Module 6: Smear Preparation and Staining

Purpose

To provide the participants with knowledge and skills to safely prepare and stain sputum smears using the Ziehl-Neelsen method.

Pre-requisite Modules

Modules 1, 2, 3, 5

Module Time

5 hours 30 minutes

Learning Objectives

At the end of the module, participants will be able to

- 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.

Module Overview

Step	Time	Activity / Method	Content	Resources Needed
1	5 min	Presentation	Module Introduction	Slides 1–3
2	30 min	Presentation	Smear Preparation	Slides 4–13
3	2 hours	Demonstration and Practical exercise	Laboratory Practical Session # 3: Good Smear Preparation Technique	Laboratory supplies
4	35 min	Presentation	Smear Staining Procedure	Slides 14 - 31
5	1 hour 30 min	Demonstration and Practical exercise	Laboratory Practical Session # 4: Smear Staining	Laboratory supplies
6	10 min	Questions and Answers	Summary	Slide 31
7	40 min	Interactive exercise	Assessment of smear quality: Troubleshooting of smear staining	Slides 32–62

Material and Equipment Checklists

- PowerPoint slides or transparencies
- Overhead projector or computer with LCD projector
- Prepared flipchart – content outline, discussion questions

Facilities Required

- A well-lit and well-ventilated laboratory
- Facilities for hand washing

Teaching Guide

Slide Number	Teaching Points
1	**Module 6: Smear Preparation and Staining** DISPLAY this slide before you begin the module. Make sure participants are aware of the transition into a new module.
2	**Learning Objectives** STATE the objectives on the slide.
3 **Flipchart** 	**Content Overview** (Suggested format) WRITE the content outline before beginning this session. REFER to flipchart frequently to orient participants to where they are in the module. EXPLAIN that these are the topics that will be covered in this module.
4	**Overview of Smear Preparation** STATE the message on the slide
5 	**Labeling frosted slides - pencil** EXPLAIN that it is important to write the Laboratory Serial Number and specimen number clearly on the slide ▪ Pencil on a slide with a slide with frosted end
6 	**Labeling slides - diamond pencil** EXPLAIN that it is important to write the Laboratory Serial Number and specimen number clearly on the slide ▪ Diamond pencil on a slide
7	**Specimen Appearance** POINT OUT the two portions of the sputum specimen ▪ The saliva portion ▪ The purulent portion at the bottom of the container

Slide Number	Teaching Points
8	**Purulent portion of the specimen** HIGHLIGHT the: ▪ Numerous inflammatory cells in background ▪ The clump of AFB
9	**Saliva potion of the specimen** HIGHLIGHT the: ▪ Lack of inflammatory cells ▪ Oral flora (cocci and bacilli) ▪ Single AFB (Scanty AFB) EMPHASIZE the importance of collecting the most purulent portion of the sputum specimen to prepare smears
10	**Consistent Quality of Smears** HIGHLIGHT the consistent quality of the smear preparation ▪ Smear centered on the slide ▪ Correct size and shape ▪ Ideal thickness
11	**Making Smear With Loop** HIGHLIGHT the importance of making small coil movements to spread the sputum into a consistent smear NOTE that gloves are not required to prepare smears
12	**Heat Fixing a Smear** EMPHASIZE that the smears must be dry before heat fixing by passing through the flame of a bunsen burner or spirit lamp ▪ Smear facing upwards ▪ Pass smear through flame three times ▪ Do not overheat
13	**Proper Thickness of Smear** EXPLAIN that a heat fixed smear is of correct thickness when words in a newspaper can just be read ▪ Hold smear 5-10cm above newspaper

Slide Number	Teaching Points
	<u>Laboratory Practical Session # 3: Preparation of Smears</u> EXPLAIN participants will go to the laboratory for a practical session on preparing smears. ADD the purpose of this practical is to familiarize participants with the materials and techniques required to produce a good smear. EXPLAIN that participants should work in pairs at each bench workstation. DEMONSTRATE smear preparation to participants before participants start preparing smears INSTRUCT the groups to make smears from specimens provided. INFORM participants that these smears will be used for the next practical sessions. After completion of exercise, continue with PowerPoint presentation *Staining of Sputum Smears*.
 ***TIPS***	**Tips for Demonstration** • 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.
14	**<u>Ziehl-Neelsen Staining Procedure</u>** STATE the message on the slide
15	**<u>Acid-Fast Principles</u>** STATE the message on the slide

