

**LABORATORY SERVICES**  
*in*  
**TUBERCULOSIS**  
**CONTROL**



**PART II:**  
**MICROSCOPY**



**WORLD HEALTH ORGANIZATION**

# **LABORATORY SERVICES IN TUBERCULOSIS CONTROL**

## **MICROSCOPY**

### **PART II**

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## PREFACE

Within the framework of National Tuberculosis Programmes the first purpose of bacteriological services is to detect infectious cases of pulmonary tuberculosis, monitor treatment progress and document cure at the end of treatment by means of microscopic examination. The second purpose of bacteriological services is to contribute to the diagnosis of cases of pulmonary and extra-pulmonary tuberculosis. Standardisation of the basic techniques for tuberculosis bacteriology has so many advantages that it has become an unavoidable need. The absence of standardised techniques complicates the activities of new laboratory services as well as the organisation of existing laboratories into an inter-related network. Standardisation makes it possible to obtain comparable results throughout a country; it facilitates staff training, delegation of responsibilities and the selection of equipment, materials and reagents to be purchased; it also facilitates the evaluation of performance and the establishment of suitable supervision in order to increase efficiency and reduce operational costs.

Standardised techniques and procedures are useful if they meet the needs of - and are prepared in accordance with - prevailing epidemiological conditions and different laboratory levels. These techniques should be simple (to obtain the widest coverage) and should be applicable by auxiliary laboratory workers. At the same time, their sensitivity and specificity must guarantee the reliability of results obtained.

While tuberculosis laboratory services form an essential component of the DOTS strategy for National Tuberculosis Programmes, it is often the most neglected component of these programmes. Furthermore, the escalation of tuberculosis world-wide, driven by the HIV epidemic and aggravated by the emergence of multidrug-resistance, has resulted in renewed concern about safety and quality assurance in tuberculosis laboratories.

The above considerations have led to the preparation of guidelines for laboratory services for the framework of National Tuberculosis Programmes. These guidelines are contained in a series of three manuals, two of which are focused on the technical aspects of tuberculosis microscopy and culture and a third which deals with laboratory management, including aspects such as laboratory safety and proficiency testing. These manuals have been developed for use in low-and middle-income countries with high tuberculosis prevalence and incidence rates. Not only are they targeted to everyday laboratory use, but also for incorporation in teaching and training of laboratory and other health care staff.

Finally, in order to adapt the functioning of bacteriological laboratories to the needs of integrated tuberculosis control programmes, information on control programme activities has been included. It is hoped that the series on laboratory services will enable National Tuberculosis Programmes to draw up national laboratory guidelines as one of their essential components.



## INTRODUCTION

Tuberculosis is a disease of global importance. One-third of the world's population is estimated to have been infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year. The tuberculosis crisis is likely to escalate since the human immunodeficiency (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases. The majority of tuberculosis patients are 15 to 45 years of age, persons in their most productive years of life. Tuberculosis kills over two million people world-wide each year, more than any other single infectious disease, including AIDS and malaria.

Transmission of tuberculosis is virtually entirely by droplet infection, created through coughing by untreated persons suffering from pulmonary tuberculosis (the most common form) in a confined environment. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible persons.

Pulmonary tuberculosis usually occurs in the apex of the lung. These develop cavities containing large populations of tubercle bacilli which can be detected in a sputum specimen. Pulmonary tuberculosis is suggested by persistent productive cough for three weeks or longer, weight loss, night sweats and chest pains. *The diagnosis can only be made reliably by demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory.*

The cornerstone of the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. The technique is simple, inexpensive and detects those cases of tuberculosis who are infectious, ie. those responsible for maintaining the tuberculosis epidemic. Currently no other diagnostic tool is available which could be implemented affordably.

Between 5 000 and 10 000 tubercle bacilli per millilitre of sputum are required for direct microscopy to be positive. Sputum specimens from patients with pulmonary tuberculosis - particularly those with cavitory disease - often contain sufficiently large numbers of acid-fast bacilli to be readily detected by direct microscopy. The sensitivity can further be improved by examination of more than one smear from a patient. Many studies have shown that examination of two smears will on average detect more than 90% of infectious tuberculosis cases. The incremental yield of acid-fast bacilli from serial smear examinations has been shown to be 80-83% from the first, 10-14% from the second and 5-8% from the third specimen. Therefore three sputum specimens are recommended for suspects of pulmonary tuberculosis. A negative smear result does not exclude the diagnosis of tuberculosis as some patients harbour fewer tubercle bacilli than can be detected by microscopy. A poor quality specimen may also produce negative results.

Sputum examination by microscopy is relatively quick, easy and inexpensive and must be performed on all cases suspected of having tuberculosis. Most patients with infectious tuberculosis have respiratory symptoms and the use of smear microscopy in those presenting to health services with suggestive symptoms constitutes the most efficient means of case detection. Tuberculosis microscopy is also performed to assess response to treatment and to establish cure or failure at the end of treatment.



Smear sensitivity is poor in extra pulmonary tuberculosis and in diseases caused by mycobacteria other than tubercle bacilli (MOTT). It is also virtually impossible to distinguish different mycobacterial species by microscopy. Nevertheless, in high-prevalence countries extra-pulmonary tuberculosis and MOTT disease are far less common than pulmonary tuberculosis and are neither rapidly progressive nor highly infectious. From a public health perspective, early diagnosis is therefore less important..

## LABORATORY LAYOUT AND EQUIPMENT

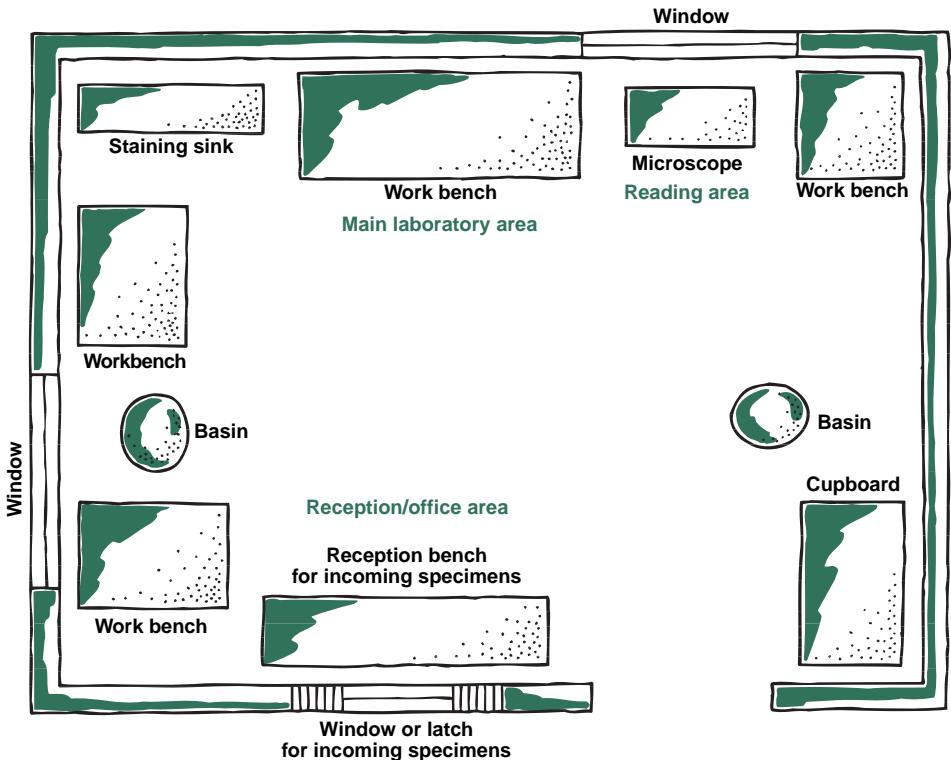
### 2.1 Plan of a peripheral microscopy laboratory

Ideally, tuberculosis microscopy should be done in a separate room. However, tuberculosis microscopy services are usually integrated in general laboratory diagnostic services of countries, which makes the design of a dedicated tuberculosis microscopy laboratory difficult. Nevertheless, in integrated laboratories a separate area should be reserved for tuberculosis microscopy. Enough space should be provided. Walls, ceilings and floors should be smooth, non-absorbent, easy to clean and disinfect and resistant to the chemicals used for microscopy. Floors should be slip resistant and lighting should be adequate. The microscopy area should contain three distinct sections, as illustrated in Figure 1:

- one area for the receipt of specimens, for completing the laboratory register and for storage
- one well-lit area for preparing and staining smears
- one area for microscopy

#### *Figure 1. Plan of a peripheral microscopy laboratory*

(Adapted from: Collins CH, Grange JM, Yates MD. *Tuberculosis Bacteriology*. 2nd ed. Organization and Practice. Oxford; Butterworth-Heinemann, 1997)



The laboratory should contain at least four benches or tables, as indicated in Figure 1:

- one bench for smear-preparation
- one bench for staining (preferably containing a staining sink)
- one bench for microscopic examination. If there is no electricity, this should be directly in front of a window
- one bench for receipt of specimens, laboratory registers and slide storage

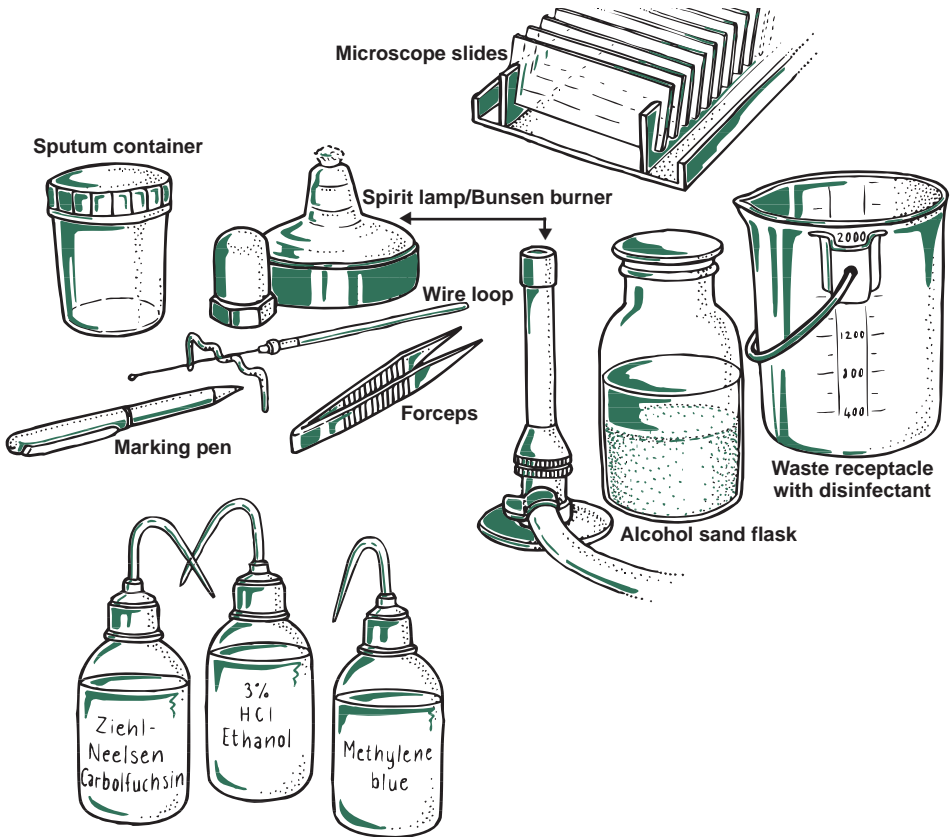
Bench tops should be wide, the correct height for work at a comfortable sitting position, smooth, easy to clean and disinfect and resistant to the chemicals used. Adequate storage facilities should be provided.

