Acid-Fast Direct Smear Microscopy

A Laboratory Training Program

This multinational training project has
been the collective effort on the part
of the following organizations:

World Health Organization (WHO)

International Union Against Tuberculosis
and Lung Disease (IUATLD)

Centers for Disease Control and
Prevention (CDC), USA

Pan American Health Organization (PAHO)

Instituto Nacional de Diagnóstico y
Referencia Epidemiológicos (INDRE), Mexico

Association of Public Health Laboratories (APHL), USA

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Table of Contents

Introduction

In most areas of the world, direct microscopy for acid-fast bacilli (AFB microscopy) is the primary diagnostic tool for the detection and control of tuberculosis (TB). The Ziehl Neelsen technique, performed directly on pulmonary specimens, is an effective method to detect TB cases, assess response to treatment and monitor cure rates.

As a crucial component of TB control, direct AFB microscopy must be performed appropriately using standard methods to accurately determine and report whether a patient is acid-fast smear positive or negative. To ensure that this diagnostic test is accessible to as many patients as possible, direct AFB microscopy is performed in many rural and urban peripheral laboratories.

In many countries, there are very few training products or courses available to the laboratory technician. This training program is intended to guide the laboratory technician by demonstrating appropriate technique and standard methods. It is not intended to replace the detailed procedures and instructions that are found in a laboratory manual for AFB microscopy. Therefore, these training materials should be used in conjunction with either the National TB Programme (NTP) manual for AFB microscopy, if available, or manuals published by WHO and IUATLD.

Target Audience

This training program is intended for laboratory technicians, at all laboratory levels, who perform or supervise direct AFB microscopy.

Program Description

This program is designed to assist laboratory technicians in performing direct AFB microscopy. The program content is intended to complement laboratory manuals by demonstrating appropriate technique, emphasizing standard methods, and promoting consistent reading and reporting of smear results.

The technical content of this training program, which was developed by an international team of experts, follows the methods and practices recommended by WHO and IUATLD.

The multiple formats of video, audiocassette, booklet and slides for projection are provided to ensure that the training material can be reviewed even if some resources, such as a videoplayer, are unavailable. The audiocassette is the exact soundtrack from the video. Projection slides contain information and pictures extracted from the video. This booklet also contains the same information and pictures from the video and may help with understanding if the video is not in the technician's primary language.

The videotape is approximately thirty minutes in duration. You may wish, however, to pause the video periodically to review the material in the booklet or discuss the topics with your colleagues. The images will also be provided in slide format.

A quiz is also included to help the technician measure what they have learned from the video and other training materials.

Learning Objectives

After viewing the video and/or reviewing the other training materials, the technician will be able to:

- Perform and report direct acid-fast smear microscopy using the WHO and IUATLD recommended methods.
- Understand the safety precautions for sputum collection and smear preparation.
- Identify the components of the laboratory register, specimen submission, and report forms.
- Identify common practices in smear preparation, staining, and reading to increase the reliability and accuracy of the smear.
- Describe the different reasons for false positive and false negative smear results.

"An Ancient disease is killing more people today than ever before. Tuberculosis, which many of us believed would disappear in our lifetime, has staged a frightening comeback.

We are at the crossroads.

We can permit the TB epidemic to become more deadly, or we can act now to end the suffering and deaths.

We are at the crossroads."

G. Harlem Brundtland, M.D., Director, WHO 1998

As we enter the twenty first century, many diseases continue to challenge the health of people around the world. Tuberculosis, both an ancient and a modern disease, looms in the foreground.

Over 8 million cases of TB continue to occur annually. Close to 2 million people still die of TB each year.

Poverty and malnutrition fuel the fire of disease in developing countries, while migrations, wars, and natural catastrophes set the scene for mass infection.

The global escalation in TB,the rise in HIV and the emergence of multiple drug resistant strains of TB have caused urgency and concern.

Several international agencies are dedicated to the control and eradication of tuberculosis worldwide.

Practical and affordable diagnosis is a key link in the chain.

ACID-FAST DIRECT SMEAR MICROSCOPY

This teaching module will focus on the Ziehl Neelsen diagnostic technique of direct smear microscopy of sputum.

Since most TB cases are pulmonary, sputum smear microscopy is the most available tool for TB diagnosis.

It is inexpensive, rapid and simple to perform.

More importantly, it can detect the most infectious cases of tuberculosis.

Identifying these cases can lead to treatment and cure while interrupting the flow of TB transmission. This procedure can also assess response to drug therapy and monitor cure rates after treatment.

