ANNEXURES
&
APPENDIX

Annexure -1

Ziehl-neelsen staining procedure

1. A new unscratched slide is selected and the slide is labelled with the Laboratory Serial Number with a diamond marking pencil.
2. A smear is made from yellow purulent portion of the sputum using a broom stick. A good smear is spread evenly, 2 cms x 3 cms in size and is neither too thick nor too thin. The optimum thickness of the smear can be assessed by placing the smear on a printed matter. The print should be readable through the smear. Smear preparation should be done near a flame. This is required, as six inches around the flame is considered as a sterile zone which coagulates the aerosol raised during smear preparation.
3. The slide is allowed to air dry for 15–30 minutes.
4. The slide is fixed by passing it over a flame 3–5 times for 3–4 seconds each time.
5. 1% filtered carbol fuchsin is poured to cover the entire slide.
6. The slide is gently heated with carbol fuchsin on it, until vapours rise. Do not boil.
7. Carbol fuchsin is left on the slide for 5 minutes.
8. The slide is gently rinsed with tap water until all free carbol fuchsin stain is washed away. At this point, the smear on the slide looks red in colour.
9. 25% sulphuric acid is poured onto the slide and allowed to stand for 2–4 minutes.
10. The slide is gently rinsed with tap water and tilted to drain off the water.
11. A properly decolourised slide appears light pink in color .If the slide is still red, sulphuric acid is reapplied for 1–3 minutes and then rinsed gently with tap water.The back of the slide is wiped clean with a swab dipped in sulphuric acid,
12. 0.1% methylene blue is poured onto the slide and left for 30 seconds. Then theslide is rinsed gently with tap water and allowed to dry.
13. The slide is examined under the binocular microscope using x40 lens to select the suitable area and then examined under x100 lens using a drop of immersion oil.
14. The results are recorded in the Laboratory Form and the Laboratory Register.
15. The slides are inverted on a tissue paper till the immersion oil is completelyabsorbed. Xylene is not to be used for cleaning the slides, as it may give falseresults at repeat examination after storage.
16. All positive and negative slides are stored serially in the same slide-box untilinstructed by the supervisor.
17. All contaminated materials are disinfected as per guidelines before discarding.

Grading of smears

The table below depicts information on grading and the number of fields to be examined in different situations:-

Examination finding	No. of fields examined	Grading	Result
No AFB in 100 oil immersion fields	100	0	Neg
1-9 AFB per 100 oil immersion fields	100	Scanty*	Pos
10-99 AFB per 100 oil immersion fields	100	1+	Pos
1-10 AFB per oil immersion field	50	2+	Pos
More than 10 AFB per oil immersionfield	20	3+	Pos

*Record actual number of bacilli seen in 100 fields – e.g. “Scanty 4”

Annexure -2

Fluorescence staining procedure

Smear Preparation-

- Mark a new, clean, grease free slide with laboratory number
- Pick the purulent portion of the sputum using the crushed end of the broom stick
- Prepare smear in an oval shape in the centre of the slide(3x2cm), for good spreading of sputum firmly press the stick perpendicular to the slide and move in small concentric circles
- Thorough spreading of sputum is very important; it should be neither too thick nor too thin. Prior to staining, hold the smear about 4-5 cm over a piece of printed paper, if letters cannot be read, it is too thick.
- Allow smear to air dry at room temperature
- Heat fix by passing the slide over flame 2-3 times for about 2-3 seconds each time. (Do not heat or keep the slide stationary over the flame or for too long or else it will be scorched)

Staining

Arrange slides in serial order on staining bridge, with smear side up, at a distance of at leastone cm between every slide

1. Flood the slide with filtered 0.1% Auramine solution
2. Do not heat
3. Keep the staining reagent for at least 20 min; make sure that the smear area iscontinuously covered with Auramine by adding more if needed
4. Rinse with water and drain
5. Apply decolourising solution, 0.5% acid alcohol for 3 minutes
6. Gently rinse with water until the macroscopically visible stain has been washed awayand drained
7. Flood smear with 0.5% potassium permanganate solution for 1 minute. Time iscritical because counter staining for longer time may quench the acid fast bacillifluorescence.
8. Gently rinse with water and drain
9. Air dry on a slide rack away from sunlight. If they are not read immediately placethem in slide box.

Reading

- Keep stained smears in the dark (box or folder), and read on the same day of staining as the fluorescence is prone to fading with time.
- To be able to focus with ease, better to read first a positive control smear stained by auramine O
- Use the objective 20x for focusing and read the slide using 40X objective (avoid using oil and immersion 100X objective, inexperienced readers should ask confirmation from a supervisor)
- Scan the stained smear systematically from one side to the other, for one length of the smear
- Acid-fast bacilli appear bright yellow against the dark background material.
- Store the slides in a slide box following the Laboratory Register Number as they will be needed for EQA. Do not write the result on the slide.

