STANDARD MANUAL FOR LABORATORY TECHNICIANS ON SPUTUM SMEAR MICROSCOPY

2nd Edition

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PREFACE

The main objective of the National Tuberculosis Reference Laboratory (NTRL), Public Health Laboratory (PHL) under the Department of Public Health is to coordinate the Hospital laboratories in Bhutan under the National External Quality Assurance Scheme (NEQAS) network for Tuberculosis (TB) Microscopy in the areas of Quality Assurance (QA) for Sputum Smear Microscopy, Culture & Drug Susceptibility Testing, Surveillance on Drug Resistant or Multi-drug Resistant Tuberculosis and Bio-safety in TB Laboratory.

This 2nd Edition of “Standard Manual for Laboratory Technicians on Sputum Smear Microscopy” is one of the great efforts to improve the QA for sputum microscopy in the Hospital laboratories performing sputum smear microscopy for TB. This is in line with the recommendation made by the Joint External Review Team from Global Drug Facility, WHO SEARO and the Supra-national TB Reference Laboratory, Thailand conducted from 9th – 18th June 2010.

The key changes in the present edition include the formulation of reagents for Ziehl Neelson (ZN) staining with the increase in the concentration of primary stain (Carbol Fuchsin) from 0.3% to 1% and decrease in concentration of Methylene blue from 0.3% to 0.1% as counter stain. It also includes the important aspects of sample estimation for Blinded Rechecking of slides based on Lot Quality Assurance Sampling (LQAS) and more in depth on Bio-Safety in the laboratory performing smear microscopy for TB.

Thanks go to all the professionals of NTRL, PHL for their support for the compilation of specific technical aspects and completion of the “Standard Manual for Laboratory Technicians on Sputum Smear Microscopy”. It is hoped that this revised manual will prove to be useful to all the laboratory technicians and will help in improved diagnosis of TB through quality assured laboratory service in Acid Fast Bacilli (AFB) sputum smear microscopy. Suggestions and recommendations are welcomed from all users so that changes can be made in the subsequent editions for better version.

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DIRECTOR GENERAL
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ACKNOWLEDGEMENTS

PHL is very much indebted to World Health Organization (WHO) for their technical and financial assistance. Their continued support and co-operation have been invaluable in the amendment of the “Standard Manual for Laboratory Technicians on Sputum Smear Microscopy” and publication of this manual.

Sincere thanks to members involved from PHL for their valuable efforts to revise and update this Standard Manual and to the Joint External Review Team for their recommendation to revise the Standard Manual for laboratory Technicians on Sputum Smear Microscopy.

NTRL, PHL in particular gratefully acknowledges the following members of the working group for reviewing and finalizing this manual:

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This 2nd Edition was discussed and finalized by members of the working group on 26th September, 2011.

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<tr>
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<td>National Tuberculosis Reference Laboratory</td>
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<td>PHL</td>
<td>Public Health Laboratory</td>
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<td>MDR</td>
<td>Multi-drug Resistance</td>
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<td>TB</td>
<td>Tuberculosis</td>
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<td>LQAS</td>
<td>Lot Quality Assurance System</td>
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<td>AFB</td>
<td>Acid Fast Bacilli</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>DOTS</td>
<td>Directly Observed Treatment Short-course</td>
</tr>
<tr>
<td>NTCP</td>
<td>National Tuberculosis Control Programme</td>
</tr>
<tr>
<td>PTB</td>
<td>Pulmonary Tuberculosis</td>
</tr>
<tr>
<td>MOTT</td>
<td>Mycobacterium other than Tubercle</td>
</tr>
<tr>
<td>IUATLD</td>
<td>International Union against Tuberculosis &amp; Lung Disease</td>
</tr>
<tr>
<td>IQC</td>
<td>Internal Quality Control</td>
</tr>
<tr>
<td>EQA</td>
<td>External Quality Assessment</td>
</tr>
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<td>QI</td>
<td>Quality Improvement</td>
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CHAPTER-1

Background

Tuberculosis is one of the major public health problems globally. Nearly one-third of the global population, i.e. two billion people, are infected with Mycobacterium tuberculosis and thus at risk of developing the disease. More than nine million people develop active TB every year and about two million die. More than 90% of global TB cases and deaths occur in the developing world, where 75% of cases are in the most economically productive age group (15-54 years).

Although Bhutan is one of the low TB burden countries in the South-East Asia Region, TB has been one of the major public health problems in Bhutan. Total number of new TB cases detected each year is around 1000 to 1100, out of which approximately 35% are new smear positive patients. TB has affected people of all age groups and mostly the economically productive age groups between 15 to 34 years. In the year 2008, the annual incidence of all TB cases was estimated at 165/100,000 population (Global TB Control Report 2009).

Since the introduction of Direct Observed Treatment Short-course (DOTS) in 1994 and coverage by DOTS in 1997, case notification rates have declined over the years from 211/100,000 population in the year 2000 to 168/100,000 in the year 2009 (NTCP cohort report, MoH). According to WHO, the estimated incidence of smear positive in Bhutan is 80/100,000 population. The case notification rate during the year 2009 was 63/100,000 population. Bhutan has already achieved the target of 85% (90.96% in 2008) treatment success rate among the detected new smear positive cases.

The WHO strategy for tuberculosis control (DOTS) relies on a network of laboratories that provide AFB sputum smear microscopy. Both diagnosis and treatment monitoring are key components of the DOTS. The establishment of a network of well functioning peripheral laboratories within the health system and readily accessible to the population is a high priority for the TB Control program in Bhutan. If the laboratory diagnosis is unreliable, all other activities will be affected. However, the quality of laboratory services are often not considered a high priority of the National TB Control Program (NTCP). Microscopy errors are likely to result in the failure to detect persons with infectious TB who then continues to spread infection in the community, or unnecessary treatment for “non-cases”. Errors in reading follow-up smears can result in patients being placed on prolonged treatment, re-treatment, or treatment discontinued prematurely. Therefore, quality assured laboratory services for AFB sputum smear microscopy is essential. The availability and the quality of AFB smear microscopy depend on National Reference Laboratory that support and monitor the performance of individual laboratories. This manual is intended to provide guidance for the laboratory personnel in achieving the quality assured and reliable laboratory services in AFB smear microscopy. This manual covers the information focusing on safety in the laboratory, sputum smear microscopy and Quality assurance programme.
1. Role of Sputum Smear Microscopy in DOTS Strategy

1. Diagnosis of Tuberculosis
   1.1 Confirmatory diagnosis for Pulmonary Tuberculosis (PTB)
   1.2 Case detection of PTB
      a. Prioritize and allocate cases to standardized treatment regimens
      b. Aid in prognosis:
         - Smear positive TB (infectious cases)
         - Smear negative TB (and smear negative culture-positive TB)

2. Monitoring and evaluation of chemotherapy
   2.1 At the end of intensive phase of standard short course chemotherapy (2\textsuperscript{nd} or 3\textsuperscript{rd} month to check on the DOTS process and treatment result).
   2.2 At the end of treatment to monitor the treatment success rate.

3. Epidemiology and surveillance
   3.1 For surveillance reporting / notification of smear positive TB case report in the index of the TB problem.
   3.2 As the screening test for infectious form of PTB for the prevention of TB in the health care setting.

2. Sputum Smear Microscopy

The most important method in the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum smear for AFB. Direct microscopy is simple and inexpensive, and detects most of the infectious form of pulmonary tuberculosis. Currently, no other diagnostic tool is available which could be implemented affordably. Direct microscopy for TB is also performed to assess the response to treatment and to establish cure or failure at the end of treatment.

