**Destinatari: Personale del Settore Micobatteri (SM)**

**CONTENT**



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## 1 SCOPE

This instruction describes methods of specimen processing and other laboratory procedures for purposes of culturing *Mycobacterium tuberculosis* culture on solid or in liquid media in order to isolate the pathogen.

## 2 APPLICATION

This instruction is to be considered part of Advanced Mycobacterial Diagnosis (DMA) as the first step of samples treatment.

## 3 DEFINITIONS AND ABBREVIATIONS

BSC Biological Safety Cabinet
RCF Relative Centrifugal Force
LJ Löwenstein-Jensen
NALC *N*-acetyl *L*-cysteine
PACT Polymyxin B ; Amphotericin B ; Carbenicillin ; Trimethoprim
PANTA Polymyxin B ; Amphotericin B ; Nalidixic Acid ; Trimethoprim ; Azlocillin

## 4 RESPONSIBILITIES

The supervision and the correct application of the following instruction is the responsibility of the area coordinator. The execution of the test is responsibility of area technicians, master students and coordinator.

## 5 EQUIPMENT AND MATERIALS

### *5.1 General Equipment and Materials*

- Biosafety Cabinet Class II or I
- Slides
- Disposable loops
- Refrigerated centrifuge with safety shield, a minimum RCF of 3000*g*, operated at 8–10 ℃
- Centrifuge tubes 50-ml capacity, clear plastic or thick-walled glass, with screw-caps, resistant to RCF of >3000*g*
- Pasteur pipettes for 1.0 ml (with graduation), sterile, single-use, plastic (non-sterile pipettes must be sterilized on site before use)
- Pipetting aids
- Disinfectants
- Separate waste containers, autoclavable, for pipettes and disposals
- Buckets, stainless steel or polypropylene

- Vortex mixer
- Timer
- Home-prepared decontamination reagents and solutions (see Annex 1)

### *5.2 Commercially Available Reagents*

**-** BD BBL™ Löwenstein-Jensen PACT ***(Cat No. 220502)*** (Approximate formula per liter Medium): Potassium phosphate 1.55 g; Magnesium Sulfate 0.15 g; Sodium Citrate 0.37 g; LAsparagine 2.23 g; Potato Starch 18.60 g; Malachite Green 0.25 g; Glycerol 7.44 ml; Whole Egg 620.0 ml; I.U. Polymyxin B 200000; Amphotericin B 10.0 mg; Carbenicillin 100.0 mg; Trimethoprim 10.0 mg; Purified Water 373 ml

**-** BD BBL™Löwenstein-Jensen ***(Cat No. 220909)*** (Approximate formula per 600 ml Purified Water): Monopotassium Phoshpate 2.5 g; Magnesium Sulfate 0.24 g; Sodium Citrate 0.6 g; LAsparagine 3.6 g; Potato Flour 30.0 g; Malachite Green 0.4 g; Glycerol 12.0 ml; Whole Egg 1000.0 ml

**-** BBL™ MGIT™ tube ***(Cat. No. 245122)*** (Approximate formula per liter of Purified Water): Modified Middlebrook 7H9 broth base 5.9 g; Casein Peptone 1.25 g. Each tube contains 110 µl of fluorescent indicator and 7 ml of broth. The indicator contains Tris- 4,7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate in a silicon rubber base. The tubes are flushed with 10% $CO_2$ and capped with polypropylene caps.

**-** BACTEC MGIT™ 960 Supplement Kit ***(Cat No. 245124)*** contains:
• 15 ml Middlebrook OADC enrichment (Bovine Albumin 50.0 g; Dextrose 20.0 g; Catalase 0.03 g; Oleic Acid 0.1 g; Polyoxyethylene Stearate POES 1.1 g)

• BBL MGIT PANTA (Approximate formula per Vial Lyophilised):
Polymyxin B 6000 I.U.; Amphotericin B 600 µg; Nalidixic Acid 2400 µg; Trimethoprim 600 µg; Azlocillin 600 µg)

