ACID FAST BACILLUS (AFB) STAINING

1.0 PRINCIPLE

The acid-fast smear plays an important role in early diagnosis of mycobacterial infections because most mycobacteria grow at a slow rate. The acid-fast stain serves to confirm the acid-fast nature of recovered organisms and monitor patients on anti-mycobacterial therapy. Acid-fast bacilli are difficult to stain due to lipid content of cell wall. The exact nature of this unique staining reaction is not completely understood, but is believed that phenol dissolves the lipid sufficiently to allow penetration of the primary stain. The cell wall retains primary stain even after exposure to acid-alcohol decolorizing agent. Resistance to decolorizing by acid-alcohol is required for an organism to be termed acid fast.

A counter stain is employed to highlight stained organisms for recognition. In the Ziehl-Neelsen carbofuchsin procedure, acid-fast organisms appear red, and in the fluorochrome procedure, Auramine O, acid-fast organisms fluoresce yellow to orange (color may vary with filter system used). The acid-fast smear should not be used in place of culture. It is an adjunct, since studies have shown that 5,000 to 10,000 bacilli per mL of sputum are required for recognition by direct microscopy; culture detects as few as 10 to 100 viable mycobacteria.

2.0 SPECIMEN

Specimens recovered from culture or made from concentration procedure spread over approximately a 2 cm^2 area on a glass slide with a disposable pipette.

3.0 REAGENTS, SUPPLIES, EQUIPMENT

3.1 REAGENTS

(see **Appendix A** for formulas and preparation instructions)

3.1.1 Carbolfuchsin

3.1.1.1 Ziehl-Neelsen

3.1.1.2 Decolorizing agent, 3% acid-alcohol

3.1.1.3 Counterstain, methylene blue

3.1.2 Flurochrome

3.1.2.1 Auramine O

3.1.2.2 Decolorizing agent, 0.5% acid-alcohol

3.1.2.3 Counterstain, potassium permanganate

3.2 SUPPLIES

3.2.1 Frosted End Slides, stock drawer #19

3.2.2 Forceps

3.2.3 Glass Pasteur pipettes

3.2.4 Disposable needles, sterile

3.2.5 Filter paper, cabinet #1

3.2.6 Staining Racks, cabinet #6

3.3 EQUIPMENT

3.3.1 Striker, cabinet #1

3.3.2 Butane fuel burner, cabinet #1.

NOTE: ***Allow burner to burn for 60 seconds on low flame prior to tilting or tipping***.

3.3.3 Slide heater, set at 67°±5°C

4.0 CALIBRATION

There is no calibration required for this procedure.

5.0 QUALITY CONTROL

A positive and negative control slide must be included with each run.

5.1 POSITIVE CONTROL SLIDE PREPARATION

5.1.1 Label 25 clean slides on metal tray with ‘Positive Control’.

5.1.2 Using a sterile needle, add small amount of inoculum from a *Mycobacterium gordonae* slant to 4mL sterile water.

5.1.3 Place one drop suspension in middle of the slide.

5.1.4 Spread drop over approximately 2 cm^2 area.

5.1.5 Allow to air dry in BSC.

5.1.6 Heat fix at 67°C on slide heater for 1½ hours.

5.1.7 Store in QC box in office.

5.2 NEGATIVE CONTROL SLIDE PREPARATION

5.2.1 Label 25 clean slides on metal tray with 'Negative Control'.

5.2.2 Using a sterile needle, add small amount of inoculum from a *Escherichia coli* slant and a Candida albicans slant to 4mL water.

5.2.3 Place one drop suspension in middle of slide.

5.2.4 Spread drop over approximately 2 cm^2 area on a glass slide.

5.2.5 Allow to air dry in BSC.

5.2.6 Heat fix at 67°C on slide heater for 1½ hours.

5.2.7 Store in QC box in office.

5.3 CONTROL SLIDE EVALUATION

New control smears should be reviewed before patient smears are read to confirm that Mycobacteria stain acid-fast.

5.3.1 EXPECTED RESULTS

5.3.1.1 Carbolfuchsin (Ziehl-Neelsen)

Positive: red or magenta against a blue background

Negative: blue background

5.3.1.2 Fluorochrome (Auramine O)

Positive: yellow fluorescence

Negative: no fluorescence

5.3.2 Record control slide results on worksheet and in STARLiMS. If acceptable, read patient smears. If control slides are unacceptable, review procedures and reagent preparations, and remake slides.