There are many facets to the process of smear microscopy.

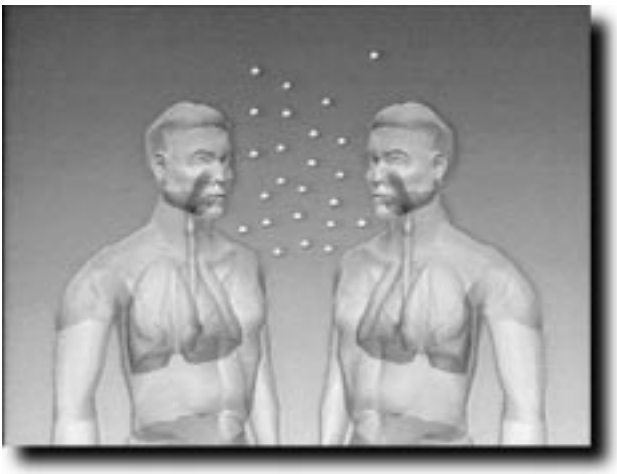
First let's discuss **Biosafety**

Working with TB requires care and special precautions.

A brief review of the mode of transmission will highlight the dangers in working with this microbe.

Mycobacterium tuberculosis

…is spread through the air from person to person via coughing, sneezing, and even singing and speaking.



Tiny invisible droplets dry quickly to form droplet nuclei that are less than 5 microns in diameter. They can contain disease organisms such as tubercle bacilli. These droplets may remain suspended in the atmosphere for several hours. Another person can breathe in the particles and become infected.

This is especially dangerous in a confined area. Keep in mind, when appropriate techniques are employed, there is very little risk of acquiring infection in the laboratory.

It is vital that all personnel be aware of potential hazards and work in a careful manner.

The greatest risk of infection in the laboratory involves sputum collection. Preparing smears presents less risk to the lab worker than exposure to direct coughing.

Since TB patients are sometimes directed to the lab for sputum collection, precautions must be taken to minimize the risk of exposure.

Never collect sputum specimens inside the clinic or laboratory.

It is safer to collect the specimens outside.

At all times, avoid creating aerosols in the lab! A daily routine that is followed exactly will help maintain safe conditions.

Strict adherence to safety regulations in the laboratory is crucial. Safe practices include:

- Washing hands frequently and always before and after performing any procedures.

- Establishing airflow in working areas that will carry infectious particles away from lab personnel. This air should be exhausted into a remote location.

- Strictly refraining from smoking and eating in the lab!!!

- Wearing protective clothing and use of protective equipment.

Surgical masks DO NOT protect against TB infection !

Effective respiratory protection, such as an N95 respirator, is expensive, and probably unnecessary if the technician uses appropriate technique.

Careful handling during smear preparation should produce minimal aerosols.

The low risk of infections during these procedures may not justify the cost of respirators and gloves where there may be limited resources for TB control.

Each country must evaluate the risks and decide on the level of protection that is appropriate with the resources that may be available.

In this training video, the technicians do not wear gloves or masks. Handwashing and careful technique are acceptable practices for most countries.

Remember, safety is everyone's concern no matter what job is being performed.

Laboratory Arrangement

To perform TB microscopy, a laboratory must have three areas:

Area 1

a well lighted space with a sink or basin with running water for preparation and staining of smears,

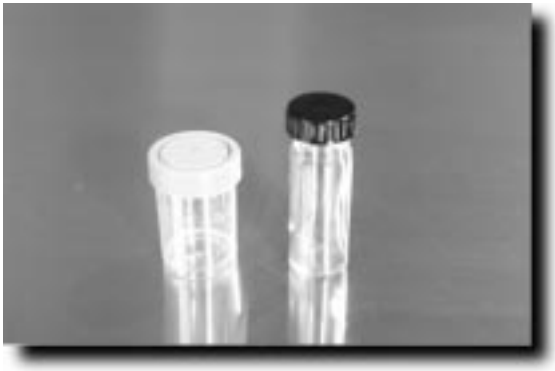
Area 2

a microscope table, (if there is no electricity, this table should be placed directly in front of a window)

Area 3

and a table for recording results and slide storage.

Specimen Collection



Two kinds of containers are recommended for sputum collection:

The first is a disposable wide-mouthed, screw-capped container. It is made of unbreakable transparent plastic. It must be hermetically sealed to keep the specimen from leaking and drying out.

The second is a screw capped "Universal container" made of heavy glass which can be first sterilized, cleaned and used again.

Many countries use their own containers.

