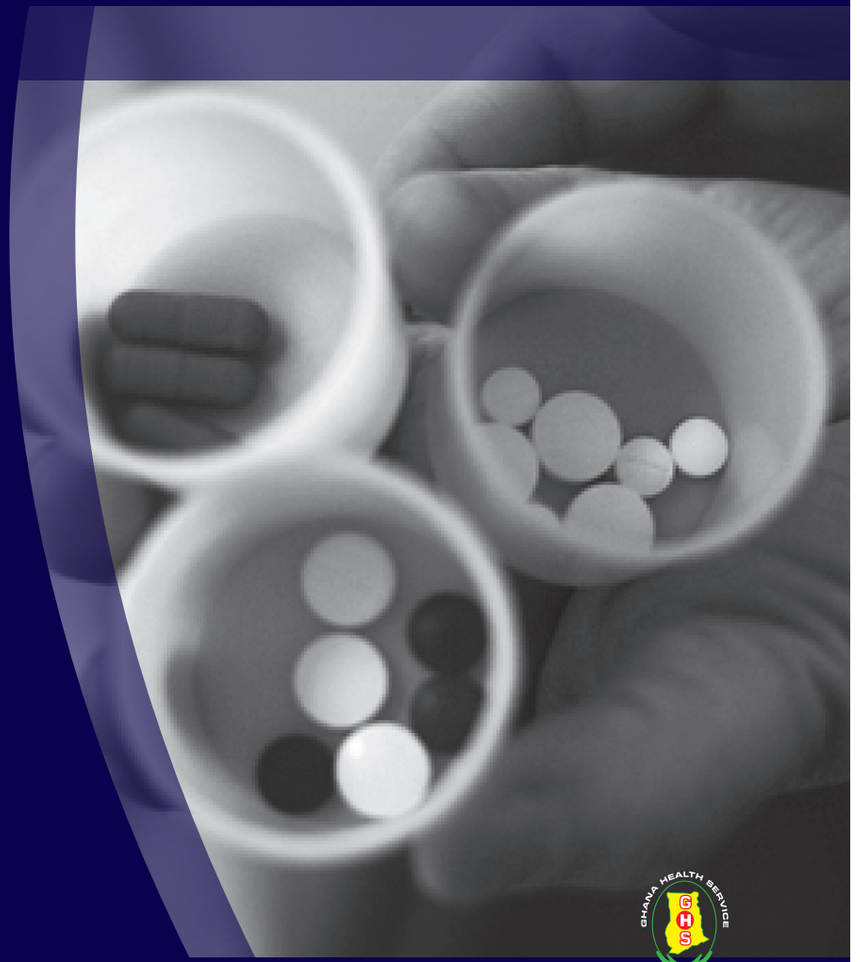
Standard Operating Procedure For Tuberculosis Microscopy For Ghana



Ghana Health Service, Ministry of Health.
Accra, Ghana.

DESIGNXPRESS

PREFACE

In spite of great success in improving treatment outcomes, Ghana's case detection is far behind the 2005 World Health Assembly global target for case detection (>70% of estimated incidence of new smear positive TB). This low case detection rates remain an obstacle to the long-term success of the NTP in Ghana. The comprehensive Programme review of the Ghana NTP in 2007 and the National Tuberculosis Health Sector Strategic Plan for Ghana (2009-2013) clearly identified low TB case detection as one of the main challenges facing TB control in Ghana. The laboratory plays a vital role in improving case detection and quality of diagnosis

One of the mandates of the Global Laboratory Initiative (GLI) is to guide and coordinate the scaling-up of TB laboratory services. In order to assist the GLI in this, the Tuberculosis Control Assistance Project (TBCAP) partners have developed generic TB Laboratory Standard Operating Procedures (SOPs), covering all the techniques that are needed to comply with the WHO Stop TB Strategy. Development and implementation of SOPs are key steps in realizing quality-assured TB laboratory services that provide reliable microscopy, services.

This SOP is modelled around the TBCAP tool.

The sops are structured as follows: Scope Definitions and abbreviations, Principles, Samples, Equipment and materials, Reagents and solutions, Detailed instructions, Reporting, Control quality

Dr. Frank Adae Bonsu
Manager
National Tuberculosis Control Programme

Acknowlegement

The Laboratory Manual is a revised edition of the first originally developed by a task team led by Dr Kwasi Addo, Dr Dorothy Yeboah Manu and others, all from Noguchi Memorial Institute for Medical Research.

In the current Edition the following experts and technical groups made tremendous contributions and are gratefully acknowledged.

Programmatic	**Laboratory Group**
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Ms Francesca Dzata	Laboratory programme management
Dr. Rehab Chimzizi	TBCARE I Country Director Laboratory Experts Task Team

The task team reviewed the laboratory Manual using all literature and documents provided by the laboratory programmatic group and the team laboratory practice experience.

• Dr Kennedy Kwasi Addo	Bacteriologist, NMMIR (Leader)
• Prof. E.H Frimpong	Bacteriologist, SMS, KNUST
• Francesca Dzata	Deputy Chief Biomedical Scientist, CTU
• Mr. Samuel Kudazawu	Biomedical Scientist, KBTH

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- Mr. Andrews Adjei Annan — Biomedical Scientist, Swedru Hospital
- Mr. Tony Basingnaa — Biomedical Scientist, UE

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Dr. Frank Adae Bonsu
Programme Manager
National Tuberculosis Control Programme

Introduction

Importance and use of SOPs
Standard Operating Procedures (SOPs) are an essential part of good laboratory practices and ensure consistency of quality in laboratory results. SOPs are also a prerequisite for accreditation of laboratories

Standard Operating Procedures provide detailed step-by-step instructions for carrying out a laboratory activity in a (bio) safe manner (for the laboratory staff, the community and the environment) and achieving accurate and reliable laboratory results. SOPs are used in the laboratory and written copies should be available (preferably displayed) at the work area or bench. They are important for the standardization of procedures, ensuring that every laboratory at each level of care performs national standard procedures and produces good-quality results.

TB laboratory SOPs:

- provide written standardized techniques for use in the laboratory;
- provide laboratory staff with instruction on how to consistently perform tests to an acceptable standard to ensure conformity in pre-analytical, analytical and post-analysis steps;
- avoid the performance of a test being changed by new staff and avoid shortcuts;
- maintain and improve the quality of TB laboratory services;
- improve the reliability of test results for clinical and epidemiological interpretation;
- Promote safe laboratory practice.

Standard Operating Procedure (Sop)
Use Of Personal Protective Equipment In An AFB Microscopy Laboratory

1. Scope

The SOP describes the use of personal protective equipment and clothing related to the handling of specimens for AFB smear microscopy.

Abbreviations

AFB: acid-fast bacilli
MDR: multidrug-resistant
NA: not applicable
PPE: personal protective equipment

4.1 Principle

Personal protective equipment (PPE) may act as a barrier to minimize the risk of exposure to aerosols, splashes and accidental inoculation.

The risks associated with smear preparation are considered to be less than those associated with manipulation of cultures. Proper laboratory ventilation, which directs potentially infectious particles away from laboratory workers, is the most appropriate control measure provided that workers adhere rigorously to good laboratory practice and good microbiological technique.

However, risk assessment should be carried out, regularly reviewed and revised when necessary to define the safest possible conditions for work with additional protection barriers – PPE and/or biological safety cabinets.

4.2 Samples

NA

4.3 Equipment and materials

4.3.1 Masks

Surgical masks do not offer significant protection to laboratory personnel performing aerosol-producing TB diagnostic techniques.

They are not designed to protect the wearer from inhaling small infectious aerosols.

Respirators (N95/FFP2) are not necessary for laboratory staff performing microscopy activities. However, if risk assessment indicates that they should be worn in exceptional circumstances, refer to SOP on protective clothing in culture/DST laboratories.

4.3.2 Gloves

In accordance to universal precautions, appropriate gloves should be worn for all procedures.
Gloves must be worn in case of hand injury/skin disease.
Gloves may give a false sense of protection.

Contaminated gloves may in fact be the source of hazards for other staff members if used to handle or operate equipment in the laboratory.
Change gloves after every session that requires their use and after every interruption of the activity.

Never wear gloves outside the laboratory.

Proper hand-washing with soap and adequate care in the handling of contaminated materials are critical elements of safe laboratory practice.
Disposable gloves (latex, vinyl or nitrile) can be used, and the correct size (small, medium or large) should be available for all individuals.
Discard used gloves as contaminated material.

The procedure for removing gloves safely is to pull the first glove by the cuff, over and off the first hand; before the tips of the fingers are completely out of the first glove,
Use the first glove to pull the second glove off the second hand completely. This should prevent the skin from contacting the outer surface of either glove.

Following the safe removal of gloves, wash hands immediately with water and soap (Annex 1).

4.3.3 Laboratory coats

Always use a laboratory coat inside the laboratory ***(never outside)***.
Laboratory coats should be fully buttoned, long sleeved and cuffed.
Laboratory coats must be stored apart from personnel clothing.

Change at least weekly. Laundering services should be provided at/near the facility.

4.3.4 Protective glasses

Protective glasses should always be worn when handling acids, alkaline and irritant chemicals during reagent preparation.

4.4 Reagents and solutions

NA

4.5 Detailed instructions

Protective clothing must be worn when working in the laboratory, as described above.

Whenever necessary, hands should be thoroughly lathered with soap, using friction, for at least 10 s, rinsed in clean water and dried using a clean paper, hand dryer or cloth towel (see Annex 1).

Before leaving the laboratory, protective clothing should be removed and hands should be washed with soap.

A hand-washing sink should be provided in each laboratory room, preferably near the exit door. Foot- or elbow-operated taps are recommended. Where not fitted, a paper/cloth towel should be used to turn off the tap handles to avoid re-contaminating washed hands (see Annex 1).

4.6 Reading, interpretation, recording and reporting

NA

4.7 Quality control

NA

4.8 Waste management and other safety precautions

Personnel should carefully adhere to good laboratory practice and GMT.

Frequent hand-washing with carbolic or liquid disinfectant soap and care when handling contaminated materials are elements of good laboratory practice (see Annex 1).

Used gloves should be discarded as contaminated material.

2. Related documents

Biosafety in microbiological and biomedical laboratories, 5th ed. Washington, DC, 2007. United States Department of Health and Human Services/Centers for Disease Control and Prevention/National Institutes of Health, 2007.

Kim SJ et al. Risk of occupational tuberculosis in National Tuberculosis Programme laboratories in Korea. International Journal of Tuberculosis and Lung Disease, 2007, 11(2):138–142.

Laboratory biosafety manual, 3rd ed. Geneva, World Health Organization, 2004. Rieder HL et al. The public health service national tuberculosis reference laboratory and the national laboratory network. Minimum requirements, role and operation in a low-income country. **Paris, International Union Against Tuberculosis and Lung Disease, 1998.**

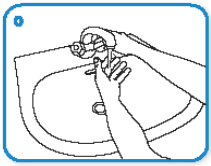
Standards Australia/Standards New Zealand. Safety in laboratories – microbiological aspects and containment facilities. **Sydney, Standards Australia International, 2002.**

Annex 1: How to handwash. *http://www.who.int/gpsc/tools/HAND_WASHING.pdf*

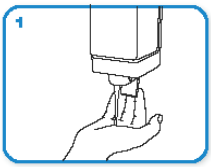
How to handwash?

WASH HANDS ONLY WHEN VISIBLY SOILED! OTHERWISE, USE HANDRUB!

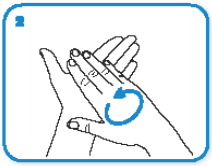
Duration of the entire procedure: **40-60 sec.**



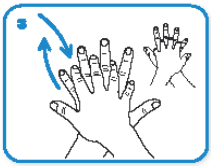
Wet hands with water



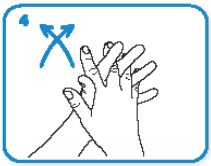
apply enough soap to cover all hand surfaces.



