



ORGANISMO ANDINO DE SALUD - CONVENIO HIPÓLITO UNANUE
PROGRAMA "FORTALECIMIENTO DE LA RED DE LABORATORIOS
DE TUBERCULOSIS EN LA REGIÓN DE LAS AMÉRICAS"

HANDBOOK

FOR THE BACTERIOLOGIC DIAGNOSIS
OF TUBERCULOSIS

PART I: Smear Microscopy Update

Cataloging carried out by the Andean Health Organization Agreement –Hipolito Unanue Agreement

HANDBOOK FOR THE BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS. PART 1: SMEAR MICROSCOPY UPDATE / Program “Strengthening of the Network of Tuberculosis Laboratories in the Region of the Americas” -- Lima: ORAS - CONHU; 2018.
xx p.; ilus, tab.
DIAGNOSIS/ TUBERCULOSIS/ LABORATORIES/SMEAR MICROSCOPY

Legal deposit done in the National Library of Peru. N°..... ..

HANDBOOK FOR THE BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS. PART 1: SMEAR MICROSCOPY UPDATE

Technical coordination

María Susana Imaz. Tuberculosis Laboratory, Instituto Nacional de Enfermedades Respiratorias, ANLIS Dr. Carlos G. Malbrán, WHO Collaborating Centre for Epidemiology and Control of Tuberculosis, Argentina.

Technical review

Lucía Barrera. Independent consultant.

Susana Balandrano and Claudia Backer. Mycobacteriology Laboratory, InDRE, Supranational Laboratory, México.

Ernesto Montoro. Tuberculosis Labs Coordinator. OPS/OMS, WDC.

Consultant

Eddy Valencia Torres. Laboratorio de Referencia Nacional de Micobacterias, INS. Lima, Perú.

First Edition of the Bacilloscopy Manual, 2008.

María Delfina Sequeira de Latini. Tuberculosis laboratory, Instituto Nacional de Enfermedades Respiratorias “Emilio Coni”, ANLIS Dr. Carlos G. Malbrán, WHO Collaborating Centre for Epidemiology and Control of Tuberculosis, Argentina.

Lucia Barrera. Instituto Nacional de Enfermedades Infecciosas, Supranational Laboratory, ANLIS Dr. Carlos G. Malbrán, Argentina.

Photographs

Gonzalo Márquez. Tuberculosis Laboratory, Instituto Nacional de Enfermedades Respiratorias “Emilio Coni”, ANLIS Dr. Carlos G. Malbrán, WHO Collaborating Centre for Epidemiology and Control of Tuberculosis. Argentina

ORGANISMO ANDINO DE SALUD – CONVENIO HIPÓLITO UNANUE, 2018

Av. Paseo de la República N° 3832, Lima 27 – Perú

Telf.: (00 51-1) 422-6862 / 611 3700

<http://www.orasconhu.org>

contacto@conhu.org.pe

Tiraje: ***** ejemplares

Imprenta:

Second Edition, June 2018

This document has been prepared in the framework of the execution of the Program “Strengthening of the Network of Tuberculosis Laboratories in the Region of the Americas” financed by the Global Fund, which has the Andean Health Organization Agreement –Hipolito Unanue Agreement as a Principal Recipient; and as Subrecipients to the Council of Ministers of Health of Central America and the Dominican Republic (COMISCA) and to the Pan American Health Organization (PAHO/WHO).

It can be reviewed, summarized or translated, totally or partially without prior authorization, with the condition of specifically citing the source and not being used for commercial purposes. Rights reserved according to Law.

PART I:
Smear Microscopy Update

ABREVIATIONS AND ACRONYMS	9
INTRODUCTION	10
THE SPECIMEN	13
THE SPUTUM	13
The container	13
Number of samples and moment of sample collection	14
For diagnosis	14
For treatment monitoring	14
To organize the patient hospitalization	14
Spontaneous collection of sputum	15
Specimen quality	15
Especial methods to obtain a sputum sample	16
Sputum induction	16
Gastric lavage	17
Bronchial lavage	18
OTHER SPECIMENS	18
Urine	18
Cerebrospinal fluid	19
Pleural, ascitic, pericardial, joint and another fluids	19
Biopsies and resected material	19
Pus	20
Blood	20

SAMPLE RECEPTION, STORAGE AND TRANSPORT	21
Patient reception	21
Specimen Storage	22
Transport	22
Reception at the lab that performs smear microscopy	23
SMEAR MICROSCOPY	26
WORK PLACE AND MATERIALS	27
SMEAR PREPARATION AND FIXATION	28
STAINING	32
Ziehl Neelsen technique	32
Stain	32
Decolourisation	33
Counterstain	33
Fluorescence staining	35
Stain	35
Decolourisation	35
Counterstain	35
Fluorescence staining by bulk method	37
MICROSCOPICAL EXAMINATION AND SMEAR READING	39
Morphological characteristics of the tuberculosis bacillus	39
Reading and results report of smears staining by Ziehl Neelsen	40
Reading and results report of smears staining by fluorochromes	40
RECORDING AND REPORTING	48
MATERIAL DECONTAMINATION AND DISPOSAL	49
REGISTER SYSTEM	52
SMEAR MICROSCOPY QUALITY ASSURANCE	53

INTERNAL QUALITY CONTROL	53
Stains, staining and microscope control	55
Preparation of non-stained positive control	55
Preparation of non-stained negative control	55
Quality control of each new batch of staining reagents	55
Quality control of staining reagents in use/coloration	56
Control of record and reports	56
Performance indicators monitoring	56
EXTERNAL QUALITY ASSESSMENT	58
SELECTED BIBLIOGRAPHY	
APPENDIX I. MINIMUM REQUIREMENTS OF BIOSAFETY	59
Information and medical control of the lab staff	61
General precautions of work	62
Precautions in the specimen collection and handling	63
Handling and use of disinfectants	64
Handling of other chemical substances	64
Procedures in case of a work accident	64
APPENDIX II: REAGENTS PREPARATION	66
For the Ziehl Neelsen staining	66
For the Fluorescence staining	67
Precautions with the staining solutions	68
Care and maintenance of the balance	69
Calculation of reagent stock	69
APPENDIX III: MICROSCOPE	72
MICROSCOPY BY ZIEHL NEELSEN STAINING	72
Mechanical part	72
Illumination and optical elements	73
Illumination centring (Kohler illumination)	73
Optical microscope focus	74

MICROSCOPY BY FLUORESCENCE STAINING	74
Microscope focus	75
GENERAL CARES OF MICROSCOPES	75
APPENDIX IV: DOCUMENTATION	77
Request for examination of biological specimen for TB	79
Lab register for smear microscopy and Xpert MTB/RIF	80
Register for staining reagents quality control	82

ABREVIATIONS AND ACRONYMS

DNA	Deoxyribonucleic acid
AFB	Acid-fast bacilli
PI	Performance Indicators
LED	Acronym in English Light Emitting Diode
FM	Fluorescence microscopy
NTP	National Tuberculosis Program
WHO	World Health Organization
PAHO	Pan American Health Organization
PT	Presumptive TB patients
MDR- TB	Multidrug resistant tuberculosis
RR-TB	Tuberculosis resistant to rifampicin
THE UNION	International Union for the Control of Tuberculosis and Respiratory Diseases
HIV	Human immunodeficiency virus
ZN	Ziehl Neelsen

INTRODUCTION

Tuberculosis bacillus spread is produced almost exclusively through suspended cores in little drops that are expelled in people's cough that are infected with pulmonary tuberculosis. These little drops can remain infectious in the air for a quite long time and can be inhaled by other people. The contact infection is more likely when they live together or stay for a very long time near a person who is expectorating bacilli and in a poorly ventilated environment



The higher the number of ill people coughing up bacilli in the community, the greater is the dissemination of the tuberculosis. The identification of the infectious cases is the beginning of the solution for the problem of the sick people and, essentially, for the public health problem.

Not every infected person (latent tuberculosis) gets ill, only 1 out of 10 approximately, which are the most susceptible. Tuberculosis can manifest in any organ, because the *Mycobacterium tuberculosis* spreads through the entire organism. However, the pulmonary disease is more infectious and frequent (80-85 % of all diagnosed cases) due to the bacillus needs plenty oxygen to develop. At the apex of the lungs, cavities are developed in which huge amounts of bacilli host and when they are coughed up, they can be detected in sputum samples. Most known symptoms of pulmonary tuberculosis are the cough and spitting that persist for 2 weeks or more. People with these symptoms are called presumptive TB patients (PT). Other manifestations can be weight loss, night sweats and chest pain.

The accurate diagnosis of TB can be performed in a reliable way at the lab showing the presence of bacilli in a specimen of the lesion by means of smear microscopy, culture or a rapid molecular test (like the Xpert MTB/Ultra RIF or the TB-LAMP).

For smear microscopy to be positive, the specimen must have at least 5,000 to 10,000 bacilli per milliliter. This high quantity of bacilli occurs in patients with pulmonary tuberculosis, especially in those whose lesions are severe, with cavitations. These patients are the ones with higher chances to spread bacilli, keeping the illness within the community. The smear microscopy, however, shows limited sensibility for extrapulmonary illness diagnosis and in some patients groups, like children and people with HIV. New diagnostic tools recommended by WHO for the past 10 years (like liquid culture and the rapid molecular tests like the Xpert MTB/RIF) allow to improve chances to detect the bacillus.

The smear microscopy is not specific for *M. tuberculosis*, but reveals the presence of the *Mycobacterium* gender, whether of *Mycobacterium tuberculosis* or environmental mycobacteria. These latter can cause illness only in particular situations. Most of the times, they can be found as colonizers or contaminants.

The main objective of the National Tuberculosis Programmes (NTPs) is to cut the transmission chain, diagnosing on an early stage the infectious cases and to treat them with successful schemes with an almost 100 % of effectiveness.

In many countries, smear microscopy is still the first diagnostic exam employed in group of presumptive TB patients not prioritized for the use of rapid molecular methods. It is simple, economic and efficient to detect infectious cases.

It is necessary to count on sufficient labs to assure to assure that patients have a fast, precise and accesible diagnosis.

Laboratory services are more efficient and potent when they become part of a National TB Laboratory Network that must involve laboratories from the public health system of all jurisdictions including the ones that provide services to prisons, health insurance system, private health system and non-governmental organizations. The management of this network must be integrated at the programming and decision level of the NTP which, in turn, must make the necessary arrangements to sustain the organization and operation of this network.

Every component of this network has responsibilities and they complement each other to assure access to a fast and reliable diagnosis through smear microscopy. All health units must receive samples from PT patients which must be studied. Primary health center labs must perform smear microscopy

and integrate to quality assurance programs. Intermediate labs add among their responsibilities to train the staff of the laboratories in their jurisdiction and to assure in them the quality of smear microscopy. Central or national reference laboratories must be capable to organize the quality assurance program all over the country, keeping under evaluation the smear microscopy offer and performance, providing tools for the lab staff training at all levels, planning and managing those supplies for which central acquisition is considered convenient. The rest of the components of the NTP have to use appropriately the smear microscopy offer and the results produced by the laboratory network.

For the smear microscopy to become a good control tool, technical quality is not good enough. It is also necessary the quality of records, the laboratory reports and the analysis of the information produced by the laboratory.

The standardization of the procedures involved in the smear microscopy is based on technical regulations which are the result of a wide experience regularly checked by international organizations like the Pan American Health Organization (PAHO)/ the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (UNION).

The first Tuberculosis Smear Microscopy Manual released by the PAHO for Latin America was written by Dr. Luis Herrera Malmsten, Head of the Tuberculosis Department at the Bacteriological Institute in Chile, edited and approved by an Advisory Committee on Bacteriological Tuberculosis and published as a CD/TB- ST/LAB document in 1973. This handbook was used until, in 1983, an Advisory Committee of the PAHO/WHO, updated the norms in the CEPANZO's n° 26 Technical Note (PAHO/WHO). The Committee was integrated by Drs. Lamberto Blancarte from the Central Tuberculosis Lab in Mexico; Omar Latini from the Epidemiology National Institute in Argentina, Adalbert Laszlo from

PART I: *Smear Microscopy Update*

the Center for Disease Control in Canada and Pedro Valenzuela from the Public Health Institute in Chile and worked under the supervision of Drs. Isabel N. de Kantor and Álvaro Yáñez from the Pan American Health Organization.

In 2008, the handbook was updated, basically incorporating elements and tools to guarantee the quality of the results and to improve people and environment's safety. In the last few years, however, the recent recommendations in relation to the biosafety of the tuberculosis labs, as the advent of new diagnose methods, especially related to the fluorescence microscopy with LED lamp, have forced to update this handbook.

THE SPECIMEN

To obtain reliable results in a laboratory, it is not only necessary to perform the techniques in the correct way, but also it is essential to receive a good specimen, meaning that:

- it comes from the anatomical site where TB is suspected
- it is an enough amount,
- it is placed in an appropriate and clean container,
- it has a proper identification,
- it is well storage and transported.

The most commonly specimen examined is the sputum, because TB disease most commonly affects the lungs. However, as TB disease can occur in almost any anatomical site, the examination of diverse specimens can be required: urine, cerebrospinal fluid, pleural fluid, ascitic fluid, blood, pus, biopsy specimens, among others. These extrapulmonary samples must be proceeded also by culture or (for some types of specimens, especially the cerebrospinal fluid and biopsy specimens) through a rapid molecular test (like the Xpert MTB/RIF), as stated by national policies.

THE SPUTUM

The container

The most suitable container must have the following characteristics:

- **Wide mouth:** not less than 50 mm of diameter
- **30 to 50 ml capacity,** so the patient can be able to easily deposit the sputum inside the container without getting their hands or the walls of the container dirty so the lab can select and take the most suitable particle in order to carry out the smear.
- **Hermetic seal:** with screw cap in order to avoid spills during the transport and the production of aerosols when it's opened at the lab. There's a higher risk of aerosols and spills with pressure caps when they are being opened.



- **Break-resistant, transparent plastic material,** in order to observe the sputum quality when the PT patient handles it, to avoid breaks and spills of infected material and to ease its disposal. It is not recommended to wash and reuse glass containers to avoid possible mistakes made in the material transfer from one sample to another and to reduce the handling of potentially infectious material.

Number of samples and moment of collection

For diagnosis

Since the bacilli elimination through the sputum is not regular, it is convenient to analyze more than one sample of every PT patient. The first specimen detects around 80% of positive cases, the second identifies 15% and the third adds 5% more. For technical and operational reasons, international organisms recommend two sputum samples per PT patient.

The first sample must always be taken at the moment of the appointment (spot sample), when the doctor or another person from the health staff identifies the PT. The second one must be collected by the patient at his house in the morning once awake (overnight sample). The collection of the spot sample assures that at least one smear microscopy of the PT can be performed. Nevertheless, it is more likely to excrete bacilli in the overnight sample, so major efforts must be done for the person to return with another sample.

For treatment monitoring

The standard tuberculosis treatment has two phases: an initial intensive one that lasts between 2 or 3 months and another one of continuation that lasts 4 to 7 months, depending on the adopted scheme of treatment.

The gradual and sustained decrease on the smear microscopy positivity scale until a negative result indicates a good progress of the patient.

For those patients who start a standard scheme treatment of 6 months, it is recommended to examine a specimen by smear microscopy at the end of the intensive phase, during the fifth month and at the end of the treatment. If the smear microscopy at or after the second month of treatment is positive, the sample will be sent for culture in case a drug susceptibility testing would be required. It is important to emphasize that the use of rapid molecular test, like the Xpert MTB/RIF, is not suitable for the treatment monitoring because these tests detect DNA residual of no-viable bacilli. However, the Xpert MTB/RIF can be useful to detect rifampicin resistance on a patient that remains smear-positive at month two or during the following months of treatment.

The treatment failure detection is more accurate when it is based on repeated positive smear microscopy results in successive patient samples. Some patients that start their treatment with highly positive smear microscopy and have a good response can still be positive at the end of the intensive phase although in a lower level. It is also possible that dead sputum bacilli can be seen in the microscopy exam. The culture allows detecting if those bacilli are alive or no-viable. If the majority is no-viable, the culture will show a few colonies or will be negative despite the positivity of the smear microscopy and this will match with a favorable clinical evolution.

Treatment of multidrug-resistant TB (MDR-TB) or rifampicin-resistant TB (RR-TB) must be monitored through smear microscopy and cultures. If resources allow, it is recommended to perform a culture per month throughout the entire treatment.

To organize the patient hospitalization

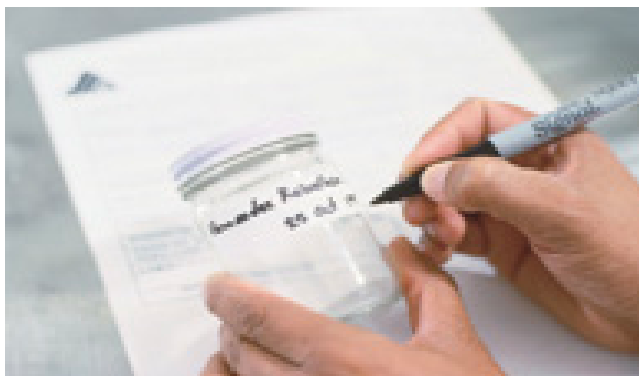
To avoid in-hospital tuberculosis transmission, the bacilliferous patient that, unusually needs to be hospitalized shall remain in isolation until a battery of three sputum samples taken in successive days is smear-negative.

