TITLE: Mycobacteria Sample Processing	Revision Date: 07-April-2017	Issue Date: 07-April-2015
Document Number: MIC80400	Status: Approved	
Distribution: Mycobacteria Manual	Page: 1 of 12	
Approved by: Gloria Badari, Director, Corporate Services and Chief Financial Officer	Signed by: (Original Signed Copy in Microbiology)	

PURPOSE:

To standardize the processing of clinical samples for Mycobacteria culture and direct smear preparation using 3% NaOH as a digestant.

INTRODUCTION:

Samples submitted for Mycobacteria culture are from a wide variety of sources, some containing superficial contamination with commensal microorganisms. The single factor that most influences the recovery of mycobacteria from clinical samples is the presence of contaminating microorganisms. The goal of sample processing is to eliminate contaminating commensal flora and break down mucous so that underlying Mycobacteria are more easily detected.

Samples from sources such as sputum, urine and gastric washings require decontamination to kill commensal flora and digestion to break up mucous. This is done with 3% Sodium Hydroxide (NaOH). At this dilute concentration, NaOH is an effective mucolytic agent and decontaminant, without killing Mycobacteria.

Samples from sterile sites do not normally contain contaminating agents and may be cultured and have a direct smear exam without processing with 3% NaOH, depending on a sterility check. Processing is performed, and exposure in NaOH is adjusted, according to the amount of bacterial growth if a normally sterile fluid contains contaminants.

Since Mycobacteria are often present in low numbers, the sample is concentrated by centrifugation before inoculating to culture or direct smear.

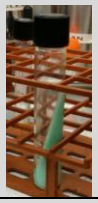
SPECIAL SAFETY PRECAUTIONS:

- Handle all patient samples and testing reagent using “Routine Practices”
- Please refer to the Northwest Territories Infection Prevention and Control Manual, March 2012
- Prior to testing all patient are to be identified as per I-0500 Use of Two Patient Identifiers.

SUPPLIES:

- 50 mL conical FALCON tubes
- 5 ml unsterile Pasteur pipettes
- “Wet” Metal discard bucket with lid
- “Dry” orange double-lined bin
- Yellow Waste bucket (1/3 full with fresh Accel TB)
- Vortex mixer
- Dedicated timer
- Alcohol wipes
- Clean frosted glass slides
- 5% phenol alcohol (fixer)
- Albumin-phenol
- Pencil
- “sterile H2O” labelled Conical FALCON tube
- “3% NaOH” labelled Conical FALCON tube
- Paper towels
- 4x4 gauze
- 50 mL beaker

MEDIA & EQUIPMENT:

Criteria	Information
List of supplies, reagents, media and equipment required for Mycobacteria processing:	
LJ slants 	• Manufacturer: Remel • Vendor: Unipath/Oxoid • Storage: 2- 8 Celsius
MGIT 	• Storage: In-use box kept by Allegra with the lid closed to protect from light (light degrades the fluorescent compound inside tube). • Refer to PANTA document for additional inf0rmaion
PANTA (reconstituted) 	• Storage: 2- 8 Celsius in Reagent fridge • Use on or before date handwritten on Vial • Refer to PANTA document for reconstitution instructions.
3% NaOH (digestant) 	• *3% NaOH working solution* • Refer to reagent preparation manual for make-up from stock NaOH.
BSC (CL-2 protection) 	• Manufacturer: LABCONCO • Refer to Equipment document for use and maintenance
Allegra X15R (centrifuge) 	• Manufacturer: Beckman Coulter • Refer to Equipment document for use and maintenance

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ORGANIZATION OF MYCOBACTERIA BIOHAZARD SAFETY CABINET:

Organization is important for proper air flow inside BSC, both to minimize contamination and for ergonomics. Follow the picture below as a guide to how to set up the Mycobacteria BSC before sample processing:

