1. Fluorescence Microscopy

2. Objectives & Scope

The Directly Observed Treatment Short-course (DOTS) strategy uses acid-fast bacilli (AFB) microscopy as the first recommended technique for diagnosis of tuberculosis as well as follow-up of patients during treatment. The auramine staining technique applies to fluorescence microscopy.

Fluorescent acid-fast staining allows more rapid examination of smears compared to the basic fuchsin procedures and is particularly indicated for high-volume laboratories. It may also be more sensitive for paucibacillary specimens.

This Standard Operation Procedure (SOP) is applicable to all employees of the National Tuberculosis Reference Laboratory, NTRL.

3. Abbreviations and definitions

For general abbreviations, definitions and terms refer to quality manual chapter 1 “General”.

- NTRL National Tuberculosis Reference Laboratory
- NTP National Tuberculosis Programme
- N/A Not applicable
- QO Quality Officer
- SOP Standard Operating Procedure
- LED Light Emitting Diode
- ZN Ziehl-Neelsen
- AFB Acid Fast Bacilli
- BSC Biosafety cabinet

4. Tasks, responsibilities and accountabilities

For general authorizations refer to the Authorization Matrix.

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<thead>
<tr>
<th>Task</th>
<th>Authorized</th>
<th>Responsible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of reagents</td>
<td>LT</td>
<td>LM</td>
</tr>
<tr>
<td>Preparation of AFB Smears</td>
<td>LT</td>
<td>LM</td>
</tr>
<tr>
<td>Stain procedure</td>
<td>LT</td>
<td>LM</td>
</tr>
<tr>
<td>Interpreting of Results</td>
<td>LA</td>
<td>LM</td>
</tr>
<tr>
<td>Reporting of Results</td>
<td>DC</td>
<td>LM</td>
</tr>
</tbody>
</table>

5. Principle

This SOP describes fluorescence acid-fast bacilli (AFB) microscopy in the National Tuberculosis Reference Laboratory.

The property of acid-fastness of mycobacteria is based on the presence of mycolic acid in their cell wall. The primary stain (auramine) binds cell wall mycolic acids. Intense decolourization (strong acid) does not release the primary stain.
from the cell wall of AFBs so they will have the fluorescent bright yellow colour of auramine. Counterstain (Methylene blue or Potassium Permanganate) provides contrasting background. Fluorescent stains are usually organic substances which absorb ultraviolet light and remit part of the energy as light of longer wavelength which can be observed through the eyepiece as fluorescence. When exposed to ultraviolet light, the fluorescent bacilli are perceived as brightly coloured organisms against a dark background.

6. Safety and environment
6.1 Waste management
Contaminated materials must be discarded in a red autoclave bag and autoclaved before incineration at the end of each day (see P35 Waste Segregation and Disposal and P35A1 Waste Segregation Chart).

6.2 Personal protection
- Surgical masks do not protect against TB infection, because they don’t fit tightly. They may give a false sense of security; TB bacilli pass through the poorly fitting masks.
- Gloves are not required for use in direct smear preparation since TB is acquired by airborne inhalation. Gloves may be used in settings where the incidence of HIV is high and lab personnel may be exposed to HIV when handling specimens. If used, gloves have to be changed at every interruption of activity and discarded as contaminated material. If supply is not adequate to do this, it is wise to reserve gloves for handling blood and other fluids than sputum, to protect against HIV, hepatitis, etc.
- Hand washing and careful techniques should be followed.
- Gowns should be worn in the laboratory, never outside, and be changed on a regular basis.
- For staining procedures, especially for decolourization with acids, protective glasses must be worn.
- Refer to the biosafety manual.

7. Sample
7.1 Sputum
- Decontaminated sputa - concentrated by centrifugation
- Spontaneous sputa. All sputa form suspects, even if they are liquid and clear as water, should be accepted. Patients should be encouraged to collect lower respiratory tract samples.
- Induced sputa. These specimens resemble saliva but have to be processed as adequate specimens.

