THE REPUBLIC OF UGANDA
MINISTRY OF HEALTH

UGANDA Supranational® Reference Laboratory
Timely Accurate Diagnostics for a TB-Free Africa

Training on Proficiency Testing Scheme (Microscopy PT)

Module 5: preparation of microscopy PT items

24th- 29th April 2018

Uganda Supranational Reference Laboratory

Content outline

- Materials required
- Checklist
- Personal protective equipment (PPE)
- Working in a Biological Safety Cabinet (BSC)
- Preparation of Microscopy PT items
- Quality control

Group exercise-5 minutes

1) Prepare a written checklist for all materials and tools required during preparation of Microscopy PT items.

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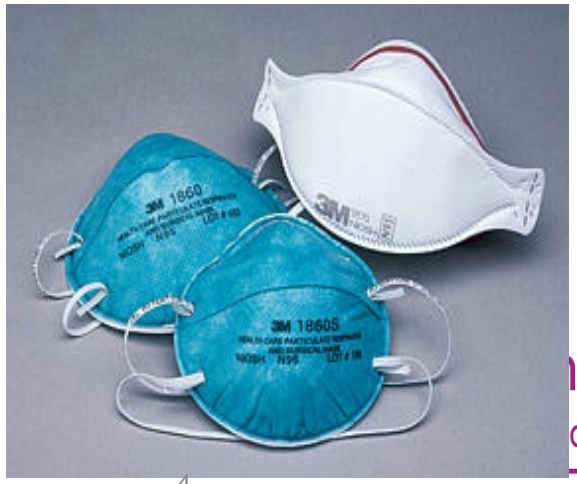
Use appropriate BSL3 PPE

- Gowns
  - Must have solid front and can be tied in the back
  - Long-sleeved with elastic cuffs
  - Must be either autoclavable/washable (soak in bleach) or disposable

- Respirators
  - N95
- Gloves
  - Single-use



Working in BSC II

- Decontaminate the BSC with freshly diluted 1% bleach or 5% lysol (phenol), followed by 70% alcohol
- Cover the working area with paper towels and spray it with suitable disinfectant
- Use a discard container with a plastic bag and freshly diluted 1% bleach or 5% lysol (phenol).
- DO NOT block the grill
- Perform all operations at least 12 cm away from the front grill on the work surface
- Place all materials and aerosol-generating equipment away from the front grill

Materials required.

- Slide mailers
- Slide boxes
- Slide transport box
- Biosafety cabinet
- Microscopes (Fluorescent and Bright field)
- Processing should be performed in a Biological Safety Cabinet.
- 50 ml plastic screw cap tubes
- 40% Formaldehyde
- 4% NaOH
- Vortex
- Water bath at 55-60°C
- Distilled water
- Centrifuge
- Slides
- Positive specimen
- Negative specimen

Materials required.

- Positive specimen

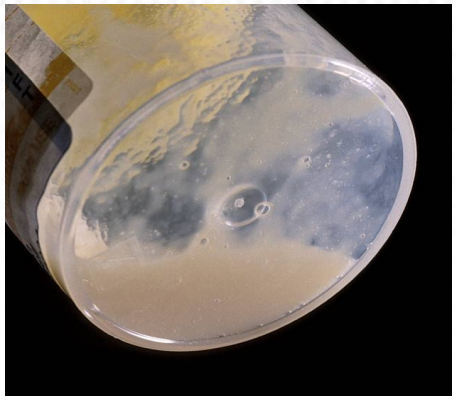
(Fresh specimens, no more than 2 days old, are preferred) **Amount**: 3 ml or more; AFB load: >2+ AFB by Ziehl-Neelsen direct smear; **Color:** White to light green; blood stained specimens should be avoided; **Thickness**: Watery (less mucous) specimens are preferred to increase consistency.

