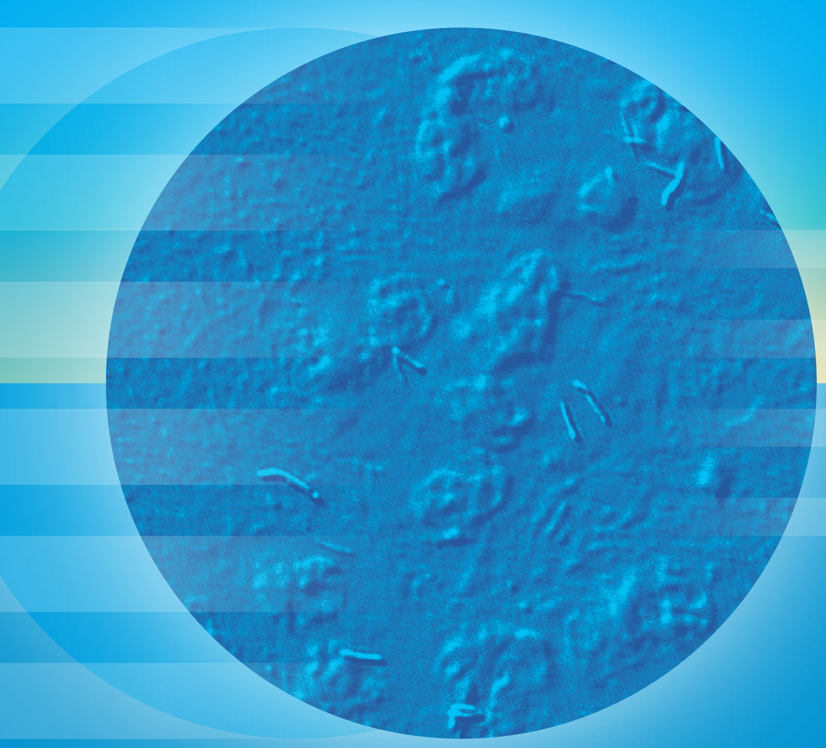
Laboratory Diagnosis of Tuberculosis by Sputum Microscopy



The handbook

Global edition

A publication of the Global Laboratory Initiative
a Working Group of the StopTB Partnership

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Laboratory Diagnosis of Tuberculosis by Sputum Microscopy

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Thanks go also to illustrator Kerry Reid and Sue Dyer Design for their long-standing contributions to earlier editions of *The Handbook*, and their enthusiastic and very professional inputs.

As we remarked in the first edition of *The Handbook*, it remains our sincere hope that the intended users of *The Handbook*, the technicians at the forefront of the international effort to contain and overcome TB, will find it useful in their daily laboratory work.

The handbook

Global edition

TB CARE I

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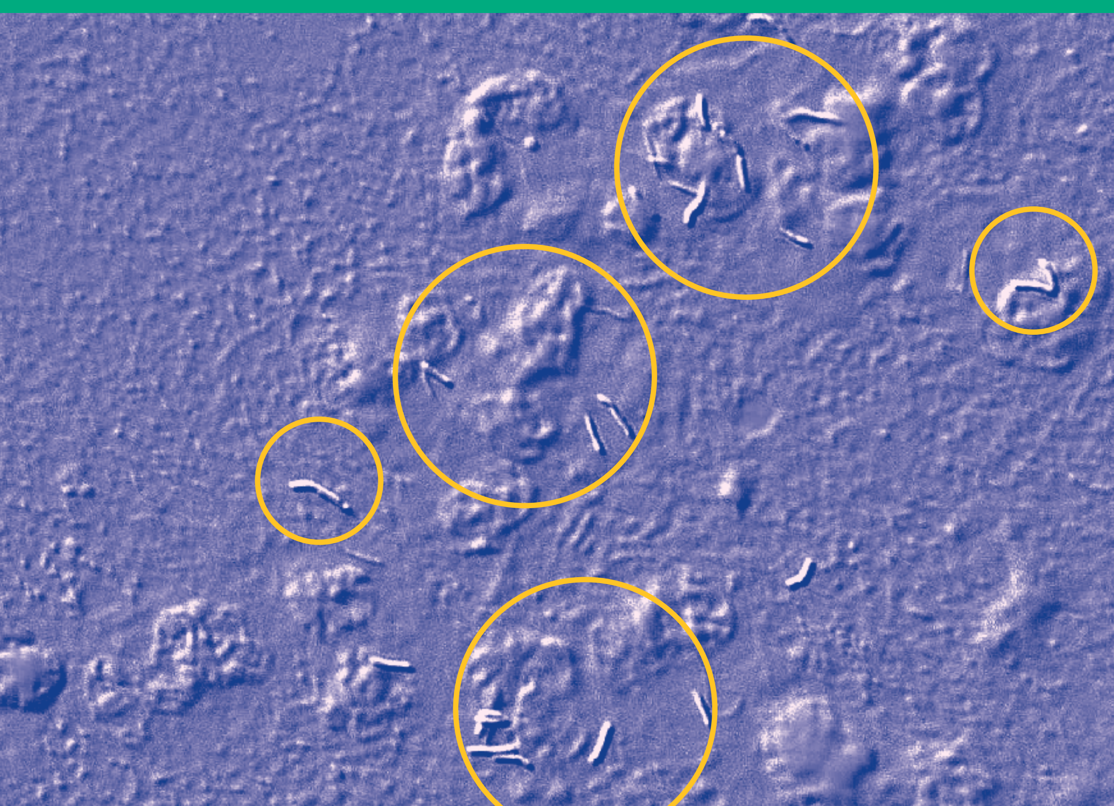
Foreword

In 2011, there were an estimated 8.7 million new cases of tuberculosis (13% coinfected with HIV). 1.4 million people died from the disease, including almost one million deaths among HIV-negative individuals and 430,000 among people who were HIV-positive. 5.8 million (67%) of these newly diagnosed cases were notified to national TB control programmes and reported to the World Health Organization. Among the 4.5 million new cases with pulmonary TB, 2.6 million (56%) had sputum smear-positive TB, and another 1.9 million were smear-negative*.



In many countries, sputum smear microscopy remains the primary tool for the laboratory diagnosis of tuberculosis. It requires simple laboratory facilities, and when performed correctly, has a role in rapidly identifying infectious cases. It has been shown conclusively that good-quality microscopy of two consecutive sputum specimens will identify the vast majority (95–98%) of smear-positive TB patients**. Moreover, microscopy can be decentralised to peripheral laboratories.

Despite its advantages sputum smear microscopy does fall short in test sensitivity, especially for certain patient groups such as those living with HIV/AIDS, and also in the laboratory diagnosis of childhood and extrapulmonary disease. New diagnostic tools endorsed by WHO (such as liquid culture, line probe assay, Xpert MTB/RIF) overcome many of the limitations of smear microscopy, especially for patients living with HIV/AIDS and those with a high likelihood of having drug-resistant TB.

WHO and The Union have previously published guidelines for sputum smear microscopy. In the decade since publication, many developments have occurred and a revised and updated text replacing both is timely.

The Handbook is a practical guide for the laboratory technician; it draws on the ideas outlined above and references best practice documents released by WHO and the GLI. *The Handbook* uses simple text and clear illustrations to assist laboratory staff in understanding the important issues involved in conducting sputum smear microscopy for the diagnosis of TB.

** WHO Global tuberculosis report 2012 WHO/HTM/TB/2012.6*
***WHO Same-day diagnosis of tuberculosis by microscopy 2011. WHO/HTM/TB/2011.7*

The purpose of *The Handbook* is to teach laboratory technicians how to safely collect, process and examine sputum specimens for the laboratory diagnosis of tuberculosis (TB).

Sputum microscopy

Sputum smear microscopy is one of the most efficient tools for identifying people with infectious TB.

Smear-positive patients are up to ten times more likely to be infectious than are smear-negative patients.

The purpose of sputum microscopy is to:

- Diagnose people with infectious TB
- Monitor the progress of treatment
- Confirm that cure has been achieved

Consistent and accurate laboratory practice helps to save lives and improves public health.

Risk of infection

Where good laboratory practices are used, risk of infection to laboratory technicians is very low during smear preparation.

A higher risk of infection exists when collecting sputum specimens from patients.

Doctors and nurses working in TB wards and clinics where aerosols are generated have a much higher risk of becoming infected with TB.

Personal safety

When performed correctly sputum examination will not place laboratory technicians at increased risk of developing TB.

Failure to follow these instructions may harm your health or cause immediate damage to equipment

Failure to follow these instructions may affect test results, or cause equipment damage over time

Correct – the preferred way to do something

Do not do this

Wear gloves for this procedure

Wear a laboratory coat for this procedure

Wash your hands

This substance is toxic

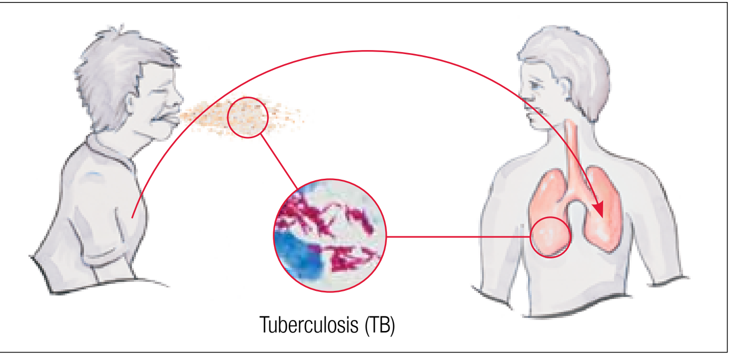
This substance is corrosive

This substance is infectious

This substance is flammable

Risks and transmission

TB is an infectious disease. Transmission occurs when small aerosols containing acid-fast bacilli (AFB) become airborne and are inhaled. When a person coughs, sneezes, sings or vigorously exhales they produce aerosols that could be infectious if the person has pulmonary TB.



Tuberculosis (TB)

Properly trained technicians, when working correctly, have a very low risk of infection in a TB laboratory. However some activities such as talking to infected patients and collecting specimens can carry a greater risk.

Assume all specimens are infectious. Do not shake or stir samples, aerosols may be generated

Specimens may contain pathogens other than TB. When working in the laboratory do not:

- Put anything in your mouth (e.g. a pen, your fingers etc.)
- Eat, drink or smoke
- Pipette by mouth
- Lick labels and envelopes etc.
- Apply cosmetics or handle contact lenses
- Store food or drinks in the laboratory
- Wear open-toed footwear or bare feet
- Use mobile telephones in the laboratory

Personal protective equipment (PPE)

Laboratory staff must be supplied with PPE that is appropriate for the microscopy laboratory.

- You must wear protective clothing at all times in the laboratory
- You must wear gloves when handling specimens
- Do not take PPE out of the laboratory
- Store PPE separately from personal clothing

Gloves

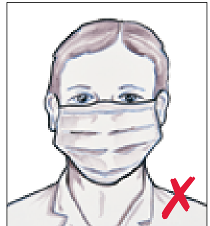
Wear gloves for all procedures that may involve direct or accidental contact with sputum, blood, body fluids and other potentially infectious materials.

- After use, remove gloves and discard into the biohazard waste bin
- Wash your hands:
  - Immediately if contaminated by a sample
  - When you finish work
  - Before leaving the laboratory

Coats

A good laboratory coat protects your skin and clothing. It has long sleeves and fastens in the front. The laboratory is responsible for supplying and cleaning laboratory coats.

Masks



Surgical masks *are not designed to protect the wearer*, they are designed to stop the wearer spreading aerosols. Respirators are not required for performing sputum smear microscopy.

Aerosols

Good work practice minimises aerosol formation and contamination of work surfaces and equipment.

- *Separate* 'clean' activities (administration, microscopy) from 'dirty' activities (specimen reception, smear preparation, staining)
- *Never shake* a sputum specimen
- *Carefully open* specimen containers, the sample may have collected around the thread of the container
- Spread the sample onto the slide *gently* in a regular motion
- *Always air dry smears* before heat fixing
- Use disposable wooden applicator sticks or transfer loops for making smears
- Always *manage laboratory waste correctly*

Ventilation

Open doors and windows help reduce the risk of infection (see page 73 Biosafety).

Two specimens

Where External Quality Assessment (EQA) is well established, and staff are limited, two sputum specimens are recommended for the laboratory diagnosis of TB.

Specimen 1

- Collect the first specimen when the patient presents to the clinic
- Give the patient a labelled sputum container for the next morning's sputum collection

Specimen 2

- Patient collects early morning sputum and takes it to the clinic

Alternatively, microscopy of two consecutive sputum specimens, collected on the same day, may be performed.

Hospital patients

If the patient is in hospital, it is better to collect a sputum specimen each morning on two consecutive days.

Safe collection

Transmission of TB occurs because infectious droplets are released into the air when an infected patient coughs.

Collect specimens outside so that infectious droplets are diluted in an open, well-ventilated area

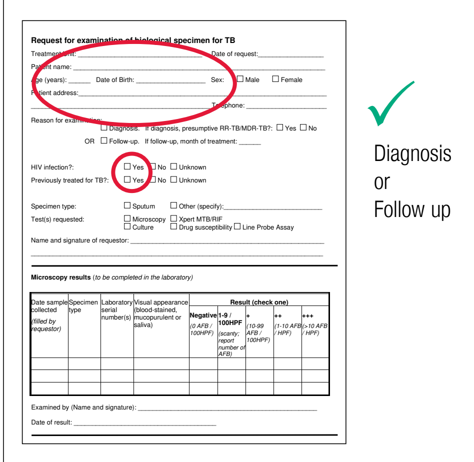
To reduce the possibility of laboratory staff becoming infected:

- Tell the patient to cover their mouth when coughing
- Collect sputum outside the laboratory, preferably outside the building and well away from other people

Do not collect sputum specimens in closed spaces like:

- Laboratories or wards
- Toilet cubicles
- Waiting rooms
- Reception rooms
- Any poorly ventilated area

Pre-collection and patient advice



Request for examination of biological specimen for TB

Treatment Unit: ______________________ Date of request: ______________

Patient name: ____

Age (years): _____ Date of Birth: _______________ Sex: ☐ Male ☐ Female

Patient address: ____

______________________________________ Telephone: ______________

Reason for examination:

☐ Diagnosis. If diagnosis, presumptive RR-TB/MDR-TB?: ☐ Yes ☐ No

OR ☐ Follow-up. If follow-up, month of treatment: _____

HIV infection?: ☐ Yes ☐ No ☐ Unknown

Previously treated for TB?: ☐ Yes ☐ No ☐ Unknown

Specimen type: ☐ Sputum ☐ Other (specify): ______________________

Test(s) requested: ☐ Microscopy ☐ Xpert MTB/RIF
☐ Culture ☐ Drug susceptibility ☐ Line Probe Assay

Name and signature of requestor: ______________________________________

Microscopy results *(to be completed in the laboratory)*

Date sample collected *(filled by requestor)*	Specimen type	Laboratory serial number(s)	Visual appearance (blood-stained, mucopurulent or saliva)	**Result (check one)**				
				Negative *(0 AFB / 100HPF)*	**1-9 / 100HPF** *(scanty; report number of AFB)*	**+** *(10-99 AFB / 100HPF)*	**++** *(1-10 AFB / HPF)*	**+++** *(>10 AFB / HPF)*

Examined by (Name and signature): ______________________________________

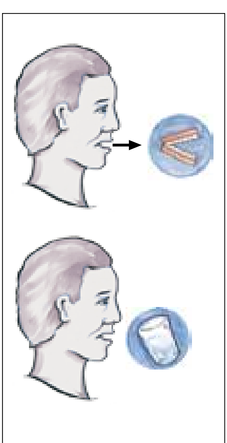
Date of result: ____________________________

Diagnosis
or
Follow up

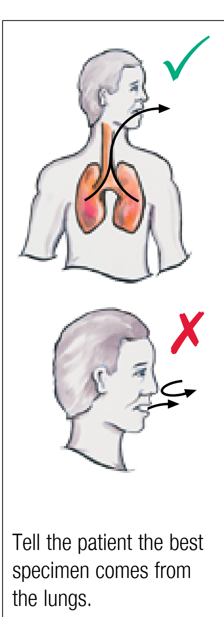
- Check the Laboratory Request Form
- Fill in any missing details
- Tick Diagnosis or Follow-up

Label the container, never the lid.