**-** BD BBL™ MycoPrep™ Kit ***(Cat No. 240862)*** contains:
• BBL Mycoprep reagent (Approximate formula per liter Purified Water):
NaOH 20.0 g; Trisodium Citrate 14.5 g; Each sealed glass ampul within the bottle contains 0.375 g NALC

• BBL Mycoprep Phosphate Buffer (Approximate formula per 500 ml Purified Water):
Disodium Phosphate 2.37 g; Monopotassium Phosphate 2.27 g; final pH 6.8

**-** 3.0% NaOH with TSCA (5 x 50ml base solution + 5 x 250mg NALC) **(Cat No.043-03)**
**-** NPC-67™ Neutralization Buffer (87 mM, 50 x 50ml) **(Cat No.039-40)**
**-** Pellet Resuspension Buffer (Cat No.045-10)

# 6 PROCEDURES

## 6.1 Principle of the procedure

Culture examination detects fewer bacilli than microscopy and increases the number of TB cases found by 20–50%, depending on local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and allow the detection of drug resistance.
Specimens for isolation of tubercle bacilli contain associated bacterial and/or fungal flora which have to be eliminated before the specimen is inoculated onto culture media. Several methods (and variations) for doing this are described in the literature and applied in practice. Contamination rates due to bacteria other than mycobacteria should fall within acceptable rates.

## 6.2 Digestion, Decontamination and Concentration

### *6.2.1 Sputum*
Proper sputum collection is extremely critical for best results, and early morning specimens are preferred. The specimen should be expectorated sputum and not saliva that often would not yield correct results. A specimen should be between 2-10 ml in volume. Sputa should not be processed in sets of more than 6– 8 because the methods described here are strictly time-dependent. Larger sets cannot be handled in time.

**NaOH-NALC procedure**
This is the standard recommended procedure to be used with BACTEC MGIT™ System, although it is recommended also for cultures on solid media.

**MycoPrep™ Specimen Digestion/Decontamination Kit**

This is the standard recommended procedure to be used with BACTEC MGIT™ System, although it is recommended also for cultures on solid media. In this procedure, the initial concentration of NaOH is 4%. This 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution (NaOH concentration in this solution is 2%). When an equal quantity of NaOH-NALC-citrate and sputum are mixed, the final concentration of NaOH in the specimen is 1%.

• Reconstitute the BBL MycoPrep™ as explained in the Annex 1

• If specimen is not collected in a 50 ml centrifuge tube, transfer it to a 50 ml centrifuge tube with a screw cap.

• Add NaOH-NALC-sodium citrate solution (Mycoprep) in a volume equal to the quantity of specimen. Tighten the cap.

• Vortex lightly or hand mix for about 15-30 seconds. Invert the tube so the whole tube is exposed to the NaOH-NALC solution.

• Wait 20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution.

Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.

• Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC solution directly to the specimen tube. Mix well.

• At the end of 15-20 minutes, add Phosphate Buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times). Addition of sterile water is not a suitable alternative for the phosphate buffer.

• Centrifuge the specimen at a speed of 3000 g or more for 20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.

• After centrifugation, put the centrifuge buckets under the safety hood and allow tubes to sit for 5 minutes to allow aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid.

• Add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.

**NAC-PAC™ *Red***

NAC-PAC™ *Red* is a NaOH and Sodium Citrate decontamination solution that includes an integrate pH indicator. Procedure:

• if specimen is not collected in a 50 ml centrifuge tube, transfer it to a 50 ml centrifuge tube with a screw cap.

• Using the NAC-PAC™ Red System, open bottle labeled “TB Base Solution”. With the plastic sleeve still attached, carefully break off the top of the ampule N-acetyl-L-cysteine (NALC) powder. Add the NALC powder to the TB Base Solution (some residual NALC powder may remain in the ampule, it is not necessary to liquefy this portion remaining in the ampule). This solution will be good for only 72 hours after mixing.

