

Module 5: Preparation of Ziehl-Neelsen Reagents

Purpose	To provide participants with knowledge and skills in the preparation of Ziehl-Neelsen reagents for AFB Staining.
Pre-requisite Modules	Modules 1, 2
Module Time	4 hours 15 minutes
Learning Objectives	<p>At the end of the module, participants will be able to</p> <ul style="list-style-type: none">• Describe the importance of using quality chemicals for reagent preparation• Prepare reagents required for the Ziehl-Neelsen method• Describe the safety requirements for reagent preparation• Use positive and negative control slides for the quality control of Ziehl-Neelsen reagents• Explain the use and frequency of routine quality control procedures.

Module Overview

Step	Time	Activity / Method	Content	Resources Needed
1	5 min	Presentation	Module introduction	Slides 1–3
2	30 min	Presentation	Reagent preparation	Slides 4–17
3	10 min	Questions and Answers	Summary	Slide 18
4	1 hr 45 min	Practical	Laboratory Practical Session #1: Reagent Preparation	Laboratory with reagent preparation capacity
5	1 hr 45 min	Practical	Laboratory Practical Session # 2: Quality Control of Reagents	Laboratory with staining facilities and microscopes

Material and Equipment Checklists

- PowerPoint slides or transparencies
- Overhead projector or computer with LCD projector
- Laboratory with facilities for reagent preparation, staining and microscopy
- Appendix 1: Example of Logbook For Quality Control Of Staining Reagents and a Blank sheet
- Appendix 2: Recording Worksheet for Laboratory Practical Session # 2

Teaching Guide

Slide Number	Teaching Points
1	<p><u>Module 5: Preparation of Ziehl-Neelsen Reagents</u></p> <p>DISPLAY this slide before you begin the module.</p> <p>Make sure the participants are aware of the transition into a new module.</p>
2	<p><u>Learning Objectives</u></p> <p>STATE the objectives on the slide</p>
3	<p>Flipchart</p>  <p><u>Content Overview</u></p> <p>(Suggested format for presentation)</p> <p>WRITE the content outline on a flipchart prior to training.</p> <p>REFER to it frequently to orient participants to where they are in the module.</p>
4	<p><u>Required Materials</u></p> <p>STATE what materials are required to make ZN staining reagent.</p>
5	<p><u>Reagent Quality: Specifications</u></p> <p>STATE the message on the slide</p> <p>EXPLAIN that the quality of basic fuchsin varies from different manufacturers in its purity and solubility. If a basic fuchsin powder is used which does not easily dissolve in alcohol/phenol the final concentration of staining reagent achieved may be unsatisfactory.</p> <p>EXPLAIN that technical grade acid and alcohol give satisfactory performance and are cheaper than analytical grade reagents.</p> <p>EMPHASIZE that the water used in reagent preparation must be purified to ensure that it is free from contamination with environmental mycobacteria which may be present in tap water.</p>

Slide Number	Teaching Points
6	<p><u>Dye Powder Specifications</u></p> <p>POINT OUT that the bottle of methylene blue on the left has detailed specifications, including dye content (bottom right corner).</p> <p>EXPLAIN that the picture on the right shows four different brands of basic fuchsin, none of which has the dye content indicated.</p>
<p>7</p> 	<p><u>Correcting for Dye Purity</u></p> <p>EXPLAIN that if the dye content is below 85% then a correction for dye purity is needed.</p> <ul style="list-style-type: none"> If the purity of basic fuchsin is low then the concentration of the prepared carbol fuchsin may not stain the AFB properly. False negative results may occur. <p>DISCUSS the example displayed on the slide.</p> <p>ENSURE that the participants understand how to calculate the correction for dye purity and its importance.</p>
8	<p><u>Weighing Balance</u></p> <p>EXPLAIN purpose of using the Weighing balance.</p> <p>STATE the messages on the slide.</p>
9	<p><u>Using the Balance</u></p> <p>STATE the messages on the slide.</p> <p>HIGHLIGHT the importance of ensuring the balance is set to zero after the weigh container or paper is placed on the balance.</p>
10	<p><u>Water Quality</u></p> <p>EXPLAIN that rain water and tap water may contain environmental mycobacteria.</p> <p>Boiling of water will kill any mycobacteria present but the organisms may still appear as AFB on staining</p>
<p>11</p> 	<p><u>0.3% Carbol Fuchsin</u></p> <p>DISCUSS the preparation of carbol fuchsin staining reagent.</p> <ul style="list-style-type: none"> The formulation may need to be adapted to meet local NTP guidelines.

