedures, references, and Internet resources are provided in the supplementary program booklet. The course objectives serve as a basic guide to the content and learning outcomes to be achieved from this program. A quiz, included in this booklet, will assess knowledge gained in both Part I and Part II of the program. The program objectives will be met by scoring 80% or higher on the quiz. Answers to the quiz are included.

 A manuscript of the audiotape may be requested from the National Laboratory Training Network for persons with hearing impairment.

OBJECTIVES

After viewing Part I and II of the sound-slide program, reading the booklet, and completing the quiz, the student will be able to:

- **define** acid-fast microscopy.
- **describe** the importance of acid-fast staining.
- **list** two advantages of fluorochrome staining over fuchsin staining.
- **state** criteria for preparing smears.
- **state** two acceptable methods for fixation of smears.
- **explain** the purpose of control slides.
- **list** laboratory practices that are important in preventing false positive and false negative results.
- **describe** the color of the acid-fast bacilli and smear background for two different fluorochromes and counter stains.
- **state** the number of microscopic fields to be examined at selected magnifications with fluorochrome stained smears.
- **list** criteria for a quantitative reporting system in fluorochrome acid-fast microscopy.

Explanation of Terms

Acid-fast organism– Acid fast organisms have mycolic acids in their cell wall. The mycolic acids are thought to bind fuchsin or fluorochrome stains tightly, making them difficult to decolorize with acid alcohol.

Decolorization– Decolorization is the removal of unbound primary stain from the cell walls of acid-fast organisms with the application of acid-alcohol or other reagents.

Primary stain– The specific stain used to detect organisms or structures.

Counter stain- A counter stain (or reagent) is applied after the primary stain and decolorization process. It provides a contrast between acid-fast organisms in the smear and background material, thereby enhancing microscopic observation of the organisms.

Fuchsin staining- Fuschin staining methods are primarily Ziehl Neelsen (hot) and Kinyoun's (cold) stains. Carbol fushin, a mixture of basic fuchsin and phenol, is the primary staining reagent.

Fluorochromes- Fluorochromes are dyes which make non-fluorescent objects fluoresce. The staining dyes are known as fluorophores or carriers of the fluorescent organic compounds.

Microscopic field- This is the entire area visible through the eyepiece of the microscope.

Magnification- This is the enlargement factor of the microscopic field which is based on the product of the power of the eyepiece and the product of the objective lens. For example, a 10x eyepiece and 20x objective equal a total field magnification of 200x.

Quench- A reagent or environment that reduces the intensity of fluorescence**.**

Barrier filter- A filter which emits light from a sample and excludes any stray excitation light.

Excitation filter- This filter absorbs unwanted wavelengths of light from the arc lamp going into the microscope.

Nonspecific staining- The undesirable staining or binding of primary stain to bacterial cells and other background debris. Organisms that are not acid-fast may be the same color as the suspected acid-fast organisms. They are usually distinguishable by specific morphological features.

Autofluorescence- The bright fluorescence of bacteria or artifacts in a smear due to the content of the specimen or stain impurities.

USE OF FLUOROCHROME STAINING FOR DETECTING ACID-FAST MYCOBACTERIA

LECTURE OUTLINE:

Patterns for Examining Slides

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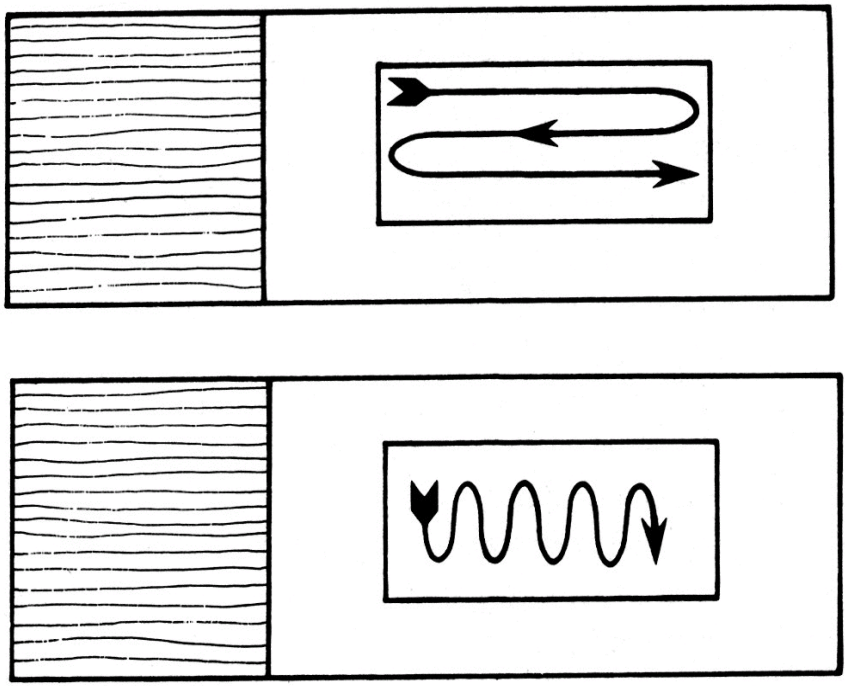

Recommended Filter Sets for Fluorochrome Stains[a]

Manufacturer	Filter Set	Excitation (nm)	Emission (nm)	Fluorochrome Stain(s)[b]
Lecia	13	450-490	>515	AO*, AR*, FITC*
	H3	420-490	>515	AO, AR, FITC
	L4	450-490	515-560	FITC
	D	355-425	>470	CW*
Nikon	B-2A[c]	450-490	>520	AO*, AR*
	B-2H	450-490	>515	AO
	B-3A	420-490	>520	CW
	BV-2A	405-445	>475	CW*
	UV-2B	380-425	>460	CW
	B-1E	470-490	520-560	FITC
	B-1A	470-490	>520	FITC*
Olympus	B	450-490	>515	AO, AR*, FITC
	IB	460-490	>515	AO*, FITC*
	UV	330-385	>420	CW*
Carl Zeiss	01	359-371	>400	CW
	02	330-390	>420	CW
	05	400-440	>475	CW*
	09	450-490	>520	AO*, AR*, FITC*
	10	450-490	520-560	AO, AR, FITC

[a] Listing of manufacturer's recommended filter sets as of September 1998. Continued improvements in this technology will determine the filter sets available in the future. Data provided by Paul Millman, Chroma Technology Corp., Brattleboro, VT (1-800-824-7662) in collaboration with the individual microscope manufacturers.

[b] AO, acridine orange; AR, auramine-rhodamine; FITC, fluorescein isothiocyanate; CW, calcofluor white; *, preferred filters, although filter selection is subjective, on the basis of the specific application and prior staining experience of the microscopist.

[c] The Nikon B-2A filter set is commonly used for FITC, although the manufacturer does not specify this application.

Reference: Murray, P.R. (Editor in Chief). Manual of Clinical Microbiology. 7th ed. 1999. American Society for Microbiology, Washington, D.C.

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Number of Fields to Examine at Selected Magnifications [a]

Magnification[b]	Number of Fields
250x	30
400x	55
450x	70

[a] The minimum number of fields to examine before reporting a smear as negative for acid fast organisms.

[b] This final magnification represents the objective lens magnification multiplied by the eyepiece magnification

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Evaluating and Reporting Acid-fast Smears

Report	Number of AFB Observed	
	250x	450x
No AFB seen	0	0
Doubtful: repeat	1-2/30 F*	1-2/70 F
1+	1-9/10 F	2-18/50 F
2+	1-9 / F	4-36/10 F
3+	10-90/ F	4-36/F
4+	>90/F	>36/F

*** number of acid-fast bacilli observed per microscopic field**

Blair Fluorochrome Acid-fast Staining Procedure (Auramine O)

Materials:
Auramine O
Phenol crystals
Hydrochloric acid, concentrated
Ethanol, 70%, 95%
Potassium permanganate
Water, distilled

Preparations:

Auramine O
1. Auramine O – dissolve 0.1 g of Auramine O in 10 ml of 95% ethanol.
2. Phenol – dissolve 3.0 g of phenol crystals in 87 ml of distilled water.
3. Mix solutions #1 and #2.

