foundation
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TRAINING MANUAL

FOR FLUORESCENCE-BASED AFB MICROSCOPY

Demonstration Project iLED

Effectiveness of the
Primo Star iLED Microscope
for Detection of Tuberculosis

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Partnering for better diagnosis for all

Based on "Acid-fast Direct Smear Microscopy" Training Introduction Module developed by WHO-CDC-RIT-IUATLD-APHL in 2004

Demonstration Project iLED

Training Manual

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Module 1: Introduction
The Possible Role of LED-based Fluorescence Microscopy in Improving the Global Tuberculosis Situation

Workshop goal

This workshop is intended for microscopists and supervisors participating in the LED demonstration projects. Training participants will already have experience in ZN microscopy and/or conventional fluorescence microscopy.
During this workshop, you will gain the knowledge and skills to perform fluorescence-based smear microscopy using the Primo Star iLED accurately and reliably in a safe, timely, and professional manner.

Certification Criteria

You will be awarded a certificate upon successfully completing the Fluorescence-based AFB Microscopy Training Workshop after you have:

- Attended and actively participated in all theoretical and practical sessions
- Successfully reported the AFB smear results by completing EQA panel test examination

Introduction

The diagnostic technology recommended in current control strategies is sputum microscopy, which was developed in the 1880s and has remained essentially unchanged since then. Smear microscopy is an attractive technology for public-health programmes, as it requires simple equipment only, can be used for more than one purpose, and provides visual evidence not only of tuberculosis, but of bacterial burden, which in most instances is specific enough that no confirmatory testing is needed.[1]

In many countries it is based only on the examination results of Ziehl-Neelsen (ZN) stained smears. However, only tiny amounts of material are examined - as little as 0·2 micro L, and hence bacteria must be present in high concentrations to be visible; typically over 10,000 acid fast bacilli per mL.

Before declaring a smear as negative, a minimum of 100 microscopic fields have to be examined under 100 x oil immersion objective, which takes about 3 to 5 minutes of a technician's time. In busy, overburdened laboratories, smears may not be examined for the recommended amount of time, resulting in lower sensitivity. A re-examination for longer periods proved that the negative smears were, in fact, positive.[2]

Since the first description of the auramine O fluorescence microscopy technique by Hagemann [3] in 1937, numerous reports have confirmed the superior diagnostic performance of fluorescence microscopy, compared with Ziehl-Neelsen (ZN) staining and light microscopy [4–8]. In a systematic review of 18 studies, Steingart et al. [9] reported that fluorescence microscopy of auramine-stained smears provides similar specificity and increased sensitivity (mean improvement of 10%), compared with light microscopy of ZN-stained smears. In addition to increased sensitivity, fluorescence microscopy also allows more-rapid screening of sputum smear specimens. From an operational perspective, this is highly advantageous, particularly when high numbers of samples are screened per day, because the majority of laboratory time is spent confirming negative smear results. According to the International Union Against Tuberculosis and Lung Disease technical guidelines for sputum microscopy, at least 5 minutes of screening time is required to correctly identify a negative smear result when conventional light microscopy is used [10]. However, under routine field conditions, the time spent per slide is often far less than the minimum required. An operational study from Cameroon demonstrated a median sputum microscopy examination time of only 2 min [11]. Almost 50% of the cases detected after a thorough 10-min evaluation were missed during routine investigation [11], which demonstrates the negative impact that conventional light microscopy may have on early case detection and diagnostic delay.

A comparative study reported that a mean time of 1 min to examine a sputum smear with fluorescence microscopy achieved higher sensitivity and equivalent specificity than did conventional light microscopy with an examination time of 4 min [12]. The auramine O stain is inexpensive, and the procedure is easier and quicker than ZN staining. Despite the clear operational advantages of fluorescence microcopy, conventional light microscopy remains the most widely used diagnostic test in resource-limited settings. The main reason that fluorescence microscopy is not used more widely is the need for a more complex and expensive fluorescent microscope, the limited lifespan (typically 200–300 hrs) and the high cost of the short-arc mercury vapor lamp (MVP), which has traditionally been used as the excitatory light source. Repeated on-and-off switching, as may occur with unreliable local power supply, shortens the lifespan even further [13]. In addition, MVPs are energy inefficient and require an extensive power supply; they may also fail catastrophically and release toxic mercury into the environment [13]. Consequently, fluorescence microscopes provided by donor agencies often fall into disuse because of high maintenance costs [14].

Light-emitting diode (LED) technology provides a cheap and reliable light source with a usable lifespan of 150,000 h; repeated on-and-off switching does not reduce its usable lifespan, and it does not pose a potential toxicity risk [13]. Initial studies indicated that LED fluorescence microscopy, with use of a royal blue LED light, offers a valid alternative to the MVP [13, 14], but data regarding its diagnostic use or operational impact remain limited.

A new generation of fluorescence microscopes has now been developed based on LED technology. A leading microscope manufacturer (Zeiss MicroImaging, Göttingen, Germany), in a joint development agreement with FIND, has developed a fluorescence microscope (Primo Star iLED) [15]. Other LED-based approaches, such as the FRAEN After device, designed to attach to a bright field microscope, are or will become available shortly. One of the major innovations of Primo Star iLED compared to others is the use of ultrabright LED as a reflected light source.

The new microscope has high-quality optics and is very robust (e.g. complete antifungal coating). It allows effortless switching from bright light to fluorescence light and can be battery operated. These innovations, in combination with the affordable price, may allow wide introduction of fluorescence microscopy and gradual replacement of conventional microscopy in the public health sector of resource-limited countries.

In collaboration with National TB Programs and International Partner Organizations, the Foundation for Innovative New Diagnostics (FIND) is undertaking this large-scale demonstration project to explore the feasibility and impact of scaling up use of LED fluorescence microscopes to improve TB control.

These training modules have been developed for the FIND LED demonstration project and are based on the 'Acid-fast Direct Smear Microscopy' Training Modules developed by WHO-CDC-RIT-IUATLD-APHL in 2004. In addition, the training modules developed by Fujiki A [16] and the Central TB Division, DGHS, MoHFW, Government of India, New Delhi [17] helped in developing module six.

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Module 2

Demonstration Project
Primostar iLED
Study Outline

Purpose	To provide you with an overview of the LED demonstration project phases and roles, and your responsibilities during the project
Learning Objectives	At the end of this module, you will be able to ▪ Explain the LED demonstration project phases and the study flow during these phases ▪ Refer to the correct documents to obtain further details
Content Outline	▪ LED demonstration project phases ▪ Study documents
Handout and Exercises	Handout: SOP and Protocol for LED Demonstration Project.

WHAT IS A DEMONSTRATION PROJECT?

Demonstration projects are carried out in the context of routine clinical services provision, either directly by the National TB Program (NTP) or by other agencies working in collaboration with the NTP. These are large studies, with 10,000 or more patients enrolled, are intended to provide the evidence that new tests that perform well in controlled settings can also have an important medical and public health impact when implemented in programmatic settings. Endpoints commonly studied include feasibility of assay implementation, comparative cost of the new versus the old technology, and impact on speed or accuracy of detection and subsequent patient management. The results of demonstration projects are compared against Customer Requirements, as stated by Ministries of Health, WHO and other international technical agencies, donors, and patients. Data from Demonstration projects are compiled, analyzed and presented to WHO for policy recommendation on the use of these tests in high-burden, low-income countries. National TB Programs in countries participating in the demonstration projects for these new tests may of course independently make a policy decision on their use.

LED DEMONSTRATION PROJECT

In collaboration with National TB programs and international organizations, this demonstration project aims at a programmatic implementation and evaluation of the Primo Star iLED fluorescence microscope system in comparison to the existing microscope standard. Participating microscopy centers will be grouped in clusters. Each cluster will consist of one supervisory site and two to three microscopy centers. The supervisory site will be responsible for training, monitoring, rechecking of slides and data management.

Hypothesis:
We postulate that the Primo Star iLED system is a feasible, advantageous and cost-effective replacement for ZN (and, where existing, conventional fluorescence) microscopy in low- to moderate-income laboratory settings. Especially in busy microscopy centers, it will increase the case detection rate while substantially decreasing the daily workload.

Endpoints:
The purpose of this demonstration project is to assess the implementation of Primo Star iLED as a ZN replacement for routine TB diagnosis in low- and moderate-income settings. Specifically, we are interested in the following:

1. To assess the feasibility of implementing Primo Star iLED for TB diagnosis at microscopy centers without prior experience with fluorescence microscopy in low- to moderate-income settings and to identify barriers to implementation
2. To determine the false positivity and negativity rate of LED fluorescence reading compared to a ZN baseline and compared to results from the supervisory site
3. To determine the development of false positivity and negativity rates of LED fluorescence reading over time (with increasing experience)
4. To assess the impact of this implementation on daily workload and case detection rates for low, middle and high-volume settings
5. Determine lab technicians' appraisal of using Primo Star iLED
6. To evaluate detailed costs associated with LED-based fluorescence microscopy in comparison with conventional methods

LED DEMONSTRATION PROJECT PHASES

Study phase	Duration	% slides re-checked	Staining reagents	Microscope for reading	Microscope for re-checking	Patient management	Frequency of retrieving slides /forms	Supervisory visits with checklist	Forms	Data transfer by courier
ZN Baseline	1 month	100%	Routine Zn stain	Conventional Brightfield (1000X)	Conventional Brightfield (1000X)	Based on ZN result of microscopy center	Once every 2nd week	Monthly	1. Result Form: ZN Baseline 2. Rechecking Form: ZN Baseline	At the end of phase
Training	5 days									
Proficiency testing & User appraisal	1 day	100%	For 10 Au and 10 ZN slides	Primo Star iLED (400X) Conventional Brightfield (1000X)	Only for discrepants: Primo Star iLED (400X) Conventional Brightfield (1000X)	-	-	-	1. Proficiency Testing Result Form; 2. User appraisal questionnaire	Scanned by e-mail following day
Validation	Minimum 1 month. Until targets met.	100%	Au staining reagents provided by supervisory site once per month	Primo Star iLED (400X)	Conventional FM (200-250X) (where not available Brightfield after restaining (1000X))	Based on conventional FM result from supervisory site (Brightlight if not available) *! Daily provision of results!*	Daily .	Every 2nd week	1. Result Form: Validation 2. Rechecking Form: Validation	Every 2nd week
Proficiency testing & User appraisal	See above									
Implementation	3 months	As per LQAS	Au staining reagents provided by supervisory site once per month	Primo Star iLED (400X)	Primo Star iLED (400X)	Based on iLED result from microscopy center	Once every 2nd week.	Monthly	1. Result Form: Implementation 2. Rechecking Form: Implementation	Monthly
Proficiency testing & User appraisal	See above									
Continuation	6 months	As per NTP	Au staining reagents by supervisory site	Primo Star iLED (400X)	Primo Star iLED (400X)	Based on iLED result from microscopy center	Monthly	Monthly	Same as implementation	Monthly

Module 3

Use and Maintenance of the Primostar iLED

Purpose	To provide you with an understanding of the components and functionalities of Primo Star iLED, its use and maintenance
Prerequisite Modules	None
Learning Objectives	At the end of this module, you will be able to: ▪ Name the essential components of a microscope and understand their function ▪ Correctly use brightfield and fluorescence applications of Primo Star iLED ▪ Maintain the instrument as per user manual
Content Outline	▪ Microscope components and operation ▪ Microscope maintenance
Handout and Exercises	Exercise: Familiarization with Primo Star iLED by reading of several ZN and fluorescent slides
Appendices	Appendix 1 – Specifications for a LED-based fluorescence microscope Appendix 2 – Questionnaire: User Appraisal of Primo Star iLED

Module 3: Use and Maintenance of Primo Star iLED

LED-based fluorescence microscopy

Replacing light microscopy with fluorescence microscopy would be one of the immediate options to improve the global TB situation.

A systematic review by WHO/TDR and FIND has shown that:

a) Fluorescence microscopy is on average 10% more sensitive than conventional light microscopy. The increased sensitivity is greatest in low grade positives.
b) The specificity is comparable.
c) Reading a fluorochrome stained smear takes only 25% of the time it takes to read a ZN stained smear.

To date, the major constraints to the broader implementation of fluorescence microscopy are the high price for fluorescence microscopes and the lack of robustness and sustainability. Conventional fluorescence microscopes use expensive and very fragile gas discharge lamps (such as Xenon- or Mercury-lamps) with high power consumption and a short lifespan of only 100-200 hours. Furthermore, the acceptability of darkrooms has generally been low.

The recent application of ultra-bright LED (light emitting diode) technology to facilitate inexpensive fluorescence microscopy is a potentially significant advance in TB diagnostics for the following reasons:

- Low cost of ultra-bright LEDs whose lifespan is ≈ 15,000-20,000 hours
- Low power consumption, plus possibility of battery operation
- Enhanced robustness
- No need for air conditioning facility
- No need for a dark room
- Fluorescence stains do not require a heating step
- Diagnostic performance ≥ standard FM
- Decreased technician workload

The Primo Star iLED combines these advantages with high-quality optics. One of its major innovations, compared to others, is the use of ultrabright LED as a reflected light source, permitting effortless switching from bright light to fluorescence light.

For complete microscope specifications, refer to Appendix 1.

MICROSCOPE COMPONENTS – PRIMO STAR iLED

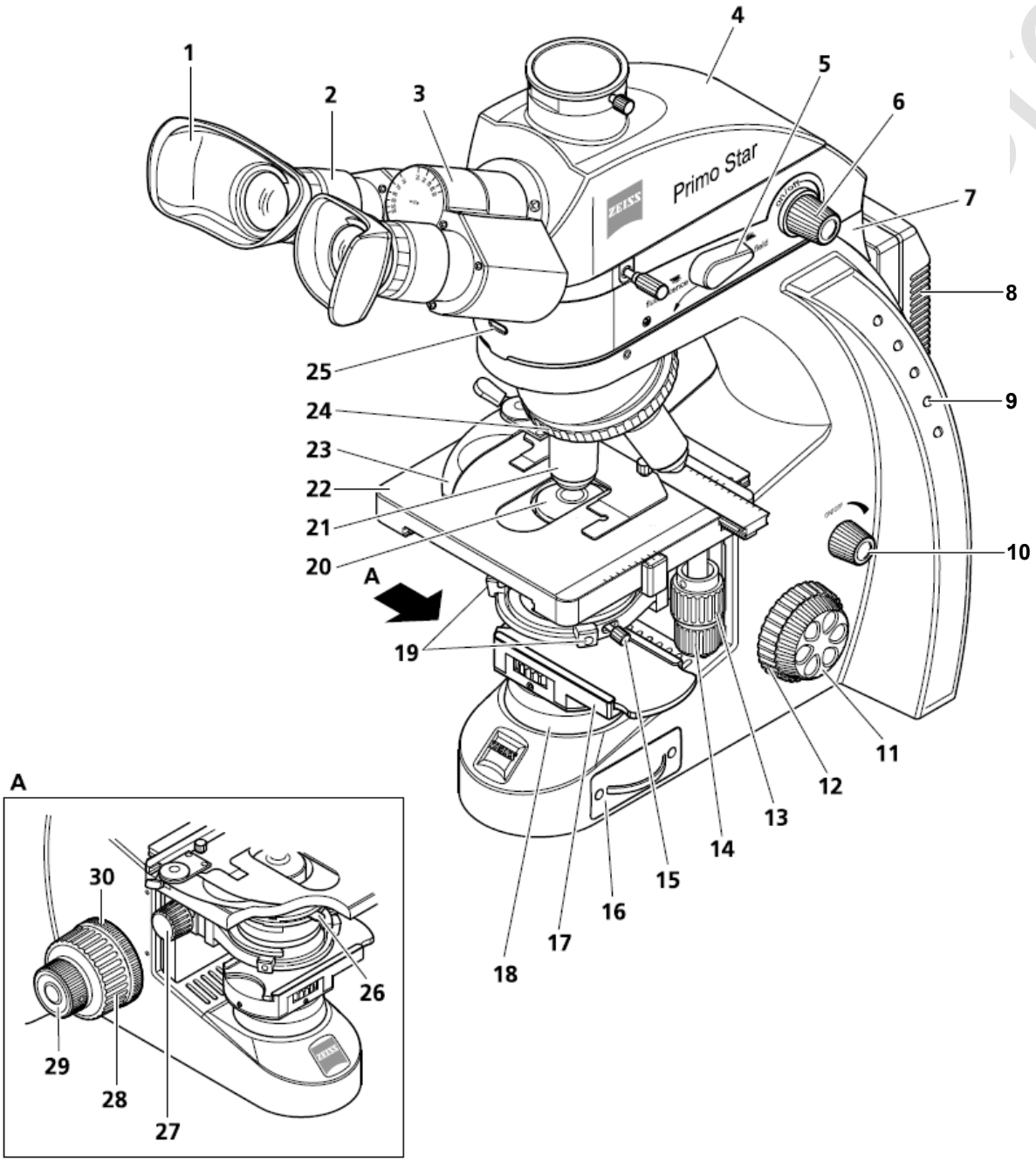


Fig. 15 Controls of Primo Star iLED

Legend:

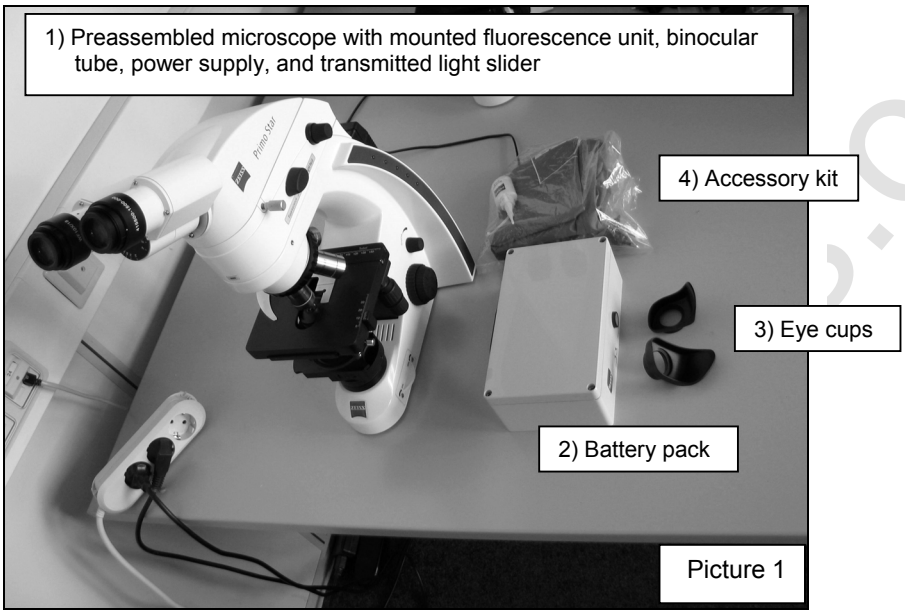
1 Special eyecups with light protection
2 Eyepieces
3 Binocular body of the tube
4 Tube
5 Transmitted light / reflected light changeover switch (Brightfield/Fluorescence)
6 Rotary knob for switching ON/OFF and adjusting the illumination intensity for reflected light
7 Carrying handle
8 Plug-in power unit
9 Illumination-intensity indicators for transmitted light
10 Rotary knob for switching ON/OFF and adjusting the illumination intensity for transmitted light
11 Fine focusing dial or knob (right side)
12 Coarse focusing dial or knob (right side)
13 Control knob for X travel of mechanical stage
14 Control knob for Y travel of mechanical stage
15 Clamping screw for condenser
16 Transmitted-light illuminator LED
17 Slider with yellow filter (with filter position for adapting the color temperature in transmitted light and with position for blocking the transmitted-light path in case of reflected-light fluorescence applications
18 Luminous-field diaphragm (fixed)
19 Centering screws for condenser on condenser carrier
20 Abbe condenser, Fixed-Köhler
21 Objective
22 Microscope stage
23 Spring level of specimen holder
24 Knurled ring of objective nosepiece
25 Pilot lamp for reflected-light fluorescence illuminator: lighting blue when switched on; brightness corresponds to intensity
26 Lever for adjusting the aperture diaphragm of the condenser
27 Knurled knob for vertical adjustment
28 Coarse focusing dial or knob (left side)
29 Fine focusing dial or knob (left side)
30 Knurled ring for adjusting the smoothness of the coarse focusing drive

Below is a list of the microscope components and their respective functions.