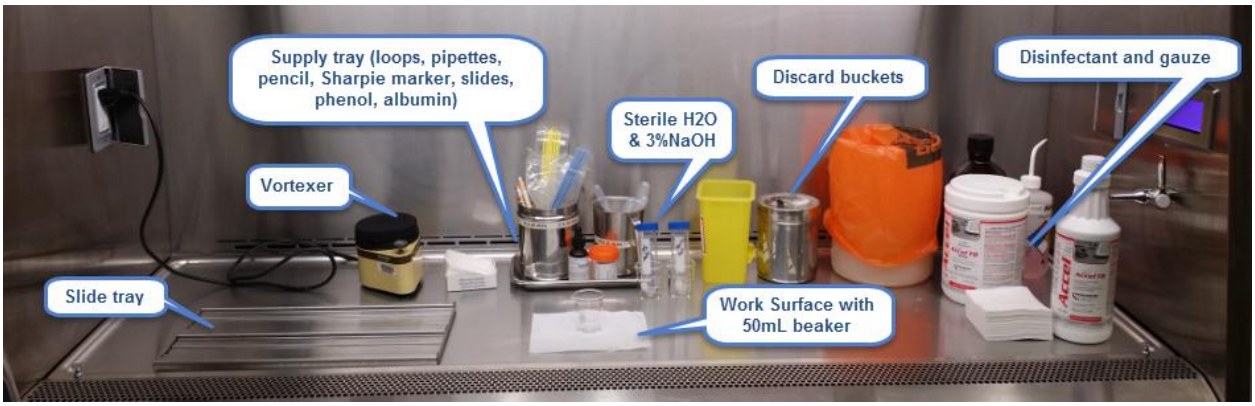


Figure 1: (above) Organization of the Mycobacteria BSC

PREPARATION OF SAMPLES AND MYCOBACTERIA BSC BEFORE PROCESSING:

Before processing, evaluate and check the following criteria:
Set the # of samples per run:
• Mycobacteria sample processing is time sensitive (20 mins max exposure to NaOH before it becomes lethal) therefore organize workflow so that only 12 samples are processed per run.
Pre-label the Direct Smear slides:
• Use plain frosted smears free of wax. Pre-etched slides are available but be aware they can cause false-positives even if cleaned with alcohol. • Using a pencil, label direct smears with the Accession #, Patient Last name, and "F" for Fluorescence stain • Place smears on metal tray inside the Mycobacteria BSC

Notes about fluids:

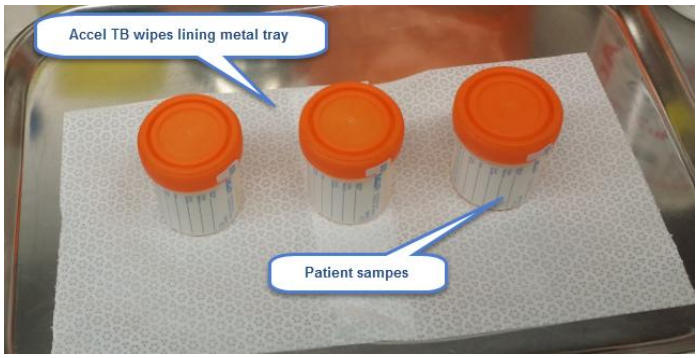
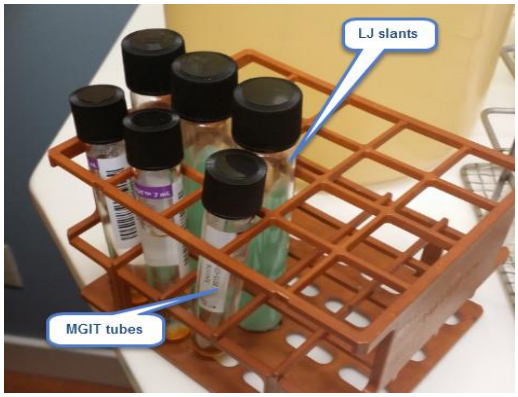
- **Concentrate before processing**: *Ensure all required samples have been concentrated by centrifugation and the supernatant poured off.*
- **Check sterility of fluids before processing:** *For sterile fluid samples: Check the 24hr C&S culture results or sterility plate for potential contaminating growth. Make a note on the sample NaOH conical tube if any processing is required. Write with a Sharpie pen the required time for NaOH exposure ie. "**5 mins**"*