Spontaneous collection of sputum

The first step to assure the smear microscopy quality consists on explain to the PT patient, with details, the importance of examining the sputum sample, the need to collect sputum and not saliva, the way to obtain a good sample, where to collect it and how to handle it until deliver to the lab.

For the collection of sputum samples, the following recommendations should be taken into account:

- Choose a well-ventilated place that offers privacy for the PT to produce expectoration. It can be a well-ventilated room with access to natural light (sun) or an open place with low flow of people in the yard of the Health Service. Do not use closed spaces like labs, doctor's office, waiting rooms or bathrooms, because this process is the riskiest among all the necessary to perform a smear microscopy.
- Give the labeled container to PT with the name or ID and, if possible, the facility that requires the smear microscopy. These data must be written at the container's walls and not on the cap to avoid mistakes, using labels that do not peel off or with a permanent ink pen.



- Ask the PT to give a good sputum sample using the proper words (phlegm, moisture, mucus, etc.), giving instructions with a simple language:

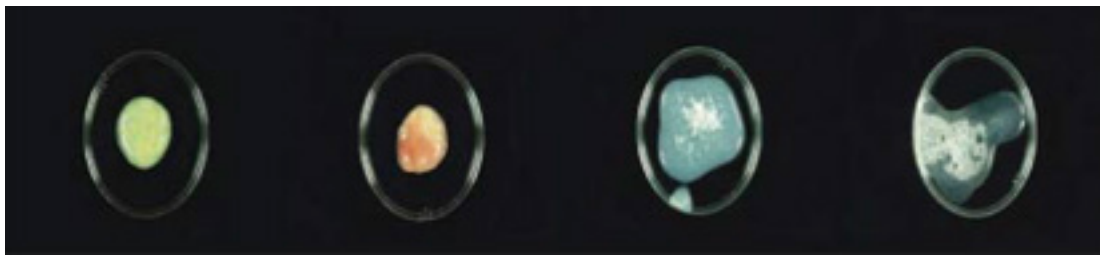
- to inhale filling their lungs with air as much as possible
- to hold breath for a moment
- to spit the sample by coughing, trying to throw out the secretions from the bronchial tree.
- to collect all the sputum sample inside the container without getting hands or external walls dirty.
- to repeat two or three times collecting all secretions in the same container.
- to clean the outside of the container with a tissue and wash hands with water and soap.



Specimen quality

The mucopurulent sputum sample that comes from the bronchial tree is the one that assures the greatest probability of observing the presence of the bacilli

A good sample has around 3 to 5 ml, is usually thick and sticky. It can be fluid with particles of purulent material. The color can change (white, yellow and even green). Sometimes they have blood. Nasal, pharyngeal secretions or saliva are not good samples to investigate for tuberculosis, although it is convenient to examine them, because there's always a chance they have part of the expectoration or bacilli expelled through cough that have remained in the mouth, nose or pharynx.



Mucopurulent

Bloody

Mucosa

Saliva

Especial methods to obtain a sputum sample

Always try to get spontaneous expectoration, because it produces the sample with the greatest richness in bacilli. Regarding to patients who are not able to expectorate, like children, mentally ill patients or the elderly, there are other less efficient ways to obtain the sputum sample such as the sputum induction or gastric lavage. These procedures require especial biosafety equipment and measures and must be performed by experienced staff.

Sputum induction

It consists in fluidizing the secretions by means of nebulization with physiological solution and then facilitating their drainage. The procedure requires well-trained personnel and, in case of applying massage and catheter, very specialized. It implies a high risk of infection with the tuberculosis bacillus for the staff nursing the patient, so this method should be used only when there's no other choice.

Perform the procedure at the sampling room or another place with good ventilation (it is highly advisable to perform the procedure in a room with UV light- turned off when the procedure is being done- and a mechanical ventilation system).

- Use disposable biosafety facemasks (N95 respirators)
- Nebulize for 15 minutes with saline solution at a temperature just above the body
- To facilitate the sputum expulsion, it may be convenient to lay the patient face down with a pillow under the chest and the head outside the stretcher and lower, and if possible, massaging with physiotherapeutic techniques
- Repeat the procedure up to three times if necessary
- Collect the first sputum produced
- Give a second container for the person to collect the produced secretions in the next 24 hours
- Discard the masks
- Sterilize the material used and then wash it with detergent and plenty of water

When dealing with children who do not know how to expectorate, after nebulization and physiotherapeutic massage, secretions must be suctioned with a manual or mechanical suction pump. For the manual procedure, it may be possible to use secretory aspirators or to connect a wet K30 nasogastric tube only until the nasopharynx with the catheter attached to a syringe, in order to aspirate the secretions. For mechanical

suction, the K30 nasogastric tube is placed in the same way, connected to a nozzle (of the type used to perfuse solutions) and the secretions are aspirated with an electric aspirator as gently as possible. The secretions will be retained in the ampoule of the tubing.

Although it is not mucous, the collected material should be examined by smear microscopy, culture and a rapid molecular test (like the Xpert MTB/RIF or the Xpert MTB / Ultra RIF), as established by national policies.

Gastric lavage

It is used to detect bacilli in the sputum ingested while they are in the stomach, especially in children who do not know how to expectorate. Gastric lavage smear microscopy has a relative value. On one hand, child patients present lesions that contain few bacilli, and therefore are unlikely to be detected by this methodology. On the other hand, it is possible that the sample contains environmental bacteria coming from food or water that may lead to false positive results.

The sample collection must be performed by a doctor or an experienced nurse. To avoid delays in the process, the sample collection must be programmed with the lab staff.

It is recommended to use this sample for diagnosis purpose only and not on the treatment monitoring. The next recommendations must be followed:

Number of samples: at least three

Container: the suitable for sputum

Moment of collection; in the morning, upon waking up, at fasting state, due to the food consumption makes the sputum ingested goes straight to the intestine. Fast must not be too long and there shouldn't be alimentary stimulation that increases heartburn (i.e.: the presence of the mother to the nursing baby).

Technique: a catheter of length and diameter suitable for the age of the patient is introduced into the stomach. Once the tube reaches the stomach, the gastric content (usually 3-5 ml) is aspirated with a syringe very gently so that the suction does not cause damage. If no material is obtained, 10 to 15 ml of distilled water or sterile saline solution are inoculated and the gastric content is collected immediately afterwards, in an appropriately sized bottle. In order to neutralize the acidity of the gastric content, and that way, prevent the bacillus destruction, it is advisable to add the same amount of sodium bicarbonate solution (8 %) to the sample collected.

Storage and transport: the material must be sent immediately to the lab (in a cooler box), because it must be cultured during the next 4 hours from the collection. If, by exception, it is not possible the prompt procedure, it should be preserved in a refrigerator but for no more than 24 hours.

Sample processing: due to the suffering that the procedure implies on a child and the low performance of the smear microscopy in this population, this method should only be performed when, besides the smear microscopy, other sensitive techniques are available like culture (or other rapid molecular test like the Xpert MTB/RIF or Xpert MTB/ Ultra RIF). It is recommended to schedule with the lab the day(s) assigned for the sample collection so as to assure the immediate processing or as fast as possible for the culture. Smear microscopy has to be performed with the sample sediment previously centrifuged for 15 minutes at 3 000g, so it is convenient to have it done at the same lab that makes the culture of the sample.

Bronchial lavage

Before collecting this type of specimen, if possible, it is important to perform a smear microscopy of at least two sputum specimens to try to detect the disease without any invasive procedures and to avoid the risks associated to this practice.

The collection of this sample is reserved for the specialized doctors. The next recommendations must be followed:

- Collect the sample in a ventilated room and using biosafety facemasks.
- Use a fibrobronchoscopy sterilized no more than 15 days before.
- Give the patient a container to collect all the sputum that, through the fibrobronchoscopy stimulation, can be produced in the next 24 hours.
- Sterilize strictly the fibrobronchoscopy with 2% glutaraldehyde activated with a bicarbonate solution, according to the provider's instructions.
- After sterilizing, wash the fibrobronchoscopy vigorously to detach dead bacillus that might remain attached.

If the fibrobronchoscopy is not sterilized correctly, it might become a way to spread the tuberculosis. If, besides, it is not correctly washed after sterilization; it can also lead to false positive results in the future samples because of the remaining dead or alive bacilli.

The material must be preserved at 2-8°C and must be sent to the lab (in a cooler box) the same day of the sample collection, if possible. This material must be cultured or processed through a rapid molecular test (like the Xpert MTB/RIF or the Xpert MTB/Ultra RIF) in order to assure the best possible performance of this hard-to-get sample. To confirm the presence of viable bacilli in the case of a positive

result of the smear microscopy, it is necessary to make a culture of this sample.

OTHER SPECIMENS

All extrapulmonary samples have to be addressed for culture and eventually processed using a technique that is more sensitive than smear microscopy, such as the Xpert MTB/RIF or Xpert MTB/ultra RIF, which have been recommended for a rapid identification in samples of cerebrospinal fluids and biopsies. This recommendation is addressed to the low number of tuberculosis bacilli present in this type of specimens, which could only be detected by tests more sensitive than smear microscopy. Additionally, in other cases, it is necessary the use of culture to confirm or rule out that the sample contains saprophytic environmental mycobacteria (as in the case of the urine that results smear-positive) or, extraordinarily pathogenic ones.

Smear microscopy of fluids with a volume higher than 1 ml should be performed after centrifuging them for 15 minutes at 3 000g and in the case of tissues after disintegrating the material. For this reason, it is advisable that the smear microscopy of these samples to be performed in the same lab where the culture is performed. Given the risk of the concentration/homogenization procedures have to produce aerosols, these processes have to be performed inside a Biological Safety Cabinet

Urine

Number of samples: minimum three and maximum six

Volume and moment of collection: previous external washing with water, the patient has to collect at least 50 ml of the first voided in the morning releasing some urine first in order to reduce the contaminant germs.

Container: between 300 and 500 ml, clean and with

a wide enough mouth to ease the direct collecting.

Storage: the sample should be processed immediately because the acid pH compromises the bacillus viability. If the sample has to be carried to another lab, it is recommended to send all the urine centrifuged for 15 minutes at 3 000g, neutralizing with 1 mg of sodium bicarbonate or trisodium phosphate anhydrous and, if necessary, preserved between 2 and 8°C for no longer than 12 hours until the moment of the dispatch.

It should be remembered that a positive smear microscopy from urine's sediment is not necessarily a conclusive diagnosis for tuberculosis, as there are saprophytic mycobacteria in the urinary tract that can produce false positive results. The diagnosis must be completed with a culture and identification of the observed bacilli.

Cerebrospinal fluid

The collection of this material is reserved for the medical staff. Next details must be considered:

Number of samples: as much as the doctor considers. The higher the amount and volume of the sample collected, the higher chance of finding bacilli.

Container: sterile, 10 to 15 ml of capability and with a screw cap of hermetic seal.

Anticoagulant use: not necessary.

Storage: when the sample is processed by culture, it is convenient to process immediately or preserved at 2-8°C for no longer than 12 hours. For use in the Xpert MTB/RIF or the Xpert MTB/Ultra Rif systems, although the preservation of this type of sample for a longer period of time wouldn't affect its performance, it is recommended to process it as soon as possible and in case it is necessary to keep it, store at 2-8°C (the maximum storage time before processing is 7 days).

Pleural, ascitic, pericardial, joint and other fluids

The collection of these materials is reserved for the medical staff. The next details must be considered:

Number of samples: as much as the doctor considers.

Container: sterile with the right size to collect the sample.

Anticoagulant use: it can be removed with a heparinized syringe or, after the liquid is removed with a syringe, it can be placed in a sterile container and add two drops of 10 % sodium citrate or potassium oxalate for every 10 ml of sample.

Whenever pleural fluid specimens are collected for adenosine deaminase (ADA) technique, the opportunity should be taken to investigate by smear microscopy and culture the sediment of the specimen.

Biopsies and resected material

The collection of these materials is reserved for the medical staff.

In case of an endometrial biopsy, the sample must be mostly of uterine scraping taken during the first stage of the menstrual cycle or when ovulating.

Container: sterile

Preservatives: 1 or 2 ml of sterile saline solution or distilled water to avoid a dryness. Do not add formalin for the bacteriological study because it is lethal to the bacillus. The portion of the specimen reserved for the histopathological study must be separated to be preserved in 10 % formalin.

Storage: refrigerated. The material should be sent immediately to the lab that will perform the culture or kept in a cool place and protected from light until its dispatch. For its use in the Xpert MTB/RIF or Xpert MTB / Ultra RIF system, although the preservation of this type of sample for a longer period wouldn't affect its performance, it is advisable to process it as soon as possible and in case it is necessary to conserve it, keep it at 2-8°C (maximum storage time before processing is 7 days).

Storage: if the sample cannot be sent immediately to the lab that will process it, place the blood extracted in a vial containing 50 ml of blood culture medium (brain heart infusion (BHI) with anticoagulant). Incubate at 37° C until the moment of dispatch.

Pus

- **Container:** sterile. It is preferable not to use swabs to avoid drying out. In case they are used, they must be moistened with sterile saline solution or distilled water before taking the sample.

- **Storage:** in a fridge. The sample must be sent immediately to the lab that will perform the culture, otherwise, keep it in cool place and protected from light until the dispatch.

Blood

Blood investigation is suitable for patients with a severe immunosuppression, as in HIV cases with a low total lymphocyte or CD4 count, and with a smear microscopy of respiratory samples repeatedly negative. Next details must be considered:

Number and moment of collection: two samples of 10 ml of venous blood in consecutive days.

Sterility and biosafety: use gloves; previously disinfect with iodine alcohol the skin area where the extraction will be performed.

Anticoagulants: use syringe with heparin (do not use EDTA).

Container: transfer the blood to a dried sterile plastic tube with screw cap of hermetic seal.

SPECIMEN RECEPTION, STORAGE AND TRANSPORT

Patient reception

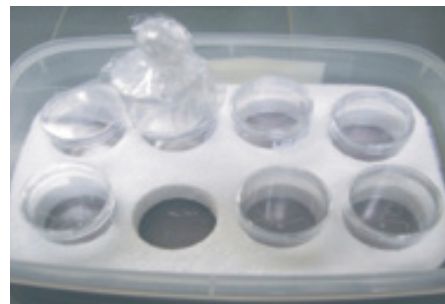
The reception of patients who deliver their samples must be organized at a place in the health facility that can be ventilated or where the air is renewed by a mechanical ventilation system. It must be agile so the patient does not have to wait. It must be considered that **the prolonged stay of patients who are expelling bacilli in a waiting room generates risk of transmission of tuberculosis** to other patients and the staff.

In order to ease the accurate identification of the tuberculosis cases, sputum samples produced by PT patients must be collected and handled at any time of the day at the most suitable moment for the patient during opening hours.

The lab must receive samples all day. Then, it can regulate the moment in which it processes them, because the sputum can be preserved for a few days, especially if it is only going to be examined by smear microscopy. Even so, the exam must be performed out as quickly as possible within a logic work routine.

At the reception moment, the following procedures must be done:

- Check that the sample containers are clearly identified on the walls of the container, not on the cap, with a hermetic seal.
- Check that the sample comes with the laboratory request form.
- Check the quality of the sample through the containers walls without open it. If it is saliva or nasal secretions it is convenient to receive it because, even if it is not a good quality sample, it may contain bacilli. Register this information in the laboratory form. Ask the patient to collect another sample.
- Place the containers in plastic boxes with a cap that can be decontaminated with sodium hypochlorite solution.



- If the patient did not collect sputum and gives the container back, put it inside the box anyways to throw it in the trash along the contaminated material just as if it had been used.
- After receiving the sample, it is necessary to streamline the procedures as much as possible. The sooner it is processed, the greater the possibility of finding *M. tuberculosis* in it by smear microscopy or culture. Room temperature and time contribute to the multiplication of germs of the bronchial tree and the mouth that denature the sputum proteins, which will difficult the picking of the useful particle to prepare the smear and, also, will contribute to the destruction of the bacillus.

The multiplication of the contaminant or regular flora of the sputum increases the chance of the culture to get contaminated.

Specimen Storage

If the sputum samples are not going to be processed during the day, it is advisable to put every container in a polyethylene bag and tie it above the cap to avoid spilling. The samples must be preserved in a fridge or a cooler, in a plastic box if possible. In case of not having a fridge, place the samples in a cool, dark place.

If the samples are going to be processed only by smear microscopy and must be inevitably stored for several days, you can add about 10 drops of 5 % phenol on the day they are received, cover the container and mix softly. This disinfectant kills all sputum germs including mycobacteria, but even then these are stained by the Ziehl-Neelsen or auramine technique.

If the samples are going to be processed by culture, they should be stored at 2-8°C for up to 7 days, although it is advisable to store them only for 3 days in order to avoid culture contamination.