7.2 Others
- Laryngeal swab, gastric lavages, bronchial washings, brushings and trans-tracheal aspirates.
- Urine
- Body fluids (spinal, pleural, pericardial, synovial, ascetic, blood, pus, bone marrow)
- Tissue biopsies

8. Equipment and supplies
8.1 Smear preparation
- BSC
- Sharps container
- Biohazard bag for waste disposal
New slides
Pencil for frosted and diamond pencil for unfrosted slides
Slides holder
Wooden sticks or disposable loops or Pasteur pipettes
Alcohol sand jar
Cotton wool (absorbent)
Metal rod (for flaming)

8.2 Staining
- Forceps
- Slides staining rack
- Timer
- Bunsen burner

8.3 Microscopy
- Fluorescent microscope
- Lens cleaning solution
- Lens cleaning paper
- Slide box

9. Reagents and chemicals
- Staining solution: Auramine O stain (0.1% Auramine stain)
- Decolorizing solution: Acid-alcohol (0.5% HCl in 70% Alcohol)
- Counterstaining solution: Methylene blue 0.125% or Potassium Permanganate 0.5%

10. Quality Control
10.1 Internal Quality Control
1. Blinded Positive and Negative control slides are made and kept in a slide box by the Laboratory supervisor. The Supervisor also keeps the blind key.
2. One positive and one negative control slide (coded and blinded) must be stained on a daily basis together with the patients’ slides to control the stains and the staining procedure. Control slides are stained at least once a day as long as the staining reagents are not changed.
3. If fresh stains are prepared later in the day and are to be used immediately, then the positive and negative control slides must be stained and examined again.
4. Stained Positive and negative control slides should be examined first before examining patients’ slides. After examination, results are forwarded to the laboratory supervisor for verification. The results of quality control slides must meet the expected results to pass, otherwise quality control fails.
5. If quality control slides fail (do not yield expected results), repeat staining the entire batch of slides stained together with the controls, and fill in a Nonconformity Form.

NOTE: A positive control slide still fails if the quantification is one scale apart from the expected result, e.g. if expected result is 1+ and the result obtained is 3+, quality control fails and should be repeated.
10.2 Internal QC Indicators
Laboratory performance is monitored on a monthly basis by counting and plotting:
- Total number of smears examined
- Number of smears examined by each technologist
- Positivity rate
- Positive cases detected
These indicators reveal an early warning of problems. These will indicate the need for corrective actions. They contribute to staff motivation and self-reliance.

10.3 External Quality Assurance
1. Blinded rechecking of slides should be done monthly in accordance to lot quality assurance checking methods as described in SOP P7 Blinded Rechecking of Slides.
2. Inter-laboratory slides will be read monthly according to SOP P8 Inter-laboratory Comparison.
3. Proficiency testing will be done twice a year according to SOP P9 Proficiency Testing.

10.4 Repeat of samples
If a sample that was referred to the laboratory with a positive smear turns out to be negative at the NTRL, the slide should be re-read. If it remains negative, another smear should be prepared from the sediment. If this turns out to be positive: repeat the whole batch.

11. Procedure
11.1 Preparation of smear
1. Label the slides properly using a unique NTRL register number.
2. Place the labeled slides, the samples and the wire loops/applicator sticks in the BSC.
3. Match each slide with the corresponding sputum or sample container.
4. Proceed to smearing, taking the labeled slides and opening containers one by one; do the smearing from the center of the slide outwards making small coil-like movements.
5. For a direct sputum smear select a small portion of purulent or muco-purulent material with the applicator stick/wire loop and transfer it to the slide; if a stick is used, break it in two pieces and use the ragged ends for dissecting sputum and for smearing.
6. If a smear is prepared after specimen decontamination, the concentrated material has to be transferred to the slide with a sterilized loop or Pasteur/transfer pipette.
7. If a loop is used, it has to be sterilized before re-use by heating until it becomes red-hot.
8. Spread the material carefully over the middle area of the slide equal to about 2x1 cm using repeated coil-like movements, without touching the margins of the slide.
9. Make the smear as even as possible by continuing this process until no thick parts remain. Remove excess material with the second stick and discard in the biohazard bag.
10. The thickness of the smear should be such that a newspaper can be read through the dried smear held about 10 cm above it (slide is translucent).

11. Warm the slides on the Fisher Slide Warmer (60°C to 70°C) in the BSC for at least two hours to dry and fix the smear.

12. If slide warmer is not functional, air dry the smear and when dry, fix the smear by passing the slide three times slowly through the flame of a spirit lamp, or quickly through the flame of a Bunsen burner (until the slide back part is hot but can still be touched without burning), smear upwards; do not over-heat or else the AFB staining will be poor.