Microscope components	Function
Eyepieces	Pair of lenses used to view the magnified image from the objective lens
Diopter adjustment ring	Used to focus by turning clockwise or anticlockwise to obtain a sharp image
Binocular tube	The part holding the eyepieces and dividing the light between them. It is used to adjust the distance between the eyes so that a single, overlapping image is obtained
Nose piece	The mechanical and revolving part that holds the objective lenses
Objective lenses	Lenses of various magnification power used to view the object
Stage	Horizontal platform for placing the object for viewing
Slide holder	Mechanical arm that is used to hold the object or slide for smooth and uniform movement
Condenser with diaphragm	The lens system that concentrates the light on the object to be magnified. It contains an iris diaphragm meant to reduce glare from dispersed light
Filter	A blue-colored glass that makes the light in the visual field to appear as natural daylight
Field diaphragm	Controls the amount of light from light source
Lamp	Light source in the base of the microscope stand
Coarse focus knob	Focusing knob that allows a coarse adjustment of the image
Fine focus knob	Focusing knob that allows a fine adjustment of the image
Power switch	Controls the power supply to microscope
Voltage regulator	Controls the amount of voltage supplied to the lamp
Stage movement knobs	Used to move the slide in x and y direction for complete coverage of object, in our case it is the smear

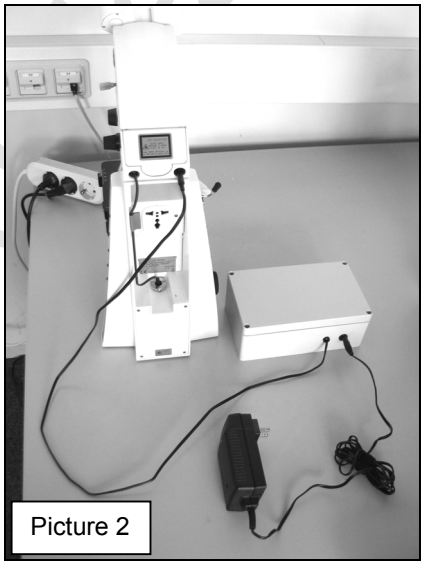
UNPACKING AND USE OF PRIMO STAR iLED (***For details, see User Manual***)

The Primo Star iLED consists of the following parts:

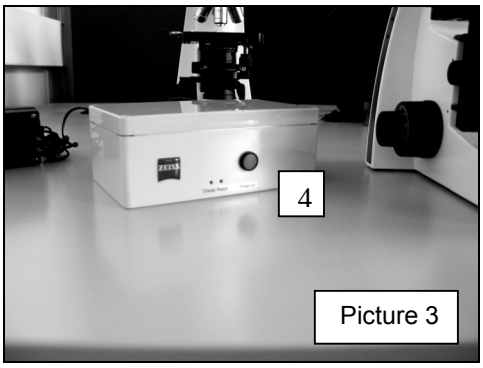


The microscope should be placed on a stable level bench, well away from the staining area.

The battery pack is setup as follows:



- Remove the power supply and its plug from the microscope and connect the battery pack as shown in picture 2.
- By connecting the power supply to the power supply line, the battery pack will automatically start charging the accumulators. While the accumulators are being charged, you can work with the microscope, which is being supplied by the power supply.
- In case of a power cut, the battery pack switches automatically to accumulator mode.



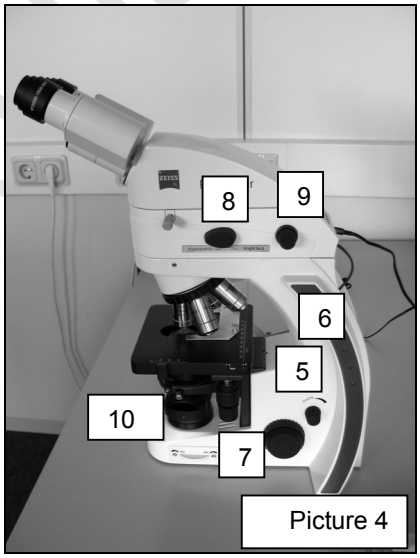
Picture 3

To switch the power supply on, push the “power on” button (4) at the front of the housing. The battery pack is equipped with two indicator LEDs to indicate the mode of operation:

- The right green LED indicates the availability of the power-supply line.
- The left yellow LED indicates the charging mode of the accumulators. When the yellow LED is on, the battery pack is charging. When it is off, charging is completed.
- The battery pack switches off automatically when the light sources of the microscope are turned off. The battery pack turns off automatically when a critically low charging level is reached. In this case, the battery pack needs to be connected to the power supply to charge.
- The battery pack will require charging after 6 - 8 hours of use.

OPERATION OF PRIMO STAR iLED (for more details, see User Manual)

The correct operation of the microscope is as important for the quality of results as applying correct smear and staining procedures.



Picture 4

1. Switch on the light (5) at low intensity (the level of light intensity is indicated by small blue LEDs (6) to the right of the switch).
2. Place a specimen slide on the stage. Be sure the slide is not placed upside down.
3. The next steps depend on whether you wish to use the fluorescent or brightfield mode.

<u>Fluorescent mode</u>:

4. When the lever on the fluorescence unit (8) is turned to the front of the microscope, the fluorescence mode is active. You can adjust light intensity using the knob (9) behind the lever.
5. The transmitted light slider (10) must remain closed in the fluorescence mode (otherwise the contrast of fluorescence is poor). The slider may be opened initially to increase the background signal and thereby facilitate focusing. By loosening the retaining ring, the slider can be turned to the desired direction for a better handling.
6. Focus the specimen with the 10× or 20x objective by turning the coarse adjustment knob (7).
7. Adjust the distance between the ocular lenses until both the right and left images become one.
8. Fine-focus the image by turning the fine adjustment knob (7).
9. Change to the 40x objective for screening the slide. Focus the specimen slide if necessary by turning only the fine-focus adjustment knob (7).
10. Scan the smear by moving across the smear in a horizontal direction.
11. Stop and observe each field before moving onto the next field.
12. Read at least 40 high power fields before reporting a negative result. (Note: Fewer than 40 fields may be read if the slide is found positive for AFB.)

Brightfield mode:

13. By turning the lever (8) clockwise to face the rear, the microscope switches automatically to brightfield illumination mode.
14. The intensity of the brightfield illumination can be adjusted using the fine adjustment knob (5).
15. Focus as in fluorescent mode (steps 6-9)
16. Put one drop of immersion oil on the smear.
17. Change to the 100× objective. Be sure the condenser is raised as high as possible to maintain the intensity of the light. Open the condenser iris to 70-80% of the aperture diameter. Focus the specimen slide by turning only the fine-focus adjustment knob.
18. Use only the 100x objective (immersion objective) for observation through immersion oil. All other objectives must be used without immersion oil and kept dry.
19. Read at least 100 high power fields before reporting a negative result. (Note: Fewer than 100 fields may be read if the slide is found positive for AFB.)
20. Usually, examining 100 fields takes about 5 minutes.
21. To view the next slide, the entire procedure does not need to be repeated. Turn away the 100x objective and take out the slide, add a drop of immersion oil on a new stained smear and insert onto stage, then turn to 100x objective.

Applying immersion oil when using the brightfield option

- Make sure that the smear is facing upwards when the slide is placed on the mechanical stage.
- Put one drop of immersion oil on the stained smear, letting it fall freely onto the slide.
- Never allow the oil applicator to touch the slide. Touching the slide with the applicator could lead to contamination of the oil with AFB and could transfer AFB to a negative slide.

MICROSCOPE MAINTENANCE

Never attempt to disassemble any part of the microscope for repair. If there is any problem with the microscope, contact the microscope company's technical support unit or a qualified technician.

Treat the microscope with care! Never expose it to sharp knocks, vibrations, moisture, dust, or direct sunlight.

Humidity causes fungal growth on the surfaces of lenses and prisms. This can cause cloudiness of the view field and rusting of metal parts of the microscope. To protect from fungus, always keep the glass surface as clean as possible and free of dirt and fingerprints. In very humid areas, keeping the microscope inside a temperature-controlled cabinet, and using silica gel (desiccant) or anti-mold strips may be useful.

Maintenance instructions:

- Cover the instrument with the dust cover after every use.
- Cover open tubes with the dust caps.
- Remove dust and loose dirt from visible optical surfaces with a brush, blower brush, cotton bud, optics cleaning tissue, or a cotton cloth. You may also use a cloth moistened with water to which you may add a mild detergent.
- For resistant dirt, use optics cleaning solution L (90 vol % gasoline and 10 vol % isopropanol). Clean optical surfaces by gently wiping the objective lens in small circles, starting in the middle and moving to the edges.
- *Never* use petroleum, benzene, acetone, or xylene to clean objective lenses.

TROUBLESHOOTING

There are several conditions that can affect good functioning of the microscope. Review these problems and their solutions.

The brightness / contrast of the viewing field is poor:

Contrast	Problem	Solution
FL	Transmitted light slider is open	Always close slider when in fluorescence mode
BF	Condenser is too low	Raise the condenser to correct its position
BF	Condenser iris diaphragm is closed	Open the diaphragm properly

The light cannot be switched on:

Contrast	Problem	Solution
FL	You are in brightfield mode	Switch to fluorescence mode by turning the lever towards fluorescence
BF	You are in fluorescence mode	Switch to brightfield mode by turning the lever towards bright light
BF/FL	The microscope has no power supply.	Plug in the cable or connect and switch on the battery pack
BF/FL	The LED bulb is defect	Replace LED by following instructions in user manual

There are dark shadows in the field which move as you turn around the eyepiece:

Problem	Solution
Surface of the eyepiece has scratches	Replace the eyepiece
Eyepiece is dirty	Clean the eyepiece

The image with the high power objective is not clear:

Problem	Solution
Slide is upside down	Turn the slide over
There is dirt on the objective	Clean the lens
There is an air bubble in the oil	Move 100x lens quickly from side to side
Oil is too sticky	Use thinner or specified immersion oil

The image with the low power objective is not clear:

Problem	Solution
There is a layer of dust on the upper surface of the objective	Clean the lens
There is oil on the lens	Clean the lens

If the view field is still dim and cloudy, consider the following possible causes:

- Massive growth of fungus on the lenses or prisms due to storage in a high humidity environment
- Penetration of immersion oil between the lenses of the objective through damaged lens cement (due to use of poor-quality oil, such as cedar oil or misuse of xylene): this is most likely the cause if a completely hazy field becomes clear after changing the objective.
- A damaged objective (due to careless focusing, dropping, rough changing of sides)

Frequently-encountered operational errors include the following:

- Focusing the first slide using the 100x immersion objective without first passing through a low power
- Changing slides from under the immersion objective without turning it away first
- Wiping lenses without first blowing away dust and sand
- Cleaning lenses or other parts with xylene
- Using cedar wood oil, liquid paraffin, or xylene-diluted oil instead of pure synthetic immersion oil
- Keeping the microscope in a confined space and without ventilation in a humid climate

LOGBOOK

A microscope logbook should be maintained to enter problems encountered in the operation of microscope, maintenance schedule, repairs done, etc.

FREQUENTLY USED TECHNICAL TERMS IN MICROSCOPY

The following terms are frequently used when judging the quality of the optics of a microscope. At the end of this training, a user appraisal questionnaire will have to be completed, for which these terms will have to be understood.

Technical Term	Definition
Contrast	The difference in brightness between the light and dark areas of a picture
Color intensity	Brightness, brilliance and saturation of colors
Signal-to-noise ratio	Compares the level of a desired signal (AFBs) to the level of background noise
Homogeneity of fluorescence illumination	Homogeneous illumination of the image with light that is bright, glare-free, and evenly dispersed in the field of view
Resolution	The smallest distance between two points on a specimen that can still be distinguished as two separate entities. The resolving power of a microscope is the most important feature of the optical system and influences the ability to distinguish between fine details of a particular specimen
Depths of focus	The range over which the image plane can be moved while an acceptable amount of sharpness is maintained

Appendix 1: Specifications for a LED-based fluorescence microscope

- **Binocular microscope** for use with electric light via power line or alternatively via battery pack. Battery pack can also be used as uninterruptible power supply, and is usually included as an accessory
- **Observation tube:** binocular, 30 deg inclination (viewing angle) and 360 deg rotation
- **Stage:** rectangular, built in mechanical stage with vernier scale (minimum: 14 mm x 135mm). No polymer belts, metal cables, timing belt systems or non-metallic components are acceptable in the drive mechanism. Coaxial controls must be low mounted for ease of use. Stage finger assembly is to be slide friendly so that it does not damage or break slides
- **Condenser:** Abbe type condenser (0.9/1.25) with iris diaphragm
- **Objective:** 10x, 20x, 40x, 100x oil immersion; colour-corrected infinity optics
- **Eyepieces:** wide field, 10x/18 mm, FOV 18mm, adjustable, can be used by spectacle wearers
- **Brightfield illumination in transmitted light mode:** White light LED, minimum 3W
- **Fluorescence illumination in reflected light mode:** Blue light LED, minimum 3W
- **Focus:** Focus drive must be a self-tensioning, three ball design. Coarse and fine focusing dials or knobs on both sides
- **Power supply:** wide range input 100-240V, 50-60 Hz
- All gears throughout the microscope: mechanical stage, focus, condenser rack and pinion must be made of metal, brass, stainless steel or aluminum – no plastic components
- **Ergonomic design**
- **Anti-fungus** treatment
- Microscope has to fulfil the following **standards**: CE, CSA, UL, IvD, ISO 9001

Appendix 2: Questionnaire – Appraisal of Primo Star iLED

Trial Site Name: _____ (where applicable)

Supervisory Site: _____

Country: _____

Date of completion: _____ (DD/ MM / YY)

Completed by: _____ (First name, last name)

Position: _____ (Microbiologist, laboratory technologist, microscopist)

Instructions:

This questionnaire should be completed by at least 2 staff members per supervisory site and 1 from each microscopy center at the end of each demonstration project phase.

Please check for each question the box of your selected evaluation category.

Please provide further details in text fields where applicable.

If you complete this form electronically, check fields by double-clicking on the selected box and by selecting “checked”. For text fields, double-click on the field and enter default text.

Please send completed forms to FIND Study Coordinator, either by fax (+41 22710 0599) or via e-mail: catharina.boehme@finddiagnostics.org

Part I: Installation and first use

Question #1:
Was the installation/first use of Primo Star iLED by a microscopist:

☐ Self-explanatory, can be done without reading the user manual
☐ Easy, but a user manual with instructions is required
☐ Rather difficult; some problems were faced during installation/first use
☐ Very difficult; cannot be expected of a microscopist

Describe difficulties that have occurred or may occur during installation: _____

Question #2:
Was the installation/first use of the battery pack by a microscopist:

☐ Self-explanatory, can be done without reading the user manual
☐ Easy, but a package insert is required
☐ Rather difficult; some problems were faced during installation/first use
☐ Very difficult; cannot be expected of a microscopist

Describe difficulties that have occurred or may occur during installation/first use: _____

Question #3:
How satisfied were you with the Primo Star iLED user manual:

☐ Easy to read and understand; covers all questions I had during installation/use
☐ Most sections easy to read and understand, with some weaknesses in sections:_____ Missing topics:_____
☐ Rather cumbersome to read (information required is not found easily; not enough pictures that allow understanding at first glance), weaknesses especially in the following sections: _____ Missing topics: _____

Comments: _____

Part II: Training

Question #1a:
For a microscopist trained in ZN microscopy, how intensive should the training for Primo Star iLED be? _____ days

Question #1b:
For someone without prior training in smear microscopy, how intensive should the training for Primo Star iLED be? _____ days

Comments: _____

Question #2: (*only to be completed by supervisory sites in phase II*)
How satisfied were you with the Primo Star iLED training manual:

☐ Can be used by NTPs for implementation of LED microscopy without major changes
☐ Can be used by NTPs for implementation of LED microscopy but requires some major changes
☐ Requires complete revision

Suggestions for changes: _____

Part III: Optics and Handling

Question #1:
How satisfied are you with contrast, color intensity and signal-to-noise (background) ratio of Primo Star iLED?