Patient advice



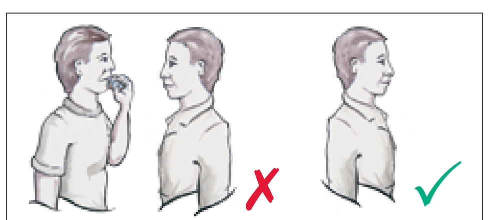
If dentures are present, remove them and rinse mouth with bottled water.



Tell the patient the best specimen comes from the lungs.
Saliva or nasal secretions are unsuitable.

How to collect a specimen

Patient Information page 84

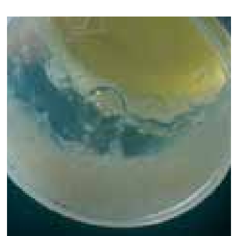


Do not stand in front of the patient during collection

Instruct the patient to:
1. Relax, take time
2. Inhale deeply 2 to 3 times, breathe out hard each time
3. Cough deeply from the chest
4. Place the open container close to the mouth to collect the sputum
5. After collection screw the lid on tightly

Several attempts may be necessary to obtain a good quality specimen.

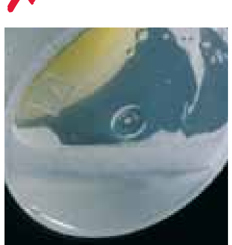
Specimen quality



Good quality specimen
Mucoid

Good quality specimen
Purulent

Good quality specimen
Blood stained



Poor quality specimens are thin and watery or composed largely of bubbles

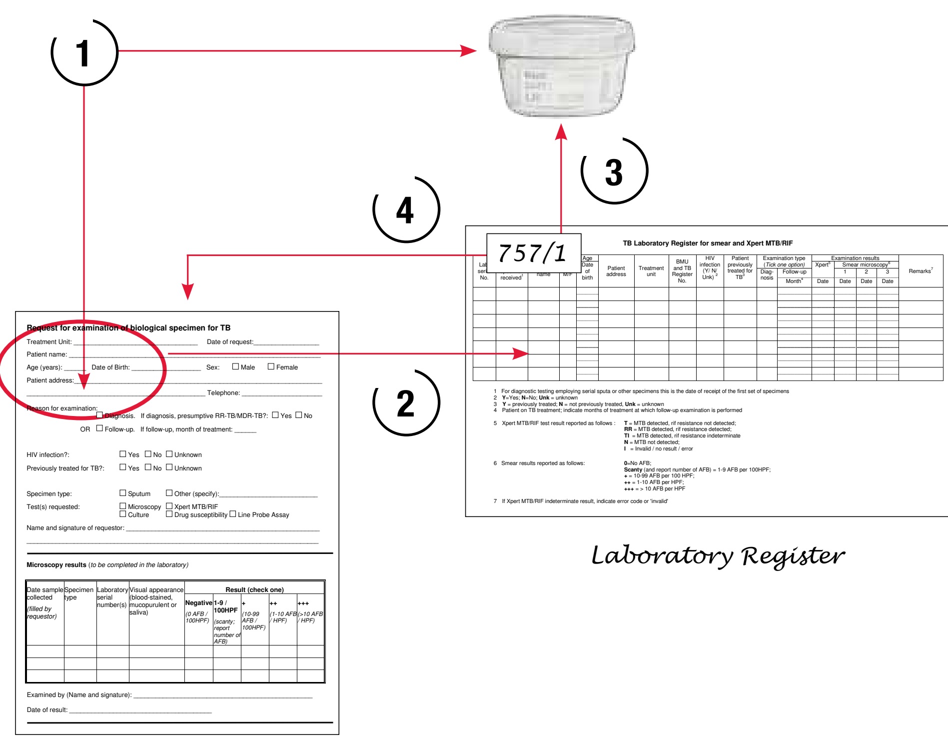
Keep the best sample

Rejection criteria

Repeat collection in the following cases:
- Broken or leaking specimen containers
- Specimen container details do not match the Laboratory Request Form
- The specimen has been collected into a fixative (e.g. formalin)
- Container unlabelled
- The specimen has been collected into tissue paper

Registration

Register the specimen before processing



1. Check patient details on the container match the Laboratory Request Form
2. Transfer patient details from the Laboratory Request Form to the Laboratory Register. For follow-up specimens copy the Patient District Number to the appropriate column of the register
3. Write the Laboratory Number (LN) on the side of the specimen container
4. Write the LN on the Laboratory Request Form

For each patient, use the same LN and the numbers 1 and 2 to identify the:

- First specimen (1)
- Second specimen (2)

Saliva specimens must be reported on the Laboratory Request Form.

Specimen storage and transport

Specimen storage and transport

Where AFB microscopy or molecular testing for TB are not available and the patient cannot be referred, appropriate specimen storage and transport is required.

Storage

To preserve specimen quality:

- If microscopy or molecular testing only is requested refrigeration is not required
- Store specimens to be cultured in a refrigerator or keep as cool as possible
  – Do not freeze

What you need

- Permanent marker to write details on the side of the container
- Plastic bag for each specimen
- Transport box
- Master List of specimens
- Laboratory Request Forms

Approved secondary packaging (transport box) must:

- Be leak proof and strong
- Contain absorbent material, bench roll etc.
- Keep Laboratory Request Forms separate from sputum specimens
- Be kept out of sunlight

Packing checklist

Is the sputum container clearly labelled with:

- Patient name
- Date of collection
- Specimen number (1 or 2)

Always label the container never the lid (see page 11).

- Are Laboratory Request Forms completed correctly?
- Are Laboratory Request Forms packed separately from specimens?

Prepare a Master List that contains the details for each specimen being transported.

Ensure the Master List contains the name and address of the laboratory sending the specimens.

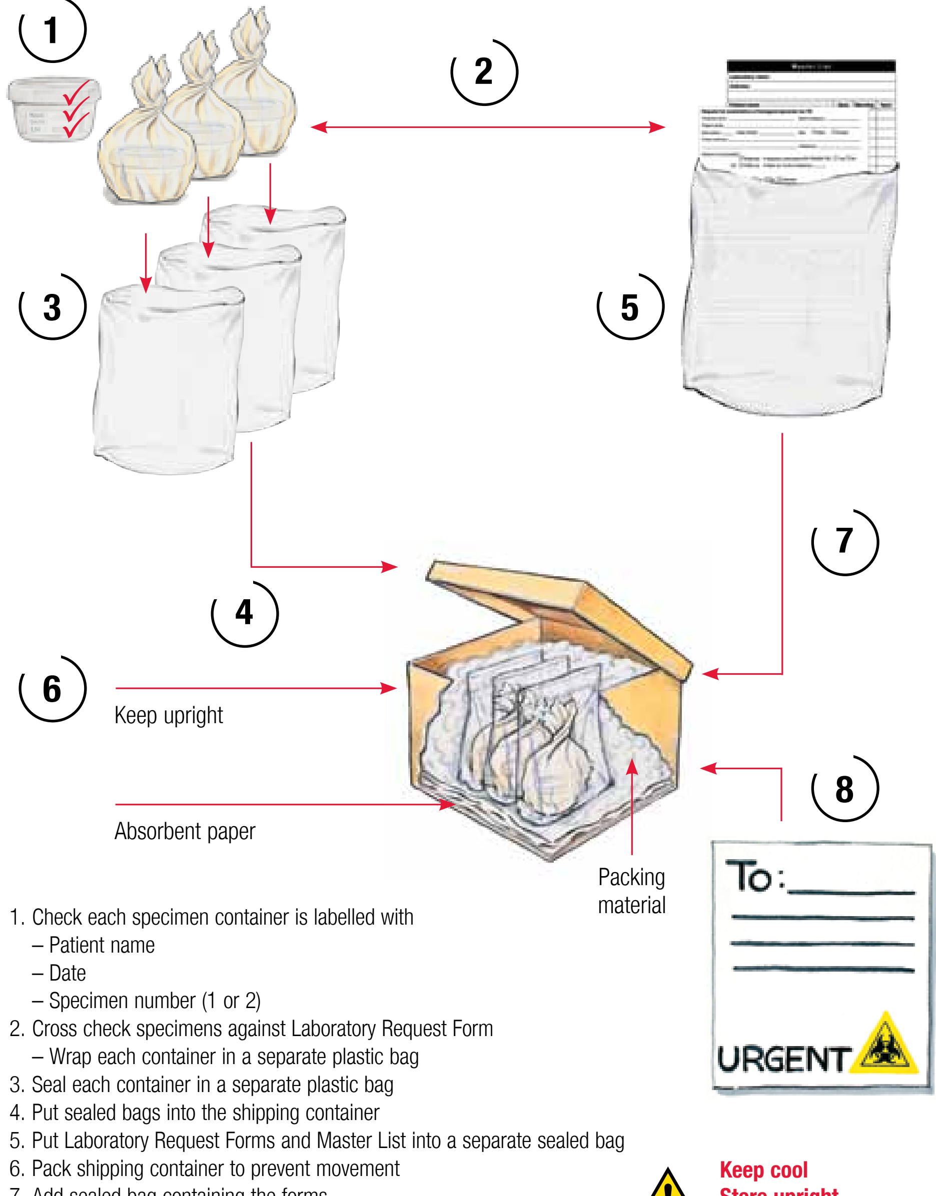
Check that the number of specimens equals that on the Master List.

Transport

- Follow local regulations for specimen transport
- Whilst delays, even in hot weather, will not affect test results, you should send packed specimens as soon as possible

Packing specimens

Put several layers of absorbent paper in the bottom of the shipping container.



1. Check each specimen container is labelled with
   – Patient name
   – Date
   – Specimen number (1 or 2)
2. Cross check specimens against Laboratory Request Form
   – Wrap each container in a separate plastic bag
3. Seal each container in a separate plastic bag
4. Put sealed bags into the shipping container
5. Put Laboratory Request Forms and Master List into a separate sealed bag
6. Pack shipping container to prevent movement
7. Add sealed bag containing the forms
8. Seal and address the shipping container

Keep cool
Store upright
Deliver urgently

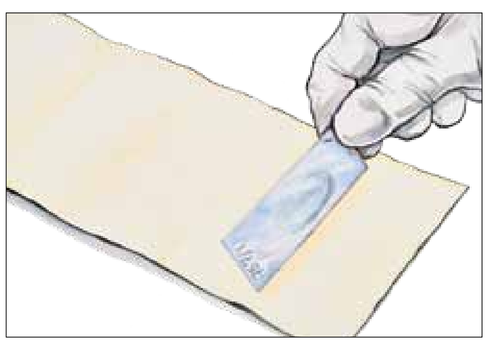
Specimen storage and transport

- For Satellite health centres preparing sputum smears:
  - Sputum smears must be prepared as soon as possible after collection
  - Smears are easier and safer to transport than specimens
  - Couriers bring sputum smears to the Microscopy Centre for examination, and return the results
  - Avoid once-weekly courier collections because they will result in unacceptable delays

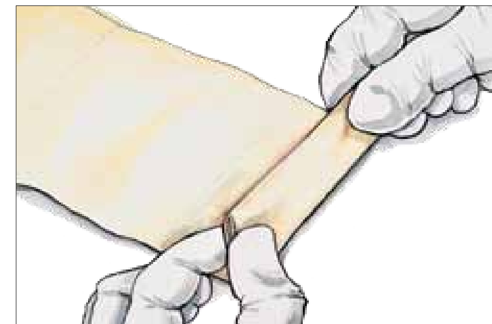
- Ensure each smear is clearly labelled and has a completed Specimen Request Form
- Keep in a slide box away from light, heat, dust, humidity, and insects
- The courier will bring the slide box and the Specimen Request Forms
- The courier should bring back an empty slide box from the Microscopy Centre
- Seal the slide box so that smears cannot fall out or break during transit

OR

- If a slide box is unavailable wrap each slide in toilet paper
- To prevent breakage put at least five slides in each bundle. Use unused slides if required

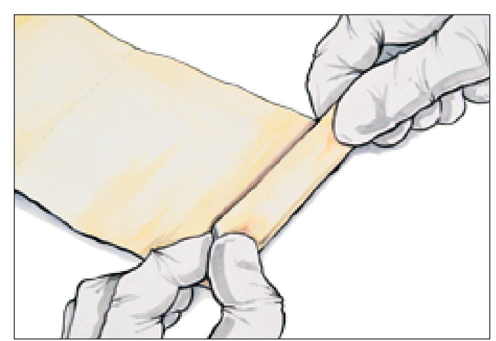


Add the first slide



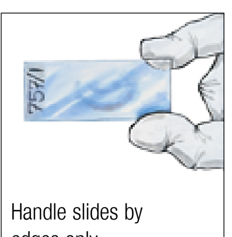
And roll twice

Add one slide at a time



Until all slides are wrapped

Use a rubber band or tape to prevent unrolling



Handle slides by edges only

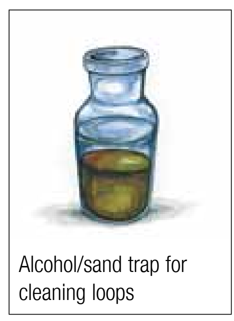
Sputum smears must be prepared promptly after collection or receipt.

To effectively prepare smears, you will need:

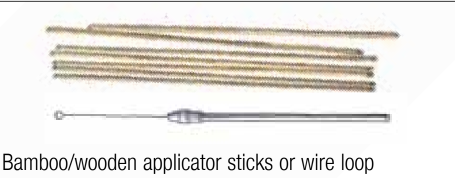
A dedicated solid bench with a non-absorbent surface that can be disinfected.

New, clean glass slides

Discard bucket with plastic liner

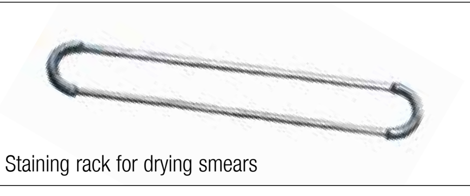


Alcohol/sand trap for cleaning loops



Bamboo/wooden applicator sticks or wire loop

Spirit lamp



Staining rack for drying smears

Never reuse sputum smear slides

Applicator stick

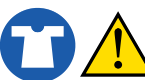
Bamboo/disposable applicator sticks are best because they:

- Separate purulent material from saliva faster
- Pick up more sputum
- Are faster, safer
- Are disposable, single use

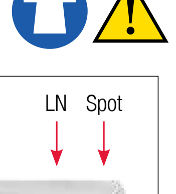
Wire loops

Some technicians prefer wire loops because they can be reused however they:

- Are more time consuming
- May collect a smaller sample volume
- Are less efficient, must be flamed and cooled between samples



Never put more than one sputum specimen on each slide

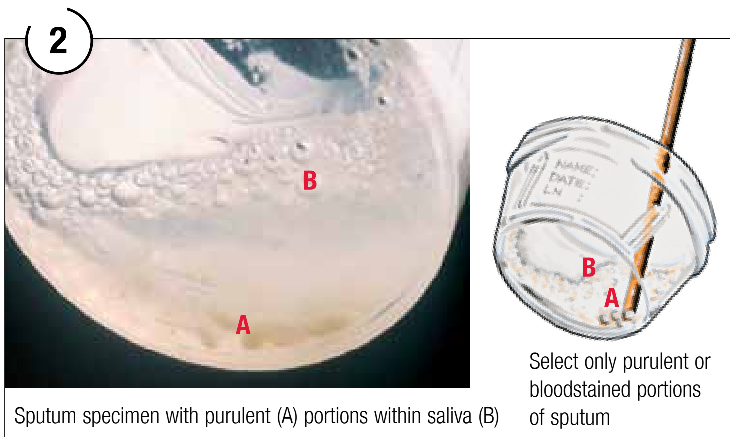


Write the LN and 1 or 2 identifier on the frosted end of each slide using a pencil

For non frosted slides use a diamond pen or stylus

Aerosols may be generated

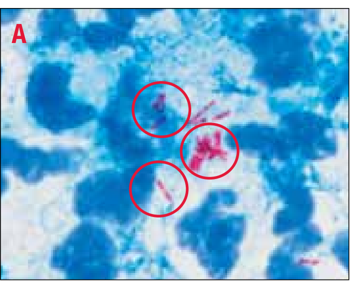
Do not mix purulent/bloodstained portions with saliva/mucous



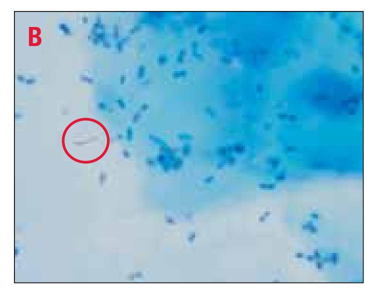
Sputum specimen with purulent (A) portions within saliva (B)

Select only purulent or bloodstained portions of sputum

More AFB will be found in the purulent portions of a specimen.