The minimum number of acid-fast bacilli necessary to produce a positive smear result has been estimated to be 5000 -10,000 per milliliter sputum (Table 1). At concentration below 1000 AFB per ml of the sputum, the chance of observing the bacilli in a smear is less than 10%.

*Table 1: Number of acid-fast bacilli observed in smears, concentrations of culturable bacilli in sputum specimens and probability of positive result*

<table>
<thead>
<tr>
<th>Number of bacilli observed</th>
<th>Estimated concentration of bacilli per ml sputum</th>
<th>Probability for a positive result</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 in 100 or more fields</td>
<td>Less than 1,000</td>
<td>Less than 10%</td>
</tr>
<tr>
<td>1-2 in 300 fields</td>
<td>5,000-10,000</td>
<td>50%</td>
</tr>
<tr>
<td>1-9 in 100 fields</td>
<td>About 30,000</td>
<td>80%</td>
</tr>
<tr>
<td>1-9 in 10 fields</td>
<td>About 50,000</td>
<td>90%</td>
</tr>
<tr>
<td>1-9 per field</td>
<td>About 100,000</td>
<td>96.2%</td>
</tr>
<tr>
<td>10 or more per field</td>
<td>About 500,000</td>
<td>99.95%</td>
</tr>
</tbody>
</table>

The sensitivity can be further improved by examining more than one specimen from a patient, as many studies have shown that the examination of two specimens will on average detect more than 90% of cases and the addition of a third specimen increases to approximately 95-98%. Therefore three sputum specimens are strongly recommended for screening suspects of pulmonary tuberculosis. However, a negative smear result does not exclude the diagnosis of TB as some patients harbor fewer numbers of bacilli which cannot be detected by direct microscopy. A poor quality specimen or smear may also produce negative results.

Smear sensitivity is poor in extra-pulmonary tuberculosis cases and in disease caused by Mycobacterium other than tubercle bacilli (MOTT). It is also virtually impossible to distinguish different mycobacterial species by microscopy.

### 2.1 Essential Equipment and Reagents

#### For Smear preparation
- Sputum container of 50 ml capacity to collect sputum
- Wire loop with an inner diameter of 3mm or applicator stick to spread sputum on the slide
- New glass slide (no grease and no scratches on the slide)
- Diamond marker/ marking pencil to enter identification number on the slide
- Forceps to hold slide with smear
- Bunsen burner or spirit lamp to sterilize wire loop, fix smeared slide and flame the same during staining
- Metal waste bin/can with disinfectant to discard infected material
- Alcohol-sand flask to remove and disinfect excess sputum on the wire loop

#### For stain & staining
- Balance
- Staining rack to hold smeared slides to stain
- 1% Carbol fuchsin to stain acid-fast bacilli
- 25% Sulphuric acid to decolorize stained smears
- 0.1% Methylene blue to counter stain decolorized material in the smear
- Water to rinse/wash the staining solution
- Slide rack to place stained slides to air dry

**For Microscopic Examination**
- Microscope
- Immersion oil for 100X objective
- Tissue paper to absorb immersion oil on stained slides before storing.
- Slide box to store stained slides for quality assurance (Blinded rechecking)
- 70% ethanol to clean lenses (*frequent use is not recommended*)
- Clean, dry lens tissue to clean microscope lenses

**For Recording & Reporting**
- TB laboratory register
- Laboratory Request/Report form
- Red pen to write positive result

### 2.2 Specimen Collection
Three specimens should be collected for the laboratory diagnosis of tuberculosis as follows:
- One spot specimen when the patient first visits the health facility
- One early morning specimen (preferably the next day)
- One spot specimen when the early morning specimen is being submitted for examination
Containers
An essential prerequisite for the safe collection and transport of satisfactory specimens is the use of a proper container. The following specifications are recommended to facilitate the choice of a proper container for sputum specimen collection:

- Wide-mouthed so that patient can expectorate easily inside the container without contaminating the outside
- Approximately 50 ml in capacity
- Made of transparent material to ease observation of specimen volume and quality without opening the container
- Screw-capped to obtain a water tight seal & to reduce the risk of leakage during transport
- Easily labeled to allow permanent identification
- Rigid, to avoid breakage during transit

Collection Technique

Explain well the reasons for sputum collection and give clear instruction on collection technique to the patient so that a good quality sputum specimen is collected. Instruct patients to produce specimens outside in the open air away from other people and not in confined spaces such as toilets, since the air dilutes the aerosols generated during coughs to produce a sputum specimen and further the UV rays from the direct sunlight deactivates the TB bacilli contained in aerosols.

Instruct the patient to obtain an early morning sputum specimen before eating & to rinse mouth prior to sputum collection to remove any food remnants, since food particles in smears make them difficult to examine.

The patient is given a container on his first visit to health facility. He/she should be instructed, with demonstration by actual actions, to

- Inhale deeply 2-3 times
- Cough out deep from the chest during exhalation
- Open the container and spit the sputum into the container
- Avoid saliva or nasal secretions
- Close the container

Instruct the patient to collect an early morning specimen in a similar manner and bring it to the laboratory. On the second attendance, a third specimen (spot) is collected.

Criteria of good quality sputum

- Physical appearance of sputum sample should be thick, purulent or and/or bloodstained.
- Sufficient quantity of at least 3-5 ml.
- Contains no food particles or other remnants.

On receipt in the laboratory, the specimens are given unique laboratory serial number and the details of the patient’s name, address, age/sex, contact number are entered in the TB laboratory register.
Sputum specimens must be processed as soon as possible; however under unavoidable circumstances, the specimen should be refrigerated or kept in cool place to inhibit the growth of unwanted microorganisms.

**Sputum Transportation**
Sputum specimens collected at health facility where there is no microscopy service, should ship the specimens accompanied with request form to the nearest laboratory where it will be examined. Transportation of heat-fixed smear is recommended for TB Microscopy. Label the slides and prepare three smears from three different sputum specimen of a TB suspect and heat fix all smears before transportation.

### 2.3 Smear Preparation Procedures
Smear should be prepared in manageable batches (maximum of 12 per batch). Labeling of smears should be done at the bench for incoming specimens using a permanent marker, e.g. a diamond marker. Avoid touching the surface of the slides. The maximum chance of finding bacilli is in the solid or moist dense particles of the sputum and the results of direct smear examination depend to a great extent on the choice of these particles. The recommended size of the sputum smear is 1 cm x 2 cm.

**Flow Chart for Smear Preparation**

1. Label a new clean, unscratched slide at one end with the laboratory serial number. Label frosted slides with pencil and non-frosted slides with diamond pencil.

2. Use muco-purulent, opaque, grayish or yellowish portion for smear preparation. Transfer an appropriate portion of the specimen to the slide by using an applicator stick or wire loop.
Smear the specimen over an area of approximately 1cm x 2cm to 2cm x 3cm. Make it thin enough to be able to read through it. Use a new slide for each specimen.

Allow smears to air-dry for 15 minutes. Do not use heat for drying.

Pass slide through a flame three or four times with the smear uppermost. Do not overheat. Allow to cool before staining.