☐ Very satisfied (better than for the available light microscope _____ [enter brand and model of microscope] and where applicable fluorescence microscope _____)
☐ Satisfied (comparable to available light microscope _____ and where applicable fluorescence microscope _____)
☐ Not satisfied (not as good compared to those of the available light microscope _____ and where applicable fluorescence microscope _____)

Comments:

Question #2:
How satisfied are you with the color impression for ZN stain of the Primo Star iLED (white LED) in comparison to a standard light microscope (halogen bulb)?

☐ AFBs can be better distinguished
☐ Same
☐ AFBs can be less well distinguished

Comments: _____

Question #3:
How satisfied are you with the resolution and depth of focus of Primo Star iLED?

☐ Very satisfied (better than for the available light microscope _____ and where applicable fluorescence microscope _____)
☐ Satisfied (comparable to available light microscope _____ and where applicable fluorescence microscope _____)
☐ Not satisfied (not as good compared to those of the available light microscope _____ and where applicable fluorescence microscope _____)

Question #4:
Was there a difference between the homogeneity of fluorescence illumination in the field of view compared to your standard microscope?

☐ Field of view of Primo Star iLED is more homogenously illuminated
☐ Same
☐ Field of view of Primo Star iLED is less homogenously illuminated

Question #5:
How satisfied are you with the overall handling features of the microscope (on/off switch, intensity regulation of bright light and fluorescence light, variable viewing height, focus mechanism (coarse and fine focus))?

☐ Very satisfied (better than for the available light microscope _____ and where applicable fluorescence microscope _____)
☐ Satisfied (comparable to available light microscope _____ and where applicable fluorescence microscope _____)
☐ Not satisfied (less good compared to those of the available light microscope _____ and where applicable fluorescence microscope _____)

Suggestions for improvements/comments: _____

Question #6:
Is the procedure for switching between brightfield and fluorescence light convenient and do you easily understand the symbols used for white light and fluorescence light?

☐ Very convenient
☐ Convenient
☐ Not convenient

Comments: _____

Sub-question: Do you consider the toggle switch to be robust enough?
☐ Yes
☐ No

Question #7:
Is focusing with the fluorescence unit (due to black background):

☐ Very difficult
☐ Difficult, but only a matter of training
☐ Easy, I quickly got used to it

Question #8:
Do you use the option of opening the slider on the white light source to focus with the fluorescence unit (dark background gets structured which makes focusing easier)

☐ Yes, I always use this to facilitate focusing
☐ Sometimes
☐ Never

Question #9:
Are the blue LEDs on both sides of the microscope that indicate the intensity level of the brightfield illumination convenient or rather disturbing/dazzling?

☐ Convenient
☐ Disturbing/Dazzling

Question #10:
Are the 4pcs objectives with magnifications: 10x, 20x 40x and 100x the best choice for the applications Auramine O fluorescence and Ziehl-Neelsen brightfield detection of pulmonary tuberculosis?

☐ Yes
☐ No; I would prefer to have a magnification
Comments:

Question #11:
Which magnification do you prefer for fluorescence detection of AFBs: 20 times or 40 times?
☐ 20x ☐ 40x

Question #12:
In your opinion, can Primo Star iLED be used without a darkroom?

☐ No darkroom is needed
☐ Darkroom is needed

Question #13:
Do you use the dazzling protection for the eyepieces?

☐ Yes, they are useful
☐ No, I do not need them (no dazzling)
☐ No, I would need them, but they are not comfortable/convenient

Question #14:
Did you have any technical problems with your microscope until now (repair, replacement)?

☐ Yes, describe: _____
☐ No

Part IV: Application questions

Question #1:
In your daily work, do you plan to switch between brightfield and fluorescence contrast using just the Primo Star iLED microscope or would you rather use the iLED for fluorescence detection only and a second microscope for brightfield detection (Ziehl-Neelsen)?

☐ I would use the Primo Star iLED for both fluorescence and brightfield and would switch between the two modes at least once per day
☐ I would use the Primo Star iLED for fluorescence only and will use a second microscope for bright light microscopy
☐ I do not think a brightfield microscope will be needed in the future anymore for TB detection, i.e. I will only use it for fluorescence

Question #2:
For which applications would you use the Primo Star iLED?

☐ for TB detection only
☐ for Malaria or HAT detection only
☐ for various applications (such as TB, Malaria, Blood Cell Counts, urine analysis, Trypanosomiasis)

Question #3:
Do you see a significant gain in speed when reading slides with Primo Star iLED (30 fields) compared to ZN (100 fields)?

☐ Yes
☐ Yes, for negative and low positive slides only
☐ No

Question #4:
If you had to decide whether to change a majority of microscopy centers in your country from light microscopy to LED based fluorescence microscopy, would you recommend to the Head of the National Health Program to switch to LED?

☐ Yes. Reasons: _____

☐ In principle, yes. But I would prefer using another microscope and not the Primo Star iLED. Reasons: _____

☐ Only for low volume microscopy centers. Reasons: _____

☐ Only for high volume microscopy centers. Reasons: _____

☐ Only in specific settings. Define setting:_____Reasons: _____

☐ No. Reasons: _____

☐ No. But I would switch centers that currently have and use a conventional fluorescence microscope to LED fluorescence. Reasons: _____

Thank you very much for helping us with your feedback!

Key messages

- Familiarize yourself with all working parts of your microscope
- Record all problems with the microscope in a logbook
- Call for help when troubleshooting any problems related to function
- Fluorescence microscopy saves time and is more sensitive than ZN microscopy
- Bulb replacement will very rarely be necessary when using LED-based microscopes (>10,000 h)
- Read at least 30 high power fields (20x) for FM smears and 100 high power fields for ZN smears before reporting a negative result with Primo Star iLED
- Key points for maintenance: Storage and cleaning

Review: Module 3

Please answer the following questions based on the use and maintenance module.

What would be possible reasons to switch from ZN microscopy to LED-based fluorescence microscopy?

_____

_____

_____

If the fluorescence contrast is reduced when using Primo Star iLED, what is the most likely cause?

_____

_____

_____

What entries are made in the Logbook?

_____

_____

_____

When and how are microscope objectives cleaned?

_____

_____

_____

Module 4

Safety Precautions for TB Microscopy, Including Collection and Transport of Sputum Samples

Purpose	To provide you with an understanding of safe handling techniques and precautions while performing AFB smear microscopy, with the knowledge and skills for proper collection and transport of sputum samples for AFB microscopy
Prerequisite Modules	None
Learning Objectives	At the end of this module, you will be able to ▪ Explain airborne transmission of TB ▪ Describe risks involved when collecting sputum ▪ Describe personal health and safety practices ▪ Describe why there should be three distinct areas in the TB laboratory ▪ Describe methods for the disposal of contaminated material ▪ Describe chemical safety precautions in the laboratory. ▪ Describe specifications of suitable containers for sputum collection ▪ Explain the collection strategy: spot–morning–spot ▪ Describe and demonstrate safe and correct collection of sputum ▪ Describe options for specimen collection, handling, and transport ▪ List features of a good sputum specimen ▪ Describe the requirements for a properly labeled specimen.
Content Outline	▪ Transmission of TB bacilli ▪ Proper collection of sputum ▪ Laboratory arrangement ▪ Safety practices in the TB microscopy laboratory ▪ Safe disposal of infectious waste ▪ Chemical safety ▪ Suitable specimen containers ▪ The number and timing of specimen collection ▪ How to collect a specimen ▪ Specimen handling and referral ▪ Assessing specimen quality
Handout and Exercises	None
Appendix	None

Module 4: Safety Precautions for Tuberculosis Microscopy

The most important factor in the prevention of laboratory-acquired infection is good technique on the part of the individual worker. Specialized equipment can support good laboratory practice but does NOT replace it.

Aerosols may be produced in the TB laboratory when handling leaking specimens, opening sample containers, and preparing smears. When care and appropriate techniques are used, handling sputum presents a minimal risk of acquiring infection to a technician.

For laboratory staff, the greatest risk of infection involves sputum collection. People with suspected TB may cough and in doing so spread TB bacilli in tiny droplets in the air, which may infect others when they are inhaled. Precautions must be taken to minimize this exposure.

The laboratory technician is at considerably more risk when sputum is processed for culture and drug susceptibility testing. These procedures require shaking and centrifugation. Consequently, special equipment such as biological safety cabinets and Biosafe centrifuges, which are costly to purchase and maintain, are required.

However, this equipment is not justified for the AFB smear microscopy laboratory.

Transmission of TB bacilli

The TB bacilli are almost always transmitted by patients with active pulmonary disease. The patient expels TB bacilli in small droplets of respiratory secretions. These secretions quickly evaporate leaving "droplet nuclei" of less than 5 μm in diameter. Droplet nuclei of this size containing 1–3 bacilli can remain suspended for long periods of time in the air and, following inhalation, are able to reach deep into the lungs to produce infection. Larger particles do not remain airborne for as long and do not transmit tuberculosis as efficiently.

The risk of infection depends on (1) the infectiousness of the source, (2) the environment (e.g., overcrowding and inadequate ventilation promote transmission of droplet nuclei), (3) the duration and intensity of exposure, and (4) the susceptibility of the recipient.

Smear-positive patients have 10^6–10^7 bacilli per millilitre of sputum whereas smear-negative patients have about 10^4 or less per millilitre. This difference in bacterial load (as determined by smear status and radiologic extent of disease) is the most significant predictor of the infectiousness of a patient. Household contacts of smear-positive patients have tuberculin positivity rates of 30%–50% compared with contacts of smear-negative patients who have tuberculin positivity rates of only about 5%.

The infectiousness of the patient may also depend on how often that person coughs. Coughing is a good mechanism for producing droplet nuclei and a higher prevalence of tuberculin reactivity has been reported among contacts of frequent coughers (i.e., people who cough >48 times per night) than among contacts of infrequent coughers (i.e., people who cough <12 times per night). Interestingly, singing produces infectious droplet nuclei as effectively as coughing and several outbreaks in choirs have confirmed that singing can spread infection. However, while coughing and singing may increase the contagiousness of a patient, the radiologic extent of disease and smear status remain the best indices of infectivity.

Proper Collection of Sputum

Collecting sputum represents the greatest hazard to a laboratory technician because infectious aerosols may be produced by coughing.

When patients come coughing into the laboratory, ask them to cover their mouth.

Wherever possible, collect specimens outside where air movement will rapidly dilute infectious droplets and UV rays from the sun will rapidly inactivate TB bacilli. NEVER collect sputum specimens in laboratories, toilets, waiting rooms, reception rooms, or any other enclosed space.

Always stand well clear and upwind when a patient is collecting a sputum sample.

LABORATORY

Ideally, the TB laboratory should be a well-ventilated area which is dedicated to microbiology with restricted access. Three separate areas are recommended for performing TB microscopy.

1. **Smear preparation and staining:** This area should be well lit and preferably near an open window to ensure adequate ventilation during smear preparation. A sink with running water and a spirit lamp are also required. An area of approximately six inches around the spirit lamp flame is considered as sterile zone as it coagulates any aerosol generated while opening of sputum containers and during smear preparation.

2. **Performing microscopy:** This area should have a flat bench or table for placing the microscope. Subdued lighting is preferred. If no electricity is available, daylight must be used as the light source; in this case, place the microscope directly in front of a window.

3. **Record keeping and storage:** This third area is for entering data in the log book for Quality Control and for storing slides.

SAFETY PRACTICES IN THE TB MICROSCOPY LABORATORY

Take the following precautions to protect yourself and all laboratory personnel:

- Assume ALL specimens are potentially infectious
- Never smoke, eat, or drink in the lab
- Wash hands frequently with soap and water at least before and after performing any procedures
- Establish airflow in working areas that will direct potentially infectious particles away from personnel. Air must be exhausted into a remote area. An extraction fan can be useful to vent air from a smear preparation area with poor ventilation that is closed off due to extreme climatic conditions.
- Do not rely on laboratory coats to protect you against infection with TB. They are useful protection against strong chemicals, staining reagents, and accidental spills but they will not prevent TB infection.
- Prepare smears near a spirit lamp flame
- Always follow safety procedures

Gloves
Gloves do not provide any appreciable protection against airborne transmission of *M. tuberculosis*. Gloves are not required to prepare sputum smears and lack of their availability does NOT mean that sputum smears cannot be prepared. Indeed, wearing gloves can give technicians a false sense of safety and may result in contaminated gloves being used to handle or operate equipment that may otherwise not become contaminated (e.g., microscope or telephone).

If gloves are used, there should always be an adequate supply. Reusing single use gloves is not advised. Never wear gloves outside the laboratory. Discard gloves at any interruption of smear preparation. All gloves should be discarded in a foot operated, closed lid, waste receptacle containing 5% phenol or 0.5% sodium hypochlorite solution.

Hand washing and careful techniques are mandatory for safe laboratory practice in all countries.

Laboratory Coats
Laboratory coats are not required when assisting in specimen collection or performing sputum microscopy. A lack of laboratory coats does NOT mean that sputum microscopy cannot be performed. If they are available, laboratory coats of various sizes should be provided (and cleaned) by the laboratory organisation. They should be tied at the back, not the front, and be made from water-resistant materials to avoid liquids soaking into the gown.

Laboratory coats must NOT be worn outside of the laboratory.

Masks
One of the greatest false beliefs is that a standard surgical mask will protect the wearer from becoming infected with TB. These masks are made from porous material that will not trap TB bacilli, and have an extremely poor fit leaving large gaps between the face and mask.

N95 “duck-bill” respirators (often incorrectly referred to as "masks") and particulate respirators are expensive and **are not necessary for laboratory technicians carrying out sputum smear preparations only.** Such equipment must be selected and fitted correctly to be functional.

Appropriate Disinfectants
Phenolic agents (5% phenol in water or a phenolic disinfectant product diluted as per label) are excellent disinfectants for cleaning up sputum spills and for decontaminating equipment and single use items prior to disposal. Fresh household bleach diluted 1:10 with water (approximately 5% sodium hypochlorite) can also be used as a general disinfectant. Bleach solution works well for cleaning up blood spills; however, it is somewhat less effective than phenolic agents against TB.

It is important that a bleach dilution be made fresh since it loses potency with time.

Seventy percent alcohol is a good agent for cleaning bench tops.

- **Surgical masks do NOT protect against TB infection as TB bacilli can pass through these masks. Therefore, surgical masks provide a false sense of protection.**
- **Effective respiratory protection, such as an N95 respirator, is expensive and unnecessary if the technician uses appropriate technique.**
- **Gloves are not required for use in smear preparation since TB infection is acquired by airborne inhalation.**
- **Each country must evaluate the risks and decide on the level of protection that is appropriate with the resources that are available.**
- **<u>Hand washing and careful techniques are mandatory for safe laboratory practice in all countries.</u>**

Take the following safety precautions <u>before and during laboratory procedures</u>.

- Reject broken or leaking containers. Request another specimen.
- Once collected, allow a sputum specimen to stand undisturbed for at least 20 minutes before opening to allow any aerosols to settle.
- Cover sputum containers with their lids at all times except when removing specimen for smear preparation.
- Open sputum containers with care and away from the face, near the spirit lamp flame. Gently open the sputum container, especially if the lid clicks or snaps on.
- Do not forcefully shake or stir the sputum in the container.
- Move slowly and carefully while sampling sputum particles and smearing onto slide.
- Avoid any rapid motion when making the smear, as infectious aerosols may be produced.

Safety practices during procedures

- Disinfect the work area before and after smear preparation. Immediately cover any sputum spills with disinfectant before cleaning up the area. A phenolic or freshly prepared hypochlorite disinfectant is sufficient.
- Where available, use disposable wooden sticks for smear preparation. Discard into a receptacle immediately after use.
- If wire loops are used, remove residual sputum on the wire loop before flaming. Do so by inserting the wire loop in a sand-alcohol flask and then moving it up and down or rotating it. Never put a wire loop into a flame when sputum is still attached to it as sputum particles containing live AFB will produce infectious aerosols.
- Always keep discard receptacles containing disinfectant in the immediate area.

After sputum is smeared onto the slide, let the slide air dry for 15–20 minutes. Wet slides can produce aerosols if disturbed. Do not flame slides to expedite drying. This can produce dangerous aerosols.

Fix smears by flaming only after they have dried completely.

SAFE DISPOSAL OF INFECTIOUS WASTE

After smears have been processed, place all infected materials including closed sputum containers in a discard bag (polyethylene, if available).

Discard applicator sticks into disinfectant containing bin used for smearing immediately after use.

Since all sputum specimens are considered potentially infectious, treat all materials in the procedure as contaminated.

Discard disinfected specimens by one of the following methods:

- Burning
- Burying
- Autoclaving

To protect the surrounding population, the laboratory must dispose of waste safely. Burning waste in an incinerator is usually the most practical way for safe destruction of laboratory waste. If safe burning can not be arranged, discard the waste in a deep pit of at least 1.5 meter depth. If an autoclave is available, place infected materials inside and follow procedures for safe and adequate sterilization.

CHEMICAL SAFETY

AFB microscopy requires the use of several hazardous chemicals. These include concentrated acids, alcohols, and phenol. Take the following precautions when working with chemicals in the TB microscopy laboratory:

- Always wear laboratory coats, gloves, and safety glasses **when handling strong acids**.
- Take particular care in diluting concentrated acids. **ALWAYS ADD THE CONCENTRATED ACID TO WATER.** This avoids splashes of acid causing burns to the skin or eyes.
- Do not use alcohols near an open flame, as they are flammable.
- Phenol is a toxic chemical. Avoid direct contact with the skin or mucus membranes. Reduce exposure to phenolic fumes by staining smears in a well-ventilated area and by limiting the number of slides in each staining batch to a maximum of 12.

HANDLING AND STORAGE

Auramine O

Storage: Tightly closed, in a well-ventilated place.
Storage temperature: +5ºC to +30ºC
Auramine prepared stain should be stored in amber coloured bottles for a maximum period of one month for the study purposes.

