



Institut Pasteur

centre d'enseignement

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TUBERCULOSIS

PROTOCOL BOOK

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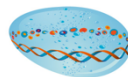
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Institut Pasteur



Milieu Intérieur
Vers une médecine personnalisée



international network
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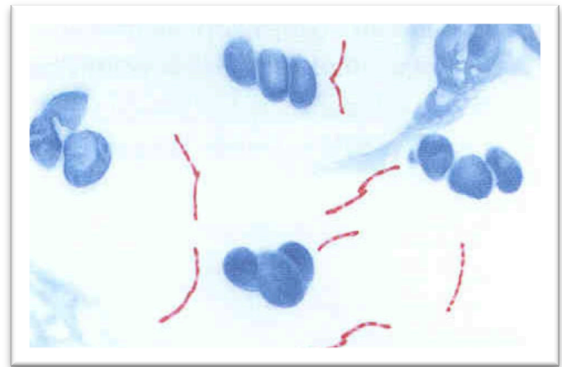
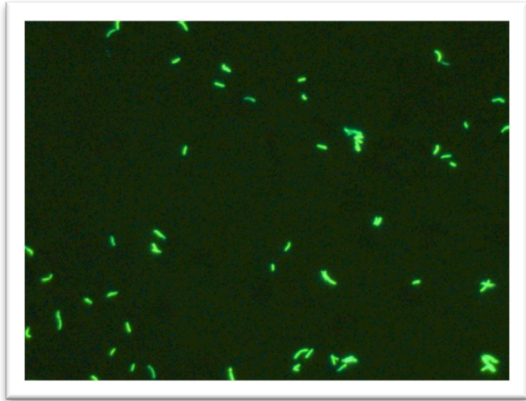


The Union
International Union Against
Tuberculosis and Lung Disease
Health solutions for the poor



AUTREMENT RÉACTIFS

Staining and Microscopy of Mycobacteria



Purpose

The purpose of AFB microscopy is to detect acid-fast bacilli (AFB) by microscopic examination of clinical specimens and cultures. Both living and dead (viable and non-viable) bacilli will stain and be counted. A semi-quantitative grading system is used to report the number of AFB observed in stained smears. Sputum smears are prepared from specimens. The smears are stained with fluorescent stains, either auramine O or auramine/rhodamine. The Ziehl-Neelsen stain can be used to confirm fluorescent smear results. The Ziehl-Neelsen stain is used to confirm the presence of AFB in positive cultures (MGIT, LJ).

Staining of mycobacteria or Acid Fast Bacilli (AFB) is based upon the characteristic structure of the mycobacteria walls that hampers discolouring agent penetration. This property allows AFB to keep the dye staining after discolouring with acid and alcohol. Other bacteria (non-AFB) and cell elements are counterstained by an against-dye.

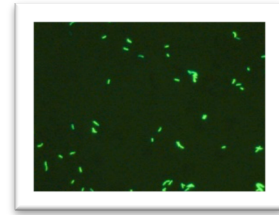
Fluorescence staining of clinical specimen allows to detect 10^3 mycobacteria/mL. It has to be confirmed by Ziehl-Neelsen (ZN) staining.

For details about the organization of microscopy laboratory see

TECHNICAL GUIDE Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries. Fifth ed. 2000. International Union Against Tuberculosis and Lung Disease

http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf

Fluorescence Staining



Principle

RAL Auramine Staining Kit is one of the kits that allows screening of the slide using a quick fluorescence detection of mycobacteria or Acid Fast Bacilli (AFB).

For the fluorochrome stain, the principle of stain, decolorizer and counterstain is the same as for Ziehl-Neelsen staining. With auramine O stain, organisms fluoresce bright yellow, non-specific debris stains pale yellow, and the background is almost black.

Fluorochrome stain is more sensitive than Ziehl-Neelsen because the smear can be examined under a lower power, thus more fields can be read in the same amount of time, and the bacilli stand out brightly.