Access to the laboratory should be restricted to authorised persons. Entry to the laboratory is via a single door which should always be closed. Specimens arriving at the laboratory are presented through a window/hatch to a separate *reception bench*. Here, specimen containers are checked for leakage and their surfaces decontaminated. Cross-checking of laboratory request forms against specimens is also done and the relevant details are entered into the laboratory register. On completion of these activities the specimens are passed into the *main laboratory area* for further processing. The main laboratory area contains all the facilities necessary for smear preparation. This area houses work benches, a wash basin and storage cabinets. The *reading area* is reserved for performing microscopy and contains work benches, a microscope and a wash basin (optional). Laboratory reports are completed here and passed to the reception bench where the laboratory register is completed and the results despatched.

## 2.2 Arranging equipment and materials

Before the preparation of smears is started, equipment and materials should be arranged to ensure a logical and safe flow of work. All manipulations in preparing smears should be standardised and the arrangement of materials should always be the same to ensure maximum safety.

*Figure 2. Equipment and reagents*



***For smear preparation***

- Sputum container to store sputum
- Wire loop with an inner diameter of 3mm to spread sputum on the microscope slide
- Microscope slide (no grease and no scratches on the slide)
- Marking pen to put identification number on the microscopy slide
- Forceps to hold smear slide
- Bunsen burner or spirit lamp to fix smear slide and to flame the smear during staining
- Metal waste can to incinerate contaminated material

***Forstaining***

- Staining rack to hold smear slide
- Forceps
- Slide rack to place stained smear slide to dry in the air
- Ziehl-Neelsen to stain acid-fast bacilli
- 3% HCL-Ethanol or 20-25% H<sub>2</sub>SO<sub>4</sub> to decolorise smear solution
- 0.3% Methylene blue to counterstain decolourised material in the smear
- Water to rinse the smear
- Bunsen burner or spirit lamp to flame the stain

**2.3 Care and maintenance of essential equipment**

Annex 1 contains a list of essential equipment and supplies for a peripheral laboratory performing Ziehl-Neelsen microscopy. Before purchasing new equipment and supplies it is worthwhile to obtain personal advice of laboratory persons who have had experience in their use. Do not rely entirely on advertisements, catalogues, extravagant claims of sales representatives and the opinion of purchasing officers.

**① Microscope**

The microscope is a precision instrument and requires careful maintenance from both the optical and the mechanical points of view. Laboratory workers should be familiar with the general mechanical and optical principles. Detailed knowledge is unnecessary and apart from basic principles on care, maintenance should be left to professionals. Most manufacturers publish manuals containing useful explanations and information.

- When not in use, the microscope should be kept in its case or protected from dust by a plastic cover.
- Avoid exposing the microscope to moisture. Humidity may allow fungi to grow on the lenses and may cause rusting of the metal components. To limit exposure to moisture, place a shallow plate containing dry blue silica gel in the microscope case whenever the microscope is stored. When silica gel is unable to absorb more moisture it changes colour (from blue to pink). In such situations the silica gel could be replaced or dehydrated in a hot air oven and re-used when its original (blue) colour reappears.
- To remove a slight film of fungi, moisten a wad of cotton wool with appropriate fungal cleaner and clean the lens by moving the cotton around in circles using moderate pressure. If necessary, repeat with a fresh wad of cotton wool. Wipe the lens with lens tissue.
- Avoid keeping the microscope in a place where there are chemical reagents, water or discharges of corrosive gas.

- The microscope should be picked up or carried with two hands, one grasping the arm firmly and the other under the base for added support. Never carry a microscope with only one hand.
- Install the microscope on a sturdy, level surface. Do not place equipment and instruments generating vibrations (eg. centrifuges) on the same table.
- If the microscope is to be used every day, do not remove it from the site of installation, but when not in use keep it covered with a polythene or plastic cover.
- Microscope lenses may be scratched by dirt or grit. The lenses should be cleaned only with clean, dry *lens tissue*. Cloth or other tissues should not be used as they may scratch the lenses. Never use soap, alcohol, or other solvents to clean the lenses.
- Immersion oil must not be allowed to fall on the stage. Immersion oil should be wiped from the objective lenses after each slide to avoid cross-contamination.
- Care must be taken that objectives for dry use do not touch the immersion oil. Should this happen, clean immediately.
- The microscope should never be dismantled; if faulty it should be entrusted to a competent person for repair.

## 2 Balance

The balance is a fragile and precision instrument and must be used with care. Always consult the accompanying manual for detailed operating instructions.

- Always keep the balance and weights clean and dry to protect them from corrosion. Any change in the surface of the parts may affect the accuracy of the balance.
- Do not put material to be weighed directly on the pan; always use a container or weighing paper. Subtract the weight of the container or weighing paper from the combined weight of the container and material weighed.
- Protect the balance from drafts of air. Air moving cross the pans will cause an inaccurate reading. The more accurate balances have individual cases which are to be closed at the final reading.
- Do not return unused substances to the stock bottle to prevent contamination of stock materials.



## SPECIMEN COLLECTION

In tuberculosis diagnosis attention tends to be focussed on the problems of microscopy, while an often overlooked problem is that of obtaining adequate specimens. Correct collection and transportation of specimens to the laboratory are important to ensure that results are accurate and reliable.

### 3.1 Containers

An essential prerequisite for the safe collection of satisfactory specimens is a robust, leak-proof and clean container. Containers must be rigid to avoid crushing in transit and must possess a water-tight wide-mouthed screw top to prevent leakage and contamination. Specimens should be collected in clean containers that are free from paraffin and other waxes or oils. These materials may appear in smears as acid-fast artefacts or may react with other bacteria and cause them to appear to be acid-fast.

To facilitate the choice of a container the following specifications are recommended:

- Wide-mouthed (at least 35mm in diameter) so that the patient can expectorate easily inside the container without contaminating the outside
- Volume capacity of 50ml
- Made of translucent material in order to observe specimen volume and quality without opening the container
- Made of single-use combustible material to facilitate disposal
- Screw-capped to obtain a water-tight seal to reduce the risk of leakage during transport
- Easily-labelled walls that will allow permanent identification

An alternative container is the 28ml Universal bottle, which is a heavy glass, screw-capped bottle. This container is reusable after thorough cleaning and sterilisation in boiling water for at least 30 minutes.



*Figure 3. Standard specimen containers*



### 3.2 Collection procedures

Although *M. tuberculosis* is capable of causing disease in almost any organ of the body, more than 85% of tuberculosis disease in high prevalence countries is pulmonary. Therefore, sputum is the specimen of choice in the investigation of tuberculosis and should always be collected. If extra-pulmonary disease is suspected, sputum should be collected in addition to any extra-pulmonary specimens if there are respiratory symptoms.

Collecting a good sputum specimen requires that the patient be given clear instructions. Aerosols containing tuberculosis bacteria may be formed when the patient coughs to produce a sputum specimen. Patients should therefore produce specimens either outside in the open air or away from other people and not in confined spaces such as toilets.

In some countries, patients may present first to the laboratory for diagnosis. It is therefore appropriate that laboratory staff know the correct way of collecting sputum specimens. This procedure is described in Annex 2. It is best to obtain a sputum specimen early in the morning before the patient has eaten since food particles in smears make them difficult to examine.

Because tuberculosis lesions in the lungs may drain intermittently, it is possible for a specimen to be negative on one day and positive the next. For this reason, three specimens should be collected for diagnosis as follows:

- one spot specimen when the patient first presents to the health service
- one early morning specimen (preferably the next day)
- one spot specimen when the early morning specimen is submitted for examination

These should not be pooled but should be sent to the laboratory as single specimens. For follow-up of treatment at regular intervals and to determine outcome at the end of treatment, one specimen should be collected.

If a patient has a productive cough, obtaining a sputum specimen is a fairly straight-forward procedure. However, if a patient finds it difficult to produce sputum, other methods may be used to obtain pulmonary secretions, such as sputum induction. Collection techniques fall outside the scope of this document and will not be discussed. However, induced sputum resemble saliva and it is important that these specimens be marked “induced” in order not to be discarded as unsuitable.

Specimens should be transported to the laboratory as soon as possible after collection. If a delay is unavoidable the specimens should be refrigerated or kept in as cool a place as possible to inhibit the growth of unwanted micro-organisms.



## SPECIMEN STORAGE AND TRANSPORT

If the health facility does not perform its own microscopy, the collected sputum specimens must be brought to the laboratory. This transport should take place once or twice a week (in isolated areas, storage time could be increased to 1 to 2 weeks). It is, however, of utmost importance that specimens be examined as soon as possible, since patient treatment depends on the results. Consequently, the specimens collected over a period of a few days must be kept at the health centre and transported together in one batch to the laboratory. A special box with the sputum containers should be kept refrigerated or in as cool a place as possible until it is dispatched.

Requirements and recommendations for the safe transport of pathological specimens are given in various national and international codes of practice and guidelines. In addition, the postal and transport authorities of most countries as well as the International Air Transport Association (IATA) have regulations about conveying such materials.