Concentrated Hydrochloric acid

Handling:
Wash thoroughly after handling. Remove contaminated clothing and wash before reuse. Use with adequate ventilation. Avoid contact with skin or eyes. Do not ingest or inhale.
Storage:
Keep away from heat and flame. Do not store in direct sunlight. Store in a cool, dry, well-ventilated area and away from incompatible substances.

Phenol

Phenol should be stored in a cool, dry, well-ventilated area in tightly sealed containers. Containers of phenol should be protected from physical damage and ignition sources, and should be stored separately from strong oxidizers (especially calcium hypochlorite), acids, and halogens.

Potassium permanganate

Keep tightly closed.
Keep away from combustible materials, heat, sparks, and open flame.
Store in a cool and dry place

Key messages

- The greatest risk to a laboratory worker is a patient coughing and not the patient's sputum specimen
- Never collect sputum in the laboratory
- Never smoke, eat, or drink in the lab
- Wash your hands frequently with soap and water at least before and after performing any procedures
- Gloves, laboratory coats, and surgical masks do not provide any appreciable protection against airborne transmission
- Protect the surrounding population by disposing of laboratory waste safely
- Avoid hazards that may occur in a TB laboratory by paying careful attention to safety procedures
- Always work carefully and in a safe manner

COLLECTION AND TRANSPORT OF TUBERCULOSIS SPECIMENS

SUITABLE SPECIMEN CONTAINERS

Use clean, wide mouthed and leak proof specimen containers. Single use disposable plastic containers (50 ml capacity) are preferred.

THE NUMBER AND TIMING OF SPECIMEN COLLECTION

To ensure optimal recovery of TB bacilli from sputum, collect and process three specimens. Consult your country's NTP for specific guidelines. At least one should be an "early morning" specimen that can be collected by the patient upon rising. Early morning specimens have the highest yield of AFB.

When TB is suspected, collect three sputum specimens from the patient as recommended for diagnosis (or by following NTP recommendations). If the first two are positive then the third sample can be omitted. For outpatients, collect one sample at the time of presentation. This is known as the spot specimen. Give the suspect a second sputum container for collection the following morning and instruct the patient to deliver the morning specimen to the laboratory. When the patient returns the morning specimen, give him or her the third container and collect another spot specimen.

Give the patient clear instructions on the proper collection of a specimen for TB. For hospitalized patients, collect early morning specimens on three successive days, since such samples often contain more bacilli and thus are more likely to be positive by microscopy.

Sputum collection for follow-up of treatment:

For patients on treatment, collect follow-up specimens at intervals specified by the NTP. Early morning sputum is the preferred specimen.

HOW TO COLLECT A SPECIMEN

Sputum collection is the most dangerous procedure in the AFB smear microscopy laboratory and must be done in the open air and at a distance from other people.

Never collect sputum in the laboratory!

Give a new sputum container to each patient from whom sputum examination for TB is requested. Demonstrate how to use it to collect a good specimen.

Clearly instruct the patient on

- the importance of sputum examination for diagnosis or follow-up of TB;
- how to open and close the containers;
- the need for collecting real sputum, not saliva;
- how to produce good sputum (i.e., by repeated deep inhalation and exhalation of breath followed by cough from as deep inside the chest as possible);
- how to avoid contaminating the exterior of the container (i.e., by carefully spitting and closing the container);
- how to collect and safely deliver the morning sputum to the laboratory; and
- the need for three sputa to facilitate diagnosis.

A good specimen should be approximately 3–5 ml. It is usually thick and mucoid. It may be fluid and contain pieces of purulent material. Color varies from opaque white to green. Bloody specimens will appear reddish or brown. Clear saliva or nasal discharge is not suitable as a TB specimen.

SPECIMEN HANDLING AND REFERRAL

Specimen handling

For optimum patient management, process the specimen as soon as possible (i.e., < 24 hours). For microscopic examination the interval between collection and staining matters little. Acceptable results can be obtained even on delayed specimens.

If the peripheral health centre does not perform microscopy, there are several options. Each has advantages and disadvantages. Depending on local circumstances, one or more options may apply:

- **Refer the patient to a health centre where microscopy is performed**
- Collect a sputum specimen in a leak proof sputum container and refer it to the microscopy centre.

Patient referral

Ideally, you can refer a patient to the microscopy centre so that a specimen can be collected under supervision. If an unsatisfactory specimen is submitted, then a repeat sample can be obtained immediately. **The disadvantage of this option is that the patient may find it expensive or impractical to travel to the microscopy center if it is in a different location from the clinic. Patients may be reluctant to seek help and diagnosis may be delayed.**

Specimen referral

Alternatively, the peripheral health center can supervise the patient in collecting an appropriate specimen, which is then forwarded to a microscopy center. Transport specimens once or twice each week, although in some remote settings this may not always be possible. To prevent leaks and breakage, place specimens carefully in the specimen container. Clearly label each specimen with the patient identification and include a completed request for sputum examination.

Slide referral

Time delays for slide referrals may occur. Training and periodic supervision is required to assess the quality of smear preparation. There are, however, several advantages. Heat fixed sputum smears are less infectious than sputum specimens are and require less packaging for transport.

ASSESSING SPECIMEN QUALITY

Upon arrival in the laboratory, assess the quality of samples. TB sputum can have various colours and aspects. If the sample is liquid and as clear as water, without particles or streaks of mucous material, process the sample but ensure that the poor quality of the sample is reported on the result form. When possible, encourage the patient to try again. Even saliva can yield positive results. All specimens should be processed, except for broken or leaking containers which should be discarded safely and another specimen requested.

Accept very small quantities if the patient has difficulty in producing sputum and if the aspect is right. Blood-streaked sputum is suitable, but pure blood should not be examined. Refer patients producing pure blood specimens immediately to a medical officer or doctor, as they require emergency medical treatment.

Key message

- Good quality sputum samples are important for the diagnosis of pulmonary TB.
- Early morning specimens provide the biggest yield of AFB.
- For patients on treatment, collect follow-up specimens at intervals specified by the NTP.
- Never collect sputum specimens in the laboratory.
- Provide patients with clear instructions on the collection of good quality samples.
- Patient referral, specimen referral, and slide referral are options for peripheral health centers not performing microscopy.
- Assess the quality of all specimens submitted to the laboratory for microscopy.

Review: Module 4

Please answer the following questions based on the safety precautions module.

How is TB transmitted from person to person?

_____

_____

_____

What are appropriate laboratory disinfectants?

_____

_____

_____

What precautions must you take when handling specimens?

_____

_____

_____

Why do surgical masks offer little protection against TB?

_____

_____

_____

What precautions should you take when preparing dilutions of strong acid?

_____

_____

_____

Review: Module 4

Please answer the following questions based on the safety precautions module.

What is the benefit of an early morning specimen?

_____

_____

_____

Why should sputum never be collected in the laboratory?

_____

_____

_____

What are the important instructions that should be given to patients for the collection of good quality sputum specimens?

_____

_____

_____

How should salivary specimens be handled in the laboratory?

_____

_____

_____

Module 5

Managing Supplies for Fluorescence-based AFB Microscopy

Purpose	To provide you with an understanding of inventory and to help you calculate your laboratory supplies for fluorescence microscopy for a given period
Prerequisite Modules	None
Learning Objectives	At the end of this module, the participant will be able to ▪ List supplies required to perform fluorescence-based smear microscopy ▪ Calculate supplies required (by completing Excel sheet) ▪ Order supplies ▪ Maintain proper records ▪ Explain use of stock book ▪ Inspect and verify supplies received ▪ Explain storage of supplies
Content Outline	▪ Supply list for smear microscopy ▪ Supply storage ▪ Stock management ▪ Recordkeeping: Stock book use and importance ▪ Calculating supplies required ▪ Placing, receiving, and storing supply orders
Handout and Exercises	Stock Management Spreadsheet Stock Book Stock Summary form Exercise 1: Calculation of quarterly supply requirements for a supervisory centre that prepares staining reagents for itself and 3 microscopy sites Not mandatory: Exercise 2: Calculation of quarterly supply requirements for a microscopy centre that receives prepared staining reagents
Appendix	None

Module 5: Managing Supplies for Fluorescence Microscopy

LABORATORY SUPPLY SYSTEMS

Laboratory supply systems vary among countries. Factors that affect how an AFB microscopy laboratory receives its supplies include whether the health care system is integrated or vertical, whether the laboratory calculates its own needs and places its own orders or whether the laboratory receives orders based on calculations performed at another level in the health care system. In any case, microscopy laboratories must know how to perform orders, how to ensure that required supplies are always available for testing, and how to store such supplies.

SUPPLY SYSTEM DURING LED DEMONSTRATION PROJECT

For the duration of the LED demonstration project, participating microscopy laboratories will receive the staining working solutions required for Auramine staining from their respective supervisory site on a monthly or quarterly basis. The supervisory sites will be responsible for timely ordering of all ingredients required to prepare the staining solutions for the 2-3 participating microscopy centers as well as for their own needs. The supply mechanism for general supplies such as sputum containers, slides etc. will not change and will be handled as per NTP guidelines.

SUPPLY LIST FOR FLUORESCENCE-BASED AFB MICROSCOPY (Auramine stain)

The following is a list of general supplies required at AFB microscopy centres using Auramine staining method:

Items	Quantity
Sputum containers and slides	1 per examination
Burning spirit (for heat fixation of smear only)	0.5 ml per smear
Marker pens or grease pencils to label sputum containers / smears	1 per 2 months
Tissue paper to clean microscope lens:	20 sheets per month
lens paper or soft toilet paper	1 roll per month
Forceps	1 per 5 years
Staining rack	1 per 5 years
Drying rack	1 per 5 years
Safety glasses (if handling concentrated acids)	1 per 5 years
Spirit lamp or Bunsen burner	1 per 10 years
Slide boxes (100 slides)	Need to have enough to store all smears collected during 12 months
Spare bulb for Primo Star iLED	1 per 10 years
Disinfectant to clean bench top	100 ml per month

General supplies requiring NTP specification

Certain supply items require a NTP policy choice. These include:

- Device used to make smear: metal wire or wooden applicators
  - For metal wire loop: Nichrome wire (1 meter per year), Wire loop holder (1 per 5 years), Sand bath (1 per 5 years), Spirit lamp (1 per 10 years)
  - For wooden applicators: disposable bamboo, coconut, or wooden sticks: 1 piece of 10-12 cm in length per smear
- Glass slides: 1 slide per smear
  - For unfrosted slides: high-quality diamond stylus 1 per technician for 5 years.
  - For frosted slides: lead pencils of HB grade are sufficient.
- Lens cleaning solutions: Refer to microscope manual for specific cleaning solution.

The following is a list of reagents required to prepare the staining solutions for Auramine staining method:

0.1% Auramine*		
	Ingredients	**Quantity per liter**
1	Auramine O, certified grade	1.0 g
2	Alcohol (denaturated ethanol or methanol), technical grade	100.0 ml
3	Phenol, crystals, analytical grade	30.0 g
4	Distilled or purified water	870.0 ml

* To be used for ≤ 1 month

Decolorizing solution (0.5 % Acid Alcohol)**		
	Ingredients	**Quantity per liter**
1	Hydrochloric acid, technical grade	0.5 ml
2	Ethanol	100 ml

* To be used for ≤ 6 months

0.5% Potassium permanganate**		
	Ingredients	**Quantity**
1	Potassium permanganate, certified grade	5.0 g
2	Distilled water	1000.0 ml

* To be used for ≤ 1 month

** To be used for ≤ 3 months

RECEIVING AND STORING SUPPLIES

When ordered items arrive at the laboratory, check to ensure that what you ordered was delivered, and that items are in good condition (i.e. expiry dates). Label each item with the date you received it (when you open and use it, be sure to mark those dates on the item also). Place items on shelves whenever possible. Items should be stored in an orderly fashion, keeping like items together. Store new shipments behind existing shipments and make sure that the oldest items are stored in front so they will be used first. Store all stock items in a well-ventilated, clean, and tidy room. Store the chemicals, staining reagents, and other reagents away from direct sunlight. Lastly, be sure to update the stock management records.

Keep staining reagents in well-closed bottles, out of direct sunlight and preferably inside a cabinet, unless they are made of dark glass or plastic. Label all bottles containing staining reagents with name and date of preparation. Well-prepared staining solutions have a shelf life of 3-6 months if they are kept in the dark. Old reagents deteriorate and may not work effectively. For demonstration project purposes, Auramine staining solution will be kept for < 1 month, Acid Alcohol and Potassium Permanganate solutions for ≤ 3 months.

When storing new microscope slides, make sure they are as dry as possible to prevent fungus growth. Keep new sputum containers in closed cartons or bags. Microscopes should be kept in a well-ventilated, dry, and safe place. Optical parts must be kept in a dry place to prevent damage from fungus.

STOCK MANAGEMENT

Stock management means properly maintaining adequate supplies to ensure uninterrupted service. It involves performing a stock count (physical inventory), maintaining proper inventory records, determining how much to order, when to order, placing orders properly, inspecting and verifying supplies received, and ensuring proper storage of stock.

Stock management ensures the availability of staining reagents and materials, avoids the use of old reagents, and minimizes waste. The availability of high-quality microscopy testing services depends on the uninterrupted availability of supplies required for testing.

It is important not to under or over stock supplies. Under stocking will result in insufficient supplies and will interrupt the testing process. Over stocking presents different problems. Laboratories have limited space that excessive stock can overwhelm, compromising safety and security. Excessive stock also requires additional management, which takes up laboratorians' valuable time. Over stocking can lead to deteriorated reagents and waste. For these reasons, proper management of your stocks is very important, and will ensure that only adequate supplies are on hand.

RECORDKEEPING: STOCK SUMMARY FORM AND STOCK BOOK

An inventory count is performed to know exactly what and how much stock is on hand. It means the physical counting of each item in the stock, and it should be performed at the end of each quarter. A designated person is responsible for performing this count. The quantities of items on hand can be recorded on a form such the "Stock Summary" form, found at the end of this module. This form is also useful to record the work performed (number of smears examined) by a microscopy centre. Determine the work performed at the end of each quarter. This can be done by reviewing the TB register.

Efficient stock management depends upon accurate recordkeeping. Keeping accurate records ultimately saves time. Proper inventory records help laboratorians determine and predict their pattern of consumption, and estimate supplies for a year for budgeting purposes. The stock book contains a list of all items in the store. It must be routinely updated when orders are placed and received. It also serves as a reference to track orders that have been placed and not received. The information recorded in the stock book regarding when orders are placed and when they arrive may help a site to adjust reserve supplies that are kept on site to ensure uninterrupted testing.

CALCULATING SUPPLIES REQUIRED

Calculations for the supplies required for a microscopy centre can be based on the actual number of smears examined during a month and a stock count of supplies on hand.

This actual supply calculation is typically performed with a spreadsheet. For the LED demonstration project, calculations for the most critical supplies will be made by the supervisory site with the help of an Excel spreadsheet provided by FIND (see below). The supervisory sites only need to enter the number of smear exams performed at a microscopy centre per month and, where applicable, the stock on hand at the supervisory and, for general supplies only, at the microscopy sites. The spreadsheet provides an accurate estimate of supplies actually required for one quarter by calculating the quantity of each item for one quarter of operation plus a reserve quantity of 20%.

Depending on how reliable the ordering system is and how quickly placed orders are received, the reserve quantity can be increased or decreased. From that estimate, general supplies you already have on hand (you should know these numbers from your physical inventory (i.e., stock count) are subtracted automatically. The result should be the amount of items you must order to insure uninterrupted testing during the next quarter of operation.

Quarterly Management of Supplies for iLED Demonstration Project

Number of slides per month	Enter
Site A	
Site B	
Site C	
Site D	
TOTAL	**0**

Product Example Merck
101301 Au O (C.I. 41000) 50 g bottle
516724 Phenol 500 g
See below
See below
107017 Ethanol absolute 2.5 l
109057 Hydrochloric acid 1 l
105080 Potassium permanganate 1 kg

Staining solution requirements	l/slide	
Auramine O per slide	0.003	liter
Acid Alcohol per slide	0.006	liter
Permanganate per slide	0.003	liter

Staining solution requirements	l/month	l/quarter	
0.1% Auramine O	0	0	liter
0.5% Hydrochloric Acid Alcohol	0	0	liter
0.5% Permanganate	0	0	liter

Estimated supplies for next quarter including 20% reserve stock	Quantity needed/l	Required for next quarter	Enter stock on hand in g or l	Actual required (stock on hand subtracted)	Product units	Actual order (round up)	Unit
Auramine O powder (g)	1	0		0	1 bottle / 50 g	0	bottles
Phenol crystals (g)	30	0		0	1 bottle / 500 g	0	bottles
Ethanol for Auramine O (l)	0.1	0					
Ethanol for Hydrochloric Acid (l)	0.995	0					
Ethanol combined (l)		0		0	1 bottle / 2.5 l	0	bottles
Hydrochloric Acid (l)	0.005	0		0	1 bottle / 1 l	0	bottles
Potassium permanganate powder (g)	5	0		0	1 bottle / 1000 g	0	bottles

Estimated general supplies for next quarter including 20% reserve	Per smear	Required for next quarter	Stock on hand	Actual Required (stock on hand subtracted)	Product Units	Actual Order (round up)	Unit
Sputum container	1	0		0	1 bag / 1000	0	bags
Slides	1	0		0	1 box / 72	0	boxes
Slide Boxes	1	0		0	1 box / 1	0	boxes
Burning Spirit for heat fixation (l)	0.0005	0		0	1 bottle / 1 l	0	bottles

MODULE 5: STOCK BOOK

Item Name:_____

Unit:_____

Physical count (units)	Date count performed	Quantity (units) requested	Date of request	Quantity received	Date received	Total stock on hand

Quarterly Report

Quarter/ year

STOCK SUMMARY FORM

Center…….. District……..