Slide Number	Teaching Points
16	**Principle of ZN Stain - 1** DISCUSS that when carbol fuchsin is added to the smear and heated, all parts of the smear take up the stain and everything is red, including non-AFB organisms
17	**Principle of ZN Stain - 2** DISCUSS that when decoloriser is added to the smear, the non-AFB parts lose the red colour HIGHLIGHT that AFB keep the red colour of the carbol fuchsin stain despite the addition of decoloriser
18	**Principle of ZN Stain - 3** DISCUSS that after the counterstain (methylene blue) has been added to the smear, the AFB still stain red HIGHLIGHT that non-AFB organisms take up the counterstain stain and now stain blue
19	**Overview of Staining Procedure** STATE the message on the slide DESCRIBE that the steps and timing are important, and errors will occur if the staining method is not followed correctly
20	**Setting up slides on staining rack** EMPHASIZE that the staining rack must be level otherwise the stain will not cover the smear properly and errors may occur ▪ If the staining rack is not level, correct the error so that the stain lies evenly over the slide EMPHASIZE that slides should be kept apart ▪ Use a finger thickness to keep smears separate from each other EMPHASIZE that the slides are placed smear upwards and should be placed in laboratory serial number order
21 	**Carbol Fuchsin** EMPHASIZE that sufficient carbol fuchsin must be added to cover the entire slide

Slide Number	Teaching Points
22	**Heating Carbol Fuchsin to Steaming** STATE the importance of heating the slides from below, continually moving the flame to avoid creating a hotspot on a slide ▪ Stop heating once steam rises from the slide ▪ Heat to steaming once only STATE the importance of keeping the heated carbol fuchsin on the slide for a minimum of 5 minutes, higher staining time of 10 minute is preferred to get better staining of AFB. ▪ Leaving the carbol fuchsin on the slide for longer is OK, provided it does not dry out on the smear.
23	**Rinsing slides** EMPHASIZE that the rinsing step should use a gentle stream of water ▪ do not splash the other slides on the staining rack as AFB from one slide may contaminate another producing a false-positive result ▪ once the rinsing step has been completed, tip the slide to one side to drain excess water
24	**Don't splash adjacent slides** EMPHASIZE the importance of not splashing adjacent slides
25 	**Decolorisation Step** EMPHASIZE that sufficient decoloriser must be added to cover the entire slide STATE that one minute is usually sufficient time for the decoloriser ▪ thicker smears may require longer, up to a maximum of three minutes
26	**Rinsing slides** EMPHASIZE that the rinsing step should use a gentle stream of water ▪ do not splash the other slides on the staining rack as AFB from one slide may contaminate another producing a false-positive result ▪ once the rinsing step has been completed, tip the slide to one side to drain excess water

Slide Number	Teaching Points
27 	**Counterstaining** EMPHASIZE that sufficient counterstain (methylene blue) must be added to cover the entire slide ▪ Leave on the slide for a maximum of one minute
28	**Rinsing off Counterstaining** EMPHASIZE that the rinsing step should use a gentle stream of water ▪ do not splash the other slides on the staining rack as AFB from one slide may contaminate another producing a false-positive result once the rinsing step has been completed, tip the slide to one side to drain excess water
29	**Drying the stained slides** EMPHASIZE that slides must be air dried and out of direct sunlight ▪ do not blot dry or heat the slides
30	**Quality Control of Routine Staining** EMPHASIZE the importance of quality control of routine staining in the laboratory.
31	**Summary** ASK the participants to answer the questions. ANSWER any questions the participants may have.

Slide Number	Teaching Points
	Laboratory Practical Session # 4: Staining Sputum Smears EXPLAIN participants will go to the laboratory for a practical session on staining smears. ADD the purpose of this practical is to familiarize students with the procedure for staining sputum smears using the Ziehl-Neelsen staining method. EXPLAIN that participants should work in groups of two to four persons at each staining sink. DEMONSTRATE the staining procedure to participants before participants start staining smears PROVIDE the participants with unstained five panel smears INSTRUCT the groups to stain their smears prepared in practical session # 3 and the panel smears. INFORM participants that these stained smears will be viewed in next practical exercises. After completion of laboratory practical session #4, CONTINUE with interactive PowerPoint presentation 'Troubleshooting Smear Preparation and Staining'.