Discard the applicator stick in disinfectant and use a new one for each specimen. Remove particles of adherent sputum from wire loop by moving it up and down through a sand alcohol bottle containing 70% alcohol. Flame wire loop thoroughly prior to re-use.
3. **Zielh Neelsen Staining Technique**

AFB resists decolourization by weak acid and retains the primary stain. The counter stain is used to stain other materials and the background which gives a contrast background for the easy visibility of the Acid Fast Bacilli. After the smear is stained with Ziehl Neelsen technique, AFB appears red against a blue background.

3.1 **Formulation of reagents for Ziehl Neelsen Staining**

*(See Annexure ii for Worksheet for ZN reagent preparation)*

**1% Carbol fuchsin**

Basic fuchsin : 10g  
Absolute alcohol : 100 ml  
Dissolved basic fuchsin in ethanol…………………………. *Solution 1*

Phenol crystals : 50 g  
Distilled water : 900 ml  
Dissolved phenol crystals in distilled water ……………….. *Solution 2*

- Weigh 10 gm of basic fuchsin dye in a balance & transfer it to 250 ml conical flask.  
- Add 100 ml of absolute alcohol and dissolve the dye by placing it in a water bath at 60° C. Avoid direct heating (Solution 1).  
- Weigh 50 gm of phenol crystals in a balance and transfer it to 1000 ml conical flask.  
- Add 500 ml of distilled water and dissolve the phenol crystal. Gentle heat may be required. (solution 2)  
- Mix solution 1 and solution 2 and transfer the contents into a 1000 ml measuring cylinder.  
- Add distilled water to make up the final volume to 1000 ml.  
- Pour the solution through filter paper (whatman No 1) and store filtered solution in an amber colored bottle.  
- Label the bottle with the name of reagent (1% Carbol Fuchsin) as well as date of preparation and expiry. It can be stored at room temperature for six months.

**25% Sulphuric acid (H\textsubscript{2}SO\textsubscript{4})**

Conc. H\textsubscript{2}SO\textsubscript{4} : 250 ml  
Distilled water : 750 ml

- Take 750 ml distilled water in a flask  
- Carefully add concentrated Sulphuric acid to the water (do not add water to the acid). This mixture will heat up, so always place the flask in a bowl of cold water while diluting the acid.  
- Mix gently and store it in amber colored bottle.  
- Label it with name of reagent (25 % Sulphuric acid) and date of preparation and expiry. It can be stored at room temperature for twelve months.
0. 1 % Methylene blue

Methylene blue chloride : 1 g
Distilled water : 1000 ml

- Weigh 1 gm of Methylene blue and transfer to a 1L flask.
- Add 1000 ml of distilled water.
- Shake well & dissolve.
- Pour the solution through filter paper (whatman No.1) and store filtered solution in an amber colored bottle.
- Label the bottle with the name of reagent (0.1 % Methylene blue) as well as date of preparation and expiry. It can be stored at room temperature for six month.

3.2. Ziehl Neelsen Staining Procedures

1. Place the numbered slides on a staining rack with the smeared side facing up (individual or in batch of maximum 12 slides). Ensure to leave a finger thickness gap between slides to prevent the transfer of material from one smear to another and the solution running off from slides.

2. Flood the entire surface of slide with Ziehl-Neelsen 1% Carbol fuchsin solution.
3. Heat each slide slowly until steam arises. Maintain steaming for **5 minutes** by using intermittent heat. (In no case must the stain solution boil or dry during staining process.)

4. Rinse each slide individually in a gentle flow of water until all free stain is washed away. Drain off excess rinse water by tilting the slide.

5. Flood the slide with the decolorizing solution (**25% Sulphuric acid**) for **3 minutes**. (If the slide is under decolorized after 3 minutes, further decolorize for 1 minute.)
6. Rinse the slide thoroughly with water. Drain off excess water from the slide. Wipe off back of slide with cotton soaked in decolorizer to clean the dried stains.

7. Rinse the slide again with water and drain off excess water from the slide by tilting the slide.

8. Flood the slide with 0.1% Methylene blue and counter-stain for 60 seconds.
9. Rinse the slide thoroughly with water and drain off any excess stain from the slide by placing the slide under gentle stream of running water.

10. Stand the rinsed slide on the slide holding block and allow the smear to air dry. 
(Do not heat or use blotting paper to dry the smear. Do not place the slide under direct sunlight to dry the smear.)

4. Microscopic Examination Procedures

To obtain excellence in smear examination, a good microscope and a comfortable work area are required. Reading of smears must be systematic and standardized to ensure that a representative area of the smear is examined. To ensure that an area is covered only once, the smear should be examined in an orderly manner and the following procedure is recommended:
• Focus the stained slide under low power objective lens (5x or 10x) and, observe the staining quality and evenness of smear. Select the field where the smear is evenly distributed and WBCs & mucus can be seen.

• Apply a drop of immersion oil on the stained smear and focus the smear using 100x objective lens. Do not touch the smear with the dropper. Doing so may contaminate the applicator whereby may spread AFB from one slide to the next resulting in false-positive results.

• Always examine ZN-stained smears with a 100x objective lens under oil immersion.

• Include a known positive slide and a known negative slide each day. The positive control ensures the staining capability of the solutions and of the staining procedure. The negative control confirms that acid-fast contaminants are not present in the stains or other solutions.

• Make a series of systematic examination over the length of the smear. After examining a microscopic field, move the slide longitudinally so that the neighboring field to the right can be examined. Search each field thoroughly.
• Examine a minimum of 100 fields before the smear is reported as negative. For skilled microscopists this will take approximately five minutes. In a smear of 2.0 cm x 1.0 cm size, the number of microscopic fields in one length of the slide corresponds to around 100. If the smear is moderately or heavily positive, fewer fields may be examined and a report of “positive” may be made even though the entire smear has not been examined.

• Before examining the next slide, wipe the immersion lens with a piece of lens tissue.

• Unexpected objects may be seen when using the microscope. If these objects move only when the slide is moved, they may be materials occasionally found in the specimen or object, precipitated stains, contaminants from the stains or contaminants in the immersion oil.

• Artifacts that move only when the eyepiece is rotated are in the eyepiece or on its lenses. Artifacts may also be caused by material on the condenser, lenses, mirror or light source.

• Keep all the slides for quality control according to established procedures.

4.1. Morphological Characteristics of Acid-Fast Bacilli

• AFB are approximately 1-4 μm long and 0.3 - 0.6 μm thick that typically appear as slender, rod-shaped bacilli, but they also may appear curved or bent.

• With carbol-fuchsin staining, AFB look like fine red rods, slightly curved, more or less granular, isolated, in pairs or in groups, standing out clearly against the blue background.

• Individual bacteria may display heavily stained areas referred to as “beads” and areas of alternating stain may produce a banded appearance.

• All Mycobacterial species cannot be differentiated by microscopy examination.

• Some Mycobacteria other than M. tuberculosis may appear pleomorphic, ranging in appearance from long rods to coccoid forms, with more uniform distribution of staining properties.

• Rapidly growing mycobacteria may vary in their abilities to retain acid-fast stains.
5. Recording and Reporting of Results

The microscopic observation must be established first of all and then if there are AFB present in the smear; the approximate average number of these bacilli per microscopic field be observed. It is recommended that a uniform pattern of reading be followed, observing up to 100 useful fields. A useful microscopic field is regarded as one in which cellular elements of bronchial origin (leucocytes, mucous fibers and ciliated cells) are observed. The fields in which there are no such elements should not be included in the reading.

Reporting

Results obtained should be entered or transcribed correctly into the laboratory register. All results are reported as earlier as possible or preferably within 24 hours. Positive results are entered in red.