Region…….. ……

Case detection	Number of suspects examined during the quarter ……	Number with at least one positive or scanty smear ……

Stock left at the end of the quarter (in liters)

Slides ……	Sputum cups ……	Burning spirit (l) ……
1% Carbol-fuchsin (l) ……	25% Sulphuric acid (l) ……	0.1% Methylene blue (l) ……
0.1% Au O (l) ……	0.5% Hydrochloric acid (l) ……	0.5% Potassium permanganate (l) ……

Key message

- Maintain an adequate inventory at all times to ensure uninterrupted service
- Quantity required is estimated based on smears performed + reserve stock – stock in hand = actual order for next quarter
- Never order more than you require in the next quarter
- Record and account for all items in the inventory
- Always inspect a new shipment before accepting it

Review: Module 5

Please answer the following questions based on the supply management module.

How do you determine reserve stock level?

_____

_____

_____

How do you determine the order for the next quarter?

_____

_____

_____

What procedure should you follow when receiving new supplies?

_____

_____

_____

How & for how long should fluorescence staining solutions be stored?

_____

_____

_____

Module 6

Preparation of Reagents for Fluorescence-based AFB Microscopy

Purpose	To provide you with an understanding of staining reagents and their preparation in acid fast staining
Prerequisite Modules	Module 4
Learning Objectives	At the end of this module, you will be able to ▪ Describe the importance of using quality chemicals for reagent preparation ▪ Prepare reagents required for Auramine O method ▪ Describe the safety requirements for reagent preparation ▪ Use positive and negative control slides for the quality control of Auramine O reagents ▪ Explain the use and frequency of routine quality control procedures
Content Outline	▪ Equipment required for staining reagent preparation ▪ Reagents required for the preparation of stains ▪ Methods for staining reagent preparation ▪ Storage of staining reagents ▪ Quality control (QC) of freshly prepared staining reagents
Handout and Exercises	Laboratory Practical Session # 1: Reagent preparation Laboratory Practical Session # 2: Quality control of staining reagents
Appendices	Appendix 1: Worksheet for preparation of Auramine O staining reagents Appendix 2: Example of logbook for quality control of Auramine O staining reagents Appendix 3: Recording worksheet for Laboratory Practical Session # 2

Module 6: Preparation of Fluorescent Microscopy Reagents

Reagent preparation requires equipment for weighing and measuring. Distilled or purified water, free of environmental mycobacteria, must also be available. Environmental mycobacteria often colonize water tanks and taps and could, on rare occasions, result in a false positive reading. Therefore, avoid using tap water.

Fluorochrome staining employs dyes which fluoresce and stain acid-fast organisms. The nonspecific background fluorescence of tissue debris may be suppressed by a counterstain such as potassium permanganate. Good staining reagents, especially those made with a high-quality Auramine O phenol dye, are essential to detect acid-fast bacilli (AFB). While it is easy to demonstrate AFB in a highly positive smear, only a good staining reagent will also be able to show the AFB when they are rare or damaged due to drug treatment and are especially difficult to stain. Poor quality staining reagents may not show these AFB and a case of TB maybe missed.

EQUIPMENT REQUIRED FOR STAINING REAGENT PREPARATION

The following list is required for preparing staining reagents:

- A balance or weight scale, with a sensitivity of 0.1 gram (g)
- Measuring cylinders of 100 mL, 500 mL, and 1000 mL capacity (one each)
- Large Erlenmeyer (conical) flasks or flat-bottomed balloon flasks, capacity at least one liter
- A spirit lamp for heating
- A stirring plate with heating and magnetic stirrers (this is preferable when preparing larger quantities)
- Containers for the newly prepared staining reagents (dark amber glass bottles are recommended, but plastic bottles or containers with tight closures may be easier to transport)
- Labels for bottles
- Brushes to clean bottles before reuse
- Funnels to fill bottles, one funnel for each solution
- AFB-positive and negative unstained control smears

REAGENTS REQUIRED FOR STAINING REAGENT PREPARATION

Preparation of Fluorescent Microscopy Reagents

Auramine O stain

For staining reagent preparations using auramine, use the following items:

- Auramine O powder of good quality
- Phenol crystals of good quality. The crystals should be almost colorless; quality must be assessed through quality control of AFB staining. AFB smears should yield solid, homogenous, and strong red-staining bacilli
- Alcohol (can be denatured 95% ethanol or methanol)
- Water (distilled or purified)

Decolorization solution
For staining reagent preparations using acid, use the following items:
- Hydrochloric acid (37%, fuming)
- Alcohol (denatured 95% ethanol or methanol)

Quenching solution: Permanganate
- Potassium permanganate certified
- Water (distilled or purified)

Prepare the final solutions according to the following guidelines:

- **1 liter of auramine stain final concentrations 0.1% Auramine O**
  - Disolve 1 g of Auramine O in 100 ml of alcohol (denatured ethanol or methanol)
  - Dissolve 30 g of phenol crystals in water
  - Mix both solutions by swirling for about one hour.
  - Filter the Auramine solution by pouring through a funnel with filter paper held over the slides while transferring to a definitive container. Working solutions have to be kept in dark bottles, or better yet in a cupboard..

  Label the bottle '0.1% Auramine', add date and sign with initials. The date first opened has to be mentioned. Stocks and solutions should not be used over 1 month.

- **1 liter of 0.5% hydrochloric acid in alcohol** (Decolorizing solution)
  Hydrochloric acid, technical grade 0.5 ml
  Ethanol 100 ml
  - Add 995 mL of 95% alcohol to a two liter Pyrex conical flask
  - Measure 5 mL of concentrated hydrochloric acid in a cylinder
  - Pour it ***slowly*** into the flask containing alcohol, directing the flow of acid gently along the inner side of the flask with constant swirling.
  - Mix well by swirling.

  Label the bottle '0.5% acid alcohol ', add date and sign with initials. The date first opened has to be mentioned. Stocks and solutions should not be used over 6 months.

- **1 liter of Quenching solution: 0.5% permanganate**
  - Weigh 5 g of potassium permanganate
  - Add the powder to 0.5 liter of distilled or purified water, which has been placed in a conical flask
  - Swirl the contents of the flask to dissolve the dye
  - Add another 0.5 liter of water and mix again

  Label the bottle '0.5%Potassium permanganate', add date and sign with initials. The date first opened has to be mentioned. Stocks and solutions should not be used over 6 months.

Next steps

Let the flasks with freshly prepared reagents stand (covered) until quality control procedures have been performed.

After these reagents have passed quality control, pour the solutions into clean bottles and label them. If bottles are reused, clean thoroughly, use acid alcohol and a bottlebrush to remove this residue. On the label of the bottle, clearly print the reagent name, concentration and the preparation date.

STORAGE OF REAGENTS

Well-prepared reagents will keep for at least six months to one year, even at higher temperatures. However, for demonstration project purposes, Auramine staining solution will be kept for < 1 month, and acid alcohol and potassium permanganate solutions for ≤ 3 months. Store all reagents in clean and tightly closed bottles with a label showing the name of reagent and the date of preparation. Keep these bottles out of direct sunlight. If clear bottles are used, keep stocks of reagents in a closed cabinet.

QUALITY CONTROL

Preparation of positive and negative controls

Quality control (QC) smears, which are supplied by FIND, are manufactured, validated and unstained slides.

After the demonstration is completed, the NRL of your country will provide these slides.

In case you need to prepare your own QC slides, make positive control smears with low positive (1+) sputum. Let this sputum stand for one or more days at room temperature to allow the sputum to liquefy. Then, with the container closed, mix the contents carefully and make as many smears as possible from this same low positive sputum. Check the average number of AFB by staining a few randomly selected smears from the entire batch. Record this number in your staining reagent logbook.

Ensure that sputum used to prepare negative control smears has been extensively examined to ensure that there is no AFB. Prepare smears and fix them.

To protect fixed unstained smears from dust and sunlight, store them in a separate and labeled slide box.

QUALITY CONTROL OF FRESHLY-PREPARED STAINING REAGENTS

After preparing staining reagents, always perform quality control for each batch of staining reagents prepared. Quality control is essential to ensure that the staining reagents work well, and that they do not contain artifacts or contaminating AFB.

It is more efficient to prepare bigger batches if very large flasks are available.

Keep accurate records in a logbook for quality control (see Appendix 2). This serves as an important reference record to ensure good staining reagents. In the logbook, identify the batches by name of reagent and preparation date (as on the bottle labels). Perform QC by using one or more freshly prepared staining reagents and the normal staining procedure as described for positive controls.

Examine all controls carefully for number, completeness, and intensity of color of AFB, as well as color and complete destaining of background. Record the results in logbook for quality control of staining reagents, as in the example given later in the document.

If unsatisfactory results are obtained in the staining of AFB, Auramine O and other reagents and if the preparation procedure seems to have been correct, the stain may be good but the staining procedure was not correctly made. Ensure that the proper staining method was followed. Repeat with few more control slides, paying attention to employing correct staining technique. If no error is found in the preparation method or staining technique, then prepare fresh staining solution(s) or reagents from a new batch of stains or reagents and perform quality control.

Report the unsatisfactory batch and discard the unsatisfactory solution(s).

QUALITY CONTROL OF STORED STAINING REAGENTS

Staining reagents may spoil with aging. In addition, the staining procedure may not have been performed correctly. For these reasons check staining periodically in all labs. Include a positive control smear (as described above and prepared by these laboratories themselves) in the routine series. *Perform the QC at least weekly and with each new batch of reagents.* Check the control smear first for properly stained AFB, and record the result in the sputum smear register. If the result is unsatisfactory, stain another control smear, making sure that the procedure is correct. If this gives a good result, use this lot to stain routine smears. If it does not, use a new lot of staining reagent to re-stain them. Make sure that the new lot has gone through proper quality control.

Key messages

- Use quality reagents to prepare staining reagents
- Accurate preparation of reagents is critical to obtain optimum staining results
- Quality control of staining reagents using control smears ensures proper performance of newly prepared staining solutions
- Record quality control results in logbook for quality control of staining reagents
- Store prepared reagents in clean bottles and out of direct sunlight

Review: Module 6

Please answer the following questions based on the preparation of FM reagents module.

Why must quality reagents be used to prepare staining reagents?

_____

_____

_____

Why is correct preparation of reagents necessary to obtain optimum staining results?

_____

_____

_____

What is the role of control smears in evaluating the performance of newly-prepared staining solutions?

_____

_____

_____

How should reagents be labelled and stored?

_____

_____

_____

Appendix 1

Worksheet for Preparation of Auramine O Staining Reagents

Laboratory Practical Session # 1

Item	Lot number	Actual amount taken
Auramine O		
Auramine O (g)		
Phenol (g)		
Alcohol (ml)		
Deionized H2O (ml)		
Decolorizing solution – Hydrochloric acid		
Hydrochloric acid (ml)		
Alcohol (ml)		
Quenching solution		
Potassium permanganate (g)		
Deionized H2O (ml)		

Appendix 2

Example of Logbook for Quality Control of Auramine O Staining Reagents

Batches checked on date 3/5/08:

Auramine O (Au O) batch 3/5/08, Hydrochloric acid batch 3/5/08, Potassium permanganate batch 3/5/08
Average grading positive controls (LED 40 x objective): no. 345 = 90/40 fields; no. 411 = 66/40 fields

Control slide	AFB color	AFB number	Decolorization	Decision
345/13	Bright yellow	80/40 (40x F)	OK	Accept Au O
411/26	Bright yellow	60/40 (40x F)	OK	Accept Au O
NEG	NA	None in 30 (40x F)	OK	Accept others
NEG	NA	None in 30 (40x F)	OK	Accept others

Note:

Batches checked on date 17/5/08:
Au O batch 17/5/08, Hydrochloric acid batch 17/5/08, Potassium permanganate batch 17/5/08
Average grading positive controls (LED 40 x objective): no. 345 = 90/40 fields; no. 411 = 66/40 fields

Control Slide	AFB color	AFB number	Decolorization	Decision
345/16	weak yellow	2/30 (40x F)	OK	Reject Au O
411/29	NA	0/30 (40x F)	OK	Reject Au O
NEG	NA	None in 40 (40x F)	OK	Accept others
NEG	NA	None in 40 (40x F)	OK	Accept others

Note: This batch of Auramine O is bad; all has been discarded. Hydrochloric acid is OK.

Batches checked on 18/5/08:
Au O batch 18/5/08, Potassium permanganate batch 18/5/08 (+ old Hydrochloric acid solution)
Average grading positive controls (LED 40 x objective): no. 364 = 151/40 fields; no. 428 = 147/40 fields

Control Slide	AFB color	AFB number	Decolorization	Decision
364/1	Bright yellow	124/40 (40x F)	OK	Accept Au O
428/1	Bright yellow	120/40 (40x F)	OK	Accept Au O
NEG	Bright yellow	15/40 (40x F)	OK	Reject others
NEG	NA	None in 40 (40x F)	OK	? Accept others

Note: contamination, probably Auramine O. To be checked further using only one of the new staining reagents on negative controls (+ old good stains of the other types).

Blank Log Book Sheet:
Quality Control of Staining Reagents
(This sheet can be reproduced for individual laboratory use)

Auramine O stains

Batches checked on (date) : _____
Au O batch :_____;Hydrochloric acid batch: _____;
Potassium permanganate batch: _____
Average grading positive controls (LED 40 x objective): no. ____= ____/40 fields;
no. ____= ____/40 fields

Control slide	AFB color	AFB number	Decolorization	Decision

Note:

Batches checked on : _____
Au O batch :_____;Hydrochloric acid batch: _____;
Potassium permanganate batch: _____; Methylene blue batch_____ (if used)
Average grading positive controls (FM 40 x objective): no. ____= ____/40 fields;
no. ____= ____/40 fields

Control Slide	AFB color	AFB number	Decolorization	Decision

Note:

Batches checked on : _____
Au O batch :_____;Hydrochloric acid batch: _____;
Potassium permanganate batch: _____; Methylene blue batch_____ (if used)
Average grading positive controls (LED 40 x objective): no. ____= ____/40 fields;
no. ____= ____/40 fields

Control Slide	AFB color	AFB number	Decolorization	Decision

Note:

Appendix 3

Recording Worksheet for
Laboratory Practical Session # 2

Batches checked on: ……

Auramine O, date prepared …… Concentration……...

Hydrochloric acid, date prepared…… Concentration…..……...

Potassium permanganate, date prepared…… Concentration……...

Average grading positive controls = ……AFB / 40 fields

Control slide ID	AFB color	AFB No.	Background	Remarks

Module 7

Smear Preparation and Fluorescence-based Staining Methods

Purpose	To provide an understanding of proper smear preparation and staining technique for AFB by fluorescence smear microscopy
Prerequisite Modules	Module 6
Learning Objectives	At the end of this module, you will be able to ▪ Safely prepare sputum smears ▪ Prepare good-quality sputum smears ▪ Identify problems with smear preparation ▪ Perform the Auramine O staining of sputum smears ▪ Troubleshoot problems with the Auramine O method
Content Outline	▪ Labeling of slides ▪ Selecting the best portion of the specimen for smear preparation ▪ Techniques for preparing smears ▪ Principles of the Auramine O method ▪ The Auramine O staining procedure
Handouts and Exercises	Laboratory Practical Session #3: Preparation of smears Laboratory Practical session #4: Staining sputum smears
Appendix	None

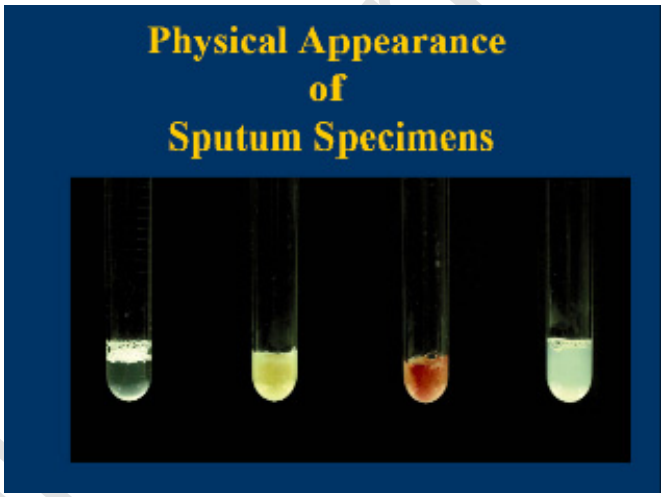
Module 7: Smear Preparation and Fluorescence Microscopy Staining Methods

The quality of work in AFB diagnostic microscopy depends on a number of factors like specimen collection, quality of reagent, staining technique, reading of smear, reporting and recording, and a technician's level of training. However, collecting a suitable specimen and making a good smear are critical as the quality of the rest of the procedure depends on it. Smear preparation must be performed carefully and with attention to detail.

Overview of smear preparation:

1. **Label each slide with the correct number (serial and order number)**
2. **Smear sputum onto slide**
3. **Allow smear to air dry**
4. **Heat fix smear**

The physical appearance of sputum specimens – salivary, mucopurulent, bloody and muco-colloidal (watery) – are shown below.



PREPARING SPUTUM SMEARS

1. Labeling the slides

- Select new, clean, grease-free, unscratched slides which are free from fingerprints.
- Using a pencil, record the laboratory register serial number and order number of the sputum specimen on the frosted end of the slide. If plain unfrosted slides have to be used, labeling is best done using a diamond pencil.
- Ensure that the number on each slide corresponds to the number on the specimen container.

2. **Sputum smearing**
   - Using the end of an applicator stick or wire loop, select and pick up the blood –specked, opaque, grayish or yellowish purulent parts of sputum.
   - Prepare the smear in an oval shape in the center of the slide (Figure 1). The smear size should be about 3 cm in length x 2 cm wide, which will allow 100–150 fields to be counted in one length.
   - For good spreading of sputum, firmly press the stick perpendicular to the slide and move in small concentric circles or coil-like patterns.
   - Place the used stick into a waste receptacle which also contains a disinfectant.
   - Use a separate stick for each specimen.
   - Alternatively, if a wire loop is used instead of a broken stick, dip the wire loop into a sand-alcohol bottle (Figure 2). Remove the excess sputum from the wire loop by moving it up and down. After each smear is completed, heat the wire loop in a flame until red-hot.
   - Thorough spreading of the sputum is very important; it should be neither too thick nor too thin. Prior to staining, hold the smear about 4-5 cm over a piece of printed paper. If letters cannot be read, it is too thick (Figure 5).