• To the sterile 50 ml centrifuge tube containing the specimen to be digested, add NALC/digestant solution in the following amounts:

- for specimens 1-5 ml add a volume equal to that of the specimen volume
- for specimens 6-7 ml add 5 ml of base solution
- for specimens 8-10 ml add equal volume of base digestant and split the specimen after step 6 equally into two centrifuge tubes, proceed with steps 7-9 and then combine the sediments from both tubes into one centrifuge tube and proceed with step 10

• Tighten the caps on the centrifuge tubes. Mix each specimen on a vortex until liquefied (30 seconds per specimen).

• Allow each specimen to stand for 15 minutes. Each specimen should stand for 15 minutes but not longer than 20 minutes before the buffer is added. Vortex every 5 minutes during this step.

• Fill each tube with NPC-67TM Neutralizing Buffer until effective neutralization is indicated by a color change from red/pink to colorless. Once a colorless point has been reached, do not continue to add buffer to the sample.

• Centrifuge the specimen tubes at 3000 g for 15 minutes. It is recommended but not required to use a refrigerate centrifuge.

• Working in a biosafety hood, poor off all supernatant into a slash-proof container holding an appropriate disinfectant. Do not allow the disinfectant to run down inside the specimen tube.

• resuspend the pellet with 1-2 ml of Pellet Resuspension Buffer.

**Sodium Hydroxide (modified Petroff) Method**

NaOH is a digestant and a decontaminant. Timing of the digestion-decontamination step is crucial for effective recovery of mycobacteria.

• Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of 4% NaOH and tighten the screw-cap.

• Vortex to digest.

• Allow to stand for 15 minutes at room temperature.

• Fill the tube to within 2 cm of the top (e.g. to the 50-ml mark on the tube) with phosphate buffer or sterile saline.

• Centrifuge the specimen at a speed of 3000 g or more for 20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.

• After centrifugation, put the centrifuge buckets under the safety hood and allow tubes to sit for 5 minutes to allow aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid.

• Resuspend the deposit in approximately 1 ml phosphate buffer or sterile saline

### *6.2.2 Specimen other than sputum*

**Bronchial washing**

All other pulmonary specimens, such as bronchial washings (BAL) may be treated as sputum. If the specimen is up to 10 ml in volume, process the whole specimen. For larger

volumes, concentrate the specimen by centrifugation (3000x g, 15-20 minutes). After centrifugation, resuspend the sediment in 5 ml sterile water and decontaminate like sputum.

**Gastric aspirates**

Concentrate by centrifugation before decontaminating. Resuspend the sediment in about 5 ml of sterile water and decontaminate with NaOH-NALC or 2-4% NaOH as recommended for sputum. After decontamination, concentrate again prior to inoculation of the sediment into culture media. Due to the low pH, gastric aspirates should be processed as soon as possible (within 4 hours of collection). If the specimen cannot be processed quickly, it should be neutralized with NaOH before transportation or storage.

**Urine**

Routine isolation method, a totally voided, early morning urine specimen is used for mycobacterial culture. Pooled or mid-stream urine specimens are not recommended. The specimen is concentrated by centrifugation using several 50 ml centrifuge tubes (with screw caps) for at least 20-25 minutes. Resuspend the sediment in each tube with 1-2 ml sterile water and then pool together (total volume 5-10 ml). Decontaminate the concentrated specimens with 4% NaOH for 15- 20 minutes. After decontamination, proceed in a manner similar to sputum.

**Tissue**

Tissue biopsies are generally collected aseptically and therefore decontamination procedures are not required. Homogenize the tissue in a tissue grinder with a small quantity of sterile saline or water (2-4 ml). All steps must be done in a biological safety cabinet (BSC) and all equipment must be sterile. Decontaminate the homogenized specimen following the same NaOH-NALC procedure as in sputum. After resuspension of the sediment with phosphate buffer, inoculate 0.5 ml. If the tissue grinder is not available, use a mortar and pestle. Tissue may also be placed in a Petri dish with sterile water (2-4 ml) and be torn apart with the help of two sterile needles.