1. Equipment, materials and reagents

Equipments

- Fluorescence Microscope with Auramine filter set

Materials

- Tuberculocidal disinfectant
- Waste receptacles (including splash proof receptacle for liquids)
- Discard bucket with biohazard bag insert, containing appropriate disinfectant
- Paper towel soaked in appropriate disinfectant
- Microscope slides, frosted at one end, new and clean
- Pencil for labeling slides
- Hot plate or slide warmer
- Bunsen burner (or spirit lamp)
- Sterile, transfer pipettes (individually wrapped)
- Sterile loop or disposable applicator stick
- Staining sink or bucket
- Staining rack
- Forceps
- Timer
- Vortex mixer

Reagents

1. Non specific reagents

- Tap water wash bottle

- Absolute alcohol

2. RAL Auramine Staining KIT description (Ref.: 359500-0000)

- Carbolic Auramine (Fluorescence Technique) 1 x 1000 mL
- Discolouring Solution (Fluorescence Technique) 1 x 1000 mL
- Methylene Blue 0.3% (Fluorescence Technique) 1 x 1000 mL

The kit allows staining between 600 and 800 slides.

Processing Time: 24 minutes

3. Available Refills:

- Carbolic Auramine (Fluorescence Technique) Ref. 361435-1000 mL
- Discolouring Solution (Fluorescence Technique) Ref. 320810- 1000 mL
- Methylene Blue 0.3% (Fluorescence Technique) Ref. 365350- 1000 mL

Recommendations and/or Note of use:

For professional, In vitro use only.

The collection and processing of chemical biological waste must be conducted by specialized and registered companies

Auramine toxicity : acute oral and dermal toxicity, carcinogenicity (safety data RAL 310220)

Storage: 15 – 25 °C away from light

2. Specimen preparation

Specimen must be treated in accordance with procedures available in the laboratory and promulgated by national authorities.

a) Preparation of smears from processed sputum

1. Label the frosted end of the slide in pencil with the laboratory accession number, screening ID number and/or subject ID number, visit number, sputum specimen number (#1 or #2, unless specimen is from V2 or V3), and date
2. Draw a circle in the center of the slide with a diamond tip
3. Use a transfer pipette to place ~100 µl (2 drops) from the specimen (10 µl for the workshop) onto the slide, spreading over an area approximately 1 x 2 cm. Air-dry the smear.
4. Place the slides on a hot plate or slide warmer at a temperature between 65°C to 75°C until the slide is dry. Do not expose slides to UV light.
5. Work systematically through the samples with slides on one side and the discard bucket in close proximity. Remember to open only one specimen tube at a time. Dispose of the transfer pipette into the biohazard discard bucket.

b) Staining procedure

Wear gloves

1. Place the slide on a staining rack with fixed smear on top, at least 1 cm apart
2. Flood the slide with absolute alcohol during 10 minutes or heat fix on a hot plate or slide warmer at a temperature between 65°C to 75°C for at least 2 hours (longer time is preferable)
3. Stain by flooding the slide with Carbolic Auramine (Bottle n) and let stand during 20 minutes
4. Rinse the stain away with tap water and tilt slide to drain. Water must be Auramine free.
5. Discolour by flooding the slide with Discolouring Solution (Bottle o) during 3 minutes
6. Wash off the Discolouring Solution with tap water
7. Counterstain by flooding the slide with Methylene Blue 0.3% (Bottle p) during 1 minute
8. Wash off the Methylene Blue with tap water
9. Air dry and keep away from light

Protect smears from light and examine immediately using the fluorescent microscope. If unable to read right away, place slides in covered box

3. Results

The microscopic examination is usually performed with x20 and x40 dry objectives, without coverslip, using a Fluorescence Microscope with Auramine filter set. Acid-Alcohol Fast Bacilli (AFB) : fluorescent green-yellow Background of the preparation: black-blue

Each time fluorescent organisms are detected, it is necessary to confirm the presence of Acid Fast Bacilli (AFB) by a ZN staining. In practice, it is possible to stain the same slide for both stainings.