Slide Number	Teaching Points
32	**Module 6: Troubleshooting Smear Preparation and Staining Interactive Exercise** DISPLAY this slide before you continue with this exercise.
 ***TIPS***	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. Take time to discuss any problems participants may have. Be available to spend extra time helping those experiencing a challenge with this critical step.
33	**Smear Preparation** STATE that the first part of the exercise will cover troubleshooting smear preparation
34	**Uniform Smear Size** EXPLAIN that with proper understanding, technicians can learn to prepare good quality smear every time. ▪ The smears seen here were prepared by young technicians with basic laboratory training – this consistency is what we are trying to achieve
35	**Size of Smear** STATE that the smear should be either ▪ 2 x 3cm OR ▪ 1 x 2cm ▪ EMPHASIZE that the smear should be in the centre of the slide
36	**Evenness of smear** STATE the importance of preparing a homogeneous smear - that is, spreading the sputum evenly across the slide ▪ Sloughing off occurs when smears are too thick and not correctly heated fixed ▪ Avoid preparing smears with uneven thickness as it makes it difficult to read the smear

Slide Number	Teaching Points
37	**Smear Thickness** The thickness of the smear should be even and not too thick or too thin ▪ Too thick will make the smear very difficult to read and to focus on AFB ▪ Too thin will make the smear difficult to read because there will be insufficient sputum in each high power field and AFB may be missed
38	**Question: Identify The Problem?** ASK the participants to identify what is wrong with smears 2–5 (Smear 1 is a good quality smear). Point to each smear (2–5) and find out what the participants think is wrong. PROVIDE an answer BEFORE moving to the next smear ▪ Smear 2: too large and too uneven ▪ Smear 3: too small and too thin ▪ Smear 4: too small, too uneven, and not central on the slide ▪ Smear 5: multiple smears on a slide. NEVER have more than one smear on each slide
39	**Staining** STATE that the rest of the exercise will cover troubleshooting staining
40	**Good quality staining of AFB** EXPLAIN that the four photographs on this slide demonstrates how AFB should appear HIGHLIGHT the strong staining of the AFB and ask the participants to remember how AFB look HIGHLIGHT that the background staining of each slide does not interfere with the intensity of AFB staining
41	**What are the Causes of Pale Staining AFB?** ASK for the participants for their reasons why AFB can be pale staining PROVIDE the participants sufficient time to ensure they can contribute answers before moving to the next slide

Slide Number	Teaching Points
42	**Pale Staining AFB** HIGHLIGHT that the photograph contains numerous AFB but that they are poorly stained This example shows the effect of using poor quality carbol fuchsin and not providing sufficient heat REMIND participants that the carbol fuchsin needs to be heated to steaming BUT NOT boiling
43	**Causes of pale staining AFB** STATE the importance of following: • low concentration of CF – the minimum concentration of carbol fuchsin is 0.3% – always check new carbol fuchsin by performing Quality Control to assure performance in satisfactory • Staining time less than 5 minutes – always ensure that the carbol fuchsin is contact with slide for at least five minutes • No heating step – ensure that the carbol fuchsin is heated to steaming
44	**Time Effect of Carbol Fuchsin on Intensity of AFB** EMPHASIZE the importance of incubating the heated carbol fuchsin on the slide for at least 5 minutes. ADD that it is OK to leave the carbol fuchsin for longer but that it must not dry out of the slide HIGHLIGHT how weak the AFB are stained in the photographs showing staining for 2 or 3 minutes only
45	**Effect of Not Heating the Carbol Fuchsin to Steaming** POINT out the circle with pale staining AFB within. The photograph is of a slide where the carbol fuchsin was not heated. All other steps were correct EMPHASIZE the importance of heating the carbol fuchsin to steaming (but not boiling)
46	**What are the Causes of Excessive Counterstaining?** ASK for the participants for their reasons how excessive counterstaining can occur PROVIDE the participants sufficient time to ensure they can contribute answers before moving to the next slide