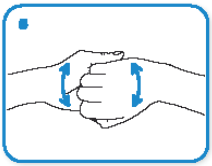
Rub hands palm to palm



right palm over left dorsum with interlaced fingers and vice versa

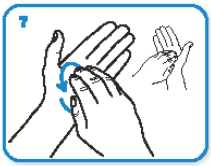


palm to palm with fingers interlaced

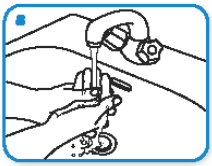


backs of fingers to opposing palms with fingers interlocked

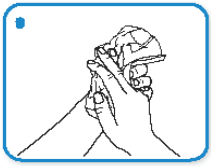
rotational rubbing of left thumb clasped in right palm and vice versa



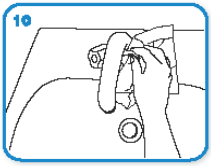
rotational rubbing, backwards and forwards with clasped fingers of right hand in left palm and vice versa.



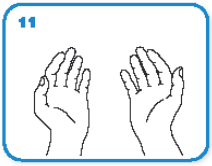
Rinse hands with water



dry thoroughly with a single use towel



use towel to turn off faucet



...and your hands are safe.

WORLD ALLIANCE FOR PATIENT SAFETY

WHO acknowledges the Hôpitaux Universitaires de Genève (HUG),
in particular the members of the Infection Control Programme, for their active participation in developing this material.

World Health Organization

Standard Operating Procedure (Sop)

Emergency procedure in case of fire

1.Scope

The SOP describes emergency procedures to be followed in case of fire and the responsibilities of workers exposed to a fire hazard in a TB laboratory.

2.Definitions and abbreviations

fire emergency
an uncontrolled fire or signs of fire hazard;
OR the presence of smoke or the odour of burning;
OR the uncontrolled release of a flammable or combustible substance;
OR a fire alarm sounding.

Manageable fire
A small or early-stage, localized fire (no larger than a waste paper basket).
accident
An undesired event giving rise to death, ill-health, injury, damage, loss or distress.
incident
An event that gives rise to an accident or has the potential to lead to an accident.
NA: not applicable

4.1 Principle

Individuals who have been trained in the proper use of fire extinguishers and are confident in their ability to cope with the hazards of a fire may use a portable fire extinguisher or sand bucket to fight small, early-stage and localized fires (no larger than a waste paper basket).
In cases of uncontrollable fire, staff should evacuate the laboratory immediately.

4.2 Samples

NA

4.3 Equipment and materials

Portable carbon dioxide extinguisher(s), well-maintained (at least once a year, recorded in a written document) and within its shelf-life.
Bucket(s) full of sand
Bucket(s) full of water
Fire blankets

4.4 Reagents and solutions

NA

4.5 Detailed instructions

4.5.1 Small fires

- Small localized fires can be extinguished without evacuating the premises, but there must be constant evaluation of the evolution of the fire and readiness to evacuate if it cannot be controlled.
- Fire involving cotton, paper, cardboard, wood, fabric: use water.
- Fire involving flammable liquids and gases or alkali metals, and electrical fires: use carbon dioxide extinguisher or sand. Only personnel trained to use fire extinguishers may use them. Always aim the extinguisher at the base of the fire.
- Fire-fighting efforts must be terminated as soon as it becomes apparent that there is risk of harm from smoke, heat or flames.

4.5.2 In all other cases of fire discovery

- Alert people in the area of the need to evacuate the premises.
- Telephone the fire service emergency number, indicating the location and extent of the fire.
- Evacuate promptly.
- Close doors behind you
- Never enter a smoke-filled room.

4.6 Reporting

Every accident/incident and all corrective action must be documented and records kept in the laboratory supervisor's archives (See Annex).

4.7 Quality control

Use a check-list (non-exhaustive) for further corrective action

- Electrical circuit overloading, frequently due to wires of inappropriate cross-section in relation to fuses used.
- Poor electrical maintenance (poor and perished insulation on cables, extension leads with unprotected plugs lying on the floor, etc).
- Inappropriate circuit-breakers or earth-fault-interrupters.
- Absence or misuse of transformers where required.
- Excessively long gas tubing or long electrical leads.
- Equipment left switched on unnecessarily.
- Equipment not designed for use in a laboratory environment.
- Open flames.
- Deteriorated gas tubing.
- Improper handling and storage of flammable materials.
- Improper segregation of incompatible chemicals.
- Sparking equipment near flammable substances and vapours.
- Improper or inadequate ventilation.
- Appropriate location of water buckets, sand buckets, extinguishers (near room doors and at strategic points in corridors) and fire blankets.

4.8 Safety precautions

The effects of fire on the possible dissemination of infectious material must be considered with the laboratory supervisor and any necessary action to maintain biosafety must be taken promptly.

3. Related documents

Biosafety in microbiological and biomedical laboratories, 5th ed. Washington, DC, 2007. United States Department of Health and Human Services/Centers for Disease Control and Prevention/National Institutes of Health, 2007.
Furr A. CRC handbook of laboratory safety, 5th ed. Boca Raton, FL, CRC Press, 2000.

Health Canada. Laboratory biosafety manual, 2nd ed. Ottawa, Minister of Supply and Services Canada, 1996.
Laboratory biosafety manual, 3rd ed. Geneva, World Health Organization, 2004.

Manual of basic techniques for a health laboratory, 2nd ed. Geneva, World Health Organization, 2003.
Standards Australia/Standards New Zealand. Safety in laboratories - microbiological aspects and containment facilities. Sydney, Standards Australia International, 2002.

Annex. Incident report form

Institution:	
Laboratory designation:	
Head of the laboratory:	
Date, time of the incident:	
Nature of the initial incident (what was the fire source?)	
Extent of the incident:	
Name of the physician in charge of the first medical aid, if requested	
List of persons injured during the incident	
Corrective action: how to prevent the start of such a fire how to limit the spread of fire how to improve staff adherence to safety and emergency procedures	
Measures for biosafety, if any	

Standard Operating Procedure (Sop)
Use of disinfectants

1.Scope

The SOP describes the use of disinfectants used in the laboratory to decontaminate surfaces and equipment and also as a pre-decontamination treatment before autoclaving, burning or incinerating waste.

2.Definitions and abbreviations :

disinfectant
Chemical or mixture of chemicals used to kill microorganisms, but not necessarily spores. Disinfectants are usually applied to inanimate surfaces or objects.

microbicide
Chemical or mixture of chemicals that kills microorganisms. The term is often used in place of “biocide”, “chemical germicide” or “antimicrobial”.

antiseptic
Substance that inhibits the growth and development of microorganisms without necessarily killing them. Antiseptics are usually applied to body surfaces.

disinfection
Physical or chemical means of killing microorganisms, but not necessarily spores.

decontamination Any process for removing and/or killing microorganisms. The same term is also used for removing or neutralizing hazardous chemicals and radioactive materials.

sterilization
Process that kills and/or removes all classes of microorganisms and spores.

inactivation
Process rendering an organism inert by application of heat, or other mean

4. Procedure

4.1 Principle

The temporal killing action of disinfectants depends on the population of organisms to be killed, the concentration used, the duration of contact and the presence of organic debris.

The proprietary disinfectants suitable for use in tuberculosis laboratories are those containing phenols, chlorine, alcohols, iodophors or glutaraldehyde. These are usually selected according to the material to be disinfected.

Note: *It is incorrect to assume that a disinfectant which has general usefulness against other microorganisms is effective against tubercle bacilli.*

A number of commercially available disinfectants have no or little mycobactericidal activity, while quaternary ammonium compounds are not effective at the recommended concentrations.

All of the above disinfectants are toxic and undue exposure may result in respiratory distress, skin rashes or conjunctivitis. However, used normally and according to the manufacturers' instructions, and national chemical safety regulations, they are safe and effective.

4.2 Samples

NA

4.3 Equipment and materials

Glass or plastic bottles of adequate volumes(e.g. 100 ml, 500ml, 10 litres, 50 litres) with leak-proof tops.
Measuring cylinders
Plastic, glass or metal funnel
Balance
Distilled water

4.4 Reagents and solutions

4.4.1. Phenol

Phenol should be used at a concentration of 5% in water. However, inhalation and dermal exposure to phenol is highly irritating to the skin, eyes, and mucous membranes.

Phenol is also considered to be very toxic to humans through oral exposure. Because of this toxicity and odor, phenol derivatives are now generally used in its place. Many phenolic compounds are used for the decontamination of surfaces and some (e.g. triclosan, chloroxylenol, orthophenylphenol) are among the more commonly used antiseptics. To have effect, commercially available solutions should be used according to manufacturer's instructions for "dirty or worst possible situations".

Phenol solutions are used for decontaminating equipment and single use items prior to disposal. They are useful for cleaning up sputum spills in soaked paper towels to cover working surfaces.

4.4.2. Chlorine

Chlorine is a widely available disinfectant.

- Sodium hypochlorite solutions, as domestic bleach, contain 50 g/l available chlorine and should therefore be diluted 1:50 or 1:10 to obtain final concentrations of 1 g/l and 5 g/l, respectively. Bleach, either in stock or in working solutions must be stored in well ventilated, fresh and dark areas. In good storage conditions, the 50g/l solution may last as long as 3 months, **while diluted solutions should be prepared daily.** However, the actual content of available chlorine in domestic bleach may not be reliable in many countries. The two alternatives below should be considered:

- Granules or tablets of calcium hypochlorite ($Ca(ClO)_2$) generally contain about70% available chlorine. Solutions prepared with granules or tablets, containing 1.4 g/l and 7.0 g/l, will then contain 1.0 g/l and 5 g/l available chlorine, respectively.

Storage of stock or working solutions in open containers releases chlorine gas thus weakening their germicidal potential.

Bleach can be used as a general purpose disinfectant and for soaking contaminated metal-free materials (it is highly alkaline and can be corrosive to metal).

4.4.3.Glutaraldehyde

Glutaraldehyde does not require dilution but an activator (provided separately by the manufacturer) must be added. Glutaraldehyde is usually supplied as a 2% solution, while the activator is a bicarbonate compound.
The activated solution should be used within two weeks and discarded if turbid. It can be reused for 1–4 weeks depending on the formulation and type and frequency of its use. Dipsticks supplied with some products give only a rough indication of the levels of active glutaraldehyde available in solutions under use.

Glutaraldehyde is toxic and an irritant to skin and mucous membranes, and contact with it must be avoided. It must be used in a fume-hood or in well-ventilated areas.
Glutaraldehyde is useful for decontaminating bench surfaces and glassware.

4.4.4. Alcohols

Alcohols, ethanol (denatured ethanol, methylated spirits) or iso-propanol , are used at 70%. Alcohols are volatile and flammable and must not be used near open flames. Working solutions should be stored in proper containers to avoid the evaporation of alcohols. Bottles with alcohol-containing solutions must be clearly labelled to avoid autoclaving.

Alcohols can be used on skin, work surfaces of laboratory benches and biosafety cabinets. A major advantage of aqueous solutions of alcohols is that they do not leave any residue on treated items. When hands become contaminated, a rinse with 70% ethanol, or isopropyl alcohol followed by thorough washing with soap and water is effective.

Note that dilutions have to be performed as mentioned below:

To prepare 70% alcohol
Mix 100 ml of 95% alcohol and 39.1 ml of distilled water
Mix 100 ml of 90% alcohol and 31.0 ml of distilled water
Mix 100 ml of 85% alcohol and 23.1 ml of distilled water
Mix 100 ml of 80% alcohol and 15.3 ml of distilled water
Mix 100 ml of 75% alcohol and 7.64 ml of distilled water

4.4.5 Iodophors

Iodophors are a combination of iodine and an inert polymers such as polyvinyl-pyrrolidone that reduces surface tension and slowly releases the iodine. Iodophors are less irritating than iodine and do not stain. Iodophor preparations should be used at concentrations of 3% to 5% and contact time should be 15-30 minutes, depending on the type and volume of material to be disinfected.

The action of these disinfectants is similar to that of chlorine, although they may be slightly less inhibited by organic matter. Iodophors are useful for mopping up spills.

4.5 Detailed stepwise instructions

Use disinfectant as indicated in the technical procedures

4.6 Reading and recording

NA

4.7 Quality control

Disinfectant solutions should be prepared fresh each day and should not be stored in diluted form because their activity will diminish.

4.8 Waste management

NA

5. Related documents

Centers for Disease Control and Prevention / National Institutes of Health. Biosafety in microbiological and biomedical laboratories. 4th ed. Washington DC; 1999.

Collins C, Grange J, Yates M. Organization and practice in tuberculosis bacteriology. London: Butterworths; 1985.