Slide Number	Teaching Points
<p>12</p> 	<p><u>0.3% Methylene Blue</u></p> <p>DISCUSS the preparation of methylene blue staining reagent.</p> <ul style="list-style-type: none"> The formulation may need to be adapted to meet local NTP guidelines.
<p>13</p> 	<p><u>Decolorising Solution</u></p> <p>DISCUSS the preparation of 25% sulphuric acid and 3% acid alcohol as decolorising reagents.</p> <p>EXPLAIN that strong acids are cheaper than alcohol based decolorising reagents</p> <ul style="list-style-type: none"> Use the decolorising agent recommended in the local NTP guidelines.
<p>14</p> 	<p><u>Alternative Decolorising Solution</u></p> <p>DISCUSS the preparation of acid alcohol</p>
<p>15</p>	<p><u>Diluting Acids Safely</u></p> <p>STATE that a lot of heat is generated in the preparation of strong acid solutions. REMIND participants to always add acid to water.</p> <p>EMPHASIZE that is necessary to add the acid slowly to the water stopping regularly to mix, swirling the flask to allow the solution to cool. The flask may be placed running tap water to cool.</p>

Slide Number	Teaching Points
16	<p><u>Quality Control of New Staining reagents</u></p> <p>EMPHASIZE the need for performing quality control on ZN reagents before using routinely.</p> <p>DISCUSS the QC protocol from the slide.</p> <p>EMPHASIZE that the positive control smears should be prepared from a sputum sample containing between 10–99 AFB per 100 fields.</p> <ul style="list-style-type: none"> • Determine the average count of AFBs in each batch of positive control smears. • Ensure that the expected number of AFB are seen in the positive control smears when stained with new reagents. • Check that the AFB are strongly stained. <p>EXPLAIN that the negative control smears need to be stained three times with the new reagents. This process is performed to check that the newly prepared reagents do not contain environmental mycobacteria.</p> <p>Check all control smears for clean and well decolorised background.</p>
17	<p><u>Reagent Labeling and Storage</u></p> <p>EMPHASIZE the need for proper labelling with the name of the reagent and date of preparation.</p> <ul style="list-style-type: none"> • An expiry date is not recommended as the shelf life of staining reagents is variable depending on reagent quality and the country setting. • EXPLAIN that staining reagents will rapidly deteriorate if continually exposed to direct sunlight. Store in a cool dark area in the laboratory.
18	<p><u>Summary</u></p> <p>ASK the participants to answer the questions on the slide.</p> <p>ANSWER any questions the participants may have.</p>

Slide Number	Teaching Points
	<p><u>Laboratory Practical Session # 1 and 2: Preparation of Ziehl-Neelsen reagents and quality control</u></p> <p>EXPLAIN participants will go to the laboratory for a practical session on preparing reagents. ADD the purpose of this practical is to familiarize participants with the materials and techniques required to prepare good quality reagents.</p> <p>EXPLAIN that participants should work in pairs at each bench workstation.</p> <p>DEMONSTRATE reagent preparation to participants before participants start preparing reagents</p> <p>INSTRUCT the groups to make reagents from chemicals provided.</p>
 <p>TIPS</p>	<p>Tips for Demonstration</p> <ul style="list-style-type: none"> • Make sure everyone can see • Show each step slowly and methodically. Move slowly enough so participants can follow what you are doing – this is slower than normal. • Talk out loud as you perform each step, but keep explanation brief and clear. Describe every step at the same time that you do it. • Refer to written procedure • Point out commonly made mistakes and teach participants how to avoid them. • Repeat steps as necessary • If you repeat the procedure, do exactly the same thing each time.

Laboratory Practical Sessions # 1 and 2: Ziehl-Neelsen Reagent Preparation And Quality Control

Trainer must plan ahead for the laboratory activity exercise depending upon the workstations, number of participants and availability of reagents and glassware at the training site.

Trainer must arrange in advance, unstained negative and 1+ AFB smears for quality control purpose.

Practical session to be conducted in groups of 2– 6 persons in a laboratory.