As a general rule, diagnostic specimens must be packaged to withstand leakage of contents, shocks, pressure changes and other conditions incident to ordinary handling practices. Pathological material intended for postal or air transport should be in approved, robust, leak-proof primary containers which are packed into secondary containers made of metal, wood or strong cardboard with enough absorbent material so that if they are damaged or leak the fluids will be absorbed. For sending material across international or state boundaries this container may have to be packed in the same way in an outer container and special administrative arrangements with the postal authorities and airlines may be necessary.

Sputum specimens comprise the majority of specimens submitted to tuberculosis microscopy laboratories and special transport boxes of metal or wood should be provided. They should be made to hold between 20 and 30 specimen containers packed vertically to avoid leaking. The lid should be securely fastened and the box should preferably contain a locking mechanism. During transport it must be kept as cool as possible and protected from sunlight.

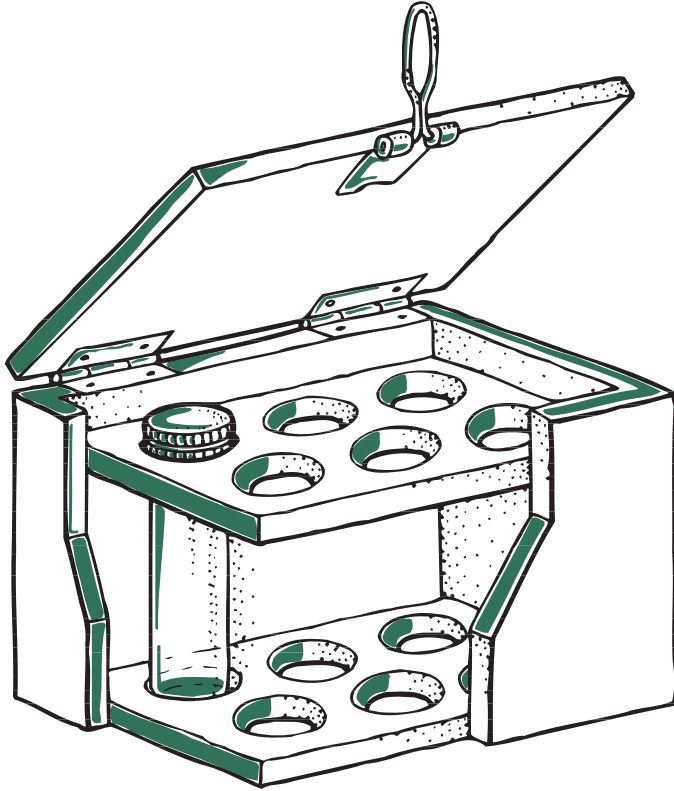
Request forms should be located separately from specimen containers. With each transport box an accompanying list must be prepared which identifies the specimens and the patients from whom the specimens were collected. Before dispatch from the health centre the following must be verified:

- that the number of specimen containers in the box corresponds to that on the accompanying list
- that the identification number on each specimen container corresponds to the identification number on the accompanying list
- that the accompanying list contains the necessary data for each patient
- that the date of dispatch and the particulars of the health centre are on the accompanying list

A model laboratory request form is presented in Annex 3.

**Figure 4. Appropriate packaging of specimens to be transported**

(Acknowledgement: Rieder HL, Chonde TM, Myking H et al. The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network. Minimum Requirements, Role and Operation in a Low-income Country. International Union Against Tuberculosis and Lung Disease, France, 1998)



## SPECIMEN HANDLING

### 5.1 Receipt of incoming specimens

Specimens should be received at a separate specimen delivery counter and the following procedures applied:

- Wear disposable gloves, if available, during receipt and inspection of incoming specimens.
- Inspect the delivery box for signs of leakage. If mass leakage is evident discard the box by autoclaving or burning.
- Disinfect the outside of the delivery box using cotton wool or paper towels saturated with a suitable disinfectant (eg. 5% phenol).
- Open the delivery box carefully and check for cracked or broken specimen containers. Autoclave or burn these without processing and request another specimen.
- Check that specimens have been adequately labelled with individual identification numbers and that these correspond with the numbers on the accompanying list.
- Note the relevant patient and specimen details into the laboratory register.
- Disinfect the inside of the delivery box, discard gloves and wash hands after handling specimen containers.

### 5.2 Safe handling of specimens

Transmission of *M. tuberculosis* results essentially from infectious aerosols, ie. droplet nuclei of 1-5µm in diameter containing tubercle bacilli, sufficiently small to reach lung alveoli and initialise an infection.

Infection control in the microscopy laboratory must aim at reducing the production of aerosols. Good ventilation is necessary for the protection of laboratory staff from infectious airborne nuclei. A simple way to ensure ventilation and directional airflow is by properly placed windows and doors.

Microscopy procedures differ considerably in their potential to create aerosols:

#### 5.2.1 Specimen collection

Tuberculosis suspects are sometimes referred directly to the laboratory for sputum collection. This practice exposes laboratory workers to a high risk of infection by aerosols produced during collection procedures. Precautions to lower this risk include instructing tuberculosis suspects to cover their mouths while coughing, standing behind (and not in front of) coughing individuals and collecting specimens outdoors where aerosols are diluted and sterilised by direct sunlight.

### 5.2.2 *Smear preparation*

While opening sputum containers and preparing smears may produce aerosols, these manipulations entail less risk of transmission than unprotected coughing. There is very little epidemiological evidence that preparing sputum smears is correlated with an increased risk of tuberculosis infection. Expensive and sophisticated equipment is no substitute for good laboratory practice, and biological safety cabinets are therefore not mandatory in peripheral laboratories performing smear microscopy only.

### 5.3 **Evaluation of sputum quality and volume**

A good sputum specimen consists of recently-discharged material from the bronchial tree, with minimum amounts of oral or nasal material. Satisfactory quality implies the presence of mucoid or mucopurulent material and is of greater significance than volume. Ideally, a sputum specimen should have a volume of 3-5ml, although smaller quantities are acceptable if the quality is satisfactory. Induced sputum resembles saliva and it is important that these specimens not be discarded as unsuitable.

## SMEAR PREPARATION PROCEDURES

### 6.1 Sputum smear preparation

Smears should be prepared in manageable batches (maximum of 12 per batch). Labelling of smears should be done at the bench for incoming specimens using a permanent marker, eg. a diamond-point stylus or wax pencil. Avoid touching the surface of the slides.

The maximum chance of finding bacilli in unconcentrated specimens is in the solid or most dense particles of the sputum and the results of direct smear examination depend to a great extent on the choice of these particles.

The procedure for smear preparation is presented in Diagram 1 on page 23.

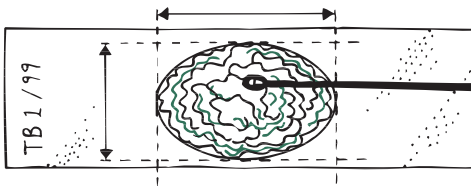
#### Diagram 1. Sputum smear preparation

##### PROCEDURE

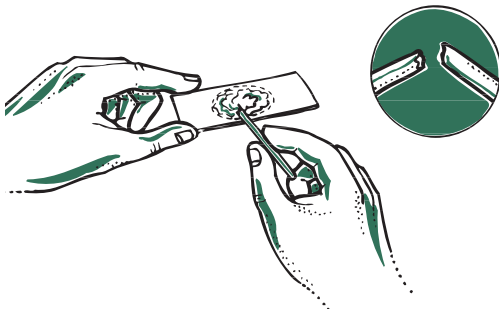
Label a new, clean, unscratched slide at one end with the relevant patient number



Transfer an appropriate portion of the specimen to the slide by using an applicator stick (recommended) or bacteriological loop. Use blood-speckled, opaque, greyish or yellowish cheesy mucus for smear preparation when it is present



Smear the specimen on the slide over an area approximately 2.0 by 1.0 cm. Make it thin enough to be able to read through it. *Do not make more than one smear per slide* (use the broken end of a wooden stick to smear the sputum)





Allow smears to air dry for 15 minutes. *Do not use heat for drying*



Fix smears to the slide by one of the following methods:

- Pass slides through a flame three or four times with the smear uppermost. Do not overheat. Allow to cool before staining
- Allow the slides to fix on an electric slide warmer (65°C - 75°C) for at least 2 hours



Discard the applicator stick in disinfectant or a biohazard receptacle, and use a new one for each specimen. Remove particles of adherent sputum from loops by moving it up and down through a flask containing sand and 70% alcohol. Flame the bacteriological loop thoroughly prior to re-use. The flame should be colourless or blue, because an orange or red flame is usually not hot enough

## 6.2

### Value of smears on extra-pulmonary specimens

Because *M. tuberculosis* may infect almost any organ in the body, the laboratory could receive a variety of extra-pulmonary specimens, eg. body fluids, tissues, pus and urine. The benefit of microscopy on these specimens is limited and it is recommended that extra-pulmonary specimens be referred for culture.

#### 6.2.1

##### *Gastric washings*

Direct smears should be avoided as the results can be misleading. Acid-fast bacilli are frequently present in food and water and hence in the stomach. There is no way of distinguishing such organisms from tubercle bacilli on microscopy and positive smears must be regarded with suspicion.

#### 6.2.2

##### *Laryngeal swabs*

Direct smears are almost useless. A negative result is meaningless and it is best to conserve what little material there is for culture.

#### 6.2.3

##### *Pus and thick aspirates*

Direct smears of these specimens should be very thin. Thick smears tend to float off the slide and even if they are retained, acid-fast bacilli may be difficult to see after staining. Problems may arise if a large amount of blood is present in the specimen since blood may sometimes produce acid-fast artefacts.

**6.2.4** *Pleural fluids*

The fluid should be centrifuged and smears prepared from the sediment. Again, these should be thin or they may float off the slide.

**6.2.5** *Cerebrospinal fluids*

Smears from cerebrospinal fluid are rarely positive and sediment from concentrated specimens should rather be cultured. If a smear is desired, two parallel marks, about 10mm long and 2mm apart, should be made on a clean glass slide. A loopful of the sediment is then spread between these marks and the smear allowed to dry. Another loopful is then spread over the first. When this is dry the process may be repeated, depending on how much sediment is available. This procedure clearly marks the area to be searched for acid-fast bacilli. It is desirable that the smears are examined by two independent microscopists. Clots should be saved for culture.