Health Canada. Laboratory biosafety manual. 2nd ed. Ottawa: Minister of Supply and Services Canada; 1996.

Smithwick RW. Laboratory manual for acid-fast microscopy. 2nd ed. Atlanta: CDC; 1979.

World Health Organization. Laboratory services in tuberculosis control. Part I: Organization and management. Geneva; 1998.

World Health Organization. Laboratory Biosafety Manual. 3rd ed. Geneva: WHO; 2004.

World Health Organization. Regional Office for the Eastern Mediterranean. Basics of quality assurance for intermediate and peripheral laboratories. 2nd ed. Cairo; 2002. Available at:
http://www.emro.who.int/dsaf/dsa190.pdf

alcohol-based handrub formulation and preparation available at http://www.who.int/gpsc/tools/faqs/abhr1/en/

Standard Operating Procedure for Sputum Smear Microscopy,
Collection of Sputum FOR AFB Microscopy

1.Scope
This SOP describes Sputum collection for AFB microscopy.

2.Definition and abbreviations
AFB: Acid Fast Bacilli
NA: Not applicable

3.Procedure
4.1 Principle
Good quality sputum is a prerequisite for the diagnosis of TB bacilli. Sputa are of the following types in terms of appearance.

• Salivary	watery
• Mucoid	sticky
• Mucopurulent	Sticky and yellowish
• Bloody	Sticky and a bit brownish

A good sputum specimen should be mucopurulent (sticky and yellowish) and should be about 3-5ml

4.2 Specimen required
NA

4.3 Materials required
1.Sputum containers; clean, leak-proof, transparent wide-mouthed container with a screw-cap lid
2.Laboratory request form(TB05)
3.Marker pen
4.Gloves

4.4Reagents and solutions
NA

4.5 Detailed instructions

1. Day – 1

a) Collect the first specimen when the patient presents to the clinic.
b) Give the patient a labeled sputum container for the next morning's sputum collection.

2. Morning Day – 2

Patient collects early morning sputum and brings it to the clinic.

In a situation where the patient comes far away from the sputum smear microscopy centre second sputum sample can be collected one hour after the first "spot" sample using a novel strategy which is now referred to as "Front-loaded" or "Same Day" or "one Stop shop" microscopy.
Procedure

- √ Label the sputum container on the side and not on the lid with patient's name, outpatient or unit number, age, sex, date of specimen collection, sample (either 1 or 2)
- √ Fill sputum request form with the following information; name of treatment centre, patient's name, age, sex, full patient's address including telephone number, date sample requested and type of request
- √ Find a suitable space or area for collecting the specimen
- √ The area should be outside
- √ OR in a well-ventilated space, away from other people
- √ Do not collect the sputum while others are watching
- √ Do not stand in front of the person producing the specimen
- √ Observe wind direction
- √ Explain in a simple language about steps for sputum specimen collection:
- √ Rinse mouth with plenty of water to remove debris such as food, cola or tobacco

√ Rinse mouth with plenty of water to remove debris such as food, cola or tobacco

√ Take in a lot of air (inhale) deeply

√ Retain the air in the lungs and exhale

√ Repeat this procedure for three times

√ After third inhale, make effort to cough in order to produce sputum

√ Spit produced sputum into container

√ Cover the sputum container tightly with the lid

√ The specimen should be delivered to the laboratory immediately; if the laboratory is far, specimen should be delivered not more than 48 hours after collection of specimen

√ Ensure that the date the patient has come to collect sputum results is recorded preferably using red pen

NEVER put the specimen on the laboratory request form.

4.6 Reporting

NA

4.7 Quality control

Ensure client has understood instructions for sputum production. The collection should be supervised.

4.8 Waste mangement

1. Add 5% Phenol disinfectant into leftover specimens before discarding
2. Incinerate or burn and bury

5. Related documents

1. International Union Against Tuberculosis and Lung Disease. Tuberculo sis Guide. Paris; 1998.

2. Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide: Institute of Medical and Veterinary Science; 2005.

3. World Health Organization. Maintenance and repair of laboratory, diagnostic imaging and hospital equipment. Geneva: WHO; 1994.

4. World Health Organization. Laboratory services in tuberculosis control. Part II: Microscopy. Geneva; 1998.

5. World Health Organization. Manual of basic techniques for a health laboratory. 2nd ed. Geneva: WHO; 2003

6. Fukuji A. AFB Microscopy Training, Tokyo, Japan; The Research Institute and Training 2005.

7. Centers for Disease Control and Prevention. Acid Fast Direct Microscopy Manual, 2000.

8. WHO, CDC, IUATLD, RIT, APHL, KNCV. External Quality Assurance for AFB Smear Microscopy.

9. WHO, CDC, IUATLD, RIT, APHL, USAID. Current Laboratory Practice Series; Acid-Fast Direct Smear Microscopy Training Package

Standard Operating Procedure For (SOP)
Preparation Of Reagents For Microscopy in a Tuberculosis Diagnostic Laboratory

1 Scope

The SOP describes the preparation of all reagents used for microscopy purposes in a TB diagnostic laboratory.

2.Definitions and abbreviations

NA: not applicable

3.Procedure

4.1 Principle

Batches of reagents should be prepared in adequate volumes according to laboratory needs, especially if batches are to be sent to peripheral laboratories.

4.2 Samples

NA

4.3 Equipment and materials

Balance, with a sensitivity of 0.1 g
Brushes to clean bottles before reuse
Containers for the newly prepared stains (dark amber glass bottles or plastic bottles)
Distilled or purified water
Flasks (conical or flat-bottomed), capacity at least 1 litre
Filter papers, large (appropriate size for funnels)
Funnels, large, for filling bottles
Labels for bottles
Stirring plate, heated, and magnetic stirrers
Chemicals see below

*4.4 Reagents and solutions****

4.4.1 For Ziehl–Neelsen staining

1) Stock alcoholic fuchsin

Fuchsin (basic)	3g
Ethanol (95%)	100ml

Dissolve the basic fuchsin in ethanol.

2) 5% Phenol solution

Phenol melted	5ml
Distilled water	95ml

To liquefy pure phenol crystals, loosen the cap of the phenol reagent bottle, place it into a warm bath. Measure it with a warm pipette to avoid re-crystallization. Mouth pipetting is prohibited.
Add the melted phenol slowly to distilled water while stirring.

3) Ziehl's solution (Working carbol fuchsin solution)

Stock alcoholic fuchsin	10ml
5% Phenol solution	90ml

Mix the stock alcoholic fuchsin with 5% phenol while stirring. Filter the solution before use to remove fuchsin crystals or particles.

4) 20% Sulphuric acid solution

Sulphuric acid (conc. H2SO4)	20ml
Distilled water	80ml

Add the sulphuric acid slowly to distilled water using a safety pipette (chilled distilled water can be used). Never add water to sulphuric acid and mouth pipetting is prohibited.

5) 0.3% Methylene blue solution

Methylene blue	0.3g
Distilled water	100ml

Dissolve the methylene blue in the distilled water. Filter the solution before use.

4.4.2 For fluorescence microscopy with auramine staining

Stain solution

Auramine	1.0 g
Alcohol (denatured ethanol or methanol)	100.0 ml
Phenol crystals	30.0 g
Distilled or purified water	870.0 ml

If liquefied phenol is to be used, adjust quantity as volume indicated by the manufacturer. First dissolve auramine in ethanol, then phenol crystals with water and mix both solutions. Mix only amounts that can be consumed within a few weeks, since the working solution is not stable in the long term, although the stock solution (1% auramine in alcohol) can be kept for longer (3 months). Thorough mixing for about one hour on a magnetic stirring plate is recommended, but the solution should not be heated.

Label the bottle "0.1% auramine", add the date and sign with initials. The date the bottle is first opened must be written on the label. Stock and working solutions must be kept in dark bottles in the dark, and working solutions should be used within 1 month.

Decolorizing solution

Hydrochloric acid	5 ml
70% ethanol	1000 ml

Use a 1-litre flask and slowly pour hydrochloric acid into alcohol.
Label the bottle "0.5% acid–alcohol", add the date and sign with initials. The date the bottle is first opened must be written on the label. This solution may be kept indefinitely.

Counterstaining solution: permanganate (preferred for LED microscopes)

Potassium permanganate	5.0 g certified grade
Distilled water	1000.0 ml

Label the bottle "0.5% potassium permanganate", add the date and sign with initials. The date the bottle is first opened must be written on the label. Solution should be used within 6 months.

Counterstaining solution: blue ink (alternative to permanganate)

Blue ink	100.0 ml
Phenol	5.0 g
Distilled water	900.0 ml

Label the bottle "10% blue ink", add the date and sign with initials. The date the bottle is first opened must be written on the label. Solution should be used within 6 months.

4.5 Detailed instructions

NA

4.6 Reading, interpretation, recording and reporting

NA

4.7 Quality control

See SOPs on Ziehl-Neelsen and auramine staining procedures for internal quality control of newly prepared batches of reagents for microscopy. Quality control must be performed by microscopists.

4.8 Storage

Storage conditions for each reagent are specified in section 4.4 above.

4. Related documents

Angra P et al. Ziehl-Neelsen staining: strong red on weak blue, or weak red under strong blue? International Journal of Tuberculosis and Lung Disease, 2007, 11:1160–1161.

Health Protection Agency. Investigation of specimens for Mycobacterium species. London, Standards Unit, Evaluations and Standards Laboratory, 2006 (National Standard Method BSOP 40 Issue 5, www.hpa-standardmethods.org.uk/pdf_sops.asp).

http://wwwn.cdc.gov/dls/ila/acidfasttraining/

Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, United States Department of Health and Human Services, Centers for Disease Control, 1985.

Laboratory services in tuberculosis control. Part II: Microscopy. Geneva, World Health Organization, 1998 (WHO/TB/98/258).

Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide, Institute of Medical and Veterinary Science, 2005.

Rieder HL et al. Priorities for tuberculosis bacteriology services in low-income countries, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.

Smithwick RW. Laboratory manual for acid-fast microscopy, 2nd ed. Atlanta, GA, Center for Disease Control, 1976.

Log-sheets: preparation of stains for microscopy

Ziehl-Neelsen method	Quantity of reagent	Volume prepared	Date	Signature
Basic fuchsin Alcohol Phenol crystals Distilled water Carbol-fuchsin 1%	10 g 100 ml 50 g 1000 ml	1 litre		
Sulfuric acid Distilled water H2SO4 25%	250 ml 750 ml	1 litre		
Methylene blue Distilled water Methylene blue 0.1%	1 g 1000 ml	1 litre		

Auramine method	Quantity of reagent	Volume prepared	Date	Signature
Auramine Ethanol Phenol Distilled water Auramine 0.1%	1.0 g 100 ml 30.0 g 1000 ml	1 litre		
Hydrochloric acid Ethanol 0.5% acid–alcohol	5 ml 1000 ml	1 litre		
Potassium per-manganate Distilled water Counterstaining	5 g 1000 ml	1 litre		

Standard Operating Procedure For (SOP)
Ziehl–Neelsen staining

1.Scope

The SOP describes the Ziehl–Neelsen (ZN) staining technique for the detection of acid-fast bacilli (AFB) by microscopy. The ZN staining technique is used with ordinary (bright-field) microscopes.

2.Abbreviations

AFB:	acid-fast bacilli
EQA:	external quality assessment
QC:	quality control
ZN:	Ziehl–Neelsen method
NTP:	national tuberculosis programme
MDR-TB.	multidrug-resistant TB

PROCEDURES

Principles

The property of acid-fastness is based on the presence of mycolic acids in the cell wall of mycobacteria. Primary stain (fuchsin) binds to cell-wall mycolic acids. Intense decolourization (strong acid) does not release the primary stain from the cell wall and the mycobacteria retain the red colour of fuchsin – hence acid-fastness. Counterstaining (with methylene blue) provides a contrasting background.

While mycobacteria are AFB, very few other bacteria possess the property of acid-fastness, and then only weakly (e.g. Nocardia). AFB found in respiratory specimens of patients from countries with high TB prevalence are almost always TB bacilli. Non-TB mycobacteria are more commonly found in countries where TB prevalence is low. In high-burden countries, however, some patients suspected of having MDR-TB may actually have disease caused by non-TB mycobacteria. AFB found in extrapulmonary specimens, particularly gastric washings, stool or urine, should never be automatically assumed to represent TB bacilli.