5.3.3 UNACCEPTABLE CRITERIA FOR CONTROL SLIDES

5.3.3.1 Carbolfuchsin (Ziehl-Neelsen)

a. Positive control is not stained red.
b. Negative control remains red after decolorization.
c. Background is not properly decolorized.

5.3.3.2 Fluorochrome (Auramine O)

a. Negative control fluoresces.
b. Positive control does not fluoresce or is dull.
c. Background is not properly decolorized or fluoresces.

NOTE: When problem is resolved, re-make all patient slides and re-stain problem run.

6.0 PROCEDURE

6.1 PREPARATION OF SMEARS

NOTE: <u>Before preparing smears for staining, add a run for Carbolfuchsin and/or Fluorochrome in STARLiMS. See Appendix B for instructions.</u>

6.1.1 CONCENTRATED OR UN-CONCENTRATED SPECIMENS

6.1.1.1 Label new, unscratched slide with patient number.

6.1.1.2 Transfer one drop of patient specimen to slide using a pipette.

6.1.1.2.1 For concentrated specimens, use sediment from concentration process.

6.1.1.2.2 For unconcentrated specimens, use bloody or necrotic material when present.

6.1.1.3 Smear specimen on slide over approximately 2 cm^2 area on a glass slide. **Do not make more than one smear per slide.**

6.1.1.4 Place pipette in discard can; use new pipette for each specimen.

6.1.1.5 Allow smear to air dry in BSC.

6.1.1.6 Heat fix slides on slide warmer for 1½ hours at 67°±5C.

<u>NOTE</u>: Heat fixing may not kill all Mycobacteria. Carefully handle and discard in a biohazard receptacle following examination.

6.2 STAINING PROCESSES

6.2.1 ZIEHL-NEELSEN (hot Carbolfuchsin)

6.2.1.1 Place strip of filter paper (approximately 2x3 cm) on slide.

6.2.1.2 Flood paper strip with Ziehl-Neelsen carbolfuchsin stain.

6.2.1.3 Using butane burner, heat paper slowly until steaming.

6.2.1.4 Allow stain to stand for 5 minutes.

<u>NOTE</u>: Add more stain but do not reheat if the smear dries.

6.2.1.5 Use forceps to remove paper strip from slide and discard strip.

6.2.1.6 Rinse slide thoroughly with water.

6.2.1.7 Flood slide with 3% acid-alcohol decolorizer for 2 minutes.

6.2.1.8 Rinse slide with water and drain excess water.

6.2.1.9 Flood slide with counterstain (methylene blue) for 1 minute.

6.2.1.10 Rinse slide with water, drain excess water.

6.2.1.11 Wipe back of slide and allow to air dry. **Do not blot.**

6.2.1.12 Examine smear with 50x oil immersion objective.

6.2.2 FLUOROCHROME (Auromine O)

<u>CAUTION</u>: Use gloves to protect skin; cancer causing agent.

6.2.2.1 Flood slide with fluorochrome stain (Auramine O).

6.2.2.2 Allow smear to stain for 15 min.

<u>NOTE</u>: Be sure stain stays on smear. Do not heat or use paper strips.

6.2.2.3 Rinse slide with water. Drain water from slide.

6.2.2.4 Flood slide with 0.5% acid-alcohol.

6.2.2.5 Allow smear to decolorize for 2 min.

6.2.2.6 Rinse slide with water. Drain water from slide.

6.2.2.7 Flood slide with counterstain (potassium permanganate).

6.2.2.8 Allow smear to counterstain for 2 min.

<u>NOTE</u>: Timing is critical during counterstaining step with potassium permanganate. Counterstaining for a longer time may quench fluorescence of acid-fast organisms.

6.2.2.9 Rinse slide with water. Drain water from slide.

6.2.2.10 Allow smear to air dry. **Do not blot.**

6.2.2.11 Examine smear with fluorescence microscope as soon as possible with a 40x objective for screening. (A 100x oil immersion objective may be needed to confirm characteristic morphology of fluorescing bacilli.)

<u>NOTE</u>: Fluorochrome-stained slides may be directly re-stained with Carbolfuchsin staining procedures. This may be done to confirm a positive fluorochrome and to study organism morphology.

6.3 SMEAR EXAMINATION

6.3.1 METHOD OF EXAMINATION

6.3.1.1 Screen carbolfuchsin-stained smears with a 50x oil immersion objective. Evaluate all positives on 100x.

6.3.1.2 Screen fluorochrome-stained smears with a 40x objective.

6.3.1.3 A minimum of 100 fields must be examined before smear is reported as negative.

6.3.1.4 Three passes along long axis of slide or nine passes along short axis of slide should provide ample area

for reading and eliminate possibility of reading same area more than once.

