



## Module 5

### Preparation of Ziehl-Neelsen Reagents

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<b>Purpose</b>	To provide you with an understanding of staining reagents and their preparation in acid fast staining
<b>Prerequisite Modules</b>	Module 2
<b>Learning Objectives</b>	<p>At the end of this module, you will be able to</p> <ul style="list-style-type: none"><li>• Describe the importance of using quality chemicals for reagent preparation</li><li>• Prepare reagents required for the Ziehl-Neelsen method</li><li>• Describe the safety requirements for reagent preparation</li><li>• Use positive and negative control slides for the quality control of Ziehl-Neelsen reagents</li><li>• Explain the use and frequency of routine quality control procedures.</li></ul>
<b>Content Outline</b>	<ul style="list-style-type: none"><li>• Equipment required for staining reagent preparation</li><li>• Reagents required for staining reagent preparation</li><li>• Methods for staining reagent preparation</li><li>• Storage of staining reagents</li><li>• Quality control (QC) of freshly prepared staining reagents</li></ul>
<b>Handout and Exercises</b>	Laboratory Practical Session # 1: Reagent preparation Laboratory Practical Session # 2: Quality control of staining reagents
<b>Appendix</b>	Appendix 1: Worksheet for preparation of reagents for Laboratory Practical Session # 1  Appendix 2: Example of logbook for quality control of staining reagents and blank sheet  Appendix 3: Recording worksheet for Laboratory Practical Session # 2

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## Module 5: Preparation of Ziehl-Neelsen Reagents

Reagent preparation requires equipment for weighing and measuring. Water, free of environmental mycobacteria, must also be available. Environmental mycobacteria often colonize water tanks and taps, and could on rare occasions result in a false positive reading. Therefore, the intermediate-level laboratory usually prepares staining reagents with quality control checks before these staining reagents are distributed to the peripheral labs.

Good staining reagents, especially those made with a high-quality basic fuchsin dye, are essential to detect acid-fast bacilli (AFB). While it is easy to demonstrate AFB in a highly positive smear, only a good staining reagent will also be able to show the AFB when they are rare or damaged due to drug treatment and are especially difficult to stain. Poor quality staining reagents may not show these AFB and a case of TB maybe missed.

### EQUIPMENT REQUIRED FOR STAINING REAGENT PREPARATION

The following list is required for preparing staining reagents:

- A balance or weight scale, with a sensitivity of 0.1 gram
- Measuring cylinders of 100 mL, 500 mL, and 1000 mL capacity (one each)
- Large Erlenmeyer (conical) flasks or flat-bottomed balloon flasks, capacity at least one liter
- A spirit lamp for heating
- A stirring plate with heating and magnetic stirrers (this is preferable when preparing larger quantities)
- Containers for the newly prepared staining reagents (dark amber glass bottles are recommended, but plastic bottles or containers with tight closures may be easier to transport)
- Labels for bottles
- Brushes to clean bottles before reuse
- Funnels to fill bottles, one funnel for each solution
- AFB-positive and negative unstained control smears

### REAGENTS REQUIRED FOR STAINING REAGENT PREPARATION

These will vary according to guidelines and supplies issued by your NTP. Always follow NTP instructions.

#### **Carbol fuchsin**

Since many brands of basic fuchsin on the market do not specify quality, it is imperative to buy reputable products from branded international companies or any other company that has been certified by the Biological Stain Commission.

For carbol fuchsin, the following items are required:

- Basic fuchsin powder of good quality
- Phenol crystals of good quality. The crystals should be almost colorless; quality must be assessed through quality control of AFB staining. AFB smears should yield solid, homogenous, and strong red-staining bacilli.

- Alcohol (can be denatured 95% ethanol or methanol)
- Water (distilled or purified)

### **Decolorization solution**

For staining reagent preparations using acid, you need the following items:

- Concentrated sulphuric acid ( $\geq 95\%$ )
  - Water (distilled or purified)
- or**
- Hydrochloric acid (37%, fuming)
  - Alcohol (denatured 95% ethanol or methanol)

Both acids can be of industrial grade, but they should not look dirty.