Labeling of Specimens

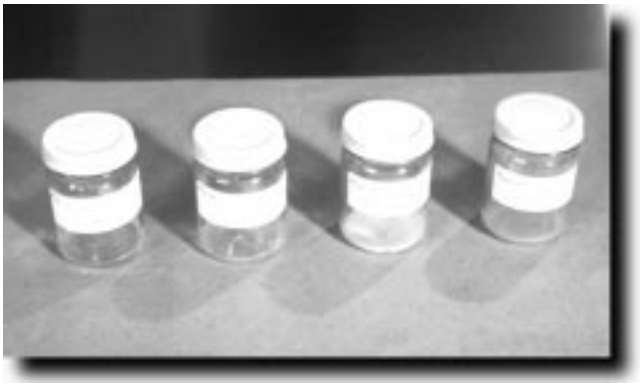
It is critical to label a specimen properly to avoid confusion and delay.

A request form must accompany each specimen. The information on the form must exactly match the information on the container. Always label the container on the side, never on the lid.

Using an indelible marking pen, write the name of the TB suspect and the date of collection.

Sputum Specimens

For best recovery of tubercle bacilli, collection of three specimens is advised. At least one should be an "early morning" specimen.



The patient should be properly instructed to ensure that the collected specimen is of sputum, not saliva.

Clear saliva or nasal discharge is not considered a suitable specimen for TB. Nevertheless they should be processed. Specific instructions for collection can be found in the TB program manual.

A good sputum specimen should be 3-5ml in quantity. It is frequently thick and mucoid, but may be fluid with chunks of dead tissue. The color may range from opaque white to green. Bloody specimens will be reddish or brown in color.

Record the visual appearance of the sputum sample on the lab form.

Washing Hands

Always wash hands with soap and water after handling specimens and containers.

Transport of Specimen

Specimens should be packed and transported according to your country's NTP guidelines.

Specimen Handling

Open the transport box carefully and examine it for cracked or broken containers. Discard any broken or leaking container and request another specimen.

All specimens must correspond with the accompanying list containing data of the TB suspect.

Once verified, record all information in the **laboratory register.**

The Laboratory Register

The WHO or IUATLD laboratory register is strongly recommended as a guideline. The format of the register should never be altered.

Information for each specimen must be complete, precise, and include:

- **Laboratory serial number**
- **Date received**
- **Patient's name, sex, age & address**
- **Name of Health Institution**
- **Reason for exam (whether as diagnosis or as chemotherapy follow up).**

Label the specimen container accordingly**.**

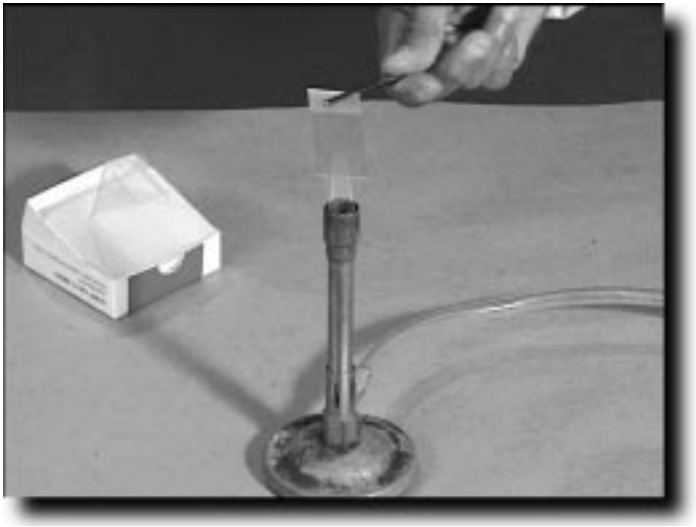
Smear Preparation

To ensure a safe and consistent workflow, always arrange equipment and materials in the same way at the workbench.

The following supplies are required:

- **Forceps,**
- **A diamond marker or lead pencil,**
- **Wooden applicator sticks or loop,**
- **A sand/alcohol jar (if a loop is used),**
- **A discard container with disinfectant,**
- **A Bunsen burner or spirit lamp,**
- **A box of new slides, and**
- **Specimen containers.**

Always use new slides when preparing AFB smears. They should be cleaned with alcohol and wiped dry… or passed briskly through a flame.



This will remove any residue of oil that could interfere with staining.

Never reuse sputum smear slides for TB work!!!

Properly identify each slide

Properly identify each slide with the laboratory and specimen number. Use a lead pencil to write these numbers on the frosted end of the slide.