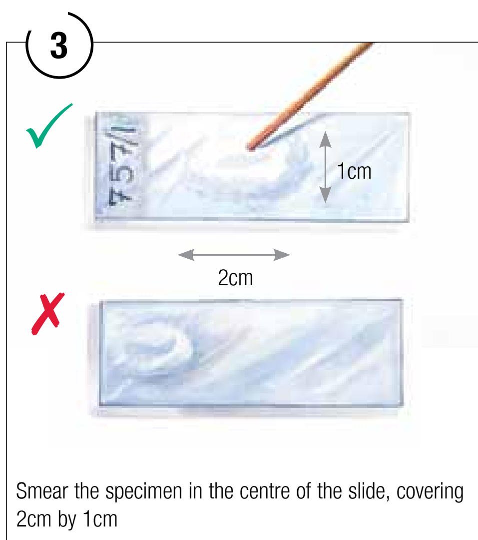
Ziehl-Neelsen stained smear – purulent



Ziehl-Neelsen stained smear – saliva

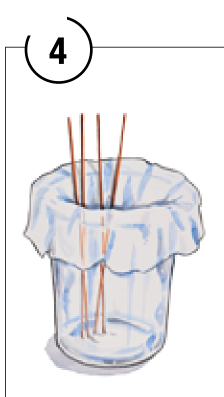
Older sputum specimens still give excellent results for microscopy.

3



Smear the specimen in the centre of the slide, covering 2cm by 1cm

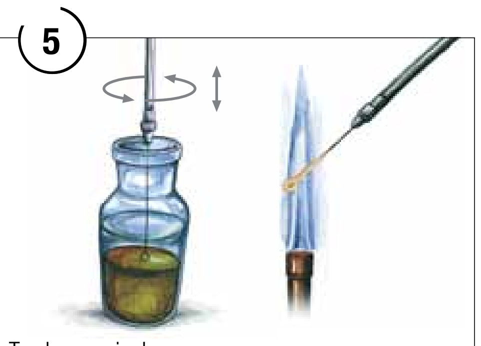
4



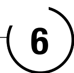
Discard the applicator stick into discard container after use, do not flame, do not reuse

Use a new clean applicator stick for each specimen

5



To clean a wire loop
- Insert loop in sand trap and rotate
- Flame the loop to red-hot and allow to cool

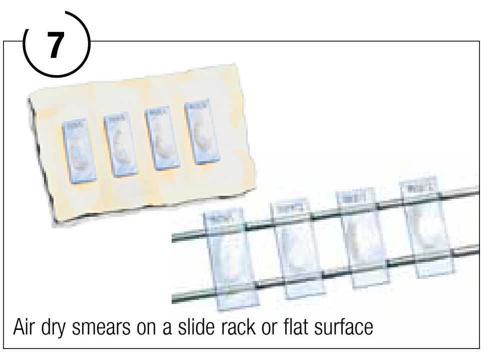


6

Retain all specimens until results are reported

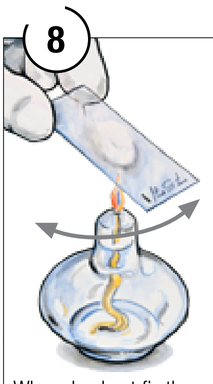
Remove gloves and wash your hands after preparing smears

7



Air dry smears on a slide rack or flat surface

8

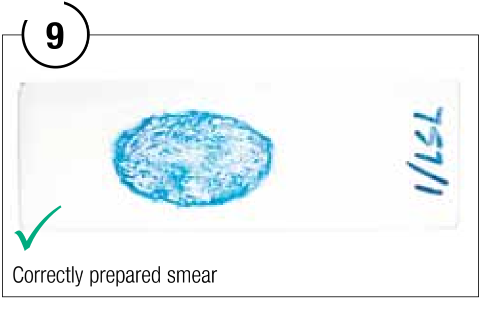


When dry, heat fix the smears:

- ensure the smear is facing upwards
- pass 3 times through the flame of a spirit lamp

Overheating will damage the bacilli

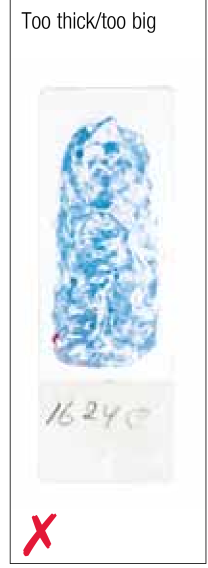
9



Correctly prepared smear

Stained smears resulting from poor smear preparation

Too thick/too big



Too thin

Not centred and too small

Multiples/confusing label

Method A | Brightfield Microscopy

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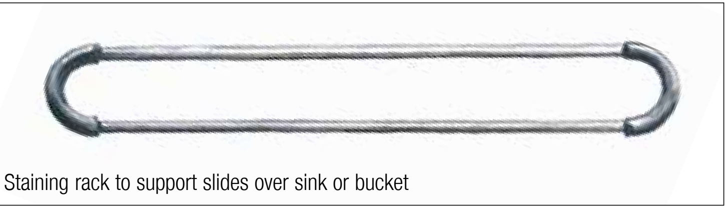
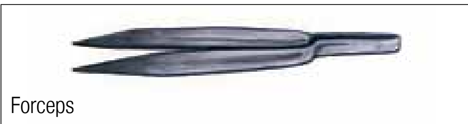
Brightfield sputum smear microscopy requires simple laboratory facilities and is a much cheaper alternative to the complex and costly process of TB culture. However, to be effective staff must be trained, follow correct standard operating procedures, be provided with good quality equipment, consumables and reagents, and be part of a Quality Assured network of laboratories.

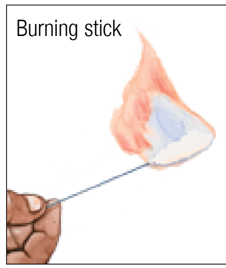
The Ziehl-Neelsen (ZN) technique has been the primary diagnostic technique for over 100 years. It is easier to learn to recognise ZN stained AFB compared with fluorescence microscopy. The detection of one AFB in a smear is sufficient to declare a positive result.

Staining What you need

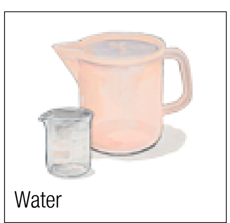
To stain smears using the Ziehl-Neelsen method you will need:

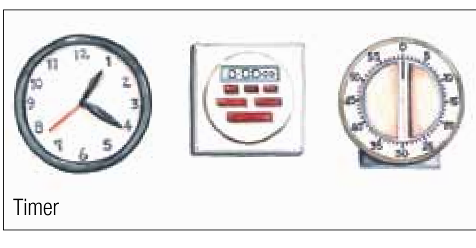

Staining rack to support slides over sink or bucket


Forceps


Burning stick

Slide rack for drying stained slides


Water

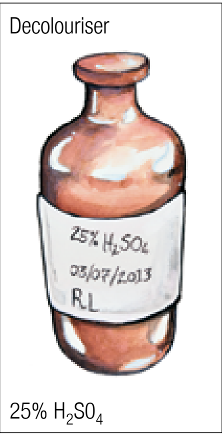

Timer

Staining bottle

Stain

1% carbol fuchsin

Decolouriser



25% H_2SO_4

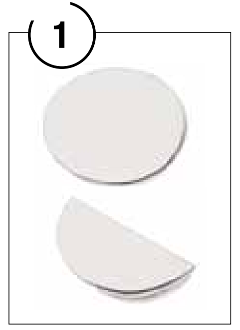
Counterstain

0.1% methylene blue

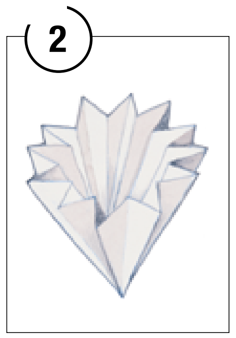
You will require 2 – 3 volumes of decolouriser for each volume of stain

How to fold a filter

1



2

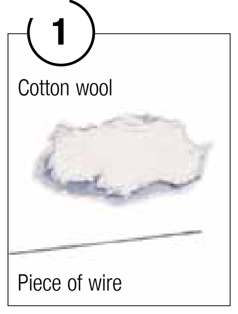


3

How to make a burning stick

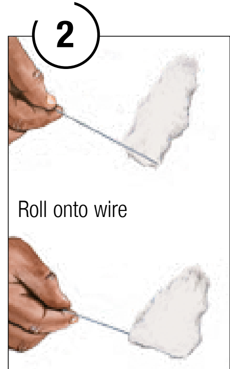
1

Cotton wool



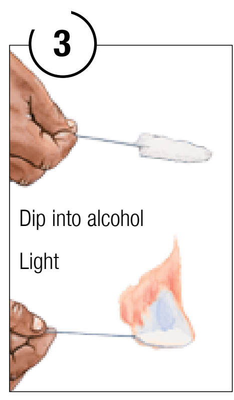
Piece of wire

2



Roll onto wire

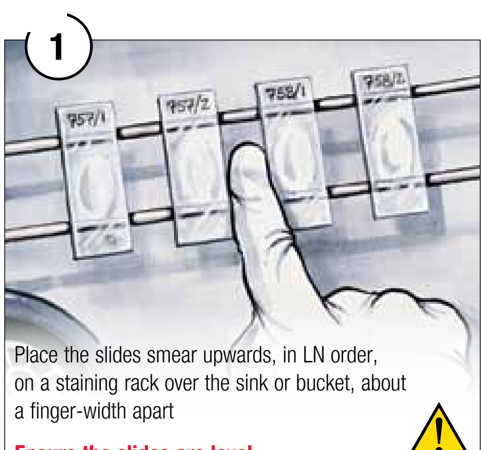
3



Dip into alcohol

Light

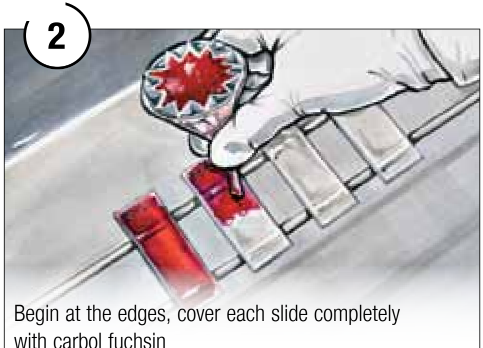
Staining The Ziehl-Neelsen method



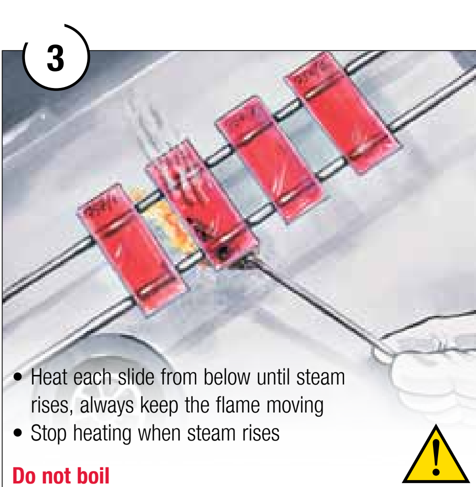
Place the slides smear upwards, in LN order, on a staining rack over the sink or bucket, about a finger-width apart

Ensure the slides are level

Filter during use

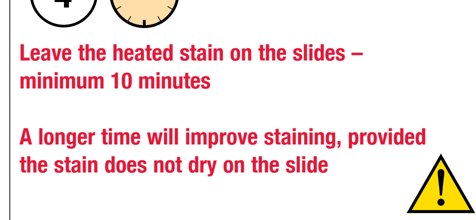


Begin at the edges, cover each slide completely with carbol fuchsin



- Heat each slide from below until steam rises, always keep the flame moving
- Stop heating when steam rises

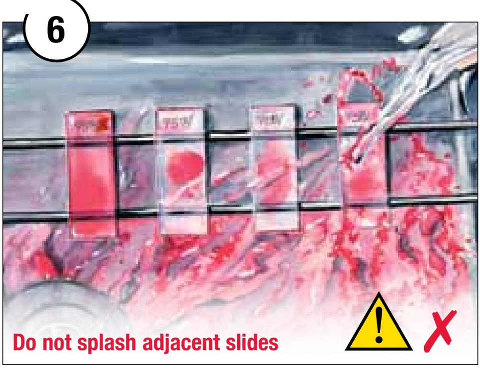
Do not boil



Leave the heated stain on the slides – minimum 10 minutes

A longer time will improve staining, provided the stain does not dry on the slide

- Gently rinse each slide with water
- Tilt each slide to drain off excess water

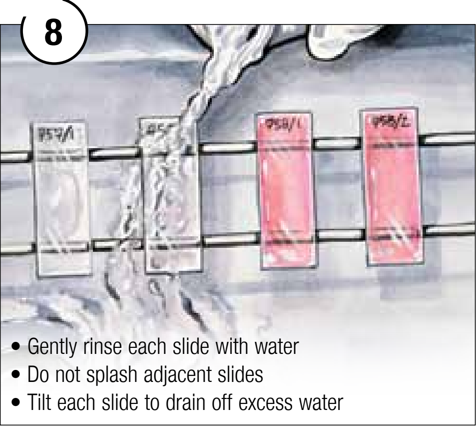


Do not splash adjacent slides

7

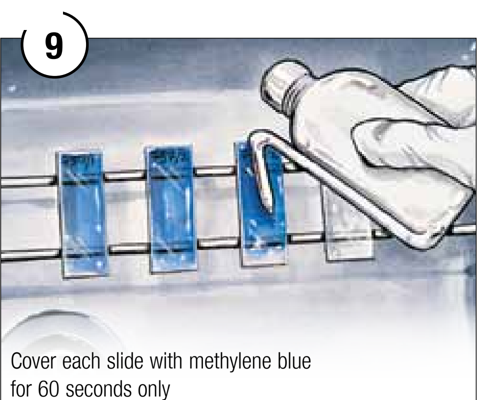
Add decolourising solution to the slide and leave for 3 minutes

8



- Gently rinse each slide with water
- Do not splash adjacent slides
- Tilt each slide to drain off excess water

9

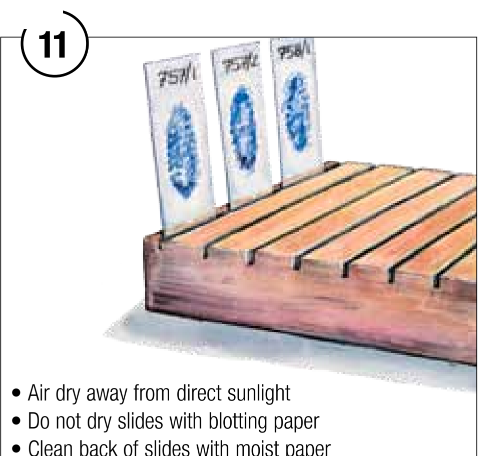


Cover each slide with methylene blue for 60 seconds only

10

- Gently rinse each slide with water
- Do not splash adjacent slides
- Tilt each slide to drain off excess water

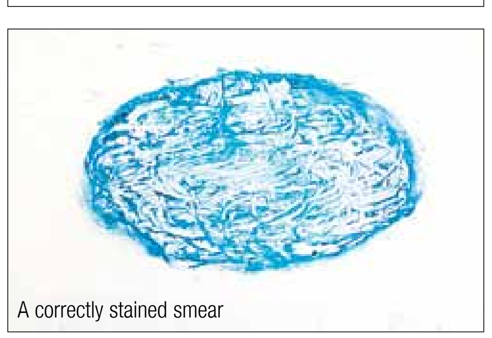
11



- Air dry away from direct sunlight
- Do not dry slides with blotting paper
- Clean back of slides with moist paper

12

Do not examine slides until they have dried

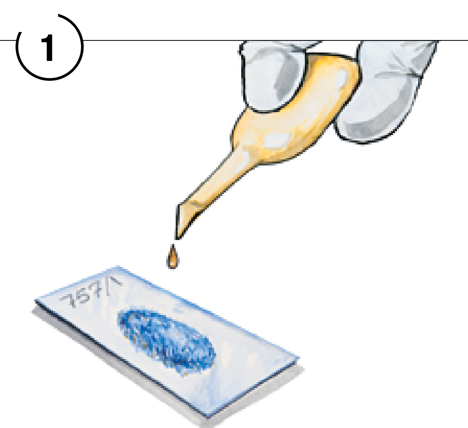


A correctly stained smear

Examination Reading smears

Smears must be consistently and systematically examined to ensure a representative area of the smear is reported.