### **Methylene blue**

For staining reagent preparations using methylene blue, you need the following items:

- Methylene blue powder of good quality
- Water (distilled or purified)

## **METHODS FOR STAINING REAGENT PREPARATION**

Quantities of reagents needed may vary according to final concentrations prescribed by your NTP guidelines (some prefer a 0.3% fuchsin concentration, while others suggest 1%; some NTPs prefer 0.1% methylene blue over 0.3%). Always follow NTP instructions.

The quality of basic fuchsin varies from different manufacturers in its purity and solubility. If dye purity is known it should be taken into account for basic fuchsin, especially if the 0.3% final stain concentration is to be used. The amount to be weighed has to be corrected by dividing the prescribed amount by the decimal equivalent of the dye content. For instance, if a brand has a 75% dye content, you must divide the amounts by 0.75. So  $3 \text{ g} / 0.75 = 4 \text{ grams}$  will be weighed for the 0.3% stain. No corrections are needed if it is 85% or higher in final concentration.

If the dye purity is unknown or if the basic fuchsin dissolves poorly or precipitates out of solution even after initial filtering of the stain, it may be prudent to use the higher concentration (1%) in the staining reagent preparation. This can help ensure that the minimal concentration of 0.3% carbol fuchsin is achieved.

Prepare the final solutions according to the following guidelines:

- **1 liter of carbol fuchsin: final concentrations 0.3% basic fuchsin and 5% phenol**
  - Weigh 3 grams of basic fuchsin and 50 grams of phenol crystals separately
  - Measure 100 mL of alcohol (denatured ethanol or methanol) and pour it in a conical flask
  - Add the phenol and swirl the flask till it is dissolved
  - Add the basic fuchsin powder and continue to swirl until the fuchsin powder completely dissolves. If this is too difficult, try adding 100 mL

water. Check for remaining powder or crystals on the bottom. If these are seen, continue swirling with occasional slight heating. It may be preferable to leave the mixture stirring with slight heating overnight.

- Only after fuchsin is completely in solution, add 850 mL of water (or the remainder) and mix by further swirling
- **1 liter of sulfuric acid 25%**
  - Add 750 mL of pure (cold) water to a two liter Pyrex conical flask
  - Measure 250 mL of concentrated sulphuric acid in a cylinder
  - Pour it **slowly** into the flask containing the water, directing the flow of acid gently along the inner side of the flask. This will generate a lot of heat. Usually, it is necessary to stop a few times, swirling the flask to let it cool off a bit, or even holding it under the tap with cold water.
  - Mix well by swirling the flask.

***Never add water to acid! This will make it boil immediately and it may even splash in your face!***

***Always wear protective laboratory coats, gloves, and safety glasses when handling strong acids.***

In case of an accident with acid, rinse the affected body part or cloth ***immediately*** with plenty of water.

- **1 liter of 3% hydrochloric acid in alcohol**
  - Add 970 mL of 95% alcohol to a two liter Pyrex conical flask
  - Measure 30 mL of concentrated hydrochloric acid in a cylinder
  - Pour it **slowly** into the flask containing alcohol, directing the flow of acid gently along the inner side of the flask with constant swirling.
  - Mix well by swirling.

***Always wear protective laboratory coats, gloves, and safety glasses when handling strong acids.***

In case of an accident with acid, rinse the affected body part or cloth ***immediately*** with plenty of water.

- **1 liter of methylene blue 0.3%**
  - Weigh 3 grams of methylene blue powder
  - Add the powder to 0.5 liter of pure water, which has been placed in a conical flask
  - Swirl the contents of the flask to dissolve the dye
  - Add another 0.5 liter of water and mix again

### **Next steps**

Let the flasks with freshly prepared reagents stand (covered) until quality control procedures have been performed.

After these reagents have passed quality control, pour the solutions into clean bottles and label them. If bottles are reused, clean them thoroughly. Carbol fuchsin crystals stick to the bottom and are hard to remove, use acid alcohol and

a bottlebrush to remove this residue. On the label of the bottle, clearly print the reagent name, concentration and the preparation date.