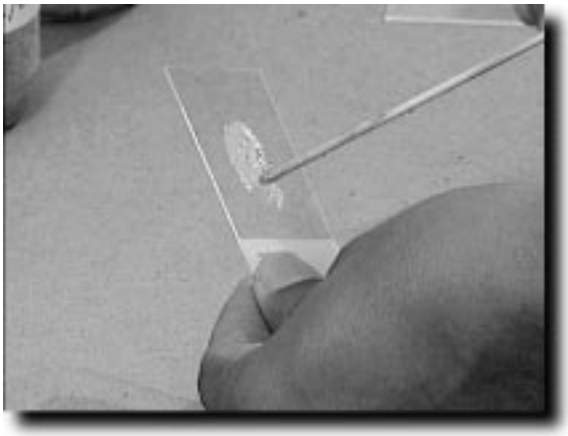
If the slide is not frosted, engrave the laboratory serial number using a diamond pointed stylus.

Never use a grease pencil, since markings could wash off during the staining process.

Open the container slowly to avoid producing aerosols.

Using either a wooden applicator stick or a loop, pick out portions of sputum for examination. Solid caseous particles often produce the highest number of bacilli.

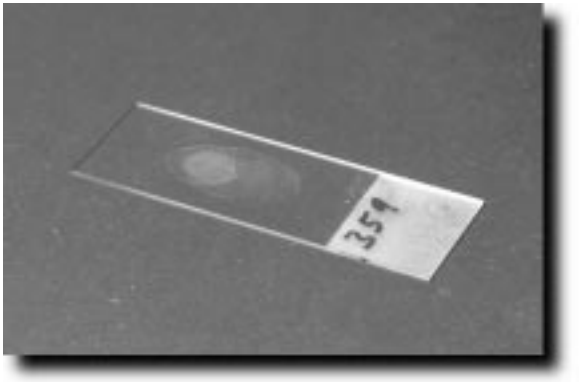
Using a continuous rotation, cover an oval area approximately 2 cm in length on the slide. Put only one smear on each slide.

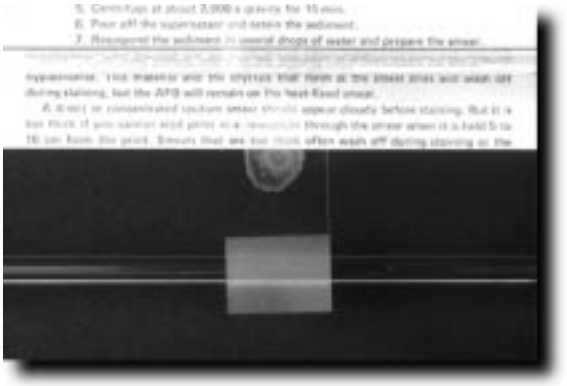


Wooden stick

Wire loop

How can you tell you have the proper thickness?





If it is too thin, the specimen may yield false negative results. If it is too thick, the smear may wash off the slide during the staining procedure. Newsprint can usually be read behind a dried smear when it is the proper thickness.

If a wire loop is used, clean it before the next use. Dip the contaminated loop into a sand and alcohol bottle and move it up and down to remove solid debris.

Next, heat the wire loop in the flame until red hot. Allow it to cool before using it again.

Wet slides can create aerosols if disturbed. Place them in a protected area where they can air dry for 15-30 minutes.

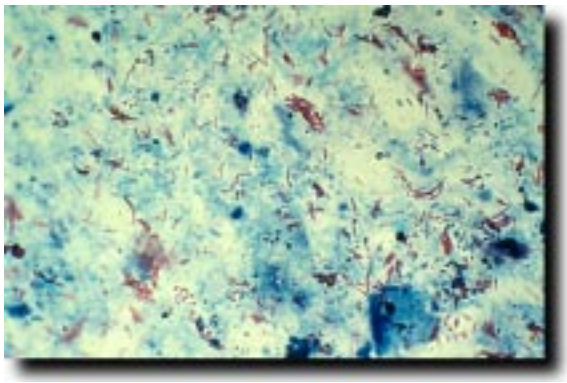
Do NOT flame them to induce drying.
This can also produce aerosols

When dried, fix the slides using the blue flame of a Bunsen burner. With forceps, pass the slide briefly through the flame 3 times, smear side up.

Heat fixation ensures that the sputum will stick to the glass slide. Excessive heating could damage the bacilli. If not sufficiently heat fixed, the acid fast bacilli may wash off during staining.