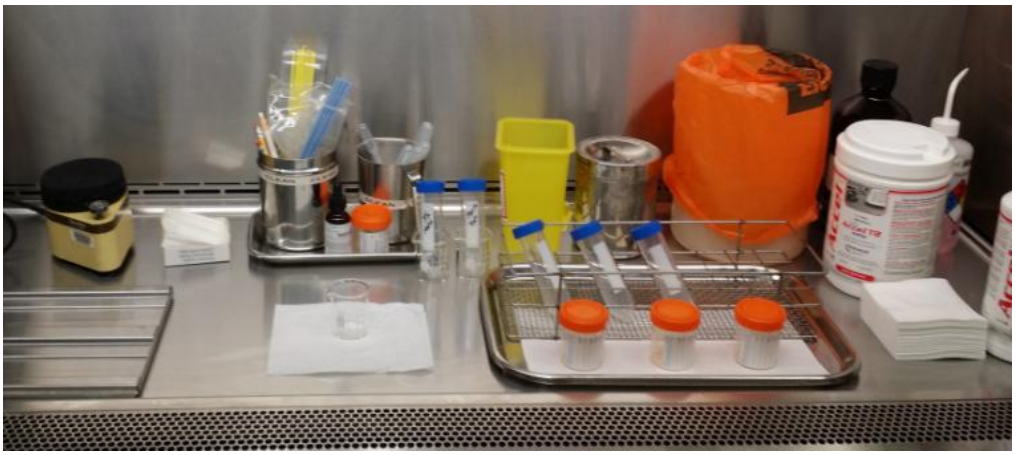
Ready the Mycobacteria BSC:

- **Set up air currents:** *Raise sash to line*
- **Clean-up from previous day**: *As the airflow sets up, scan BSC for fixed smears from the previous day (move them to stain rack in the Mycobacteria sink) and scan for waste in yellow biohazard bucket and orange bin.*
- **Prepare the yellow disinfectant bucket**: *Remove any used pipettes and loops from the previous day (depress liquid from pipettes into yellow bucket), and place into the "dry" discard bin (double lined with two orange autoclave bags). Spray/wipe down yellow bucket with Accel TB before removal from BSC. Dump old Accel TB down sink with plenty of running water. Return to BSC and refill 1/3rd with fresh Accel TB.*
- **Prepare the "dry" discard bin:** *If the orange-lined dry discard bin is full, completely close to seal. Spray/wipe down with Accel TB before removal from the BSC. Place in yellow biological cardboard bin in Bacteriology Room. Replace with fresh autoclave bags in bin.*
- **Prepare the "wet" discard bucket:** *Open the stainless steel tin and check fill. If 2/3rd full, spray/wipe down with Accel TB before removal from BSC. Place a piece of autoclave tape over the top and place in autoclave. Place a fresh clean tin in BSC.*
- **Restock Supply Tray:** *Check loops, pipettes, etc. and re-stock if necessary. Re-fill sterile water and NaOH tubes if required. Refill phenol alcohol vial. Check fill levels of Accel TB bottle and Accel TB wipes.*
- **Ensure work surface is clean**. *Wipe down cabinet with Accel TB before daily work begins.*
- *When proper airflow in BSC is established, begin processing following the steps outlined below:*

MYCOBACTERIA SAMPLE PROCESSING:

Follow the steps below to process samples for Mycobacteria culture & direct smear.

Step	Action
	Gather samples and material. *Patient samples:* Obtain a metal tray (large enough to fit all patient samples in run). Line with several Accel TB wipes or several 4x4 gauze pads soaked in Accel TB or several paper towels soaked in Accel TB. Place samples in tray on the wet surface. Place tray in BSC. 
1	*Prep PANTA:* Reconstitute PANTA if required (follow MIC80900 AFB PANTA for instructions). *Label media and NaOH tubes:* Label and organize media (set aside on workbench in a plastic culture rack) and conical centrifuge tubes for NaOH aliquots (set aside on workbench in a metal rack).  

2	**Aliquot 3% NaOH in labelled centrifuge tubes.** On the work bench, dispense pre-measured 2.5 mL 3% NaOH into each labeled conical FALCON centrifuge tubes. Take all tubes into the BSC in a metal rack and place by patient samples.
3	**Prepare work surface in BSC.** Lay out several Accel TB wipes or several 4x4 gauze pads soaked in Accel TB or several paper towels soaked in Accel TB. This will be the work-surface for all Mycobacteria sample processing. *Keep this work surface moist at all times with Accel TB to catch and disinfect any splatter or drip when processing.* Place a clean 50 mL beaker in the centre of the work surface. This will hold the specimen NaOH conical tube steady while handling the patient specimen. You are now ready to begin sample digestion.  *Figure: (above) BSC lay-out with samples; ready to begin sample digestion.*
4	**Add sample to 3% NaOH aliquot.** Work with only one sample at a time (only have ONE patient specimen open at a time to prevent cross-contamination). Remove sample container from metal tray. Place on work surface. Match-up patient name and accession # on the NaOH tube and place the tube in the 50 mL beaker to hold it steady.