**6.2.6** *Urine*

Smears of centrifuged urine deposits are very unreliable and should be avoided. Mycobacteria other than tubercle bacilli are sometimes present in urine, either when it is voided or as a result of poor collection techniques. The presence of acid-fast bacilli in urine should be viewed with suspicion.



## ACID-FAST STAINING PROCEDURES

Mycobacteria retains the primary stain even after exposure to decolorising acid-alcohol, hence the term “acid-fast”. A counter-stain is employed to highlight the stained organisms for easier recognition. There are several methods of determining the acid-fast nature of mycobacteria. In the carbolfuchsin (Ziehl-Neelsen) procedure, acid-fast organisms appear red against a blue background, while in the fluorochrome procedures (auramine-O, auramine-rhodamine), the acid-fast organisms appear as fluorescent rods, yellow to orange (the colour may vary with the filter system used) against a paler yellow or orange background.

Stain powders are often not pure and a corrected weight should be used to ensure proper staining. Most manufacturers print the % available dye content on the label. The corrected weight is determined by calculating a correction factor and multiplying the amount of dye required by this factor:

### *Example*

75% available dye and 3g of dye needed

- Divide 1 by the decimal equivalent of the available dye to obtain the correction factor

$$\begin{aligned} \text{Decimal equivalent of 75\%} &= 0.75 \\ 1/0.75 &= 1.33 \text{ (correction factor)} \end{aligned}$$

- Multiply 1.33 by 3g = 3.99
- 3.99 of impure dye is required to obtain 3g usable dye

If powder with a dye content of 88% or more is used, no correction factor needs to be calculated.

Pure phenol crystals are colourless; brown-tinted crystals should not be used since they may cause unsatisfactory staining.

### 7.1 Ziehl-Neelsen staining

- **Reagents**

#### *Fuchsin*

|  |            |
|--|------------|
| Basic fuchsin                          | 3.0g       |
| 95% ethanol (technical grade)          | 100ml      |
| Dissolve basic fuchsin in ethanol..... | Solution 1 |

*Phenol*

|                 |       |
|-----------------|-------|
| Phenol crystals | 5g    |
| Distilled water | 100ml |

Dissolve phenol crystals in distilled water (gentle heat may be required).....Solution 2

*Working solution*

Combine 10ml of solution 1 with 90ml of solution 2 and store in an amber bottle. Label bottle with name of reagent as well as preparation and expiry dates. Can be stored at room temperature for six to twelve months and filter before use.

- ***Decolourising agent: 3% acid-alcohol***

|  |      |
|--|------|
| Concentrated hydrochloric acid (technical grade) | 3ml  |
| 95% ethanol (technical grade)                    | 97ml |

*Carefully* add concentrated hydrochloric acid to 95% ethanol. *Always add acid slowly to alcohol, not vice versa.* The mixture will heat up. Store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

In countries where the acquisition of alcohol may be problematic, a solution of 25% sulphuric acid may be used as decolourising agent. This is prepared as follows:

- ***Decolourising agent: 25% sulphuric acid***

|   |       |
|---|-------|
| Concentrated sulphuric acid (technical grade) | 25ml  |
| Sterile distilled water.                      | 100ml |

*Carefully* add concentrated sulphuric acid to water. *Always add acid slowly to water, not vice versa.* The mixture will heat up. Store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

- ***Counterstain: Methylene blue***

|                         |       |
|-------------------------|-------|
| Methylene blue chloride | 0.3g  |
| Distilled water.        | 100ml |

Dissolve methylene blue chloride in distilled water and store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

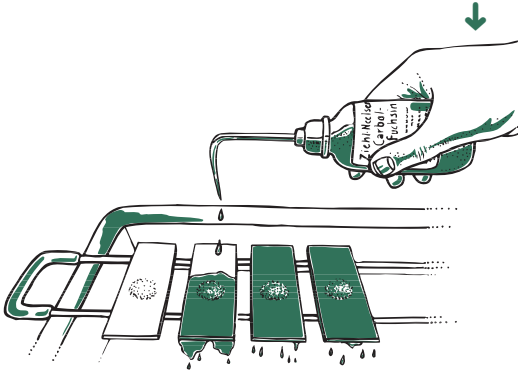
- ***Procedure***

Refer to Diagram 2 on page 29.

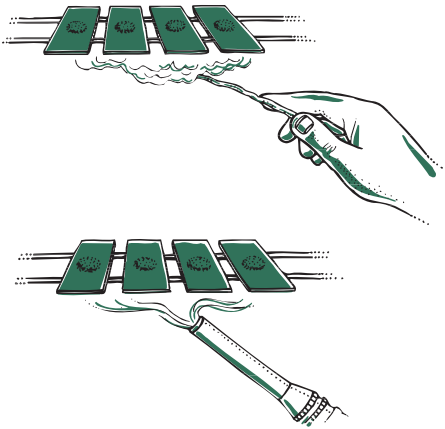
*Diagram 2. Ziehl-Neelsen staining*

**PROCEDURE**

Place the numbered slides on a staining rack in batches (maximum 12).  
Ensure that slides do not touch each other



Flood entire slide with Ziehl-Neelsen carbolfuchsin, which has been filtered prior to use, or cover each slide with a piece of filter paper if unfiltered carbolfuchsin is used



Heat the slide slowly until it is steaming. *Do not boil.* Maintain steaming for three to five minutes by using low or intermittent heat. *In no case must the stain boil dry*

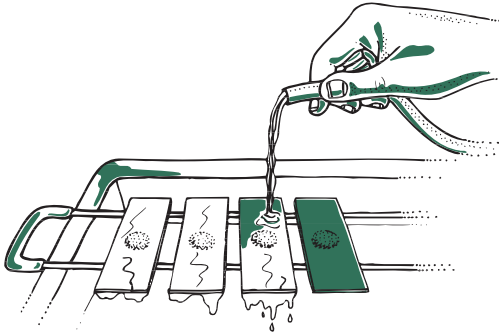
If filter paper strips have been used, remove them with forceps. Rinse each slide individually in a gentle stream of running water until all free stain is washed away

Flood the slide with the decolourising solution for a maximum of three minutes

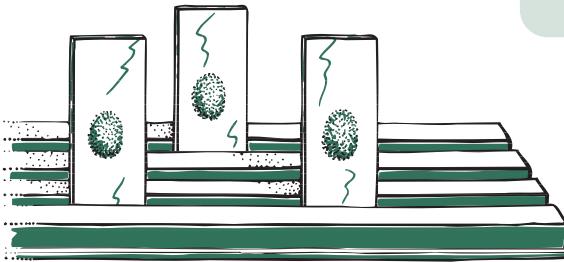
Rinse the slide thoroughly with water. Drain excess water from the slide

Flood the slide with counterstain

Allow the smear to counterstain for 60 seconds



Rinse the slide thoroughly with water. Drain excess water from the slide. Allow smear to air dry. Do not blot



## 7.2 FLUOROCHROME STAINING

Fluorescence microscopy uses illumination from either a quartz-halogen lamp or a high-pressure mercury vapour lamp. The advantage of fluorescence microscopy is that a low magnification objective is used to scan smears, allowing a much larger area of the smear to be seen and resulting in more rapid examination. However, one drawback in using a low magnification is the greater probability that artifacts may be mistaken for acid-fast bacilli. It is therefore strongly recommended that suspect bacilli be confirmed at higher magnification, and that positive fluorochrome stains be confirmed by Ziehl-Neelsen microscopy.

Much money can be wasted in purchasing very expensive and sophisticated fluorescence microscopes that are not necessary for tuberculosis microscopy. The following technical specifications will suffice:

- Halogen lamps are adequate. They are usually built-in and are much less expensive than mercury vapour lamps which also have a limited life span. Halogen lamps also warm up immediately.
- Good light-passing ability is required, including high numerical apertures for the condenser and for two objectives. Three objectives are necessary, eg. 10x and 25x for scanning and 45x for morphological definition.

- Objectives should be uncorrected since smears are examined without cover glasses.
- Fluorescence oil should always be used because of its better refraction index.

- **Reagents**

*Auramine O*

|                               |      |
|-------------------------------|------|
| Auramine                      | 0.1g |
| 95% ethanol (technical grade) | 10ml |

Dissolve auramine in ethanol.....Solution 1

*Auramine is carcinogenic and direct contact with the powder or the solution should be avoided.*

*Phenol*

|                 |      |
|-----------------|------|
| Phenol crystals | 3.0g |
| Distilled water | 87ml |

Dissolve phenol crystals in water.....Solution 2

Mix solutions 1 and 2 and store in a tightly stoppered amber bottle away from heat and light. Label bottle with the name of the reagent and dates of preparation and expiry. Store at room temperature for three months. Turbidity may develop on standing but this does not affect the staining reaction.

- **Decolourising solution**

|                                |       |
|--------------------------------|-------|
| Concentrated hydrochloric acid | 0.5ml |
| 70% ethanol (technical grade)  | 100ml |

*Carefully* add concentrated hydrochloric acid to the ethanol. *Always add acid slowly to alcohol, not vice versa.* Store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for three months.

- **Counterstains**

Either potassium permanganate or acridine orange may be used as counterstains.

*Potassium permanganate*

|   |       |
|---|-------|
| Potassium permanganate (KMnO <sub>4</sub> ) | 0.5g  |
| Distilled water.                            | 100ml |

Dissolve potassium permanganate in distilled water in a tightly stoppered amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for three months.



*Acridine orange*

|  |       |
|--|-------|
| Anhydrous dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) | 0.01g |
| Distilled water  | 100ml |
| Acridine orange  | 0.01g |

Dissolve sodium phosphate in distilled water. Add acridine orange and dissolve. Store in a tightly stoppered amber bottle away from heat and light. Label bottle with name of reagent and dates of preparation and expiry. Store at room temperature for three months.

- **Procedure**

Refer to Diagram 3 on page 33.

7.3

**Precautions during staining**

- Avoid under-decolourisation with acid-alcohol. Organisms that are truly acid-fast are difficult to over-decolourise.
- Avoid making thick smears. This may interfere with proper decolourisation, and counterstains may hide the presence of acid-fast bacilli. Additionally, thick smears may flake, resulting in loss of smear material and possible transfer of material to other slides.
- Strong counter-staining may mask the presence of acid-fast bacilli.
- Smears that have been examined by fluorescence microscopy may be re-stained by Ziehl-Neelsen staining to confirm observations (recommended). However, once smears have been stained by Ziehl-Neelsen staining they cannot be used for fluorescence microscopy.