Staining Procedure

Since tubercle bacilli retain the primary stain even after exposure to strong acid solutions, they are called acid-fast. In the Ziehl Neelsen staining procedure, using carbol fuschsin and methylene blue, the acid-fast organisms appear red.



Prepare the area for staining.

On the table counter next to the sink, place the following items: Staining reagents, clock, forceps, cotton/alcohol holder or Bunsen burner, and tray containing fixed slides to be stained. A slide rack should be placed in the sink.

To assure quality and consistency, many countries produce reagents in a central lab. If preparation instructions are required, consult the WHO and IUATLD manual for standard guidelines.

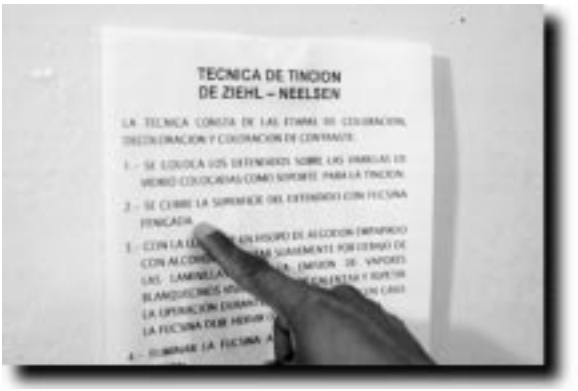
Standard reagents are:

- carbol fuchsin
- a decolorizing solution of acid/alcohol or sulfuric acid
- the counterstain methylene blue.

All reagents must have expiration dates.
Discard all expired dates.

Filter carbol fuchsin prior to use. If precipitate is still detected, discard the stain.

The staining procedure should be posted above the staining area.



To avoid cross contamination, never stain AFB smears in a staining dish.

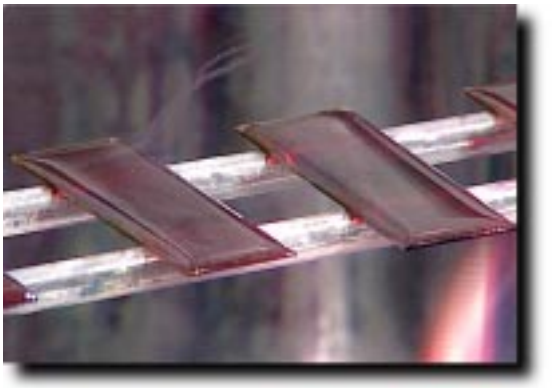
Now, let's study the important steps of the **staining procedure.**

Using forceps, place slides on a staining rack with the smear side up. Place all slides in uniform orientation.

Slides must not touch each other. This avoids the possibility of stain running over and causing cross-contamination. Include a + and - control slide daily for quality control purposes. Never stain more than 12 slides at a time.

Cover the entire surface of each slide with carbol fuchsin.

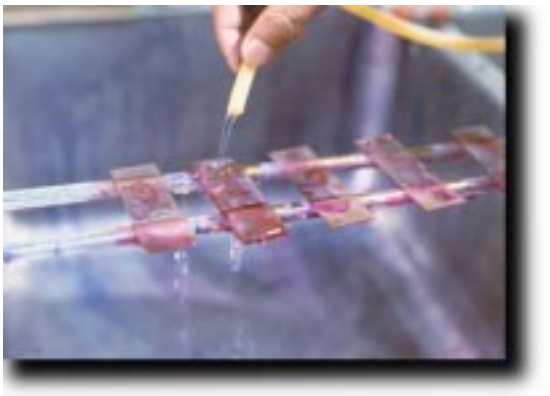
Using a Bunsen burner or cotton and alcohol flame, gently heat the slides until vapor rises.



Do not allow them to boil or dry. Boiling will alter the shape of the TB bacilli and could result in a false negative reading.

Allow the stain to remain on the slides for 5 minutes. Maintain heat throughout this period. Adequate time is required for the carbol fuchsin to penetrate and stain the cell wall.

Gently wash the stain from each slide with a stream of cold water until all free stain has washed away. Always rinse gently so the smear is not washed off of the slide.



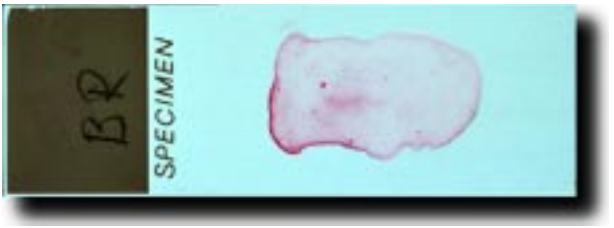
Individually tilt each slide to drain off any excess rinse water. This prevents puddles remaining on the slide which can dilute the next reagent.