- Negative specimen –

Artificial sputum prepared at NTRL

fresh specimens, no more than 2 days old, are preferred) **Amount:** 5 ml or more; **Color**: white to green; **Thickness**: Watery (less mucous) specimens are preferred to increase consistency. **Note**: An AFB negative specimen with 20 or more white blood cells per field is preferred.

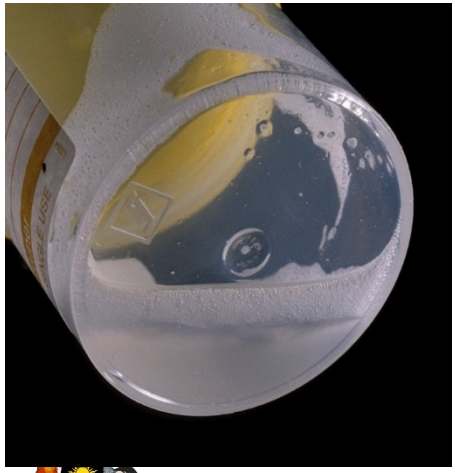
Materials required.



Pulurent

mucoid

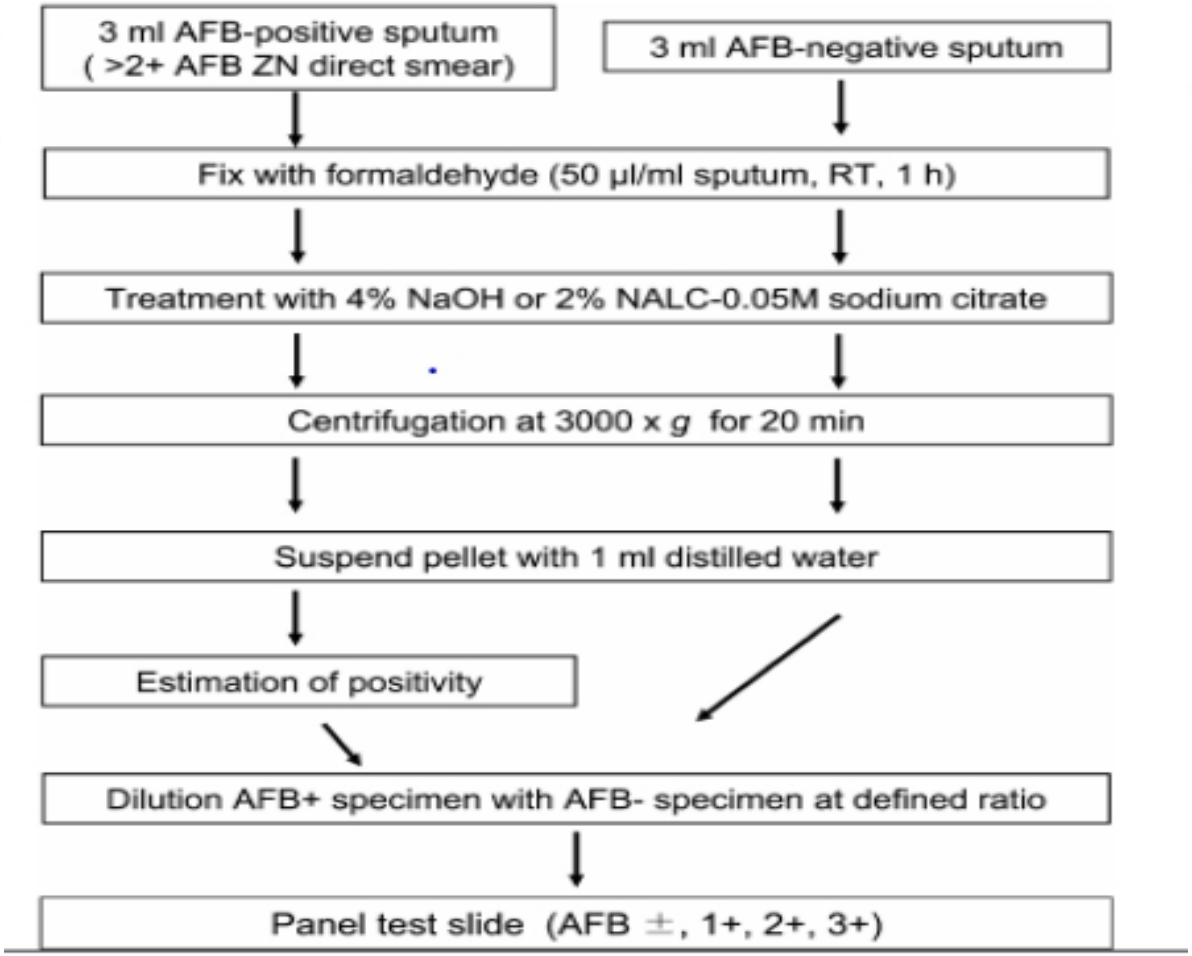
Obtaining adequate good quality specimens is critical to ensure accurate and Reliable AFB microscopy results