Grading of smears

The table below depicts information on grading and the number of fields to be examined in different situations:-

200-250x magnification: 1 length = 30 fields = 300 HPF	**400x magnification: 1 length = 40 fields = 400 HPF**	**Grading**	**Result**
No AFB per 1 length	No AFB per 1 length	0	Neg
1-29 AFB per 1 length	1-19 AFB per 1 length	Scanty*	Pos
30-299 AFB per 1 length	20-199 AFB per 1 length	1+	Pos
10-100 AFB per 1 field on average	5-50 AFB per 1 field on average	2+	Pos
More than 100 AFB per 1 field on average	More than 50 AFB per 1 field on average	3+	Pos

Annexure 3

Specimen collection and Transport of samples to C & DST laboratory (including CBNAAT laboratory)

Specimen Collection

An often-overlooked problem is that of obtaining adequate good quality specimens at theperipheral laboratories. Unless specimens are collected with care and promptly transported tothe laboratory under temperature control, diagnosis may be missed, and the patient couldmiss the chance to be detected and put on the correct treatment. A good sputum specimenmay literally make the difference between life and death, and allow containment of thedisease and prevent spread to others in the family and community.

The Laboratory technician needs to explain the process of collecting “a good quality sputumspecimen” and avoid using vernacular terminologies that convey the meaning as salivainstead of sputum. In addition though the general guideline for collection of sputa is one spotand one morning, this does not preclude from collecting 2 spot specimens that need to becollected with a gap of at least one hour (60 minutes) if the patient is coming from a longdistance or there is a likelihood that the patient may default to give a second specimen.

A good sputum specimen consists of recently discharged material from the bronchial tree,with minimum amounts of oral or nasopharyngeal material. Satisfactory quality implies thepresence of mucoid or mucopurulent material. Ideally, a sputum specimen should have avolume of 3-5ml. The patient must be advised to collect the specimen in a sterile container(falcon tube) after through rinsing of the oral cavity with clean water.

Specimens should be transported to the laboratory as soon as possible after collection. Ifdelay is unavoidable, the specimens should be refrigerated up to 1 week to inhibit the growthof unwanted micro-organisms.

Specimen transportation to culture-DST laboratories

Fresh sputum samples will need to be transported from the DMC to the RNTCP-certified CDSTlaboratory in cold chain within 72 hours. Ideally an agency (courier /

speed post) with apan district presence should be identified for this purpose. Two innovative models forspecimen collection and transport using fresh samples in falcon tubes to be transported incold chain using gel packs and their technical specifications have been developed by Gujarat(from peripheral DMCs) and Andhra Pradesh (from high burden DMCs at TUs/DTCs).

All states and districts should establish sample transport system in cold chain irrespective ofthe time taken for transport considering the hot climatic conditions in most of the statesduring most of the year. An appropriate courier / speed post service with pan district presenceshould be identified and contracted by the DTO of every district for prompt transport of thespecimen cold box on the same day from the DMC linked to the courier / speed post office inthe locality to the assigned RNTCP-certified C-DST laboratory.

The following points are critical for the collection of fresh sputum samples at DMCs:

- The falcon tubes and the 3 layer packing materials like thermocol box, ice gel pack (pre-freezed at -20 degree for 48 hours), request for C-DST forms, polythene bags, tissue paper roll as absorbent, para-film tapes, brown tape for packaging box, permanent marker pen,labels, bio-hazard sticker, scissors, spirit swab etc. should be supplied to the DMCs forcollection of sputum through the DTO.
- The falcon tubes should carry a label indicating the date of collection of the samples andthe patient’s details like name, date of sample collection, name of DMC/DTC, Lab. No:-XYZ, specimen A or B
- The Lab technicians at DMCs should be trained to carefully pack the sputum samples in thecold box to avoid spillage of the samples.
- The LT of DMC issuing the falcon tubes to the patients should also give clear instructionsto the patients on correct technique of collection of the sputum. Also the date of issue of thefalcon tubes to the patient should be recorded.
- The LT of the DMC should ensure that the request for C-DST form is packed in a separateplastic zip pouch and placed in the cold box before sealing the lid of the box. Also, the biohazardsymbol should be pasted on the external side of the cold box along with the labelindicating the postal address of the RNTCP-certified C-DST Lab assigned.

- The LT of the DMC should promptly inform the sample transport agency like a courier /speed post service, speed post or a human carrier to collect and transport the samples

As per the national guidelines for biomedical waste management the containers used fortransporting sputum samples to the RNTCP-certified laboratory should be labelled with a “BIO-HAZARD” sticker.

For every presumptive DR TB referred by the MO-DMC, the date of referral and transport ofsputa samples to the Culture & DST laboratory should be entered in the “Remarks” columnof the respective DMC Lab register and the TB notification register**.** Alternatively the presumptive DR-TB patients referred to nearby DMCselected for sample collection and transport for C-DST may be provided two falcon tubes bythe concerned DMC LT/MO and instructed on collecting two samples (one early morningand one supervised spot). These samples will be taken by the patient / relative to the DMCselected for sample collection for C-DST from where these will be packed in cold boxes andtransported to the RNTCP-certified laboratory for culture and DST. Once the sputum hasbeen transported to the RNTCP-certified laboratory, the p should return tocontinue their RNTCP DOTS treatment.