4. References

- GROSSET J., CATALAN F., LEVENTIS S., Le problème actuel des mycobactéries, Biologiste et Praticien, n°39, 1979, p. 1-22
- TECHNICAL GUIDE Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries. Fifth ed. 2000. International Union Against Tuberculosis and Lung Disease

http://www.ups.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf

- GLI Mycobacteriology Laboratory Manual, First Ed 2014
<http://www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf>
- Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. K R Steingart, M Henry, V Ng, P C Hopewell, A Ramsay, J Cunningham, R Urbanczik, M Perkins, M Abdel Aziz, M Pai, The Lancet Infectious Diseases. 2006; 6, 570–581

Cold Ziehl-Neelsen Staining



Principle

The Ziehl-Neelsen method uses a carbol fuchsin stain, acid alcohol decolorizer, and methylene blue counterstain. Acid-fast organisms stain red, while the background of debris stains blue. The ZN stain confirms the acid-fast property of mycobacteria.

QUIK-TB Kit is a Ziehl-Armand staining that is a cold variation of the Ziehl-Neelsen technique of mycobacteria or Acid Fast Bacilli (AFB) staining. The use of Armand Solution offers the advantage that all bacteria are discoloured, except the mycobacteria, and the background of the coloration is colored at the same time.

It is possible to stain the same slide for both stainings (first Auramine Staining and then stained with ZN).

1. Equipment, materials and reagents

Equipments

- Optic Microscope

Materials

- Tuberculocidal disinfectant
- Waste receptacles (including splash proof receptacle for liquids)
- Discard bucket with biohazard bag insert, containing appropriate disinfectant
- Paper towel soaked in appropriate disinfectant
- Microscope slides, frosted at one end, new and clean
- Pencil for labeling slides
- Hot plate or slide warmer
- Bunsen burner (or spirit lamp)
- Sterile, transfer pipettes (individually wrapped)
- Sterile loop or disposable applicator stick
- Staining sink or bucket
- Staining rack
- Forceps
- Timer

Reagents

4. Non specific reagents

- Tap water wash bottle
- Absolute alcohol

5. KIT QUICK-TB description (Ref.: 361560-0000)

- Carbolic Fuschin RAL 1 x 125 mL (bottle 1)
- Armand Solution 1 x 125 mL (bottle 2)

The kit allows staining between 125-200 slides.

Processing Time: 7 minutes

For professional, In vitro use only.

Specialized and registered companies must conduct the collection and processing of chemical biological waste

Fushine toxicity: acute oral toxicity, skin corrosion, serious eye damage, and germ cell mutagenicity

Physical hazards: Flammable liquids

Storage: 15 – 25 °C

2. Specimen preparation

Specimen must be treated in accordance with procedures available in the laboratory and promulgated by national authorities.

a) Preparation of Smears from processed sputum

1. Label the frosted end of the slide in pencil with the laboratory accession number, screening ID number and/or subject ID number, visit number, sputum specimen number (#1 or #2, unless specimen is from V2 or V3), and date
2. Draw a circle in the center of the slide with a diamond tip
3. Use a transfer pipette to place ~100 µl (2 drops) (10 µl for the workshop) from the specimen onto the slide, spreading over an area approximately 1 x 2 cm. Air-dry the smear.
4. Place the slides on a hot plate or slide warmer at a temperature between 65°C to 75°C until the slide is dry. Do not expose slides to UV light.
5. Work systematically through the samples with slides on one side and the discard bucket in close proximity. Remember to open only one specimen tube at a time. Dispose of the transfer pipette into the biohazard discard bucket.

b) Preparation of Smears from Positive Cultures

1. Label frosted end of slide in pencil with laboratory accession number, screening and/or subject ID number, sputum specimen number (if applicable), and date.
2. Work in a BSC as described in GLI Mycobacteriology Laboratory Manual, 11/04/2014
3. If examining a positive MGIT culture: vortex tube well, unscrew MGIT tube cap and sample an aliquot of broth using a disposable pipette. Place ~100 µl (2