Transport

In a **Health Center that does not have a lab**, the staff must know **to which lab they should send the samples, how often and** by what means of transport. **For both** smear microscopy and culture and rapid molecular test, it is recommended that the transport be done **at least twice a week**. If possible, schedule the dispatch days, transportation and departure and arrival time should be established. If the dispatches are not regularly done, it is convenient to notify in advance the lab that is going to receive the specimens.

Valid regulation must be considered in every country for sample transportation or dispatch by mail. **At least** two important conditions must be considered:

- Over heat and sun light protection
- Zero risk of spilling, if possible.

It can be used for transportation a metal or opaque plastic box, with some mechanism that locks its cover, and with a handle to facilitate its transport, such as those used to move refrigerated material or tools. They are also useful plastic boxes with airtight lid, of the type used in the home to store food or other items, slightly higher than that of sample containers. These boxes are easily decontaminated by washing with sodium hypochlorite solution. In the interior of the boxes a plate is adapted in which circles of suitable diameter are cut so that they fit in them the containers of the samples inside their bags. Then fill the spaces between the containers with absorbent paper. Paper destined to be discarded can be used.

Each submission must be accompanied by the corresponding examination request forms and a list of the patients' data: full name, service, clarification on whether it is a diagnostic sample (1st, 2nd) or for treatment monitoring indicating the month. These forms and lists must be in an envelope or nylon bag, separate from the sample containers.

It must be verified that the address of the laboratory to which the box is sent is correct, that the number of containers corresponds to that of the list and the number of application forms, that the identification of each container matches that of the list and request forms and that the list clearly shows the date of dispatch and the name and address of the health center that sends it.

Reception at the lab that performs the smear microscopy

The lab staff must:

- Use disposable gloves
- Open the box on a table exclusively for that purpose
- Inspect the samples looking for spills.
- Disinfect the outside of the containers with cotton wool with phenol 5% or sodium hypochlorite 1% if small spills have occurred during transport. If the spill has been massive, sterilize the entire box in an autoclave or incinerate it.
- Check that the samples are correctly identified.



- Disinfect the box with 1% sodium hypochlorite.
- Discard the disposable gloves.
- Wash hands after taking the gloves off.
- Record the data of each patient, type and quality of the sample received, the objective of the study

(diagnosis or treatment monitoring in the lab's register.



- Inform to the service that dispatched the samples, if necessary, the inconveniences that have been observed, especially with the quality and quantity of sputum and way of the dispatch.

The reasons to reject a sample are that:

- the container is broken or the sample overturned
- the identification of the sample in the container does not match with that of request forms for bacteriological studies
- the container has no identification
- the sample was collected inadequately (i.e.: in a piece of paper)
- If the lab that is receiving the sample does not perform culture and/or molecular tests (like the Xpert MTB/RIF, Xpert MTB/Ultra Rif or the hybridization in strips systems) it must have a connection with a reference lab that does perform it, arranging a regular transport.

- The detection of sensitive and resistant tuberculosis requires the use of diagnostic algorithms that consider the group with higher risks in order to assure the efficient use of the diagnostic resources. Such algorithms are specific for each country and, depending on them; biological specimens will be addressed for culture and/or rapid molecular test. The generic recommendations for the use of these tests on biological samples are detailed in the table. Depending on the established diagnostic algorithm, the same patient could be studied by one or more of these techniques.

Test/ procedure	Description	Referred specimens
<p style="text-align: center;">Cultures</p>	<p>Solid media</p> <p>Automated systems with commercial liquid media</p>	<p>- Samples for diagnosis:</p> <ul style="list-style-type: none"> • from patients with persistent respiratory symptoms and two or more previous samples with negative smear microscopy. • from patients suspected to have extra pulmonary TB • from children • from immunosuppressed especially from patients living with HIV • from patients with previous treatment and positive smear microscopy, especially if there was a loss of follow-up or failure. • of patients with exposure to infection with drug-resistant bacilli (contacts of cases with resistant tuberculosis, hospitalized or workers from health institutions or prisons where there are resistant tuberculosis cases) • from gastric and bronchioalveolar lavage <p>- Treatment monitoring:</p> <ul style="list-style-type: none"> • with positive smear microscopy at the end of the second month of chemotherapy or in a further control • Diagnosed cases with negative smear microscopy that become positive during the treatment
<p style="text-align: center;">Molecular methods</p>	<p>Hybridization systems in strips system that allows to identify that the bacilli present in the samples correspond to <i>M. tuberculosis</i> complex and to study the rifampicin resistance (alone or combined with isoniazid)</p>	<p>Diagnosis samples with positive smear microscopy coming from:</p> <ul style="list-style-type: none"> • patients with higher risk of having resistant TB (patients with history of antituberculosis treatment or exposure to infection by multi-drug resistant bacilli) • TB patients living with HIV

Test/ procedure	Description	Referred specimens
Molecular methods		<p>-Positive smear microscopy samples received for treatment monitoring (only to detect rifampicin resistance emergence- alone or combined with isoniazid):</p> <ul style="list-style-type: none"> • with positive smear microscopy of the second month of chemotherapy or in a further control • Diagnosed cases with negative smear microscopy that turn to positive smear microscopy during the treatment
	<p>Hybridization systems in strips that allow the identification of resistance to fluoroquinolones and second line injectable drugs in patients already identified as MDR or RR</p>	<p>- Sputum samples with positive/negative smear microscopy coming from patients identified as MDR/RR</p>
	<p>Xpert MTB/RIF or Xpert MTB / Ultra RIF assay that allows to identify the presence of the <i>M. tuberculosis</i> complex and evaluates the rifampicin resistance.</p>	<p>- Diagnosis sample</p> <ul style="list-style-type: none"> • respiratory samples of patients with a higher risk of having TB resistant (patients with history of tuberculosis treatment or exposure to multi-drug resistant bacilli) y related to HIV infection. • cerebrospinal fluid samples and biopsies coming from patients suspected to have TB . <p>- Positive smear microscopy samples collected for treatment monitoring (only to investigate sensitivity to rifampicin)</p> <ul style="list-style-type: none"> • at the control of the second month of chemotherapy or in a further control • diagnosed cases with negative smear microscopy that become positive during the treatment

SMEAR MICROSCOPY

The technique is based on the acid-alcohol resistance, which is the property that mycobacteria's wall has to bind carbol-fuchsin or auramine and hold them against the action of discolorants like the acid and alcohol mix. This characteristic is due to the high amount of lipids, particularly to the mycolic acids that mycobacteria have at the cellular wall. This way, using a right technique, it is possible to recognize the acid fast bacilli (AFB) in the patient's sample as a red/fuchsia or fluorescent stick on a background color that facilitates its visualization.

This property is not specific of the tuberculosis bacillus, but to the bacilli of the *Mycobacterium* gender, even to environmental mycobacteria and a few other microorganisms.

In any case, in countries with high tuberculosis rate, a positive smear microscopy of a respiratory sample from an immunocompetent patient has a very high predictive value for the diagnosis of tuberculosis. That is, the risk of making mistakes when diagnosing tuberculosis in this circumstance is very low.

Ziehl-Neelsen staining (ZN) has been the most used technique for the TB diagnosis in Latin America countries for the past 100 years. Comparing to the fluorescence microscopy (FM), conventional microscopy has the advantage of requiring a much simpler training, because the capacity to identify the bacillus through this method is much simpler to acquire.

In 2011, WHO recommended the use of FM with LED lamp. FM is at least 10% more sensitive than ZN microscopy. Besides, the use of this technique speeds work, since reading time of the smear reduces almost in half. Compared to the conventional FM (with a mercury lamp), the FM with LED lamp offers considerable advantages, because it does not require a totally dark room for the smear reading and it also has important functional advantages over the standard mercury lamp, as a long useful life, it does not generate heat and does not have contamination risk to the environment in case of breaking. In order to perform it with an acceptable quality, it is necessary to have personnel trained in the reading of smears stained with auramine. Given that it reduces the needed time for the reading performance and it requires a specialized staff, it is especially recommended for labs with a high load of work.

WORK PLACE AND MATERIALS

Smear microscopy can be performed at any lab that has a microscope with immersion lens in good condition, some low-cost materials and simple lab facilities. Simple and basics norms must be followed to assure quality and minimize risks.

It is advisable that the work place be exclusive, although it is not always possible. In case of sharing a lab area, it is necessary to choose an exclusive place for the smear preparation, far off the entrance to avoid airflow and the in-and-out of the staff during the sample procedure. Also, it is highly advisable to perform the smear and staining at the time of less work in the laboratory.

Basic laboratory requirements are:

- Good lightning
- Natural ventilation (windows) or mechanical (air extractor) that allows renewing lab air. In case of using an air extractor, this must have an approximate flow of 6 to 12 air changes per hour and if it is a wall air extractor, this must have an air exit to a low-flow street of the service and situated at least two and a half meters above ground. Although natural air is suitable, mechanical ventilation is more acceptable, because it assures the one-way air flow. In all cases, it must be ensured that the air flow is not directed to the table/bench in which the smears are prepared.
- Air conditioning equipment must be correctly place considering the air flow direction and must always be constantly checked that the air flowing does not point directly to the table/bench where the smears are being prepared.
- Painted walls, no peeling and washable floors that can get disinfected with a sodium hypochlorite solution.
- A 1 x 0.50 m table to place the received samples and to perform the smear covered by a flat material that resists germicide solutions (formica, stainless

steel, ceramic, tiles with minimum joints or similar materials). In case of not having this type of test table, you can use trays or cover the table with glass or paper.

- A sink with a dish and water drain in which you can wash your hands and do the staining.
- A shelf or cabinet for the reagents, slides and other materials.
- A table for the microscope, placed closed to a window in case of no electric light during the day.
- A table to write reports and laboratory records.
- A cupboard to store the smears.



PART I: Smear Microscopy Update

The basic equipment to perform smear microscopy is composed by

- Two coats or overalls exclusively used for the staff performing the smear microscopy.
- Microscope with immersion lens in good conditions, with a spare lamp. In case of performing the staining with fluorochromes, LED fluorescence or an optic microscope with an adaptor that allows to transform it into a LED fluorescence microscope with 20x and 40x objectives is needed.
- Containers for sample collection.
- Wood or cane/bamboo applicators.
- New slides, cleaned with alcohol and air-dried.
- Amber bottles for staining solutions.
- A support to hold 12 slides during the smear preparation.
- Glass sticks or other stainless support with the right dimensions to hold 12 slides during the staining
- A lighter, preferably a gas one, although an alcohol one can be used.
- A glass marker pen: oily or permanent ink (not red, to avoid mistakes), with a diamond tip, the one used to mark ceramic.
- Filter paper.
- Lens clean paper: it can be disposable paper tissue.
- A clamp.
- A swab.
- A pot to dispose the sample containers, with a cap, made with material that can be disinfected with sodium hypochlorite solution or that has a bag for pathological waste.

- 70 % etanol
- Immersion oil: it is not recommended to use cedar oil, but synthetic hydrocarbon-based oil or polymers with refractive index >1.5 because they do not dry out, get hard nor dissolve the fuchsin.
- Disinfectant solutions:
 - 5 % phenol
 - 1 % sodium hypochlorite

If the lab receives staining solutions and reagents from the References Lab ready to use, it does not require additional equipment.

If the lab receives reagents and dyes in amounts necessary to prepare a certain volume of staining solutions, it requires glass containers in order to do the preparation.

If, instead, the lab has to prepare reagents and staining solutions, it requires:

- A balance.
- Graduated glass containers to prepare solutions.
- A funnel.
- Chemical substances with quality for analysis (see appendix II).

SMEAR PREPARATION AND FIXATION

If the recommended biosafety measures are observed, the risk of getting tuberculosis associated with the preparation of a smear is much lower than that of health personnel who are close to a patient who coughs. The best measure to avoid risks and errors that can cause false results is the systematization of the activities following the following indications:

- Wash hands
- Put a coat or overall and disposable gloves.
- Put on the flat surface table, stray or paper soaked in 1% sodium hypochlorite only what is needed to perform the smear:
 - burner
 - applicators
 - supports to hold the smear
 - pen to mark the slides
 - new slides, previously soaked in alcohol and air-dried
 - no more than 12 containers with the samples



- Put on a side of the table the container to eliminate material with cap
- Organize samples and number them with their register number.



For each sample, number a slide always on the same side. I must have the same number from the lab register, the request form of the exam and the container wall. Do not touch with your fingers the part of the slide intended for the smear.

- Arrange the samples to the left of the operator or the right, always in the same position in increasing number order. Place each marked slide in front of the corresponding sample.
- Use a slide for each sample. Do not place the smear of more than one sample on a slide.
- If the samples were in movement, let them set for 20 minutes before open them.

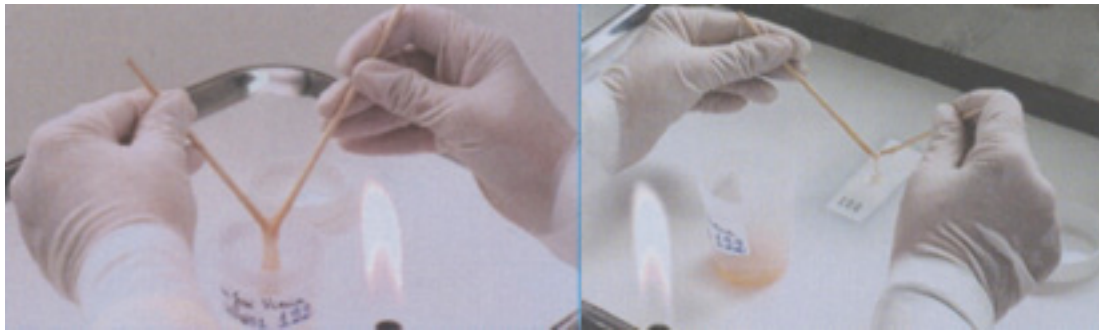
• Take the first sample and the appropriate slide and put them behind the burner so the flame stays between the operator and the container. This position will protect the technician of possible aerosols formations at the moment of opening the container.

• Carefully open the container in order to avoid aerosols formation.

• Split an applicator in two, trying to make the ends rough.

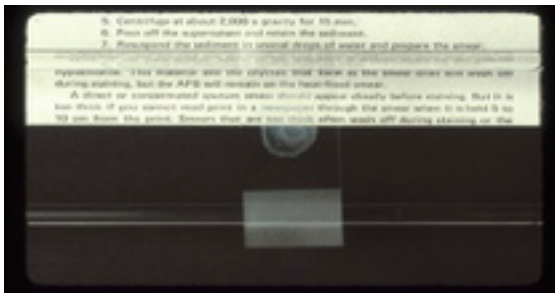
• Take each part of the applicator between the thumb and the index of each hand and with the irregular ends of each piece select the thickest or purulent particle of the sputum sample and roll it into one of the two side of the applicator with the help of the other. If the sample contains several mucopurulent portions, try to mix them with soft movements of the stick and then take a portion of the sample.

If there are only small purulent particles, choose three or more and mix them on the same slide to homogenize them



The selection of the most purulent particle from the sample is one of the most important steps in order to increase the probability to identify tuberculosis cases through direct smear microscopy

Place the selected particles on the slide and spread them with the applicator with soft, circular movements trying to spread in an even form in the center of the slide, drawing an oval of 2 cm long x1 or 2 cm wide, without reaching the edges of the slide in order to avoid the technician to get contaminated when handle it.

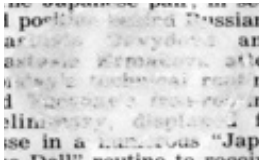


Good



Too thick

Check that the smear has a uniform and suitable thickness. If it is too thin, it is possible to have a false negative result. If it is too thick, the material can fall apart during the staining or it can be difficult to see the bacillus under a thick layer of mucus.



Good

To practice, put a printed piece of paper under the smear at a distance of 10 cm. The suitable thickness is the one that allows to see **but not read** a printed text through the mixture. Once the practice is done, it is preferable not to repeat routinely this process in order to avoid touching and transferring samples with the printed papers.



Too thin

- Leave the smear on a support placed on the side of the test table to dry at room temperature. The smear should not be heated near the flame while it's humid thus the heat alters the structure of the bacilli and its subsequent staining; besides, it can produce aerosols.

- Dispose the applicators in a disposable container; this flask will go to the autoclave or directly be incinerated.

- Close the container of the sample used to perform the smear and leave it on the opposite to the place where the containers with the specimens that have not yet been processed are, to avoid confusions.

- Continue in the same way with each of the following samples

- Keep the samples until finishing the readings of the smear microscopy and check if it is necessary or not to perform new smear or send it to culture or molecular tests.

- Clean the work surface with a paper or cotton towel soaked in 1% sodium hypochlorite to disinfect it.

- Discard the disposable gloves and samples

- Wait until the slides have dried in the air.

- Take one at a time each smear with a clamp keeping the face containing the sample up and pass them quickly over the flame of a burner three or four times taking care not to get too hot, since overheating can damage the wall of the bacillus.

The smears must be stained as soon as possible, since some bacilli remain alive after being fixed with heat until they incorporate the fuchsin



STAINING

Ziehl Neelsen (ZN) technique

Stain:

0.3 % Carbol fuchsin

Decolourising solution

3 % hydrochloric acid in ethyl alcohol or 25 % sulphuric acid.