11.2 Staining method
1. Place the slides on the staining rack over a sink.
2. It is a must to keep distance of at least 1 cm between every slide. Otherwise there is a possibility that acid-fast bacilli might float off one slide and become attached to the next slide.
3. Cover the smears completely with 0.1% auramine solution (with simultaneous filtration by pouring through a small funnel with filter paper held over the slides).
4. Do not heat.
5. Leave for 15 minutes.
6. Wash the slides well with distilled or running water.
7. Pour the acid alcohol solution over the slides.
8. Allow to react for 3 minutes.
9. Gently rinse each slide with distilled or tap water.
10. Repeat decolourization if macroscopically visible stains are still present.
11. Flood smear with 0.5% Potassium permanganate counterstain solution for 1 minute. Time is critical because counterstaining for a longer time may quench the acid-fast bacilli fluorescence.
12. Wash off with distilled or running water.
13. Stand the slide on edge to drain, and air dry on the slide rack out of the direct sunlight.

If using the counter stain solution the stained smear should show a light blue color. If the smear is dark blue, it usually indicates that the smear is too thick or over-counterstained.

11.3 Reading, recording and reporting
11.3.1 Reading
1. Keep stained smears in the dark (in a box or folder) until reading and read as soon as possible (within 24 hours) since fluorescence fades quickly.
2. Use objective 40x for scanning and confirmation.
3. Scan the stained smear systematically from one side to the other.
4. One length has to be scanned before reporting a negative result.
5. Acid-fast bacilli appear bright yellow against the dark background material.
6. With good staining (always check first a freshly stained positive control), there may still be fluorescing (sometimes green) artifacts. Also non-fluorescing bacillary shapes must be considered as artifacts. In case of doubt, change to a higher magnification than used for scanning (i.e. use 40X objective).
7. Store the slides in a slide box according to the study, following the Laboratory Register Number as they will be needed for EQA. Record the position on the worksheet. Do not write the result on the slide.

Note: Acid-fast bacilli appear bright yellow against a dark background. Report as positive for AFB if at least one acid-fast bacillus was seen in a well stained smear, even if you think they might be other mycobacteria than a
tubercle bacilli. Tubercle bacilli are quite variable in shape, from very short fragments to elongated types. They may be uniformly stained or with one or many gaps, or even granular. They occur singly or in small groups (coded), and rarely in large clumps. The typical appearance of bacilli are usually rather long and slender, straight or slightly curved rods.

11.3.2 Recording
Because the fluorochrome-stained smear are examined at 200x to 400x the following should noted:
1. Note that for the per length scales, the surface observed by 20x objective (200x magnification) will correspond to 3 streaks by the high power objective if using ZN and by 40x objective (400x magnification) will correspond to 2 streaks by the high power objective if using ZN).
2. Note that for the per field scales, the number of AFB observed per field under 20x objective can roughly be divided by a factor 10 to make them equivalent to AFB seen by a high power objective if using ZN. While the AFB number observed under 40x objective is divided by 5 to get the equivalent number if seen by high power objective if using ZN.

<table>
<thead>
<tr>
<th>IUATLD/WHO SCALE (1000x field = HPF)</th>
<th>MICROSCOPY SYSTEM USED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRIGHTFIELD (1000x magnification; one length = 2cm = 100 HPF)</td>
</tr>
<tr>
<td>Negative</td>
<td>Zero AFB / 1 length</td>
</tr>
<tr>
<td>Scanty</td>
<td>1-9 AFB / 1 length</td>
</tr>
<tr>
<td>Scanty</td>
<td>10-99 AFB / 1 length</td>
</tr>
<tr>
<td>1+</td>
<td>1-10 AFB / 1 length</td>
</tr>
<tr>
<td>2+</td>
<td>&gt;10 AFB / 1 HPF on average</td>
</tr>
</tbody>
</table>

11.3.3 Reporting
1. The results have to be reported on the ZN/FM microscopy worksheet (See SOP A1F1) and thereafter entered in the laboratory register and the database.
2. Use a red pen for positive results.
3. Use a blue or black pen for Negative results

Report:
Negative result: “acid-fast bacilli were not seen”
Positive result: report the quantification of AFB seen. (It should not be assumed that acid-fast bacilli are tubercle bacilli.) **NEVER REPORT “NO TB” OR THE LIKE.**

### 11.3.4 Slide storage

1. At the end of the day, store the slides in a slide box following the Laboratory Identification Number as they will be needed for EQA. Do not write the result on the slide.
2. Store slides according to the study and record slide positions on the Slide Storage worksheet (See SOP A1).

### 12. Related document

- SOP A1 “Ziehl Neelsen staining and MicroscopySOP

### 13. Related Forms

- SOP A1F1, ZN/FM Microscopy worksheet
- SOP A1F2, Slide Storage worksheet
- SOP A1F3, Flow chart for Smear Microscopy

### 14. References

- International Union Against Tuberculosis and Lung Disease. The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network. Paris; 1998.

### 15. Attachments

- N/A