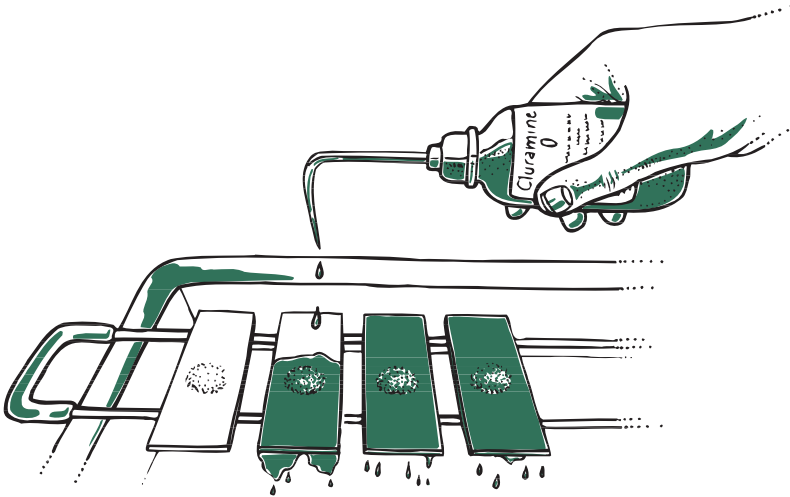
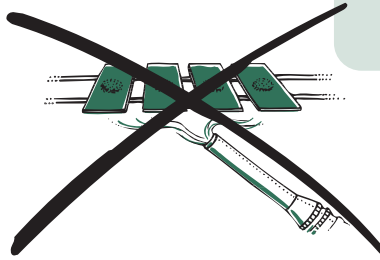
*Diagram 3. Fluorochrome staining*

**PROCEDURE**

Place the numbered smears on a staining rack in batches (maximum 12).  
Ensure that the slides do not touch each other



Flood entire smear with auramine O and allow to stain for 15 minutes, ensuring that staining solution remains on smears.  
*Do not heat and do not use filter paper strips*



Rinse with *distilled water* and drain. Tap water contains chlorine which may interfere with fluorescence



ACID-FAST STAINING PROCEDURES

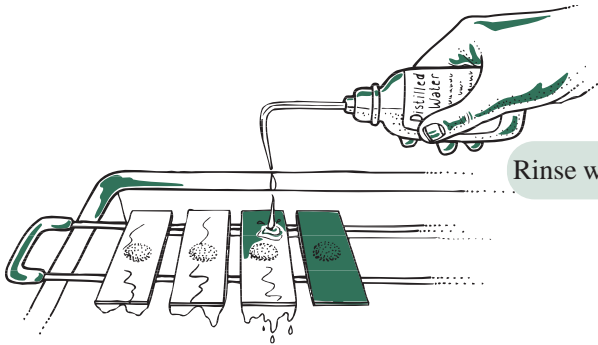
Decolourise with 3% acid-alcohol for two minutes



Rinse with distilled water and drain



Flood smears with either potassium permanganate or acridine orange and allow to counterstain for two minutes. Time is critical with potassium permanganate because counterstaining for longer may quench the fluorescence of acid-fast bacilli



Rinse with distilled water and drain



Allow smears to air-dry. Do not blot.  
Read as soon as possible after staining

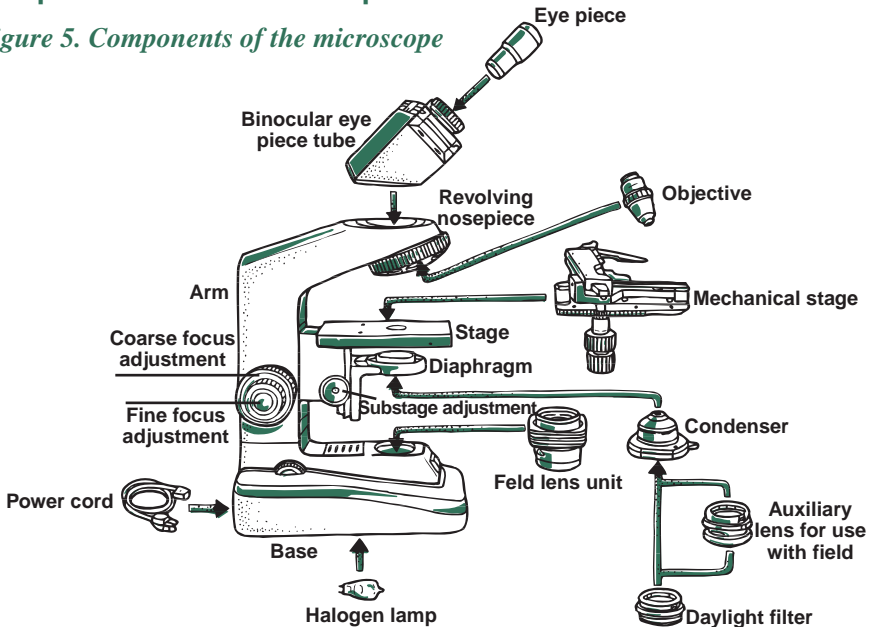
## SMEAR EXAMINATION PROCEDURES

Because the human eye cannot see objects with a diameter of less than 0.1mm, individual bacteria can only be detected with a microscope. The function of the magnifying lens system of the microscope is to magnify the objects within the microscopic field to a size which can be detected by the human eye. In addition to magnification, two other factors, contrast and resolution, are of great importance. In order to be perceived through the microscope, an object must possess a certain degree of contrast with its surrounding medium: in order to produce a clear magnified image, the microscope must possess a resolving power sufficient to permit perception of individual objects. The degree of contrast can be greatly increased by staining procedures: treatment with dyes that bind selectively either to the whole cell or to certain cell components. The acid-fast stain is used to obtain information on the composition of the cell walls of tubercle bacilli. The types of microscopy most commonly used for observing acid-fast bacilli are bright-field and fluorescence microscopy. In bright-field microscopy the light passes through the bacilli and the variation in colour due to staining show the form of the organisms. Fluorescence stains are usually organic substances which absorb ultraviolet light and remit part of the energy as light of longer wavelength which can be observed through the eyepiece as fluorescence. When exposed to ultraviolet light, the fluorescent bacilli are perceived as brightly coloured organisms against a dark background.

A binocular microscope (ie. one with two eye pieces) is recommended for the examination of stained smears. If a monocular microscope is used, both eyes should be kept open while looking into the eyepiece, to prevent eye fatigue. If no electricity is available, daylight must be used as light source and the table with the microscope be placed in front of a window.

### 8.1 Components of the microscope

Figure 5. Components of the microscope



### 8.1.1 Mechanical part

The *base* or foot of the microscope should be sufficiently heavy to be stable as it supports the *stage* on which the slide is placed. The slide is kept steady by clips attached to the stage; movements are guided by a micrometric screw with vernier to help relocate a field of examination.

The *limb* supports the eyepiece holder and the objective holder. In the monocular microscope the two devices are separated by a tube with the distance between the eyepiece and the objective approximately 170mm. In binocular microscopes the tube is replaced by a system of prisms.

In the monocular model, the eyepiece holder is a simple tube, while in the binocular model, the prisms are placed within the eyepiece holder. The two eyepieces can be moved to adjust to the distance between the observer's pupils; one of the eyepieces moves around its axis to correct any difference in convergence between both eyes.

The *objective holder* can be rotated as well as replaced, if necessary.

### 8.1.2 Optical outfit

The *mirror*, which has two faces, plane and concave, sends parallel rays from the light source to the optical axis of the microscope.

The *light source* is usually incorporated in the foot of the microscope. The fixed light source keeps a constant distance and alignment. Usually small halogen bulbs of great intensity are used. Tungsten filament lamps can also be employed.

The *condenser* is a lens that focuses the light on the slide. It is centered by moving the centering screws until the iris is concentric with the opening of the back of the objective. The greater the aperture of the diaphragm the wider the angle of vision. The light opening must be smaller than the opening of the objective used.

### 8.1.3 Objectives

Objectives are classified according to their numerical aperture (NA) and magnification. The NA describes the properties of the objective lens. Up to a certain limit an increase in the NA of the objective lens increases resolving power.

The passage of light through curved lenses produces the separation of the spectrum in the different wave lengths. This results in coloured bands in the periphery of the lens due to a chromatic aberration. The greater the magnification, the greater the aberration will be. The different colours (wave lengths) do not focus on the same place, producing spherical aberration. These are compensated for by using a combination of lenses. *Achromatic objectives* are corrected for two colours of the chromatic aberration (yellow and green) and for one of the spherical aberration. *Apochromatic objectives* do not present chromatic aberrations throughout the whole scale of visible

spectrum provided there is an optimal distance between the object and the lens of the objective. They are classified by distance in mm or fractions of mm. They give clearer and crisper images than the achromatic lenses and can be used with higher power oculars. The flat apochromatic lenses have a correction for spherical aberration.

#### 8.1.4 Immersion objectives

Dry objectives are those where the front lens is in contact with no other medium than the air (refraction index nil). The refraction index of glass (as used for microscope slides) is 1.51. As a result, light rays are refracted in passing from the glass to the air; some fall beyond the visual scope of the objectives. With oil-immersion objectives, the oil is used to fill the space between the front lens and the slide. To achieve high magnification, immersion oil is placed between the slide and the immersion objective lens. Unlike air, immersion oil has the same refractive index as glass and consequently improves the resolving power of the lens.

The *eyepiece* or ocular is composed of two lenses mounted on two ends of a tube. The lower lens is called the collector or field glass; it flattens and clears the real image and therefore completes the action of the objectives. The upper lens enlarges the image and therefore gives a virtual image of the object.

In brief, the objective produces an image of the preparation inside the microscope, and the observer looks at the image through the eyepiece. To form a clear image the lens must focus each point in the slide to give a point in the image.