Cover each slide with decolorizing solution such as acid alcohol.

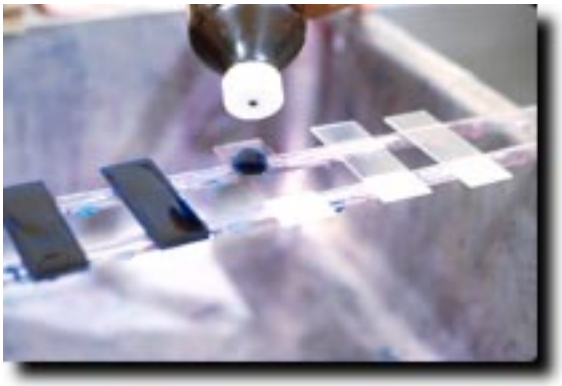
Leave this on the slides for 3 minutes. If under-decolorized, sputum contents other than the TB bacilli may remain stained. This could lead to a false positive result.

Rinse slides again carefully with water and tilt each slide to remove excess water.

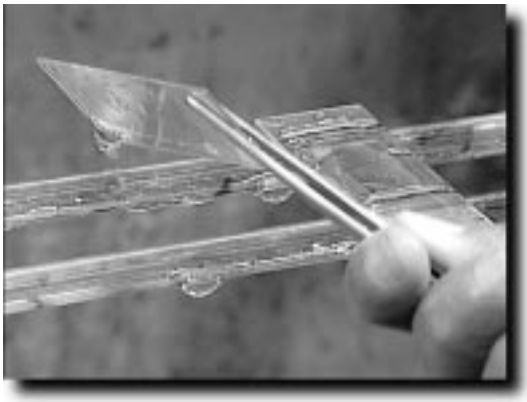
If the slide is still pink, an additional amount of decolorizing solution can be reapplied for 1 to 3 minutes.



Next, counterstain with methylene blue for l minute.



Rinse again with a gentle stream of water and tilt each slide to remove excess water.



Finally, tilt each slide and place in a slide block to air dry. **Do NOT blot.**

After smears are stained, clean off the back of each slide if necessary with some alcohol on a paper towel.

Do not examine slides until they are thoroughly dried.

Cover the stained slides to protect them from sunlight which can fade acid-fast bacilli.

Microscopic Examination

A good quality binocular microscope is recommended for reading TB smears.

It must be equipped with an electrical light source or mirror, an oil immersion or 100X objective, eyepieces of 8-10X magnification and a mechanical stage.

Daily care and cleaning are essential to avoid dust, oil build up and moisture.

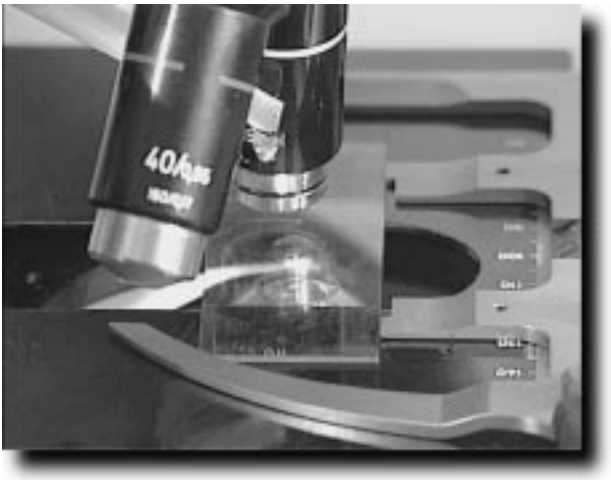
An area of subdued lighting is preferable for reading slides.

However, if no electricity is available, daylight must be used as a light source and the microscope should be placed in front of a window.

Arrange the following supplies next to the microscope: a bottle of immersion oil, organic solvent, lens paper, laboratory register and a slide box for storage.

Use the 40X objective to focus and determine a suitable reading area of the slide.

Place a drop of immersion oil on the stained smear. Let the drop fall freely onto the slide.



Never touch the slide with the oil applicator. This could lead to carry over of AFB in the immersion oil to the next slide.

Turn the nosepiece to bring the 100X objective into place. Now, gently lower the 100X objective.