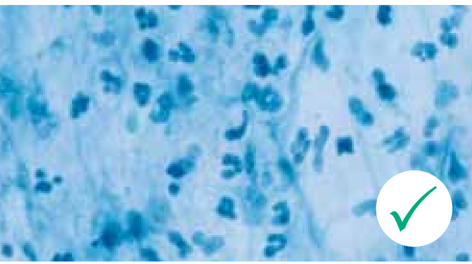
1



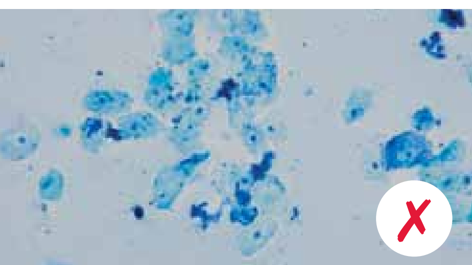
- Check the smear is facing upwards
- Apply one drop of immersion oil
- The drop must fall freely onto the smear so that the oil applicator does not become contaminated with TB organisms

Never allow the oil applicator to touch the slide

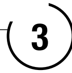
Use the 10X objective to focus the first smear, avoiding the oil drop. Scan the smear, looking for purulent or mucoid material. Where the smear is too thick, too thin, or contains epithelial cells only, move up or down to find purulent or mucoid material; continue scanning.



Inflammatory cells (high power) – look for areas like this



Avoid areas containing epithelial cells (low power)

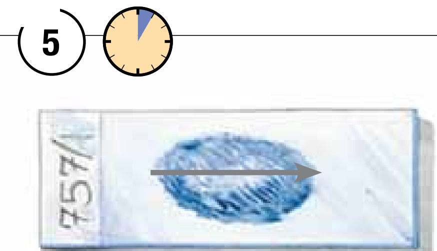


Carefully rotate the 100X oil objective lens over the slide

Carefully adjust the fine focus until cells are sharp

Never allow the lens to touch the glass slide

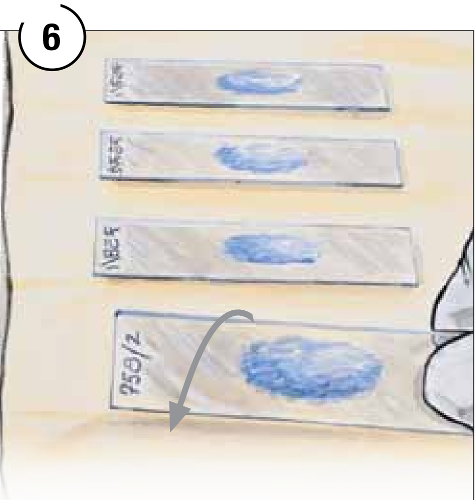
5



Direction of traversing the stained slide

Examine at least 100 high power fields (one length) before recording a negative result
You should take approximately 5 minutes to read a negative smear

6



Place the slides smear down on a clean piece of paper, leave overnight

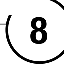
Avoid contamination, always use a clean piece of toilet paper

7

✓ Store the slides in LN order in a closed box. They will be needed for EQA

✗ Do not write the result on the slide

✗ Do not treat slides with xylene

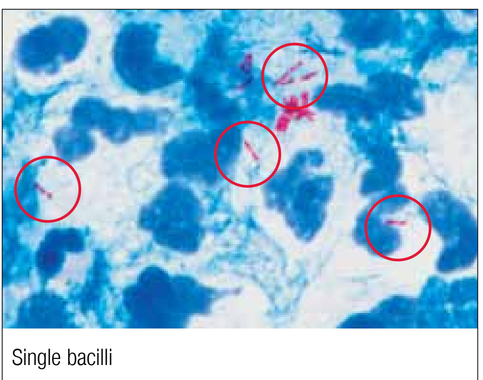


Wipe the microscope lens gently with tissue paper to remove immersion oil after each positive slide and when you have finished examining a batch of slides (for cleaning agents see page 38)

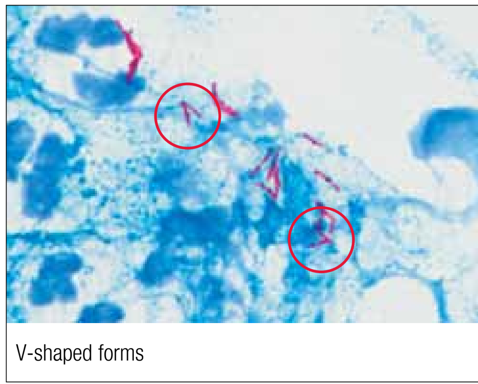
Examination Appearance of acid-fast bacilli

- Viewed with an oil immersion lens, AFB are red, slender rods, sometimes with one or more granules
- Tubercle bacilli may occur singly, as V-shaped forms, or as clumps of bacilli
- Report fragments of bacilli – often seen during treatment

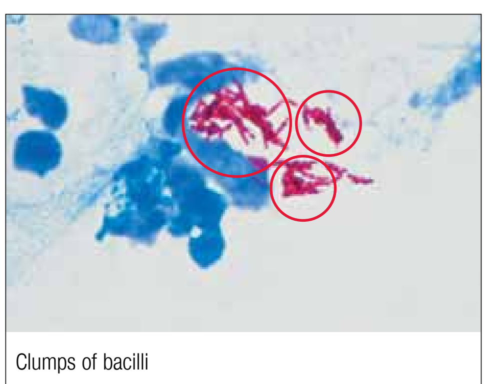
Typical morphological characteristics of *Mycobacterium tuberculosis*



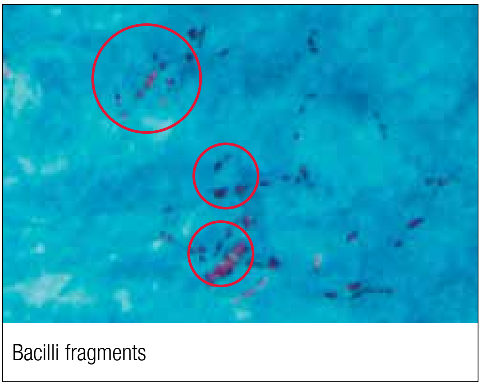
Single bacilli



V-shaped forms



Clumps of bacilli



Bacilli fragments

Where possible, all positive smears should be reviewed by another technician

The number of AFB indicates how infectious the patient is. It is important to record exactly what you see.

What you see	What to report
No AFB in 100 fields	No AFB observed
1 – 9 AFB in 100 fields	Record exact number of bacilli
10 – 99 AFB in 100 fields	1+
1 – 10 AFB per field, check 50 fields	2+
More than 10 AFB per field, check 20 fields	3+

1 757/1

2 Read the smear

Laboratory Register

757/1

TB Laboratory Register for smear and Xpert MTB/RIF

3 Record results

757/1

Date/Sign

4 Transfer the result to the Laboratory Register

5 → 6 Return to doctor or clinic

Laboratory Request Form

1. Use the LN to find the correct patient Request Form
2. Read the smear
3. Immediately record the result on the Request Form
4. Transfer the result to the Laboratory Register
   use red pen for positive results
5. Date and sign the Laboratory Request Form
6. Return the completed Laboratory Request Form to the Doctor or Clinic

Do not give results to the patients as lost reports may delay treatment
Do not write the results on the slide as they are needed for EQA checking

Summary

False-negative means reported negative but truly smear-positive

Be accurate and consistent in all your work, lives depend on you

False-negatives – consequences

- Patients with TB may not be treated resulting in on-going disease, disease transmission, or death

Prevention

- Label sputum containers, slides and laboratory forms accurately
- The specimen must contain sputum not saliva
- Select purulent material to make the smear
- Smear preparation – centred, spread evenly, 2cm x 1cm in size
- Use good quality basic fuchsin powder and reagents
- Heat carbol fuchsin until steaming
- Do not boil during fixation
- Stain with carbol fuchsin – minimum 10 minutes
- Do not overheat the carbol fuchsin
- Decolourise until no more carbol fuchsin is released, maximum 3 minutes
- Counterstain – maximum 60 seconds
- Keep the microscope well maintained and the lenses clean
- Perform regular QC on stains and reagents
- Check the slide LN matches the Laboratory Request Form before recording the result

False-positive means reported positive but truly smear-negative

Don't rush – examine at least 100 fields in one length before reporting 'No AFB observed'

False-positives – consequences

- Patients are treated or retreated unnecessarily
- Medications will be wasted

Prevention

- Ensure laboratory staff can reliably recognise acid-fast bacilli
- Label sputum containers, slides and laboratory forms accurately
- Always use new unscratched slides
- Use bamboo/wooden sticks once only
- Do not allow carbol fuchsin to dry on the smear
- Decolourise adequately
- The oil applicator must not touch the slide
- Keep the microscope well maintained, the lenses clean, store appropriately
- Perform regular QC of stains and reagents
- Check the slide LN matches the Laboratory Request Form before recording the result

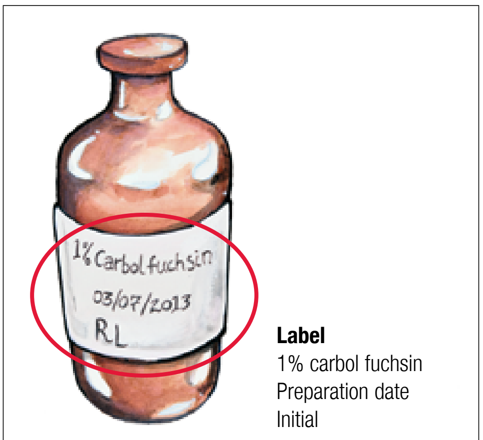
Stain

Carbol fuchsin – 1.0%		Grade
Basic fuchsin powder	10g	Certified
Ethanol (or methanol)	100ml	Technical
Phenol crystals*	50g *use colourless not tinted crystals*	Analytical
Distilled water	900ml	

Phenol crystals and vapour are corrosive, toxic and may cause burns
Use care, prepare in a well ventilated area

Preparation
1. Add 100ml of ethanol (or methanol) to a one litre glass flask
2. Add 50g of phenol crystals and dissolve
3. Add 10g of basic fuchsin powder
4. Mix well until dissolved
5. Add distilled water to make one litre
6. Label the bottle – **"1% carbol fuchsin", date and initial**
8. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Wash your hands after preparing reagents



Label
1% carbol fuchsin
Preparation date
Initial

Perform a Quality Control check and record results in the QA log book
Filter solution at time of use

Decolourising solution

Always add the acid to ethanol or water. Solutions will generate heat.

25% H_2SO_4		Grade
Concentrated sulphuric acid (H_2SO_4)	250ml	Technical
Distilled water	750ml	

Preparation
1. Carefully add the H_2SO_4 to the water
2. Label the bottle **"25% H_2SO_4", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

3% HCl in ethanol (acid alcohol)		Grade
Fuming hydrochloric acid (HCl)	30ml	Technical
95% ethanol	970ml	Technical

Preparation
1. Carefully add the HCl to the ethanol
2. Label the bottle **"3% HCl in ethanol", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

6% HCl		Grade
Fuming hydrochloric acid (HCl)	60ml	Technical
Distilled water	940ml	

Preparation
1. Carefully add the HCl to the water
2. Label the bottle **"6% HCl", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Counterstain

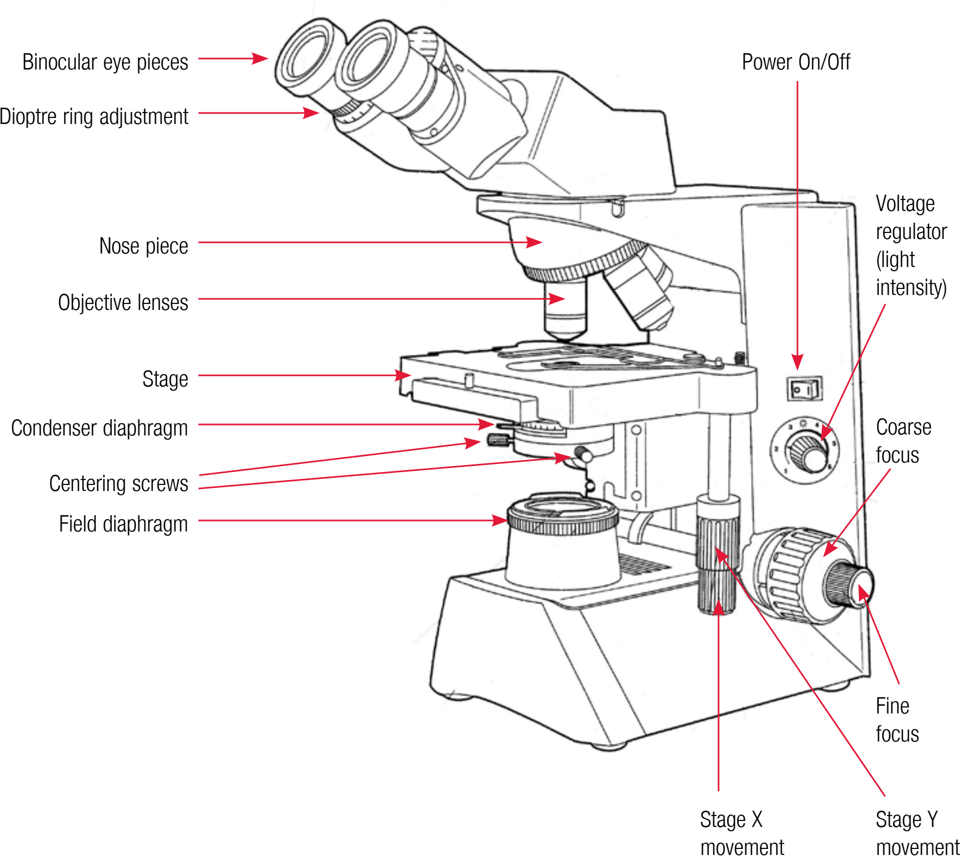
0.1% methylene blue	
Methylene blue chloride	1.0g
Distilled water	1000ml

Preparation
1. Dissolve the methylene blue chloride in distilled water
2. Label the bottle – **"0.1% methylene blue", date and initial**
4. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Perform a Quality Control check and record results in the QA log book.