The number of AFB found is an indication of the degree of infectivity of the patient as well as the severity of tuberculosis disease. Results should therefore be quantified. For Ziehl-Neelsen stained smears the following semi-quantitative method of reporting is recommended:

Table 2: Grading of AFB smears as per WHO and IUATLD recommendation

<table>
<thead>
<tr>
<th>No of acid-fast bacilli (AFB)</th>
<th>Fields</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>No AFB</td>
<td>In 100 immersion fields</td>
<td>Negative</td>
</tr>
<tr>
<td>1-9 AFB</td>
<td>In 100 immersion fields</td>
<td>Positive scanty</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Record exact figure</td>
</tr>
<tr>
<td>10 to 99 AFB</td>
<td>In 100 immersion fields</td>
<td>1+</td>
</tr>
<tr>
<td>1 to 10 AFB</td>
<td>Per field (examine 50 fields)</td>
<td>2+</td>
</tr>
<tr>
<td>More than 10 AFB</td>
<td>Per field (examine 20 fields)</td>
<td>3+</td>
</tr>
</tbody>
</table>
6. Causes of Error in Microscopy

Connected with the specimen

- Inadequate specimen volume and/or poor quality.
- Carelessness in marking the container. Marking should be done on the body of the container and not on its lid.

Connected with the preparation of the smear

- Insufficient or poorly lit work surface.
- Mixing-up of slides.
- Preparing too many slides at once. A maximum of 12 is recommended per batch.
- Re-use of slides that had been positive earlier. These should be discarded.
- Specimen contamination due to careless use of loops/wooden applicators.

Connected with the staining

- Using scratched slides on which deposits of stain may look like bacilli.
- Using unfiltered fuchsin, which may contain crystals.
- Carelessness in heating the fuchsin, allowing it to dry and crystallize on the smear.
- Inadequate decolorizing of the smear, which may leave red stain on saprophytic bacilli which then appear to be acid-fast.

Connected with the microscopic examination

- Failure to check the slides and renumber them, if the number is obscured during staining. This may lead to substitutions.
- Failure to clean the immersion lens with lens tissue after each examination, especially after a smear was found to be positive.
- Carry-over of bacilli from a positive smear due to immersion oil dropper touching the smear. The oil drop should be allowed to fall without any contact between the dropper and slide.
- Erroneous recording of the results.
Table 3: Troubleshooting guide for microscopy

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible causes</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>The field is dim</td>
<td>Condenser may be too low</td>
<td>Raise the condenser.</td>
</tr>
<tr>
<td></td>
<td>Condenser iris may be closed</td>
<td>Open the diaphragm</td>
</tr>
<tr>
<td>Dark shadows in the field which move when eye</td>
<td>Eyepiece may be dirty.</td>
<td>Clean the eye piece</td>
</tr>
<tr>
<td>piece is moved</td>
<td>Eyepiece or objective may be contaminated with fungi.</td>
<td>Eye piece or objective may need cleaning</td>
</tr>
<tr>
<td></td>
<td>Surface of eyepiece may be scratched.</td>
<td>A new eye piece may be needed</td>
</tr>
<tr>
<td>The image is not clear</td>
<td>The smeared portion of the slide may be upside down</td>
<td>Turn the slide over</td>
</tr>
<tr>
<td></td>
<td>There may be an air bubble in the oil</td>
<td>Move the 100x lens from side to side</td>
</tr>
<tr>
<td></td>
<td>The oil may be of poor quality</td>
<td>Use only good quality immersion oil.</td>
</tr>
<tr>
<td></td>
<td>There may be dirt on the lens</td>
<td>Clean the lens</td>
</tr>
<tr>
<td>The image through low power is not clear</td>
<td>There may be oil on the lens</td>
<td>Clean the lens</td>
</tr>
<tr>
<td></td>
<td>There may be dust on the upper surface of the lens</td>
<td>Clean the lens</td>
</tr>
<tr>
<td></td>
<td>The lens may be broken</td>
<td>A new lens may be needed</td>
</tr>
</tbody>
</table>

7. Consequences of False-Positive and False-Negative Smears

False positive results

- Patients are started on treatment unnecessarily.
- Tuberculosis medicines are wasted.
- In follow-up examinations, the intensive phase of treatment is continued longer than necessary.
- Patients may lose confidence in the programme.

False negative results

- Patients with tuberculosis are not treated, resulting in suffering, spread of tuberculosis and death.
- Intensive phase treatment is not extended for the required duration, resulting in inadequate treatment.
- Patients may lose confidence in the programme.
8. Storage of the Stained Slides for Quality Assurance

The laboratory must store all examined slides in a way that allows easy retrieval of every slide identified for the rechecking sample. Instruction for peripheral laboratory staff regarding storage of examined slide includes:

- Remove immersion oil from the slides by facing the smear part on the tissue paper before placing them in the storage boxes.
- Store the slides in the slide box in a way that they do not touch each other (slides should not be stacked or pressed together).
- Do not write the result of the smear examined on the slide.
- Label the slides in a manner consistent with the laboratory register to ensure that the correct slide is matched to the result.
- Store the slides sequentially by laboratory serial number regardless of positivity in the slide boxes in the same order as they are listed in the laboratory register (Positive and negative slides should not be stored separately in different boxes).
- To maintain consistency with the laboratory register, leave two blank spaces behind the first slide from a patient so that the second and third slides can be added after they are read.
- Always store slides in closed boxes away from direct sunlight.
CHAPTER-2

QUALITY ASSURANCE PROGRAMME

Rationale:
- Ensure that the reported results are accurate
- Identify any practices that are potential sources of error
- Ensure that appropriate corrective actions are initiated

Quality assurance activities include:
- Validation of samples submitted for testing
- Regular checking of reagents used in test procedures (including expiry dates)
- Inclusion of standards (or samples of known positivity) in routine test runs
- Periodic review and updating of procedure manuals
- Regular maintenance and calibration of equipment
- Data collection and analysis
- Regular meetings with the laboratory’s clients

Elements/Components of Quality Assurance
- Internal Quality Control (IQC)
- External Quality Assessment (EQA)
- Quality Improvement (QI)

Internal Quality Control (IQC)
- Effective and systematic monitoring of the performance of bench work, technical procedures, equipments and supplies, including the quality of stains in the tuberculosis laboratory.
- Ensures accurate, reliable and reproducible laboratory information and serves as a mechanism by which tuberculosis laboratories can validate the competency of their diagnostic services.

External Quality Assessment
- A process to identify laboratories with problems resulting in poor performance by an identified reference laboratory.
- Assess overall laboratory performance in sputum microscopy.
Quality Improvement

- Process by which the components of tuberculosis laboratory service are analyzed continuously to improve their reliability, efficiency and utilization.

EQA Methods

- On-site Evaluation
- Panel Testing
- Blinded Rechecking

1. On-Site Evaluations

On-site evaluation is an essential component of a meaningful EQA program to:

- cover administrative as well as technical aspects
- Check that the laboratory is following the guidelines for QC
- Respond to errors found in a recent round of blinded rechecking (In some cases, the visit needs to be specifically planned for this purpose, for instance high frequency of false positives found in rounds of blinded rechecking or panel testing)

On-site evaluation activities include:

- Evaluating sputum collection procedure.
- Observing and evaluating procedures for smear preparation, staining, and reading.
- Assuring that positive and negative control slides are used with all newly made/received batches of stains as well as with every batch of smears daily.
- Re-checking several positive and negative smears to evaluate quality of smear and stain, and results.
- Providing suggestions for corrective action or implementing corrective action as needed after reviewing results of blinded rechecking and panel testing.
- On-site training.
2. Panel Testing

Panel testing is conducted to improve and maintain proficiency in AFB smear examination in all peripheral laboratories that provide sputum microscopy service.