3. **Air drying of smear**
   - Allow the smear to air dry completely at room temperature (Figure 4).
   - Do not dry smears in direct sunlight or over a flame.

4. **Heat fix smear**
   - After the slide is completely dry, use forceps to hold the slide upwards.
   - Pass the slide over the flame 2–3 times for about 2–3 seconds each time (Figure 3). Do not heat or keep the slide stationary over the flame for too long or else it will be scorched.

A well stained smear should have more than 20 leucocytes of uniform size of 2 x 3 cm, with even, good thickness and should be properly decolorized.

Graphic representations of smear preparation are shown below.

Figure 1

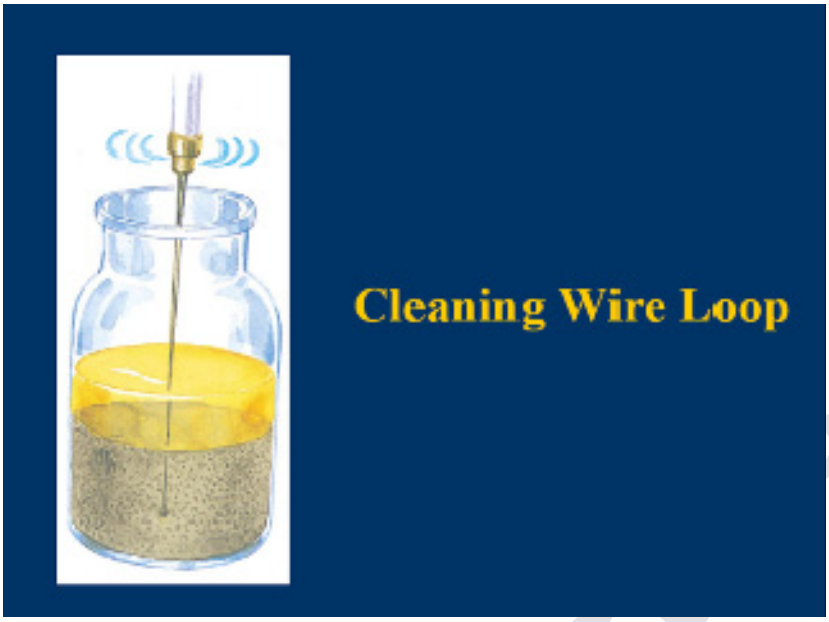


Figure 2

Figure 3

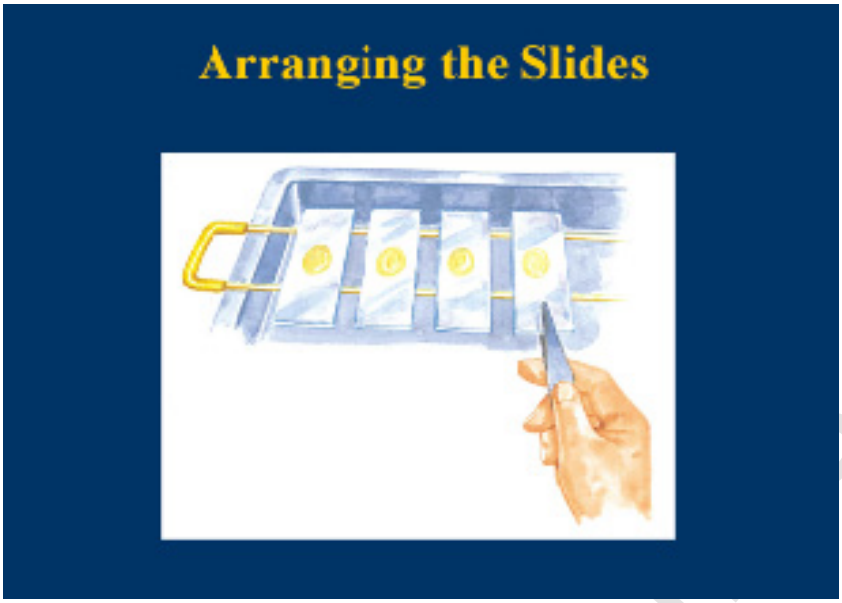


Figure 4

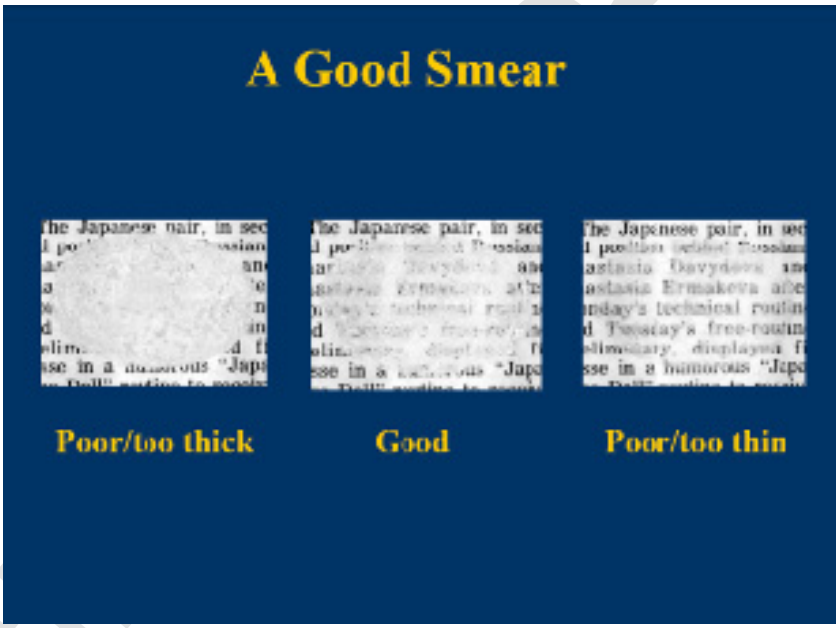


Figure 5

STAINING WITH AURAMINE O SOLUTION

Principle of acid fastness

The nucleic acids of the cell become stained by Auramine O, which later does not lose its color despite the action of acids or alcohol.

Auramine O staining procedure:

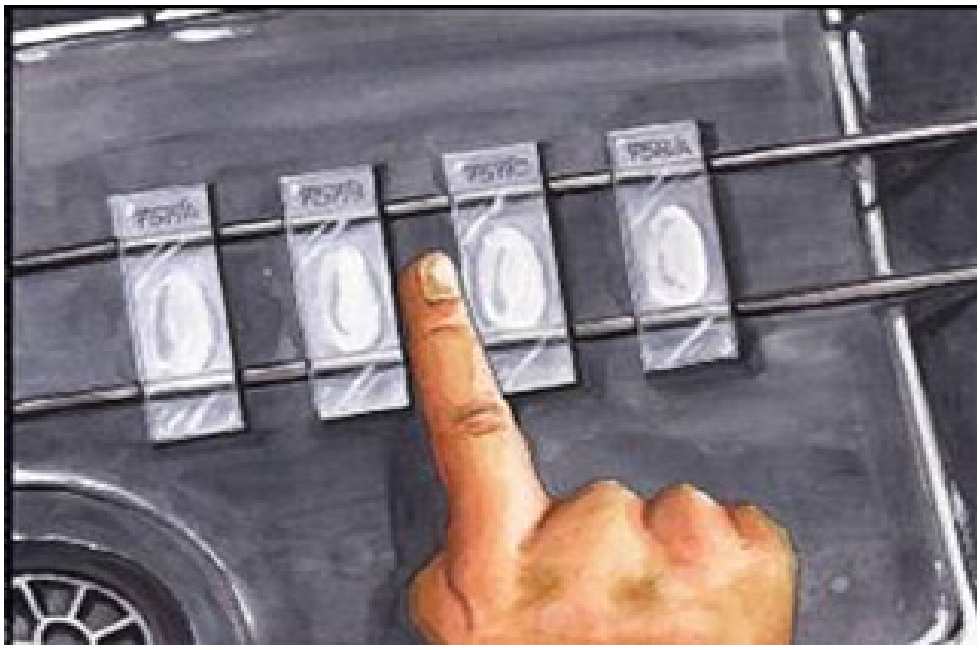
1. Arrange slides in serial order on staining bridge, with smear side up, at a distance of at least 1cm between every slide.
2. Flood slides with filtered 0.1% Auramine O solution.
3. Do not heat.
4. Keep the staining reagent for at least 20 minutes. Make sure that the smear area is continuously covered with Auramine solution by adding more if needed.
5. Rinse with water and drain.
6. Apply decolorizing solution, 0.5% Acid alcohol, for 3 minutes.
7. Gently rinse with water until the macroscopically visible stain has been washed away and drained.
8. Flood smear with 0.5% Potassium permanganate solution for 1 minute. Time is critical because counterstaining for a longer time may quench the acid-fast bacilli fluorescence.
9. Gently rinse with water and drain.
10. Air dry on a slide rack.

The staining procedure, steps 1 – 7, is shown graphically in the next pages.

STAINING PROCEDURE

Step # 1

Place slides 1 cm apart on a staining rack with the smeared side facing up

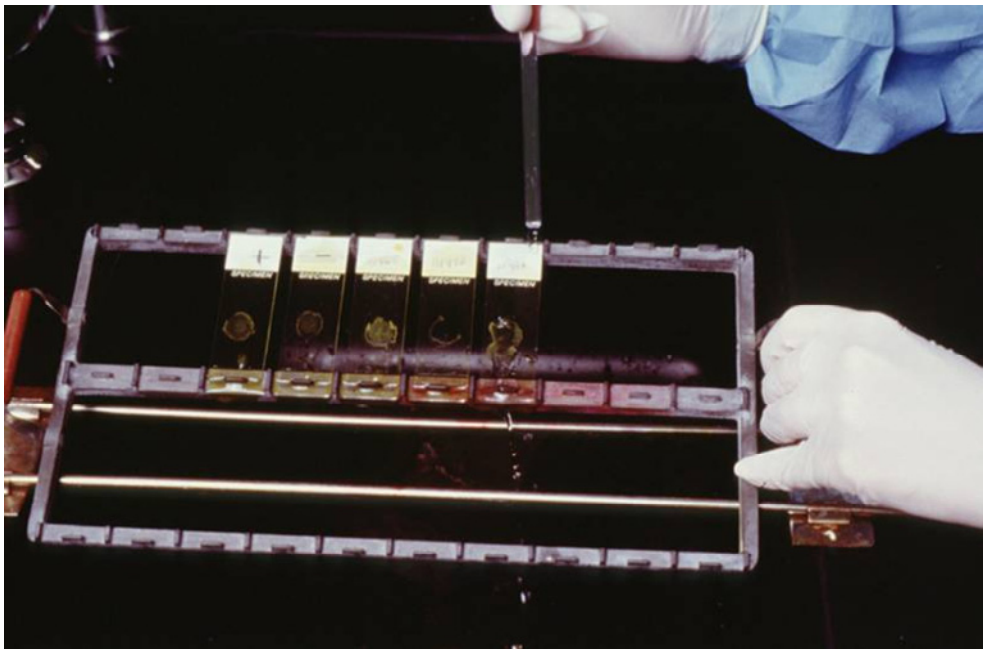


Step # 2

Flood the slides with freshly filtered auramine-phenol. Let stand for 20 minutes

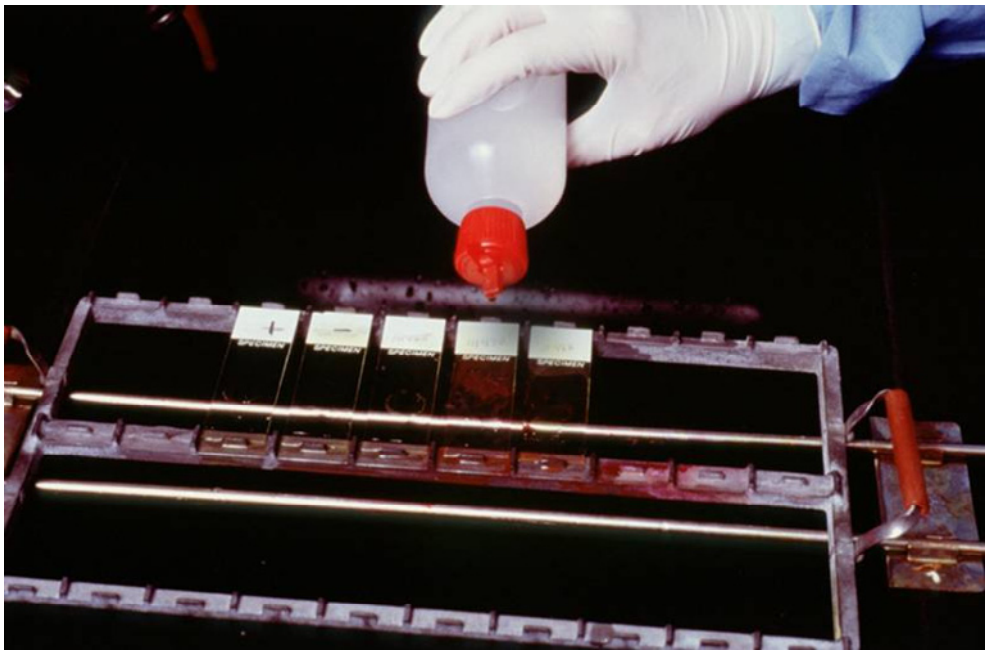
Step # 3

Rinse well with running water, taking care to control the flow so as not to wash away the smear



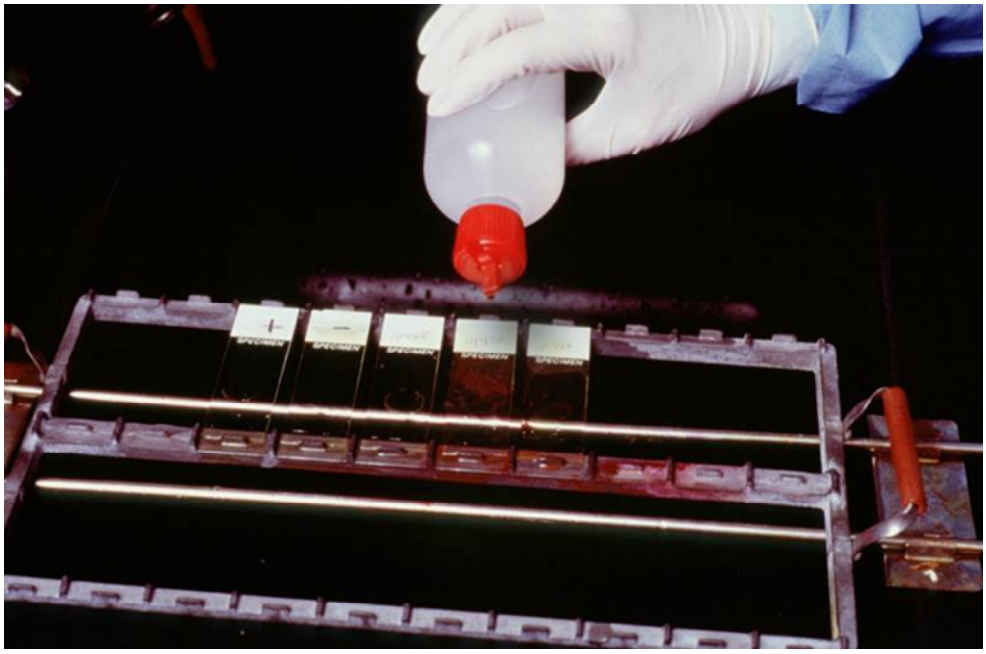
Step # 4

Decolorize by covering completely with acid-alcohol solution for 3 minutes



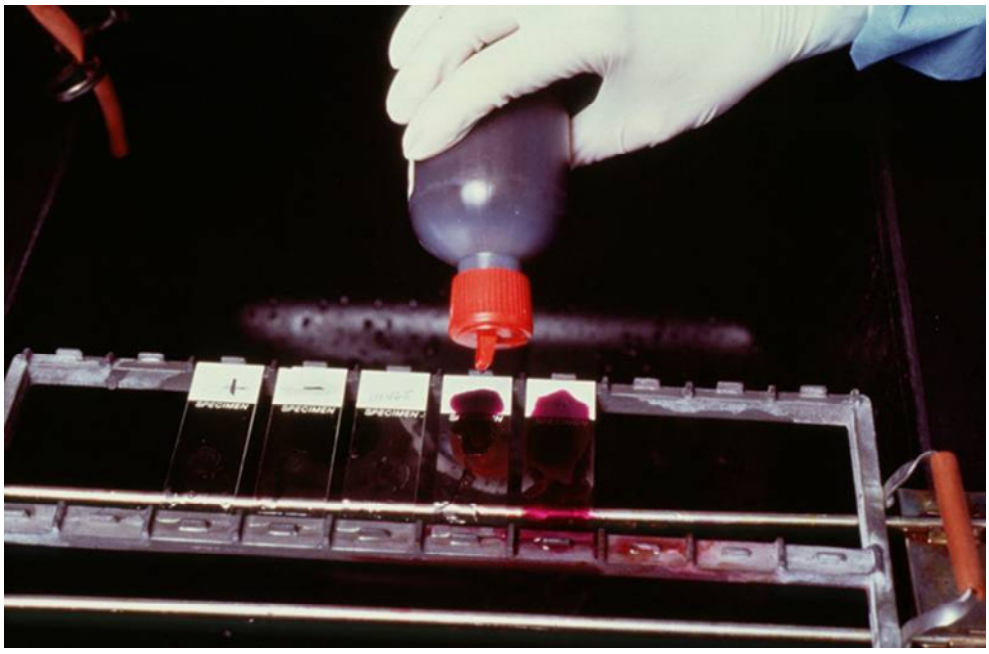
Step # 5

As before, rinse well with water so as to wash away the acid alcohol



Step # 6

Quench with 0.5% potassium permanganate for 1 minute



Step # 7

Rinse with water and slope the slides to air dry

EVALUATING SMEARS

Spend time evaluating good and bad smears. Without a quality smear, the procedure of diagnostic microbiology is seriously impeded. Bad smears can lead to false results. The quality of examination depends on making good smears. In this training, the preparation of good smears is a very important process.