Slide Number	Teaching Points
47	**What are the Causes of Excessive Counterstaining?** STATE the causes from slide
48	**Good Quality Counterstain** EMPHASIZE the importance of correct timing of the counterstain step HIGHLIGHT the clean, lightly stained background and the visibility of the AFB in the smear
49	**Excessive Counterstain** HIGHLIGHT the differences in the staining intensity of the methylene blue counterstain ▪ Top two slides have excessive counterstain time ▪ The lower slide (with tick) shows the intensity of colour when the correct counterstaining time was used
50	**Effect of excessive counterstain-1** HIGHLIGHT the two means of identifying an excessive counterstain ▪ Nuclei of inflammatory cells are too dark ▪ Background detail is too obvious
51	**Effect of excessive counterstain-2** Here is another example of excessive counterstaining ▪ Background detail is too obvious
52	**Combination Of Excessive Counterstain & Poorly Stained AFB** EMPHASIZE how important it is to ensure that AFB are correctly stained ▪ Photograph shows poorly stained AFB in a smear with excessive counterstain SHOW the participants where the AFB are in the smear and remind them of how AFB should stain

Slide Number	Teaching Points
53	**Good Quality Staining Of AFB** HIGHLIGHT how strongly the AFB are stained in these photographs REMIND the participants that we are trying to achieve this level of AFB staining intensity
54	**What are the Causes of Insufficient Decolorisation?** ASK for the participants for their reasons why insufficient decolourisation can occur PROVIDE the participants sufficient time to ensure they can contribute answers before moving to the next slide
55	**Assessment of Smear Quality** POINT out the areas of these smears where insufficient decolourisation has occurred (pink areas)
56	**Insufficient Decolourisation** POINT out that this is a higher power field (x1000) view of an area of insufficient decolourisation
57	**Importance of Correct Decolourisation** HIGHLIGHT the difference between the two photographs. MENTION that the photos are taken from the same smear ▪ The photo labeled 'insufficient' was found during a supervisory visit The photo labeled 'correct' is the same smear after the decolourisation step was performed correctly
58	**Troubleshooting - Miscellaneous** INFORM the participants that problems with smears can also result from other reasons. STATE the causes from slide

Slide Number	Teaching Points
59	**Excessive Heat Fixing of Smear** The photograph demonstrates what can happen if excessive heat fixing of the smear occurs ▪ Arrows point to damaged AFB ▪ The AFB are so damaged that they are unable to retain the carbol fuchsin. POINT out that this smear contained 3+ AFB
60	**Glass Slide Scratch & AFB** The photograph shows a scratch on the surface of the slide HIGHLIGHT the difference between the scratch and AFB present in the smear
61	**Soot Deposit On Underside Of Smear** The photograph shows soot on the underside of stained smears. STATE the remedy of removing the soot by rubbing the underside of the slide with a moist tissue or cloth
62	**Summary** STATE from the slide

Laboratory Practical Session # 3: Preparation of Smears

Materials and Equipment (per pair of participants)

- At least 3 freshly-collected sputum specimens per pair of students. Refer to Section 2 for smear and specimen requirements.
- Ten glass slides, clean with frosted end per participant. Each participant will make 10 smears.
- Disposable wooden or bamboo applicator sticks (15–20 per participant)
- Forceps
- Spirit lamp (burning spirit)
- Bench disinfectant (5% phenol or 0.5 % sodium hypochlorite)
- Discard container
- Ruler marked in centimeters
- Newsprint

Recommended Biosafety Practices

EMPHASIZE to participants to work deliberately and cautiously, and avoid any rapid movements that can produce aerosols. This practical exercise is for training purposes only, any improper handling of sputum samples by participants may cause safety concerns

EMPHASIZE that particular attention must be given to:

- Opening the container
- Manipulating the specimen
- Smearing the specimen on the slide

PROVIDE participants with appropriate personal protective equipment (e.g., laboratory coat).

Preparation of Smears 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. REMIND participants that smears are potentially infectious until stained.

15. ANSWER any questions or concerns participants have before proceeding to the next session.

16. Make sure that all participants WASH their 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 (per group of participants)

- A set of 10 smears prepared in Module 6, practical session # 3 and a set of 5 unstained panel smears (Refer to Section 2 for details).
- Staining sink
- Running water
- Beaker
- 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
- Forceps
- 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.

10. ANSWER any questions or concerns participants have before proceeding to the next session.