4.2 Specimens

Any incoming specimen must be properly labelled, as a minimum with a unique identification number. This identification is also written on the request form (Annex 1), and must correspond with the identification in the laboratory AFB-microscopy register.

4.2.1 Sputum

- Spontaneous sputum
  Sputum from suspects should be rejected only if they are liquid and clear as water, with no particles or streaks of mucous material. However, it should be accepted if the patient cannot produce a better specimen on a repeated attempt.

Sputum from follow-up patients should be accepted and examined even if it looks like saliva, since these patients often cannot produce mucoid specimens.

- Induced sputum
  This specimen resembles saliva but has to be processed as adequate specimen.
- Decontaminated sputum, concentrated by centrifugation.

4.2.2 Other specimens

- Laryngeal swabs, gastric lavages, bronchial washings, brushings and transtracheal aspirates.

- Body fluids (cerebrospinal, pleural, pericardial, synovial, fluids from ascites, blood, pus, bone marrow).

- Tissue biopsies.

4.3 Equipment and materials

Bunsen burner or spirit lamp
Gas or burning spirit torch
Diamond pencil or lead pencil (if frosted-end slides are available)
Filter paper; small, and appropriate for funnel size
Funnels; small, for filtering solutions in use
Forceps

Lens paper or soft tissue paper
Plastic bag for waste disposal
Wooden applicators / sticks
Microscope, preferably binocular, with perfocal lenses, electric light source or mirror, mechanical stage, 100x objective, 10x eyepiece (see Annex 2)
Immersion oil, synthetic, refractive index 1.5180 ± 0.0004 (according to DIN/ISO recommendations). ***Do not use cedarwood oil.***
Slide drying rack
Slide staining rack
Slide boxes
New, clean, grease free and frosted-end slides (rinse in alcohol and dry if necessary)
Timer
Staining reagents
Staining bottles, with spout or wash bottle
Beaker for rinsing water
Sink and water supply
Oil-absorbing paper
Disinfectant solution (see relevant SOP)

4.4 Reagents and solutions

See SOP for preparation of staining and reagent solutions.

4.4.1 Carbol fuchsin staining solution, 0.3%

4.4.2 Acid decolourizing solution, 20% H2SO4

4.4.3 Methylene blue counterstaining solution, 0.3%

4.5 Detailed instructions

4.5.1 Preparation of smears

√ Disinfect the working area.
Label the slides properly with diamond pencil or lead pencil (if frosted slides are available) using the laboratory register serial number marked on the sputum container.

Frosted slide:

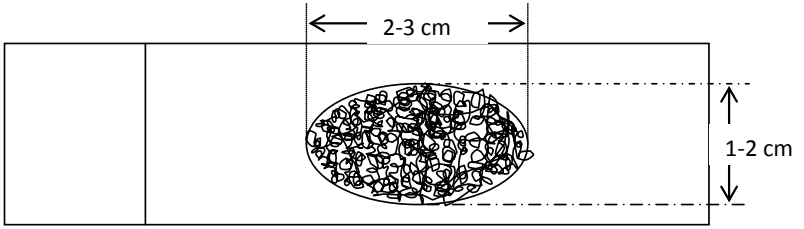
For frosted slides, ordinary lead pencil is used on the frosted section of the slide.

Non- frosted slide:

For non-frosted slides diamond pencil is used to label the slides.

- √ First record the yearly serial number as recorded in the laboratory register (e.g. 100)
- √ For diagnosis cases indicate the order number (e.g. A, B or I, II) corresponding to the 1st or 2nd specimen.
- √ Write the date of smear preparation (e.g. 20/03/10)
- √ Place each slide on its corresponding container.
- √ Proceed to smearing, taking the labelled slides and opening containers one by one; using aseptic techniques.
- √ For a direct sputum smear, select a small portion of purulent or mucopurulent material with the stick and transfer it to the slide;
- √ If a smear is prepared after specimen decontamination, the concentrated material must be transferred to the slide with a sterilized loop to avoid splashing.
- √ Spread the material carefully over an area equal to about 2–3 cm x 1–2 cm using repeated circular movements, without touching the edge of the slide.
- √ Make the smear as even as possible by continuing this process until no thick parts remain.

√ The thickness of the smear should be such that a newspaper held under the slide can barely be read through the dried smear
Do not smear 2/3 of the slide nor dry the smear on the flame



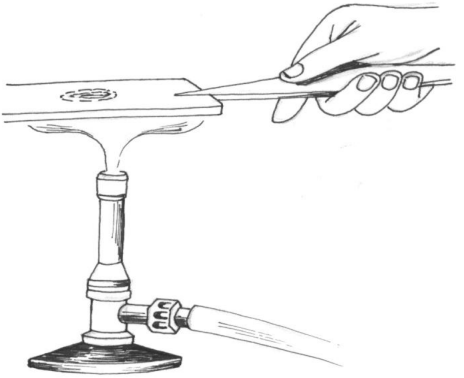
Disinfect the working area after smear preparation.

√ Let the smears air-dry at room temperature; do not use heat to speed the drying.

√ Where humidity is high, gentle warming will be needed on a slide warmer (or locally made box with glass top under which there is a 20-W light bulb

Fixing of smears

Fig 17: With a forceps, hold the slide and pass it over a flame 2-3 times. Never heat-fix a wet smear.
All the biosafety measures discussed earlier should be strictly followed



Always keep smears out of direct sunlight

4.5.2 Staining method

√ Place the slides, smear upwards, on the staining rack over a sink, about 1 cm apart.

√ Flood slides with the carbol-fuchsin solution.

√ Heat the underside of the slides with a flame (spirit lamp / Bunsen burner / cotton stick) keeping the flame a little below them and moving it continuously back and forth along the line until steam comes out. Then leave the slides for about 5 minutes to cool down.

NB: Do not boil the solution nor allow it to dry off.

√ Wash off with water to remove the excess carbol-fuchsin.

√ Cover the slide with 20% H2SO4 for about 5 minutes and drain. If traces of carbol fuchsin are seen, flood again with the decolouriser to clear.

√ Afterwards wash with water.

√ Counter stain with 0.3% methylene blue for about 1 minute.

√ Rinse and drain the slides.

√ Leave the slides to air-dry on a slanting rack. If a rack is not available, slant the slides (on a wall) to air-dry completely.

Note: The stained smear should show a light blue colour. A dark blue colour usually indicates that the smear is too thick or that the methylene blue staining time was too long; this will hide the red AFB in the background.

4.6 Reading, recording and reporting

4.6.1 Reading (see Annex 2)

√ Set the variable voltage regulator to minimum and switch the power on.

- √ Slowly adjust the light until the desired intensity is reached.
- √ Ensure that the lenses, mirrors and other light-conducting surfaces are clea
- √ Turn the coarse adjustment knob to move the stage away from the objective lens.
- √ Place a stained slide on the stage, smear upwards.
- √ Rotate the nosepiece to the 10x objective and adjust the light intensity as required
- √ Adjust the inter-pupillary distance until the right and left images merge.
- √ Focus the image with the right eye by looking into the right eyepiece and adjusting with the fine focus knob.
- √ Focus the image with the left eye by looking into the left eyepiece and turning the dioptre ring.
- √ Open the condenser iris diaphragm so that the field is evenly lit (about 80%
- √ Turn the coarse focus knob to bring the 10x objective lens close to the slide; do not allow the objective lens to touch the smear.
- √ While looking into the eyepieces, slowly turn the coarse focus knob to separate the objective lens and the stage. The smear should come into focus within a few turns. Then turn the fine focus knob until the smear is seen most clearly.
- √ Always use the focusing adjustment knobs to lower the stage away from the lens.
- √ Place a drop of immersion oil on the left edge of the smear; do not touch the slide with the oil applicator but allow the drop of oil to fall freely onto it. Then rotate the 100x objective into place.
- √ It is a professional error to focus directly with this objective.

√ With perfocal lenses, the immersion objective will now be in the oil; if not lower it slightly until it just touches the oil (looking from the side).

√ Raise the condenser as high as possible. Increase the brightness of the light until the field is well-lit but still comfortable for the eye.

√ Focus by adjusting with the fine focus knob. Use a maximum of one turn in one direction; if this is not successful, repeat in the other direction.

√ Scan the stained smear systematically from left to right side, covering one length (100–150 microscopic high-power fields, depending on the length of the smear – 2 or 3 cm). This is the minimum that must be scanned before reporting negative result; the process should take about 5 minutes.

√ Count AFB in positive smears for quantification. Always search for useful areas, i.e. those containing mucoid threads and pus cells; do this by moving up or down when arriving at an almost empty area, until another useful zone has been found, then continue moving to the right.

√ Acid-fast bacilli appear bright red against the background material counterstained in blue.
√Report as positive for AFB when the background is bluish and at least one red AFB is seen in a well stained smear.

√ Once the smear has been read, rotate the 100x objective away, without changing focus, and remove the slide.

√ Place the slide smear-down on a piece of absorbent paper (e.g. folded toilet paper, tissue paper, paper towel) to soak up the oil; do not move the slide once it is on the absorbent paper.

√ When all slides have been examined, reset the voltage regulator control to minimum and turn the power off.

√ Store the slides in a slide box in order of the numbers of the laboratory register; they will be needed for external quality assessment. Do not write results on the slides.

√ Clean the objective lens at the end of each day using lens tissue or other suitable soft tissue.

4.6.2 Recording

Finding	**Recording**
No AFB found in at least 100 fields	negative
1–9 AFB per 100 fields	exact figure/100
10–99 AFB per 100 fields	+
1–10 AFB per field (count at least 50 fields)	++
More than 10 AFB per field (count at least 20 fields)	+++

√Results must be recorded in TB laboratory register (TB04). Use red ink for positive results (Annex 3).

4.6.3 Reporting

Results must be reported in TB laboratory request form (TB05) Annex 1. Reports must be provided as soon as possible.

√ For a negative result report: "Acid-fast bacilli were not seen."

√ For a positive result: report quantification of AFB seen. (It should not be assumed that AFB is tubercle bacilli.)

√ Never report "No TB" (or equivalent wording).

4.7 Quality control and evaluation of smear quality

4.7.1 Internal QC

Internal QC of freshly made / newly received staining solutions

√ Prepare batches of control slides from suitable sputum specimens. These are negatives that have been thoroughly examined, and low positive sputum 1+ homogenized after liquefaction by standing overnight at room temperature. Prepare at least 10 smears of each, as nearly identical in size and thickness as possible, giving each series the same QC identification number.

- √ Check every newly prepared staining solution with unstained control smears, using at least one positive, with known approximate number of AFB, and one negative slide.
- √ Examine the control smears and record the results in the QC logbook, under the batch number (and/or preparation date) of the new solutions.
- √ Unacceptable control results include the following:
  Positive controls AFB are not stained strongly red or are clearly too few in number.
- √ Positive control background remains red or contains precipitates.
- √ Negative control shows AFB (possibly from contaminated water).
- √ Stain deposit is present on the QC slides.
- √ If one or more of these are found, check whether something went wrong with the solution preparation. If this seems unlikely, repeat the controls with two more slides of each control, paying attention to correct staining technique
- √ Accept if these controls give the expected results.
- √ If the repeat controls also give unacceptable results, discard the staining solutions and prepare new ones.

Internal QC of staining solutions in use and of staining procedure

- √ Include positive and negative controls weekly. Read control slides before patient smears.
- √ If results are unacceptable (as described above), re-stain smears of that day together with new controls, paying attention to correct technique;
- √ If these controls are also unacceptable, prepare new staining solutions and repeat the staining.

Internal QC indicators

Monitor laboratory performance by monthly counts – plotted on a graph – of:

- number of smears,
- positivity rate,
- positive cases detected.

These indicators provide an early warning of problems and signal the need for corrective actions. They contribute to staff motivation and self-reliance.

Among the possible reasons for ***false-positive*** results are:

- √ re-use of containers or positive slides;
- √ contaminated stain prepared with water containing environmental mycobacteria;
- √ use of scratched slides;
- √ AFB floated off one slide and became attached to another during the staining procedure because there was no space between adjacent slides;
- √ inadequate decolourization;
- √ lack of experience, confusing with artefacts (especially if stains are not or poorly filtered);
- √ microscope (lamp) in poor condition or poorly adjusted: interpreting glitter as AFB;
- √ poor quality of staining solutions.