Key messages

- Label slides with the laboratory serial number and specimen number (e.g., 562/2)
- Select the most purulent portion of the sample for smear preparation
- The size of the smear should consistently be 2-3 cm by 1-2 cm; the smear should be thin enough that you can read newsprint through it
- Use gentle heat fixation for smears
- In ZN staining, carbol fuchsin is heated to steaming at least twice. Leave it on the smear for 10 minutes
- Do not extend the time for counterstaining with methylene blue
- In Auramine staining, leave the Auramine solution on the smear for 20 minutes
- **DO NOT HEAT AURAMINE SOLUTION**
- Do not extend the time for quenching solution with potassium permanganate

Review: Module 7

Please answer the following questions based on the smear preparation module.

What labelling information is needed on a slide?

_____

_____

_____

What portion of the specimen should be used for smear preparation?

_____

_____

_____

How can you determine the correct size and thickness of a sputum smear?

_____

_____

_____

What are critical steps in the Auramine stain?

_____

_____

_____

Laboratory Practical Session #3: Preparation of Smears

Materials and Equipment:

- Sputum specimens
- Glass slides, clean with frosted end
- Disposable wooden or bamboo applicator sticks
- Spirit lamp (burning spirit)
- Bench disinfectant (5% phenol or 0.5 % sodium hypochlorite)
- Discard container
- Newsprint, Forceps, and Ruler marked in centimeters

Procedure:

Review safety procedures in module 2 before beginning this procedure.

1. USE a pencil to label the frosted end of a slide with the laboratory serial number on the sputum container.
2. OPEN the sputum container carefully and place the lid face up on the work surface.
3. EXAMINE the specimen to select the best portion to sample. Choose yellow (purulent) or bloodstained particles if present.
4. PREPARE the smears near the flame of a spirit lamp. The heat around the flame creates a sterile zone for aerosols generated during smearing of the specimen.
5. USE a wooden applicator stick to select the most purulent material from the specimen container.
6. USE the applicator stick to transfer the selected specimen particles/fluid to the glass slide.
7. SMEAR the specimen over a 2 x 3 cm area in the middle of the unfrosted part of the slide.
8. USE the applicator stick to crush, break up, and spread out particles.
9. USE small circular motions to distribute the specimen evenly.
10. DISCARD the applicator stick into a waste receptacle containing a suitable disinfectant.
11. RESEAL the sputum container and set aside.
12. ALLOW the smear to air dry completely (*never* use heat to reduce smear drying time).
13. AFTER the slide is completely dry, hold the slide using forceps, with the smeared slide facing upwards. Pass the slide over the flame 2-3 times, about 2-3 sec each time.
14. EVALUATE the fixed smear for the proper thickness.
15. *Consider smear to be potentially infectious until after it has been stained.*
16. Make sure you WASH your hands before leaving the laboratory.

Note: Use these smears for Practical Session# 4 “Staining Sputum Smears”.

Laboratory Practical Session #4: Staining Sputum Smears

Materials and Equipment:

- Ten smears prepared in Practical Session # 3 and a set of 5 unstained panel smears
- Staining sink
- Running water
- Small funnel with filter paper
- Set of Auramine stain reagents for each staining area
- Spirit lamp
- Beaker, forceps, and gloves

Procedure:

1. ARRANGE the slides by placing them in serial order on the leveled staining bridge, smear side up. Leave enough space between slides to prevent the transfer of material and/or staining solution from one smear to another.
2. APPLY Auramine stain. Cover the entire surface of the slide with filtered Auramine solution. If the staining solution drains off, add more stain to cover the entire slide.
3. DO NOT HEAT
4. Leave for 20 minutes and do not let the solution dry.
5. RINSE the slide. Tilt the slide to drain off excess stain and then rinse the staining solution off with a gentle stream of water. It may be convenient to use a beaker, flask, or squeeze bottle to pour the water onto the slides. When rinsing slides, avoid getting water stream directly on the smear; vigorous washing may cause the smear to lift. Tilt the slide to drain off excess rinse water.
6. DECOLORIZE the smear by covering the whole slide with 0.5% hydrochloric acid-alcohol solution and leave it for a maximum of 3 minutes.
7. WASH the slide again with a gentle stream of water. Tilt the slide to drain off excess water.
8. QUENCH the smear by covering the entire surface of the slide with potassium permanganate solution and leave it for a maximum of 1 minute.
9. DRAIN off the permanganate solution. Rinse the slide again with a gentle stream of water. Make sure the stained smear is free from stain deposits, dirt, debris, and crystals produced by overheating during staining. Underside of the smear should be wiped, if possible with alcohol.
10. PLACE on the slide rack to air-dry. Do not allow the stained slide to dry in direct sunlight. When the slides are completely dry, they are ready for microscopy. If they are not read immediately, place them in a slide box.

Module 8

Reading, Recording and Reporting of Fluorescent Smears

Purpose	To provide you with an understanding of smear reading, recording and reporting of fluorescent smears
Prerequisite Modules	Module 3 and 7
Learning Objectives	At the end of this module, you will be able to ▪ Describe the method for observing AFB using 40x objective for Auramine O stained smears ▪ Recognize the appearance of AFB in a stained smear ▪ Describe the quantification scheme for reporting results ▪ Appropriately quantify results in the study forms
Content Outline	▪ Required materials ▪ Reading the smear ▪ Recording of results: WHO/ IUATLD grading scale ▪ Storing smears ▪ Essential elements of record keeping ▪ Reporting results
Handout and Exercises	Exercise: Laboratory Practical session # 5: Reading and reporting of panel slides from Laboratory Practical session # 4
Appendices	Appendix 1 - Results form

Module 8: Reading, Recording and Reporting of Fluorescent Smears

Examination of sputum smears for acid-fast bacilli requires a good microscope and a motivated, trained technician.

Required Materials

The following materials are required to perform the microscopy of AFB smears:

1) Primo Star iLED microscope
2) Electric power or power pack
3) Lens paper or fine tissue paper
4) Lens cleaning solution
5) Laboratory register
6) Slide storage boxes
7) Red and blue writing pens

Microscope Components

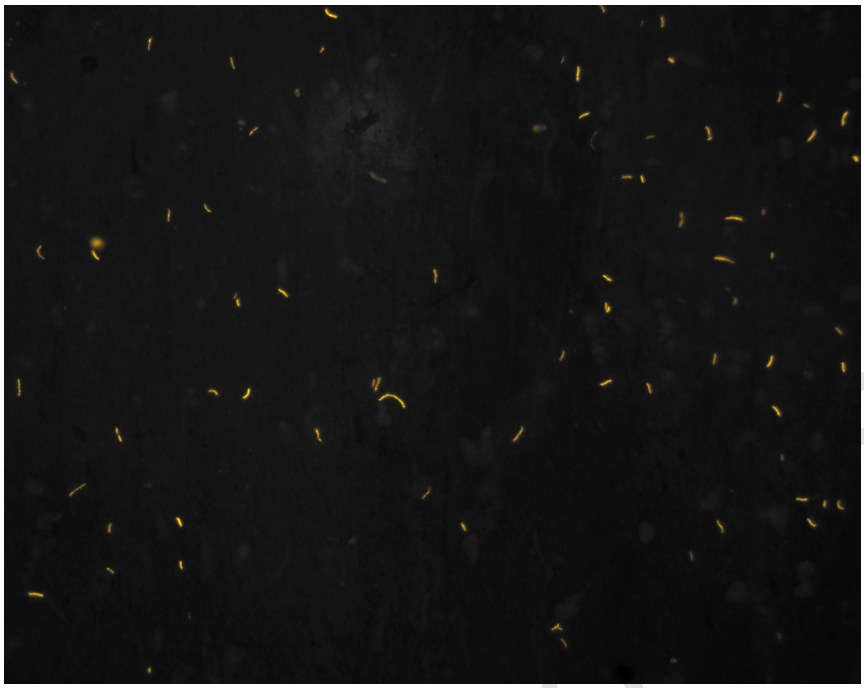
Review the components and functions of each part of the Primo Star iLED microscope (Module 3).

Cleaning the objectives

- Unlike ZN microscopy, immersion oil is not used for reading FM smears, hence the objective lenses do not require wiping with lens or fine tissue paper to remove traces of oil.

Reading the smear

- Keep stained smears in the dark (in a box or folder) until reading time, and read them as soon as possible, since fluorescence fades quickly.
- Make sure that the smear is facing upwards when the slide is placed on the mechanical stage.
- Focus the smear using low power objective 10x or 20X.
- Use the objective 40x objective to systematically examine the smear
- Scan the stained smear systematically from one side to the other and move back; at least one length/40 fields have to be scanned before reporting a negative, corresponding to 200 high-power fields and taking approximately 2 minutes (40x objective).
- Acid-fast bacilli appear bright yellow against the dark background material.



Appearance of AFB in Auramine O Smear

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, and rarely in large clumps. The typical appearance is of bacilli that are rather long and slender, slightly curved rods. With good staining (always check first a freshly stained positive control), there may still be fluorescing (sometimes green) artifacts, which do not have the typical shape. Also non-fluorescing bacillary shapes must be considered as artifacts.

Recording of Results

Semiquantitative results will be recorded according to the NTP guidelines. If no local guidelines are available it is recommended to follow the below grading scale.

Table 1: Recommended number of AFBs and fields for grading of ZN and fluorescent stained slides

IUATLD/WHO SCALE (1000x field=HPF) Result	MICROSCOPY SYSTEM USED		
	BRIGHTFIELD (1000x magnification; 1 length = 2cm = 100 HPF	FLUORESCENCE (200-250x magnification; 1 length = 30 fields = 300 HPF	FLUORESCENCE (400x magnification; 1 length = 40 fields = 200 HPF
Negative	Zero AFB/1 length	Zero AFB/1 length	Zero AFB/1 length
Scanty (actual count)	1-9 AFB/1 length or 100 HPF	1-29 AFB/1 length	1-19 AFB/1 length
1+	10-99 AFB/1 length or 100 HPF (=1-9 AFB/10 fields)	30-299 AFB/1 length	20-199 AFB/1 length
2+	1-10 AFB/1 HPF on average	10-100 AFB/1 field on average	5-50 AFB/1 field on average
3+	≥10 AFB/1 HPF on average	≥100 AFB/1 field on average	>50 AFB/1 field on average

The table below provides the number of fields to be read before providing results using different magnifications of FM.

Table 2: Magnification of FM objectives and minimum number of fields to be read for FM results using the Primo Star iLED during the project

FM objective magnification (power)	Minimum number of fields for FM results				
	Negative	**Scanty**	**1+**	**2+**	**3+**
40x	40	40	40	20	8

For the purpose of uniform examination and quantitative reporting of results, a method has been suggested (ref 1-WHO Manual on Microscopy Part II) whereby the number of acid-fast bacilli observed under fluorochrome staining could be divided by a "magnification correction factor" to yield an approximate number that might be observed if the same smear were examined under 1000x after carbol fuchsin stain. The magnification correction factors for the two FM objectives used in this study are given in Table 3 below:

Table 3: Magnification correction factor

FM objective magnification (power)	Magnification correction factor
20x	10
40x	5

Essential Elements of Recordkeeping

Accurate recordkeeping in the TB laboratory is essential. Recording means keeping the register up-to-date. Lives depend upon it, and so does the proper management of the tuberculosis control program. Records should include information about the following events:

- What type of specimens were received by the laboratory
- How were the specimens identified
- How results are reported
- When specimens are sent to higher-level laboratories for culture and drug susceptibility testing

Laboratories should use a standardized record keeping system that is simple, practical, and limited to recording only essential information.

Accurate recordkeeping is based on four fundamentals:

- Completeness
- Consistency
- Credibility
- Timeliness

During the project, supervisors should review 5-10% of the study results forms for correctness compared to source data (Laboratory Register) and ensure that laboratory recordkeeping meets the above elements.

- Record results in the laboratory register and study forms immediately after reading smears.

LABORATORY REQUEST AND REPORT FORMS

Patient details

In many countries, the Laboratory Request Form and the Microscopy Report Form are combined into a single sheet of paper. This enables better tracking of reporting and not only reduces the time it takes to transcribe patient and sample related information on separate report forms but also reduces transcription errors.

A Laboratory Request Form must be submitted with the first sputum specimen (or patient). Information on the form must exactly match the information on the slide of the specimen container. If the form is incomplete, and the patient is available, ask the patient for the required information. Also, the NTP needs to know whether the specimens are for diagnosis or follow-up.

A completed Laboratory Request Form should give the following information (See Appendix 3):

- Name of health center
- Date
- Patient's name, address, age, and sex
- Source of specimen
- Reason for exam (diagnosis or follow-up)
- Specimen ID number
- Signature of person requesting exam

Microscopy report

After the sputum smear has been read, the result should be written immediately into the result form. **Whenever possible, use a RED pen for positive results.** Check that the number on the slide matches the number on the Laboratory Request form. Subsequently, the results are written onto the Laboratory Register, again checking to make sure that the laboratory serial number matches for both.

The microscopy report should include the following information:

- Specimen ID number (laboratory serial number)
- Date of specimen collection
- Evaluation of the quality of the specimen (e.g., bloody, mucopurulent, saliva)
- Smear result
- Date of examination
- Name and Signature of technician, who performed the microscopy

Once completed, the microscopy report should be made available as soon as possible, preferably no longer than 24 hours after the laboratory receives the specimen.

The Laboratory Register

It is recommended to use the WHO or IUATLD laboratory register as a guideline. The format of this register should never be altered by laboratory staff.

This is a record book maintained by the technician/technologist responsible for sputum smear examination of patients with suspected TB and their follow-up examinations. The TB laboratory register must include the following data for each patient with suspected TB:

The laboratory register should include:

- Laboratory serial number
- TB registration number
- Date the specimen was received
- Patient's name, sex, age, and address
- Reason for exam (diagnosis or follow-up)
- Smear results
- Signature of person responsible for tests

Make sure all necessary columns are filled in. However, if patients with suspected TB do not deliver three sputa (in those settings where three sputa are required according to NTP guidelines), leave the data field blank. A blank space is not a negative result. Results must be accurate; scanty results should be entered as such and not changed to negative or positive. A positive follow-up result must also be registered accurately, whatever the conversion or cure targets may be.

- Reset the laboratory register number to one on January first each year. DO NOT reset at the end of each day, week, or month.
- Enter patients successively, increasing the line number by one each time. The line number is sufficient for identification of the request form.
- Slides require an extension to identify the first (spot), second (morning), or third (spot) sputum of a series (in those settings where three sputa are required according to NTP guidelines). Add "/a" or "/b" or "/a" or "/b" or "/c" if two or three sputa are required, after the line number.
- Allocate a separate line and serial number in the register to specimens submitted for follow-up examination.

Always fill in the reason for examination (i.e., diagnosis or follow-up). Use a tick mark to indicate whether the specimen is "diagnostic" and indicate the TB number for “follow-up” patients. A diagnostic patient with specimens with negative results in all smears will submit specimens after completion of two weeks of antibiotic therapy (or according to NTP guidelines), and these are labeled as ‘RE’.

Supervisors should analyze the register when conducting a laboratory review as it provides a simple, easy, and rapid summary of the work conducted in a laboratory and assessment of its performance. In positive diagnostic samples, it can be helpful to obtain the patient's registration number from the NTP; this number should be added either under the tick mark or in the remarks column.

False-negative: consequences

False-negative means that the result reported as negative was actually positive.

- Patients with TB may not be treated, resulting in ongoing disease, disease transmission, or death.
- Intensive phase treatment may not be extended, resulting in inadequate treatment and potential drug resistance.

False-positive: consequences

False-positive means that the result reported as positive was actually negative.

- Patients are treated unnecessarily.
- Treatment may be continued longer than necessary.
- Medications will be wasted.

Storing Smears

- Store ALL slides in slide boxes in the order they were recorded in the laboratory register. This will allow easy sampling of slides for external quality assessment using blinded slide rechecking.
- Do not write the result on the slide.

Exercise: Laboratory Practical session # 5

Reading and reporting of panel slides from Laboratory Practical session # 4

Read and report the smears prepared in Practical Session # 4.

Use:

- Appendix 1: Microscopy recording form
- Appendix 2: Grading chart, and
- Appendix 3: Panel testing report form

Appendix 1

Results Form
(For Laboratory Practical Session# 5)

Microscopy Center ID # _____
Supervisory Site ID # _____

Slide ID	Date of sample reception	Date of slide reading	Results*		Lab Tech ID**
			Neg	Pos	

* If negative, tick “neg”; if positive register “scanty”, “1+”, “2+” or “3+” according to IUATLD/ WHO scale.
** Number assigned by the supervisory site.

Key messages

- Use the recommended grading scale for the FM smears
- Systematically scan the slide by moving across the smear in a horizontal direction
- Examine each field before moving on to the next field
- Read at least 30 high power fields (20x) for FM smears before reporting a negative result
- Store all examined smears in the order they appear in the laboratory register
- Accuracy is critical at all levels of reporting and recording
- Each field must be filled out carefully
- Recording of results must adhere to guidelines provided in this module for FM smears
- The number of AFB found indicates how infectious the patient is, so it is important to record exactly what you see

Review: Module 8

Please answer the following questions based on the reading, recording and reporting module.

How many AFBs are required for a 1+, 2+, and 3+ FM smears?

_____

_____

_____

How many fields need to be examined when reading FM smears for AFB?

_____

_____

_____

Which smears must be stored after examination?

_____

_____

_____

When and how are microscope objectives cleaned?

_____

_____

_____

What are the 4 elements required for accurate recordkeeping?

_____

_____

_____

What are the essential data required in the Laboratory Register?

_____

_____

_____

Why is it important to identify whether a specimen is for diagnosis or follow-up?

_____

_____

_____

What are the quantifying categories of smear reading?

_____

_____

_____

What are the consequences of reporting a false-positive result?