Salivary

bloodstained

Preparation of PT item for Microscopy



Infectious material: all steps have to be performed in a BSC at least in an appropriate BSL2 Laboratory

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Preparation of PT item for Microscopy

B. Sodium hydroxide (NaOH) method

Preparation of AFB Positive stock

- Place 3 ml of AFB positive specimen into a 50 ml screw cap plastic tube. If volume of the specimen is more than 3 ml, aliquot it into separate tubes.
- Add 1 drop (approx. 50 µl) of 40% Formaldehyde per 1 ml of sputum, vortex well. Incubate for 1 hour at room temperature (25- 30^{o}C).
- Add 1 ml of 4% NaOH (if the sputum is too thick, add up to 2 ml of NaOH solution so that the final concentration of NaOH is always 1-2%).
- Vortex thoroughly for 4-5 minutes.

Infectious material: all steps have to be performed in a BSC at BSL3 Laboratory

Preparation of PT item for Microscopy

- Add up to 20 ml of distilled water, mix well.
- Incubate in a water bath for 30 min. at 55-60^{o}C, mix occasionally by inverting the tube during incubation. If there is no water bath available, boil a beaker of water, cool to 90-95^{o}C and place the tube in the beaker for 20-25 min. It is important to maintain the incubation temperature in the 55-90^{o}C range.
  - **NB. This step may not apply in situations where the water bath is not available. Boil water and monitor the temperature using an external thermometer.**
- Add distilled water to a total volume of 40 ml, mix by inversion.
- Centrifuge @ 3,000 x g for 20 minutes at room temperature (25-30^{o}C).
- Decant supernatant carefully, add 0.5-1 ml of distilled water to re-suspend pellets. If initial sputum was aliquoted into portions, pellets from the same specimen are combined, prior to re-suspending.

Infectious material: all steps have to be performed in a BSC at BSL3 Laboratory

Preparation of PT item for Microscopy

- **Preparation of AFB Negative Stock**
- Distribute 3-4 ml aliquots of artificial sputum into 50 ml screw cap tubes.
- Note: Several good quality negative sputa can be pooled together and then split into 3 ml aliquots. Sputa should be checked for AFB prior to pooling.
- Add 1 drop (approx. 50 µl) of 40% Formaldehyde per 1 ml of sputum, vortex well.
- Incubate for 1 hour at room temperature (25-30^{o}C).
- Add 1 ml of 4% NaOH (if the sputum is too thick, add up to 2 ml of NaOH solution so that the final concentration of NaOH is always 1-2%).
- Vortex for 2-3 min.
- Add up to 20 ml of distilled water, mix well.
- Incubate in a water bath for 10 min. at 55-60^{o}C (Note: the negative specimen should be heated for a shorter period than the positive specimen to preserve white blood cells). If there is no water bath available, boil a beaker of water, cool to 90-95oC and place the tube in the beaker for 5-10 min.