The microscopy area should be:

- Free from dust
- On a stable level platform
- Away from centrifuges and refrigerators
- Away from water, sinks or chemicals to avoid splashes or spills
- Ergonomically correct work position (see page 77)



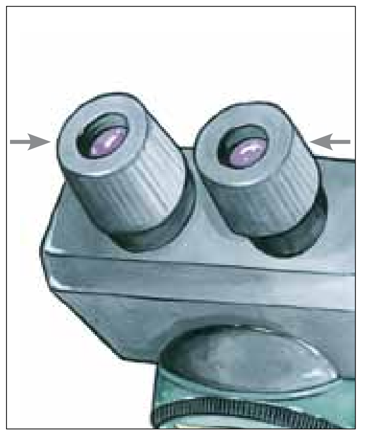
Setting up the microscope

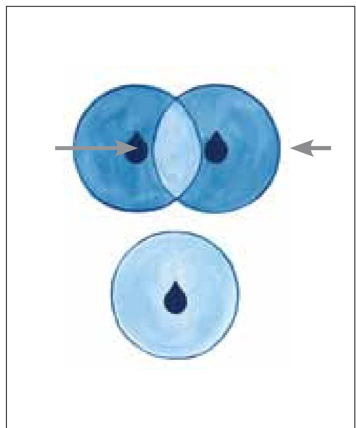
For binocular microscopes with pre-centred and fixed condensers:

1. Rotate the nose-piece to the 10X objective
2. Set the variable voltage regulator to minimum
3. Turn the power on
4. Slowly adjust until the desired light intensity is reached
5. Place a stained slide onto the stage
6. Bring the smear into focus with the coarse and fine-adjustment knobs

Always use the focusing adjustment knobs to lower the stage away from the lens

7. Adjust the interpupillary distance until the right and left images merge





8. Focus the image with the right eye by looking into the right eye-piece and adjusting with the fine focus knob
9. Focus the image with the left eye by looking into the left eye piece and turning the dioptre ring
10. Open the condenser iris diaphragm so that the field is evenly lit
11. Place one drop of immersion oil onto the smear and rotate the 100X objective into it

12. Focus using the fine adjustment knob
13. Use the variable voltage regulator to achieve a comfortable illumination
14. Once the smear has been read, rotate the 100X objective away, locate the 10X objective over the slide, and then remove the slide
15. When finished, reset the voltage regulator to a minimum, and turn the power off
16. At the end of each day, use fine tissue paper to carefully remove immersion oil from the 100X lens, do not use gauze. Cover the microscope, or put it in the microscope box or return to the humidity controlled cupboard

Do's and Don'ts

- The 100X objective is the only lens requiring immersion oil
- Keep immersion oil away from other lenses
- Immersion oil must have medium viscosity and a refractive index (RI) greater than 1.5. Any synthetic, non-drying oil with an RI > 1.5 is suitable (refer to manufacturer's instructions)
- Do not use cedar wood oil as it leaves a sticky residue on the lens

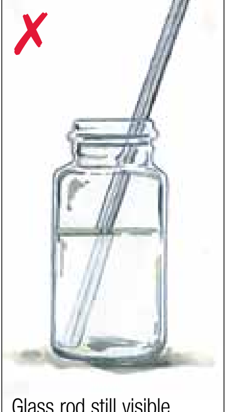
Never use cedar wood oil diluted with xylene instead of immersion oil, as it will quickly destroy the lens

Immersion oil – a simple test

Good immersion oil

A clear glass rod 'disappears' RI > 1.5

Poor immersion oil



Glass rod still visible below the surface RI < 1.5

Maintenance

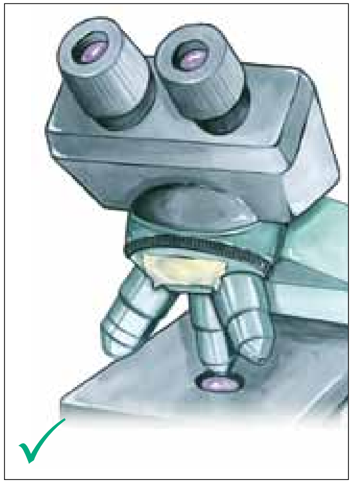
Do not use xylene

Cleaning lenses

Some cleaning agents will damage lenses over time – for daily cleaning use tissue paper

Cleaning Agent	Long term use	Infrequent use
Manufacturer's recommendation	✓	✓
Ethyl ether/alcohol (80/20)	✓	✓
Alcohol	✗	✓
Benzene/petrol	✗	✓
Acetone/ketones	✗	✓
Xylene	✗	✗

- Never use xylene to clean any part of a microscope
- Remove dust and sand from dry lenses before using cleaning fluid
- When ever possible use the cleaning fluid recommended by the manufacturer
- Use a minimum amount of cleaning fluid, never dip a lens into cleaning fluid
- Fine tissue paper is best for cleaning optical surfaces as it does not scratch the lens
- Alternatively use fine quality toilet paper
- Do not use ordinary paper, or cotton wool or gauze to clean lenses
- Keep the microscope covered when not in use
- Keep the eye-pieces in place
- Fungus or dust may enter through holes where objectives in the nose-piece are missing

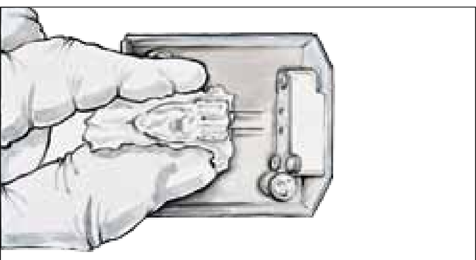


Cover holes from missing objectives

- If the image appears hazy with black dots, check for dust or dirt on the lenses (eye-pieces, objectives, condenser and illuminator lens). If:
  – The black dot moves when the eye-piece is rotated, then the dust is on the eye-piece
  – The black dot moves when the slide is moved, then it is on the slide
  – These two are ruled out, then assume the dust is on the objective (if inside the objective, it appears as dots; if on the outside, then as a hazy image)
- Dust can be removed using a camel-hair/artist brush or by blowing over the lens with an air brush

A simple air brush made using a Pasteur pipette and rubber bulb

Light source
- Never touch the glass bulb surface as skin oils will burn, reducing light intensity
- Use paper to hold the bulb when inserting into the microscope



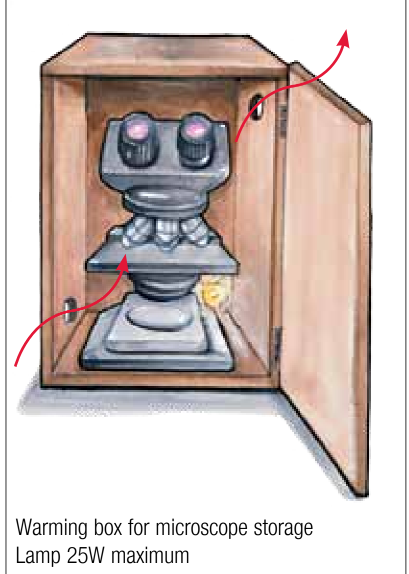
Use a tissue, do not touch the bulb with your fingers

Mechanical parts
- Never disassemble the microscope – send to a specialist technician

Fungal growth

Fungus growing inside the eyepiece tube

- Fungus growth on the lenses, the eye-piece tube and prisms causes the microscope image to become hazy and unclear
- To check for fungus turn the microscope on:
  - Rotate the 10x objective into the light path
  - Take out both eyepieces, look down the eyepiece tubes for fungus
- To prevent fungal growth, the microscope should be kept in a warm cupboard or box. A cupboard with a tightly fitting door, heated by a light globe (maximum 25W), works well
  - Always leave the cupboard light on, even when the microscope is not in the cupboard

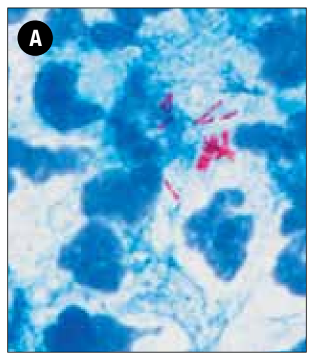


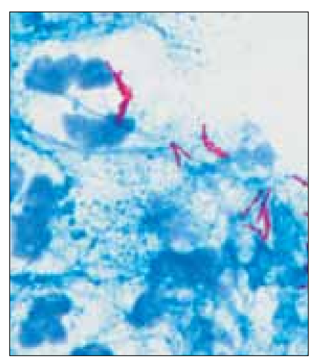
Warming box for microscope storage
Lamp 25W maximum

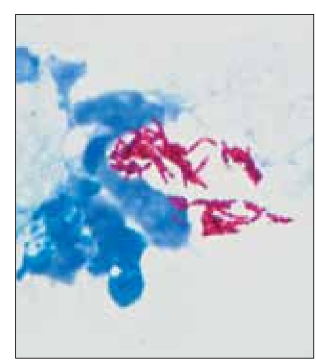
- If proper storage is not available, keep the microscope in the shade and with good air circulation

Trouble-shooting Staining

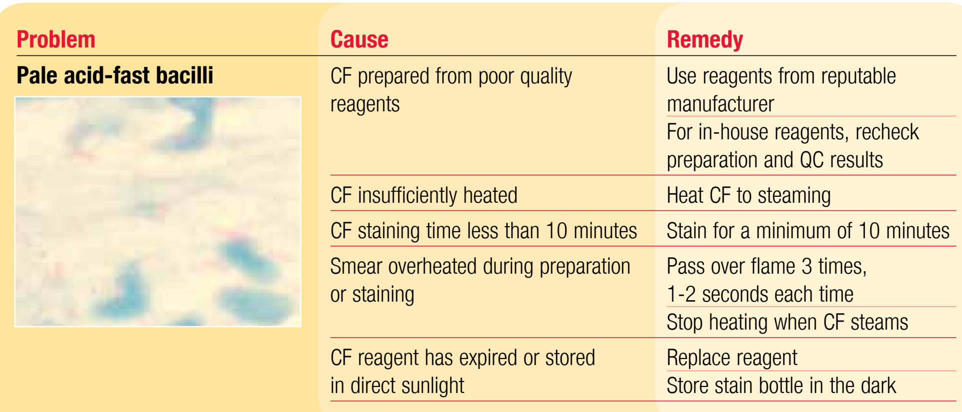
Correctly stained slides







Problem	Cause	Remedy
Smear too pink	Insufficient decolourisation	Decolourise for longer
	Acid concentration very low, or applied for too short a time	For commercial reagents, check with NTP
		For in-house reagents, recheck stain preparation and QC results
	Carbol fuchsin (CF) has dried on smear	Check smears are level over sink
		Add sufficient CF
	Smear too thick	Prepare new smear
		When correctly stained this slide looks like **A** above



Problem	Cause	Remedy
Pale acid-fast bacilli	CF prepared from poor quality reagents	Use reagents from reputable manufacturer For in-house reagents, recheck preparation and QC results
	CF insufficiently heated	Heat CF to steaming
	CF staining time less than 10 minutes	Stain for a minimum of 10 minutes
	Smear overheated during preparation or staining	Pass over flame 3 times, 1-2 seconds each time Stop heating when CF steams
	CF reagent has expired or stored in direct sunlight	Replace reagent Store stain bottle in the dark

Problem	Cause	Remedy
Counterstain too dark	Excessive counterstaining time	Do not exceed 60 seconds
	Inadequate washing step after counterstaining	Extend washing step
	Methylene blue concentration too strong	For commercial reagents, check with NTP For in-house reagents, recheck preparation and QC results
	Smear too thick	Prepare new smear

Problem	Cause	Remedy
Deposit on slide	Stains not filtered	Filter stains
	Soot deposit on underside of smear	Clean with a moist tissue paper

Trouble-shooting Microscopy

Problem	Cause	Remedy
Light flickers or does not turn on	Loose plug or connection	Check wall sockets, transformer, power supply
	Loose light bulb	Reinstall the bulb – Do not touch bulb with fingers
	Dirty bulb contacts	Clean contacts with 70% alcohol and retry or replace bulb
	Erratic voltage supply	Use a voltage stabiliser
	Faulty on-off switch	Replace the switch
	Fuse blown or transformer blown	Replace the fuse
	Discoloured bulb/burnt out	Replace the bulb – Do not touch bulb with fingers

Problem	Cause	Remedy
Uneven illumination	Field of view partially blocked	Rotate the nose-piece until it clicks into position
	Iris diaphragm is almost closed or condenser is not aligned	Recalibrate microscope
	Dirty lenses	Gently wipe the lenses with lens paper/soft cloth. If the trouble persists clean with lens paper soaked in the recommended lens cleaning fluid (see page 38)
	Heavy fungal growth on lenses	Clean the lens using lens cleaning fluid as recommended by the manufacturer

Problem	Cause	Remedy
Excessive image contrast	Iris diaphragm is almost closed	Open diaphragm

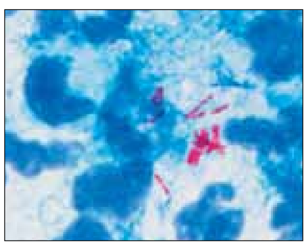
Problem	Cause	Remedy
Unclear image with glare	Iris diaphragm too far open	Close the iris diaphragm to make the opening smaller

Problem	Cause	Remedy
Specimen focused at 10x but not at higher magnification	Slide upside down	Turn it over

Problem	Cause	Remedy
Specimen goes out of focus more than usual at high magnification	Slide is not flat on the stage	Clean the stage and underside of slide

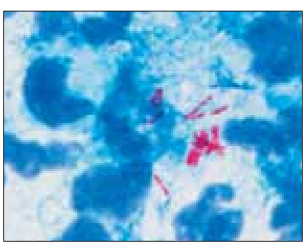
Problem	Cause	Remedy
Mechanical stage cannot be raised	Lock set too low	Adjust to proper height and lock

Problem	Cause	Remedy
Mechanical stage is not moving, too stiff or does not stay up	Poor tension adjustment on the mechanical stage	Adjust tension with tension adjustment knob (if present)
	Solidified lubricants	Microscope requires service



Problem	Cause	Remedy
Oil immersion objective does not give a clear image	Insufficient oil on slide	Add immersion oil
	Light source or condenser collector lens dirty	Clean using lens paper and cleaning fluid
	Poor quality immersion oil (low refractive index)	Use quality immersion oil (see page 37)
	Surface of the lens is dirty	Clean lens with tissue paper
		If oil/fungus inside the objective, replace lens
	Water on slide	Air dry slides
	Bubbles in immersion oil	Remove oil from slide and carefully reapply oil
	Oil inside lens	Clean or replace lens

Good



Problem	Cause	Remedy
Dust/dirt visible in the field of view	Dust on the collector lens of the light source	Clean all surfaces
	Dust on the top-most lens of the condenser	Clean all condenser surfaces
	Dust on the eye-piece	Clean all surfaces

Problem	Cause	Remedy
Cracked objective lens	Lens has been dropped	Replace lens
	Lens forced into slide or stage	Replace lens

Problem	Cause	Remedy
Regular or semi regular crescent shapes that maybe confused for AFBs	The glass slide is scratched	Learn to recognise glass artefacts

Problem	Cause	Remedy
Headaches/incomplete binocular vision	Improper adjustment of interpupillary distance	Adjust the interpupillary distance
	Dioptre adjustment was not done	Adjust dioptre settings
	Eye-pieces are not matched	Use matched eye-pieces

Problem	Cause	Remedy
Fuse blows frequently	Fuse incorrectly rated	Replace with correctly rated fuse
	Unstable line voltage	Use voltage protection device

Method B | Fluorescence Microscopy

Method B

Fluorescence Microscopy

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– Consequences	
– Prevention	
False-positives	
– Consequences	
– Prevention	
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– Staining	66

In 2011, WHO released a new policy on Light Emitting Diode (LED) based Fluorescent Microscopy (FM) for diagnosing TB. FM is equally accurate, at least 10% more sensitive and has qualitative, operational, cost and workload advantages for all laboratories performing sputum smear microscopy.
WHO recommended a phased approach to change from brightfield microscopy to LED-based FM across the microscopy network.

LED FM offers considerable advantages over conventional FM, which requires a darkened room to read smears. Conventional FM relies on expensive mercury vapour lamps that have a limited life span, generate large amounts of heat, and are a safety hazard if broken.

For a laboratory with a high workload, bulk staining is an acceptable option and protocols are described on page 56-57.

Reporting

Due to an historical inaccuracy, the FM reporting scale for positive smears has been revised because the actual field observed is larger than previously calculated.

Low scanty positives, 1-4 AFB in one length at 200x magnification, or 1-2 in one length at 400x magnification should be confirmed by:

- viewing additional fields
- having another technician check the AFB morphology or
- collecting another sputum sample

Confirmation of FM low-positive smears by re-staining with ZN should not be done.

Quality control

AFB in FM-stained smears fade rapidly; for FM re-stain all smears.
Auramine reagent must be prepared as 10X concentrated stock that keeps well for 12 months. Diluted staining solution may deteriorate within a few months, and should be prepared monthly from stock.

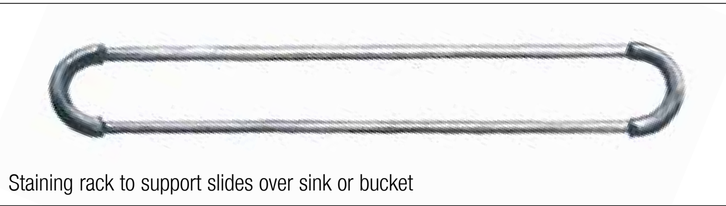
Introducing LED FM methods

The switch to LED FM should be carefully phased in at country level, with LED technology that meets WHO specifications. Countries using LED microscopy should retrain laboratory staff with strong emphasis on practical training of longer duration. EQA should be introduced for individual laboratories; technique validated for the network as a whole, and the effect on TB case detection rates and treatment outcomes monitored.