Among the possible reasons for false-negative results are:

- √ poor quality of specimen;
- √ not taking proper portion of specimen for smear preparation;
- √ excessive decolourization;

- √ poorly prepared staining solution;
- √ too little time staining with carbol fuchsin;
- √ over-staining with methylene blue;
- √ overheating during fixing;
- √ reading less than one length
- √ slide exposed to daylight for too long;
- √ Too long an interval between staining and reading, particularly if slides were poorly stained or not kept in the dark.

4.7.2 External quality control

EQA is described in a separate SOP.

4.8 Waste management

At the end of each day, seal contaminated material (used sputum containers, sticks, etc.) in a bag and incinerate as soon as possible. Keep the bag in a safe, closed bin or large bucket until it can be incinerated.
In intermediate or central laboratories where there is an autoclave, infectious waste should be collected in an autoclavable bag and should be autoclaved before incineration.

6 Related documents

Angra P et al. Ziehl-Neelsen staining: strong red on weak blue, or weak red under strong blue? International Journal of Tuberculosis and Lung Disease, 2007, 11:1160–1161.

Health Protection Agency. Investigation of specimens for Mycobacterium species. London, Standards Unit, Evaluations and Standards Laboratory, 2006 (National Standard Method BSOP 40 Issue 5, www.hpa-standardmethods.org.uk/pdf_sops.asp).
http://wwwn.cdc.gov/dls/ila/acidfasttraining/

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Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide, Institute of Medical and Veterinary Science, 2005.

Rieder HL et al. Priorities for tuberculosis bacteriology services in low-income countries, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.

Smithwick RW. Laboratory manual for acid-fast microscopy, 2nd ed. Atlanta, GA, Center for Disease Control, 1976.

Annex 1. Request and reporting form for sputum examination

SPUTUM EXAMINATION REQUEST FORM, GHANA NTP TB05

Health facility:_____ Date ___/___/20____

Name of Patient: _____ Age: _____ Sex: M ☐ F ☐

Address of Patient _____

_____ District _____

Reason for Examination: Diagnosis ☐ TB Suspect No: _____

OR

Follow-up ☐ Months on treatment:____; Patient's District TB. No: _____

Specimen Identification No: _____ Number of Specimens sent with this form _____

Date of first Sputum collection: ___/ ____/20____

Signature _____ Name of person who requests Examination

RESULTS **(To be completed at Laboratory)**

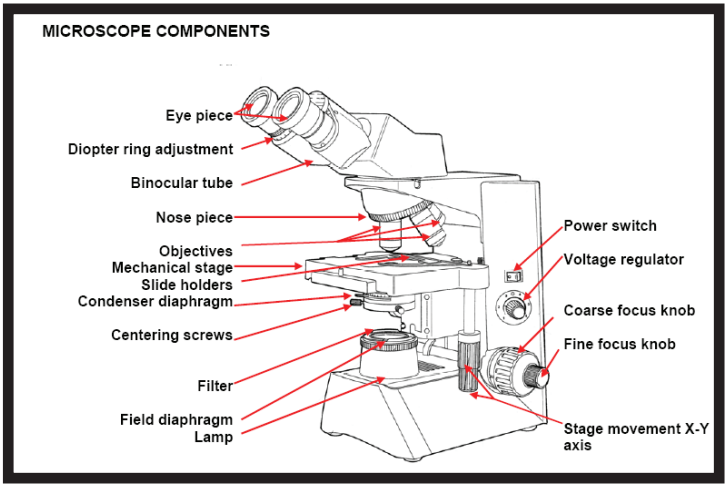
Lab. Specimen No.: _____

Visual appearance of specimen: Muco – purulent= a Blood stained = b Muco – Salivary = c Saliva = d

Date	Specimen	Appearance: write a, b, c, or d as indicated above	Result grading (tick ✓ appropriate boxes)				
			Negative	Scanty (1-9)	+	++	+++
	1						
	2						
	3						

Date: ___/____/20____ Examined by (signature) _____

Annex 2. Microscope components



Annex 3 : TB Laboratory Register

Lab Serial No	Date Specimen Recieved	Name of Patient	Age	Sex M/F	Address of Patient	Referring Health Facility	Reason for Examination 1	District TB/Suspect No.2	Microscopy Results		Remarks
									1	2	

[1]For Diagnosis ebter 0 and for Foll-up cases enter the number of months (2,3,4,5, 6 0r 8) on treatment

[2]For TB suspects (New cases) enter the Tb Suspect Number, if any, and for follow-up cases enter the District TB number

Standard Operating Procedure For (SOP)

Auramine staining

Content

Scope

This SOP describes the auramine staining technique for detection of acid-fast bacilli by microscopy. The auramine staining technique applies to fluorescence microscopy.

1. Definitions and abbreviations

microscope magnification

individual objective magnification x eyepiece magnification

AFB:	acid-fast bacilli
HPF:	high-power fields
LED:	light-emitting diode
MDR-TB:	multidrug-resistant TB
QC:	quality control

➢ .

2. Procedure

4.1 Principle

The property of acid-fastness is based on the presence of mycolic acids in the mycobacterial cell wall. Primary stain (auramine) binds cell-wall mycolic acids. Intense decolourization (strong acids, alcohol) does not release primary stain from the cell wall and the mycobacteria retain the fluorescent bright yellow colour of auramine. Potassium permanganate is used to quench fluorescence in the background; however, it provides little contrast for focusing and stains are therefore sometimes preferred, of which blue ink may be the best.

All mycobacteria are acid-fast, but very few other bacteria possess this property and then only weakly (e.g. Nocardia). AFB found in respiratory specimens of patients from countries with high TB prevalence are almost always TB bacilli. Non-TB mycobacteria are more commonly found in countries where TB prevalence is low. In high-burden countries, some patients suspected of having MDR-TB may actually have disease caused by non-TB mycobacteria. AFB found in extrapulmonary specimens, particularly gastric washings, stool or urine, should never be automatically be assumed to represent TB bacilli.

Fluorescence microscopy allows smears to be examined more rapidly than is possible with the basic fuchsin procedures and is particularly indicated for high-volume laboratories. It may also be more sensitive for paucibacillary specimens, since it allows examination of more fields with less effort. However, it requires a stable power supply, greater expertise in reading and microscope adjustment, and a regular supply of the costly and short-lived bulbs. Cheaper systems using halogen lamps have less stringent requirements, but performance does not entirely match that of the standard mercury vapour lamps.

Note: Newly developed of blue LED light sources adjusted to fluorescence microscopes may overcome these difficulties in near future, because a 5-W lamp is sufficient, can be operated with simple batteries and has a life of at least 15 000 hours.

4.2 Specimens

Any incoming specimen must be properly labelled, as a minimum with a unique identification number. This identification is also written on the request form (see Annex), and must correspond with the identification in the laboratory AFB-microscopy register.

4.2.1 Sputa

- Spontaneous sputa
  Sputa from suspects should be rejected only if they are liquid and as clear as water, with no particles or streaks of mucous material. However, they should be accepted if the patient cannot produce a better specimen on a repeated attempt. Sputa from follow-up patients should be accepted and examined even if they look like saliva, since these patients often cannot produce mucoid specimens.

- Induced sputa
  These specimens resemble saliva but have to be processed as adequate specimens.

- Decontaminated sputa, concentrated by centrifugation.

4.2.2 Other specimens

- Laryngeal swabs, gastric lavages, bronchial washings, brushings and transtracheal aspirates.

- Urine.

- Body fluids (spinal, pleural, pericardial, synovial, fluids from ascites, blood, pus, bone marrow).

- Tissue biopsies.

4.3 Equipment and materials

Diamond pencil or lead pencil (if frosted-end slides are available)
Filter paper, appropriate for funnel size
Funnels, small, for filtering solutions in use

Forceps
Lens paper or soft tissue paper
Plastic bag for waste disposal
Bamboo or wooden sticks or wire loops
Fluorescence microscope with objectives of 20x or 25x, and 40x (ideally specific for fluorescence microscopy), and eyepieces of 10x
Slide staining rack
Slide boxes
New, clean slides (rinse in alcohol and dry if necessary)
Timer
Staining reagents
Staining bottles, 250 ml, with spout
Beaker for rinsing water
Sink and water supply
Disinfectant solution

4.4 Reagents and solutions

See SOP for preparation of staining and reagent solutions

NOTE: Here the preparation of staining solutions and/or (if staining solutions are provided centrally) method used or recommended by the NTP should be inserted or described.

4.4.1 Auramine staining solution, 0.1%

4.4.2 Acid-alcohol decolourizing solution, 0.5%

4.4.3 Counterstaining solution

Potassium permanganate, 0.5%, or blue ink, 10%

4.5.Detailed instructions

4.5.1 Preparation of smears

- Disinfect the working area.
- Label the slides properly using the laboratory register serial number marked on the sputum container.

- Place each slide on its corresponding container
- Proceed to smearing, taking the labelled slides and opening containers one by one; do the smearing behind the flame of a Bunsen burner or spirit lamp.
  - *for a direct sputum smear, select a small portion of purulent or mucopurulent material with the stick/loop and transfer it to the slide;*
  - *if a smear is prepared after specimen decontamination, the concentrated material must be transferred to the slide with a sterilized loop to avoid splashing.*
- Spread the material carefully over an area equal to about 2–3 cm x 1–2 cm using repeated circular movements, without touching the edge of the slide. Make the smear as even as possible by continuing this process until no thick parts remain. The thickness of the smear should be such that a newspaper held under the slide can barely be read through the dried smear.
- Disinfect the working area after smear preparation
- Let the smears air-dry at room temperature; do not use heat to speed the drying. Where humidity is high, gentle warming will be needed on a slide warmer (or locally made box with glass top under which there is a 20-W light bulb).
- When dry, hold the slides in forceps and fix them by passing three times slowly through the flame of a spirit lamp or quickly through that of a Bunsen burner, smear upwards; do not overheat or AFB staining will be poor.
- Always keep smears out of direct sunlight.

4.5.2 Staining method

- Place the slides, smear upwards, on the staining rack over a sink, about 1 cm apart.

- Place a new filter paper in a small funnel, keep it over the first slide and fill it up with auramine staining solution.
- Let the solution filter through the paper, covering each slide completely. Do not heat. Leave for 20 minutes.
- Using forceps, tilt each slide to drain off the stain solution. Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap).
- Pour the acid solution over the smears, covering them completely, and allow to act for 3 minutes.
- Using forceps, tilt each slide to drain off the acid-alcohol solution. Gently rinse each slide again with distilled water or clean tap water from a beaker (not directly from the tap).
- Flood smears with potassium permanganate solution for 1 minute. Time is critical because counterstaining for longer may quench the AFB fluorescence.
- Using forceps, tilt each slide to drain off the counterstain solution. Gently rinse each slide again with distilled water or clean tap water from a beaker (not directly from the tap).
- Using forceps, take each slide from the rack and let the water drain off. Stand the slide on edge on the drying rack and allow to air-dry.

4.6 Reading, recording and reporting

4.6.1 Reading

- Keep stained smears in the dark (in a box or folder) and read as soon as possible –fluorescence fades quickly when exposed to light.
- Switch on fluorescent lamp 5 minutes before use; leave the lower ordinary lamp off.
- Rotate the nosepiece so that the 20x (or 25x) objective is in the light path.

- Select the filter set position suitable for auramine stain (see manufacturer's manual)

- Check that there is a strong blue light; if not, open shutters and/or the fluorescent light beam diaphragm

- Load the positive control slide on the stage and move the stage to position the slide under the objective.

- Use the coarse adjustment first, and then the fine adjustment, to focus the objective. If this fails (i.e. in thin negative smears), turn the filter set to transmitted light, switch on the lower normal lamp and focus as with a light microscope. Then switch off the lower lamp and return to the required filter position. The field should now be in focus.

Note: Focusing and maintaining focus while moving the smears may prove quite difficult if the permanganate-quenched background is too dark. If the lamp works well (strong blue light seen from the side), try background staining with blue ink.

- Check that bright yellow fluorescent AFB are clearly seen. If not, adjust the lamp and/or the mirror position. Check that the whole field is evenly lit. If not, centre the diaphragm after partially closing it (see manufacturer's manual).

- Exchange the positive control for the first routine smear without changing focus or rotating the objective. Repeat the procedure with each smear to be examined.