Standard Operative Procedure for collection, transport and processing and inoculation of Extra-pulmonary specimens

1. *Introduction:*

Mycobacteria may not be suspected as the causative agent of an extra pulmonary disease because the chest X-ray or the tuberculin test is negative or both. However, based on clinical symptoms and because mycobacteria can infect almost any organ in the body, the laboratory should expect to receive a variety of extra pulmonary specimens such as body fluids, surgically excised tissues, aspirates or draining pus and urine.

Extra pulmonary specimens are divided in to two groups based on the site and mode of collection and the extent of contamination.

- Aseptically collected specimens, usually free from other microorganisms (sterile) **–** fluids like spinal, pleural, pericardial, synovial, ascitic, blood, bone marrow, tissues (lymph node, tissue biopsies) and fine needle aspirates (FNAs)
- Specimens contaminated by normal flora or specimens not collected aseptically (not sterile) **–** gastric lavage, bronchial washings, urine , pus and stool (in case of disseminated TB in HIV infected patients and infants)

2. *Collection of extra pulmonary specimens*

Body fluids (spinal, pleural, pericardial, synovial, ascitic, bone-marrow) should be aseptically collected in a sterile container by the physician using aspiration techniques or surgical procedures. Specimens should be transported to the laboratory as quickly as possible.

2.1 Pleural fluid

Considered a suboptimal specimen as tubercle bacilli are mainly in the pleural wall and not within the fluid. The minimum volume for pleural fluid required for processing for culture is 20**–**50ml. The fluid is collected using pleural tap or thoracocentesis.

2.2 Pericardial fluid

Should be collected using ultra sonogram

2.3 Blood

Blood as a specimen for isolating *M. tuberculosis* should be generally discouraged for the low diagnostic yield and high possibility of contamination with respect to the technique required for its culture. However, if there are specific indications when a physician suspects disseminated TB in a HIV infected patient, blood can be collected provided, the culture systems for recovery of

mycobacteria is available in that laboratory (BacTAlert, MB Bact or MycolyticF medium on BACTEC 9050 systems)

2.4 Tissues

The aseptically collected tissues are placed by the physician in sterile containers preferably without fixatives or preservatives. If the specimen is to be shipped, it should be protected from drying by adding sterile saline or ideally in selective Kirchner's liquid medium and maintaining a temperature of 4- 15^{0}C. Specimens should be transported to the laboratory as quickly as possible.

2.5 Swabs

Swabs are always sub optimal specimens and not recommended because of risk of infection for specimen collector. They may be useful in children and patients who cannot produce sputum or may swallow it. A sterile absorbent cotton swab should be used for collection. The best time for the collection is early morning before food and drinks are taken. The swab should be placed in a screw capped container containing normal (0.9%) saline to prevent drying. Swabs except for laryngeal swabs or from discharging sinus should be avoided.

2.6 Urine

Among specimens expected to be contaminated, urine is the most common. To minimize excessive contamination of urine specimens, special instructions for collecting urine with adequate cleansing of external genitalia to prevent contamination by commensals should be given. Early morning sample should be collected in 500 ml screw capped sterile containers. Once received in the laboratory, urine must be immediately processed or centrifuged and the pellet refrigerated for further processing. As excretion of tubercle bacilli in urine is intermittent, three early morning specimens must be collected on different days.

2.7 Bronchial secretions

Other respiratory specimens that can be submitted to the laboratory for mycobacteria culture are bronchial secretions (minimum volume: 2- 5ml) and bronchial alveolar lavage (BAL) (minimum volume of 20 – 50 ml). Trans-bronchial and other biopsies should be collected under sterile conditions and placed in 0.5-1.0 ml of sterile normal (0.9%) saline to prevent drying during transportation to the laboratory.

2.8 Gastric Lavage

In children, who rarely produce sputum, the aspiration of the early morning (gastric content) may be used for TB diagnosis. This is done as an inpatient procedure. This should be transported immediately to the lab and processed (nor more than 4 hours) to prevent the killing action of the acid content in the gastric lavage on the tubercle bacilli. In the event of delay, the sample can be neutralised using 1-2 ml of sterile 10 % sodium bicarbonate solution depending on the volume of gastric aspirate. Trisodium phosphate at a final concentration of

25% can be used but it may affect the viability of tubercle bacilli with prolonged storage.

NOTE:

- Samples for culture should **never be** collected in formalin.
- If histo pathological examination is required, two samples should be collected
- No preservative should be used for any extra-pulmonary specimen for culture. Necessary instructions are to be given to the concerned staff for sending the biopsy specimen in normal saline for culture and NOT IN FORMALIN as it will kill the bacilli.
- Extra pulmonary specimens should never be collected or transported in CPC.