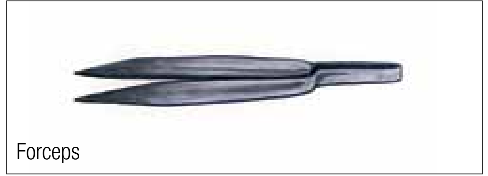
Staining solutions can deteriorate quickly – the solution becomes lighter

Staining What you need

To stain smears using the Auramine method you will need:



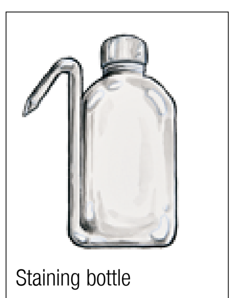
Staining rack to support slides over sink or bucket



Forceps

Water

Slide rack for drying stained slides



Staining bottle

Timer

Stain

0.1% auramine

Decolouriser

0.5% acid-alcohol

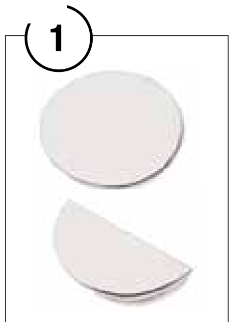
Counterstain

0.3% methylene blue or 0.5% potassium permanganate

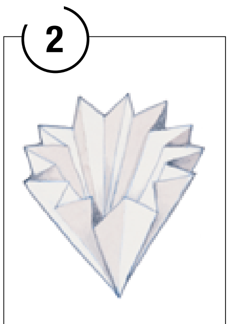
You will require 1 – 2 volumes of decolouriser for each volume of stain

How to fold a filter

1



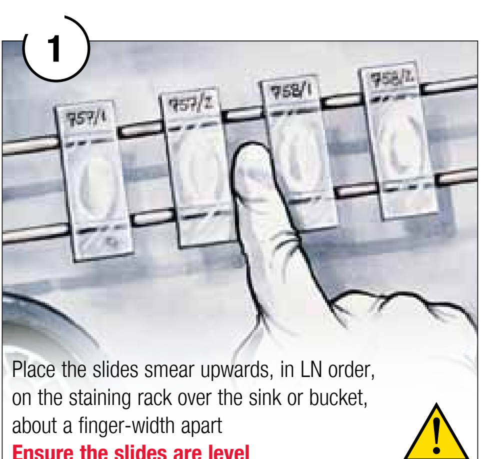
2



3

Staining Auramine method

Filter during use



1

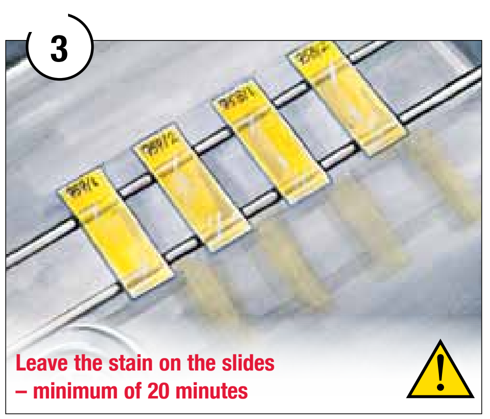
Place the slides smear upwards, in LN order, on the staining rack over the sink or bucket, about a finger-width apart

Ensure the slides are level

2

Cover the slides completely with filtered auramine

Do not heat



3

Leave the stain on the slides – minimum of 20 minutes

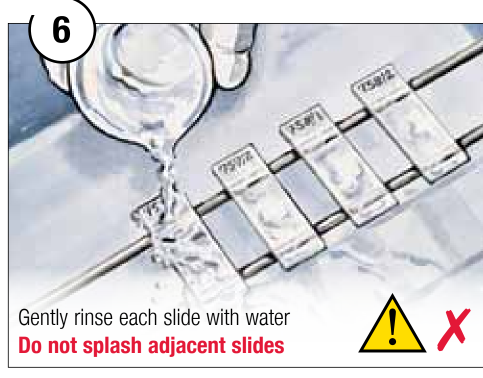
4

- Gently rinse each slide with water
- Tilt each slide to drain off excess water

Do not splash adjacent slides

5

Add acid-alcohol to each slide and leave on for 1-2 minutes

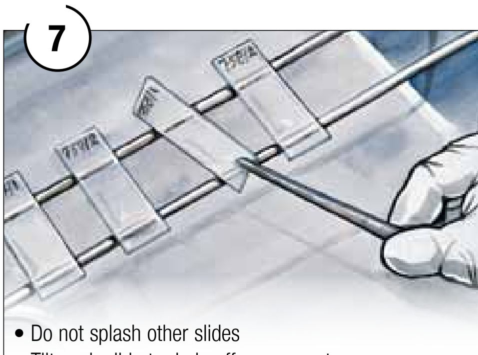


6

Gently rinse each slide with water

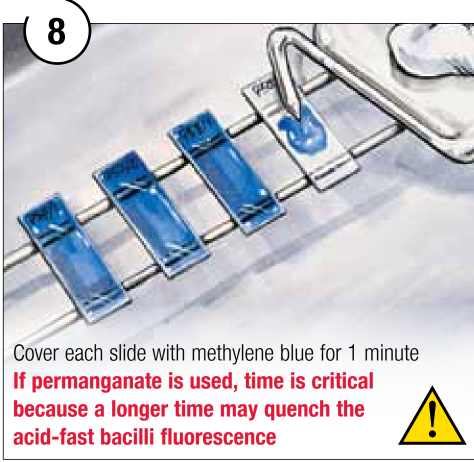
Do not splash adjacent slides

7



- Do not splash other slides
- Tilt each slide to drain off excess water

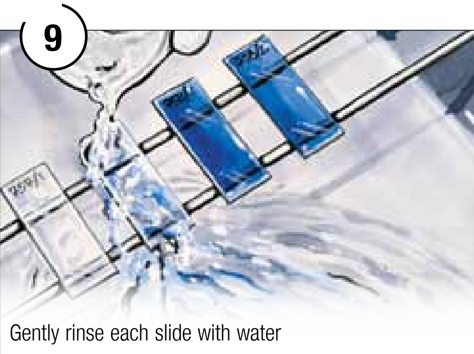
8



Cover each slide with methylene blue for 1 minute

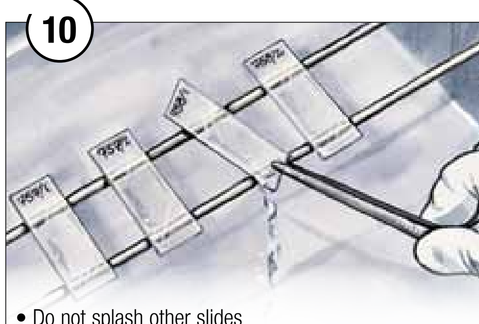
If permanganate is used, time is critical because a longer time may quench the acid-fast bacilli fluorescence

9



Gently rinse each slide with water

10



- Do not splash other slides
- Tilt each slide to drain off excess water

11

- Air dry away from direct sunlight
- Do not dry slides using blotting paper

12

Do not examine slides until they have dried

A correctly stained smear

Staining Auramine bulk method

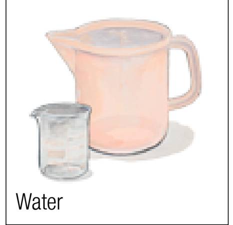
Bulk staining

Consider this method when workload exceeds 10 smears per day.

What you need

Timer

Slide basket

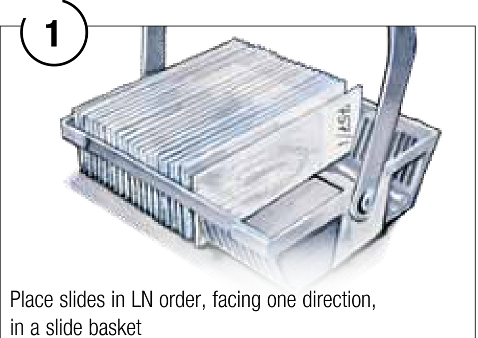


Water

Four ~600 ml glass containers able to hold the slide basket

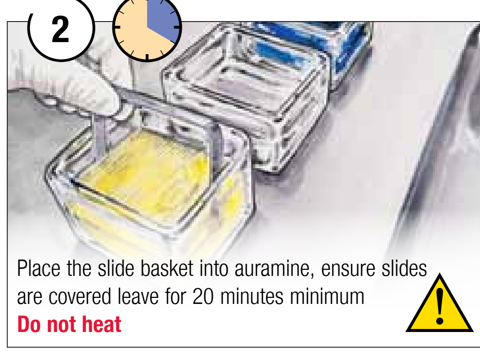
- Stain 0.1% auramine
- Decolouriser 0.5% acid-alcohol
- Counterstain 0.3% methylene blue

1



Place slides in LN order, facing one direction, in a slide basket

2



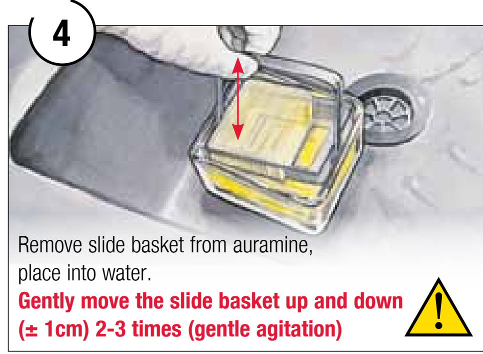
Place the slide basket into auramine, ensure slides are covered leave for 20 minutes minimum

Do not heat

3

Fill the wash container with water

4



Remove slide basket from auramine, place into water.

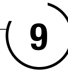
Gently move the slide basket up and down (± 1cm) 2-3 times (gentle agitation)

5

Remove from water place into decolouriser for 2 minutes – gentle agitation

7

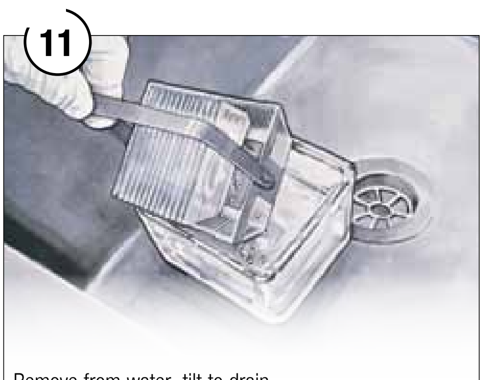
Remove the slide basket from the decolourising solution and place into the water; gentle agitation



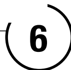
9

Discard the water from the container: refill and discard twice

11



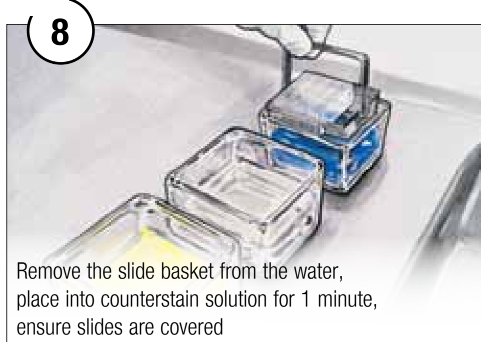
Remove from water, tilt to drain



6

Rinse the wash container. Discard and refill twice

8

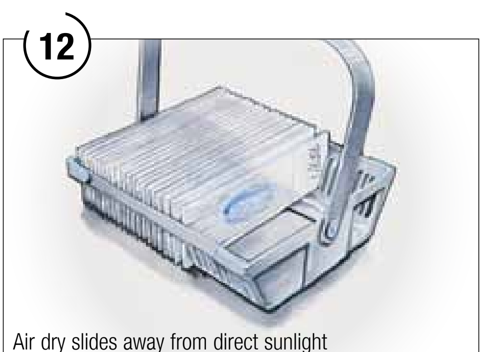


Remove the slide basket from the water, place into counterstain solution for 1 minute, ensure slides are covered

10

Remove the slide basket from the counterstain solution and place into the water; gentle agitation

12



Air dry slides away from direct sunlight

A correctly stained smear

Examination Reading smears

Keep stained smears in the dark using a slide box or folder as fluorescence quickly fades when exposed to light

Read the smears on the same day they were stained.

AFB are stained bright yellow against a dark background, but with some filter systems they will appear green.

Use the 20X objective to scan the smear and the 40X objective for confirming suspicious objects.

Smears must be examined in a consistent way to ensure a representative area of the smear is reported. At least one length of the smear must be examined before reporting a negative result.

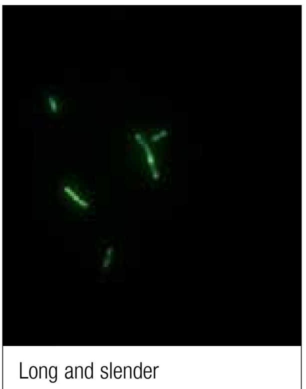
When the smear has been read, store the slides immediately in LN order in a closed box, as they will be needed for EQA.

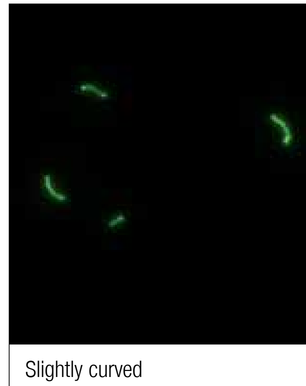
Do not write the result on the slide.

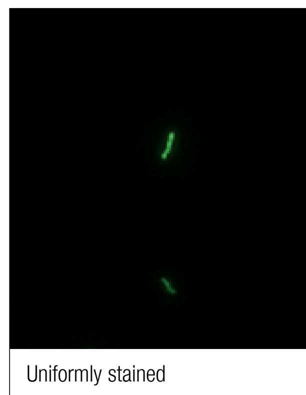
Do not restain scanty smear positives with ZN

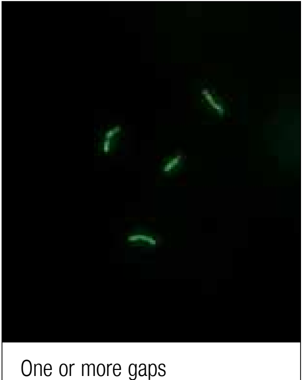
The typical appearance of AFB is a long, slender, slightly curved rod, but variable in shape and staining intensity

They may be uniformly stained or may contain one or more gaps, or have a granular appearance. AFB occur singly, in small groups containing a few bacilli, or more rarely, as large clumps.


Long and slender


Slightly curved

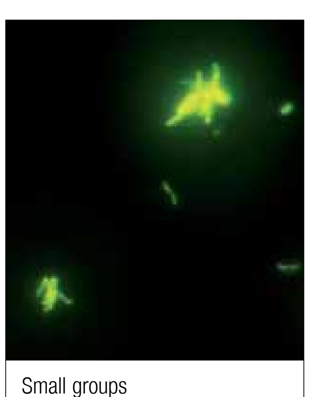

Uniformly stained

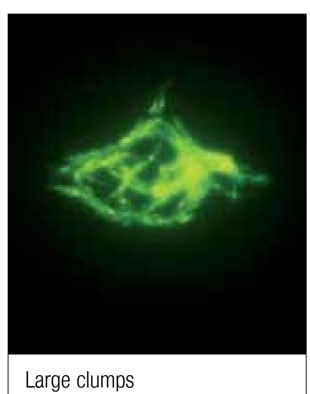

One or more gaps

Granular

Examination Appearance of AFB

Single AFB


Small groups


Large clumps

Stained smears may contain fluorescing artefacts which do not have a typical bacillary shape, and sometimes also a different colour.

Non-fluorescing yellow or green coloured bacillary shapes should not be accepted as AFB

The number of AFB indicates how infectious the patient is. It is important to record exactly what you see.