Counterstain

0.1 % methylene blue

Stain

Put two glass sticks in parallel, separated 5 cm from each other on a support inside a coloration sink/ lavatory.

Filter the amount of fuchsin needed for the stains to be made during the day. If the amount of smears to stain is low, fuchsin can be filtered directly when it is deposited on the smear through a small funnel with filter paper



- Put the fixed smears on the support keeping the numerical order with the smear upwards and maintaining a separation of at least 1 cm between them.
- Completely cover the smear surface with the filtered carbol-fuchsin. Dispense the dye gently, without splashing and without touching the dropper or funnel with the smears.
- With the flame of a swab soaked in alcohol, heat

gently bellow the slide with swinging movements until you notice that the first white steams are released. Do not heat with a burner.

- In case of spillage of the dye, restore the fuchsin; do not let the dye dry on the smear.
- In the term of approximately five minutes, heat three times until vapor emission. This is enough for the fuchsin properly penetrates the bacilli and binds to its lipids. Make sure to keep the hot dye

on the slide for at least five minutes. Do not boil the fuchsin, because the wall of the bacilli can get destroyed and bad colored.

- With a clamp, carefully lift the slide from the end closest part to the operator. Rinse with plenty of water at low pressure (preferably distilled or purified), wash very softly (do not splash the rest of the slides) and carefully the surface of the slide completely eliminating the fuchsin solution. Turn the slide and carefully wash the back.

Note: If possible, use distilled or purified water for washing. The tap water may contain environmental mycobacteria that could adhere to the smears and become evident during the restaining of smears performed in the external quality assessment of the smear microscopy by rereading, which would lead to false positive results

- Tilt the slide to eliminate the excess of water and avoid diluting the reactants that will be used next.

Decolourisation

- Fully cover the slide with decolorizing solution and let set for three minutes.
- Rinse with plenty of water (preferably distilled or purified) at low pressure. It is considered to be decolourised when the thickest parts of the smear at most retain slight pink color. In case of the intense pink color remains, let cover again with decolouring solution, let it act from 1 to 3 minutes and rinse again.
- Eliminate the excess of water by tilting the slide

Counterstain

- Fully cover the slide with methylene blue solution.
- Let set for a minute.
- Rinse both sides of the slide with water at low

pressure and clean the bottom with cotton if colored.

- Check if the slides have the numbering clear and visible. If not, number them again.
- Let the smears dry at room temperature leaving them in vertical position on an absorbent paper support. Do not lean the absorbent paper on the smear.

TINCIÓN DE ZIEHL NEELSEN

	Cover with filtered fuchsin
	Heat until vapor emission; three times for 5 minutes (make sure to keep the dye warm over the slides for at least 5 minutes).
	Wash with water (preferably distilled or purified)
	Cover with decolouriser for 3 minutes (Repeat this step if necessary)
	Wash with water (preferably distilled or purified)
	Counterstaining with methylene blue for 1 minute
	Wash with water (preferably distilled or purified)
	Air dry

Fluorescence staining

Stain

0.1 % auramine-O

Decolourising solution:

0.5 % hydrochloric acid in ethyl alcohol

Counterstain:

0.5 % potassium permanganate or 0.3 % methylene blue

Due to the acquisition skills to recognize the bacillus through this technique requires a higher training than the ZN technique, the induction of the fluorescence technique into the lab network must assure the staff retraining with emphasis in a practical formation of moderate duration.

When the staining of the smears is performed with auramine-O, AFB are like yellow fluorescence rods. In order to observe them, the WHO advises the handling of fluorescence microscopes with LED lamps. The smears are examined with an objective of lower augmentation (20x and 40x) than in the ZN technique (100x), which allows to observe a much bigger surface of the smear in less time.

Stain

- Avoid performing thick smears. It can interfere with a suitable decolouration and the counterstain can hide the presence of AFB.
- Put the slides on a staining support in batches of no more than 12 in the same way as the ZN staining. Make sure that the slides are in level.
- Filter the amount of auramine-O necessary for the stains to be made during the day. If the number of smears to be stain is small, Auramine-O can be filtered directly when it is deposited on the smear through a small funnel with filter paper

- Fully cover the smears with auramine-O solution and let set the dye for 20 minutes minimum, making sure that the dye solution remains on the smear. Do not heat.

- Rinse with distilled water (do not splash the rest of the slides) and let drain. Do not use tap water, because normally contains chlorine and it can interfere the fluorescence.

Decolourisation

- Decolourise with alcohol-acid for 1 or 2 minutes. Care should be taken that the decolouration is as complete as possible.

- Rinse with plenty of water at low pressure (do not splash on the rest of the extended ones). If yellow clusters or intense yellow coloration is observed, cover again with decolouring solution, let set for 1 or 2 minutes and rinse again

- Tilt each slide to drain excess water.

Counterstain

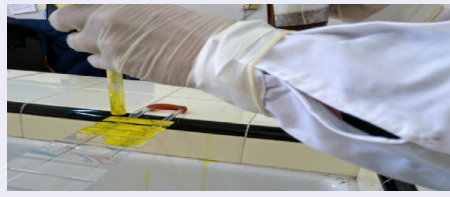
- Cover the smears with the potassium permanganate or the methylene blue solution and let set for 1 minute. If potassium permanganate is used, this time is critical, because if it is left longer than the established, AFB fluorescence can be masked.

- Rinse with distilled water and let drain by tilting the slants.

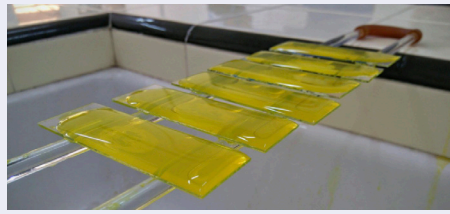
- Let the smears to air dry and protected from light. Do not dry with filter paper.

- Examine the smears microscopically as soon as possible after the staining, because AFB can lose the fluorescence, but not before the smears are fully dry.

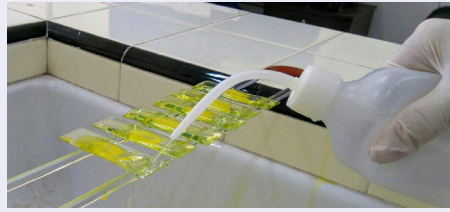
FLUORESCENCE STAINING



Cover with filtered 0.1 % auramine-O solution



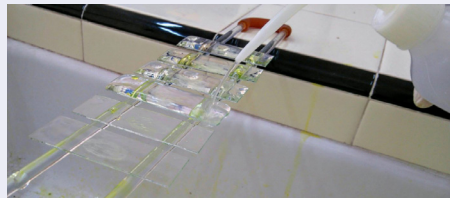
Let set for at least 20 minutes (do not heat)



Rinse with distilled water



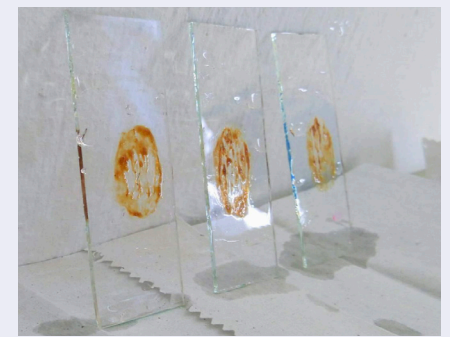
Cover with decolorant solution for 1 or 2 minutes (repeat if necessary)



Rinse with distilled water



Counterstaining with contrast colorant (0.5 % potassium permanganate or 0.3 % methylene blue) for 1 minute



Rinse with distilled water and let air dry protected from light

Fluorescence staining by bulk method

This kind of staining is suitable when the amount of smears to stain per day is higher than 10.

It is necessary to have a metallic/glass basket for smears and at least 4 glass containers suitable for the coloration baskets.



FLUORESCENCE STAINING BY BULK METHOD

	<p>Place the slides in the basket, placing the face of all the smears in the same orientation.</p> <p>Place the basket inside the container with Auramine-O and leave a minimum of 20 minutes. Make sure that the liquid covers the entire slides</p>
	<p>Fill the corresponding container with distilled water.</p> <p>Remove the basket of the auramine-O container and place in the container with water. Softly move the basket up and down (± 1 cm) two or three times (shake softly).</p>
	<p>Remove from the distilled water container and put the basket in the decolourant for 2 minutes with gentle agitation.</p>
	<p>Remove the water of the corresponding container, fully rinse and fill it again with distilled water.</p> <p>Remove the basket from the decolourant solution and put it in the container with water. Shake gently.</p>
	<p>Remove the basket from the water and put it in the counterstaining solution for 1 minute. If potassium permanganate is used, remember that this time is crucial, because if you leave more time than the established, the AFB fluorescence can be masked.</p>
	<p>Remove the water of the corresponding container, fully rinse and fill it again with distilled water.</p> <p>Remove the basket from the counterstaining solution and put it in the container of water. Shake gently</p>



Remove the basket of the water and let dry the smears protected from the light.

The auramine-O solution, kept with a cap in a cabinet protected from the light, can be used for 3 days. The permanganate solution must be daily replaced, since its oxidation creates a background colour that makes the reading more complex.

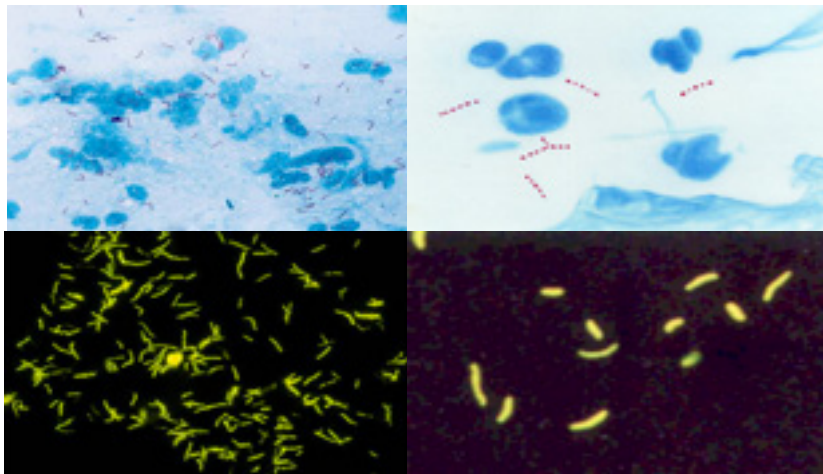
MICROSCOPICAL OBSERVATION AND SMEAR READING

The microscopic observation must fulfill these main two objectives:

- Determine if there are AFB
- If there are, quantify approximately the abundance in bacilli

Morphological characteristics of the tuberculosis bacillus

The AFB is between 1 and 10 μm long. With the ZN staining, thin, slightly curved, fushsin-red rods are observed clearly highlighting against the blue background. In the smears stained with auramine-O, rods have a fluorescence yellow color. Highly coloured granules or beads are sometimes observed. In the sputum samples they can be isolated, paired or grouped.



It is very difficult to distinguish the tuberculosis bacillus from other mycobacteria through the microscopic exam. Some mycobacteria that are not *M. tuberculosis* can appear like very long rods or bacillococci.

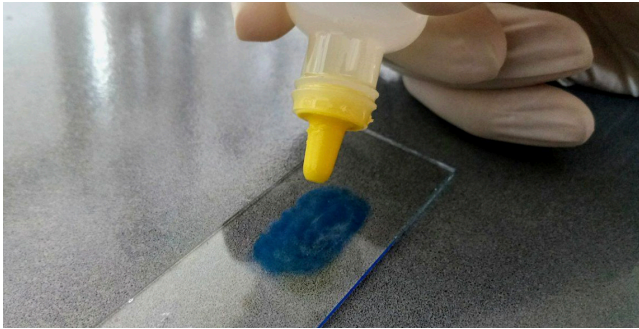
Other microorganisms can present different degrees of acid resistance, such as *Rhodococcus* spp., *Nocardia* spp., *Legionella* spp. and the *Críptosporidio* and *Isospora* spp. cysts. They look like cocci, bacteria with different forms (pleomorphics), filaments that are sometimes cut, or like big spheres, compared to bacteria.

Anyway, it is unlikely to find more than 10 acid-alcohol resistant microorganisms different from *M. tuberculosis* in the sputum samples of the PT patient. If the microscopist observes one that does not have a rod shape, he or she must consult the supervisor.

Reading and results report of the smears staining by Ziehl Neelsen

For reading, it is necessary to take into account the following indications:

- Put every element that will be used for the reading near the microscope:
 - Immersion oil
 - Tissues or soft pieces of paper
 - Lab register
 - A pen
 - A box to store the slides
- Put a drop of the immersion oil (with a refraction index higher than 1.5, see note) on one of the smear's edge without touching the smear with the dropper.



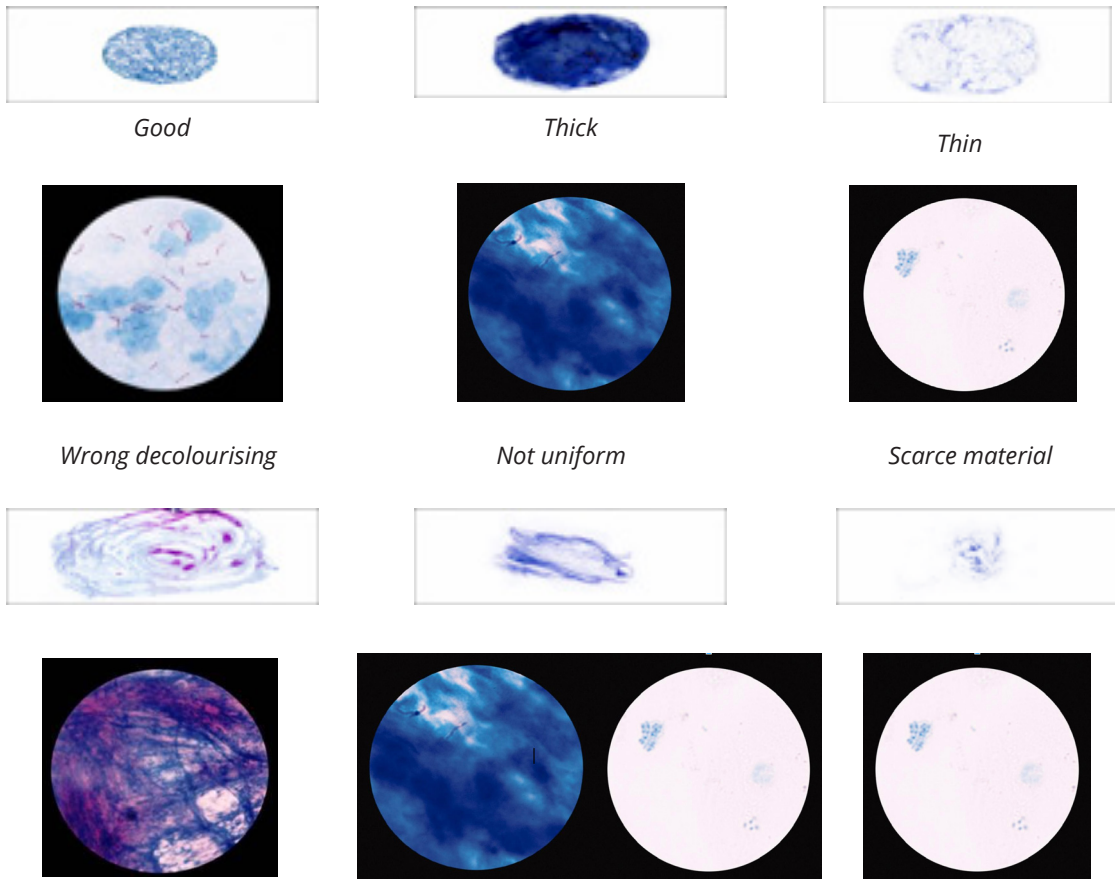
Note: to check that the refraction index of the immersion oil is higher than 1.5, place a portion of the oil in a glass container. If it turns invisible after introducing a glass stick, it means that the refraction oil is higher than 1.5.



- Using a 10x objective, focus the smear avoiding the area where the immersion oil was deposited. Explore the smear looking for the mucous or mucopurulent material.
- Carefully change the 100x objective and move the stage until getting in the area of the slide where the immersion oil was set.
- Carefully adjust the thin focus until the cells look clear.
- Observe each microscopic field in a superficial and deep way, always moving the micrometer screw before moving to the next field.
- Follow a straight line path to explore the smear avoiding repeating the reading in some fields. I.e.: from left to right.



- Observe the quality of smear and the staining. If they were not good, repeat the smear of that sample.



In case of abnormalities, identify the causes:

- If you observe AFB that move in abnormal way, they can be bacilli from another smear microscopy that were dragged by the immersion oil and it is necessary to repeat the smear microscopy.
- If there are strange bodies (artifacts) that move when the slide does, they can be food, precipitates or crystals residuals. If they only move when the eyepiece is rotated, it is dirt that is in the eyepiece and it must be cleaned.
- If they do not move, contaminant dirt or bacilli can be in the objectives, the condenser, the mirror or the illumination source; proceed to clean.