_____

_____

_____

Module 9

Assuring Quality of Fluorescence-based AFB Microscopy

Purpose	To provide you with an understanding of quality assurance and external quality assurance of AFB smear microscopy
Prerequisite Modules	Modules 1–8
Learning Objectives	At the end of this module, you will be able to: ▪ Describe the elements of Quality Assurance ▪ Explain why internal Quality Control is important in assessing laboratory performance ▪ Describe the three components of External Quality Assessment (EQA) ▪ Prepare for a supervisory visit ▪ Describe the process of sampling slides for blinded smear rechecking
Content Outline	▪ What is Quality assurance? ▪ Quality Control in the TB laboratory ▪ EQA and why it is important ▪ EQA components • Panel Testing • On-Site Evaluation • Blinded Rechecking
Handout and Exercises	Exercise 1, 2 and 3
Appendix	Appendix 1: Onsite Evaluation Checklist

Module 9: Assuring Quality of Fluorescence-based AFB Microscopy

For many countries with a high burden of TB, direct smear microscopy remains the most cost-effective tool for laboratory diagnosis of patients with infectious TB (smear-positive pulmonary disease). However, if the laboratory diagnosis is unreliable, then patients with infectious TB may not be diagnosed, resulting in ongoing transmission of disease in the community and more severe disease in the individual. Alternatively, patients without TB may be treated unnecessarily. Therefore, quality assurance of AFB sputum smear microscopy is essential.

WHAT IS QUALITY ASSURANCE?

Accuracy and reliability of laboratory testing are critical to the success of TB control programs. All parts of the testing system must be monitored to ensure the quality of the overall process, to detect and reduce errors, and to improve consistency between testing sites. To ensure reliability and to reduce errors, a quality system must address all parts of laboratory testing.

Quality Assurance (QA) is a system designed to improve the reliability and efficiency of laboratory services. WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) have defined several components for a quality assurance program for AFB smear microscopy:

- **Quality Control (QC):** A systematic internal monitoring of work practices, technical procedures, equipment, and materials including quality of stains.
- **External Quality Assessment (EQA):** A process to assess laboratory performance. EQA includes onsite evaluation of laboratories, panel testing, and blinded smear rechecking.
- **Quality Improvement (QI):** A process by which the components of smear microscopy diagnostic services are analyzed with the aim to identify and permanently correct any deficiencies. Data collection, data analysis, and creative problem solving are skills used in this process.

QUALITY CONTROL IN THE TB LABORATORY

Quality Control helps to ensure that the results produced by a laboratory are accurate, reliable, and reproducible. The QC program should be performed regularly, and to be effective, the process must be practical and readily included in standard laboratory reporting practices. All laboratory technicians are responsible for performing, recording, and reporting results of QC.

Many components of QC are either performed in conjunction with routine testing or as part of the regular management of the laboratory.

Laboratory arrangement and administration

- Ensure that doors into the laboratory are always closed. Work areas, equipment, and supplies should be arranged for logical and efficient workflow.
- Work areas should be kept free of dust. Benches should be cleaned at least daily with an appropriate disinfectant.
- Use laboratory procedures that comply with NTP guidelines.
- Every procedure performed in the laboratory must be performed as per the SOP.
- The procedures must be kept in the laboratory and be readily available.
- Any changes to procedures must be dated and initialed by the laboratory supervisor.

- Staff should have appropriate training and have their performance monitored.

Laboratory equipment

- The operating manual and cleaning instructions for all equipment must be readily available.
- Dated service records must be kept for all equipment.
- Microscope and balance must be monitored regularly to ensure consistent performance.

Specimens and request forms

- Perform microscopy only upon written request of authorized persons. Do not allow oral requests without a completed, follow-up request form.
- Insist on adequately completed request forms and proper labeling of specimens. This ensures positive identification of patients.
- Reject specimens that can not be properly identified, are leaking, or in broken containers. Request a repeat specimen.
- Record the date specimens arrive in the laboratory. Document on the request form any delays in the delivery of specimens to the laboratory.
- Evaluate the quality of sputum specimens. Record and monitor the number of salivary specimens received by the laboratory.
- Keep laboratory request forms separate from specimens. Forms that have been contaminated during transportation or otherwise by specimens should be discarded either by autoclaving or burning. Accurately make duplicate form from the original form before discarding.

Reagents and stains

- All staining reagents should be labeled with the name, date of preparation, and date first opened.
- If staining reagents are prepared in another laboratory, indicate date received.
- Record any material found to be unsatisfactory and remove it from the laboratory immediately so it is not used.
- Limit stocks to three months' supply. For study purposes, Auramine solution supply will be limited to one month. Rotate stock to ensure that oldest material is used first.

Staining and smear examination

FM staining

In addition to the above-mentioned activities, the following should be observed:

- Do not heat Auramine O solution
- Do not use oil for reading
- Do not keep stained slides in direct sunlight

- Unacceptable control slides mean:
  - positive control is not stained bright yellow
  - negative control remains fluorescent even after decolorization
  - background is not dark

Resolve any problems with control smears before reporting patient smears. Some problems may require repeating patient smears in a failed staining batch.

Recording and Reporting

- Send microscopy results out as soon as they are available, preferably within 24 hours after the sputum specimen is received. Monitor any delays or turn around time in delivery on the report form.
- Analyze microscopy results on a monthly basis to detect changes which may indicate a problem.
- All microscopy results must be recorded in standard format in laboratory register.
- It is recommended that all records be retained for at least two years.
- For study specific forms see Table 1 for frequency of completed forms retrieval.

EXTERNAL QUALITY ASSESSMENT (EQA) AND WHY IT IS IMPORTANT

The purpose of EQA is to help laboratories identify errors and improve practices for better performance.
EQA does not identify individual slide errors nor validate individual patient diagnoses. Involvement in an EQA activity should not be seen as a threat, but rather as an opportunity to strengthen skills. Most laboratory technicians want to provide accurate testing. Good performance in EQA activities reassures them that their results are contributing to TB diagnosis and control.

During this study, three methods will be used to evaluate laboratory performance:

- Onsite evaluation
- Panel testing
- Blinded smear rechecking

Table 1: Evaluation of laboratory performance

Study phase	Frequency of retrieving slides and forms	Supervisory visit with checklist	Panel testing	% slides re-checked by supervisory site
ZN Baseline	Once every 2nd week	Once a month		100%
iLED Training	NA	NA	Proficiency Testing 20 slides (10 ZN, 10 Auramine)	NA
Validation	Daily	Once every 2nd week	Proficiency at the end of this phase determined by analysis of rechecking results and by proficiency panel testing (10 Auramine slides)	100%
Implementation	Once every 2nd week	Once a month		As per LQAS
Continuation	Once a month	Once a month	NA	According to National TB Guidelines

Onsite evaluation

Monitoring visits will be performed by the Supervisory Site and frequency will vary according to the study phase (see Table 1 above). During some of these visits, the laboratory will receive an onsite evaluation (OSE) by the supervisor (Table 1). These visits allow the worker to be observed under actual working conditions. The state of equipment, laboratory safety, and the adequacy of supplies are also assessed. During OSE, the technician observes the process for specimen collection, smearing, staining, reading, recording, and reporting. Stained smears will be reviewed during the visit. When problems are detected, solutions will be suggested and, if possible, implemented immediately.

It is the responsibility of the participating microscopy centers to assist the visiting supervisory staff. The laboratory should make records available, demonstrate routine performance, and provide information.

The supervisor uses a standard checklist of items to be reviewed. These visits provide an opportunity to learn about standards, techniques, and methods. In addition, they provide an opportunity for basic supervision including assessment of laboratory supplies, basic procedures, and performance of internal QC. The supervisor will collect slides for rechecking, deliver slides for panel testing, or deliver results of EQA activities.

A format of OSE is given in Appendix 1 (see also the SOP Phase 2).

Panel testing

A panel test (PT) exercise usually involves sending a PT with an identical composition (of negatives and positives) to many laboratories at the same time. It is useful as an initial gauge of the current level of laboratory performance, as well as to determine critical priorities for expanding EQA. While it also measures the ability of a technician to stain and/or read smears, it does not assess routine laboratory performance.

It is the responsibility of the laboratory technicians to read the PT slides in the same way they read patient slides. They also need to take the same amount of time as for routine smears. Panel testing is an opportunity to compare performance with other laboratories, and reassures technicians that they can attain the same results as other laboratorians.

If discrepant results are found during PT, this may require returning slides to the supervisory laboratory for rereading

The proficiency testing during the project will be performed at the end of each study phase (see Table 1).

Slide re-checking

Re-checking is the best method for evaluating performance and motivating staff to improve. Re-checking programs are intended to assess overall laboratory performance, not to confirm any individual patient's diagnosis. This process occurs when controllers at the Supervisory Site re-read a 100% or a sample of routine smears from the microscopy centers.

During proficiency testing, validation and validation phase 100% slides will be re-checked. A minimum sample of slides will be rechecked as per LQAS method during implementation phase. The sample will be based on 80% sensitivity, 100% specificity and '0' acceptance number for a pre-selected ranges of slide positivity rate and annualized negative slide volumes (see Table 2 below).

Table 2: Lot quality assurance sampling (LQAS) for implementation phase (80% sensitivity, 100% Specificity and '0' Acceptance number)

Annualized no. of negative slides (ANSV) at the demonstration site	Slide positivity rate (SPR%)				
	2.5-4.9	**5.0-7.49**	**7.5-9.9**	**10-14.9**	**≥15**
	Monthly sample size[1] of randomly selected slides to be re-checked				
301-500	22	14	12	10	8
501-1000	28	18	12	10	8
>1000	40	20	14	10	8

Slide positivity rate and annualized negative slide volumes are calculated based on the data available for one month from each of the microscopy centers during 'ZN baseline' phase.

The sample size for each microscopy center is selected based on SPR and ANSV, as per Table 2 given above.

Re-checking

Re-staining will only be required for Auramine O fluorescent stained smears if staining is considered of low quality by the supervisor.

Feedback on the results of discordant slides, along with the slides, must be returned to the supervisor during monitoring visits, and action taken to resolve any performance problems identified.

Types of Errors

It is important to re-emphasize that random blinded **rechecking (RBRC) is not a method for validating individual patient diagnosis**, but rather of assessing overall laboratory performance, detecting unacceptable levels of errors so that corrective action can be taken, and providing continuous motivation for good performance**.**

For the purposes of EQA, the types of errors are classified on the basis of expected laboratory performance (Table 3), not on the potential impact of patient management.

[1] The monthly sample size has been rounded off to the next higher number and annually adds up to equal or more than the annual sample size.

Table 3: Classification of Errors

Result by MC LT	Result of Controllers				
	Neg	Scanty	1+	2+	3+
Neg	Correct	LFN	**HFN**	**HFN**	**HFN**
Scanty	LFP	Correct	Correct	QE	QE
1+	**HFP**	Correct	Correct	Correct	QE
2+	**HFP**	QE	Correct	Correct	Correct
3+	**HFP**	QE	QE	Correct	Correct

Correct: No errors
QE: Quantification error: Minor error
LFN: Low False Negative: Minor error
LFP: Low False Positive: Minor error
HFN: High False Negative: **Major error**
HFP: High False Positive: **Major error**

Investigation of errors

Any error that is detected during rechecking should be investigated by identifying the probable causes based on the results of the checklist and possible corrective actions are suggested. Table 4 gives some of the causes and corrective actions for the errors identified.

Feedback

The primary purpose of the re-checking is to improve the overall quality of smear microscopy; therefore regular and timely feedback to the microscopy center is essential if any improvements in performance are to be expected. The preliminary observations, feedback and remedial action will often be possible at the end of each sampling period.

Potential sources of errors are to be investigated during the on-site evaluation visits. Appropriate corrective actions and/or remedial training are to be provided during the next visit by the supervisor.

Critical components of an accurate and practical re-checking system include:

- A sufficient number of randomly selected slides to represent work performed
- The inclusion of minor errors, representing false positive or false negative interpretations of 'scanty' results, with major errors (the smaller sample size aids implementation and sustainability of rechecking programs)
- A system to provide prompt, continual feedback and improvements to the laboratories that are supervised

Discrepant results will be resolved by Supra National Reference Center.

Table 4: Investigation of Errors

Pattern of errors	Possible causes	Suggested investigation steps
HFP and HFN	Unusable microscope	Examine a 3+ using that microscope
	Staining problems, poor stains, insufficient staining time or heating	Check stains and staining procedure
	Technician cannot recognize AFB	Test with clear-cut positive & negative slides and good microscope
	Gross neglect, overworked, lack motivation	Exclude other causes
HFP with or without LFP	Administrative error	Compare lab-register and verify correct slide number and result? Exclude causes of more frequent HFP, such as low concentration of sulphuric acid, unusable microscope, untrained or inexperienced LTs.
	Poor registration routine	Check accuracy of lab-register and other record keeping
	Staining problems/Fading	Check stains and staining procedure, consider re-staining for rechecking. Assess concentration of phenol, basic fuchsin/ Auramine O and methylene blue.
	Technician unclear on AFB appearance	Look for inconsistent results of suspects (regularly single pos / low positive) in lab register
Many LFP, with or without occasional HFP	Problem with controllers Technician unclear on AFB appearance Contaminated stain/ reagents	Evaluate controllers Recheck sample of LFP from laboratory register Test stain with known negative smears, check the distilled water used for stain preparation
HFN with or without LFN	Administrative error	Compare lab-register with QC-listing: correct slide number & result?
	Very thick smears and/or poor light	Evaluate quality of smear preparation, check microscope
	Gross neglect	Exclude other causes
	Staining problems	Check stains and staining procedure, consider re-staining for rechecking. Assess concentration of phenol, basic fuchsin, Auramine O, potassium permanganate and methylene blue
	Poor smearing-technique	Test stain with known negative smears
	Problems with microscope	Check microscope with known positive smear
	Careless microscopy	Exclude other causes
Very high proportion LFN	Reading error	Less than recommended fields are being read, probably due to high workload or inadequate training
	Concentrated Methylene blue for ZN method	Check the concentration of Methylene blue
Many QE (too low grading)	Poor staining	Check the concentrations of basic fuchsin in ZN stain, Auramine O in FM stain and phenol in both
	Problems with microscope	Check microscope with known positive smear

Responsibilities of the Laboratory Technician:

1. **Store all slides in a way that allows retrieval of every slide identified for the rechecking sample when 100% re-checking is not required.** They must be stored in the slide boxes in the same order as they are listed in the laboratory register.

2. **Label slides in a manner consistent with the laboratory register** to ensure that the correct slide can be matched to the result. The labeling must be legible. The result of the smear examination must not appear on the slide.

3. Always **store slides in closed boxes away from direct sunlight**. This procedure is important for FM smears.

4. Label the slide box with the name of Demonstration site, and date.

5. The lab technician, if possible in consultation with Lab Head and Medical Director, should address the problems identified by the Supervisor (OSE Summary)

Key messages

- Everybody is responsible for ensuring that laboratory results are accurate and reliable
- To demonstrate and maintain high-quality results, a laboratory's performance needs to be monitored by the following regular QA activities:
  - Internal Quality Control (QC)
  - External Quality Assessment (EQA)
- QC is the process of effective and systematic internal monitoring of routine laboratory work
- EQA is the systematic and independent assessment of laboratory performance

Review: Module 9

Please answer the following questions based on the FM quality assurance module.

What are the components of Quality Assurance?

_____

_____

_____

What is Quality Control?

_____

_____

_____

What is blinded slide rechecking?

_____

_____

_____

For blinded slide rechecking, how does the technician store the slides?

_____

_____

_____

What should you do to make sure that the ZN & FM reagents are working properly?

Which method are used for identifying AFB during ZN baseline phase, validation phase, implementation phase and continuation phase?

List the microscopes used for each of the phases; ZN baseline, validation, implementation and continuation phases

List the frequency of OSE during each of the phases; ZN baseline, validation, implementation and continuation phases

In which phase the decision to start the patients on treatment is based on results of supervisory site?

List the frequency and number of slides for Proficiency Panel testing for each of the phases

ON-SITE EVALUATION CHECKLIST: SUPERVISORY VISIT

(User: Supervisory Site*)

iLED Demonstration Project

I General Information

Supervisor:	
Supervisory Site (name/ID):	
Microscopy Center (name/ID):	
Date of Visit:	

II Laboratory infrastructure and equipment:

- Uninterrupted power supply	No problems ☐ Rare and short interruptions ☐ Regular or sometimes long interruptions ☐
- Running water supply	No problems ☐ Rare and brief interruptions ☐ Regular or sometimes long interruptions ☐
- Primo Star iLED microscope	No technical problems observed ☐ Technical problems observed ☐, Specify:

III Adequate stock (within expiry dates) and supply of:

Item	Adequate	Comments / Requirement
- Slides	Yes / No	
- Lens tissue	Yes / No	
- Smearing/staining equipment	Yes / No	
- 0.1% Auramine	Yes / No	
- 0.5% Acid alcohol	Yes / No	
- 0.5% Potassium permanganate	Yes / No	
- Slide boxes	Yes / No	
- Study forms	Yes / No	
- Other:		

**To be completed monthly during each study phase, except during Validation phase every 2nd week*

IV Study procedures and documentation

Item	Adequate / Acceptable	Problems identified / Requirement
Study-specific SOP followed		
- Smear preparation observation	Yes / No	
- Staining procedure observation	Yes / No	
- Slides properly stained** (thickness, field size)	Yes / No	
- Slides reading following grading chart	Yes / No	
- Slide boxes stored	Yes / No	
- All slides are available and stored as per lab register***	Yes / No	
- Storage of reagents: reagent bottles labeled with content, date of preparation, date of expiry	Yes / No	
- Storage of slides in boxes	Yes / No	
- Study forms have correctly completed	Yes / No	
- Completed study forms properly filed	Yes / No	
- Review of 5-10% of results forms for correctness compared to source data	Yes / No	

**Check 4 recent positive slides

***Check 20 IDs per visit

V Internal Quality Control

- Control smears are used for each new batch of staining solutions (register are available)	Yes / No	Comments:
- Control positive slides are used at least once a week	Yes / No	

VI Supervisory site tasks

- Supply study forms	Yes ☐	Not required ☐
- Supply slide boxes	Yes ☐	Not required ☐
- Supply staining solutions (Auramine at least once a month)	Yes ☐	Not required ☐
- Completed forms and slides retrieved for re-checking	Yes ☐	

VII Overall remarks

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