Panel slide preparation

- Sputum specimen used for panel slide preparation is not more than 2 days old. Panel slides prepared are of negative and positive of different grades.

Characterization of panel slides

- Each panel set consists of 10 known smears; five ZN stained and five unstained. Both stained and unstained comprises of negative and positive slides of different grades with code numbers.

Dispatch of panel slides

- Panel slides are packed in slide boxes and sent to districts.
- Laboratories are given one month deadline for reporting the result to PHL from the date of panel slides receipt.

Instruction for test laboratories

- Standardized form for reporting and recording of panel results are provided to the technicians in the peripheral laboratories.
- Panel slides must be examined individually and not as part of a group effort.

Time for reading panel slides

- Equal time must be spent for the slides as that of routine slides and if possible, supervisors should monitor the time spent for reading panel smears.

Evaluation of Panel test results

It evaluates the proficiency of the laboratory technician in TB microscopy using the best of smears and thereby the best results are evaluated using the standard method.

Feedback

The reference laboratory must provide feedback to the peripheral laboratory, including scoring for accuracy of the results.
3. Blinded Rechecking

It’s a process by which a random selection of slides (representative of the total slides screened) is done using Lot Quality Assurance System (LQAS) from the routine workload at a peripheral laboratory and is re-examined at NTRL.

Sampling size:

Lot Quality Assurance Sampling (LQAS) method is used to determine the sample size for blinded rechecking and depends on the:

- Slide Positivity rate (SPR)
- total number of negative slides processed each year, and the
- performance (sensitivity) compared to the controllers

Considering SPR and total number of negative slides examined, the number of slides to be blinded is statistically determined keeping minimum controllers sensitivity at 75% and acceptance number as zero. Using the statistic table No. 3 reference lab need to recheck 105 blinded slides annually for each district laboratories which accounts to 26-27 slides per quarter in a year.

Table 3: Annual sample size based on LAQS

<table>
<thead>
<tr>
<th>Negatives Examined Annually</th>
<th>Acceptance Number</th>
<th>Slide Positivity Rate(SPR)</th>
<th>Total Sample Required</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.5%</td>
<td>5.0%</td>
</tr>
<tr>
<td>100</td>
<td>d=0</td>
<td>78</td>
<td>63</td>
</tr>
<tr>
<td>200</td>
<td>d=0</td>
<td>123</td>
<td>91</td>
</tr>
<tr>
<td>300</td>
<td>d=0</td>
<td>154</td>
<td>105</td>
</tr>
<tr>
<td>400</td>
<td>d=0</td>
<td>175</td>
<td>115</td>
</tr>
<tr>
<td>500</td>
<td>d=0</td>
<td>192</td>
<td>121</td>
</tr>
<tr>
<td>700</td>
<td>d=0</td>
<td>215</td>
<td>129</td>
</tr>
<tr>
<td>1000</td>
<td>d=0</td>
<td>236</td>
<td>136</td>
</tr>
<tr>
<td>2000</td>
<td>d=0</td>
<td>267</td>
<td>145</td>
</tr>
<tr>
<td>5000</td>
<td>d=0</td>
<td>289</td>
<td>152</td>
</tr>
<tr>
<td><strong>10000</strong></td>
<td><strong>d=0</strong></td>
<td><strong>297</strong></td>
<td><strong>154</strong></td>
</tr>
<tr>
<td>20000</td>
<td>d=0</td>
<td>302</td>
<td>155</td>
</tr>
<tr>
<td>50000</td>
<td>d=0</td>
<td>305</td>
<td>156</td>
</tr>
</tbody>
</table>
Evaluation of Blinded rechecking slide results:

Table 4: Reports tabulated and analyzed by using the following guideline

<table>
<thead>
<tr>
<th>Result of Laboratory</th>
<th>Result of Controlling Laboratory (PHL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>Correct</td>
</tr>
<tr>
<td>1-9 AFB/100 F</td>
<td>LFP</td>
</tr>
<tr>
<td>1+</td>
<td>HFP</td>
</tr>
<tr>
<td>2+</td>
<td>HFP</td>
</tr>
<tr>
<td>3+</td>
<td>HFP</td>
</tr>
</tbody>
</table>

Correct = No error
QE = Quantification error = Minor Error
LFN = Low False Negative = Minor Error
LFP = Low False Positive = Minor Error
HFN = High False Negative = Major Error
HFP = High False Positive = Major Error

Feedback

The reference laboratory must provide feedback to the test laboratory, including scoring for accuracy of the results as well as suggestions as to the likely explanations for any errors.
CHAPTER-3

SAFETY PRECAUTIONS IN TB MICROSCOPY LABORATORY

The most important factor in the prevention of laboratory-acquired infection is good technique on the part of the individual worker. Specialized equipment may aid good laboratory practice but does NOT replace it.

*M. tuberculosis* is included in Risk Group III in the WHO classification of risk, likely to infect laboratory workers by the airborne route. The number of tubercle bacilli required to initiate infection is as low as 10 bacilli. Aerosols may be produced in the TB laboratory when handling leaking specimens, opening sample containers, and preparing smears. When aerosolized material dry out, droplet nuclei are formed creating infective particles, which may remain in the air for long periods of time. When care and appropriate techniques are used, handling sputum presents a minimal risk of acquiring infection to a technician.

**Transmission of TB bacilli**

The TB bacilli are almost always transmitted by patients with active pulmonary disease. The patient expels TB bacilli in small droplets of respiratory secretions. These secretions quickly evaporate leaving “droplet nuclei” less than 5 μm in diameter. Droplet nuclei of this size containing 1–3 bacilli can remain suspended for long periods of time in the air and, following inhalation, are able to reach deep into the lungs to produce infection. Larger particles do not remain airborne for as long and do not transmit tuberculosis as efficiently.

The risk of infection depends on

1. the infectiousness of the source,
2. the environment (e.g., overcrowding and inadequate ventilation promote transmission of droplet nuclei),
3. the duration and intensity of exposure, and
4. The susceptibility of the recipient.

**Aerosol Formation: Spread of droplets**

- Coughing
- Singing
- Sneezing
- Talking
Relative Risk from Exposure to Infectious TB Case

Procedural Hazards

Inhalation hazards

Most infections in the tuberculosis laboratory arise from potentially infectious aerosols containing tubercle bacilli. Many microbiological techniques generate aerosols, e.g., when bubbles burst or when liquids are squirited through small openings or impinge on surfaces.

The following microbiological activities generate aerosols:

a. Collecting sputum specimens

Practices that generate aerosols are:

- coughing without covering mouth,
- Standing in front of suspected patients while they are coughing.
- Collecting specimens in confined areas.
- Reckless unscrewing of cap generates aerosols since a film of liquid is often present between the rim of the container and the cap during collection.

Precautions to lower these risks include:

- Instructing the patient to cover their mouth while coughing
- Standing behind the patient while the patient is coughing
- Collecting samples in open air
- Opening the sputum containers containing specimen with care
b. Working with bacteriological loops

Practices that generate aerosols are:
- A hot wire, when plunged into a specimen.
- Direct flaming, causes spattering which is very hazardous.