Count the number of fields you have read and the identified AFB. A 10x10 grid, that represents the 100 microscopic fields, can be used as an aid to register

the counting. In each space, write the number of the observed AFB. If there is not AFB observed, notify as 0.

0	0	1	0	4	0	0	0	2	5
7	3								

- The number of fields to observe depends on whether bacilli are found and in what concentration:

Average of AFB found	Minimum amount of useful fields to exam
None	100
Less than 1 per field	100
1 to 10 per field	50
More than 10 per field	20
From 1 to 4 in the smear	200

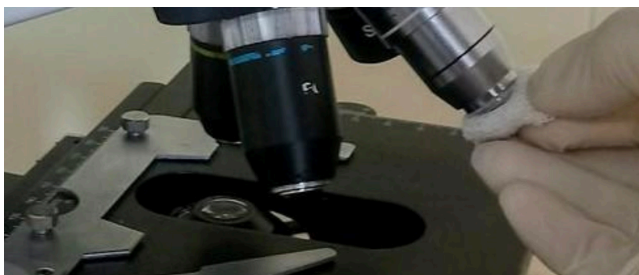
- To calculate the average of AFB found per field, add the total AFB found and divide it with the total number of fields observed. When the bacilli are grouped, an estimative amount of present bacilli in the heap is enough to calculate this average.

- The fields must be “useful microscopic fields”. A useful microscopic field is the one in which bronchial cells (leukocytes, hair cells) or mucous fibres are observed, which appeared stained blue. Fields without these elements should not be considered in the total of observed fields, unless they have AFB.

- A microscopist with experience completes a reading of 100 fields in around 5 minutes.

- At the end of the reading, turn the revolver of the lenses, remove the slide from the stage, check the identification number and record the result.

- Before checking the next slide, clean softly the immersion lens with a piece of absorbent paper. This helps to avoid the transference of material to the next smear.



Procedures to follow in case of observing less than 5 AFB in 100 observed fields

Due to the possibility that they are colored artifacts, it is recommended:

- Amplify the reading to 200 fields.

- If no more bacilli are found with this reading, make another smear with the same sample trying to choose purulent particles.

- If the reading of the second smear does not modify the previous result, the sample must be registered with the exact number of observed AFB, write this information in the Laboratory Register and request a new sample.

- If the Xpert MTB/RIF or Xpert MTB/Ultra Rif test is available and the resources allow it, process these samples by this method. Besides, in case of not having this option, cultivate or send these samples for cultivation.

The next table is the internationally adopted scale to report the results of the smears stained by the Ziehl Neelsen technique.

Result of the microscopic exam	Report
No AFB in the 100 fields	No AFB observed
1 to 9 AFB in the 100 fields observed	Exact number of AFB
10 to 99 AFB in the 100 fields	Positive (1+)
1 to 10 AFB per field in 50 fields	Positive (2+)
More than 10 AFB per field in 20 fields	Positive (3+)

Common problems

Problem	Reason	Solution
Smear too pink	Insufficient decolouration	More time to decolouration
	Very low acid concentration	Check the decolourising solutions and the results of the quality control
	Fuchsin dried on the slide	Check the amount of fuchsin poured on the slide and that the smears are leveled in the coloration tray
	Smear too thick	Prepare a new smear
Pale pink AFB	Fuchsin without a suitable quality (including the expired solutions or kept at sunlight)	Check the staining solutions and the results of the quality control Control the fuchsin's expiration date and keep it in a dark place
	Fuchsin insufficiently heated	Heat the fuchsin until white vapors emission (do not boil)
	Hot fuchsin on the smear for less than 5 minutes	Keep the primary staining for at least five minutes from the first heating
	Overheated smears during the fixation	To fix the smears, put it on the flame three times for 1 or 2 seconds each time
Counterstain too dark	Overtime with the contrast staining solutions	Do not exceed 60 seconds
	Incorrect rinse of the counterstain process	Check that the rinse is performed carefully making sure the elimination of the contrast solution
	Very high methylene blue concentration	Check the counterstain solutions and the results of the quality control
	Smear too thick	Prepare a new smear
Material deposits on the sample when observed the smear in the microscope	Unfiltered stains or filtered colorant flasks with colorant mixtures	Filter the dyes and periodically clean the dye bottles where they are placed after daily filtration.
	Material deposited on the down face of the slide	Clean the down face of the slide with a soft tissue after the staining

Reading and results report of the smears staining with fluorochromes

For reading, it is necessary to take into account the following indications:

- Exam the smears coloured with fluorochromes as soon as possible after the staining, because the fluorescence quickly vanishes; if it is not possible to read them immediately, they must be kept at room temperature (22°C) in a dark place for no more than 24 hours.
- Use the 20x objective to scan the smears and the 40x to confirm the suspicious objects. This reading scheme is the one that assures the higher performance of this method. Nevertheless, there are devices in the market that only allow reading in one magnification (200x or 400x), so the quantification results can be reported using both magnifications.
- Observe the staining quality. If it wasn't good (see the Common problem Table at next), repeat the preparation of the smear and the staining of that sample.
- Read at least one line before reporting a result as negative.
- Do not restrain the slide with low AFB through ZN.
- Smears may contain artifacts that fluoresce, but do not have typical shape of a bacillus and sometimes they have a different color (generally green).
- Bacillary green or yellow forms that do not fluoresce should not be considered as AFB.

Steps to follow when less than 5 AFB are found in one length at 200x amplification or less than 3 AFB in one length at 400x amplification

Due to the possibility that they are colored artifacts, it is recommended:

- Amplify the reading to another length of smear and confirm that observation through another technician reading.
- If after this procedure, no more bacilli are found, perform another smear of the same sample trying to choose purulent particles.
- If the reading of the second smear do not modify the previous result, the sample must be reported as "Confirmation is required", asking for a new sample and writing this finding in the laboratory Register.
- If the Xpert MTB/RIF or Xpert MTB/Ultra RIF test is available and resources allow it, process these samples by this method. In addition, in case of not having this possibility, cultivate or send these samples for cultivation.

The next table is the internationally adopted scale to report the results of the smears stained by the fluorescence technique.

Result of the microscopic exam at 200x	Result of the microscopic exam at 400x	Report
No AFB found in one length	No AFB found in one length	No AFB observed
1-4 AFB in one length (*)	1- 2 AFB in one length(*)	“Confirmation is required”(**)
5-49 AFB in one length	3-24 AFB in one length	Scanty
3-24 AFB per field	1-6 AFB per field	Positive (1+)
25-250 AFB per field	7-60 AFB per field	Positive (2+)
>250 AFB per field	>60 AFB per field	Positive (3+)

(*) See procedure to follow when less than 5 AFB in one amplification length of 200x or less than 3 AFB in one amplification length of 400x are found

(**) Ask for a new sample

Common problems

Problem	Reason	Solution
Smear with an unspecific intense fluorescence	Insufficient decolouration	More time to decolourise
	Very low acid concentration in the decolourising solution	Check the reactants mixture and the results of the quality control
	Unfiltered auramine	Filter daily the auramine solution
	Concentration of the counterstaining solution lower than the established	Check the reactants mixture and the results of the quality control
	Dried auramine on the smear	Check that the slides are levelled in the decolourising tray
	Smear too thick	Prepare a new smear
AFB with low fluorescence	Expired auramine solution or kept at sunlight	Control the auramine's expiration date and keep it in a dark place.
	Auramine solution concentration lower than 0.1%	Check the reactants mixture and the results of the quality control
	Auramine kept on the smear less than 20 minutes	Keep the dye at least 20 minutes
	Overheated smears during the fixation	To fix the smears, put it on the flame three times for 1 or 2 seconds each time
	Very decolourised smears	Put the decolourising solutions for no more than 2 minutes
	Stained smears exposed to light	Keep the stained smears at dark Read in the 24 following hours since the staining
	Smears too thick	Prepare a new smear
Contrast coloration too dark	Overtime with the counterstain solutions	Do not exceed 60 seconds
	Incorrect rinse after the counterstaining	Check that the rinse is performed carefully making sure the elimination of the contrast solution
	Very high dye concentration	Check the reactants mixture and the results of the quality control
	Smears too thick	Prepare a new smear

RECORDING AND REPORTING

The recording and reporting of the results by the standardized semi-quantitative scale assures the reproducibility of the results and allows evaluating:

- *The severity of the disease*
- *The infectivity level of the patient*
- *The patient's evolution under treatment*

- Immediately record the results of the smear reading in the Laboratory Register. Mark the positive results in red to quickly identify them.
- Write the result in the form adopted for the report by the National Tuberculosis Program.
- Check that the report has:
 - Patient's name
 - Identification number of the sample
 - Staining method used
 - The result of the microscopic exam according to the standardized scale
 - Date
 - Every observation considered relevant, like the wrong quality of the sample
 - Signature of the responsible for the microscopic exam
- Send the result as soon as possible to the health center or to the doctor who requested the exam. The time it takes to send the results is an indicator of the lab's efficiency.

***Every delay of a positive result can postpone the beginning of the treatment, extend the period in which the patient stays infectious or determine the loss of a patient.
It is necessary to do the best possible effort so the smear microscopy results arrive to the health unit within the 24 hours of the sample being delivered to the laboratory***

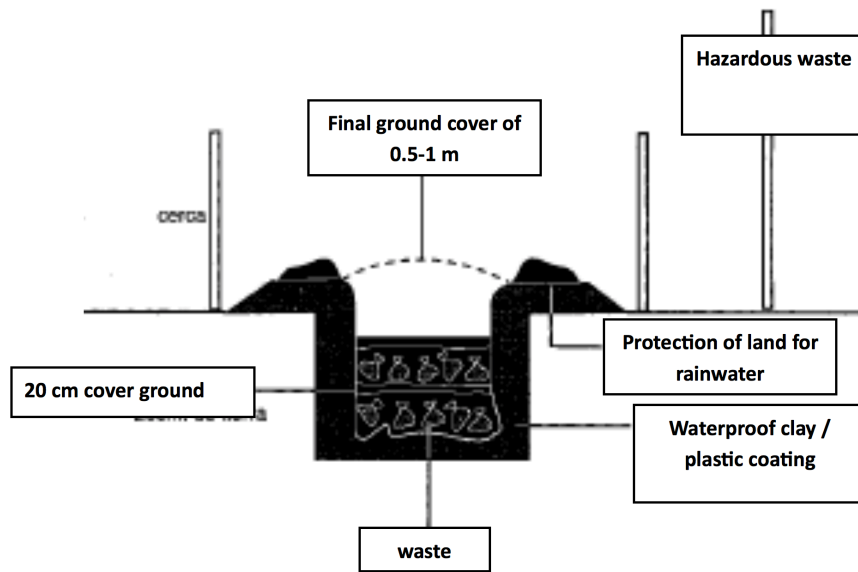
MATERIAL DECONTAMINATION AND DISPOSAL

- The principle that rules the waste handling of labs that perform smear microscopy is that every potentially infecting material must be preferably decontaminated inside the lab service, because they can be dangerous for those who transport them for the final disposal.

Based on these principles, the recommended procedures for material decontamination and disposal are:

- Dispose the samples putting them in the container inside a bag for pathological wastes together with the applicators and papers that eventually would have been used during the journey.
- Decontaminate the material inside the container by autoclaving (at 121°C for 1 hour). Then dispose it along with the pathogen wastes from the lab so they can be gathered by the waste collecting and management service that performs this task in the service.
- If the previous treatment was not available, add the same volume of 1% sodium hypochlorite to the container with the remaining of the samples, leave the containers with the cap on until the next day and then, remove them with the rest of the regular pathogen waste from the lab so they can be managed by the waste collecting service that performs this task.
- In case the health service does not have a qualified pathogen waste collection system, the material previously treated by autoclaving or with sodium hypochlorite will have to be buried in a ditch at a non floodable field in a zone where there is no people, animal or vehicle transit. The ditch must:
 - Have a minimum distance between the bottom and the ground water of 4 meters.
 - Have an approximated depth of 2 m.
 - The bottom of the ditch must be covered with a water-resistant material made of clay or plastic. The upper part of the ditch can be covered with a concrete tile.
 - The ditch will be used until waste reaches 0.6 m height (measured from the upper plane); a layer of lime will be spread on them and the rest will be filled with soil only, after removing the tiles. The upper cover will be on high and with a slope that will allow the rainwater runoff, putting before it a layer of impermeable material at ground level.

There are likely to be special provisions for controlled burial in different countries. It is recommended to consult the corresponding body on the current legislation in each of them.



If none of these decontamination and disposal procedures for potentially infectious material are available, it is possible to burn the material. In this case, dispose the potentially infectious material inside a water-resistant plastic bag placed inside the disposal container. At the end of the tasks, close the bag with a knot, cap the container and transport the material inside the disposal container to the place where it will be burned. Place the bag inside a container of empty gasoline type, in an open area with no people transit. The operator must step aside when the material is burning, because the smoke of the plastic containers is toxic and the sprays are dangerous. When it is cold, bury the contents at least 1.5 m deep. This procedure should be made at least once a week.

In order to produce precise and accurate results, avoid aerosol formation and transference of material between different samples; check whether the next routine habits has been incorporated:

- Control the accuracy and clarity of the identification of each sample in the container, request form, slide, Lab register and report of the result.
- Do not work with more than 12 samples in each sequence.
- Keep the order, placing each numbered slide in front of the matching sample in ascendant order from left to right.
- Process the samples by one; do not open the next container before closing the last one.
- Handle carefully the sample container.
- Do not put in the sample container, the applicators used in another.
- Use new slides, with no marks and no oil.
- Select the mucopurulent portion of the sputum.
- Spread in an even way enough portions of the sputum, without excess, on the slide.
- Keep the slides separated from each other at all times.
- Do not touch the smear with the hands, dropper, sticks or faucets.
- Avoid splashes with the solutions or the water.
- Filtrate the carbol-fuchsin when used, heat it until the vapours are released, no boiling and let it set for 5 minutes (making two more warm-ups during this time).
- Filtrate de Auramine O solution when used, let it set for 20 minutes, no heating.
- Eliminate the remaining water from washing.
- Dispose and prepare again the smear that, by accident, had overlapped or stained wrong.
- In case of the ZN microscopy, clean the microscope lens after reading each slide and dedicate not less than 5 minutes for the reading of each one.
- Count the bacilli in the smear using the standardized scale.
- Avoid every delay that can be eliminated
- Route for rapid molecular test/culture the samples of the cases that requires it.

REGISTER SYSTEM

The lab register does not only works for microscopic result registration, but it also gives information that, added to the ones from another labs, it is useful to evaluate the epidemiologist situation and the quality of the activities destined to the tuberculosis control and to plan such activities. Besides, it allows knowing and monitoring the development, use and efficiency degree of the Lab Network services.

The lab has to be able to track in their registers the samples collected, processed and derived for molecular tests, cultures and drug susceptibility testing, the investigated PT patients, the diagnosed and controlled cases, smear microscopy results of each patient, staining solutions and supplies received and consumed, the batches of staining and decolourising solutions prepared and consumed, the disinfectant solutions prepared and consumed and the internal quality control results.

The laboratories of the Network must have the following standardized instruments by the NTP: Bacteriology Request Form and Register for Bacteriological Investigation of Tuberculosis. Annex IV presents models of these instruments and the staining preparation / quality control register.

The registers have to be kept as long as every country legislation indicates and if it is not regulated, for at least two years.

- ***Accuracy in documentation is critical for tracking results, evaluation and appropriately planning the activities.***
- ***The registration instruments must follow the norms of the NTP***
- ***Records must be complete and contain consistent and reliable information***

SMEAR MICROSCOPY QUALITY ASSURANCE

The program of quality assurance is the part of the management system in charge of giving confidence about an organization that fulfills the quality requirements.

It allows to evaluate if the information produced by the laboratory is accurate, reproducible and timely.

It installs an alarm system that allows to prevent, discover and correct errors

Regular activities of a smear microscopy quality assurance program include:

- Internal quality control,
- External quality assessment, and
- Continuous improvement.

In this manual, we will describe mainly the procedures for the internal quality control of smear microscopy, because those are the ones under the lab responsibility that performs this technique.

INTERNAL QUALITY CONTROL

It is responsibility of each lab that performs smear microscopy. Particularly, the person in charge of the lab must establish a system of regular and continuous controls of the critical points in the work routine.

Internal quality control includes:

- the evaluation of
 - materials, equipments, reactants
 - the performance of the staff
 - procedures
 - accuracy and precision of records/reports
 - offer and appropriate implementation of the smear microscopy
 - smear microscopy performance
- the monitoring of the results of the internal controls
- corrective measures to apply when the result inaccuracy exceeds the limits considered acceptable or produces inevitable delays.