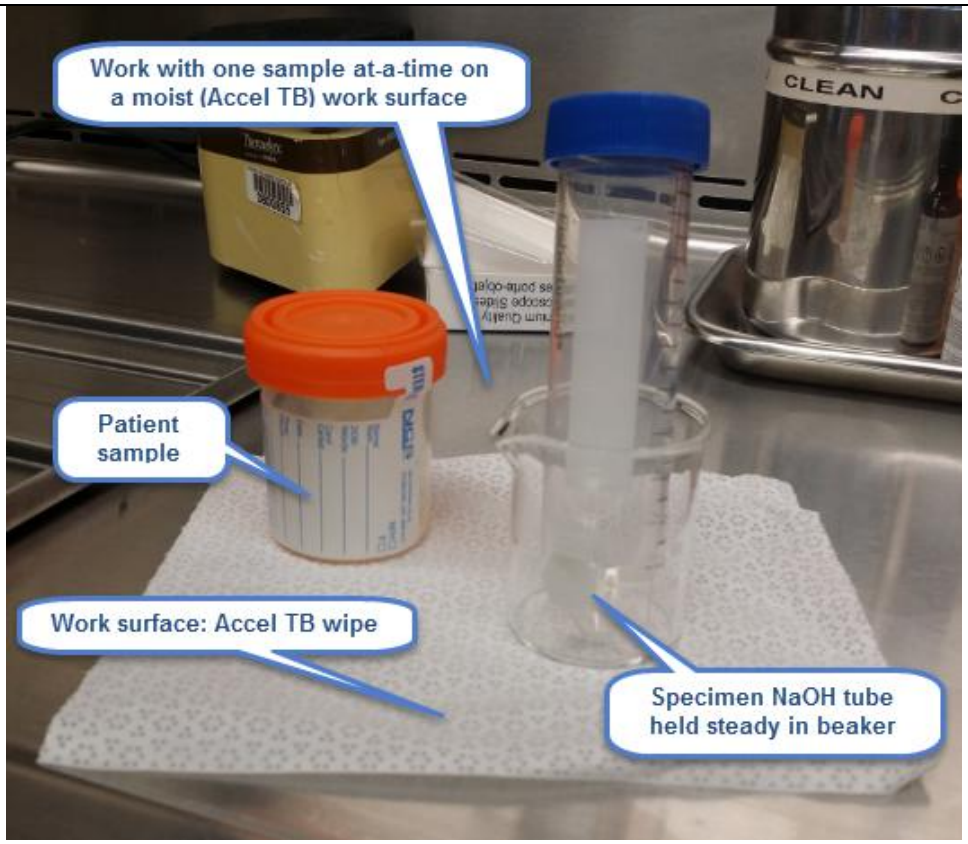


Figure (above): Work surface with patient sample

When opening, place tops of containers upside down (the inside surface facing upwards) on the wet work surface. Add sample to the NaOH conical tube using the larger 5mL Pasteur pipettes. Fluids should have already been concentrated and poured off to the proper volume. Sputum samples or low volume fluids may require adjustment.

If sample is < 2.5 mL, add sterile water to sample to equal 2.5mL sample volume. If sample >2.5 mL, select the most mucoid, bloody or purulent material (sputums) **OR** use the entire sample given and add equal volume of 3% NaOH.

Note 1: if >2.5mL of NaOH is added to sample, make a note on the cap of the conical tube such as a star or V (for extra volume) so you can remember to add an extra amount of phosphate buffer to the sample at a later step.
Note 2: If fluid or sputum sample is very thick, add a 1-2 mL of sterile water to help lower the viscosity. Sterile water should be added first to "thin out" the sample before resorting to additional 3% NaOH.