Acid Alcohol
Carefully add 0.5 ml of concentrated hydrochloric acid to 100 ml of 70% ethanol.

Potassium Permanganate
Dissolve 0.5 g of potassium permanganate ($KMn0_4$) in 100 ml of distilled water.

Procedure:
1. Prepare smears and allow to air dry.

2. Fix smears on electric slide warmer at 65-75 ^{B}C for at least 2 hours, or use a Bunsen Burner flame. Do not overheat.

3. Flood smear with Auramine O solution and allow to stain for 15 minutes. Be sure that the staining solution remains on the smear. Do not apply heat to smear. Do not use filter strips.

4. Rinse smear with chlorine free water and drain. Chlorine may interfere with fluorescence; therefore, rinse with distilled or deionized water.

5. Flood smear with acid alcohol and allow to de-stain for 2 minutes.

6. Rinse again and drain smear.

7. Flood smear with potassium permanganate* and counter stain for 2 minutes. Time is critical with potassium permanganate because counter staining for a longer time may quench fluorescence of acid-fast bacilli.

*Acridine Orange may be used in place of potassium permanganate as the counter stain. Dissolve 0.01g of anhydrous dibasic sodium phosphate (Na_2HPO_4) in 100 ml of distilled water; add and dissolve 0.01 g of Acridine Orange. Counter staining time remains 2 minutes.

Truant Fluorochrome Acid-fast Staining Procedure
(Auramine O – Rhodamine B)

Materials:
Auramine O
Rhodamine B
Phenol crystals
Hydrochloric acid, concentrated
Ethanol, 70%, 95%
Potassium permanganate
Water, distilled

Preparations:
Auramine O-Rhodamine B
Dissolve 1.5 g of Auramine O and 0.75 g Rhodamine B in a solution of 75 ml glycerol (glycerine), 10 ml heated phenol crystals, and 50 ml of distilled water. This solution is usually cleared by filtering through glass wool.

Acid alcohol
Carefully add 0.5 ml of concentrated hydrochloric acid to 100 ml of 70% ethanol.

Potassium Permanganate
Dissolve 0.5 g of potassium permanganate ($KMn0_4$) in 100 ml of distilled water.

Procedure:
1. Prepare smears and allow to air dry.

2. Fix smears on electric slide warmer at 65-75 ^{B}C for at least 2 hours, or use a Bunsen Burner. Do not overheat.

3. Flood smears with Auramine O-Rhodamine B solution and allow to stain for 15 minutes, making certain that the staining solution remains on the smear. Do not apply heat to smear. Do not use filter strips.

4. Rinse smears with chlorine free water and drain. Chlorine may interfere with fluorescence; therefore, rinse with distilled or deionized water.

5. Flood smears with acid alcohol and allow to de-stain for 2 minutes.

6. Rinse again and drain smears.

7. Flood smears with potassium permanganate and counter stain for 2 minutes. Time is critical with potassium permanganate because counter staining for a longer time may quench fluorescence of acid-fast bacilli.

Acridine Orange is not recommended as a counter stain with Auramine O-Rhodamine B because of decreased contrast in stained acid-fast organisms and background material.

QUIZ

Directions: Please select the best response to the following statements.

1. A definition of "acid-fast microscopy" is the examination of stained smears for the presence of organisms which:

a. stain a red color with basic fuchsin dye regardless of a decolorization step.

b. retain the primary stain after the smear is decolorized with an acid alcohol solution.

c. do not retain primary stains after decolorization with acid alcohol solution.

d. form fluorescence complexes only with fluorochrome dyes prior to decolorization.

2. Acid-fast microscopy provides important information in all of the following ways <u>except</u> to:

a. screen for the most infectious cases of presumed tuberculosis.

b. serve as an adjunct to culture and for selection of other tests.

c. differentiate *Mycobacterium tuberculosis* from other species of mycobacteria.

d. make decisions regarding isolation of patients.

e. initiate treatment and monitor therapy.