		False positive errors	False Negative errors
Inherent to the sample	<ul style="list-style-type: none"> - Non representative of the lesion - Collected in the wrong moment - Not enough - Poorly storage (bacteriolysis) 		<ul style="list-style-type: none"> X X X X
Inherent to the operator	<ul style="list-style-type: none"> ▪ Wrong selection of the suitable portion of sputum ▪ Defects on the smear preparation <ul style="list-style-type: none"> - Thin and thick smears (especially in the fluorescence technique) or barely uniform -Use of scratched or dirty slides -Fixation of wet smears or at temperatures higher than 60°C ▪ Defects in the ZN staining : <ul style="list-style-type: none"> - smears maintenance with the fuchsin for a period of less than 5 minutes hot - poor quality of fuchsin - poor or over heating - not enough decolouration - precipitation of crystals due to the use of non-filtered reactants or overheating - excessive decolouration ▪ Defects in the fluorescence staining <ul style="list-style-type: none"> - smears maintenance with auramine for no more than 20 minutes -use of auramine solution after a month of its preparation - not enough decolouration - precipitation of crystals for the use of non-filtered reactants - excessive decolouration - counterstaining for over a minute - delay in the reading or storage of the slides out of the light shelter ▪ Defects of the reading <ul style="list-style-type: none"> ▪ Use of microscope in bad condition ▪ Reading an insufficient number of fields ▪ Observation of only one level of the smear ▪ Low capacity to differentiate bacilli from staining artefacts ▪ Transference of the bacilli from one smear to another, contaminated immersion oil dispenser. ▪ Confusion of samples or smears ▪ Errors in the transcription of results 	<ul style="list-style-type: none"> X 	<ul style="list-style-type: none"> X X X X X X X X X X X X X X X
Inherent to the technique	<ul style="list-style-type: none"> ▪ Sensitivity limit of 5.000-10.000 bacilli/ml ▪ Specificity: AFB are detected that can be non-pathogenic and nocardia. 	X	X

Stains, staining and microscope control

For the quality control of stains, staining and microscope, the next procedures are carried out:

- Prepare smears with positive and negative samples following the next procedures:

Preparation of non-stained positive controls:

- Use sputums with low positivity (1+).
- Leave these sputums for one or more days at room temperature so the sputums get liquid.
- Mix (with the sputum container closed). Let it set for at least 20 minutes, add 5 to 10 drops of 5 % phenol and let it set for an hour.
- Perform at least 50 smears, let them air dry and fix by heat
- Check the average AFB number by staining a few randomly selected smears (i.e.: 6) from the batch. Record the result of the average AFB checked in the 6 smears.
- Keep the smears in a box marked as "Positive control smears"

Note: If enough positive samples are not received to prepare the positive controls, request smears or a low-positivity sputum sample to prepare controls for the reference laboratory.

Preparation of non-stained negative controls

- Make sure that the sputum used for the preparation of negatives smears has been strictly examined to certify that there is no AFB.
- Add 5 to 10 drops of 5% phenol, leave for an hour

and then prepare as much smears as possible.

- Let the smears air dry and fix by heat.
- Keep the smears in a box marked as "Negative control smears"

Keep the smear prepared in boxes designed to keep smears or inside a box wrapped in soft paper, well conditioned, in a dry place.

Quality control of each new batch of staining reagents

- Control the quality of each new batch of stains by staining two negative and two positive slides.
- Negative smears must be stained three times in order to increase the possibility of identifying contaminants coming from the preparation water of the stains that might attach to the smears.
- Record the result of this control in the register of preparation/control of reactants (see appendix IV).
- Check that AFB are completed and fully stained with fuchsin or auramine and that the counterstaining is uniform, of the expected colour and that has a good contrast. Check if the assigned quantification of the bacilli matches with that consigned at first for the sample used to prepare the positive smear controls.
- If the result is not the expected for the AFB staining, repeat again the staining using other controls to make sure that the mistake was not on the staining technique.
- If in this second control the staining was defective or the observed number of AFB is not the expected, discard the fuchsin, auramine and/or one of the other reagents.

Register the batch that was anomalous and discard the unsatisfactory solutions.

Quality control of staining reagents in use/staining

- If more than 10 smears are performed per day, it is convenient to stain one positive and negative control at least once a week. If less than 10 smears are performed per day, the positive and negative smear must be included as daily control. **Record these results in the register of bacteriological investigation.**

Control of record and reports

- A person not related to the performance and register of the smear microscopy has to randomly check once a week that the data and results in the reports produced on that day match exactly with the ones on the Lab Register. This must be performed by the person in charge of the lab in case that himself does not perform and register samples. Register this activity.

- Check that the samples are being processed the same day they were received or on the next day. If the lab cannot perform sputum smear every day, or if it receives samples from peripheral centers, check that no more than three days have passed since the samples were taken until they are processed.

- Control that the smear microscopy results are being delivered regularly 24 hours (48 hours top) after the sample has been processed.

- Check that the results are received in the health service in which the patient delivered the sample or in the doctor's office who requested the study in the shortest time possible, no matter how distant it is.

- Check that the samples that require to be processed by rapid molecular tests/ culture have been delivered.

Performance indicators monitoring

- Key Performance Indicators (KPIs) are useful for

the internal evaluation of the microscopy quality. They must be calculated per month or every three months from the counts in the Lab Register. Each lab is responsible to calculate its own KPI.

- The monitoring of the trend of these KPI allows recognizing a change in the regular patterns. The identification of very high or low values can indicate a problem. Although there are ranges of values established as acceptable for these indicators, the average value of these depends, in some cases, on local specific conditions.

- Perform an analysis of the following data:

- N° of suspect smears (a)
- Total n° of positive suspect smears(b)
- N° of suspect smears of low range (countable positives and 1+) (c)
- N° of smear of follow-up treatment (d)
- N° of positive follow-up smears (e)

From these values, calculate the following indicators:

Indicator	Calculation	Suggested reference value
Workload	N° of suspect smears + N° of follow-up smears a + d	Set on local level
Percentage of positive suspect smears among the suspect smears	N° of positive suspect smears x 100 / Total n° of suspect smears $b \times 100 / a$	Set on local level
Percentage of positive follow-up smears among follow-up smears	N° of positive follow-up smears x 100 / Total n° of follow-up smears $e \times 100 / d$	5-10%
Percentage of positive suspect smears of low range (pos 1+ and countable) among all the positive suspect smears	N° of positive suspect smears of low range x 100 / Total n° of positive suspect smears $c \times 100 / b$	30-50%

- Plot this data and indicators monthly or quarterly in order to obtain a trend line (the variability of the indicators can be very large if the denominators – the totals- of the percentages are small). If these values get roughly apart from the usual, the causes should be investigated.

- If a succession of positive results is detected in one or few days of work, investigate if cross-contamination of bacilli has not occurred, from a highly positive sample to the following ones.

- Ask the reference lab if abnormalities are detected and the causes of this cannot be detected.

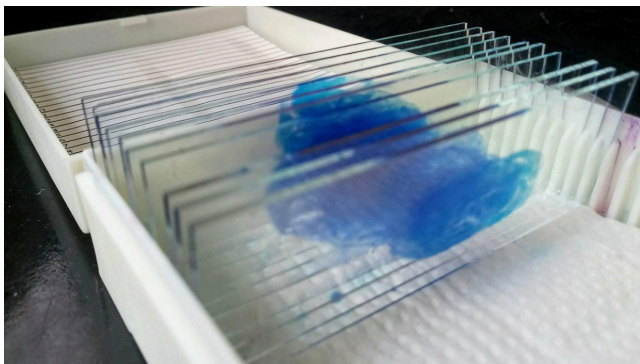
Note: these and other indicators of performance can be requested by the coordination of the lab network to contrast the lab performance with other labs placed in the same geographic area.

EXTERNAL QUALITY ASSESSMENT Proficiency tests

In order to be able to participate in the external quality assessment program by means of the rereading method, it is required that all the smears made in the local laboratories to be conserved until a sample of smears has been selected for re-reading.

In order to conserve them, it is necessary to follow the next indications:

- If they were stained by ZN, remove the oil from the read smears, leaving the slides upright on an absorbent paper (newspaper or kitchen paper) until the next morning. Then support the slide gently “upside down” on another strip of absorbent paper. Never attempt to remove the remaining oil by rubbing the smear. To avoid contamination, always use a new piece of adsorbent paper.



- Check that the numbering is visible on the smears.
- Store them in slide boxes or inside a regular box wrapped each one on a paper in packs that cluster the ones of a day or a week with a label with the date of performance. Do not put on the labels the result of each one.
- Keep in a dry, fresh place.

Eventually, the reference lab can send a panel of smears to stain, read and report. This panel must be introduced in the routine work of the laboratory without performing special procedures for this control.

Keep in a file the results of the external quality assessments. Analyse each result and apply corrective measures following the recommendations made by the reference lab.

Usually, the lab records and results of the internal and external quality assessment are required during the visit of technical assistance. They should be available.

SELECTED BIBLIOGRAPHY

1. Aziz MA, Ba F, Becx-Bleumink M, Bretzel G, Humes R, Iademarco MF, SangJae Kim, Lamothe F, Paramasivan CN, Ridderhof J, Sloutsky A, Van Deun A, Shah KV, Weyer K. External Quality Assessment for AFB Smear Microscopy. WHO, APHL, CDC, IUATLD, KNCV, RIT. Washington DC, 2002. Available at: https://www.aphl.org/aboutAPHL/publications/Documents/External_Quality_Assessment_for_AFB_Smear_Microscopy.pdf
2. Blancarte L, de Kantor, I, Latini O, Laszlo A, Valenzuela P, Yáñez A. INPPAZ. Bacteriología de la tuberculosis. La muestra. El examen microscópico. Nota Técnica N° 26 / Rev1. OPS/OMS. Martínez (Buenos Aires, Argentina), 1988.
3. Fujiki A. TB microscopy. Tokio, Japan: The research Institute of Tuberculosis, Japan Antituberculosis Association, Japan International Corporation Agency, Hachioji International Training Centre, 1998.
4. International Union Against Tuberculosis and Lung Disease. Technical guide for sputum examination for tuberculosis by direct microscopy. Bull Int Union Tuberc 1978:4-16. Available at: http://www.tbonline.info/media/.../iuatld_afb_microscopy_guide.pdf
5. Laszlo A. et al. Guía Técnica para los países con escasos recursos económicos. Diagnóstico de la tuberculosis por examen microscópico directo de la expectoración. Quinta edición. París. IUATLD, 2000.
6. Lumb R, Van Deun A, Bastian I, Fitz-Gerald M. Laboratory Diagnosis of Tuberculosis by Sputum Microscopy. The handbook. Global Laboratory Initiative. Australia, 2013. Available at: http://www.stoptb.org/wg/gli/assets/documents/TBLabDiagnosisSputum%20Microscopy_Handbook.pdf
7. Organización mundial de la salud. Los servicios de laboratorio en el control de la tuberculosis. II Microscopía. WHO Global Programme. WHO/TB/98.258. Ginebra 1998.
8. Organización Mundial de la Salud. Manual de bioseguridad en el laboratorio de tuberculosis. WHO/HTM/TB/2012.11. 2012. Available at: http://apps.who.int/iris/bitstream/10665/92661/1/9789243504636_spa.pdf?ua=1
9. Organización Panamericana de la Salud. Manual de Diagnóstico Bacteriológico de la Tuberculosis. Parte 1: Baciloscopía. OPS, Washington. 2008. Available at: <http://www1.paho.org/Spanish/AD/DPC/CD/tb-labs-baciloscopia.pdf>
10. Rieder HI, Van Deun A, Kam KA, Kim SJ, Chonde TM, Trébucq A, Urbancik R, Priorities for tuberculosis bacteriology services in a Low-Income Country. IUATLD. París, 2007.

- 11.** Stop TB Partnership (2014) Mycobacteriology Laboratory Manual. Disponible en: http://www.stoptb.org/wg/gli/assets/documents/gli_mycobacteriology_lab_manual_web.pdf
- 12.** Stop TB Partnership (2017) GLI Guide to TB Specimen Referral Systems and Integrated Networks. Available at: http://www.stoptb.org/wg/gli/assets/documents/GLI_Guide_specimens_web_ready.pdf
- 13.** Van Deun A, Aung KJM, Hamid Salim A, Gumusboga M, Nandi P, Hossain MA. Methylene blue is a good background stain for tuberculosis light-emitting diode fluorescence microscopy. *Int J Tuberc Lung Dis* 2010; 14:1571-1575.
- 14.** Van Deun A, Hossain MA, Gumusboga M, Rieder HL. Ziehl-Neelsen staining: theory and practice. *Int J Tuberc Lung Dis* 2008; 12:108-110.
- 15.** WHO. Proposed reduction of number of smears for the diagnosis of pulmonary TB: Background document. Geneva, 2008. Available at: <http://www.who.int/tb/dots/laboratory/Reduction%20of%20smears.pdf>
- 16.** World Health Organization. Xpert MTB/RIF implementation manual. Technical and operational “how-to”; practical considerations. WHO. Geneva, 2014. Available at: http://apps.who.int/iris/bitstream/10665/112469/1/9789241506700_eng.pdf
- 17.** World Health Organization. Policy statement: Fluorescent light-emitting diode (LED) microscopy for diagnosis of tuberculosis. WHO/HTM/TB/2011.8, 2011. Available at: http://whqlibdoc.who.int/publications/2011/978921501613_eng.pdf

APPENDIX I

MINIMUM REQUIREMENTS OF BIOSAFETY

The available information strongly demonstrates that the health staff with a higher risk of infection by inhalation of droplet nuclei containing AFB is the closest to the patients (doctors, nurses, staff that receives the samples from the patients). The risk of personnel working within the laboratory is lower.

No personal protective element is as necessary as the information, the organization at the work place, concentration, alert state and implementation of the precautions measures that are very simple and inexpensive.

Information and medical control of the lab staff

- People with reduced immunity, like the one caused by HIV, diabetes or pregnancy, can have a higher risk of getting TB if they become infected with the bacillus, so in these cases, precautions may need to be taken, such as assigning tasks in an area of low risk of infection. People who have chronic lung diseases must have the authorization of their doctor to be incorporated to the lab tasks.
- At the moment of the admission, the staff will have to be precisely instructed about how tuberculosis is transmitted and the biosafety measures that should be applied in their daily work. For this, the lab will have to have a written document that the staff should read. They have to be tested in comprehension, registered the evaluation and filed on its record.
- Regular meetings are necessary with all the lab staff aimed to recall the biosafety measures, analyzing if they are being fulfilled, elucidating the causes that might have provoked the accidents and making the necessary corrections in the work routine.
- The technicians must be incorporated to a regular medical control program for the health workers following the current standards in the country and the ones established by the National Tuberculosis Programs. In case of no adopted policy or if this does not consider the surveillance of respiratory infections, the supervisor must assure that the staff have at least one annual medical control that includes chest x-ray exam.
- When the lab staff presents respiratory symptoms for over 15 days, their medical examination, chest x-ray and smear and culture examination and / or rapid molecular test of sputum samples must be arranged, according to the national policies on the use of TB diagnosis tests.

- Each lab must have written and widespread instructions on the handling of patients with tuberculosis and the biological samples obtained from them. On each section, basic specific norms on biosafety must be displayed for each task.
- Staff must know who is the supervisor to whom they should immediately notify in case of any work accident.

General precautions of work

Basically, it is necessary to apply all these logic measures in order to avoid the creation and movement of sprays that are the most dangerous vehicle to transmit and transfer bacilli.

- Handle the potentially infectious material in areas apart from people. Restrict the lab access of the external staff of the work place to avoid movements, air flow, distractions and exposure of people not involved in the handling of potentially infectious materials. Serve the staff of the health center and the patients out of the manipulation area of samples at the lab.
- At the end of the task, disinfect the test table surface where the smears were made and where containers with potentially infectious material were supported.
- Clean the floor every day and the walls every week with a 0.1 % sodium hypochlorite. Do not sweep or clean in dry, always use a wet cloth. Do not wax. Do not raise dust when clean.
- Do not place unnecessary equipment in the work area and do not take from it the register books or used equipment.
- Use preferably a long-sleeve robe and close in the back. Long-sleeve lab robes closed in the front to cover clothes are also acceptable.
- Do not take it out from the health center, where it

must be cleaned with hot water and soap. Robes are useful to protect clothes from chemical substances, stains and accidental splashes with samples, but they do not protect from getting infected through air.

- Do not put purses, cell phones or clothes on the test table of the lab where diagnosis procedures are being performed.

- It is not necessary to use facemasks to perform smear microscopy. Its use can be considered if the resources are sufficient. In case of using, choose an N95 (USA regulation) or an FFP2 (European regulation) respirators, that holds particles from the 0.3 microns that assure at least 95% of protection. They should have secure closure over the nose and around the mouth. Surgery facemasks allow the tuberculosis bacillus get through and provide a false security sensation. They can be reused until they are uncomfortable to breath due to the pore saturation, they resist for about 30 hours. They must be kept in a non-hermetic box (cardboard) to prevent breakage and to clog their pores with environmental dust. So that they do not remain humid, it is necessary to avoid placing them inside plastic wrappers.

- Train the staff with the use of respiratory protectors.

- If respirators are used in a lab, the entire staff must know how to use and adapt them correctly and their limitations. Follow the manufacturer's instructions for handling and use of respirators.

- To put on a **new** disposable mask

- First put on a mob cap (if necessary)
- Take with one hand the external part of the mask leaving the elastic bands free
- Put the mask covering the nose and mouth
- Take the upper elastic band and adjust it in the back of the neck and above the ears
- Take the inferior elastic band, slip it off surrounding

the head until adjust it in the neck

- Adjust the metallic piece placed on the nose, pressing with the fingers from the nose to the ears
- Check the mask adjustment

- To remove the mask

- Remove the gloves first (see instructions bellow) and wash hands

- Then remove the mask by only touching the elastic bands without touching the external part of the mask, because it is potentially contaminated, and without touching the internal part to avoid contaminate it.