Precautions to lower these risks include:
- Adequate cooling of the loop after sterilization and before introducing into the specimen.
- Remove the sputum sticking to wire loops in a sand-alcohol jar before flaming.

c. Smear preparation and heat fixation

Practices that generate aerosols are:
- Vigorous smearing
- Heat fixing the smear before air drying.

Precautions to lower these risks include:
- Gentle movement of hands while smearing
- Complete air drying of smear before heat fixing.

Laboratory Hygiene

- Restrict entry to the laboratory only to the authorized personnel.
- Prohibit eating, drinking, smoking or applying make-up inside the Laboratory
- Do not allow licking labels or sucking pencils
- Wash hands with a suitable soap upon entering the laboratory, after handling potentially contaminated specimen containers, after any bacteriological procedure and before leaving the laboratory
- Regard all surfaces and equipment within the laboratory as potentially infectious and cleaned regularly before starting and after finishing the work using 5% phenol or 70% alcohol.
- Floors should not be swept but should be mopped with a disinfectant to limit dust formation.

Disinfectants

Disinfectant solutions should be prepared fresh every day and should not be stored in diluted form because their activity will diminish.

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Surfaces</th>
<th>Spills</th>
<th>Prepare</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol 5%</td>
<td>✓</td>
<td>✓</td>
<td>Every 2 days</td>
</tr>
<tr>
<td>Alcohol 70% v/v</td>
<td>✓</td>
<td>X</td>
<td>Weekly</td>
</tr>
<tr>
<td>Hypochlorite 5% *</td>
<td>X</td>
<td>✓</td>
<td>Every 2 days</td>
</tr>
</tbody>
</table>

* Corrosive to metal

5% Phenol

After disinfecting the working area and laboratory materials leave for 15-30 minutes before starting the work.
5 % Hypochlorite  
After disinfecting the working area and laboratory materials leave for 15-30 minutes before starting the work.

70 % Alcohol (ethanol)  
When hands become contaminated, rinse with 70% ethanol followed by thorough washing with soap and water.

All of the above disinfectants are toxic and undue exposure may result in respiratory distress, skin rashes or conjunctivitis. However, used normally and according to the manufacturers’ instructions, they are safe and effective.

Personal Protective Equipments

Masks  
Use of masks is not necessary. Only special masks such as the N95 respirator with a perfect fit are truly effective.

Gloves  
Gloves are not really needed either, since infection through the skin is rare. If used, they should be changed frequently; otherwise they will only serve to spread contamination around.

Apron  
Apron should be worn every time a technician performs work inside the laboratory. The same apron should not be worn outside the laboratory.

Laboratory Accidents

Spills inside biological safety cabinet:  
Ensure absorbent materials (gauze/cotton), 5% phenol and 70% alcohol is kept inside the cabinet. In case of spillage inside the BSC, immediately decontaminate the BSC using the procedure given below:

- Wear double pair of gloves during decontamination procedure.
- Spread 5% phenol soaked gauze/cotton immediately to cover the spillage, while the biological safety cabinet continues to operate. Wait for 15-20 minutes.
- Using gauze/ cotton, wipe up the spill from the edges to the center.
- Wipe the area again with a gauze/ cotton soaked in 70 % alcohol.
- Discard the used gauze/cotton and gloves in a biohazard bag to be autoclaved.
- Wash hands using soap after the disinfection
Spills on equipments, laboratory benches, walls, floors, clothing, etc.:

- Immediately indicate to all personnel working in the laboratory and evacuate for 1 hour to allow dissipation of aerosols created by the spill.
- Leave the contaminated laboratory, close laboratory doors and post warning signs to prevent others from entering the laboratory.
- Thoroughly wash hands and other apparently contaminated areas with soap and water.
- If personal clothing is contaminated, remove all outer clothing and place it in the autoclave or container for autoclaving. Put on clean garments.
- Wear the N95 mask, fresh laboratory coat and double pair gloves and re-enter.
- Upon returning to the laboratory, cover the spill area with gauze/ cotton soaked in 5% Phenol solution (Do not pour decontamination solution directly onto the spill in order to avoid additional release of aerosols).
- Let stand for 20 minutes then wipe up with gauze/ cotton.
- Wipe the area again with a gauze/ cotton soaked in 70 % alcohol.
- Discard the used gauze/ cotton and gloves in an autoclave bag and autoclave.
- Wash hands and other contaminated areas again with soap and water.

**Waste Disposal**

No infected material should leave the laboratory unless it is properly packed for transport. All specimens, contaminated containers, applicator sticks, pipettes and other laboratory supplies should be sterilized by autoclaving at 121oC at 15 lbs for 20 minutes before disposal or re-use.

| All clinical materials must be considered to be infectious and must be handle with exactly the same precautions with hazard warning labels. |
**Laboratory Request/Report Form:**

**NATIONAL TB REFERENCE LABORATORY**  
**PUBLIC HEALTH LABORATORY**  
**THIMPHU : BHUTAN**

**LABORATORY FORM FOR AFB EXAMINATION**

Name of Hospital: __________________________ Date: _________________________________

Name of Patient: ___________________________ Age: ________ Sex M/F: ________________

Complete Address: _____________________________________________________________________

Patient’s TB No.: _______________________________

Disease classification:  
□ Pulmonary  
□ Extra-pulmonary, Site: _______________ Specimen: __________________

Reason for Examination:  
□ Diagnosis  
□ Follow up of chemotherapy

Date of specimen collection: _________________________________

---

**RESULTS (To be completed in the laboratory)**

Lab. Serial No.: _______________________________________________

a) Visual appearance of sputum

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Mucopurulent</th>
<th>Blood stained</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen 1</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

b) Microscopy

<table>
<thead>
<tr>
<th>Date</th>
<th>Specimen</th>
<th>Results *</th>
<th>Positive (grading)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Write No AFB seen or Positive in the result column and tick under appropriate grading.

Date: ________________________________________ Examined by (Signature): ________________________________
Worksheet for ZN reagent preparation:

Reagent Preparation [Ziehl Neelsen Staining-1]

<table>
<thead>
<tr>
<th>Name of reagent:</th>
<th>Lot number:</th>
<th>Date of preparation:</th>
<th>Total Amount prepared:</th>
<th>Expiration Date:</th>
<th>Prepared by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Carbol fuchsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Supplier/catalog number</th>
<th>Lot number</th>
<th>Amount added</th>
<th>Technician/Technologist</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td></td>
<td></td>
<td>1 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td></td>
<td></td>
<td>10 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol crystal</td>
<td></td>
<td></td>
<td>5 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water up to</td>
<td></td>
<td></td>
<td>100 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Instructions for preparation of 1% Carbol fuchsin:
1. Using a digital balance weigh out 1 g of Basic fuchsin in a sterile 100 ml flask.
2. Add 10 ml of absolute alcohol and dissolve the dye by placing it in a water bath at 60° C. Avoid direct heating (Solution 1).
3. Using a digital balance weigh 5 gm of phenol crystals in a balance and transfer it to 100 ml Erlenmeyer flask
4. Add 50 ml of distilled water and dissolve the phenol crystal. Gentle heat may be required. (solution 2)
5. Mix solution 1 and solution 2 and transfer the contents into a 100 ml measuring cylinder
6. Add distilled water to make up the final volume to 100 ml.
7. Pour the solution through filter paper (whatman No 1) and store filtered solution in an amber colored bottle.
8. Label bottle with name of reagent (1% Carbol Fuchsin), lot number, date of preparation, expiration date & storage temperature.
9. Tighten caps & store at room temperature.