5	**Vortex (minimum 10 seconds).** Invert tube so the digestant makes contact with the entire inner surface of the tube. Vortex for a minimum 10 seconds. Return NaOH specimen tube to metal rack. *Note: Frequent and thorough mixing is an important step in digestion. To completely digest mucous without adding additional NaOH to the sample, thorough and repeat mixing is required during the digestion stage.*
6	**Discard waste into appropriate buckets and bins.** Pipette – discard into the yellow waste bucket. Suck-up a bit of Accel TB before discarding so the disinfectant makes contact with the inside of the pipette. Patient sample - discard container in orange discard bin.
7	**Set timer for 5 mins.** This will help you stay within the 15 → 20 mins NaOH exposure limits.
8	**Repeat Steps 4 → 6 for all samples.** **Watch your maximum time.** Sample addition, Vortexing and waste discard should take a MAXIMUM of 5 minutes for ALL SAMPLES in the run. If the timing is prolonged, some samples will have been exposed to NaOH for longer than 20 minutes. After this point, exposure to sodium hydroxide becomes lethal to Mycobacteria. *This is why there is a limit of 12 samples per run, and fluid samples should be pre-processed (ie. spun down and pellet ready) before any samples make contact with 3% NaOH.* **Add any sterile fluid samples that require 2+ (10 mins) to full (15 mins) digestion during this step**, following the processing chart above in the Pleural Fluid section.
9	**Reset timer and allow for digestion with frequent mixing.** Set the timer for 7 minutes 30 seconds. Vortex tubes one-by-one for 10 seconds. Let tubes digest until the time is up. When the time is up vortex samples again one-by-one. Repeat time for another 7 ½ minutes. **Add any sterile fluid samples that require 1+ (5 mins) digestion during this step,** following the processing chart above in the Sterile Fluid section. Digestion is done for approx 15 minutes but do not exceed 20 minutes. This is why It is important to process a small number of AFB samples per run.

10	After 2nd digestion, briefly Vortex all samples individually for 5 seconds. The samples should no longer "bounce" around in the FALCON tube; all should easily be spun around the inside of the tubes.
11	**Neutralize the digestant with phosphate buffer.** Obtain a phosphate buffer aliquot. The aliquots are pre-made by the manufacturer and are stored at Room Temp beside the BSC. Working one-by-one: • Add the entire volume of the phosphate buffer tube (20 mL) into one patient sample tube. This neutralizes the NaOH and brings the sample volume up to a higher specific gravity for proper centrifugation. Discard phosphate buffer tube into Biohazard waste bucket. Pour the buffer carefully down the side of the falcon tube to avoid generating aerosols. • If a tube requires additional buffer, add another 20 mL aliquot. • Close cap on patient sample conical tube and invert sample tube so the buffer makes contact with the entire inside of the tube. • Vortex briefly for 5 seconds. Repeat with the remaining samples.
12	**Load sample tubes into centrifuge buckets.** Wipe or spray conical tubes with Accel TB. Load samples into centrifuge buckets. Close lids tightly. Wipe the buckets with Accel TB. Remove buckets from BSC and load into centrifuge.
13	**Centrifuge. Record QC.** Close centrifuge lid. Select Program 1 → press enter → press start. Samples will spin at 3000xg for 15 minutes. Check RPM and record on Allegra X-15R maintenance.
14	**Dispense PANTA.** While the tubes are spinning take prepared PANTA supplement from the reagent fridge, or make up fresh solution according to directions. Dispense 800 uL with the pipette into each MGIT tube.

15	**When cycle is complete bring centrifuge buckets back to BSC.** Leave conical tube unopened for 5 mins to let the aerosols settle after centrifuging before opening to decant off the supernatant.
16	**Prepare concentrate.** Working one by one: • Carefully decant the supernatant into the metal discard bucket. Leave about 1.5 mL liquid behind to re-suspend pellet. • Close cap and Vortex briefly for 5 seconds. Repeat for all patient samples.
17	**Processing is complete.** Patient samples are now ready for AFB culture and smear.

RELATED DOCUMENTS:

- MIC80900 - AFB Panta
- MIC80700 - Allegra X-15R
- MIC80710 - Allegra X-15R Maintenance Sheet
- MIC80600 - Mycobacteria CL-2 BSC
- MIC80300 – Prep of Fluid Samples for Mycobacteria Culture.
- MIC80410 – Job Aid Mycobacteria Processing

REFERENCES:

- Central Public Health Laboratory. (2003). *Mycobacteriology Bench Manual.* "Pretreatment Preparation of Specimens for Mycobacterial Culture". Ottawa.
- Job Shadow at Prov Lab Edmonton Alberta. Mycobacteria Processing. 2014. Edmonton.

REVISION HISTORY:

REVISION	DATE	Description of Change	REQUESTED BY
1.0	28-JAN-2015	Initial Release	L. Driedger
	03Feb2015	Review	S. Webber