- Using the 20x (or 25x) objective, scan the stained smear systematically from one side to the other and back again – at least one length must be scanned before reporting a negative. At 200x magnification, this corresponds to three lengths or 300 high-power fields (HPF) using the oil-immersion 100x objective; at 400x it equals two lengths or 200 HPF with the oil-immersion objective.

The process will take 1–2 minutes.

Acid-fast bacilli appear bright yellow against the dark background material.

Tubercle bacilli are quite variable in shape, from very short fragments to elongated types, and may be uniformly stained or with one or many gaps, or even granular. The typical appearance is of bacilli that are rather long and slender, slightly curved rods. They occur singly or in small groups, and rarely in large clumps. With good staining (always check a freshly stained positive control first), there may also be fluorescing (sometimes green) artefacts, which do not have the typical shape. Non-fluorescing bacillary shapes must also be considered as artefacts.

- Use the 40x objective for confirmation of AFB
- Store the slides in a slide box in order of the numbers of the laboratory register; they will be needed for external quality assessment. Do not write results on the slides.
- When finished, turn the power off. When work needs to be interrupted for just a few minutes only, block the light using the shutter but do not switch off the light source. After switching off a mercury lamp, wait at least 15 minutes before switching it on again. Other types of lamps for short periods of time without problem.

4.6.2 Recording

Because fluorochrome-stained smears are examined at magnifications of 200x to 400x, the number of AFB can roughly be divided by a factor 10 or 5, respectively (depending on the objective) to make them equivalent to fields seen on examination of fuchsin-stained smears at 1000x.

IUATLD/WHO scale (1000x field = HPF) Result	Microscopy system used		
	Bright-field (1000x magnification: 1 length = 2 cm = 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	1–9 AFB / 1 length or 100 HPF	Zero AFB / 1 length	1–19 AFB / 1 length
1+	10–99 AFB / 1 length or 100 HPF	1–29 AFB / 1 length	20–199 AFB / 1 length
2+	1–10 AFB / 1 HPF on average	30–299 AFB / 1 length	5–50 AFB / 1 field on average
3+	>10 AFB / 1 HPF on average	10–100 AFB / 1 field on average	>50 AFB / 1 field on average

Doubtful results

If there is uncertainty about the presence of a bacillus because of the lower magnification, it is best to inspect this carefully with the 40x objective or, if unavoidable, with a 100x oil-immersion objective.

This is more efficient than re-staining by the Ziehl-Neelsen technique (sometimes recommended), which may result in bacilli being washed off or simply not found again. Inexperienced personnel should seek advice from a supervisor

4.6.3 Reporting

Results must be reported in a TB laboratory register.
Use red ink for positive results. Reports must be provided as soon as possible.

- For a negative result report: "Acid-fast bacilli were not seen."
- For a positive result: report quantification of AFB seen. (It should not be assumed that AFB are tubercle bacilli.)
- Never report "No TB" (or equivalent wording).

4.7 Quality control and evaluation of smear quality

4.7.1Internal QC of freshly made staining solutions

- Prepare batches of control slides from suitable sputum specimens. These are negatives that have been thoroughly examined, and a low positive (1+, 10–99 AFB/100 fields) sputum homogenized after liquefaction by standing overnight at room temperature

- Prepare at least 10 smears of each, as nearly identical in size and thickness as possible, giving each series the same QC identification number. Check 2–3 of each after good staining, and note the average number of AFB for the 1+ in the QC logbook

- Check every newly prepared staining solution with unstained control smears, using at least one positive, with known approximate number of AFB, and one negative slide.

- Stain the positive smear(s) as in section 4.5.2; repeat the cycle for the negative(s) at least once to ensure that contaminants present in decolourizer or quenching/counterstaining solution will be visible.

- Examine the controls as in section 4.6.1, and note the results in the QC logbook, under the batch number (and/or preparation date) of the new solutions.

- Unacceptable control results include the following:

  - Positive control AFB are not stained bright yellow or are too few in number.

  - Negative control remains bright yellow after decolourization.

  - Background is too dark or contains too many fluorescent artefacts. If one or more of these are found, check whether something went wrong with the solution preparation. If this seems unlikely, repeat the controls with two more slides of each control, paying attention to correct staining technique.

    Accept if these controls give the expected results. If the repeat controls also give unacceptable results, discard the staining solutions and prepare new ones.

  - Negative control shows AFB.
    If the negative control shows AFB, repeat the negative controls (2 smears) using the same reagents and technique, but use distilled water for rinsing.

    If AFB are still observed, discard the batches of staining solutions and prepare new ones, making sure to use absolutely clean glassware and distilled water for dissolving the dyes.

4.7.2 Internal QC of staining solutions in use and of staining procedure

- Include positive and negative controls with each day's reading. Read control slides before patient smears; this will also help with focusing and to check proper functioning of the instrument.

- If results are unacceptable (as described above), re-stain smears of that day together with new controls, paying attention to correct technique; if these controls are also unacceptable, prepare new staining solutions and repeat the staining.

4.7.3 Internal QC indicators

Monitor laboratory performance by monthly counts – plotted on a graph – of:

- number of smears,
- positivity rate,
- positive cases detected.

These indicators provide an early warning of problems and signal the need for corrective actions. They contribute to staff motivation and self-reliance.

Among the possible reasons for false-positive results are:

- re-use of containers or positive slides;
- contaminated stain prepared with water containing environmental mycobacteria;
- use of scratched slides;
- AFB floated off one slide and became attached to another during the staining procedure because there was no space between adjacent slides;
- inadequate decolourization;
- lack of experience, confusion with artefacts (especially if stains are not or poorly filtered);
- microscope (lamp) in poor condition or poorly adjusted: interpreting glitter as AFB;
- poor quality of staining solutions.

False-negative results

Among the possible reasons for false-negative results are:

- poor quality of specimen;
- not taking proper portion of specimen for smear preparation;
- excessive decolourization;

- poorly prepared staining solution;
- too little time staining with auramine;
- over-staining with permanganate;
- overheating during fixing;
- reading less than one length;
- slide exposed to daylight for too long;
- too long an interval between staining and reading, particularly if slides were poorly stained or not kept in the dark.

4.8 Waste management

At the end of each day, seal contaminated material (used sputum containers, sticks, etc.) in a bag and incinerate as soon as possible. Keep the bag in a safe, closed bin or large bucket until it can be incinerated.
In intermediate or central laboratories where there is an autoclave, infectious waste should be collected in an autoclavable bag and should be autoclaved before incineration.

If a burning drum is used, collect contaminated material (containers with tightened caps, sticks, etc…) from the laboratory in a bucket containing phenol 5%. Burn contents weekly. When cool, bury the residue at a depth of at least 1 metre.

5Related documents

Basics of quality assurance for intermediate and peripheral laboratories, 2nd ed. Cairo, WHO Regional Office for the Eastern Mediterranean, 2002

Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, United States Department of Health and Human Services, Centers for Disease Control, 1985.

Laboratory services in tuberculosis control. Part II: Microscopy. Geneva, World Health Organization, 1998.

Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide, Institute of Medical and Veterinary Science, 2005.

Maintenance and repair of laboratory, diagnostic imaging and hospital equipment. Geneva, World Health Organization, 1994.

Manual of basic techniques for a health laboratory, 2nd ed. Geneva, World Health Organization, 2003.

Rieder HL et al. Priorities for tuberculosis bacteriology services in low-income countries, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.

Annex. Request and reporting form for sputum smear examination form

Health facility: _____ Date __/__/20___

Name of Patient: _____ Age: _____ Sex: M ☐ F ☐

Address of Patient & Tel (mobile): _____

_____ District _____

Reason for Examination: Diagnosis ☐ TB Suspect No: _____

OR

Follow-up ☐ Months on treatment: _____ Patient's District TB. No: _____

Specimen Identification No: _____ Number of Specimens sent with this form _____

Date of first Sputum collection: __/ ___/20___

Signature _____ _____

Name of person who requests Examination

RESULTS (To be completed at Laboratory)

Lab. Specimen No.: _____

Visual appearance of specimen: Muco – purulent= a Blood stained = b Muco – Salivary = c Saliva = d

Date	Specimen	Appearance: write a, b, c, or d as indicated above	Result grading (tick √ appropriate boxes)				
			Negative	Scanty (1-9)	+	++	+++
	1						
	2						

Date: __/___/20___ Examined by (signature) _____

Standard Operating Procedure For (SOP)
Maintenance of a fluorescence microscope

1.Scope
The SOP describes the optimal operation of a fluorescence microscope through regular servicing and preventive maintenance.

Procedures
The fluorescence microscope is a precision instrument intended for microscopic detection of tubercle bacilli in specimens in the routine diagnostic TB laboratory.

The use of fluorochromes for staining allows smear examination at lower magnification than is used for light microscopy. Because of the lower magnification, each field examined under fluorescence microscopy is larger in area than that seen with light microscopy, thus reducing the time needed to examine a slide.

High-volume laboratories, dealing with more than 25 slides per day, could use a fluorescence microscope.

4.2 Samples
NA

4.3 Equipment and materials
A binocular microscope equipped with a fluorescent light source and suitable filter set for auramine-stained smears. Fluorescent light is provided by a mercury vapour lamp, a halogen lamp or a light-emitting diode (LED). The mercury vapour lamp provides the strongest light but it has a limited life of about 100-200 hours, which must be monitored with a timer.

LED fluorescence microscopes do not require a completely dark room. In hot climates, the room should be permanently air conditioned both for operator comfort and to protect the microscope from fungal growth when humidity is high.

Do not place the microscope where it could be exposed to direct sunlight, dust, vibration (e.g. from centrifuges), water (sink, spray from a tap), chemical reagents, or humidity.

Install the microscope on a rigid, flat, level surface. It is too large and sensitive to be moved regularly; thus, any protection from humidity or dust that is needed should be provided for the microscope on site.

4.4 Reagents and solutions

Cleaning fluid, as recommended by the manufacturer, or 80/20 ethylether/ alcohol or 70% alcohol.
Lens paper, muslin or silk cloth, or fine-quality toilet paper to clean lenses without scratching.
Microscope cover, plastic or cloth.
Immersion oil, if needed (does not have to be special non-fluorescing quality).

4.5 Detailed instructions for use

See SOP on auramine staining

4.6 Reading and recording

NA

4.7 Quality control and maintenance

4.7.1 General maintenance

The fluorescence microscope requires careful maintenance from both optical and mechanical points of view. Laboratory workers must be familiar with its general mechanical and optical principles. Record maintenance in the logbook (Annex 1).

- A mercury vapour lamp has a life of 100–200 hours and should be replaced after 100 hours of use. This time can be slightly exceeded, but the risk of the lamp exploding increases. Lamps from different manufacturers will have different lamp lifespans. They are expensive, so it is important to check the lifespan of each lamp and ensure that it is not exceeded (Annex 2).

- It may be possible to repair a faulty microscope by replacing easily removable parts (objectives, eyepieces, light bulbs, fuses); if this does not work, the microscope should be entrusted to a competent person for repair. Never dismantle the microscope – its operational maintenance efficiency and accuracy may be severely impaired.

- In climates with relative humidity in excess of 70% for more than just a few weeks a year, fungal growth may damage the microscope. Fungal growth occurs almost exclusively on the prisms in the binocular tube, causing haziness and then dimness, and finally obscuring the view completely. Check the microscope for fungal growth from time to time and whenever the view gets hazy. With the light on and the 10x objective in place, fungal growth can be seen easily by removing the eyepieces and looking into the binocular tube.
  Fungal growth is best removed by a trained person.

The correct procedure for changing mercury lamps must be used, by trained technicians:

- do not touch the lamp with the fingers;
- check which end should point downwards (described on package insert) and respect this way of mounting;
- adjust the position (horizontal and vertical) of the new lamp and of the lamp-house mirror by using the adjustment knobs or refer to the microscope manual; replacement of halogen lamps does not require these adjustments.

- If the blue light remains weak after a new lamp has been fitted and properly adjusted, there may be another problem such as a blackened heat filter. Discontinue use of the microscope and request repair by a specialist.

4.7.2 Daily maintenance.

- In a humid climate without continuous air conditioning and if the microscope has no dedicated antifungal protection (a special device inserted under the binocular tube), stand a dish of silica gel (250g)

on the microscope stage and cover the microscope with a plastic cover. Renew the silica gel whenever it turns pink, which may be daily; regenerate the gel by heating until it turns blue again.