Preparation of PT item for Microscopy

Preparation of AFB Negative Stock

- If foam has formed on top of the stock solution, pipette the contents from beneath the foam into a fresh tube.
- Using a standard microbiological loop make 2-3 test smears (approx. 1x2 cm in size) from the suspension for evaluation of the stock preparations.
- Use a well leveled surface for drying the smears.

Dilution Procedure

- Using negative preparation as a diluent make dilutions according to WHO Guidelines for AFB quantification: 0 AFB/100 fields: negative, 1-9 AFB/100 fields: exact number of AFB required, 10-99 AFB/100 fields: 1+ , 1-10 AFB/field: 2+ >10 AFB/field : 3+
- Choose suitable AFB concentration on a case-to-case basis within suggested range. For better results, however, it may be recommended using 20 AFB/field for 3+ smears, 5 AFB/field for 2+ smears, 50 AFB/100 fields for 1+ smear, and 5 AFB/ 100 fields for “exact” smears.

Preparation of PT item for Microscopy

Dilution Procedure

- Make 3-4 ml of each suspension in order to be able to generate sufficient amount of smears. For easy calculations both AFB-positive and AFB-negative aliquots are measured in drops. Calibrate one typical disposable Pasteur pipette by measuring the number of drops in 1 ml of sputum suspension. Note: do not use water for calibration since the amount of drops may be different from sputum due to the lack of viscosity.
- For calculation of the dilution factor use the following formula :

- N = (DC / AC) * A
- Where: N - is amount of drops of positive sputum to be added.
- DC - is desired AFB concentration.
- AC - is actual AFB concentration.
- A - Is the amount of drops in a given volume that was estimated during calibration?

Infectious material: all manipulations have to be performed in a BSC at BSL3 Laboratory

Preparation of PT item for Microscopy

Example: AFB concentration in the stock suspension (AC) is 65 AFB/field and we have to prepare 4 ml (A = 60 drops) of 2+ suspension (DC=5 AFB/field). In this case N = (5 AFB /65 AFB)*60 drops N = 4.6 drops (approx. 5 drops). So, 5 drops of the positive prep is mixed with 55 (60 - 5 = 55) drops of the negative prep.

Preparation of PT item for Microscopy

A. From routine processed samples received at NTRL*(Modified method from External Quality Assessment for AFB smear Microscopy manual by IUATLD)*

There is need to carry vigorous Quality control to ensure that all slides prepared from such a sample is consistent.

NB. This may work if the number of panel slides required is small i.e. like 20 PT panels per round

Labelling containers is key

Infectious material: all steps have to be performed in a BSC at BSL3 Laboratory

Cleaning up- decontamination

- Decontaminate any materials (vials, tubes etc.) before removal from the BSC
- Clean pipettes, racks, instruments and the BSC with freshly diluted 5% Lyol (20 min), followed by 70% alcohol
- Use area-dedicated spray flasks or beakers (separate beakers for surface cleaning and instruments)
- Do not take anything from this area to Microscopy room.

Assessment

1. What type/ forms of samples can Microscopy PT items be prepared?
2. Mention 4 ways to minimize cross contamination item preparation.
3. How can one quality control the process during microscopy PT preparation.

References

- ISO 13528:2005, *Statistical methods for use in proficiency testing by interlaboratory comparisons*
- ISO 15189, *Medical laboratories — Particular requirements for quality and competence*
- ISO Guide 34, *General requirements for the competence of reference material producers*
- ISO Guide 35, *Reference materials — General and statistical principles for certification*
- *ISO/IEC 17043 First edition 2010-02-01*
- Guide 34, ISO Guide 35 and ISO 13528 (homogeneity and stability)
- ISO/IEC Guide 98-3, *Uncertainty of measurement — Part 3: Guide to the expression of uncertainty in measurement* (GUM:1995)
- ISO/IEC 17011:2004, *Conformity assessment — General requirements for accreditation bodies accrediting conformity assessment bodies*
- ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

Acknowledgments

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