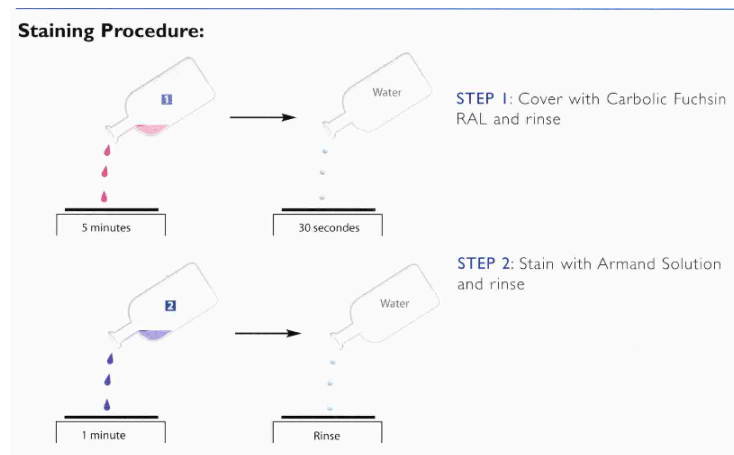
drops) of broth onto the slide, spreading it to cover an area approximately 1 x 2 cm. Dispose of transfer pipette into the biohazard discard bucket.

4. If examining colonies on solid medium, transfer 2 to 3 colonies to the slide, using a sterile loop or disposable applicator stick. Dispose of applicator stick into the biohazard discard bucket.
5. Air-dry smear.

3. Staining procedure

Wear gloves

1. Place the slide on a staining rack with fixed smear on top, at least 1 cm apart
2. Flood the slide with absolute alcohol during 10 minutes or heat fix on a hot plate or slide warmer at a temperature between 65°C to 75°C for at least 2 hours (longer time is preferable)
3. Stain by flooding the slide with Carbolic Fuchsin RAL (bottle 1) during 30 seconds
4. Rinse the stain away with tap water and tilt slide to drain. Water must be Fuchsin free.
5. Cover the slide with Armand Solution (bottle 2) during 1 minutes
6. Wash off the Armand Solution with tap water
7. Air dry



4. Results

The microscopic examination is usually performed with x100 oil objectives, without coverslip.

Acid-Alcohol Fast Bacilli (AFB): pink

Background of the preparation: black-blue

A negative result should follow from reading 200 (or 100) microscopic fields.

A positive result must provide quantitative data (table).

The observation of a single bacillus on a given slide is a dubious result and should always lead to a new investigation of another sample.

IUATLD-recommended grading of sputum smear microscopy results	
AFB counts	Recording/reporting
No AFB in at least 100 fields	0/negative
1 to 9 AFB in 100 fields*	Actual AFB counts**
10 to 99 AFB in 100 fields***	+
1 to 10 AFB per field in at least 50 fields**	++
>10 AFB per field in at least 20 fields***	+++
* A finding of 1 to 3 bacilli in 100 fields does not correlate well with culture positivity. The interpretation of the significance of this result should be left to the NTP and not to the microscopist. It is recommended that a new smear be prepared from the same sputum and re-examined	
** The reporting of actual AFB counts is recommended to allow a competent authority to determine whether the number fits the TB case definition of the NTP	
*** In practice most microscopists read a few fields and confirm the finding by a quick visual scan of the remaining fields	

5. References

- TECHNICAL GUIDE Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries. Fifth ed. 2000. International Union Against Tuberculosis and Lung Disease

http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/IUATLD_afb%20microscopy_guide.pdf

- GLI Mycobacteriology Laboratory Manual, First Edition, April 2014

A publication of the Global Laboratory Initiative a Working Group of the Stop TB Partnership <http://www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf>

- AUBERT E., "Cold" Stain for Acid-Fast Bacteria, Canad. J. Public Health, N°41, 1950, p. 31-32.
- CLARK G., Staining Procedures, Williams and Wilkins, 4th ed., 1981, p. 384-385.