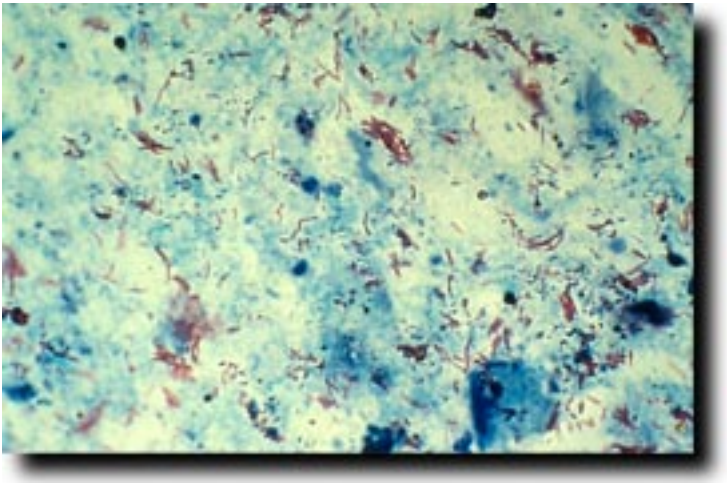
It should barely touch the oil. Never allow the lens to touch the slide. This can damage the lens and possibly break the slide.

While looking through the eye-piece, adjust the immersion lens slowly and focus until the image on the smear appears. To fine focus, turn the fine adjustment knob carefully.

At least 100 microscopic fields should be examined before reporting as a negative smear. Examining fewer than 100 fields may cause a false negative report. Fewer than 100 fields can be read if the slide is positive for AFB.

Read systematically to avoid overlap, moving the slide lengthwise so that the next field to the right can then be examined.

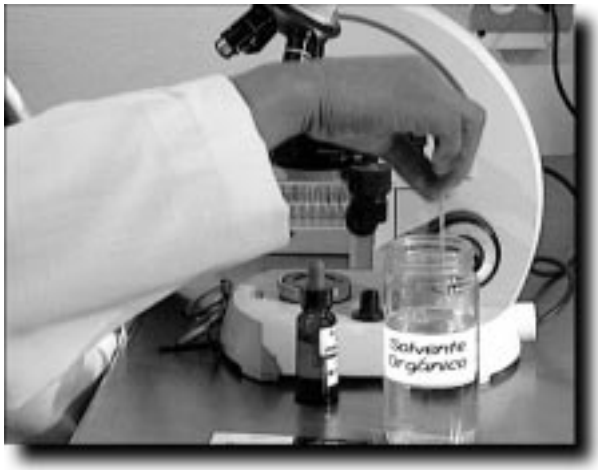
Tubercle bacilli resemble fine red rods standing out against the blue background. They may be slightly curved, granular, and may occur singly, in pairs, or groups.



Count the number of AFB per field and record this figure on the lab report.

After examining the slide thoroughly, remove it carefully from the microscope stage.

Check the slide number again and enter the result immediately into the lab register.



After completing the reading, remove the oil from the slide with organic solvent.

When dried, place the slides carefully in a slide storage container for transfer to a referral laboratory for quality control. This must be done on a regular basis as determined by the National Tuberculosis Programme.

Before examining the next slide, wipe the immersion lens with lens paper. This will protect from carry over of organisms that could create a false positive reading.

Handle specimens carefully!

All sputum specimens are considered potentially infectious. Save all specimens until the smears have been examined and recorded; then sterilize and discard them. Disposable containers must be used only once.

Discard of Specimens

- Burning
- Boiling
- Autoclaving

Recording and Reporting

It is vital that results of all sputum exams performed be entered correctly into the Tuberculosis Laboratory register. Enter positive results in red, for quick reference.

If acid-fast bacilli are seen, report the observation as “smear positive for AFB”. These patients are infectious and must be registered for treatment with an effective regimen.

Testing and reporting should be done as soon as possible, preferably within 24 hours.

The WHO and IUATLD recommended method for reporting results is as follows:

Negative: Report: “Negative for acid-fast bacilli” where no organisms have been observed in 100 fields.

Positive: Report: “Positive for acid-fast bacilli.” Provide AFB quantitation.

The number of AFB found is an indication of the degree of infectivity as well as severity of disease. Results must be quantitated.

The following semiquantitative reporting method is recommended for the Ziehl Neelsen technique.