Divide the participants into groups. Every group must work individually for exercise 1 and 2.

Laboratory Practical Session # 1: Ziehl-Neelsen Reagents Preparation

Materials and Equipment

- White board or flipchart
- Worksheet to record amounts of reagents in Appendix 1
- Sufficient space and benches
- Tap water and water for reagent preparation

Materials and equipment

- Weighing balance with a sensitivity of 0.1 to 0.01 g and a capacity of at least 50 g, with paper sheets or trays for weighing
- 2 spatulas or spoons for chemicals
- 1 set of chemicals as supplied routinely by the NTP:
 - Basic fuchsin powder
 - Phenol crystals
 - Methylene blue
 - Alcohol 95% or methanol
 - Sulfuric acid (minimum 95%) or hydrochloric acid (fuming)
 - Purified water

Per group of participants:

- 1 laboratory coat per participant
- 3 conical flasks (2 L)
- 1 measuring cylinder (1L)
- 1 measuring cylinder (50 or 100 mL)
- 3 reagent bottles (1 L)
- labels for the bottles
- 1 marker pen
- Gloves
- Safety glasses (if available)

Instructions for the trainer

EXPLAIN the safety aspects of staining reagent preparation, particularly in handling strong acids, which are corrosive and will cause burns.

ASK groups to prepare one liter of each reagent.

With the participants, **DETERMINE** the quantities of ingredients to be measured or weighed for each staining reagent.

DEMONSTRATE how to check the dye content of the basic fuchsin powder used, and **ASK** participants if this needs to be taken into account.

WRITE the quantities on the board or flipchart.

DEMONSTRATE correct method of diluting acids

DEMONSTRATE correct handling of weighing scale

DEMONSTRATE correct sequence for dissolving basic fuchsin and checking if basic fuchsin is completely dissolved before topping up with water

INSTRUCT each group to share the same balance and chemicals.

PROVIDE each group with a set of glassware.

INSTRUCT each group to record in the worksheet (Appendix 1), the amount of reagents taken for preparation of reagents

INSTRUCT groups to make one set of reagents.

INSTRUCT the groups to prepare each of the staining reagents one after the other, as a group. It is best to begin with carbol fuchsin.

SUPERVISE participants during this preparation phase and point out errors in technique, paying particular attention to

- Correct handling of weighing scale
- Correct method of diluting acids
- Correct sequence for dissolving basic fuchsin and checking if basic fuchsin is completely dissolved before topping up with water

INSTRUCT groups to label each reagent bottle with the reagent name, date of preparation and name of preparer.

Laboratory Practical Session # 2: Quality Control of Ziehl-Neelsen Staining Reagents

Materials and equipment

- 1 Binocular microscope
- 1 set of ZN reagents prepared in Exercise 1
- 1 set of squeeze bottles or pipettes for ZN reagents
- 1 washing bottle or beaker
- 1 dropper bottle with immersion oil
- 1 staining rack and a sink or basin
- 1 set of fixed two positive and two negative control smears.
- 1 spirit lamp (to fix the smear)
- 1 spirit torch (to heat the carbol fuchsin reagent laden smears)
- 1 slide drying rack
- Recording sheet

Instructions for the trainer

DESCRIBE briefly the correct staining procedure using a board or flipchart. The staining procedure will be discussed in detail in the next modules.

DEMONSTRATE the participant the staining procedure and appearance of AFB under the microscope

PROVIDE each group with heat fixed two positive and two negative control smears.

ASK each group to use their reagents to stain two positive and two negative control smears.

EXPLAIN that positive controls are to be stained once. Stain the negative control smears three times.

REMIND the participants that negative smears are stained three times to check for the presence of environment mycobacteria in the water used to prepare the carbol fuchsin.

INSTRUCT each group to read their stained control smears examining 100 fields.

PROVIDE each group with the Exercise 2 Recordng Sheet (Appendix 3) to record the number of AFB observed, the color of the AFB, and the background for each smear.

WRITE the results of each group on a board/flipchart.

COMPARE the results of each group.

DISCUSS numbers and color of the AFB observed in the positive control smears with the expected result (refer to Appendix 2)

EXPLAIN each set of data

WRITE down the conclusions on quality of the reagents prepared by each group on the board/flipchart.

Discuss any questions and concerns