- Check for broken or damaged parts
- Check the counter to ensure that the lifespan of the lamp has not been exceeded.
- Check the lenses for dirt or grit; they may easily becomes scratched if they are wiped without first blowing away dust and small sand particles. First blow the lens clean, using a rubber blower brush (bentoa), then clean the lenses with clean, dry lens paper (or suitable equivalent). If this does not produce a clear image, try again using the cleaning fluid provided by the manufacturer, or 80/20 ethylether/ alcohol or 70% alcohol on the tissue paper.
- It is best not to remove eyepieces or objectives from their fixation holes but to clean only their external surfaces as needed.
- It is equally important to make sure those holes for the eyepieces and objectives are never left open for more than a few minutes.

4.7.3 Monthly maintenance

- Blow dust off the lenses, using a rubber blower, before cleaning them. Then apply cleaning fluid (or suitable equivalent), NOT xylene, to the lens paper (or suitable equivalent) and clean the lenses.
- Wipe dust off the body of the microscope with soft tissue paper moistened with water.

4.7.4 Yearly maintenance

- Thorough inspection and service by a qualified service technician.

4.8 Waste management and other safety precautions

Worn or broken mercury vapour lamps should be disposed off as toxic waste. In case of explosion, leave the room immediately and arrange for thorough air change before entering again –mercury vapour is toxic.

2.Related documents

Manufacturer's manual, specific to each microscope.
Basics of quality assurance for intermediate and peripheral laboratories, 2nd ed. Cairo, WHO Regional Office for the Eastern Mediterranean, 2002.

Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, United States Department of Health and Human Services, Centers for Disease Control, 1985.

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Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide, Institute of Medical and Veterinary Science, 2005.

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Maintenance manual for laboratory equipment, 2nd ed. Geneva, World Health Organization, 2008 (available at www.who.int/entity/diagnostics_laboratory/documents/guidance/guidance2/en/)

Manual of basic techniques for a health laboratory, 2nd ed. Geneva, World Health Organization, 2003.

Rieder HL et al. Priorities for tuberculosis bacteriology services in low-income countries, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.

Annex 1. Maintenance logbook

ITEM IDENTIFICATION		
Equipment:	MICROSCOPE Brand name:	
Purchase date:	Model/type:	
Location within the laboratory:	Serial no.	
Warranty expiry date:		
Manufacturer:		Tel:
Address:.		
Contact person:		
Technical service representative:		Tel:

PERIODICITY:		
Date	Maintenance operation	Operator

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

Annex 2. Fluorescence microscope log-sheet

The mercury vapour lamp has a life of approximately 100-200 hours. Replace the lamp after 100 hours of use

Operator's name	Date	Time	Duration of use (hours)	Cumulative duration of use (hours)

Standard Operating Procedures for Sputum Smear Microscopy

Storage of Slides for External Quality Assessment Slides

1.Scope

This SOP describes the procedure for the proper storage of slides for re-checking.

2.Procedure

4.1 Principle

A process which allows participating laboratories to assess their capabilities through blinded rechecking.

4.2 Specimen required

Stained and examined sputum smears.

4.3 Materials required

1. Stained examined slides
2. Storage boxes for slides
3. Absorbent paper

1.4 Reagents and solutions

NA

1.5 Detailed instructions

1 After examination of the slides remove immersion oil by placing the slide on the absorbent paper face down.

2. Check that all the oil immersion has been absorbed from the slides.
3. Check to make sure that the slide is well labelled with the laboratory number and date, consistent with that in the laboratory register.
4. Store the slides in the slide box in the same order as they are in the laboratory register.

5. Leave one blank space in the slide box behind the first slide so that the next slide can be added once it is examined. This is to maintain consistency with the laboratory register.

6. Store all the slides in the slide box irrespective of the smear results.

6.4 Reporting

NA

6.5 Quality control

1. Slides must be labelled in a manner consistent with the laboratory register to ensure that the correct slide is matched with the results.

2. Prior to placing slides in the slide box, oil immersion must be removed

3. Always store slides in closed boxes away for direct sunlight.

6.6 Waste management

1. Discard used materials in 5% Phenol, izal, lysol

2. Incinerate or burn and bury.

Reference

1. International Union Against Tuberculosis and Lung Disease. Tuberculosis Guide. Paris; 1998.

2. Lumb R, Bastian I. Laboratory diagnosis of tuberculosis by sputum microscopy. Adelaide: Institute of Medical and Veterinary Science; 2005.

3. World Health Organization. Maintenance and repair of laboratory, diagnostic imaging and hospital equipment. Geneva: WHO; 1994.

4. World Health Organization. Laboratory services in tuberculosis control. Part II: Microscopy. Geneva; 1998.

5. World Health Organization. Manual of basic techniques for a health laboratory. 2nd ed. Geneva: WHO; 2003

6. Fukuji A. AFB Microscopy Training, Tokyo, Japan; The Research Institute and Training 2005.

7. Centers for Disease Control and Prevention. Acid Fast Direct Microscopy Manual, 2000.

8. WHO, CDC, IUATLD, RIT, APHL, KNCV. External Quality Assurance for AFB Smear Microscopy.

9. WHO, CDC, IUATLD, RIT, APHL, USAID. Current Laboratory Practice Series; Acid-Fast Direct Smear Microscopy Training Package

Standard Operating Procedure (SOP)
Maintenance of a light microscope

1. Scope

The SOP describes the optimal operation of a light microscope through regular servicing and preventive maintenance.

2. Definitions and abbreviations

microscope magnification
Individual objective magnification x eyepiece magnification.
NA: not applicable

3. Personnel qualifications

3.1 Medical fitness

In accordance with codes of practice, arrangements should be made for appropriate health surveillance of TB laboratory workers:

- before working in the TB laboratory;
- at regular intervals thereafter, annually or bi-annually;
- after any biohazard incident;
- at the onset of TB symptoms.

Individual medical records must be kept for up to 10 years following the end of occupational exposure.

Laboratory workers should be educated about the symptoms of TB.
Confidential HIV counselling and testing should be offered to laboratory workers.

3.2 Education and training

Education and training must be given on the following topics:

- hygiene requirements;
- wearing and use of protective equipment and clothing;

- good laboratory practice;
- organization of work flow;
- use of equipment (operation, identification of malfunctions, maintenance).

The training shall be:

- given before a staff member takes up his/her post;
- strictly supervised;
- adapted to take account of new or changed conditions; and
- repeated periodically, preferably every year.

4. Procedure

4.1 Principle

The light microscope is a precision instrument intended for microscopic detection of tubercle bacilli in specimens in the routine diagnostic TB laboratory (see Annex 1).

4.2 Samples

NA

4.3 Equipment and materials

- A binocular microscope.

Install the microscope on a rigid, flat, level surface.
Do not place the microscope where it could be exposed to direct sunlight, dust, vibration (e.g. from centrifuges), water (sink, spray from a tap), chemical reagents, or humidity.

If the microscope is to be used every day, try to keep it in the same place in the laboratory. In humid climates, however, where fungal growth is likely, the microscope is best kept overnight in a light box or cabinet equipped with a 20 W light bulb. Silica gel desiccant may be used. Silica gel is blue when dry but turns pink when wet. Regenerate the gel by heating until it turns blue again.

- Lens paper, muslin or silk cloth, or fine-quality tissue paper to clean lenses without scratching.
- Plastic microscope cover. An intact cover is required in dusty areas and whenever the microscope is not in use.
- Storage cabinet or light box, for humid climates. The cabinet should have holes to permit circulation of air and should be fitted with a 20-W light bulb.

4.4 Reagents and solutions

- Synthetic, high-grade immersion oil, of medium viscosity and with a refractive index >1.5.
- Do not use cedarwood oil as it dries onto the oil immersion objective lens and will rapidly spoil the optical quality.
- Any dilution with xylene or other organic solvents is absolutely forbidden since it will very quickly spoil the oil immersion objective.
- Cleaning fluid as recommended by the manufacturer, or an 80:20 mixture of ethylether and alcohol, or 70% ethanol.

4.5 Detailed instructions for use

See SOP on Ziehl-Neelsen microscopy

4.6 Reading and recording

NA

4.7 Quality control and maintenance

4.7.1 Daily maintenance

The modern light microscope needs no particular maintenance, but considerable care is required in its use, regular cleaning, and protection from dust, sand and fungus.

- Each day before use, check for broken or damaged parts and ensure that the lenses, mirrors and other light-conducting surfaces are clean.

  Check the lenses for dirt or grit; they may easily become scratched if they are wiped without first blowing away dust and small sand particles. First blow the lens clean, using a rubber blower (bentoa), then clean the lenses with clean, dry lens paper (or suitable equivalent). If this does not produce a clear image, try again using the cleaning fluid, 80/20 ethylether/alcohol or 70% alcohol on the lens paper (or suitable equivalent).

  It is best not to remove eyepieces or objectives from their fixation holes but to clean only their external surfaces as needed.

  At the end of each day, use lens paper (or suitable equivalent) to carefully remove immersion oil from the 100x lens.

  In humid climates, put the microscope into a light box or closed cabinet overnight to minimize fungal growth. If the microscope is stored in this way, do not place the plastic cover over it. About 250 g of silica gel should be placed in a dish in the bottom of the box to absorb humidity.

  If the microscope is not stored in a box, cover it completely with a suitable plastic cover.

4.7.2 Monthly maintenance

Blow dust off the lenses, using a rubber blower, before cleaning them. Then apply cleaning fluid ***(not xylene)*** to the lens paper (or suitable equivalent) and clean the lenses.
Wipe dust off the body of the microscope with soft tissue paper moistened with water.

Record maintenance in the logbook (Annex 2).

4.7.3 Quarterly maintenance

In climates with relative humidity in excess of 70% for more than just a few weeks a year, fungal growth may damage the microscope. Fungal growth occurs almost exclusively on the prisms in the binocular tube, causing haziness and then dimness, and finally obscuring the view completely. Check the microscope for fungal growth from time to time and whenever the view gets hazy. With the light on and the 10x objective in place, fungal growth can be seen easily by removing the eyepieces and looking into the binocular tube.

Fungal growth is best removed by a trained person.

4.7.4 Troubleshooting

It may be possible to repair a faulty microscope by replacing easily removable parts (objectives, eyepieces, light bulbs, fuses); if this does not work, the microscope should be entrusted to a competent person for repair. Never attempt to dismantle any part of the microscope for repair.

Record troubleshooting and corrective action in the logbook (Annex 2).
It is equally important to make sure that holes for the eyepieces and objectives are never left open for more than a few minutes. If a lens is missing lens, close the fixation hole using the plug provided or by sticking adhesive tape over it, otherwise dust will enter and cause haziness of the remaining objectives.

Problem: the viewing field is too dim

Likely cause	***Solution***
Condenser is too low	Raise condenser to correct its position
Condenser iris diaphragm is closed	Open diaphragm properly

Problem: there are dark shadows in the field that move as you turn the eyepiece

Likely cause	***Solution***
Surface of eyepiece is scratched	Replace the eyepiece
Eyepiece is dirty	Clean the eyepiece

Problem: the image with the high power objective is not clear

Likely cause	***Solution***

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

Problem: the image with the low-power objective is not clear

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

Problem: the viewing 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 because of damaged lens cement *as a result of using poor-quality oil such as cedarwood oil or of the misuse of xylene*. This is almost certainly the cause if a completely hazy field becomes clear after changing the objective.
- A damaged objective (as a result of careless focusing, dropping, rough changing of slides).

4.8 Waste management

NA

5.Related documents

Manufacturer's manual, specific to each microscope

Basics of quality assurance for intermediate and peripheral laboratories, 2nd ed. Cairo, WHO Regional Office for the Eastern Mediterranean, 2002.

Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, United States Department of Health and Human Services, Centers for Disease Control, 1985.