NOTE: ***No matter which procedure for enumerating acid-fast bacilli on smears is chosen, if only one or two bacilli are seen on an entire smear, this should be noted on log sheet, but not reported.***

6.4 MORPHOLOGICAL CHARACTERISTICS

6.4.1 Acid-fast bacilli are approximately 1 to 10 µm long and appear as slender, rod-shaped bacilli, but may appear curved or bent.

6.4.2 Individual bacteria may display heavily stained areas referred to as beads and areas of alternating stain producing a banded appearance.

6.4.3 Some mycobacteria other than *M. tuberculosis* may appear pleomorphic, ranging in appearance from long rods to coccoid forms, with more uniform distribution of staining properties.

6.4.4 On initial positive smear, check request for current TB medications and call TB control before reporting. If patient is on TB medications or known to TB control, report. If not, remake and re-stain sediment before reporting AFB found on smear.

6.4.5 Confirm positive smears by having them reviewed by another experienced reader.

7.0 CALCULATIONS

There are no required calculations for this procedure.

8.0 RESULT REPORTING

8.1 Upon completion of the staining procedure, record the results onto the worksheet and transfer the results to STARLiMS. See Appendix B for instructions.

8.2 Possible Results for Carbolfuchsin

- AFB found
- No AFB found

8.3 Possible Results for Fluorochrome

A. No AFB found (-)
B. 1-2/300 fields (±)
C. 1-9/100 fields (1+)
D. 1-9/10 fields (2+)
E. 1-9/ field (3+)
F. > 9/field (4+)

8.4 Only Fluorochrome stain reports are delivered separately by fax to the submitter upon completion of the test (within 24 hours of receipt). Final reports are managed by Central Accessioning and delivered either by fax or mailed hard copy depending on how the submitter has requested their reports be delivered.

9.0 PROCEDURE NOTES

9.1 Avoid under decolorization with acid-alcohol. Organisms that are truly acid fast are difficult to over decolorize.

9.2 Avoid producing thick smears which may interfere with proper decolorization, and counterstain may hide the presence of acid-fast bacilli. Additionally, thick smears may flake, resulting in loss of smear material and possible transfer of material to other slides

9.3 If an unusual number of smear-positive, culture-negative results occur, be aware of potential sources of false-positive smears.

9.3.1 Water and water reservoirs may be contaminated with acid-fast organisms.

9.3.2 Transfer of material from slide to slide during staining process may occur. Be sure to keep slides separated during all steps of staining procedures.

9.3.3 Transfer of acid-fast organisms in oil used for oil immersion microscopy may occur. Be sure to wipe oil from lens between slides.

9.4 Timing is critical during the counterstaining step with potassium permanganate. Counterstaining for a longer time may quench fluorescing bacilli.

9.5 To avoid fading of fluorescence, smears for fluorochrome staining should not be stained unless they can be observed within 24 h. If a problem is encountered, fading can be minimized by storing slides in the dark overnight at 2 to 8°C.

9.6 Strong counterstaining may mask the presence of acid-fast bacilli.

9.7 The following safety concerns should be considered

9.7.1 Handle slides carefully. Heat fixing may not kill all mycobacteria. Be sure to dispose of all slides in a biohazard container.

9.7.2 Avoid aerosols by performing all vortexing and mixing of specimens in tightly capped containers.

9.7.3 Use disposable bacteriological loops.

9.7.4 Thoroughly clean all work surfaces with a disinfectant solution before and after use. Work over lab mat soaked with disinfectant.

9.7.5 On initial training, all personnel should review the MSDS on all reagents in order to be aware of potential hazards.

9.7.6 Remember to always add acid slowly and carefully to alcohol, not vice versa.

10.0 LIMITATIONS OF THE PROCEDURE

10.1 Organisms other than mycobacteria may demonstrate various degrees of acid fastness. Such organisms include ***Rhodococcus* spp.**, ***Nocardia* spp.**, ***Legionella micdadei*,** cysts of **Cryptosporidium spp.** and **Isopora spp**.

10.2 Rapidly growing mycobacteria may vary in their abilities to retain acid-fast stains. Most will not appear fluorescent on fluorochrome-stained smears.

11.0 REFERENCES

H.D. Isenberg 1992. Clinical Microbiology Procedures Handbook p 3.5.1-3.5.11 American Society for Microbiology, Washington, D.C.

APPENDIX A
REAGENT FORMULAS

CARBOLFUCHSIN

Ziehl-Neelsen
Remel Catalog #40202; Hardy Diagnostics Catalog #CF008
Store at room temperature until expiration.