3. TRANSPORTATION OF EXTRA PULMONARY SPECIMENS

As for pulmonary samples, extra pulmonary specimens will need to be transported in cool boxes which maintain temperatures below 20^0C for specimens to be compatible for solid, liquid culture systems as well as molecular methods. Triple packing system should be utilised for transportation. All precautions that are followed for transporting pulmonary samples should be followed. For sending material across international or state boundaries this container may have to be packed in the same way with an additional outer container; in such cases, special administrative arrangements with postal authorities and/or airlines may be necessary.
When sending out specimens or when receiving them, check that:

- Request forms are located separately from the specimen containers
- Containers are labelled not on the cap but on the wall of the container
- Each transport box has an accompanying list which identifies the specimens and the patients; the information on the specimen containers should correspond to that on the accompanying list.
- Accompanying list contains the necessary data for each patient
- Date of dispatch and particulars of the health centre are on the accompanying list.

3.1 Specimens and request forms

All specimen transported to the laboratory must be accompanied by the request form for C & DST in hard and soft copy formats (See C & DST request form). For quality control reasons, the tests must be performed only upon written request of authorized persons and oral requests without follow up written instructions should not be allowed. It is also important that specimen request forms are kept separate from the specimens themselves. Forms that have been contaminated by specimens should be sterilized by autoclaving. If mistakes in filling request forms and labelling of specimens are found, reject specimens and do not register them. Document the arrival of specimens in the laboratory and note any delays in

delivery in the remarks column of the specimen register and on the report form, particularly for negative/contaminated results. The packaging material should be autoclaved before discarding.

4. REGISTRATION OF SAMPLES

4.1 Receipt of incoming specimens

For safety and work-flow reasons, specimens should be received in the office area of the laboratory and delivery boxes should be opened using all the applicable biosafety procedures inside the lab.

To minimize risk of infection, the following procedures should be applied:

1. The specimen package received should be opened only in a biosafety cabinet which may be located in a small area within the reception or in the culture room, as they could potentially be MDR or XDR Tuberculosis. (DO NOT OPEN ON AN OPEN BENCH AT THE LAB RECEPTION)
2. Before opening the packet, inspect the delivery box for signs of leakage; if there is gross leakage evident, discard the box by autoclaving or burning; do not try to open and retrieve any specimen.
3. If on gross inspection there is no leakage, disinfect the outside of the delivery box using cotton wool or paper towels saturated with a suitable disinfectant (5% phenol)
4. Open carefully and check for cracked or broken specimen containers or leakage within the packaged container. If there is minimal leakage without any gross loss of specimen, they may be processed with an asterix that leakage was noted on receipt. (This will assist in identifying reasons for contamination used in lab performance indicators). In case of gross leakage, with only very little sample being available, accept the sample and process after carefully making a note of the same – as extrapulmonary specimens are precious and repeat collection may not be possible.
5. Check labelling of specimens with individual identification numbers and correspondence with numbers on the accompanying list or Clinical information forms (CIF) that are accompanying the specimens.
6. Disinfect the inside of the delivery box, wash hands after handling specimen containers
7. Autoclave the packaging material before discarding.
8. Assign unique lab serial number to each patient.
9. Evaluate the quality of specimens and make a note as to volume (in case of fluids), leakage, blood mixed etc. Always register the incoming specimen in the laboratory register; each specimen receives a serial number that should be used to label every test for the specimen. Other data that should be reported on the laboratory register are: the date of the receipt of the specimen, patients name, age, sex and address, the name of the referring health centre, the reason for DST. The signature (with the name in capitals) of the person requesting the examination should always be present.

4.2 Decontamination of extra pulmonary samples

Most of the extra pulmonary specimens are paucibacillary in nature. Hence, they require milder decontamination. When using solid culture for primary isolation of tubercle bacilli from these specimens, it is preferable to use multiple media including one liquid medium made selective by the use of specific antibiotics that inhibit the growth of other micro organisms. The media include, LJ, LJ with sodium pyruvate (LJ–P) and selective liquid Kirchner's medium (SK). Sodium pyruvate facilitates the growth of *M. bovis*. Antibiotics incorporated in the liquid medium include polymixinB, amphotericin B, carbenicillin and trimethoprim (PACT) and vancomycin.

Preparation of media

LJ MEDIUM WITH SODIUM PYRUVATE

LJ medium is enriched with 0.5% sodium pyruvate. In the preparation of the mineral salt solution, glycerol is omitted and 8.0g sodium pyruvate is added for every 600 ml. This is added to 1 litre of egg fluid, mixed well and distributed.

SELECTIVE KIRCHNER'S MEDIUM (For culture of extra-pulmonary specimens**)**

Disodium hydrogen phosphate, $Na_2HPO_4.12H_2O$, A.R.	19.0 g (7.5g of anhydrous salt)
Potassium dihydrogen phosphate, KH_2PO_4, A.R.	2.0 g
Magnesium sulphate, $MgSO_4.7H_2O$, A.R.	0.6 g
Sodium citrate	2.5 g
L-asparagine	5.0 g
Casein hydrolysate (Bactocasitone)	0.5 g
Glycerol	20.0ml
Phenol Red, 0.4% solution	3.0 ml
Distilled water, to	1 litre

Check pH to 6.9 – 7.2

Autoclave at 15 lbs/15 minutes

Then add aseptically the following:

Polymyxin B (20,000 units)	31 mg
Carbenicillin	100 mg
Trimethoprim	10 mg
Amphotericin B, solubilised	10 mg

Dissolve the above in 5 ml sterile distilled water before addition

Also, add sterile calf serum 100 ml

Mix the above carefully and distribute, under sterile conditions, in 10 ml amounts. Check sterility by overnight incubation at 37^{o}C and store in the cold.