## 8.2 Use of the microscope

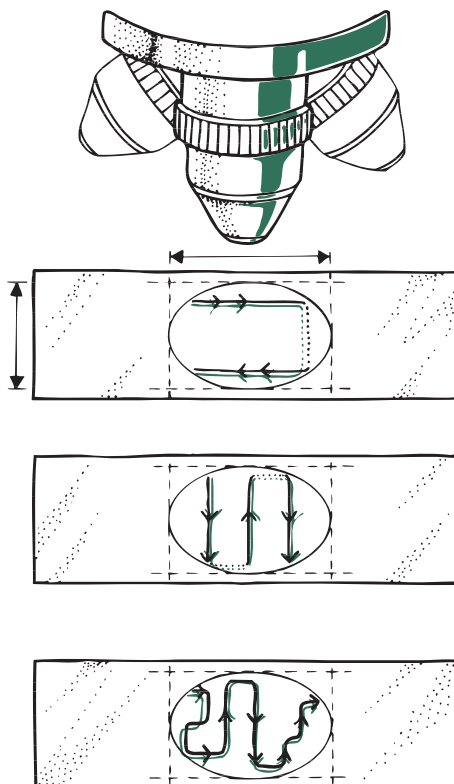
- Check for broken or damaged parts.
- Ensure that the light source is well regulated and focused.
- Ensure that the lenses, mirrors and other light-conducting surfaces are clean.
- Ensure that the condenser is in the upper position with the diaphragm open.
- Adjust the light, mirror, condenser and diagram so that a strong beam of light is directed towards the objective lens.
- Turn the coarse adjustment knob to move the objective lens away from the stage.
- Rotate the nosepiece so that a low power objective lens (5x or 10x) is directly over the condenser.
- Place the slide on the stage so that the smear is directly under the objective lens.
- Look from the side of the stage to observe the space between the slide and the objective lens. Slowly turn the coarse focus knob to bring the objective lens close - but not touching - the smear.

- Adjust the light intensity so that it is bright but not uncomfortable when looking into the eyepiece. This may be done by changing the intensity of the lamp, changing mirror surfaces, using dark filters, adjusting the diaphragm or adjusting the condenser. Usually, adjusting the diaphragm is sufficient.
- While looking into the eyepiece, slowly turn the coarse focus knob to separate the objective lens and the stage. The smear should come into focus within a few turns.
- Turn the fine focus knob until the smear is seen most clearly.
- While looking from the side, turn the nosepiece to select a higher power lens. Ensure that the lens does not touch the slide. The smear should almost be in focus. The best focus can be achieved by adjusting the fine focus knob. A light adjustment may also be helpful.
- To use the oil immersion lens, turn the nosepiece so that the lens is over the smear. Put a drop of immersion oil on the smear - do not touch the slide with the oil applicator but allow the drop of oil to fall freely onto the smear.
- Lower the 100x immersion lens so that it comes into contact with the oil. Slowly bring the immersion lens upwards until the image of the smear appears. Adjust the fine focus knob to focus.

### 8.3 Examination procedures

To obtain excellence in microscopic examination, a good microscope and a comfortable work area is required. Reading of smears must be systematic and standardised to ensure that a representative area of the smear is examined. To ensure that an area is covered only once, the smear should be examined in an orderly manner and the following procedure is recommended:

- Always examine carbolfuchsin-stained smears with a 100x oil immersion objective.
- Examine fluorochrome-stained smears within 24 hours of staining as the fluorescence may fade with time. Smears that cannot be examined immediately after staining should be kept in the dark, preferably in a refrigerator, for a maximum of 24 hours.
- If possible include a *known positive* slide and a *known negative* slide with each day's work. The positive control ensures the staining capability of the solutions and of the staining procedure. The negative control confirms that acid-fast contaminants are not present in the stains or in other solutions.
- Make a series of systematic sweeps over the length of the smear. After examining a microscopic field, move the slide longitudinally so that the neighbouring field to the right can be examined. Search each field thoroughly.



- Examine a minimum of 100 fields before the smear is reported as negative. For a skilled microscopist this will take approximately five minutes. In a smear of 1.5cm x 1.5cm the number of microscopic fields in one length of the slide corresponds to around 100. If the smear is moderately or heavily positive fewer fields may be examined and a report of “positive” may be made even though the entire smear has not been examined.
- At the end of examination, take the slide from the microscope stage, check the identification number, and note the result. Dip the slide into xylene to remove the immersion oil and place it in a box for examined slides.
- Before examining the next slide, wipe the immersion lens with a piece of lens tissue.
- Unexpected objects may be seen when using the microscope. If these objects move only when the slide is moved, they may be materials occasionally found in the specimen or object, precipitated fixatives or stains, contaminants from the stains or contaminants in the immersion oil.
- Artefacts that move only when the eyepiece is rotated are in the eyepiece or on its lenses. Artefacts may also be caused by material on the condenser lenses, mirror, or light source.
- Keep all the slides for external quality control, according to established procedures (see I. Organisation and management).



- Analyse results on a weekly and monthly basis for percentage of positive results. Investigate any sharp differences from the norm.

#### 8.4 Morphological characteristics of acid-fast bacilli

- Acid-fast bacilli are approximately 1-10µm long and typically appear as slender, rod-shaped bacilli, but they also may appear curved or bent
- With carbolfuchsin staining, tubercle bacilli look like fine red rods, slightly curved, more or less granular, isolated, in pairs or in groups, standing out clearly against the blue background.
- Individual bacteria may display heavily stained areas referred to as “beads” and areas of alternating stain may produce a banded appearance.
- With fluorochrome staining, tubercle bacilli are rod shaped and emit a bright yellow fluorescence against a pale yellow (potassium permanganate) or orange (acridine orange) background.
- Some mycobacteria other than *M. tuberculosis* may appear pleomorphic, ranging in appearance from long rods to coccoid forms, with more uniform distribution of staining properties.
- Organisms other than mycobacteria may demonstrate various degrees of acid-fastness. Such organisms include *Rhodococcus* spp., *Nocardia* spp., *Legionella* spp., and the cysts of *Cryptosporidium* and *Isospora* spp..
- Rapidly growing mycobacteria may vary in their abilities to retain acid-fast stains.

#### 8.5 Causes of error in microscopy

Connected with the *specimen*

- X Inadequate specimen quality and/or volume.
- X Inefficient washing of re-usable containers may leave residual bacilli which give rise to false positives.
- X Carelessness in marking the container. Marking should be done on the body of the container and not on its lid.

Connected with the *preparation of the smear*

- X Insufficient or poorly lit work surface.
- X Mixing-up of slides.
- X Preparing too many slides at once. A maximum of 12 is recommended.
- X Utilisation of slides that have been positive. These should be discarded.
- X Specimen contamination due to careless use of loops, pipettes or wooden applicators.

Connected with the *staining*

- X Using scratched slides on which deposits of stain may look like bacilli.
- X Using unfiltered fuchsin which may contain crystals.
- X Carelessness in heating the fuchsin, allowing it to dry and crystallise on the smear.
- X Inadequate decolourising of the smear which may leave red stain on saprophytic bacilli which then appear to be acid-fast.

Connected with the *microscopic examination*

- X Failure to check the slides and renumber them should the number be obscured during staining. This may lead to substitutions.
- X Failure to clean the immersion lens with lens tissue after each examination, especially after a smear was found to be positive.
- X Presence of bacilli in the immersion oil owing to the practice of touching the smear with the neck of the bottle. A dropper bottle should be used and the drop allowed to fall without any contact between bottle and slide.
- X Erroneous recording of the results.

**Table 1. Troubleshooting guide for microscopy**

| Problem  | Possible causes   | Solution   |
|--|---|--|
| Field is dim   | Condenser may be too low<br>Condenser iris may be closed  | Raise the condenser<br>Open the diaphragm  |
| Dark shadows in the field which move when eye piece is moved | Eye piece may be dirty<br>Eye piece or objective may be contaminated with fungi<br>Surface of eye piece may be scratched  | Clean the eye piece<br>Eye piece may need repair<br><br>A new eye piece may be needed                                    |
| The image is not clear                                       | The smeared portion of the slide may be upside down<br>There may be an air bubble in the oil<br>The oil may be of poor quality<br><br>There may be dirt on the lens | Turn the slide over<br><br>Move the 100x lens from side to side<br>Use only good quality immersion oil<br>Clean the lens |
| The image through low power is not clear                     | There may be oil on the lens<br>There may be dust on the upper surface of the lens<br>The lens may be broken  | Clean the lens<br>Clean the lens<br><br>A new lens may be needed   |

## 8.6 Consequences of false-positive and false-negative smears

### *False-positive results*

- X Patients are started on treatment unnecessarily.
- X Tuberculosis medications are wasted.
- X In follow-up examinations the intensive phase of treatment is continued longer than necessary.
- X Patients may lose confidence in the National Tuberculosis Programme.

### *False-negative results*

- X Patients with tuberculosis are not treated, resulting in suffering, spread of tuberculosis and death.
- X Intensive phase treatment is not extended for the required duration, resulting in inadequate treatment.
- X Patients may lose confidence in the National Tuberculosis Programme.

## RECORDING AND REPORTING OF RESULTS

The microscopic observation must establish, first of all, if there are acidfast bacilli present in the smear and, if so, the approximate average number of these bacilli per microscopic field observed. It is recommended that a uniform pattern of reading is followed, observing 100 useful fields. A useful microscopic field is regarded as one in which cellular elements of bronchial origin (leucocytes, mucuous fibres and ciliated cells) are observed. The fields in which there are no such elements should not be included in the reading.

### 9.1 Negative

Indicate the staining method. Report “negative for acid-fast bacilli” for all smears in which no acid-fast bacilli have been seen in 100 fields.

