



Module 6

Smear Preparation and Staining

Purpose	To provide an understanding of proper smear preparation and staining technique for AFB smear microscopy
Prerequisite Modules	Module 5
Learning Objectives	<p>At the end of this module, you will be able to</p> <ul style="list-style-type: none">▪ Safely prepare sputum smears▪ Prepare good-quality sputum smears▪ Identify problems with smear preparation▪ Perform the Ziehl-Neelsen (ZN) method on sputum smears▪ Troubleshoot problems with the ZN method.
Content Outline	<ul style="list-style-type: none">▪ Labeling of slides▪ Selecting the best portion of the specimen for smear preparation▪ Techniques for preparing smears▪ Principles of the Ziehl-Neelsen method▪ The Ziehl-Neelsen staining procedure
Handouts and Exercises	<p>Laboratory Practical Session #3: Preparation of smears</p> <p>Laboratory Practical session #4: Staining of sputum smears form</p> <p>Laboratory Prctical Session #3 and panel smears</p>
Appendix	None

Module 6: Smear Preparation and Staining

The quality of work in AFB diagnostic microscopy depends on a number of factors like specimen collection, quality of reagent, staining technique, reading of smear, reporting and recording and training of technician. However, collecting a suitable specimen and making a good smear are critical as quality of rest of the procedure depends upon it. Smear preparation must be performed carefully and with attention to detail.

Overview of smear preparation:

- 1. Label each slide with the correct number (serial and order number)**
- 2. Smear sputum onto slide**
- 3. Allow smear to air dry**
- 4. Heat fix smear**

PREPARING SPUTUM SMEARS

1. Numbering the slides

- Select new, clean, grease-free, unscratched slides which are free from fingerprints.
- Using a pencil, record the laboratory register serial number and order number of the sputum specimen on the frosted end of the slide. If plain unfrosted slides have to be used, labeling is best done using a diamond pencil.
- Ensure that the number on each slide corresponds to the number on the specimen container.

2. Sputum smearing

- Using the end of an applicator stick or wire loop, select and pick up the yellowish purulent particles of sputum.
- Prepare the smear in an oval shape in the center of the slide. The smear size should be 2–3 cm in length x 1–2 cm wide, which will allow 100–150 fields to be counted in one length.
- For good spreading of sputum, firmly press the stick perpendicular to the slide and move in small concentric circles or coil-like patterns.
- Place the used stick into a discard container.
- Use a separate stick for each specimen.
- Alternatively, if a wire loop is used instead of a broken stick, dip the wire loop in an sand-alcohol bottle. Remove the excess sputum from the wire loop by moving it up and down. After each smear is completed, heat the wire loop in a flame until red-hot.
- Thorough spreading of the sputum is very important; it should be neither too thick nor too thin. Prior to staining, hold the smear about 4-5 cm over a piece of printed paper. If letters cannot be read, it is too thick.

3. Air drying of smear

- Allow the smear to air dry completely at room temperature.
- Do not dry smears in direct sunlight or over a flame.

4. Heat fix smear

- After the slide is completely dry, use forceps to hold the slide upwards.
- Pass the slide over the flame 2–3 times for about 2–3 seconds each time. Do not heat the slide for too long or keep it stationary over the flame, or else the slide will be scorched.

STAINING WITH ZIEHL-NEELSEN CARBOL FUCHSIN SOLUTION

Principle of acid fastness

The cell wall of acid fast bacilli contains fatty acids known as mycolic acids, which makes them resistant to the action of many chemicals. Because of this, the bacilli cannot be stained easily like in Gram's stain. Strong dye concentration, application of heat, addition of phenol and longer staining time are required to stain the bacilli. Once stained it is difficult to destain them. This property is used to differentiate the AFB from all other materials like bacteria, cells and mucus will get decolorized by the action of strong acid or acid-alcohol, leaving the acid fast bacilli stained with primary stain, which is basic fuchsin in case of ZN staining or the fluorescent dye like auramine in case of fluorescent staining method.

A good stained smear by ZN method shows strong red AFB, against weak blue background.

Contact time of carbol fuchsin is very important during staining, minimum five minutes are required for good quality stains, however 10 minutes are preferred to get a strong AFB staining. The color and intensity of background is important so that it does not mask the AFB. Destaining must be complete, if required, destaining step should be repeated to ensure complete absence of red color from the background. It is not possible to destain the AFB by using cold watery acids.

Overview of Ziehl-Neelsen staining procedure:

1. Arrange slides in serial order on staining bridge, with smear side up
2. Flood slides with filtered carbol fuchsin stain
3. Gently heat to steam
4. Keep the staining reagent for at least 10 minutes
5. Rinse with water and drain
6. Apply decolorizing solution for 3 minutes
7. Rinse with water and drain
8. Apply methylene blue counter stain for NOT MORE THAN 1 minute
9. Rinse with water and drain
10. Air dry on a slide rack

EVALUATING SMEARS

Spend time evaluating good and bad smears. Without a quality smear, the procedure of diagnostic microbiology is seriously impeded. Bad smears can lead to false results. The quality of examination depends on making good smears. In this training, the preparation of good smears is a very important process.