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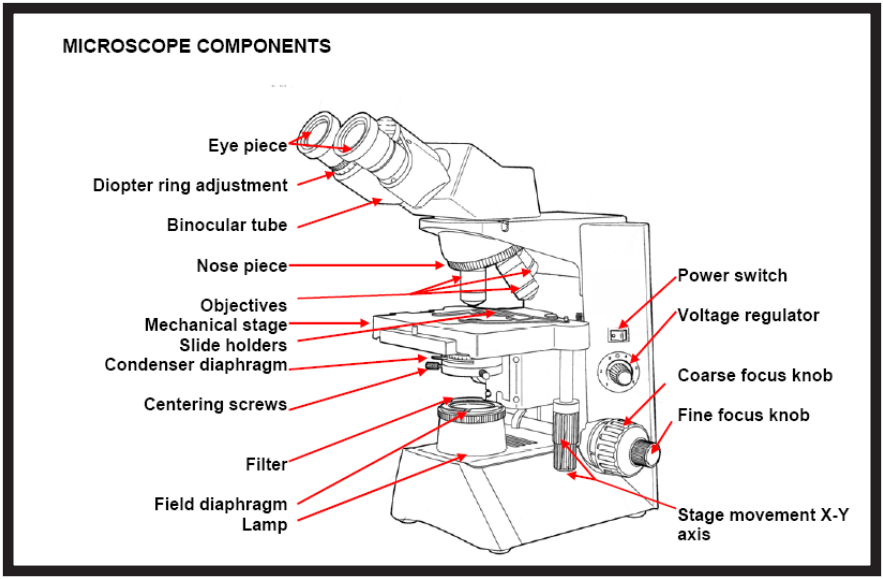
Manual of basic techniques for a health laboratory, 2nd ed. Geneva, World Health Organization, 2003.

Rieder HL et al. Priorities for tuberculosis bacteriology services in low-income countries, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.
http://wwwn.cdc.gov/dls/ila/acidfasttraining/

Annex 1. Microscope components



Annex 2. Maintenance logbook

ITEM IDENTIFICATION		
Equipment:	MICROSCOPE Brand name:	
Purchase date:	Model/type:	
Location within the laboratory:	Serial no.	
Warranty expiry date:		
Manufacturer:		Tel:
Address:.		
Contact person:		
Technical service representative:		Tel:

PERIODICITY:		
Date	Maintenance operation	Operator

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

Standard Operating Procedure (SOP)

Rechecking acid-fast bacilli smears for external quality assessment

This SOP describes external quality assessment of acid-fast bacilli smears stained by the Ziehl–Neelsen or fluorescent dye technique by rechecking of a random and representative sample of the routine work.

2. Definitions and abbreviations

AFB: acid-fast bacilli
EQA: external quality assessment
FM: fluorescence microscopy staining technique
HFN: high false-negative errors
HFP: high false-positive errors
LFN: low false-negative errors
LFP: low false-positive errors
LQAS: lot quality assurance sampling
NTP: national tuberculosis programme
QE: quantification errors
ZN: Ziehl–Neelsen staining technique

3. Procedure

4.1 Principle

To guarantee representativeness, all examined smears are kept by each laboratory. Regardless of results, a sample of smears is periodically selected at random by a visiting supervisor (TB Coordinator). The samples are given to assessors for blinded re-checking.

The supervisor compares the results obtained by the laboratory and by the assessors, and makes a list of smears for which results are discordant.
The list and the discordant smears are given to a second assessor for more careful examination and correct results.

The supervisor then uses the correct results to identify errors and determine whether they were made by the original laboratory or by the first assessor.

Routine feedback is given as frequently as possible during this process by returning results and any slides with serious errors.

Further investigation of failing laboratories is needed for confirmation and to identify and solve the underlying problems. In the worst cases this will be done during a supervisory visit by a laboratory expert.

4.2 Materials and personnel

The following personnel are required:

- Supervisors for (quarterly) sample collection and routine feedback. Ideally these are the regular NTP (district) supervisors.
- Assessors. These should be individuals with good experience – but it is the availability of their time for rechecking that is the main requirement.

All microscopy laboratories to be checked need:

- sufficient slide boxes to store all slides for at least one quarter;
- tools for permanent identification of slides: either pencils (for slides with frosted ends) or a diamond marker (for plain slides).

Supervisors collecting slides need:

- small slide boxes (20 slots) for slide transport, one per laboratory supervised;
- forms for sampling;
- transport.

Assessor /expert laboratory supervisors need

- a good microscope;
- sufficient time;
- 80/20 ethylether/ethanol mixture;
- staining facilities and equipment;
- transport.

4.3 Detailed instructions

4.3.1 Sample size and collection; keeping slides

All laboratories performing AFB smear microscopy under the NTP must be sampled. Sampling – as well as routine feedback in the apparent absence of problems – must be confided to NTP supervisors, according to a system defined at the national level.

Keeping slides at microscopy laboratories

- Keep all slides from all patients, regardless of results, until the sample has been taken. Thereafter, discard the remaining slides and start a new collection.

- Label the slides with the laboratory register number use a pencil (frosted-end slides) or diamond marker (plain slides). Do not write result on slides.

- After reading the slides, let the oil soak into absorbent paper (newspaper, toilet paper) until the end of the day or the following morning. Do not use xylene or any other solvent to remove the remaining oil and never rub the smears.

- Arrange the slides in boxes according to their numbering, leaving empty slots for those expected to arrive the following day. If there is a shortage of slide boxes, roll slides in toilet paper taking care that they cannot touch each other, and store them in their original packaging.

Collection of samples (by well trained programme supervisors)

- Sample slides during each supervision visit according to instructions from national level. In principle, one-quarter of the number needed per year is collected every 3 months; however, if the previous quarterly supervisory visit did not take place for any reason, twice as many slides should be collected.

- Record the period covered by the sample in the sputum smear register. From the register, or from a quarterly laboratory performance report, note the total number of smears examined during this period.

Divide this total by the sample size of 15 (rounding down to the next whole number); this yields the sampling step.

- Fill in a rechecking sampling form with details of laboratory identity, the period rechecked
- Ask the technicians to retrieve the selected slides from the boxes; check these against the list. Note any slides that are missing and replace each with the next slide in the register (even if the result was different), adding its identification and result at the bottom of the form until you get 15 slidess
- If none of the selected slides has a positive or scanty AFB result, add the first one or two found in the register searching from the start of the period.
- Ensure that the remaining slides are properly discarded, and instruct the technicians to start a new collection.
- Arrange the slides in a transport box in the order listed on the form (not all positives together) and deliver them as soon as possible to the assessors without results. Give the form with results to the coordinator.

4.3.2 Rechecking process

- Ensure that reading of slides by the assessor is completely blinded. The assessor should have no access to the results, only a list of slide identification numbers.
- Clean all oil-immersion examined slides with 80/20 ethylether/ ethanol mixture and allow to dry.
- For each slide, make notes on the form about the sputum quality, size, thickness, evenness, cleanness and background staining of the smear. For smears that are not re-stained, add notes on AFB colour and artefacts after microscopic examination.
- Restain all FM-examined smears.
- Restain all ZN slides if a staining or fading problem is suspected.

- For restaining, use the routine staining cycle and a certified quality auramine (FM) or carbol-fuchsin solution (ZN). Do not decolourize first (FM Smears); make a note on the form stating that restaining was done.

- Check the smears using the original microscopy system (brightfield for ZN, fluorescence for FM) and standard magnification. Check the number of fields prescribed for routine work in the NTP guidelines; do not check more fields unless there is doubt about the quantification after one length

- Do not recheck slides that lack clear identification or that have severely damaged smears. Report them as "excluded/ID" or "excluded/damaged".

- After rechecking, let the oil on the slides soak into absorbent paper as for routine work, then replace the slides in their original box.

- Rechecked FM slides – and ZN slides if fading is a problem – should be kept in a closed box, and in a cool place (refrigerator) if possible, until discordants have been taken out.

Registered result being controlled	negative	scanty	1+	2+	3+
negative	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

HFP: high false-positive, negative versus clearly positive result
HFN: high false-negative, positive versus totally negative result
LFP: low false-positive, negative versus scanty result
LFN: low false-negative, scanty versus totally negative result
QE: quantification error, at least two steps difference in quantification

4.3.3 Interpretation, recording and reporting

Final interpretation is possible only after all the annual samples have been processed, However, very poor results may already be evident after one quarter, and these need immediate action.

4.3.4 Feedback and problem-solving

Regional coordinator

Provide feedback to the laboratory sending the analysis sheet and list of substandard performance.

Laboratory supervisor

- When visiting laboratories to identify and solve problems, take with you the rechecking results for the quarter and any slides with serious errors that are still available.

- Allow the type and extent of the problems to dictate the nature of the investigation, taking account of remarks on smear and staining quality and any observed quantification error.

- Start by reviewing the error slides with the local technician:

➢ Use the local microscope, to check that it is functioning properly.

➢ To assess knowledge in case of false-positives, ask the technician to show what he or she considered to be AFB.

➢ For false-negatives in particular, check the staining solutions. Are the dates within limits? Is carbol-fuchsin a dark red colour when poured on a slide? Is there excess precipitate in the auramine solution? Any aberrant finding may indicate bad staining solutions as the cause of the problem.

➢ If there is no problem with the staining solutions, check the colour of AFB in recently stained positives. If the staining is not strong red and solid, suspect a problem with staining technique and request a demonstration.

➢ Look macroscopically at the recent slide collection to judge the quality of smearing, decolourization and counterstaining.

- Check the laboratory register for further clues (e.g. excessive proportion of isolated positives, or too many/too few scanty results). Any problem that is identified must be corrected at the very earliest opportunity, by on-the-spot training (e.g. in good smearing or staining technique), replacement, repair or adjustment of the microscope, replacement of staining solutions.

Investigation of errors detected in rechecking, by error pattern.

Pattern of errors	Possible causes	Checks to be done
HFP +++ and HFN +++ (nonsense results)	1. Unusable microscope 2. Has no knowledge of AFB 3. Doesn't look	1. Examine a 3+ using that microscope. 2. Test with clear-cut positive/negative and a good microscope. 3. Exclude other causes.
A single HFP	1. Administrative error 2. As for more frequent HFP (below)	1. Compare lab. register with QC listing – correct no.and result? 2. Exclude causes of more frequent HFP.
Regularly HFP with or without LFP	1. Poor registration routine 2. No systematic restaining before QC 3. Not quite clear on what AFB is 4. Selling positive results	1. Check lab. register (up to date?), use of sputum examination form, labelling of sputum containers. 2. Restain and re-examine HFP; again positive? 3. Look for inconsistent results of suspects in lab. register: regularly isolated positive/scanty? 4. Exclude other causes.
Rare LFP	Errors of QC	Ignore if they occur at comparable frequency for controllers.
Many LFP, with or without low-grade	1. Poor controls and counter-checks 2. Not quite clear on what AFB is 3. Contaminated carbol-fuchsin stain	1. Almost no LFN detected? Feedback of slides to laboratories – can they show the AFB? 2. Cross-check especially large sample of scanty smears 3. Test laboratory's stain on known negative smears/central laboratory records on stain QA?

Pattern of errors	Possible causes	Checks to be done
Single HFN (2–3+)	1. Administrative error as for single HFP 2. Very thick smear and/or poor light 3.Not looked at at all	1. Exclude other causes, check register as above (up to date? complete?) 2. Look at recent smears. Thickness? Too dark blue? AFB clearly seen in thicker parts (if applicable, use mirror)? Position of condenser and diaphragm? 3. Exclude other causes
More HFN and/or many LFN, quantification too low	1. Bad stain and/or poor staining (and restaining done) 2. Poor smearing technique 3. Problems with microscope 4. Careless microscopy 5. Contaminated methylene blue or rinsing water (and restaining done)	1. Check carbol-fuchsin stain – dark red, shiny (= well concentrated)? Check whether AFB are well stained in fresh (not restained) positive smear – solid, strong red colour? Observe staining procedure – sufficient time, heating? 2. As for single HFN above. 3. Use same microscope to examine a known positive – light, brightness? Image clear? See above. 4. Exclude other causes. 5. Aspect of the AFB typical? use methylene blue (distilled water for rinsing) on known negatives in repeat staining cycles, then check for atypical AFB.
Very high proportion of LFN	Contaminated methylene blue or rinse water (restained)	As above
Serious QE (too low	1. Poor stain/staining (restained) 2. Problems with microscope	1. As above 2. As above

4. Related documents

Aziz MA et al. External quality assessment for AFB smear microscopy. Washington, DC, Association of Public Health Laboratories, 2002.

Rieder HL et al. Priorities for tuberculosis bacteriology services in low-income countries, 2nd ed. Paris, International Union Against Tuberculosis and Lung Disease, 2007.