### 9.2 Positive

Indicate the staining method. The number of acid-fast bacilli found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis disease. Results should therefore be quantified. For *Ziehl-Neelsen stained* smears the following semi-quantitative method of reporting is recommended:

| No of acid-fast bacilli (AFB) | Fields                   | Report  |
|-------------------------------|--------------------------|---|
| No AFB                        | per 100 immersion fields | No acid-fast bacilli observed<br><i>(No AFB per 100 fields)</i> |
| 1-9 AFB <sup>1</sup>          | per 100 immersion fields | record exact figure<br><i>(1 to 9 AFB per 100 fields)</i>       |
| 10 to 99 AFB                  | per 100 immersion fields | 1+<br><i>(10 to 99 AFB per 100 fields)</i>                      |
| 1 to 10 AFB                   | per field                | 2+<br><i>(1 to 10 AFB per field in 50 fields)</i>               |
| more than 10 AFB              | per field                | 3+<br><i>(More than 10 AFB per field in 20 fields)</i>          |

<sup>1</sup>A finding of three or fewer bacilli in 100 fields does not correlate well with culture positivity

### 9.3 Quantification of fluorochrome smear results

When fluorochrome staining methods are used, smears are examined at much lower magnifications (typically 250x to 630x) than those commonly used for carbolfuchsin-stained smears (1 000x). Each field examined under fluorescence microscopy, therefore, has a larger area than that seen with bright field microscopy. Thus, a report based on a fluorochrome-stained smear examined at 250x may contain much larger numbers of bacilli than a similar report from the same specimen stained with carbolfuchsin and examined at 1 000x. To minimise confusion that conceivably could occur when different magnifications are used for smear examination and quantitative reporting of results, a method has been suggested whereby the number of acid-fast bacilli observed under fluorochrome staining could be divided by a “magnification factor” to yield an approximate number that might be observed if the same smear were examined under 1 000x after carbolfuchsin stain (see illustrations).

A simple table using the magnification factors enables reports to be comparable from laboratory to laboratory regardless of the stain or magnification used:

| 1<br>Carbol Fuchsin<br>1 000x | 2<br>Report               | 3<br>Fluorehent microscopy magnification* |                            |                            |
|-------------------------------|---------------------------|---|----------------------------|----------------------------|
|                               |                           | 250x                                      | 450x                       | 630x                       |
| 0                             | No acid-fast bacilli seen | 0   | 0                          | 0                          |
| 1-9 / 100 fields              | Report exact count        | Divide observed count by 10               | Divide observed count by 4 | Divide observed count by 2 |
| 10-99/100 fields              | +1                        |   |                            |                            |
| 1-10/field                    | +2                        |   |                            |                            |
| >10 / field                   | +3                        |   |                            |                            |

\* To adjust for altered magnification of fluorescent microscope divide the number of organisms seen by the factor provided and refer to column 1 for range and column 2 for what to report.

*Example:* Suppose 20 acid-fast bacilli are observed per field using the 450x magnification. If this number is divided by the magnification factor of 4 according to the table, the comparable number of bacilli that would have been observed under 1 000x is 5 per field. The laboratory result should therefore read 2+, and not 3+, as originally indicated by 20 acid-fast bacilli per field.

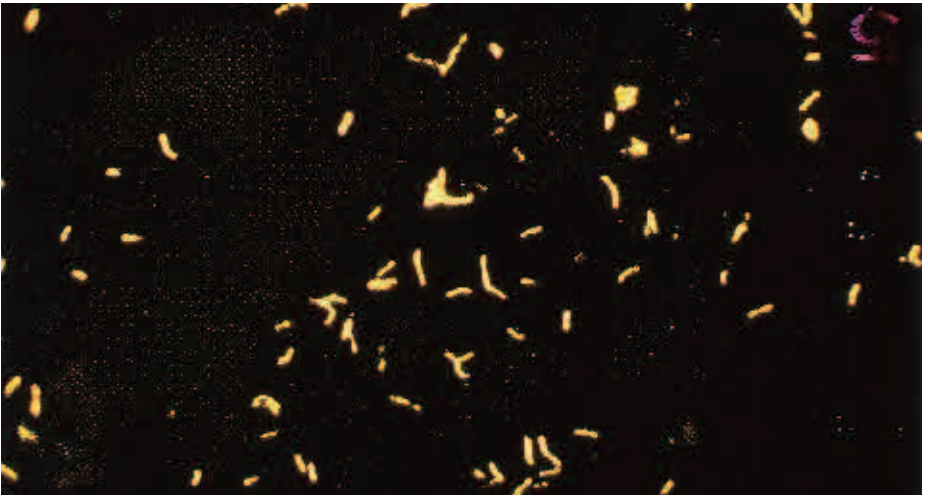
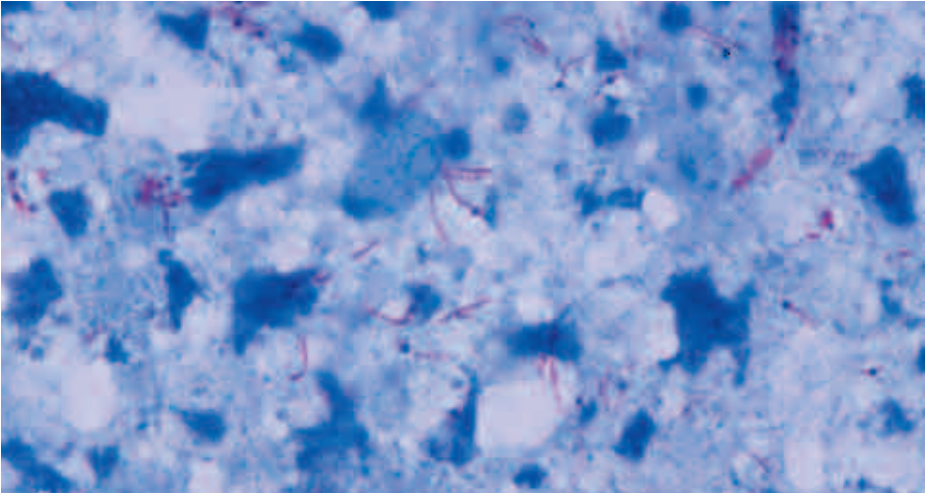
The microscopy report should be made available as soon as possible, preferably with no more than 24 hours delay from the moment of receipt of the specimen in the laboratory. The final microscopy report should contain the following information:

- evaluation of the quality of the sputum specimen
- the staining method used
- the average number of acid-fast bacilli seen on the smear
- indication of any large numbers of clumps, which means that the actual number of acid-fast bacilli may be larger than reported
- report *only* the number of acid-fast bacilli seen: *do not try to identify the mycobacterial species*
- the date of examination and the microscopist's signature
- always send a report to the referring health centre. Never give the results only to the patient. If s/he fails to bring the results to the health centre, s/he may not receive the necessary treatment

A model microscopy report form is presented in Annex 4.

All smear results should be recorded in the laboratory register, which should contain the following information: patient name, sex, age, name of health centre and patient number, reason for examination (diagnosis or follow-up of chemotherapy), microscopy results and remarks (if necessary). It is recommended that positive results be written in red ink. Individual patient reports should then be prepared from the laboratory register.

A model laboratory register is presented in Annex 5.



Acid-fast bacilli in sputum appearing as red rods against a blue background (Ziehl-Neelsen staining) or as bright yellow rods against a dark background (Auramine O fluorescent staining).

## QUALITY CONTROL

*Quality assurance* with regard to tuberculosis microscopy is a system designed to continuously improve the reliability, efficiency and use of microscopy as diagnostic and monitoring option. The purpose of a quality assurance programme is to improve the efficiency and reliability of smear microscopy services. The components of a quality assurance programme are:

- quality control (see part I, page 41),
- quality improvement (see part I, page 41),
- proficiency testing (see part I, page 43).

\* see reference 15.

The following section will focus on aspects of *quality control* in the microscopy laboratory. For a discussion on quality improvement and proficiency testing please refer to the Management Series.

Quality control of microscopy is a process of effective and systematic *internal* monitoring of the performance of bench work in the microscopy laboratory. Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible. This is accomplished by assessing the quality of specimens, by monitoring the performance of microscopy procedures, reagents and equipment against established limits, by reviewing microscopy results and by documenting the validity of microscopy methods.

Quality control should be performed on a regular basis in the microscopy laboratory to ensure reliability and reproducibility of laboratory results. For a quality control programme to be of value, it must be practical and workable.

*Quality control is the responsibility of all laboratory workers*

Quality control must be applied to:

- laboratory arrangement
- equipment
- collection and transport of specimens
- handling of specimens
- reagents and methods
- reporting of results



The keys to successful quality control are:

- adequately trained, interested and committed staff
- common-sense use of practical procedures
- a willingness to admit and rectify mistakes
- effective communication

Quality control measures which must be in place in all tuberculosis microscopy laboratories include:

***Laboratory arrangement and administration***

- Ensure that doors in the laboratory are always closed. Work areas, equipment and supplies should be arranged for logical and efficient work flow. Work areas should be kept free of dust. Benches should be swabbed at least once a day with an appropriate disinfectant (eg. 5% phenol)
- Every procedure performed in the laboratory must be written out exactly as carried out and be kept in the laboratory for easy reference. Any changes must be dated and initialised by the laboratory supervisor
- All records should be retained for two years
- Laboratory procedures used routinely should be those that have been published in reputable microbiological books, manuals or journals

***Laboratory equipment***

- Equipment should meet the manufacturers claims and specifications.
- Written operating and cleaning instructions must be kept in a file for all equipment.
- Dated service records must be kept for all equipment.
- Equipment must be monitored regularly to ensure the constant accuracy and precision necessary. For the microscope this entails the following:

*After daily use*

- Wipe oil from objective, condenser and stage with lens paper soaked in xylene.
- Turn off the microscope light source. Adjust the variable voltage regulator setting to minimum before turning off the lamp.
- Replace microscope cover.

*Monthly*

- Use an airbrush to blow away dust. (A simple airbrush can be made by attaching a Pasteur pipette to a rubber bulb). Clean objectives, eye-pieces and condenser with lens paper soaked in xylene.
- Remove slide holder from the stage and clean.
- Wipe dust off the body of the microscope and the window of the illuminator in the base using a water-moistened paper towel.

*Six-monthly*

- Have the microscope inspected, cleaned and lubricated by professional service personnel.

*Specimens and request forms*

- Perform microscopy only upon written request of authorised persons and do not allow oral requests without follow-up written instructions.
- Insist on specimen request forms being kept separate from the specimens themselves. Forms that have been contaminated by specimens should be sterilised by autoclaving or burning.
- Insist on adequately completed request forms and proper labelling of specimens to ensure positive identification of patients. Reject specimens that cannot be properly identified.
- Evaluate the quality of sputum specimens and make a note if a specimen resembles saliva. The report should state “specimen resembled saliva - interpret a negative result with caution” (to facilitate reporting a rubber stamp with the comment can be made).
- Discard leaking and broken specimen containers by autoclaving and request a repeat specimen.
- Document the arrival time of specimens in the laboratory and note any delays in delivery on the report form, particularly with negative results.

