Ministry of Health

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Acknowledgment

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Acronyms:

ZN	Ziehl-Neelsen
AFB	Acid-Fast Bacilli
ATCC	American Type Culture Collection
H&S	Health and Safety
ID	Identification
IQC	Internal Quality Control
MOH	Ministry of Health
PHL	Public Health Laboratory
SOP	Standard operating procedure
WHO	World Health Organization
BAL	Bronchoalveolar lavage
ET	Endotracheal
CSF	Cerebral Spinal Fluid
TB	Tuberculosis

1. **Purpose**

This document describes the procedure for Ziehl-Neelsen (ZN) stain for Acid-Fast Bacilli (AFB).

2. **Scope**

This document is applicable for all medical laboratories under MOH and other collaborative governmental and non-governmental health institutions.

3. **Definitions**

3.1 Pulmonary : TB infections within lung parenchyma or bronchioles.

3.2 Extrapulmonary infections: TB infections involving organs other than the lungs.

4. **Procedure**

4.1. Clinical background:

Mycobacteria can infect any part of the body. Infections are divided into pulmonary and extrapulmonary infections. Mycobacteria are difficult to stain with gram stain due to presence of mycolic acid in their cell wall and hence there are other staining modalities like ZN stain. *Mycobacteria tuberculosis*. ZN stain is an important step in infection control isolation/deisolation of infected pulmonary Tb patients. The WHO is working in international effort to eliminate Tb in the End Tb program.

4.2. Principle:

Mycobacteria is different from other bacteria in the component of their cell wall. Their cell wall is rich in mycolic acid which makes it difficult to be stained with gram stain (GS). In ZN stain heat is used to help drive the primary stain into the waxy cell walls of these difficult-to-stain cells.

4.3. Pre – analytical stage:

4.3.1. Sample:

- Sample type: Any PULMONARY samples that is thought to contain mycobacteria can be used. This includes respiratory samples (sputum, BAL or ET secretions), gastric aspirate, CSF, urine or tissues.
- Early morning sputum sample is preferred.
- Any extrapulmonary sample has to be sent to PHL.
- Transportation media: leak-proof sterile container.

- Transportation of TB samples to another laboratory, should be in triple bio bottle packing and maintained in cold conditions (2 -8C).
- In case of delay, store the sample in 2-8 C.

4.3.2. Material:

Reagents	Consumables/Supplies	Equipment
Carbol fuchsin 3% acid-alcohol Methylene blue or malachite green sodium bicarbonate	Microscopic slides Sterile loops Oil immersion	Microscope Slide dryer Heating source / candle Safety cabinet class II

4.3.3. Safety precaution:

- All specimens need to be treated as potentially infectious. Standard procedures for handling of biohazard material must be followed at all times. Universal Precautions must be practiced at all stages of these procedures.
- All suspected Mycobacterium species must be processed in a Class II exhaust
  protective cabinet.
- Heat fixing does not kill Mycobacterium species and slides should be handled
  with care.
- Specimen fixed with 5% phenol in ethanol are safe to handle outside the safety cabinet.

4.3.4. Quality control:

- Check the expiry dates of all media, reagents and stains before use.
- All reagents, kits, and stains **MUST** be quality controlled before use.
- Identification tests should be run with appropriate controls.
- Use positive slides of known positive TB sample which is fixed with 70% alcohol. As a control.
- Use known negative control slide.

- Run the positive and negative control slides together with each batch of patient sample.
- Each preparation of new reagents of ZN stains must be QC with positive and negative control before use.
- Record the quality control results in the appropriate QC sheet.

4.4. Analytical stage:

4.4.1. Smear preparation:

- Pulmonary specimens can be processed at local hospitals provided that safety cabinet class II is available,
- Extra-pulmonary samples should be referred to Public Health Laboratory (PHL) in triple packaging.
- Allow smears to air dry in a biological safety cabinet
- Smears must be heat fixed before staining by using an electric staining rack (slide drier) or with a Bunsen burner.

4.4.2. Ziehl-Neelsen staining:

- Prepare a smear that is air dried, and heat fixed.
- Flood the specimen with carbol fuschin stain, heat under the slide until flames formed but not boiling. Leave the heated stain for 10 minutes. Longer time improves staining provided that stain does not dry.
- Rinse the slides with gentle running water. Drain out excess water.
- Decolorize with 3% acid alcohol for 3 minutes, then wash under running water. Drain out excess water.
- Counter stain with methylene blue or malachite green and leave for 1-2 minutes only
- Rinse with running water, remove excess water and leave the slide to air dry.
- Examine the slide under 100x oil immersion objective.

4.5. Post – analytical stage:

4.5.1. Interpretation / Results / Alerts:

- Positive: red-stained rods against blue or green background, depending on the counterstain used
- Negative: no red-stained rods observed

4.5.2. Reporting:

Number of AFB seen	**Method of reporting**
No AFB seen in at least 100 fields	No AFB seen
1-9 AFB/100 fields	Report the exact number seen
10-99 AFB/100 fields	AFB seen +
1-10 AFB/field in at least 50 fields	AFB seen ++
More than 10 AFB/field in at least 20 fields	AFB seen +++

- All positive AFB results is considered as critical results and should be communicated by calling the requester and infection control department immediately.
- All positive slides should be sent to PHL.
- Send random 5% of negative slides to PHL for quality assurance.

4.5.3. Limitation:

- Ziehl-Neelsen's staining is less sensitive than Auramine-phenol staining and molecular methods.
- Accuracy of ZN stain results depends on the quality of specimen submitted, the proper heating procedure, competency of the staff, and the regular maintenance of the light microscope.

5. Responsibilities

5.1. Responsible staff:

- To ensure the adherence to critical result communication procedure
- To facilitate the alternative channels once needed

5.2. Quality manager /officer

- To follow up the implementation of the procedure

- To monitor regularly communication of critical results and raise non-conformance with corrective action once needed.

5.3. All lab staff:

- To adhere to the procedure.
- To document record and release results as recommended
- To report test failures or incident

6. Document History and Version Control

Version	Description	Review Date
1	Initial Release	May 2026

7. References

Title of book/ journal/ articles/ Website	Author	Year of publication	Page
Clinical Microbiology Procedures Handbook			
UK SMI: Investigation of specimens for Mycobacterium species		5.10.2020	
UK SMI: Staining procedures			
Laboratory Diagnosis of Tuberculosis by Sputum Microscopy	WHO	2013	
CPHL TB reporting flow charts	CPHL		
Methods for inactivating and fixing unstained smear preparation of mycobacterium TB for improved laboratory safety	JCM	2002	4077 to 4080