- **If No AFB are found in 100 fields, report: No acid-fast bacilli observed.**
- **If 1-9 are found in 100 fields: record the exact figure.**
- **If 10-99 AFB are found in 100 fields: report as 1+.**
- **If 1-10AFB are found per field, report as 2+.**
- **If greater than 10 AFB per field, report as 3+.**

Include the following information in the report:

- **Evaluation of the quality of the specimen**
- **Staining method used (Ziehl Neelsen)**
- **Smear result**
- **Date of exam**
- **Signature of microscopist**

There are many species of AFB in addition to the tubercle bacilli. Do not try to identify species by microscopy. Report only the number of AFB seen.

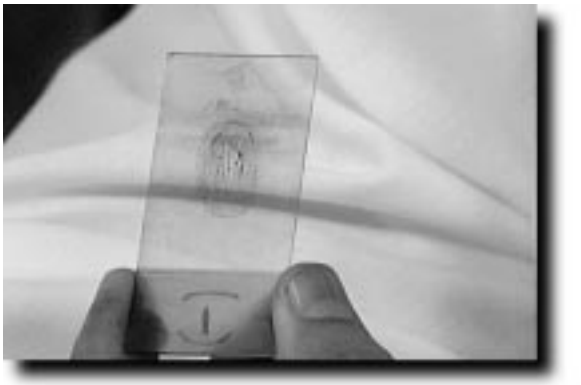
After completing the lab report, send it back to the health care center for further action.

Quality Control in the TB Laboratory

Quality Control (QC) should be routinely performed in the laboratory to ensure reliability as well as reproducibility of results.

Perform QC on each new batch of stain. Observe these results before reading patient slides. This important step will verify correct staining procedure and microscope function.

When controls are not demonstrating proper staining, determine the cause and correct it.



If the negative control appears red after the staining procedure, this is the result of incomplete decolorization.

If the positive control slide does not show AFB, either the staining reagents are defective or the procedure has been performed incorrectly. When the problem has been identified and corrected, repeat the staining procedure with new slides and controls.

Each phase of smear microscopy must be carefully performed. Errors made during any step can lead to incorrect results.

Some reasons for false positive results include:

- **Errors in specimen handling or recording information**
- **Re-use of containers or positive slides**
- **Unfiltered fuchsin**
- **Contaminated immersion oil**
- **Inadequate decolorization**

Some reasons for false negative results include:

- **Errors in specimen handling or recording information**
- **Poor quality sputum**
- **Excessive decolorization**
- **Reading less than 100 microscopic fields**

AN ERRONEOUS REPORT CAN HAVE DEVASTATING RESULTS!

If a False positive result is reported, a patient will be placed on treatment unnecessarily.

If it is a Follow-up, treatment is lengthened. Valuable medication is wasted and, sadly, it can cause emotional trauma to patients and their families. Patients may lose confidence in the program itself.

If, on the other hand, a False negative result is reported, a catastrophic set of events can occur.

- A patient with TB is not treated.

- If the sample was a follow-up specimen, the intensive phase of therapy would not be extended causing inadequate treatment. This can lead to additional suffering, spread of TB to family and community, and even end in patient death.

Accuracy at every stage is vital to the health and well being of many people.

As a member of the global campaign to control this dreaded disease, the role of the laboratory technician is as significant as every other member of the TB control team.

If the important control and prevention programs are to be successful worldwide, accurate smear microscopy is a critical link in the chain of tuberculosis control.

We are at the Crossroads.

Post Test

1. What is the greatest safety risk in a clinic performing AFB microscopy?

2. List several safety precautions that are used when performing AFB microscopy.

3. Describe a good sputum specimen.

4. What should be done with leaking or broken specimen containers?

5. Describe the size and thickness for a smear.

6. Why is a slide allowed to air dry before heat fixation?

7. How can you determine when the Carbol Fuchsin is heated appropriately?

8. How can you tell when a slide is under-decolorized?

9. How many microscopic fields should be reviewed before a smear is reported as negative for AFB?

10. When are positive and negative control slides performed?

Post Test Answers

1. *Aerosols.*

2. *Collect specimen outside; wash hands frequently; take care not to produce aerosols; refrain from smoking and eating; use protective clothing and equipment as necessary.*

3. *3 to 5 mls; thick and mucoid; may be fluid with chunks of dead tissue; opaque white to green; bloody specimens may be reddish or brown.*

4. *Carefully place in a discard container and request a new specimen.*

5. *2 centimeter oval on the slide; can read newsprint through dried smear.*

6. *To avoid aerosols.*

7. *It steams but does not boil.*

8. *It remains pink.*

9. *100 fields.*

10. *Per batch*

Use of trade names is for identification only and does not constitute endorsement by APHL, WHO, IUATLD, the US Department of Health and Human Services and the Mexican Ministry of Health.

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