Carbol fuchsin staining reagent can be filtered in the laboratory that prepared it, but this is not sufficient since it may precipitate again. Filter carbol fuchsin again during the process of staining, using a funnel with filter paper. The other staining reagents do not need to be filtered.

## **STORAGE OF REAGENTS**

Well-prepared reagents will keep for at least six months to one year, even at higher temperatures. Store all reagents in clean and tightly closed bottles with a label showing the name of reagent and the date of preparation. Keep these bottles out of direct sunlight. If clear bottles are used, keep stocks of reagents in a closed cabinet.

## **QUALITY CONTROL**

### **PREPARATION OF POSITIVE AND NEGATIVE CONTROLS**

Make positive control smears with low positive (1+) sputum. Let this sputum stand for one or more days at room temperature to allow the sputum to liquefy. Then, with the container closed, mix the contents carefully and make as many smears as possible from this same low positive sputum. Check the average number of AFB by staining a few randomly selected smears from the entire batch. Record this number in your staining reagent logbook.

Ensure that sputum used to prepare negative control smears has been extensively examined to ensure that there is no AFB. Prepare smears and fix them.

To protect fixed unstained smears from dust and sunlight, store them in a separate and labeled slide box.

### **QUALITY CONTROL OF FRESHLY-PREPARED STAINING REAGENTS**

When preparing staining reagents, always perform quality control for each batch of staining reagents prepared. Quality control is essential to ensure that the staining reagents work well, and that they do not contain contaminating AFB.

It is more efficient to prepare bigger batches if very large flasks are available.

Keep accurate records in a logbook for quality control (see Appendix 2). This serves as an important reference record to defend against possible complaints on bad staining reagents. In the logbook, identify the batches by name of reagent and preparation date (as on the bottle labels). Perform QC by using one or more freshly prepared staining reagents and the normal staining procedure as described for positive controls. Test the performance of carbol fuchsin by staining and examining two low positive smears stained once, and two negative smears stained three times.

***Negative smears are stained three times to check for the presence of environment mycobacteria in the water used to prepare the reagents.***

Examine all controls carefully for number, completeness, and intensity of red color of AFB, as well as color and complete destaining of background with absence of crystals. Record the results in logbook for quality control of staining reagents as in the example.

*If unsatisfactory results are obtained in the staining of AFB, check for the method of preparation of carbol fuchsin and other reagents. If preparation procedure seems to have been correct, the stain might be good but the staining procedure not correctly used. Ensure that proper staining technique was followed. Repeat few more control slides, paying attention to correct staining technique. If no error found in the preparation method or staining technique, then prepare fresh staining solution(s) or reagents from a new batch of stains or reagents and perform quality control.*

*Report the unsatisfactory batch and discard the unsatisfactory solution(s).*

## **QUALITY CONTROL OF STORED STAINING REAGENTS**

Staining reagents may spoil with aging. In addition, the staining procedure may not have been performed correctly. For these reasons check staining periodically in all labs. Include a positive control smear (as described above and prepared by these laboratories themselves) in the routine series. Perform the QC at least weekly and with every new batch of reagents. Check the control smear first for properly stained AFB, and record the result in the sputum smear register. If the result is unsatisfactory, stain another control smear, making sure that the procedure is correct. If this gives a good result, use this lot to stain routine smears. If it does not, use a new lot of staining reagent to restain them. Make sure that the new lot has been properly quality controlled.

### **Key messages**



- Use quality reagents to prepare staining reagents.
- Accurate preparation of reagents is critical to obtain optimum staining results.
- Quality control of staining reagents using control smears ensures proper performance of newly prepared staining solutions.
- Record quality control results in logbook for quality control of staining reagents.
- Store prepared reagents in clean bottles and out of direct sunlight.



## Module Review: Module 5

Find out how much you have learned by answering these questions.