[Note: Store the reagent upto 6 months from preparation date. Occasionally filter Carbol fuchsin if precipitate forms.]
Reagent Preparation [Ziehl Neelsen Staining-2]

<table>
<thead>
<tr>
<th>Name of reagent:</th>
<th>Lot number:</th>
<th>Date of preparation:</th>
<th>Total Amount prepared:</th>
<th>Expiration Date:</th>
<th>Prepared by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% Sulphuric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Supplier/catalog number</th>
<th>Lot number</th>
<th>Amount added</th>
<th>Technician/ Technologist</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. H₂SO₄</td>
<td></td>
<td></td>
<td>25ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td>75 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Instructions for preparation of 25% Sulphuric acid:**

1. Using a 100 ml measuring cylinder measure out 75 ml of distilled water in 500 ml conical flask and submerged the flask in a bowl of cold water.
2. Using a 50 ml measuring cylinder measure out 25 ml of conc. Sulphuric acid and carefully add concentrated Sulphuric acid to the water. This mixture will heat up, so always place the flask in a bowl of cold water while diluting the acid.
3. Mix gently and store it in amber colored bottle glass bottles with caps.
4. Label bottle with name of reagent (25% Sulphuric acid), lot number, date of preparation, expiration date & storage temperature.
5. Tighten caps & store at room temperature. Acid prepared can be stored upto 12 months from the date of preparation.

[Note: Always add acid to water. Never add water to acid.]
Reagent Preparation [Ziehl Neelsen Staining-3]

<table>
<thead>
<tr>
<th>Name of reagent:</th>
<th>Lot number:</th>
<th>Date of preparation:</th>
<th>Total Amount prepared:</th>
<th>Expiration Date:</th>
<th>Prepared by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% Methylene blue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Supplier/catalog number</th>
<th>Lot number</th>
<th>Amount added</th>
<th>Technician/Technologist</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue</td>
<td></td>
<td></td>
<td>0.1 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td>100 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Instructions for preparation of 0.1% Methylene blue:**
1. Using a digital balance weigh out 0.1 g of methylene blue in a sterile 500 ml flask and dissolve it in 100 ml of distilled water.
2. Use a stirring bar or swirl solutions to mix.
3. Pour the solution through filter paper (whatman No.1) and store filtered solution in an amber colored glass bottles with caps.
4. Label bottle with name of reagent(0.1 % Methylene blue), lot number, date of preparation, expiration date & storage temperature.
5. Tighten caps & store at room temperature.

[Note: Store the reagent upto 6 months from preparation date.]

**Quality Control**

Stain a positive control and a negative control slide using the new batch of staining reagent and observe for reagent quality. Smear quality good= pass, Smear quality poor= Fail.

<table>
<thead>
<tr>
<th>Performed by:</th>
<th>Date stained:</th>
<th>Staining quality [Good/poor]</th>
<th>Final results (Smear quality Good or poor)</th>
<th>Pass/Fail &amp; Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Color of AFB stained</td>
<td>Background</td>
<td>Counter staining</td>
</tr>
<tr>
<td>Positive Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Annexure 3

**Essential Equipment and Supplies for a Laboratory Performing Ziehl-Neelsen Microscopy**

Supplies for a laboratory performing Ziehl-Neelsen Microscopy (1000 specimens per year) are as follows:

<table>
<thead>
<tr>
<th>Item</th>
<th>Equipment</th>
<th>Quantity/volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bunsen burner for use with butane gas</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Butane gas cylinders or</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>Spirit lamp</td>
<td>1</td>
</tr>
<tr>
<td>4.</td>
<td>Binocular microscope, for use with daylight or/and electric power, Oil immersion lens (100x), eye pieces (10x), spare bulbs</td>
<td>1</td>
</tr>
</tbody>
</table>

**Supplies:**

1. Amber colored bottle, glass, 100ml
2. Apparatus for distilled water
3. Boiling flask, glass, 1000ml capacity
4. Bowl, plastic, 50x30 cm
5. Bucket, plastic, 12 liters capacity
6. Diamond marker
7. Disinfectant, bactericidal e.g., 5% phenol, 5% sodium hypochlorite
8. Drop bottle, plastic, 10 ml capacity
9. Drop bottle, glass, 100ml capacity
10. Filter paper, 15cm diameter, No.1
11. Forceps, stainless steel, 15cm
12. Funnels, glass, 45mm or 60mm diameter
13. Funnels, glass, 90mm or 125mm diameter
14. Laboratory request/report form
15. Laboratory registers
16. Lens tissue
17. Measuring cylinder, glass, 100ml capacity
18. Overall, laboratory coat
19. Paper towels, disposable
20. Pens, ball point, red ink
21. Pens, ballpoint, black or blue ink
22. Autoclave
23. Scissors, stainless steel, 25cm
24. Slide rack, plastic, 12-25 slides
25. Slide storage box, cardboard or plastic, 50 slides capacity
26. Staining rack
27. Timer, 0-60 minutes, with alarm
28. Volumetric flask, glass, 500ml capacity
29. Wash bottle, plastic, 500ml capacity
30. Wire loop holder
31. Nickel-chromium wire, 1mm diameter

**Butane Gas Cylinders or Bunsen Burner for use with Butane Gas:**

- 2 boxes
- 1 box

**Boiling Flasks:**

- 4 boxes

**Forceps:**

- 1 box

**Nets and Filters:**

- 2 boxes

**Cupboards and Shelves:**

- 5 metres
Consumable supplies:
1. Cotton wool, white absorbent ................................................................. 1kg
2. Sputum containers, screw-capped .......................................................... 1500
3. Microscope slides, 25mm x 75mm, 1.1-1.3mm thick ................................ 1500
4. Wooden applicators .............................................................................. 1500

Reagents
1. 25% Sulphuric acid, for Ziehl-Neelsen staining ....................................... 5 liters
2. 0.1% Methylene blue .............................................................................. 5 liters
3. 1% Carbol-fuchsin, for Ziehl-Neelsen staining ....................................... 6 liters
4. Immersion oil .......................................................................................... 200 ml
5. Methylated spirits .................................................................................. 4 liters

Classification of errors:

Classification of Errors

Errors
- Major
  - HFN: High False Negative
    - 1+, 2+ & 3+ reported as negative
  - HFP: High False Positive
    - Negative reported as 1+, 2+, & 3+

- Minor
  - LFN: Low False Negative
    - Scanty +ve reported as negative
  - LFP: Low False Positive
    - Negative reported as scanty +ve
  - QE: Quantification Error
    - Reported as one grade above or below

Annexure 4
Slide Receive Form for Quality Assurance:

National Tuberculosis Reference Laboratory
(SLIDE RECEIVE FORM FOR QUALITY ASSURANCE)

Name of Hospital: ……………………………………      District: ……………………………..
Slide shipped by: ……………………………………... Designation: ……………………………
Total number of positive slide: ………………. Total number of Negative slide: ……………….

[Note: Attach a copy of Smear Result Sheet for QC with Q.A.1 form for the selected quarter]

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Gender</th>
<th>Total No. of people examined for Diagnosis (A)</th>
<th>Total No. of people with positive sputum smear. (B)</th>
<th>Laboratory Indicator: Smear positive rate (B/Ax100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(To be completed by NTRL, PHL)

Date of receive (DD/MM/YY): …… / …… / ……
Total number of slide received: ………………
Total number of slide missing: ………………
Total number of Negative slide: ……………… No. of positive (random selection): ………
Total number of positive slide: ……………… No. of Negative (random selection): ………
(Intervals, All slide/no. of slide for random = ……….. / …… = ……..)