What you see (200x)	What you see (400x)	What to report
No AFB in one length	No AFB in one length	No AFB observed
1-4 AFB in one length	1-2 AFB in one length	Confirmation required*
5-49 AFB in one length	3-24 AFB in one length	Scanty
3-24 AFB in one field	1-6 AFB in one field	1+
25-250 AFB in one field	7-60 AFB in one field	2+
>250 AFB in one field	>60 AFB in one field	3+

** Confirmation required by another technician or prepare another smear, stain and read*

1 757/1

2 Read the smear

Laboratory Register

TB Laboratory Register for smear and Xpert MTB/RIF

757/1

4 Transfer the result to the Laboratory Register

757/1

Laboratory Request Form

3 Record results

757/1

Date/Sign

5 → 6 Return to doctor or clinic

1. Use the LN to find the correct patient Request Form
2. Read the smear
3. Immediately record the result on the Request Form
4. Transfer the result to the Laboratory Register
   use red pen for positive results
5. Date and sign the Laboratory Request Form
6. Return the completed Laboratory Request Form to the Doctor or Clinic

Do not give results to the patients as lost reports may delay treatment
Do not write the results on the slide as they are needed for EQA checking

Summary

False-negative means reported negative but truly smear-positive

Be accurate and consistent in all your work, lives depend on you

False-negatives – consequences

- Patients with TB may not be treated resulting in on-going disease, disease transmission, or death

Prevention

- Label sputum containers, slides and laboratory forms accurately
- The specimen must contain sputum not saliva
- Select purulent material to make the smear
- Smear preparation – centred, not too thick or too small
- Use auramine solution as fresh as possible; do not prepare large quantities
- Stain with auramine – minimum 20 minutes
- Decolourise for 1-2 minutes only
- Counterstain – maximum 1 minute
- Read smears as soon as possible and keep them protected from light
- Keep the microscope well maintained and the lenses clean
- Perform QC – use positive controls every day to check staining procedure and microscope function
- Check the slide LN matches the Laboratory Request Form before recording the result

False-positive means reported positive but truly smear-negative

Don't rush – examine at least one length of a smear before recording a negative result

False-positives – consequences

- Patients are treated or retreated unnecessarily
- Medications will be wasted

Prevention

- Ensure laboratory technicians can reliably recognise acid-fast bacilli
- Label sputum containers, slides and laboratory forms accurately
- Always use new unscratched slides
- Use bamboo/wooden sticks once only
- Filter auramine staining solution during use
- Do not allow auramine to dry on the smear
- Decolourise adequately
- Keep the microscope well maintained, the lenses clean, store appropriately
- Perform QC – use positive controls every day
- Check the slide LN matches the Laboratory Request Form before recording the result

Stain

Auramine is a potential cancer causing agent – always wear gloves and clean any spills immediately

Phenol crystals and vapour are corrosive, toxic, and may cause burns; avoid contact with skin and mucous membranes, prepare in a well ventilated area

0.1% Auramine		**Grade**
Auramine	10.0g	Certified
Ethanol (denatured) or methanol	1000ml	Technical
Phenol crystals*	30g* *use colourless not tinted crystals*	Analytical
Distilled water	900ml	

Preparation
To ensure solutions are fresh, laboratories examining low numbers of smears should prepare smaller volumes.

Solution A

1. Add 1000ml of ethanol (or methanol) to a one-litre glass flask

2. Add 10.0g of auramine powder, mix until dissolved completely

Do not use heat since this can inactivate the auramine

3. Label **"1.0% auramine in alcohol", date and initial**
4. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Solution B

1. Dissolve 30g of phenol crystals in 900ml distilled water, mix
2. Label the bottle **"3% phenolic solution for auramine", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

Preparation of 0.1% auramine solution

1. Add 50ml of solution A (1% auramine in alcohol) to a 500ml dark glass bottle
2. Add 450ml of solution B (phenolic solution for auramine) and mix
3. Label the bottle **"0.1% auramine", date and initial**
4. Store in a cupboard at room temperature (expiry 2 months)

Filter auramine solution when applying to smears or filling bulk staining containers.

Wash your hands after preparing reagents

Perform a Quality Control check and record results in the QA log book.

Correctly prepared auramine is a rich golden colour – discard if pale

Auramine reagent preparation

Decolouriser

Always add the acid to ethanol. Solutions will generate heat

0.5% acid-alcohol		Grade
Fuming hydrochloric acid	5ml	Technical
Ethanol (denatured) or methanol	1000ml	Technical

Preparation

1. Carefully add the hydrochloric acid to the alcohol
2. Label the bottle **"0.5% acid alcohol", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
4. Perform QC and record in the QA log book

1% HCl in 10% alcohol in water		Grade
Fuming hydrochloric acid	10ml	Technical
Ethanol (denatured) or methanol	100ml	Technical
Distilled water	890ml	

Preparation

1. Carefully add the alcohol (or methanol) to the distilled water
2. Carefully add the hydrochloric acid to the 10% alcohol (or methanol) in water
3. Label the bottle **"1% HCl in 10% alcohol in water"**, date and initial
4. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
5. Perform QC and record in the QA log book

Counterstain

Two counterstains are described; 0.3% methylene blue (preferred) is a true counterstain, whilst 0.5% potassium permanganate acts as a quenching agent.

The choice of counterstaining solution depends on the microscope system used: permanganate produces a very dark background in some systems, making it hard to keep focus. If this occurs, then 0.3% methylene blue is a better choice counterstain, although there is slightly less contrast.

0.3% methylene blue		Grade
Methylene blue	3.0g	Analytical
Distilled water	1000ml	

Preparation

1. Add the methylene blue to the distilled water
2. Label the bottle **"0.3% methylene blue", date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
4. Perform QC and record in the QA log book

Potassium permanganate is a powerful oxidising agent and may cause burns

0.5% potassium permanganate		**Grade**
Potassium permanganate	5.0g	Technical
Distilled water	1000ml	

Preparation

1. Add the potassium permanganate to the distilled water
2. Label the bottle **“0.5% potassium permanganate”**, **date and initial**
3. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)
4. Perform QC and record in the QA log book

The solution should be bright purple; if it is brick-red in colour it is oxidised discard it – rinse the bottle before refilling

Wash your hands after preparing reagents

Trouble-shooting Staining

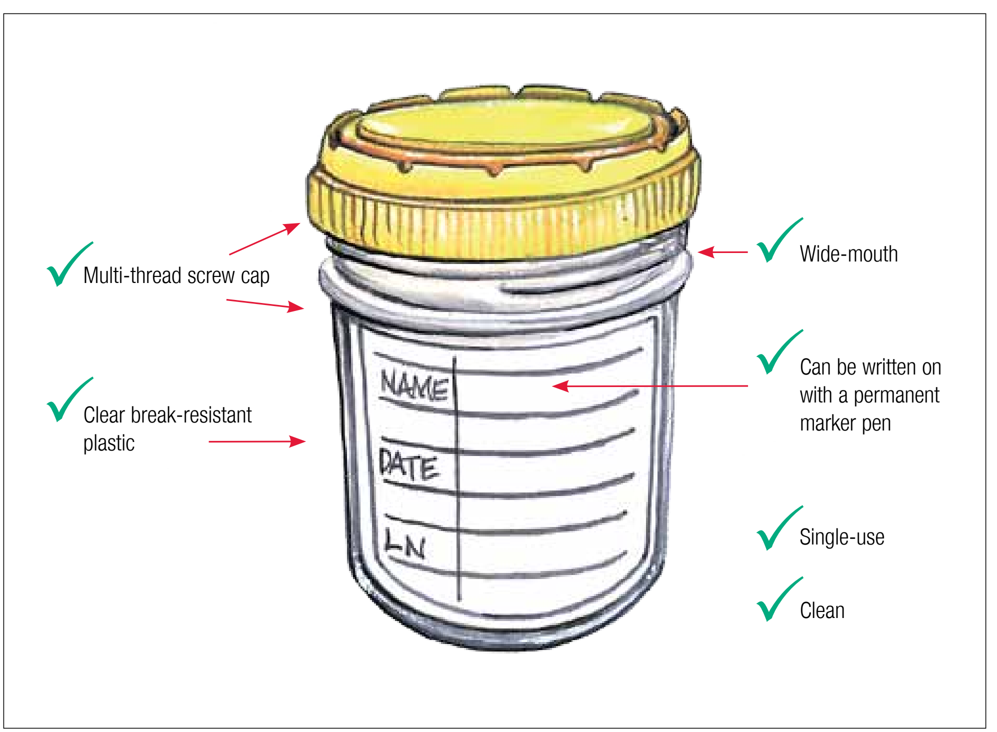
Problem	Cause	Remedy
Too much fluorescence	Insufficient decolourisation	Check decolourisation time
	Counterstain too weak or no alcohol	Prepare new reagent
	Auramine has dried on the smear	Check smears are level over sink Add sufficient stain
	Auramine not filtered	Filter auramine at time of use
	Smear too thick	Prepare new smear
	Do not heat during staining	

Problem	Cause	Remedy
Pale acid-fast bacilli	Auramine has expired or stored in direct sunlight	Replace reagent Store bottle in the dark
	Auramine <0.1%	Recheck stain preparation and QC results
	Staining time <20 minutes	Stain for at least 20 minutes
	Smear overheated during fixation step	Pass smear through flame 3 times, 1-2 seconds each time
	Overdecolourised	Do not exceed the maximum time (1-2 minutes only)
	Stained smears exposed to daylight	Keep slides in the dark using slide box or similar Read smears as soon as possible
	Smear too thick	Prepare new smear

Problem	Cause	Remedy
Background too dark	Counterstained too long (or) Decolourised too long	Do not exceed 1 minute Do not exceed 2 minutes
	Inadequate washing step after counterstaining	Extend washing step Ensure washing step is complete
	Counterstain concentration too strong	Recheck stain preparation and QC results
	Smear too thick	Prepare new smear

For microscope problems refer to manufacturer's instructions

Ideal specimen container



Laboratory Request Form

This example shows the type of information required on a Specimen Request Form.

Request for examination of biological specimen for TB

Treatment Unit: ___________________________________ Date of request:__________________

Patient name: ____

Age (years): ______ Date of Birth: ___________________ Sex: ☐ Male ☐ Female

Patient address:____

____ Telephone: ______________________

Reason for examination:

☐ Diagnosis. If diagnosis, presumptive RR-TB/MDR-TB?: ☐ Yes ☐ No

OR ☐ Follow-up. If follow-up, month of treatment: ______

HIV infection?: ☐ Yes ☐ No ☐ Unknown

Previously treated for TB?: ☐ Yes ☐ No ☐ Unknown

Specimen type: ☐ Sputum ☐ Other (specify):___________________________

Test(s) requested: ☐ Microscopy ☐ Xpert MTB/RIF ☐ Culture ☐ Drug susceptibility ☐ Line Probe Assay

Name and signature of requestor: ____

____

Microscopy results (*to be completed in the laboratory)*

Date sample collected *(filled by requestor)*	Specimen type	Laboratory serial number(s)	Visual appearance (blood-stained, mucopurulent or saliva)	Result (check one)				
				Negative *(0 AFB / 100HPF)*	**1-9 / 100HPF** *(scanty; report number of AFB)*	**+** *(10-99 AFB / 100HPF)*	**++** *(1-10 AFB / HPF)*	**+++** *(>10 AFB / HPF)*

Examined by (Name and signature): ____

Date of result: ___________________________________

Laboratory Register

This example shows the type of information required on a Laboratory Register.

TB Laboratory Register for smear and Xpert MTB/RIF

Lab. serial No.	Date specimen received[1]	Patient name	Sex M/F	Age	Patient address	Treatment unit	BMU and TB Register No.	HIV infection (Y/ N/ Unk) [2]	Patient previously treated for TB[3]	Examination type (*Tick one option)*		Examination results				Remarks[7]
				Date of birth						Diag-nosis	Follow-up	Xpert[5]	Smear microscopy[6]			
													1	2	3	
											Month[4]	Date	Date	Date	Date	

1 For diagnostic testing employing serial sputa or other specimens this is the date of receipt of the first set of specimens
2 **Y**=Yes; **N**=No; **Unk** = unknown
3 **Y** = previously treated; **N** = not previously treated, **Unk** = unknown
4 Patient on TB treatment; indicate months of treatment at which follow-up examination is performed

5 Xpert MTB/RIF test result reported as follows :
T = MTB detected, rif resistance not detected;
RR = MTB detected, rif resistance detected;
TI = MTB detected, rif resistance indeterminate
N = MTB not detected;
I = Invalid / no result / error

6 Smear results reported as follows:
0=No AFB;
Scanty (and report number of AFB) = 1-9 AFB per 100HPF;
+ = 10-99 AFB per 100 HPF;
++ = 1-10 AFB per HPF;
+++ = > 10 AFB per HPF

7 If Xpert MTB/RIF indeterminate result, indicate error code or 'invalid'

The Laboratory Numbering system

The LN begins at number "1" at the start of each year.
It increases by one with each patient, until the end of the year.

Do not return to LN 1 at the end of each day, week, or month

Master List

Include a completed Master List whenever you send specimens to the smear microscopy laboratory.

Master List		
Laboratory name:		
Address:		
Patient name	**Specimen 1**	**Specimen 2**

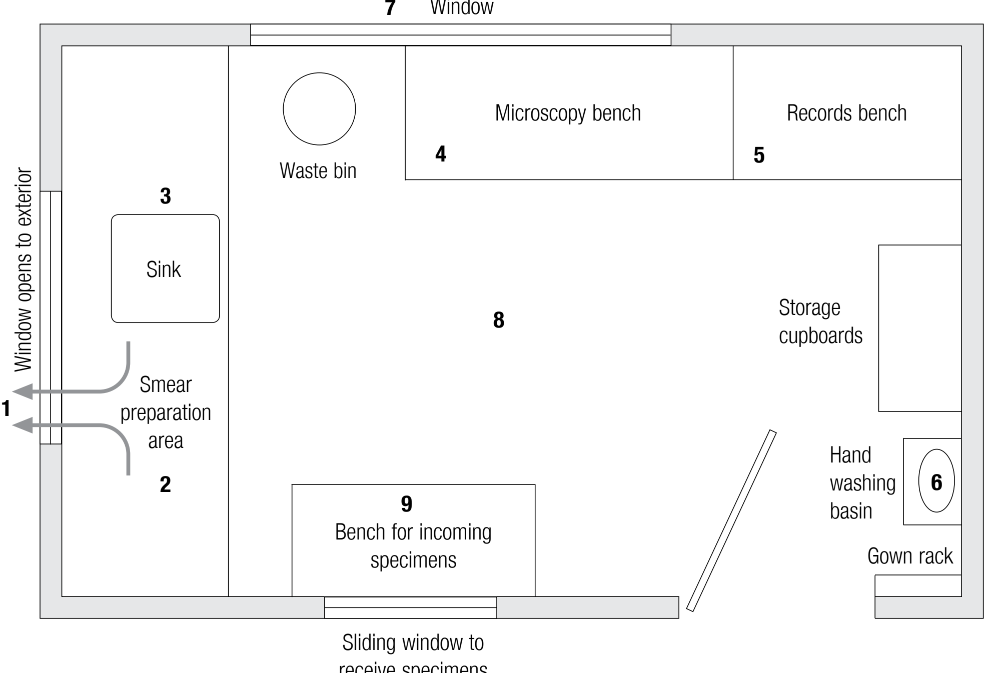
Packed by	
Name	*Signature*
Dispatched Date / /	*Time : AM/PM*

Abbreviation	Name in full
AFB	Acid-Fast Bacilli
BSC	Biological Safety Cabinet
CDC	Centre for Disease Control and Prevention
CF	Carbol Fuchsin
EQA	External Quality Assessment
FM	Fluorescence Microscopy
GLI	Global Laboratory Initiative
JATA	Japan Anti-Tuberculosis Association
KNCV	KNCV Tuberculosis Foundation
KPIs	Key Performance Indicators
LED	Light Emitting Diode
LN	Laboratory Number
NTP	National Tuberculosis Programme
PPE	Personal Protective Equipment
QA	Quality Assurance
QC	Quality Control
RI	Refractive Index
TB	Tuberculosis
The Union	International Union Against Tuberculosis and Lung Disease
v/v	Volume for volume
VWS	Ventilated Work Station
WHO	World Health Organization
ZN	Ziehl-Neelsen

Laboratory design

The basic requirements for a sputum microscopy laboratory include:

1. Good ventilation
   Directional ventilation provides healthy air for breathing. Air that may be contaminated by laboratory processes should flow away from staff and out of the laboratory.
2. A strong table/bench to prepare smears
3. A sink or plastic basin to stain smears
4. A table/bench to examine smears
5. A table/bench for paperwork
6. Basin for hand washing
7. Good lighting
8. Non-slip flooring
9. An area for receiving specimens



Biological Safety Cabinets

A biological safety cabinet (BSC) is not required for sputum smear microscopy.

- Only laboratories performing culture and drug susceptibility testing need a functioning BSC
- Never use a clean air cabinet, it can blow TB organisms into the laboratory

Contamination and infection control

Assume all samples are potentially infectious

Aerosols

Good work practice minimises aerosol formation and contamination of work surfaces and equipment. (See page 8 Personal Safety).

Disinfection and Spills

Disinfection

Disinfectants recommended for use in TB laboratories contain phenols, chlorine or alcohol.