*Reagents and stains*

- All containers of stains and reagents should show the date received and the date first opened. Any material found to be unsatisfactory should be recorded as such and removed from the laboratory immediately.
- Stocks should be limited to six months' supply and regular stock rotation should take place to avoid unnecessary expiry.

***Staining and smear examination***

- Stain slides in batches with a maximum of 12 slides per batch.
- Include positive and negative controls with each day's reading, especially if fewer than 10 slides are examined per day.
- Read control slides before patient smears are read. Unacceptable control slides include the following:

*Carbolfuchsin*

- positive control is not stained red
- negative control remains red after decolourisation
- background is not properly decolourised

*Fluorochrome*

- negative control fluoresces
  - positive control does not fluoresce or is dull
  - background is not properly decolourised or fluoresces
- When the problem has been resolved, re-stain control slides as well as all patient slides from the problem run.
  - Clean the slides with xylene and preserve them in separate slide boxes for external quality control. Pour 2-3ml of xylene onto the stained side of the slide and allow to dry. Do not clean too vigorously or the stain may come off.
  - Retain all positive and negative slides, in the order in which the slides have been examined, for external quality control according to established procedures (see I. Organisation and management).

***Reporting and administration***

- Send microscopy results out as soon as they become available and preferably within 24 hours of receipt of the sputum specimen.
- Analyse microscopy results on a weekly and monthly basis for the percentage of positive results. Investigate any sharp differences from the norm. To identify excessive numbers of positive results, compare the number of suspects examined to the number of smear-positives identified: on average, for each smear-positive patient found there should be around 10 suspects examined. If there is a marked difference there may be a problem with the quality of microscopy or health workers may not be identifying suspects properly. The suspect to case ratio of 10:1 may vary considerably from setting to setting and it is recommended that the average ratio be determined locally. Any deviation from the norm should then be investigated.
- Always check multiple positive smear results following on each other. This may suggest transfer of organisms during smear preparation or during staining.

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## ESSENTIAL EQUIPMENT AND SUPPLIES FOR A PERIPHERAL MICROSCOPY LABORATORY PERFORMING ZIEHL-NEELSEN MICROSCOPY (4000 SPECIMENS PER YEAR)

| Item  | Quantity/volume required |
|---|--------------------------|
| <i>Equipment</i>  |                          |
| <b>Bunsen Burner</b> for use with butane gas .....  | 1                        |
| <b>Butane gas cylinders or</b> .....  | 2                        |
| <b>Spirit lamp</b> , cotton wool plug or metal wire .....   | 1                        |
| <b>Microscope</b> , binocular, for use with daylight or/and electric power,<br>oil immersion lens (100x), eye pieces (8x or 10x), spare bulbs ..... | 1                        |
| <i>Supplies</i>   |                          |
| <b>Adhesive labels</b> for sputum containers .....  | 5 000                    |
| <b>Amber bottle</b> , glass, 100ml .....  | 3                        |
| <b>Apparatus</b> for distilled water .....  | 1                        |
| <b>Boiling flask</b> , glass, 1 000ml capacity .....  | 2                        |
| <b>Bowl</b> , plastic, 50x30cm .....  | 4                        |
| <b>Bucket</b> , plastic, 12 liters capacity .....   | 4                        |
| <b>Cotton wool</b> , white absorbent .....  | 1kg                      |
| <b>Diamond pen</b> .....  | 2                        |
| <b>Disinfectant</b> , bactericidal eg. 5% phenol, 5% sodium hypochloride .....  | 20 liters                |
| <b>Drop bottle</b> , plastic, 10ml capacity .....   | 2                        |
| glass, 100ml capacity .....   | 1                        |
| <b>Filter paper</b> , 15cm diameter, No 1 .....   | 8 boxes                  |
| <b>Forceps</b> , stainless steel, 15cm .....  | 1                        |
| <b>Funnels</b> , glass, 45mm or 60mm diameter .....   | 3                        |
| glass, 90mm or 125mm diameter .....   | 3                        |
| <b>Gloves</b> , disposable .....  | 400                      |
| <b>Laboratory request form</b> .....  | 5 000                    |
| <b>Laboratory register</b> .....  | 5                        |
| <b>Laboratory report form</b> .....   | 5 000                    |
| <b>Lens tissue</b> .....  | 4 boxes                  |
| <b>Measuring cylinder</b> , glass, 100ml capacity .....   | 1                        |
| <b>Methanol</b> , for burner .....  | 2.5 liters               |
| <b>Microscope slides</b> , 25mmx75mm, 1.1-1.3mm thick .....   | 5 000                    |
| <b>Overall, laboratory coat</b> .....   | 2 per person             |
| <b>Paper towels</b> , disposable .....  | 2 boxes                  |
| <b>Pens</b> , ball point, red ink .....   | 4                        |
| <b>Pens</b> , ball point, black or blue ink .....   | 8                        |
| <b>Pressure cooker</b> .....  | 1                        |
| <b>Scissors</b> , stainless steel, 25cm .....   | 1                        |
| <b>Slide rack</b> , plastic, 12-25 slides .....   | 2                        |
| <b>Slide storage box</b> , cardboard or metal, 12-25 20 slide capacity .....  | 20                       |
| <b>Sputum containers</b> , plastic, disposable, 45-50ml, wide mouthed,<br>screw-capped, combustible .....   | 5 000                    |
| <b>Staining rack</b> .....  | 1                        |
| <b>Timer</b> , 0-60 minutes, with alarm .....   | 1                        |

|   |       |
|---|-------|
| <b>Volumetric flask</b> , glass, 500ml capacity ..... | 2     |
| <b>Wash bottle</b> , plastic, 500ml capacity .....    | 2     |
| <b>Wire loop holder</b> .....                         | 5     |
| <b>Wooden applicators or</b> .....                    | 5 000 |
| <b>Nickel-chromium wire</b> , 1mm diameter            |       |

*Reagents*

|   |           |
|---|-----------|
| <b>Acid-alcohol</b> , for Ziehl-Neelsen staining .....  | 6 liters  |
| <b>Aqueous methylene blue</b> .....                     | 4 liters  |
| <b>Carbolfuchsin</b> , for Ziehl-Neelsen staining ..... | 12 liters |
| <b>Immersion oil</b> .....                              | 400ml     |
| <b>Methylated spirits</b> .....                         | 4 liters  |
| <b>Xylene</b> .....                                     | 400ml     |

## SPUTUM COLLECTION

### PROCEDURE

- Give the patient confidence by explaining to him/her the reason for sputum collection.
- Instruct the patient to rinse his/her mouth with water before producing the specimen. This will help to remove food and any contaminating bacteria in the mouth.
- Instruct the patient to take two deep breaths, holding the breath for a few seconds after each inhalation and then exhaling slowly. Ask him/her to breathe in a third time and then forcefully blow the air out. Ask him/her to breathe in again and then cough. This should produce a specimen from deep in the lungs. Ask the patient to hold the sputum container close to the lips and to spit into it gently after a productive cough. Sputum is frequently thick and mucoid, but it may be fluid, with chunks of dead tissue from a lesion in the lung. The colour may be a dull white or a dull light green. Bloody specimens will be red or brown. Thin, clear saliva or nasopharyngeal discharge is not sputum and is of little diagnostic value for tuberculosis.
- If the sputum is insufficient encourage the patient to cough again until a satisfactory specimen is obtained. Remember that many patients cannot produce sputum from deep in the respiratory track in a few minutes. Give him/her sufficient time to produce an expectoration which s/he feels is produced by a deep cough.
- If there is no expectoration, consider the container used and dispose of it in the appropriate manner.
- Check that the container is securely closed and label the *container* (not the lid) clearly.
- Wash hands with soap and water.
- Give the patient a new sputum container and make sure that s/he understands that a specimen must be produced as soon as s/he wakes up in the morning.
- Demonstrate to the patient how the container should be securely closed.
- Instruct the patient to bring the specimen back to the health centre or laboratory.





## LABORATORY REQUEST FORM

Name of Health Centre \_\_\_\_\_ Date \_\_\_\_\_

Name of patient \_\_\_\_\_ Age \_\_\_\_\_ Sex M  F Complete address: \_\_\_\_\_  
\_\_\_\_\_

Patient's register number\* \_\_\_\_\_

Source of specimen  Pulmonary  
 Extra-pulmonary Site \_\_\_\_\_Reason for examination  Diagnosis  
 Follow-up of chemotherapy

Specimen identification number \_\_\_\_\_ Date \_\_\_\_\_

Signature of person requesting examination \_\_\_\_\_

*\* Be sure to enter the register number for the follow-up of patients on chemotherapy*



**MICROSCOPY RESULTS**

Laboratory serial number: \_\_\_\_\_

*Visual appearance of sputum*

|            | Mucopurulent             | Blood-stained            | Saliva                   |
|------------|--------------------------|--------------------------|--------------------------|
| Specimen 1 | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Specimen 2 | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Specimen 3 | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

*Microscopy results*

Staining method       Ziehl-Neelsen       Fluorochrome

| Date | Specimen | Results* | Positive (grading) |    |    |         |
|------|----------|----------|--------------------|----|----|---------|
|      |          |          | 3+                 | 2+ | 1+ | 1-9 AFB |
|      | 1        |          |                    |    |    |         |
|      | 2        |          |                    |    |    |         |
|      | 3        |          |                    |    |    |         |

*\*Write negative or positive*

Date: \_\_\_\_\_ Examined by (signature): \_\_\_\_\_



## LABORATORY REGISTER

| Lab Serial no. | Date | Name (in full) | Sex M/F | Age | Complete adress (for new patients) | Name of referring Health Centre | Reason for examination* |           | Microscopy Results |   |   | Signature | Remarks |
|----------------|------|----------------|---------|-----|------------------------------------|---------------------------------|-------------------------|-----------|--------------------|---|---|-----------|---------|
|                |      |                |         |     |                                    |                                 | Diagnosis               | Follow-up | 1                  | 2 | 3 |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |
|                |      |                |         |     |                                    |                                 |                         |           |                    |   |   |           |         |

\* If sputum is for diagnosis, put a tick (✓) mark in the space under "Diagnosis"

If sputum is for follow-up of patients on treatment, write the patient's register number in the space under "Follow-up".

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