Date of Random slide: …………… Name of person: ……………………………………………

Signature: ………………………………………………

Designation: …………………………………………
Annexure 6
Q.A.1.1

National Tuberculosis Reference Laboratory
Smear Result Sheet for Quality Control

<table>
<thead>
<tr>
<th>Lab. S1.No.</th>
<th>Date</th>
<th>Sex M/F</th>
<th>Age</th>
<th>Reason for examination</th>
<th>Results</th>
<th>Signature</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diagnosis Follow-up</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
Annexure 7

Blinded Rechecking Feedback Form

NTRL EQA OF SPUTUM MICROSCOOPY

Blinded Rechecking of Sputum Smear Examinations for Acid-Fast Bacilli

Feedback form

Name of Hospital: …………………………… District: ………………………………………


Second Level Controller: ………………………… Third Level Controller: ……………………

<table>
<thead>
<tr>
<th>Report of District Laboratory</th>
<th>Result of the controller (PHL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>1-9 AFB/100F 1+ 2+ 3+</td>
</tr>
<tr>
<td>Negative Correct</td>
<td>LFN HFN HFN HFN</td>
</tr>
<tr>
<td>1-9 AFB/100 F</td>
<td>LFP Correct Correct QE</td>
</tr>
<tr>
<td>1+ HFP</td>
<td>Correct Correct Correct QE</td>
</tr>
<tr>
<td>2+ HFP</td>
<td>QE Correct Correct Correct</td>
</tr>
<tr>
<td>3+ HFP</td>
<td>QE QE Correct Correct</td>
</tr>
</tbody>
</table>

Goal Met Yes__________ No__________

Summary of Errors Identified (nos.)

<table>
<thead>
<tr>
<th>Major Errors</th>
<th>Minor Errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFP</td>
<td>HFN</td>
</tr>
<tr>
<td>LFP</td>
<td>LFN</td>
</tr>
<tr>
<td>QE</td>
<td></td>
</tr>
</tbody>
</table>

Total Major Errors: Total Minor Errors:

HPF: High False Positive; HFN: High False Negative; LFP: Low False Positive; LFN: Low False Negative; QE: Quantification Error;

Sl. No. Slides Received/ Quarter No. of slides Rechecked Total Error OAR% Total Score

OAR: Overall Agreement Rate

Recommendations:

___________________________
Incharge
National Tuberculosis Reference Laboratory
PHL, DoPH, Thimphu
Panel Testing Result Reporting Form:

QUALITY ASSURANCE NETWORK IN SPUTUM MICROSCOPY

National Tuberculosis Reference Laboratory
Public Health Laboratory, Thimphu

Lab ID No.: ............... 

Stained slides

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Slide ID No.</th>
<th>AFB Result</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Unstained slides

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Slide ID No.</th>
<th>AFB Result</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>8.</td>
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<td></td>
<td></td>
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<tr>
<td>9.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Name of the District Laboratory: _______________________________________________________

Date Panel slide received: _____ / _____ / _____   Date of Examination: _____ / _____ / _____

Examined by: Name: ___________________________   Designation: ____________________________

Name and signature of the Head of Lab: ___________________________________________________
### Panel Testing Worksheet:

**QUALITY ASSURANCE NETWORK IN SPUTUM MICROSCOPY**

**Smear Result Sheet for Quality Assurance**

**Name of Hospital:** ……………………………..  
**District:** ……………………………

**Date of report received:** …… / …….. / ……..

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Lab. ID No.</th>
<th>Result including grade for positive smear</th>
<th>PHL result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7.</td>
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<tr>
<td>8.</td>
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<td></td>
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<tr>
<td>9.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total**  | **No. True Positive** | **No. False Positive** | **No. True Negative** | **No. False Negative** |
|-----------|-----------------------|------------------------|-----------------------|------------------------|

**Report of District Laboratory**

<table>
<thead>
<tr>
<th>Report of District Laboratory</th>
<th>Result of the controller (PHL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>Correct</td>
</tr>
<tr>
<td>1-9 AFB/100 F</td>
<td>LFP</td>
</tr>
<tr>
<td>1+</td>
<td>HFP</td>
</tr>
<tr>
<td>2+</td>
<td>HFP</td>
</tr>
<tr>
<td>3+</td>
<td>HFP</td>
</tr>
</tbody>
</table>

**Rate of False positive (FP) = FP according to Supervisor or PHL / Total positive by the LT or District = ___%**

**Rate of False negative (FN) = FN according to Supervisor or PHL / Total negative by the LT or District = ___%**

[Upper limit of false positive and false negative is fixed at <5% as acceptable result on sputum microscopy]

Number of (-) ____ & (+) ____ consistencies within range by grading

**Overall Agreement Rate (OAR): __________________________ x 100 = ____%**

Grand Total _______

**[Rating of OAR: □ ≥95% (Excellent), □ ≥90% (Satisfactory), □ <90% (Poor performance)]**

**Compiled by:** ……………………………

**Date:** …… / …….. / ……..
Panel Testing Recording and Feedback Form:

National Tuberculosis Reference Laboratory, PHL
Feedback Report of Proficiency Testing

The report was tabulated and analyzed by using the following guidelines.

<table>
<thead>
<tr>
<th>Report of Laboratory</th>
<th>Result of the controller (PHL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Correct</td>
</tr>
<tr>
<td>1-9 AFB/100 F</td>
<td>LFN</td>
</tr>
<tr>
<td>1+</td>
<td>Correct</td>
</tr>
<tr>
<td>2+</td>
<td>HFP</td>
</tr>
<tr>
<td>3+</td>
<td>HFP</td>
</tr>
<tr>
<td>Negative</td>
<td>Correct</td>
</tr>
<tr>
<td>1-9 AFB/100 F</td>
<td>QE</td>
</tr>
<tr>
<td>1+</td>
<td>Correct</td>
</tr>
<tr>
<td>2+</td>
<td>QE</td>
</tr>
<tr>
<td>3+</td>
<td>QE</td>
</tr>
</tbody>
</table>

Types of Errors
Correct No error
QE Quantitative Error Minor Error
LFN Low False Negative Minor Error
LFP Low False Positive Minor Error
HFP High False Positive Major Error
HFN High False Negative Major Error

Criteria for acceptable performance
Panel of 10 slides, each slide of known worth 10 points, total possible score= 100
a. Scores of Major Errors= 0
b. Scores of Minor Errors= 5
c. Passing Score= 90

Performance of: ____________________________  Lab. ID No.: ________

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Slide No.</th>
<th>Result obtained</th>
<th>Expected Result</th>
<th>Error Type</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>2.</td>
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<td>3.</td>
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<tr>
<td>10.</td>
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</tr>
</tbody>
</table>

Note: If more than one technician performs AFB microscopy in the laboratory, each technician should read all 10 smears and record their results on a separate form. Technicians should not discuss results or share forms until all results have been sent back to the Public Health Laboratory. Forms for all technicians should be sent to the PHL for evaluation.
References

1. Regional Guidelines for Countries in the Western Pacific, WHO, Office for the Western Pacific Region – 2003
4. The public health service national TB reference laboratory and the national laboratory network IUATLD, 1998
7. Standard operating procedures for Culture & DST labs RNTCP India.
TB is Curable! DOT ensures Cure!
National Tuberculosis Control Programme