- It is essential the use of gloves when working with the biological samples. Gloves will be changed regularly and should not be reused.

- Teach the lab staff how to remove gloves following the next instructions:

- Remove one glove grabbing it from the back and wrapping it to take it off the hand in order to avoid contamination spreading.

- Grab the used glove with the other hand with the glove. Slide carefully the naked fingers without touching the surface of the contaminated glove. Roll the glove out, over the other glove used to make a small bag of gloves used with the inward contamination.

- Throw the gloves properly and safely.

- Wash hands regularly even when gloves are being used.

- Wash hands always before abandoning the lab.

- Do not touch amenities, desk material or lab equipment without taking the gloves off and washing hand.

- Do not drink, eat or smoke in the work place where the potentially infectious material is processed. Do not use cell phones or personal music players.

- Do not put in the mouth, under no circumstance, elements used in the lab.

Precaution in the specimen collection and handling

- Collect the sputum samples in a well ventilated area, never at the lab, using wide mouth containers with tight caps.

- Avoid, as possible, sprays, use masks for the fibrobronchoscopies or nebulisation performance.

- Check that there are no spills in the samples; disinfect the exterior of the container if any.

- Prepare and transport the samples in boxes that can be disinfected, resistant and tight caps.

- Make sure that the containers are always in vertical position.

- In case of movement, let the containers settle for at least 20 minutes before opening the caps. Open the containers carefully and close tightly after collecting the sample.

- Systematize the sample procedure.

- When preparing the smear, it is advisable to use wood sticks or disposable grips instead of reusable grips, which is necessary to sterilize by heat.

- In case of using reusable grip, they must be sterilized by heat with a closed microincinerator or a Bunsen burner. The reusable grips will get clean in a sand and alcohol bottle before the sterilization.

- Make soft and slow movements when the smears are being made.

- Always have a bottle with 5 % phenol or 1 % sodium hypochlorite.

- Work with lighter between the samples and the

PART I: Smear Microscopy Update

technician when making the smears.

- Keep the slide edges clean, without sample.
- Do not move or heat fix the smears until they have completely dried in the air.
- Stain the smears as soon as they get dry and fixed by flames.
- Organize the safe disposal of the used materials in containers with disinfectants and caps.

Handling and use of disinfectants

To treat the samples and everything that has been in contact with them (applicators, spills), use 5% phenol or 1% sodium hypochlorite (bleach). The minimum contact time on samples that eventually have bacilli is 30 minutes.

For the surface disinfection, use 1% sodium hypochlorite. Only for the floor cleaning use 0.1% sodium hypochlorite.

The sodium hypochlorite solution for domestic use contains 55g/l (5.5%) of chlorine and it can be between 3 y and 6%. Therefore, each solution is usually prepared in the following way:

- *1%: 1 part concentrated solution and 4 parts of water*
- *0.1%: 25 ml of concentrated solution for each liter of water*

Choose bleach of good quality. Keep it protected from light, in a cool place and with the lid tightly closed to prevent it from deteriorating. The packaging date is on the packaging, it must be checked when it is purchased. Dilutions should be made daily, because it loses activity quickly. Due to its extremely alkaline nature, it can corrode metals.

Keep in mind that phenol is corrosive and toxic:

- Keep the concentrated phenol in bottles with tight caps, in a dry place, protected from the light and

preferably in a place dedicated to storage away from the work area.

- Keep the 5% phenol in bottles with tight cap that avoids sprays.
- Avoid the direct contact with phenol to the skin or mucosa. Use gloves to manipulate it.
- Reduce phenol vapours that detach the fuchsin, placing the staining of smears in a well ventilated area and limiting the numbers of smears to stain to 12.

Handling of other chemical substances

- Handle carefully the concentrated acids. Add always the acid into the water and not backwards.
- Do not use alcohol near the lighter flame in order to avoid fire and possible burnings.

Procedure in case of a work accident

- Although it is not necessary to use respirators to perform smear microscopy, these must be available in case of accidents.
- In case of breakage of containers or spillage with potentially infectious material, immediately remove the personnel from the area, for at least one hour to allow the aerosols to be eliminated by the ventilation system of the laboratory and to deposit the heavier particles
- Signs will be placed to indicate that the entry is forbidden during this time and the cleaning process.
- When this time has expired, put on the respirators, cover immediately the zone with paper and soak it with 5% phenol or 1% sodium hypochlorite and let set in the established time (at least half an hour).

- After an appropriate time has elapsed, collect the contaminated material in a closed bag to properly dispose of it.
- In case of broken glass or other sharp objects, use a piece of hard cardboard to gather the material and put it in a perforation resistant container to dispose.
- Clean and disinfect the spilling area.
- In case of sharp or cut injuries when handling the samples, smears or disposal material, wash hands or the affected area immediately with abundant water and soap and put 70% ethanol.
- In case of spills in the eye with potentially infectious material or a reactant, wash with sterile distilled water using a sterile container applied to the eye.
- In case of contact with a concentrated acid, wash the affected zone and clothes with abundant water.
- Notify the supervisor the accident after taking the measures described.
- Each time there is contact between a sample with a wound or cutaneous penetration or through mucosa, after the emergency wash and local cleaning, check with a doctor to control the worker and dispose the chemoprophylaxis administration if necessary.
- Keep a register of incidents and accidents.

REAGENTS PREPARATION

FOR THE ZIEHL NEELSEN STAINING

0.3 % basic carbol-fuchsin

Basic fuchsin (*)	3 g
95° ethanol	100 ml
Phenol crystals (**)	50 gr.
Distilled water	900 ml

(*)Minimum content of active pure dye 85%. If the stated content was less than this, the correction of the weighed must be made (see Precaution with the colorants), if it is 85 % or more, the correction is not necessary.

(**) Phenol crystals must be colourless. Phenol crystals are corrosives, toxics and may cause burns. Avoid contact with skin and mucosa. Prepare in a well ventilated place.

In a 1-liter Erlenmeyer, dissolve 50g of phenol crystals in 100 ml of ethanol. Add the 3g of basic fuchsin, mixing until dissolve well.

Add the distilled water until complete 1 liter. Keep the solution in amber bottle with a good close. Label with the reactant name and with the elaboration and expiration dates. Keep in room temperature protective from light for no more than 12 months.

Filter when used.

Decolourising solution

Add always softly the acid to the ethanol and not backwards, because there can be spills produced for the intense overheat when the procedure is otherwise.

Hydrochloric acid p.a.	30 ml
95°ethanol	970 ml

Label the amber bottle with the reactant name and with the elaboration and expiration dates. Keep in room temperature for no more than 12 months.

If it is hard to obtain the alcohol, the following decolourising solution on sulphuric acid basis can be used:

Concentrated sulphuric acid (technical quality)	250 ml
Distilled water	750 ml

Add slowly the acid into the water and shake gently. Label the amber bottle with the reactant name and elaboration and expiration date. Keep in room temperature for no more than 12 months.

Counterstaining solution

0.1% methylene blue

Methylene blue chloride	1 g
Distilled water	1000 ml

Dissolve the methylene blue in the water, shaking gently.

Save the resulting solution in an amber bottle. Label the bottle with the reactant name and the elaboration and expiration date. Keep in room temperature inside a closet for no more than 12 months. Filter before use.

FOR THE FLOURESCENCE STAINING

Auramine-O

Solution 1 (1 % Auramine-O)

Handle the auramine with gloves. It is carcinogenic and all direct contact with the powder or the solution must be avoided.

Auramine-O	10 g
95° ethanol	1000 ml

Dissolve the auramine-O powder in the ethanol. It is advisable to use a magnetic shaker. In case of not having one, let set the auramine-O in ethanol overnight.

Label the bottle with the reactant and the elaboration and expiration dates. This is a stable solution for 12 months if kept in an amber bottle protected from light.

Solution 2

Phenol crystals (*)	30 g
Distilled water	900ml

(*) Phenol crystals must be colourless. Phenol crystals are corrosives, toxics and may cause burns. Avoid contact with skin and mucosa. Prepare in a well ventilated place.

Dissolve the phenol crystals in the water.

Label the bottle with the reactant name and the elaboration and expiration dates. This solution is stable for 12 months if kept in an amber bottle protected from light.

Working solution: 0.1 % Auramine-O

In an amber bottle, put 50 ml of solution 1 and 450 ml of solution 2.

Adjust the bottle cap.

Mix well and let set overnight.

The auramine-O just prepare has an intense gold yellow colour. If the colorant is pale, reject.

Label the bottle with the reactant name and the elaboration and expiration dates. Keep in room

PART I: Smear Microscopy Update

temperature away from the heat and protected from light for no more than a month.

Filter the auramine when applied on the smears during the staining process.

Decolourising solution

Hydrochloric acid p.a	5 ml
Ethanol	1000 ml

Add always softly the acid into the alcohol and not backwards, because the solution temperature bluntly rises.

Place in an amber bottle; label the bottle with the reactant name and the elaboration and expiration dates. Keep in room temperature for no more than 12 months.

If it is hard to obtain the alcohol, the following decolourising solution can be used:

Hydrochloric acid p.a	10 ml
Ethanol	100 ml
Distilled water	890 ml

Carefully add the alcohol in the distilled water.

Carefully add the acid to the 10 % ethanol solution in water.

Place in an amber bottle; label it with the reactant name and the elaboration and expiration dates. Keep in room temperature for no more than 12 months.

Counterstaining

A 0.5 % potassium permanganate or 0.3 % methylene blue solution can be used. The choice depends on the type of the microscope used. In some systems, the permanganate produces a very dark

background that makes difficult the maintenance of the focus during the reading; in these cases it is advisable to use the methylene blue solution, even when the contrast is low.

Potassium permanganate	5 g
Distilled water	1000 ml

Place the potassium permanganate inside an Erlenmeyer of 2 liters holding 500 ml of distilled water.

Shake until dissolve.

Add the remaining 500 ml of water and shake. Keep in a well-closed amber bottle. Label the name and the elaboration and expiration dates. Keep in room temperature for no more than 12 months.

The solution must be purple. In case of turning red, it means that the permanganate has rusted and the solution must be rejected.

Methylene blue	3 g
Distilled water	1000 ml

Place the methylene blue inside a 2 liter Erlenmeyer holding 500 ml of distilled water.

Shake until dissolve.

Add the remaining 500 ml of water and shake.

Keep in a well-closed amber bottle. Label the name and the elaboration and expiration dates. Keep in room temperature for no more than 12 months.

PRECAUTIONS WITH THE STAINING SOLUTIONS

- Stains, especially the basic fuchsin, must be of good quality. It can be convenient to make a centralized purchase in order to assure a good quality of all services of one jurisdiction. Check that the purity of

the fuchsin is higher than 85 %. If this is not the case, the quantities must be adjusted taking into account the purity degree. For example: if the purity of the fuchsin was 75 %, divide 3 g into $0.75 = 4$ g. Instead of 3 g, 4 g shall be weighed.

- Verify that the phenol is not pigmented. It should be kept away from light to avoid oxidation.

- Although Ziehl Neelsen's staining solutions can be used for 12 months, it is convenient to prepare the volumes that will be used in a month. Every reactant with abnormal characteristics (clearly precipitated, turbid, etc.) or kept for over 12 months, should be discarded.

- Keep every solution preferably in amber bottles, tight closed and at dark. In case of not having amber bottles, they can be wrapped with metallic foil. Wash the bottles before reusing them, by rinsing with alcohol or with the same decolourising solution to dissolve the crystals that may have formed.

- In case of using staining solutions ready to use prepared by the industry, ask the responsible of the Labs Network about their quality, since it is very variable.

- Keep a register with the date and the volume of each reactant that has been prepared and the result of the respective quality control (see model in the appendix IV).

CARE AND MAINTENANCE OF THE BALANCE

The balance in which the stains are weight is a sensitive and precise instrument that must be used carefully. It is advisable to weigh up to 200 g with an accuracy of 0.1g. The user manual should always be consulted.

- Place the balance in a firm table, with no vibrations and well levelled.

- Protect the balance from air flow.

- Keep always the balance and weights (in case of using a double tray scale) clean and dry to protect them from corrosion. Any change in the surface of any part can affect the accuracy.

- Take into account that dyes can intensely stain any surface where they fall. Do not place the material to be weighed directly on the saucer but on an appropriate container or paper. Subtract the weight of the paper or container from the total weight with reagents.

- Withdraw slow and softly the dyes or any other drugs until having the required weight. Do not put back again the surplus in the original container in order to avoid the contamination of the products in it.

- Clean the balance with a soft and clean brush after its use. Read the manufacturer's instructions for other cleaning instructions. The balance trays and the work place can be disinfected with 70 % ethanol.

- When the balance is not in use, keep it under a tight plastic drape to protect it from dust. Put a tray with blue silica below the drape in order to eliminate the air humidity (when the desiccant silica turns red, it must be regenerated through heat).

- Using a proper milligram/gram scale, check daily that the weight recorded by the balance matches the calibration weights.

- The balance calibration must be performed annually or after any reparation or relocation by a qualified technical service and must be settled in the corresponding register. The balance reparation must be performed by a qualified technical service.

CALCULATION OF REAGENT STOCK

In order to ensure a continuous supply of laboratory material, the services must plan the requests for

PART I: Smear Microscopy Update

inputs for a period of time. It is possible to estimate the necessary reagents by checking in the lab register the amount of cases diagnosed through smear microscopy during a determined period.

The following is an example of how the number of smears to be performed during a trimester is calculated.

Assuming that:

- The proportion of cases detected by smear microscopy among the symptomatic investigated is 5 % (this means that among 20 PT patients examined, there is one case with a positive smear microscopy).
- Each symptomatic is investigated by two smear microscopy examinations.
- Each tuberculosis case with positive smear microscopy is follow-up with 3 control exams.
- 10 cases of positive smear microscopy have been diagnosed in a trimester.

It will be necessary to perform 43 smears for each case detected through smear microscopy, according to the following calculation:

$(1 \text{ case of tuberculosis} + 19 \text{ negative PT}) \times 2 = 40$
diagnosis smears

$1 \text{ case of tuberculosis} \times 3 \text{ smears} = 3$ follow-up smears

This means that for a trimester it will be necessary to calculate that the stock of reagents is enough to perform 430 sputum smear microscopy exams.

If there is no change in the case-finding activities, the total amount of smears to perform can also be estimated by just checking the total of smears performed during the last trimester.

It is convenient to calculate the necessary stock

adding one month of work to cover up some contingencies in the workload or delays in the input delivery. In the previous example, to the 430 smear microscopy exams calculated, 143 more would be added, which makes a total of 573 smear microscopy exams.

	Necessary amount for each smear a	Stock for a trimester b = a x 573	Amount to request (rounding)
Containers	1	573	600
Slides	1	573	600
Applicators	1	573	600
Immersion oil	0.05 ml	29 ml	30 ml
Filter paper	One roll per month		
Sodium hypochlorite solution	0.1 ml	57.3 ml	60 ml
ZIEHL NEELSEN STAINING			
Basic fuchsin (0.3%)	0.009 g or 3 ml if you receive the prepared dyes	5.16 g or 1719 ml	5 g or 2 liters
95° ethanol	5.15 ml	2951 ml	3 liters
Phenol crystals	0.15 g	85.9 g	100 g
Hydrochloric acid	0.15 ml or 5 ml if you receive the prepared dyes	85.9 ml or 2865 ml	100 ml or 3 liters
Sulphuric acid	1.25 ml	716 ml	1 liter
Methylene blue (0.1%)	0.003 g or 3 ml if you receive the prepared dyes	1.7 g or 1719 ml	2 g or 2 liters
Microscope lamp	One per year (only for optical microscope with halogen lamp)		
FLUORESCENCE STAINING			
Auramine O (0.1%)	0.003 g or 3 ml if you receive the prepared dyes	1.7 g or 1719 ml	2 g or 2 liters
95° ethanol	5.25 ml	3008 ml	3.5 liters
Crystal phenol	0.09 g	51.6 g	60 g
Hydrochloric acid	0.025 ml or 5 ml if you receive the prepared dyes	14.3 ml or 2865 ml	15 ml or 3 liters
Potassium permanganate (0.5%)	0.015 g	8.6 g	10 g
Methylene blue (0.3%)	0.009 g	5.2 g	6 g

APPENDIX III

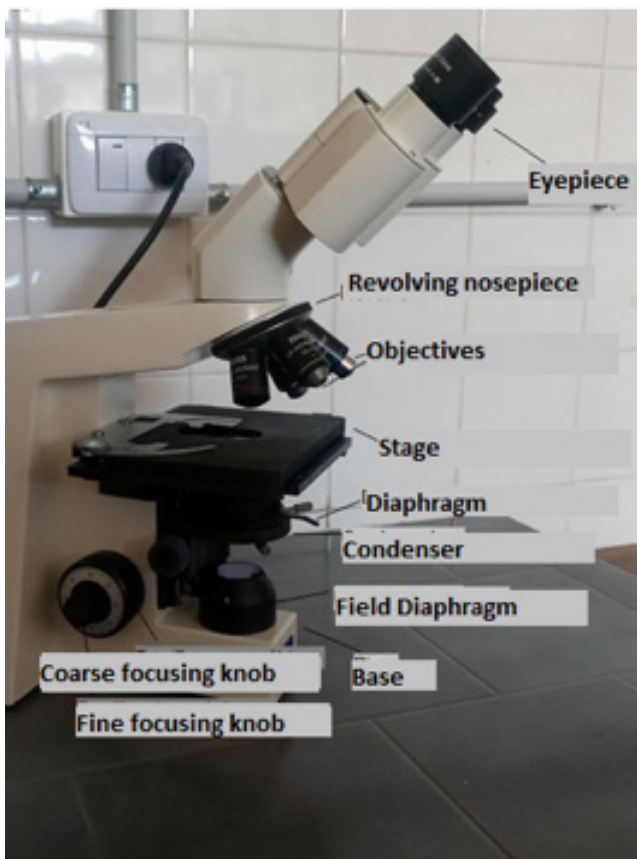
MICROSCOPE

As the human eye cannot see objects smaller than 0.1 mm, it is necessary to amplify the bacteria so they can be detected. This is possible with a microscope. They are magnified as many times as the result of multiplying the magnification of the objective by the magnification of the microscope eyepiece.