**Other body fluids**

Body fluids, such as Cerebral Spinal Fluid, synovial fluid and pleural fluid are collected aseptically and thus can be inoculated into the medium without decontamination (with the addition of PANTA or on Lowenstein-Jensen plus PACT). However, since sterility is not guaranteed, it is recommended these specimens should be lightly decontaminated. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000-3500 g for 15-20 minutes. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC. After centrifugation, resuspend the sediment in about 5 ml of saline and then decontaminate following the procedure similar to that for sputum.

***Important points about specimen processing procedures***

• Process clinical specimens as soon as possible.

• Properly label the media to be inoculated to avoid any mix-up of the specimens.

• Minimize aerosol production by opening specimen containers slowly, letting the tubes stand for a few minutes after shaking and before opening, and avoiding expulsion of the last drop from the pipette.

• Process only one specimen at each time. Do not allow open containers or open centrifuge tubes in the BSC. Use aliquots of buffer and decontamination solutions. Use a fresh pipette at every step to avoid transfer of bacilli from one specimen to the other.

• Aseptic technique is important to avoid contamination by bacteria other than tubercle bacilli and especially cross-contamination by tubercle bacilli from other specimens.

• Remember that most techniques require exposure time to disinfectant to be strictly controlled.

• If liquid media are used, it is recommended that solid media are also inoculated to provide back-up cultures in case of contamination of liquid media or in case of malfunction problem if an automated system is used.

### 6.3 Inoculation

Despite the method used for decontaminating specimen, the result of all these procedures is the sediment resuspended in 1-2 ml phosphate buffer or sterile saline. NALC-NaOH method is advisable for all test media (BBL™ MGITTM tube 7-ml and BBL™ Löwenstein Jensen with or without PACT). Sediments from Sodium Hydroxide method (Petroff's) should be inoculated on solid media only.

### Inoculation of BBL™ MGIT™ tube 7ml

• Label MGIT tubes with specimen number.

• Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipettor.

• Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well mixed processed/concentrated specimen to the appropriately labelled MGIT tube. Use separate pipette or pipette tip for each specimen.

• Immediately recap the tube tightly and mix by inverting the tube several times.

• Leave inoculated tubes at room temperature for 30 minutes.

### Inoculation of BBL™ Löwenstein-Jensen tubes

Using a calibrated pipette, dispense 0.1-0.2 ml of the sediment BBLTMLöwenstein-Jensen tube. The rest of the sediment is used for a smear and the remainder is stored at -20 ℃

### *6.4 Incubation*

#### Incubation of BBL™ Löwenstein-Jensen tubes

Incubate tubes at 37 ±1 ℃. The tubes should be inc ubated in a slanted position, with screw-caps loose, for at least 1 week to ensure even distribution and absorption of the inoculum. After 1 week of incubation, caps are tightened to minimize evaporation and drying of the media. Tubes may then stand upright to save space in incubators.
Check colony formation every week, preferably twice within the first week, to allow rapid detection of contamination and a timely request for another specimen if necessary. Contaminated cultures and rapidly growing mycobacteria (colonies apparent in less than 7 days) are removed. *M. tuberculosis* colonies should be well developed within 3–4 weeks. Cultures should be kept for up to 8 weeks before being reported as negative.

#### Incubation of BBL™ MGIT™ tube 7ml

All inoculated MGIT (7mL) tubes should be entered in the BACTEC MGIT 960 instrument after scanning each tube (please refer to IOS EBP-DMA 009). It is important to keep the cap tightly closed and not to shake the tube during the incubation. This helps in maintaining the oxygen gradient in the medium. The instrument maintains 37ºC + ±1 ℃ temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37ºC, make sure the temperature is close to 37ºC. If a specimen is suspected of containing mycobacteria which require an optimum temperature other than 37℃ (for example, *M. haemophilum, M. marinum, M. chelonae* and *M. ulcerans* require 30℃), then two sets of media should be inoculated, one in the instrument at 37ºC and the other in an outside incubator at 30ºC. These tubes can be monitored by using a UV light source (Wood's lamp) and can also be checked visually. Specimens from skin and open wounds should always be inoculated into duplicate MGIT tubes, one for 37ºC and the other for 30ºC. MGIT tubes should be incubated until the instrument flags them positive. After a maximum of six (6) weeks, the instrument flags the tubes negative if there is no growth. Some species such as *M. ulcerans* and *M. genavense* may require extended incubation time. If such species are expected to be present, incubate further for 2-3 weeks.