Disinfection methods	Surfaces	Spills	Prepare
Phenol 5%	Yes	Yes	Every 2 days
Alcohol 70% v/v	Yes	No	Weekly
Hypochlorite 0.5%	No	Yes	Every 2 days

If your skin is contaminated with phenol, bleach or alcohol, wash thoroughly with soap and water

Phenol

- Toxic if swallowed
- Phenol is highly irritating to the skin, eyes and mucous membranes (e.g. lungs)
- Due to its toxicity and smell synthetic phenol derivatives are generally used in place of phenol

Chlorine

- Bleach is highly alkaline and will corrode metal
- Sodium hypochlorite solutions (domestic bleach) contain 35-150 g/l available chlorine – store in a well ventilated dark area
- Dilute in water to obtain a final concentration of 0.5%

Alcohol

- Volatile and flammable
- Keep away from open flames
- Store in proper containers to avoid evaporation
- Label bottles clearly – do not autoclave

Spills

Treat all spills as potentially infectious

1. Put on a laboratory coat and gloves
2. Place paper towel or cloth over the spill area and liberally apply disinfectant solution
3. Leave covered – minimum 15 minutes
4. Clean up the contaminated material and put into the waste container
5. Clean with a final wash using 70% v/v alcohol
6. Wash your hands after the clean up is complete

Waste management

Treat all laboratory waste as infectious

Laboratory staff are responsible for waste management and ensuring that anyone who must handle waste, including cleaners, drivers etc. is properly trained.

Where available autoclave laboratory waste before disposal.

Place potentially infectious waste into bins that have a disposable plastic lining with disinfectant added. When moving waste within or outside the laboratory, put it into a larger leak-proof plastic bag, tied at the top.

Laboratory staff are responsible for ensuring safe movement of laboratory waste.

When moving waste outside of the laboratory, the waste should be sealed in a container with a lockable lid.

Waste disposal

Locate the burning drum away from people in an open area as the fumes are toxic

1

Tighten caps, add specimen containers and contents of the discard bucket to the waste bucket

2

Burn bucket contents weekly

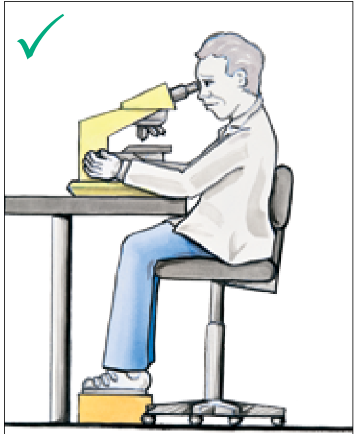
3

When cool, bury burning drum contents at least 1.5 metres deep

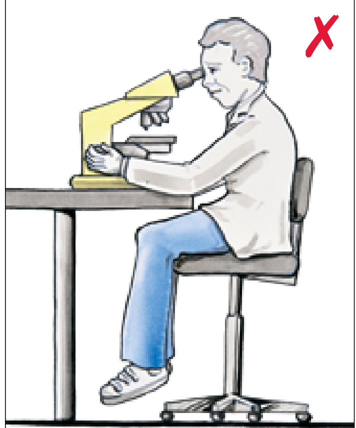
1.5m

Ergonomics

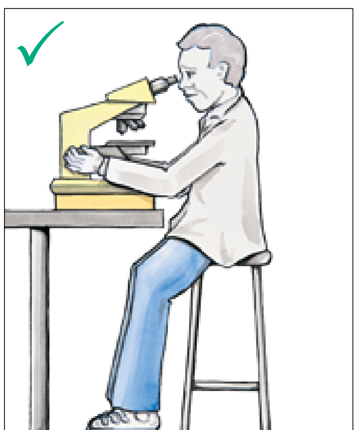
Good ergonomics reduces fatique and injury



Good posture – supporting your feet straightens your back



Poor posture – feet unsupported



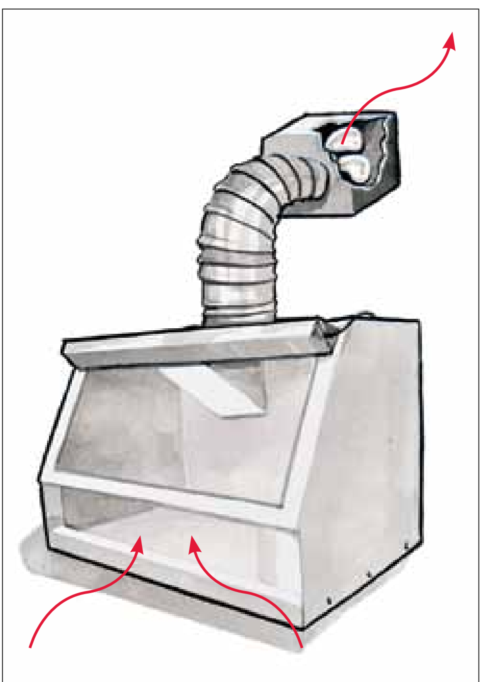
Good posture – raise the microscope to help straighten your back and keep your feet flat on the floor

Poor posture – seat too high or bench too low – feet not flat

Ventilated workstation

A ventilated workstation (VWS) is a partially enclosed workspace. Air is drawn inward, away from the technician and exhausted outside the laboratory, VWS are inexpensive to build and require little maintenance. VWS do not replace careful attention to risk minimising laboratory methods.

For more information on VWS see *Ventilated Workstation Manual for AFB Smear Microscopy* (see page 83).



Accurate laboratory results rely on internal monitoring (Quality Control and Key Performance Indicators) and EQA.

Why do Quality Control?

The purpose of Quality Control (QC) is to ensure that staining solutions work well and that they are not contaminated with AFB. Good quality solutions and staining technique make reading and reporting easier and more reliable. Accurate record keeping of preparation and testing provides confidence in your results.

Technicians preparing new staining solutions are responsible for QC before the solutions are used.

Technicians performing AFB-staining are responsible for regular QC using positive control smears.

Technicians who prepare control smears are responsible for their QC.

Preparing unstained control smears

Positive control smears

Ideal positive control smears are easy to count low-positives in the 1+ range.

1. Confirm a 1+ result for the selected specimen on 2 or 3 stained smears:
   - After liquefaction (standing overnight) and
   - Mixing – with sputum pot closed
2. Make at least 50 even equally sized smears from this confirmed 1+ sample, and air dry
3. Heat fix
4. On each slide write the positive control batch number and serial number within the batch
5. Check the number of AFB:
   - Randomly select six smears from this batch
   - Stain and carefully count the AFB
6. Start a separate page in your logbook for quality control of staining solutions
7. Record the batch number and results for each of the six smears then calculate the average number of AFB per smear length or per field
8. Store smears in a closed slide box labelled "*Positive control smears*"

Negative control smears

Make negative control smears from egg white diluted 5% in distilled water.

1. To assist focusing mix with a little sputum or saliva (containing cells)
2. After staining check a few smears to make sure there is no contamination with AFB
3. Make at least 50 even equally sized smears from this sample, and air dry
4. Heat fix
5. On each slide write the negative control batch number and serial number within the batch
6. Store smears in a closed slide box labelled "*Negative control smears*"

Testing solutions

To test the performance of a *freshly prepared solution* stain and examine:

- Two positive control smears *stained once* and
- Two negative control smears *stained three times*

The other solutions required can also be those already in routine use.

1. Stain negative smears *three times* to check for environmental mycobacteria
   - Only repeated staining makes these contaminants visible
2. Examine control smears carefully for:
   - Number and intensity of AFB colour
   - Complete de-colourisation of background
   - Absence of crystals and primary stain coloured artefacts
3. Compare your count with the number of AFB expected for the batch of positive control smears
   - There should be no negative or very low counts
   - AFB should show strong, solid colour
4. Accept the batch if it passes on all these points

Unsatisfactory results

1. Check the preparation technique, the quantities and reagents used:
   - If results are uncertain, stain a few more control smears making sure your technique is correct
2. Accept the batch if results are good
3. If results are again unsatisfactory:
   - Discard the bad batch of staining solution
   - Record the reason for rejection
   - Prepare fresh solution and perform QC

Keep accurate QC records in the logbook for all solutions prepared. Good records serve as an important reference to defend against possible complaints.

Monitoring

Key Performance Indicators (KPIs) are useful for internal and external evaluation of AFB-microscopy quality. They should be calculated monthly or quarterly from the Laboratory Register counts, and the results recorded in a chart.

Each laboratory is responsible for calculating its KPIs.

Monitoring trends within the laboratory should alert staff to identify a shift from normal patterns. Values that are too high or low may indicate a problem, however the acceptable range depends on the setting.

The TB Programme should collect individual laboratory KPIs and compare them across the laboratory network. This allows each laboratory to compare their performance with similar laboratories in the same area.

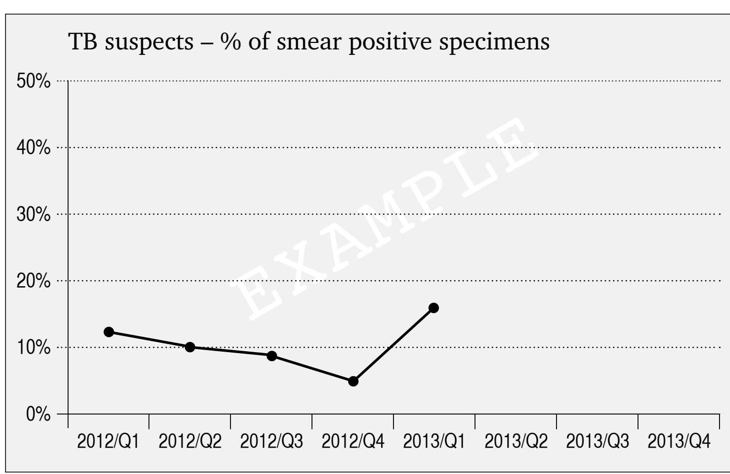
Reporting the data

	Negative	Scanty	1+	2+	3+	Total positive or scanty	Total smears
Suspect smears	a	b	c	d	e	f=(b+c+d+e)	g=(a+f)
Follow-up smears	h	i	j	k	l	m=(i+j+k+l)	n=(h+m)

Calculations

Workload	g+n
% positive suspect smears	f/g
% positive follow-up smears	m/n
% low positive suspect smears	(b+c)/f

Plot KPI's monthly or quarterly to obtain a trend line.
Plotting may not be effective if denominators (totals) are very small.



Target values

Laboratories should aim for:

- TB suspects – about 10% positives
- Follow-ups – about 5-10% positives
- Low positive suspect smears – about 30-50% of all positive suspect smears

EQA

EQA of AFB-microscopy commonly includes rechecking a randomly selected subset of routine smears by an external agency. For EQA to be effective technicians should keep all smears until the subset of smears has been selected and removed for rechecking.

The EQA process

When preparing slides for examination:

- Label all slides clearly with the LN and sample number
- Let oil soak into absorbent paper overnight after reading
- Store in numerical sequence leaving a space for the smear of the second sample
- Never write results on the slide

After the subset of routine smears has been selected for EQA and removed for rechecking, the remaining slides can be discarded.

Reuse the slide racks to start a new collection of routine slides. Store slides in numerical order leaving a space for the smear of the second sample.

Leave a space for second sample

Angra P, Becx-Bleumink M, Gilpin C, Joloba M, Jost K, Kam KM, Kim SJ, Lumb R, Mitarai S, Ramsay A, Ridderhof J, Rieder HL, Selvakumar N, van Beers S, van Cleeff M, Van Deun A, Vincent V. Ziehl-Neelsen staining: strong red on weak blue, or weak red under strong blue? Int J Tuberc Lung Dis 11: 1160-1161, 2007

Aziz MA, Ba F, Becx-Bleumink M, Bretzel G, Humes R, Iademarco MF, Kim SJ, Lamothe F, Paramasivan CN, Ridderhof J, Sloutsky A, Van Deun A, Shah KV, Weyer K. External quality assessment for AFB smear microscopy, Washington, DC:Association of Public Health Laboratories, 2002

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International Union Against Tuberculosis and Lung Disease. Technical guide: Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries. 2000

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Van Deun A, Hossain MA, Gumusboga M, Rieder HL. Ziehl-Neelsen staining: theory and practice. Int J Tuberc Lung Dis 12:108-110, 2008

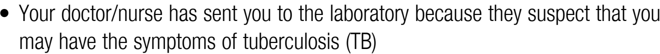
World Health Organization. Laboratory services in tuberculosis control. Microscopy. Part II 1998. WHO/TB/98.258 http://www.who.int/entity/tb/publications/who_tb_98_258/en/index.html

World Health Organization. Policy statement: Same-day diagnosis of tuberculosis by microscopy. 2011. WHO/HTM/TB/2011.7 http://whqlibdoc.who.int/publications/2011/9789241501606_eng.pdf

World Health Organization. Policy statement: Fluorescent light-emitting diode (LED) microscopy for diagnosis of tuberculosis. 2011. WHO/HTM/TB/2011.8 http://whqlibdoc.who.int/publications/2011/9789241501613_eng.pdf

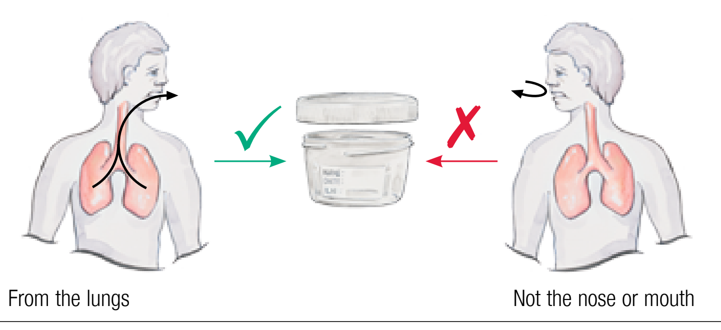
World Health Organization. Global tuberculosis report 2012. WHO/HTM/TB/2012.6 http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf

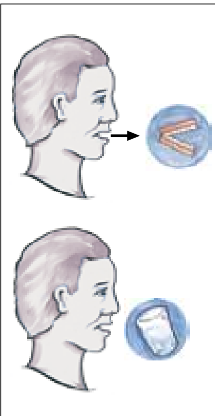
World Health Organization. Tuberculosis laboratory biosafety manual. 2012 WHO/HTM/TB/2012.11 http://apps.who.int/iris/bitstream/10665/77949/1/9789241504638_eng.pdf



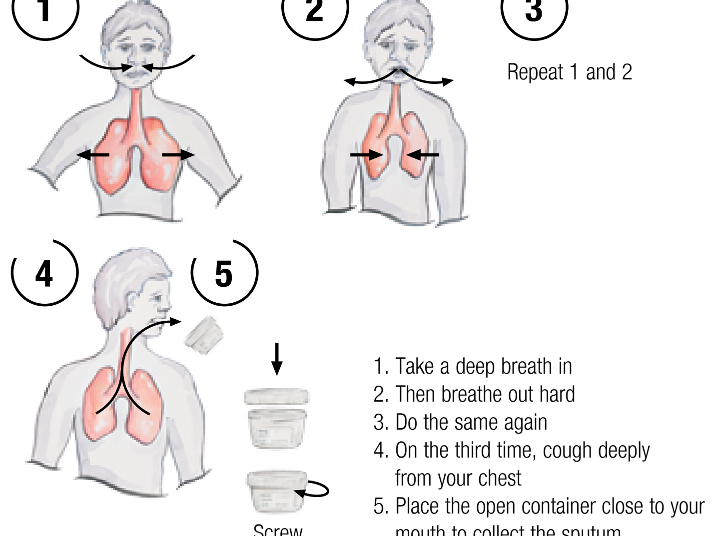
- Your doctor/nurse has sent you to the laboratory because they suspect that you may have the symptoms of tuberculosis (TB)
- To diagnose TB two sputum specimens are needed and they will be collected:
  1. At first presentation
  2. Next morning before breakfast
- Collect specimens in the open air
- Good quality specimens from the lungs are required **not** saliva or nasal secretions
- Rinse your mouth out with bottled water if you have recently eaten, or if you have dentures (remove them first)

Please cover your mouth when coughing!





If dentures are present, remove them and rinse mouth with bottled water.



1. Take a deep breath in
2. Then breathe out hard
3. Do the same again
4. On the third time, cough deeply from your chest
5. Place the open container close to your mouth to collect the sputum
   You may be asked to try again for a better specimen