3. Select the one statement that is <u>not</u> an advantage of fluorochrome staining over fuchsin staining.

a. Fluorochrome stained smears quench on exposure to light.

b. Lower magnifications are used for examination of smears.

c. Less time is required to examine smears.

d. Fluorochrome stained smears may be over-stained with a basic fuchsin method.

e. Oil immersion is not required for examination of fluorochrome-stained smears.

4. An important criterion for consideration in preparing smears for acid-fast staining is the:

a. thickness and size of the smear.

b. brand of slides used.

c. shape of the smear on the slide.

d. barrier and excitation filters used on the microscope.

5. One can prevent false positive acid-fast smear results by:

a. not using water that has been stored in large quantities for prolonged periods of time for rinsing slides and preparation of staining reagents.

b. conducting periodic monitoring and quality control of laboratory water distribution systems for the presence of acid-fast organisms.

c. positioning slides so they do not touch during the staining process.

d. a and c only.

e. a, b and c.

6. A counter stain:

a. is used to provide a contrast between acid-fast organisms and other background material.

b. may quench fluorescence of background material so that fluorescing acid-fast organisms are more easily seen by the microscopist.

c. is applied after the decolorization step.

d. a, b and c.

e. b and c only.

7. The counter stain recommended for use with Auramine O-Rhodamine B is:

a. potassium permanganate.

b. methylene blue.

c. acridine orange.

d. malachite green.

8. Important criteria in a quantitative reporting system for fluorochrome acid-fast microscopy include:

a. number of organisms seen per field.

b. magnification at which the smears were examined.

c. number of microscopic fields examined.

d. a, b and c.

e. a and c only.

Please indicate whether the following statements are true (T) or false (F).

9. T F ____ Positive and negative control slides assess the quality of staining reagents and let you know if the staining procedure was performed properly.

10. T F ____ Negative control slides may assist in detecting the presence of environmental contaminants in reagent water as well as in rinse water during the staining procedure.

11. T F____ Negative control slides will assist in finding the microscopic plane of focus when examining patient smears.

12. State the color of acid-fast bacilli and smear background for two fluorochrome and counter stains commonly used in acid-fast microscopy.

13. What is the minimum number of microscopic fields recommended to be examined at 200x and 250x magnifications in fluorochrome acid-fast microscopy?

14. What is the minimum number of microscopic fields to be examined at 400x and 450x magnifications?

15. List two acceptable methods for fixation of smears.

ANSWERS TO THE QUIZ

1. **b**
2. **c**
3. **a**
4. **a**
5. **e**
6. **d**
7. **a**
8. **d**
9. **T**
10. **T**
11. **F**
12. The color of acid-fast organisms with **Auramine O** stain is green-yellow. Acid-fast organisms will appear primarily yellow to orange when **Auramine O-Rhodamine B** is used. Some may appear yellow-green.

    When potassium permanganate is used as a counter stain with either **Auramine O** or **Auramine O in combination with Rhodamine B,** fluorescence of human cells, bacteria and other debris is quenched and the background of the smear appears dark.

    Acridine Orange is used primarily with **Auramine O** as a counter stain. Background material such as non acid-fast bacteria, yeasts, human cells and other debris will stain yellow to orange with **Acridine Orange**.

13. The minimum number of microscopic fields to examine when viewing slides at magnifications of 200x or 250x is 30 fields.

14. The number of fields to examine when viewing slides at 400x is 55, at 450x the minimum number of fields is 70.

15. Two acceptable methods for fixation of smears for acid-fast staining are as follows: The electric slide warmer may be used at 65-75 ^{B}C for a minimum of two hours. An alternate method is to pass the dried slide, smear facing upward, through the blue cone of a burner flame 2-3 times.

REFERENCES AND INTERNET RESOURCES

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14. Package Insert: TB Auramine O, TI No. 40086- 1997. REMEL, Lenexa, KS.

15. Package Insert: TB-Quik-Stain 88-2020-1, 1998, Becton Dickinson Company, Sparks, MD.

Internet Resources

http://www.omegafilters.com

http://www.cdc.gov/nchstp/tb/links.htm

http://www.phppo.cdc.gov/dls/lta/afb_fluoromx.asp