5. CULTURE BY SOLID CULTURE METHODS

5.1 CSF and pericardial fluid

Smear:

1. Label a clean dry slide with the lab number and place the slide and the sample container inside the cabinet
2. Mix well and aseptically remove one loopful of the fluid and place in the centre of the slide; close the container and allow the drop to air-dry
3. Place one more drop of the CSF on the same spot and let dry.
4. Place the third drop after processing the sample as below:

Culture:

Culture of CSF is done in two steps:

1. Direct inoculation in media 2. Inoculation after decontamination

Direct

1. Place one loopful of CSF on to one slope each of LJ and LJ-P
2. Add 0.2 ml of CSF in to one bottle containing SK medium
3. Label the set as 'A'

Decontamination

1. Add 1ml of 5% H_2SO_4 to CSF
2. Mix well and let stand for 15 minutes
3. Fill the container with sterile distilled water and centrifuge at 3000 x g for 15 minutes
4. Aspirate the supernatant carefully without disturbing the deposit or discard carefully in to a disinfectant bin containing 5% phenol or any other mycobactericidal solution
5. Inoculate one slope each of LJ and LJ-P with one loopful of deposit for each slope
6. Transfer the remaining deposit in to one bottle of SK
7. Label the set as 'B'
8. Incubate both set A and B at 37^{o}C

5.2 BAL

1. Make a direct smear
2. Process using 5% H_2SO_4 as in CSF
3. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit using 5mm twisted wireloop
4. Transfer the remaining deposit in to one bottle of SK
5. Incubate the slopes and SK medium at 37^{o}C

5.3 Gastric Lavage

1. Gastric Lavage should be processed immediately upon arrival in the lab to prevent the killing action of the gastric pH (due to HCl) on the tubercle bacilli
2. Make a direct smear and process by modified Petroff's method
3. Place one drop of the final pellet on the direct smear
4. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
5. Transfer the remaining deposit in to one bottle of SK
6. Incubate the slopes and SK medium at 37^{o}C

5.4 Tissue / Biopsy

1. Ideally, biopsy specimens should be collected and transported in SK medium
2. Carefully place the tissue inside a sterile petriplate inside the BSC
3. Using sterile scissors and forceps, cut the tissue in to tiny pieces
4. Transfer to a sterile tissue grinding tube – add a little water to the petriplate to facilitate transferring
5. Add sterile distilled water to the tube (not more than 5 ml)
6. Homogenise using a sterile Teflon grinding rod using a foot operated tissue grinder
7. Make a direct smear from the homogenate
8. Centrifuge the homogenate at 3000 x g for 15 minutes
9. Decant the supernatant carefully in to the disinfectant bath
10. To the deposit add 1 ml of sterile distilled water
11. Add one drop to the direct smear, air dry, fix and stain
12. To the remaining pellet, add 1ml of 5% H_2SO_4
13. Proceed as for CSF
14. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
15. Transfer the remaining deposit in to one bottle of SK
16. Incubate the slopes and SK medium at 37^{o}C, along with the SK medium used for transporting

5.5 Fine Needle Biopsy specimen

1. Fine needle specimens should be collected and transported only in SK medium or any other liquid medium
2. The medium is incubated as such at 37^{o}C, since only a very tiny piece of the tissue is obtained as sample

If the sample is received without SK

1. Add the contents of two SK medium bottles to the specimen
2. Shake vigorously and let stand for 10 minutes
3. Divide the medium in to two aliquots and incubate both at 37^{o}C

5.6 Pus

1. Make a direct smear, air dry, fix and stain

2. If the pus is thick or purulent, process by modified Petroff's method using 4% NaOH
3. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
4. Transfer the remaining deposit in to one bottle of SK
5. Incubate the slopes and SK medium at 37°C
6. If the pus is thin or dilute, proceed with decontamination using 5% H_2SO_4
7. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
8. Transfer the remaining deposit in to one bottle of SK

5.7 Urine / Ascitic fluid

1. Distribute the entire specimen in to 20 or 40 ml volumes in Universal containers / Falcon tubes inside a BSC
2. Centrifuge at 3000 x g for 15 minutes

Process the supernatant and deposit independently as follows:

Supernatant:

3. Aspirate carefully 1ml of the top layer from each tube and pool
4. Process by 5% H_2SO_4 as for CSF
5. Transfer 1ml of the final supernatant on to two bottles of SK each – Label the set as DSS (Decontaminated Supernatant Supernatant)
6. Decant the supernatant carefully in to the disinfectant bath
7. From the deposit transfer about 0.2 ml and the remaining in to 2 bottles of SK respectively – Label as DSD (Decontaminated Supernatant Deposit)

Deposit:

8. Pool all the deposit in to one tube
9. Process using 5% H_2SO_4 as for CSF
10. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
11. Transfer the remaining deposit in to one bottle of SK