Unopened bottles are stored in cabinet #5; opened bottles are stored in cabinet #2.

Decolorizing agent, 3% acid-alcohol
Store at room temperature.

15mL concentrated hydrochloric acid (HCl)
485mL of 95% ethyl alcohol
Prepare by carefully adding HCl to the ethyl alcohol.

Label bottle with name of reagent and dates of preparation and expiration; expires in 3 months.

Large quantities are stored in the fire cabinet; open bottles are stored in cabinet #2.

Counterstain, methylene blue
Store at room temperature. Made in Media Section.

2.4g methylene blue
300mL ethyl alcohol
700mL water
Prepare by adding the ethyl alcohol to the water and then add the methylene blue. Stir with stir bar for at least one minute and dispense in 250mL bottles.

Label the bottles with name of reagent and dates of preparation and expiration; expires 3 months.

Unopened bottles are stored in cabinet #5; open bottles are stored in cabinet #2.

APPENDIX A
REAGENT FORMULAS (cont.)

FLUROCHROME

Auramine O
Remel Catalog #40186; Hardy Diagnostics Catalog #418D8

Store at room temperature until expiration.

Unopened bottles are stored in cabinet #5; opened bottles are stored in cabinet #2.

Decolorizing agent, 0.5% acid-alcohol
Store at room temperature.

4.75mL of concentrated hydrochloric acid (HCl)
700mL of 95% ethyl alcohol
250mL of distilled water
Prepare by adding the HCl to the ethyl alcohol and then add the water.

Label with name of reagent and dates of preparation and expiration; expires 3 months.

Counterstain, potassium permanganate
Store at room temperature for a maximum of 3 months. Made by Media Section.

5g potassium permanganate
1000mL water
Prepare by adding the potassium permanganate to the water and stir with stir bar for a minimum of one minute. Dispense in 250mL bottles.

Label bottle with name of reagent and dates of preparation and expiration. Expires 3 months.

Unopened bottles are stored in cabinet #5; opened bottles are stored in cabinet #2.

APPENDIX B
STARLiMS ENTRY INSTRUCTIONS

ADDING RUNS TO STARLiMS

A. To add a run for **Carbolfuchsin:**
1. Assign Work/Micro Run Creation/Tuberculosis/By Plate/Carbolfuchsin.
2. Add Run.
3. Add the samples to Run using ► button.
4. Add QC to Run.
5. Highlight Positive Control.
6. In Reagents box double-click Inventory ID box.
7. Select correct reagent from list, Choose. Repeat for each reagent.
8. In runs box double-click Assigned To box, choose User ID, OK.
9. Send Run to Enter Results button.
10. Confirm box will appear, select Yes.
11. **Print Work list button.**
12. Use printer icon on top bar when sheet is generated to print work list
13. Close window, back.

B. To add a run for **Fluorochrome:**
1. Assign Work/Micro Run Creation/Tuberculosis/By Step/Microscopy.
2. Add Run.
3. Add the samples to Run using ► button.
4. Add QC to Run.
5. Highlight Positive Control.
6. In Reagents box double-click Inventory Id box.
7. Select correct reagent from list, Choose. Repeat for each reagent.
8. In Runs box double-click Assigned To box, choose User ID, OK.
9. Send Run to Enter Results button.
10. Confirm box will appear, select Yes.
11. **Print Work list button.**
12. Use printer icon on top bar when sheet is generated to print work list.
13. Close window, back.

APPENDIX B
STARLiMS ENTRY INSTRUCTIONS (cont.)

ADDING RESULTS TO STARLiMS

A. Add the results for Carbolfuchsin
   1. Results Entry/Micro Batch Results Entry/Tuberculosis/By Step/Carbolfuchsin.
   2. Enter the results for any positive smears by double-clicking in results column.
   3. Enter the results for QCs and Negative smears by: Update Default QC result button, All Samples.
   4. Finish Carbolfuchsin, All Samples.

B. Add the results for Fluorochrome Smear
   1. Results Entry/Micro Batch Results Entry/Tuberculosis/By Step/Microscopy.
   2. Enter the results for any positive smears by double-clicking in results column.
   3. Enter the results for QCs and Negative smears by: Update Default QC result button, All Samples.
   4. Finish Microscopy, All Samples. Select yes to approve run.
   5. Samples will go to Approve automatically.

C. Approval
   1. Preliminary Culture Report
   2. Highlight each sample and check results.
   3. Select Batch Release Clinical
   4. Release Reports