The quality of smear microscopy depends on the good condition and use of the microscope. It must be operated with great delicacy. All the laboratory personnel that use it must be trained to manage and maintain it, knowing for what each of the component parts are. Sudden or unnecessary movements, dust, any kind of dirt and humidity affect the equipment and compromise the accuracy of the microscopic examination.

MICROSCOPY BY ZIEHL NEELSEN STAINING

PARTS OF THE OPTICAL MICROSCOPE



Mechanical part

The base or foot of the microscope that works to hold the stage where the slide is placed must be heavy enough for the equipment to be steady. The stage has clamps to hold the slide and a vernier that allows locating a specific field. The movements in horizontal and vertical line of that clamp are controlled by two screws. The arm supports the eyepiece holder and a revolving nosepiece that allows locating lenses with several magnifications and can be replaced if necessary. The two eyepieces can be approximated or separated to adapt their position to the distance between the observer's pupils.

A thick screw (coarse focusing) allows to turn up and down the arm with wide movements and other smaller (fine focusing), with slow movements, in a way that is possible to adjust the objective position during the exam.

Illumination and optical elements

For the examination of a smear stained by the Ziehl Neelsen technique, it is advisable to use a binocular microscope, that is to say, a microscope with two eyepieces of 8 or 10x. It is required an objective with an immersion retractable lens 100x (preferably flat achromatic), in a way that, combined, magnify 800 or 1000 magnifications.

A mirror with two faces, one plane, another concave, directs a beam of light from the source of illumination to the optical axis of the microscope.

Generally, the lightning source is attached to the base of the microscope and it uses a halogen or a high intensity LED bulb. Tungsten filament bulbs can also be used. Halogen lamps resist a limit of use and so you should always have at least one spare. LED lamps have the advantage of having a high durability. The intensity of the lighting can be regulated. It also has a diaphragm (field diaphragm) that can be opened or closed, to disperse or concentrate the light beam, respectively.

In case of not having electricity, natural light must be used as an illumination source placing the microscope in front of a window that allows the sunlight comes inside.

The condenser is a lens placed under the stage that concentrates a light in the slide. In this part, there is a screw that allows raising and lowering this lens, and other two, generally smaller, that allows moving the beam of light that goes through the condenser. The condenser has its own diaphragm that allows scattering or concentrating the beam of light that goes through it and, generally, is handled with a lever.

Illumination centring (Kohler illumination)

With the following procedure it is possible to

centre the microscope illumination on the smear observation field in order to avoid the refraction and obtain the best image possible.

- Plug the microscope, if it needs power, or otherwise, capture the natural light with the mirror.
- Focus the smear with an object of low amplification, generally of 10x.
- Raise the condenser using the matching screw.
- Close fully the diaphragm of the lightning source.
- Identify a circle of light when looking through the eyepieces. If the field is totally dark, the beam of light is very off-center.
- Follow the next steps:
 - Soft and slowly, turn the condenser screws until the beam of light shows up or it is centred in the observation field.
 - Gently and slowly, move the position of the condenser up and down until you find the position in which the edge of the beam of light is as clear as possible.
 - Open the condenser diaphragm two thirds of the total opening.
 - Open the diaphragm of the lightning source until the beam of light reaches the edges of the observation field, no more than that.
- The intensity of the lamp light can be controlled according to the preference of the microscopist. It is also possible to add a filter over the lightning source to smooth it.
- Once the illumination is centred, do not move the diaphragm or the condenser until detects that is necessary to repeat the procedure, because the image is refracting or vague. If the microscope is handled carefully, it is not necessary to repeat the centring after several days or even months.

Optical microscope focus

Place a drop of immersion oil in the preparation taking care that the dropper does not touch the surface of the preparation to avoid transferring material from one smear to another and generate false positive results.

- Focus the sample on an area outside of where you placed the immersion oil using the 10x objective by turning the coarse adjustment knob.
- Adjust the distance between the eyepieces until both the images on the right and on the left become one.
- Fine-tune the focus of the image by turning the fine adjustment.
- Change to 100 x. Focus the sample using fine adjustment.
- Use only the 100x objective for observation with immersion oil and keep the rest of the objectives dry.
- Check if there are “artefacts”, that is, foreign bodies, or AFB that move abnormally:
 - If the artefacts or AFB move freely and pass under the gaze of the observer without being able to stop them, they can be remains from another smear microscopy that were dragged by the immersion oil. Clean the oil from the lens. Verify that the oil contained in the bottle in use is not contaminated by re-focusing another preparation with a new drop of oil
 - If the artefacts move when the slide is moved, they can be precipitates or dirt accidentally incorporated in the smear.
 - If they only move when the eyepiece is turned, it is dirt that is in the eyepiece, proceed to clean it.
 - If they do not move, dirt or contaminating bacilli can be in the lenses, the condenser, the mirror or the light source, proceed to clean them.

- Read at least 100 fields before reporting a negative result.

- Usually observation of 100 fields takes 5-10 minutes.

- To see the next smear, you do not need to perform all the previous steps. Turn the 100x objective towards the 10x objective and remove the slide, clean the lens with soft paper, place another drop of oil on the next slide and insert it into the stage. Then return the 100x objective to its place.

- When finished, reduce the light intensity
At the end of the day, clean the objective with a soft paper to eliminate the immersion oil residual. Cover the microscope.

MICROSCOPY BY THE FLUORESCENCE STAINING

For the reading of the smears stained with auramine, the WHO recommends the use of fluorescence microscopes with LED lamps replacing the regular fluorescence microscopes with mercury lamp. It has been shown that the efficiency of these microscopes is similar to the conventional ones, but they have the advantage that its lamp has a higher useful life (around 50 000 hours comparing to the 200 hours estimated for the mercury lamp), it generates almost no heat and does not have the contamination risks in case of breaking. Besides, the quality of the reading is good even with the absence of total darkness, which is an advantage in small labs.

There are microscopes with these characteristics on the market, and, in addition, adapters have been developed, which allow transforming an optical microscope into a fluorescence microscope at a lower cost than is necessary to acquire a fluorescence microscope. Some of these adapters involve the removal of the optical microscope objectives and its replacement for an equipped

objective with a LED lamp connected to an external source of energy, in such a way that they are transformed in fluorescence microscopes with reflected light (epifluorescence). Others, on the other hand, involve the installation of a source of light and a condenser in the base of the equipment and a barrier filter in the head of the microscope, in such a way that the excitation light comes from the lower part of the smear, it excites the fluorochrome and then goes through the objective to the eye of the user (transfluorescence). This illumination creates a background a little lighter than that observed when a smear is read through a microscope of reflected light. Ideally, the microscope can be focused using the 20x and 40x lenses, since the recommended strategy for reading the slides is to observe the smears at 200x, confirming the identification of the bacilli in 400x.

Microscope focus

- Place the slide on the microscope
- Focus the sample with the 20x turning the thick adjust of command.
- Adjust the distance between the eyepieces until the images on the right and left becomes one.
- Tune the focus of the image turning the thin adjust.
- Exam the smear moving it in horizontal.
- Stop and watch the field moving on to the next one.
- Read at least one 200x length before reporting a result as negative.
- Confirm the suspicious observations using the 40x objective.
- At the end of the day, clean the lens of the objective and the eyepiece with lens paper.
- Cover the instrument with the cover after its use.

GENERAL CARE OF MICROSCOPES

- The microscope must always be placed in a dry environment (mould can grow on the lenses), with no dust and on a surface with no vibrations. In wet geographic areas it is recommended to use a microscope which lenses have an antifungal treatment, because this process helps to protect the optical part of the equipment.
- It is also convenient to place the microscope in a place away from water fountains or chemical reactants in order to avoid splashes or spillsc.
- The immersion oil must have a refraction index higher than 1.5 (do not use cedar oil, because the residual remains attached to the lenses).
- In case of Ziehl Neelsen microscopy, do not withdraw the slides without changing the 100x objective to the 10x to avoid scratching the lenses.
- At the end of the reading of the day, cover the microscope with its cover.
- If it is not being used, store it in a box with silica gel; When the gel changes its colour from blue to pink it has been moistened, it must be restored by heating it in a dry atmosphere oven. It can also be stored in a box equipped with a lamp of 25 W maximum, which, when kept on, generates a dry environment.
- Clean the optic of the microscope with a soft lens paper or a tissue. Do not use solvents (alcohol, xylene, benzene, and acetone) to clean the objectives. These solvents can dissolve the adhesives of the lenses and allow the immersion oil goes into them.
- Use the cleaning solution recommended by the manufacturer only when there are finger prints or grease in the optic of the microscope.
- To clean the eyepieces: blow with a blow pear to eliminate the excess of dust. Clean the eyepieces

PART I: *Smear Microscopy Update*

with a cotton swab soaked in cleaning solution in circles from inside out. Then dry the eyepieces with soft paper.

- To clean the objectives: blow with a blow pear to eliminate the excess of dust. Soak the lens paper with the cleaning solution and softly clean with a circular movement from inside out. Then clean with a soft paper.

- The preventive maintenance should be scheduled to interfere as little as possible with routine work. The corrective (repairs and replacement of damaged parts of the microscope) is possible and must be carried out by specialized personnel.

APPENDIX IV

DOCUMENTATION

Request for examination of biological specimen for TB

Two models of forms are presented below, one prepared for laboratories that perform ZN staining and the other for those that perform staining with auramine.

Service:..... Date of request:.....

Patient name:.....

Age:.....Date of birth:..... Gender: Male Female

Patient address:.....

..... Phone:.....

Analysis reason:

Diagnosis. Is a presumptive TB RR/MDR diagnosis? Yes No

Monitoring. If it is monitoring, treatment month:.....

HIV Yes No Unknown

Previously treated for TB? Yes No Unknown

Specimen type Sputum Other.....

Test(s) requested: Microscopy Xpert MTB/RIF

Culture Drug susceptibility testing

Line Probe assay

Requested by (Name and signature).....

.....

Smear microscopy results (Ziehl Neelsen staining)(To be filled at the lab)								
Date sample collected (filled by the requester)	Type of specimen	Laboratorial serial number	Visual appearance (bloody, mucopurulent or saliva)	Result (tick one)				
				Negative (0 AFB/100 fields)	1-9 AFB/100 fields (Report number of AFB)	Pos(+)	Pos(2+)	Pos(3+)

Examined by (name and signature);.....

Date of the result:.....

Service:..... Date of request:.....
 Patient name:.....
 Age:.....Date of birth:..... Gender: Male Female
 Patiente address:.....
 Phone:.....

Analysis reason:

- Diagnosis. Is a presumptive TB RR/MDR diagnosis? Yes No
 Monitoring. Is it is monitoring, treatment month:.....

- HIV Yes No Unknown
 Previously treated for TB? Yes No Unknown
 Specimen type Sputum Other:.....
 Test(s) requested Microscopy Xpert MTB/RIF
 Culture Drug susceptibility testing
 Line probe assay

Requested by (name and signature)

Microscopy result (auramine staining)(to be filled at the lab)

Date specimen collected (filled by the requester)	Type of specimen	Laboratory serial number	Visual appearance (bloody, muco purulent or saliva)	Result (tick one)					
				Negative (0 AFB / Lengh)	It requires confirmation (*)	Scanty	Pos(+)	Pos(2+)	Pos(3+)

(*) Ask a new specimen

Examined by (name and signature);.....

Date of the result:.....

Request for examination of biological specimen for TB

- Service: name and address of public or private service that sends the specimen (hospital, health service, prison, social welfare, clinic, sanatorium, other)
- Date of request: day, month and year of the application request.
- Patient name: the one in the ID document.
- Age: at the moment of the analysis.
- Date of birth: the one in the ID document.
- Gender: Select with an X on M= Male or F= Female.
- Patient address: street and number of the patient's home. If the patient is in a prison or other institution, indicate the name and location.
- Phone: home or cell phone with the code area.

Analysis reason

- Diagnosis: select with an X if the specimen is for diagnosis.
- If it is presumptive diagnosis of TB resistant to rifampicin/multidrugresistant (TB RR/MDR): select with an X the right information.
- Monitoring: select with an X if it is a specimen is for treatment monitoring.
- If it is for monitoring, treatment month: write the number of month of treatment corresponding to the specimen.
- HIV infection: select with an X in the right option, according to the HIV state at the moment of the diagnosis. Yes= positive HIV infection, No= negative HIV infection, Unknown= unknown HIV state.

- Previously treated for TB: select with an X the right option. Yes= if the patient received the complete or incomplete treatment for TB before this application. No= if the patient did not received antituberculosis treatment before the application. Unknown= unknown state of previous treatment.
- Type of specimen: select the option corresponding to sputum. Yes= if it is this type of specimen. If it is other type of specimen, select the corresponding option and name the type of specimen for the bacteriological study.
- Tests requested: select microscopy, Xpert MTB/RIF, culture, drug susceptibility testing or Line probe assays, if applied.
- Requested by: Indicate the name of the health staff in charge of the application. Sign the request.

Microscopy result (to be filled at the lab)

- Date specimen collected: the applicant of the study must indicate the date of the sample gathering.
- Type of specimen: indicate the type of the specimen according to the upper part of the application form.
- Laboratory serial number: indicate the corresponding number of each specimen in the lab register.
- Visual appearance: If it is sputum, indicate if it is mucopurulent, bloody or saliva.
- Result: select the result of each specimen according to the staining performed and the standardized scale by the National Tuberculosis Reference Laboratory
- Examined by: full name and signature of the person that performed the microscopic exam.
- Date of the result: day, month and year of the report

(a) Y= yes; N= no; UNK= unknown

(b) Y = previously treated; N = not previously treated; UNK= unknown

(c) Patient on TB treatment; indicate month of treatment at which follow-up examination is performed.

(d) Xpert MTB/RIF test result reported as follows :

T = MTB detected, rifampicin resistance not detected

RR = MTB detected, rifampicin resistance detected

TI = MTB detected, rifampicin resistance indeterminate

N = MTB not detected

I = invalid / no result / error

If the Xpert MTB / Ultra RIF test is used, the report must be carried out as follows,

T = MTB detected, rifampicin resistance not detected

RR = MTB detected, rifampicin resistance detected

TI = MTB detected, rifampicin resistance indeterminate

TT = MTB detected (trace), rifampicin resistance indeterminate

N = MTB not detected

I = invalid / no result / error

(e) Smear results reported as follows:

If the smear microscopy was performed by Ziehl Neelsen method, results should be reported as follow:

Neg = 0 AFB

(1 to 9) AFB/100 fields = exact amount of AFB

10 to 99 AFB/100 fields = +

1 to 10 AFB/field = 2+

>10 AFB/field = 3+

If the smear microscopy was performed by the auramine method, results should be reported as follow:

Reading at 200x of magnification

Neg = 0 AFB

1 to 4 AFB/length = confirm

5 to 49 AFB /length= Scanty

3 to 24 AFB/ field = 1+

25 to 250 AFB/field = 2+

>250 AFB/field = 3+

Reading at 400x of magnification

Neg = 0 AFB

1. to 2 AFB/length = confirm

3 to 24 AFB /length= Scanty

1 to 6 AFB/ field = 1+

7 to 60 AFB/field = 2+

>60 AFB/field = 3+

(f) If the Xpert MTB/RIF result is indeterminate (I), indicate error code or "Invalid". This space can be reserved to register the date when the sample is sent to the reference laboratory, if it is the case.

INTERNAL QUALITY CONTROL STAINING SOLUTIONS CONTROL FORM

Staining solution	Date			Microscopic observation								Implemented measures		
	Preparation	Expiration	Control	Positive smear				Negative smear						
				Reading	Bacilli staining ₁	Crystals or precipitates ₂	Background color ₁	Reading	Crystals or precipitates ₂	Background colour ₁				

1 Indicate good or bad

2 Indicate yes or no