5.8 Swabs:

If two swabs are available, use one for smear and one for culture; if only one is available do only culture

1. Immerse the swab in 5 ml of 4% H_2SO_4 for 1 minute
2. Transfer the swab to another tube containing 5 ml of 4% NaOH
3. Directly inoculate two slopes each of LJ, LJ-P
4. Transfer the swab finally to a tube containing SK medium
5. Incubate all tubes at 37°C

5.9 Culture Reading

1. Read all cultures used for isolating *M. tuberculosis* from extrapulmonary specimens every week for up to 8 weeks using the same methodology used for pulmonary samples
2. If the solid media show typical growth report immediately after confirmation

3. Read SK medium up to 6 weeks
4. MTB appears as whitish granular or flaky growth that settles down at the bottom
5. If the SK medium shows growth or contamination (in the form of turbidity) within 6 weeks, decontaminate as sputum by modified Petroff's method and inoculate deposit on LJ medium alone and read up to 8 weeks
6. Even if the SK medium shows no growth within 6 weeks, proceed with decontamination using modified Petroff's method and inoculate deposit on LJ medium alone and read up to 8 weeks
7. If LJ shows typical MTB growth within 8 weeks, report immediately after confirmation
8. Report as negative only after LJ completes 8 weeks (a total of 14 weeks)

6. Processing of extra pulmonary samples for MGIT960

Isolation of M. tuberculosis by MGIT system requires the final inoculum to be in an ideal condition that will not interfere with the fluorescence.

6.1 Pus and other muco-purulent specimens

1. Thick pus of volume >10 ml is decontaminated using the NALC – NaOH method as sputum
2. If the volume is < 10 ml, either aliquot and process only 10 ml by NALC – NaOH method or concentrate the initial volume by centrifugation for 15 – 20 minutes and resuspend the pellet in 5 ml of sterile distilled water. If the pus is too thick, add about 50-100 mg of NALC powder; mix well and decontaminate using NaOH. Resuspend the final pellet in buffer to reduce the pH
3. If the pus is not thick, decontaminate using 2-4% NaOH. The concentration of NaOH can be changed based on the expected level of contamination in the specimen which depends on the site of collection

6.2 Gastric aspirates

1. Distribute the volume in smaller aliquots and centrifuge the tubes at 3000 x g
2. Pool the deposits, add 5ml distilled water and decontaminate it using NALC-NaOH or 2-4% NaOH

6.3 Bronchial washings

1. Process using NALC-NaOH like sputum
2. If the specimen is >10 ml in volume, process the whole specimen.
3. If <10ml, concentrate the specimen by centrifugation (3000x g, 15-20 minutes)
4. Add 5 ml sterile water to the pellet and decontaminate as for sputum

6.4 Laryngeal swabs

1. Transfer the swab into a sterile centrifuge tube and add 2 ml sterile water.
2. Add 2 ml of NaOH-NALC solution and mix well in a vortex mixer.

3. Let stand for 15 minutes. Remove the swab with forceps, squeezing the liquid out of the swab and discarding it.
4. Fill the tube with phosphate buffer and mix
5. Centrifuge at 3000xg for 15-20 minutes.
6. Discard the supernatant fluid and resuspend the sediment in 1-2 ml sterile buffer. Use this suspension for smear and culture.

6.5 Tissue

1. Homogenize the tissue in a tissue grinder with a small quantity of sterile saline or water (2-4 ml).
2. Decontaminate the homogenized specimen using NALC-NaOH procedure as in sputum.
3. Resuspend the sediment with phosphate buffer
4. If the tissue grinder is not available, use a mortar and pestle.
5. 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.

6.6 Urine

Isolation of mycobacteria from urine specimens using MGIT has not been validated.

1. Aliquot the entire volume in several centrifuge tubes
2. Concentrate the specimen by centrifugation for at least 20-25 minutes
3. Resuspend the pellet in each tube with 1-2 ml of sterile water and pool together
4. Decontaminate using 4% NaOH as for sputum

6.7 Other body fluids (CSF, synovial fluid and pleural fluid)

As these fluids are collected usually under aseptic conditions, they require only milder decontamination

1. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000x g for 15-20 minutes
2. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC powder (50-100 mg).
3. Resuspend the sediment in about 5 ml of saline
4. Mix and decontaminate as for sputum

6.8 Blood

Isolation of mycobacteria from blood specimens by MGIT 960 has not been evaluated thoroughly. A few published studies have used blood after lysis centrifugation. Ideally BACTEC Myco/F Lytic medium is recommended for isolation of mycobacteria from blood samples.

Institution **Laboratory name** Location Head/Responsible person	Standard Operating Procedure (SOP) Specimen processing of CSF, lymph nodes and other tissues for Xpert MTB/RIF	Code: Version: no. Date: of release Page: **1** of **7**

Content

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Laboratory area:		No of copies:	Reason for change:		

1. Scope

This SOP describes methods of specimen processing CSF, lymph nodes and tissues for testing in the Xpert MTB/RIF assay and for purposes of culturing *Mycobacterium tuberculosis* culture on solid and / or liquid media.