Key messages



- Label slides with the laboratory serial number and specimen number (e.g., 562/2).
- Select the most purulent portion of the sample for smear preparation.
- The size of the smear should consistently be 2–3 cm by 1–2 cm; the smear should be thin enough that you can read newsprint through it.
- Use gentle heat fixation for smears.
- Carbol fuchsin is heated to steaming. Leave it on the smear for 10 minutes.
- Do not extend the time for counterstaining with methylene blue.



Module Review: Module 6

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

What labelling information is needed on a slide?

What portion of the specimen should be used for smear preparation?

How can you determine the correct size and thickness of a sputum smear?

What are critical steps in the Ziehl Neelsen stain?

Laboratory Practical Session #3: Preparation of Smears

Materials and Equipment

- Sputum specimens
- Glass slides, clean with frosted end
- Disposable wooden or bamboo applicator sticks
- Spirit lamp (burning spirit)
- Bench disinfectant (5% phenol or 0.5 % sodium hypochlorite)
- Discard container
- Newsprint, Forceps, and Ruler marked in centimeters

Procedure:

Review safety procedures in module 2, before beginning this procedure.

1. USE a pencil to label the frosted end of a slide with the laboratory serial number on the sputum container.
2. OPEN the sputum container carefully and place the lid face up on the work surface.
3. EXAMINE the specimen to select the best portion to sample. Choose yellow (purulent) or bloodstained particles if present.
4. USE a wooden applicator stick to select the most purulent material from the specimen container.
5. USE the applicator stick to transfer the selected specimen particles/fluid to the glass slide
6. SMEAR the specimen over a 1 x 2 cm or 2 x 3 cm area centered in the middle of the unfrosted area of the slide.
7. USE the applicator stick to crush, break up, and spread out particles.
8. USE small circular motions to distribute the specimen evenly.
9. DISCARD the applicator stick into a discard container containing a suitable disinfectant.
10. RESEAL the sputum container and set aside.
11. ALLOW the smear to air dry completely, (never use heat to reduce smear drying time).
12. AFTER the slide is completely dry, hold the slide using a forceps with the smeared slide facing upwards. Pass the slide over the flame 2-3 times, about 2-3 seconds each.
13. EVALUATE the fixed smear for the proper thickness.
14. *Consider smear to be potentially infectious until after it has been stained.*
15. Make sure you WASH your hands before leaving the laboratory.

Note: Use these smears for staining in Practical session# 4 “Staining sputum smears”.

Laboratory Practical Session #4: Staining Sputum Smears

Materials and Equipment

- Ten smears prepared in Practical Session # 3 and a set of 5 unstained panel smears
- Staining sink
- Running water
- Small funnel with filter paper
- Set of ZN stain reagents for each staining area
- Spirit lamp, or spirit torch, or equivalent to heat carbol fuchsin on smears
- Beaker , Forceps, and Gloves

Procedure

1. ARRANGE the slides. Place them in serial order on the leveled staining bridge smear side up. Leave enough space between slides to prevent the transfer of material and/or staining solution from one smear to another.
2. APPLY carbol fuchsin stain. Cover the entire surface of the slide with filtered carbol fuchsin* solution. If the staining solution drains off, add more stain to cover the entire slide.
(*Filter carbol fuchsin prior to staining or filter directly onto the slide using a funnel containing filter paper.)
3. HEAT the slide with the flame of an alcohol-soaked cotton swab, an alcohol lamp, or a Bunsen burner until steam rises from the stain. Do not boil or allow the slide to dry or it will scorch and re-crystallized substances will develop in the smear. Leave it for 10 minutes and do not let the solution dry.
4. RINSE the slide. Tilt the slide to drain off excess stain and then rinse the staining solution off with a gentle stream of water. It may be convenient to use a beaker, flask, or squeeze bottle to pour the water onto the slides. When rinsing slides, avoid getting water stream directly on the smear; vigorous washing may cause the smear to lift. Tilt the slide to drain off excess rinse water.
5. DECOLORIZE the smear by covering the whole slide with an acid solution (25% sulfuric acid or 3%hydrochloric acid-alcohol solution) and leave it for a maximum of 3 minutes. If the carbol fuchsin stain is retained in the smear, it is considered underdecolorized. Repeat the decolorization, if necessary.
6. WASH the slide again with a gentle stream of water. Tilt the slide to drain off excess water.
7. COUNTERSTAIN the smear by covering the entire surface of the slide with methylene blue solution and leave it for a maximum of 1 minute.
8. DRAIN off the methylene blue solution. Gently rinse the slide again with a gentle stream of water. Make sure the stained smear is free from stain deposits, dirt, debris, and crystals produced by overheating during staining. Underside of the smear should be wiped, if possible with alcohol.
9. PLACE the slide on the slide rack to air-dry. Do not allow the stained slide to dry in direct sunlight. When the slides are completely dry, they are ready for microscopy. If they are not read immediately, place them in a slide box.