## 7 RECORDING AND REPORTING

All cultures must be reported into the specific database and on the Workflow summary if any as follows: POSITIVE (after identification of mycobacteria); NEGATIVE (if no growth); CONTAMINATED (if the culture is contaminated with bacteria other than mycobacteria)

## 8 RELATED DOCUMENTS

Barrera L, B. López, N. Simboli , M. D. Sequeira, O. Latini , M. Aziz, A Laszlo. *Quality control of the culturing of mycobacteria*. Reviewed, adapted and translated from the Spanish original by A. Laszlo.

Health Protection Agency. *Investigation of specimens for Mycobacterium species.* London, Standards Unit, Evaluations and Standards Laboratory, 2006 (National Standard Method BSOP 40 Issue 5, www.hpa-standardmethods.org.uk/pdf_sops.asp).

Kudoh S, Kudoh T. A simple technique for culturing tubercle bacilli. *Bulletin of the World Health Organization*, 1974, 51:71–82.

Kent PT, Kubica GP: Public Health Mycobacteriology: a guide for the level III laboratory. Atlanta, Ga, U.S. Department of Health and Human Services, Centers for Disease Control,, 1985

*Laboratory services in tuberculosis control. Part III: Culture*. Geneva, World Health Organization, 1998 (WHO/TB/98.258).

## 9 ANNEXES

### Annex 1

### Home-made Decontamination Reagents

| | | |
|---|---|---|
| NaOH<br>Distilled Water | 4 g<br>100 ml | NaOH solution 4% |
| NaCl<br>Distilled Water | 0.85 g<br>100 ml | Sterile Saline |

These two working solutions are used with both the Petroff's method. They should be then autoclaved at 121 ℃ for 15 minutes.

| | | |
|---|---|---|
| Potassium Phosphate Monobasic<br>Sodium Phosphate Dibasic | 45.4 g /L<br>47.4 g /L | Phosphate Buffer 10x |

Autoclave the solutions at 121 ℃ for 15 minutes. T he pH should be adjusted at 6.8
The working solution, used both in the Petroff's method and with NALC-NaOH decontamination procedure, is 1x. Thus, it should be diluted 1:10 in Sterile Water. Working solutions should be fresh: Phosphate Buffer 1x could be used up to 24 hours whereas the Phosphate Buffer 10x could be stored at 2-8℃ for u p to 1 month.

**Reconstitution of BBL™ MycoPrep™ working solutions**
In the BBLTM MycoPrep™ reagent bottle, the NALC is combined with 2% NaOH. When the reagent is diluted with an equal volume of specimen, it provides effective digestion and decontamination with a final concentration of 1% NaOH, which is less toxic to mycobacteria.
Sodium citrate is included in the reagent to bind heavy metal ions that may be present in the specimen and which can inactivate NALC.

Because NALC loses mucolytic activity on standing, the BBLTM MycoPrep™ reagent contains the NALC component in a sealed glass ampoule within the NaOH-citrate solution. The ampoule is broken and the reagent gently mixed before use.
Packets of pre-weighed, powdered phosphate buffer, pH6.8, are included in the BBL™ MycoPrep™ kit for use in washing the digested-decontaminated specimen.

• Prepare the BBLTM MycoPrep™ Phosphate Buffer as needed by pouring contents of one packet into a 500 ml volumetric flask and fill to line with purified water. Transfer the buffer solution to a screw-capped container and, with cap loosened, autoclave at 121℃ for 15 min. Cool to room temperature and tighten cap.

• Using caution not to spill, loosen screw-cap on the MycoPrep Reagent bottle. Locate ampoule in bottle, squeeze excess air from the bottle and tighten cap. With bottle in the upright position, squeeze the bottle until the ampoule breaks. Shake gently to dissolve the NALC. Avoid excessive agitation. Once ampoule is broken, use reagent within 24 h.