2. Definitions and abbreviations

BSC : biological safety cabinet
CSF: cerebrospinal fluid
ID: patient's specimen identification, usually laboratory number
LJ: Löwenstein–Jensen
NTP: national tuberculosis programme
PBS: Phosphate buffer 0.067 mol/ litre, pH 6.8
RCF: relative centrifugal force

3. Procedure

3.1 Principle

WHO has issued policy recommendations for the use of Xpert MTB/RIF in the diagnosis of extrapulmonary TB and rifampicin resistance detection

- Xpert MTB/RIF should be used in preference to conventional microscopy and culture as the initial diagnostic test in testing **cerebrospinal fluid specimens** from patients presumed to have TB meningitis (strong recommendation given the urgency of rapid diagnosis, very low quality of evidence);

- Xpert MTB/RIF may be used as a replacement test for usual practice (including conventional microscopy, culture, and/or histopathology) for testing of specific non-respiratory specimens (**lymph nodes and other tissues**) from patients presumed to have extrapulmonary TB (conditional recommendation, very low quality of evidence).

For CSF specimens, Xpert MTB/RIF should be preferentially used over culture if the sample volume is low or additional specimens cannot be obtained, in order to reach quick diagnosis. If sufficient volume of material is available, concentration methods should be used to increase yield;

Individuals presumed to have extrapulmonary TB but with a single Xpert MTB/RIF - negative resultshould undergo further diagnostic testing and hence processing of tissue samples (lymph nodes and other tissues) for Xpert MTB/RIF should include a decontamination step to enable samples to be concurrently cultures

Pleural fluid is a suboptimal sample for the bacterial confirmation of pleural TB, using any method. A pleural biopsy is the preferred sample.

These recommendations do not apply to stool, urine or blood, given the lack of data on the utility of Xpert MTB/RIF on these specimens.

3.2 General considerations

Important points about specimen processing procedures

- Process all specimens as soon as possible, for an optimal culture recovery of MTB. Longer transport should not affect Xpert positivity
- Ensure that the Xpert MTB/RIF cartridge and any culture media to be inoculated are labelled correctly and clearly.
- **Tissues must be processed within a BSC** given the risk of aerosol production while grinding and homogenizing samples.
- **CSF samples are paucibacillary** and can be processed using the same precautions as for sputum EXCEPT when concentrated by centrifugation
- It is important to use Safe Working Practices to avoid contamination by bacteria other than tubercle bacilli and especially cross-contamination by tubercle bacilli from other specimens.
- When sufficient sample is available, culture should be performed concurrently
- Samples requiring decontamination must have the exposure time to decontamination reagents strictly controlled.
- **Decontaminate samples** for culture using either 4% NaOH or NaOH-NALC **depending on usual practice** in the laboratory. The example below uses 4% NaOH.

3.3 Specimen processing

The Xpert MTB/RIF assay can be used directly for CSF specimens and homogenised extrapulmonary samples (lymph node biopsies and other tissues) or on decontaminated specimens if culture is performed concurrently.

Whenever possible, specimens should be transported and stored at 2 to 8°C prior to processing (a maximum of 7 days).

3.3.1 Lymph nodes and other tissues (for Xpert MTB/RIF only)

1. Cut the tissue sample into small pieces in a sterile mortar (or homogenizer / tissue grinder) using a clean, sterile pair of forceps and scissors
2. Add approximately 2ml of sterile phosphate buffer (PBS)
3. Grind tissue/PBS-solution with a mortar and pestle (or homogenizer / tissue grinder) until a homogeneous suspension is obtained
4. Transfer approximately 0.7 ml of homogenized tissue sample to a sterile conical, screw-capped tube using a transfer pipette

NOTE: Avoid transferring any clumps of tissue which have not been properly homogenized.

5. Add a double volume of Xpert MTB/RIF Sample Reagent (1.4 ml) to 0.7 ml of homogenized tissue using a transfer pipette
6. Vigorously shake 10 to 20 times or vortex for at least 10 seconds
7. Incubate for 10 minutes at room temperature, and again shake the specimen vigorously 10 to 20 times or vortex for at least 10 seconds
8. Incubate the sample at room temperature for an additional 5 minutes
9. Using a fresh transfer pipette, transfer 2ml of the processed sample to the Xpert MTB/RIF cartridge
10. Load the cartridge into the GeneXpert instrument as per manufacturer's instructions

3.3.2 Lymph nodes and other tissues (Non-sterile collections – Xpert MTB/RIF and culture)

1. Cut the tissue sample into small pieces in a sterile mortar (or homogenizer / tissue grinder) using a clean, sterile pair of forceps and scissors
2. Add approximately 2ml of sterile phosphate buffer (PBS)
3. Grind tissue/PBS-solution with a mortar and pestle (or homogenizer / tissue grinder) until a homogeneous suspension is obtained
4. Use a sterile transfer pipette to add the suspension into a 50ml conical tube
5. Add an equal volume of 4% NaOH and tighten the screw-cap
6. Vortex thoroughly to homogenise the suspension
7. Stand for 15 minutes at room temperature.
8. Fill the tube to within 2 cm of the top (e.g. to the 50-ml mark on the tube) with PBS
9. Centrifuge at 3000*g* for 15 minutes
10. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant
11. Re-suspend the deposit in approximately 1-2 ml PBS
12. Use another sterile transfer pipette to inoculate deposit into liquid media and/or onto two slopes of egg-based medium labelled with the sample ID number.
13. Label a Xpert/MTB/RIF cartridge with the sample ID

14. Using a transfer pipette, transfer approximately 0.7 ml of homogenized tissue sample to a conical, screw-capped tube for the Xpert MTB/RIF.