**Why must quality reagents be used to prepare staining reagents?**

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**Why is correct preparation of reagents necessary to obtain optimum staining results?**

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**What is the role of control smears in evaluating the performance of newly-prepared staining solutions?**

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**How should reagents be labelled and stored?**

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**Worksheet for Preparation of Reagents****Carbol fuchsin**

<b>Item</b>	<b>Lot number</b>	<b>Actual amount taken</b>
<b>Basic Fuchsin (in grams)</b>		
<b>Phenol (in grams)</b>		
<b>Alcohol (in ml)</b>		
<b>Water (in ml)</b>		

**Counter stain**

<b>Methylene Blue (in grams)</b>		
<b>Water (in ml)</b>		

**Decolorizing solution**

<b>Sulphuric acid (in ml)</b>		
<b>Water</b>		
<b>Hydrochloric acid (in ml)</b>		
<b>Alcohol (in ml)</b>		

Appendix 2

Example of Logbook for Quality Control of Staining Reagents

<p>Batches checked on date 1/5/02:            Carbol fuchsin (CF) batch 1/5/02, sulfuric acid batch 1/5/02, methylene blue batch 1/5/02            Average grading positive controls: no. 345 = 30/100 fields; no. 411 = 22/100 fields</p>				
Control slide	AFB color	AFB number	Destaining	Decision
345/12	strong red	20/100 F	OK	Accept CF
411/25	strong red	50/100 F	OK	Accept CF
NEG	NA	none	OK	Accept others
NEG	NA	none	OK	Accept others
<p>Batches checked on date 1/15/02:            Carbol fuchsin (CF) batch 1/15/02, sulfuric acid batch 1/15/02 (+ old methylene blue solution)            Average grading positive controls: no. 345 = 30/100 fields; no. 411 = 22/100 fields</p>				
Control Slide	AFB color	AFB number	Destaining	Decision
345/13	weak red	2/100 F	OK	Reject CF
411/26	NA	0/100 F	OK	Reject CF
NEG	NA	none	OK	Accept others
NEG	NA	none	OK	Accept others
<p>Note: This batch of carbol fuchsin is bad; all has been discarded. Sulfuric solution is OK.</p>				
<p>Batches checked on 1/16/02:            Carbol fuchsin (CF) batch 1/16/02, Methylene blue batch 1/16/02 (+ old sulfuric acid solution)            Average grading positive controls: no. 345 = 30/100 fields; no. 411 = 22/100 fields</p>				
Control Slide	AFB color	AFB number	Destaining	Decision
345/14	strong red	34/100 F	OK	Accept CF
411/27	strong red	40/100 F	OK	Accept CF
NEG	strong red	3 clumps	OK	Reject others
NEG	NA	none	OK	Accept others
<p>Note: contamination, probably methylene blue. To be checked further using only 1 of the new staining reagents on negative controls (+ old good stains of the other types).</p>				

Blank Log Book Sheet:  
 Quality Control of Staining Reagents  
 (This sheet can be reproduced for individual laboratory use)

Batches checked on date : _____ Carbol fuchsin (CF) batch _____, sulfuric acid batch _____, methylene blue batch _____ Average grading positive controls: no. ____ = ____/100 fields; no. ____ = ____/100 fields				
Control slide	AFB color	AFB number	Destaining	Decision
Note:				
Batches checked on date: _____ Carbol fuchsin (CF) batch _____ sulfuric acid batch _____ methylene blue Batch _____ Average grading positive controls: no. ____ = ____/100 fields; no. ____ = ____/100 fields				
Control Slide	AFB color	AFB number	Destaining	Decision
Note:				
Batches checked on _____ Carbol fuchsin (CF) batch _____, Sulfuric acid solution _____, Methylene blue batch _____ Average grading positive controls: no. ____ = ____/100 fields; no. ____ = ____/100 fields				
Control Slide	AFB color	AFB number	Destaining	Decision
Note:				

**Recording Worksheet for  
Laboratory Practical Session # 2**

Batches checked on:

Carbol fuchsin, date prepared ..... Concentration.

Decolorizer, date prepared. Type .....

Methylene blue, date prepared ..... Concentration.

Average grading positive controls = .....AFB / 100 fields

Control slide ID	AFB color	AFB number	Background	Remarks