NOTE: Avoid transferring any clumps of tissue which have not been properly homogenized.

15. Using another transfer pipette, add a double volume of Xpert MTB/RIF Sample Reagent (1.4 ml) to 0.7 ml of homogenized tissue.
16. Vigorously shake 10 to 20 times or vortex for at least 10 seconds
17. Incubate for 10 minutes at room temperature, and again shake the specimen vigorously 10 to 20 times or vortex for at least 10 seconds
18. Incubate the sample at room temperature for an additional 5 minutes
19. Using a fresh transfer pipette, transfer 2ml of the processed sample to the Xpert MTB/RIF cartridge
20. Load the cartridge into the GeneXpert instrument as per manufacturer's instructions

3.3.3 Lymph nodes and other tissues (Sterile collection – Xpert MTB/RIF and culture)

1. Cut the tissue sample into small pieces in a sterile mortar (or homogenizer / tissue grinder) using a clean, sterile pair of forceps and scissors.
2. Add approximately 2ml of sterile phosphate buffer (PBS)
3. Grind tissue/PBS-solution with a mortar and pestle (or homogenizer / tissue grinder) until a homogeneous suspension is obtained and adjust to a final volume of approximately 2ml with PBS
4. Transfer the suspension with a sterile transfer pipette to a 50ml conical tube
5. Use a another transfer pipette to inoculate suspension into liquid media and/or onto two slopes of egg-based medium labelled with the sample ID number
6. Label an Xpert/MTB/RIF cartridge with the sample ID
7. Transfer approximately 0.7 ml of homogenized tissue sample to a conical, screw-capped tube for the Xpert MTB/RIF using a transfer pipette

NOTE: Avoid transferring any clumps of tissue which have not been properly homogenized.

8. Transfer a double volume of Xpert MTB/RIF Sample Reagent (1.4 ml) to 0.7 ml of homogenized tissue using a transfer pipette

9. Vigorously shake 10 to 20 times or vortex for at least 10 seconds
10. Incubate for 10 minutes at room temperature, and again shake the specimen vigorously 10 to 20 times or vortex for at least 10 seconds
11. Incubate the sample at room temperature for an additional 5 minutes.
12. Using a fresh transfer pipette, transfer 2ml ml of the processed sample to the Xpert MTB/RIF cartridge
13. Load the cartridge into the GeneXpert instrument as per manufacturer's instructions

3.3.4 CSF

The preferred processing method for CSF in Xpert MTB/RIF depends on the volume of sample available for testing.

NOTE. Blood stained and xanthochromic CSF samples may cause false negative Xpert MTB/RIF results

More than 5 ml of CSF

1. Transfer all of the sample to a conical centrifuge tube and concentrate sample at 3000*g* for 15 minutes
2. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant
   NOTE: Decanting concentrated CSF should be performed within a BSC
3. Re-suspend the deposit to a final volume of 2ml with Xpert MTB/RIF sample reagent.
4. Label an Xpert/MTB/RIF cartridge with the sample ID
5. Using a fresh transfer pipette, transfer 2ml ml of the concentrated CSF sample to the Xpert MTB/RIF cartridge
6. Load the cartridge into the GeneXpert instrument as per manufacturer's instructions

1-5 ml of CSF (including blood-stained or xanthochromic samples)

1. Add an equal volume of the CSF to the sample reagent
2. Add 2ml of the sample mixture directly to the Xpert MTB/RIF cartridge
3. Load the cartridge into the GeneXpert instrument as per manufacturer's instructions

0.1-1ml of CSF

1. Re-suspend the CSF to a final volume of 2 ml with Xpert MTB/RIF sample reagent.
2. Add 2ml of the sample mixture directly to the Xpert MTB/RIF cartridge
3. Load the cartridge into the GeneXpert instrument as per manufacturer's instructions

Less than 0.1ml

1. Insufficient sample for testing in the Xpert MTB/RIF assay

4. Related documents

1. Xpert MTB/RIF system for the diagnosis of pulmonary and extrapulmonaryTB and rifampicin resistance in adults and children. A pre-publication version of the policy guidance may be accessed at: http://www.stoptb.org/wg/gli/assets/documents/WHO Policy Statement on Xpert MTB-RIF 2013 pre publication 22102013.pdf

2. The full Expert Group meeting report is available at: http://www.stoptb.org/wg/gli/assets/documents/Xpert%20Meeting%20Report%2024102013%20%20Pre%20publication%20FINAL.pdf