Standard Operating Procedure (SOP) for Processing Extrapulmonary Specimens

(CSF, Lymph Nodes, Other Tissues and Human Waste Product) for Xpert MTB/RIF Assay and Culture

Version 1.0

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INFECTIOUS DISEASE DETECTION AND SURVEILLANCE (IDDS)

Scope

This standard operating procedure (or SOP) describes methods for processing specimens of cerebrospinal fluid (CSF), lymph nodes and tissues, and waste product of human (stool) for testing using the Xpert MTB/RIF assay and for purposes of culturing Mycobacterium tuberculosis culture on solid media or liquid media.

Definitions and abbreviations

BSC	Biological Safety Cabinet
CSF	Cerebrospinal Fluid
ID	patient's specimen identification, usually laboratory number
LJ	Löwenstein–Jensen
MTB	*Mycobacterium Tuberculosis*
NALC	N-acetyl-L-cysteine
NaOH	sodium hydroxide
PBS	Phosphate buffer 0.067mol/ liter, pH 6.8
RCF	Relative Centrifugal Force
RIF	Rifampicin
SR	Sample reagent
WHO	World Health Organization

ACKNOWLEDGEMENTS

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SOP for Extrapulmonary Tuberculosis

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Contents

Introduction

It is estimated that extrapulmonary tuberculosis (EPTB) accounts for 15–25% of all cases of TB. In general, EPTB is more difficult to diagnose than pulmonary TB and often requires invasive procedures to obtain tissue and/or fluid specimens. Most common form of EP TB are lymph node TB, Bone and joint TB. Other forms of EPTB are tubercular pleural effusion, meningitis, abdominal tuberculosis (usually with ascites), and genitourinary tuberculosis.

Purpose

The purpose of the SOPs is to the train laboratory staff on processing EPTB and stool samples for detection of child TB using GeneXpert and culture methods.

1. Lymph nodes, CSF and other specimen (except stool)

Principle

WHO has issued recommendations about using of Xpert MTB/RIF to diagnose extrapulmonary TB and to detect rifampicin resistance:

- Xpert MTB/RIF should be used in preference to conventional microscopy and culture as the initial diagnostic test for CSF specimens from patients suspected of having TB meningitis (strong recommendation given the urgency of rapid diagnosis, very low- quality evidence).
- Xpert MTB/RIF may be used as a replacement test for usual practice (including conventional microscopy, culture, and histopathology) for testing specific non-respiratory specimens (lymph nodes and other tissues) from patients suspected of having extrapulmonary TB (conditional recommendation, very low- quality evidence).

In order to reach a quick diagnosis using CSF specimens, Xpert MTB/RIF should be preferentially used instead of culture if the specimen volume is low or if additional specimens cannot be obtained. If a sufficient volume of material is available, concentration methods should be used to increase yield.

Individuals being evaluated for EPTB who had tested negative by Xpert MTB/RIF should undergo further diagnostic testing; the processing of their tissue specimens (lymph nodes and other tissues) for Xpert MTB/RIF should include a decontamination step to enable specimens to be cultured concurrently.

General considerations

Important points about specimen processing procedures

- All specimens should be processed as soon as possible to obtain higher yield of *M. tuberculosis.*
- 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 certified biological safety cabinet, given the risk of producing aerosols while grinding and homogenizing samples.
- Body fluids can be processed using the same precautions as those used for sputum except when they are concentrated by centrifugation.
- It is important to use Safe Working Practices to avoid contamination by bacteria other than tubercle bacilli and specially to avoid cross-contamination with tubercle bacilli from other specimens.
- Collect sufficient volume of samples so that culture can be performed when necessary.
- Exposure time to decontamination reagents must be strictly controlled for samples requiring decontamination.
- Decontaminate samples for culture using either 4% NaOH or NaOH-NALC depending on usual practice. The example below uses 4% NaOH.

Major Equipment and materials

- Sterile pair of forceps and scissors \ surgical blade
- 4% NaOH or NaOH-NALC (for Culture only)
- MGIT 960 System (for culture only)
- Phosphate buffer
- Surgical Blade Handle
- Tissue grinder
- Refrigerated centrifuge
- Biosafety Cabinet (Class II-A)
- Disposable gloves
- Laboratory coat
- Protective eyewear
- Timer
- Permanent marker pen
- 0.5% sodium hypochlorite solution and 70% alcohol or other tuberculocidal disinfectant
- Xpert MTB/RIF (Ultra) kit, including:
- single-use, disposable, Xpert MTB/RIF (Ultra) cartridges
- sterile disposable transfer pipettes
- bottles with sample reagent (SR)
- Spare sterile transfer pipettes with 2 mL marking (in case of many liquid stool samples)
- GeneXpert instrument with appropriate infrastructure, equipped with a computer, ASPECT software and barcode reader.

Sample Collection and Referral

EPTB samples (lymph node tissues, CSF, Pleural fluid, BAL and others) will be collected by trained personnel and referred to EPTB testing sites as soon as possible(preferably on the same day, if not possible store at 2–8 °C and send the sample on the following day). The maximum time from collection to testing is 7 days. Preservatives should not be added to samples.

Specimen processing

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

1. Using sterile pair of forceps and scissors, cut the tissue specimen into small pieces in a sterile petri dish and transfer the small pieces into tissue grinder.
2. Add approximately 2 ml of sterile phosphate buffer (PBS).
3. Grind the solution of tissue and PBS using a tissue grinder until a homogeneous suspension has been obtained.
4. Use a transfer pipette to transfer approximately 0.7 ml of the homogenized tissue specimen to a sterile, conical screw-capped tube.

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

5. Use a transfer pipette to add a double volume of the Xpert MTB/RIF Sample Reagent (for ex.1.4 ml sample reagent is mixed to 0.7 ml of homogenized tissue.
6. Vigorously shake the tube 10 to 20 times or vortex for at least 10 seconds.
7. Incubate for 10 minutes at room temperature, and then shake the specimen vigorously again for another 10–20 times or vortex for at least 10 seconds.
8. Incubate the specimen at room temperature for an additional 5 minutes.
9. Using a fresh transfer pipette, transfer 2 ml of the processed sample to the Xpert MTB/RIF cartridge.
10. Load the cartridge into the Gene Xpert instrument following the manufacturer's instructions.

1.2. Lymph nodes and other tissues (non-sterile collection for Xpert MTB/RIF and culture)

1. Using a sterile forceps and surgical blade, cut the tissue sample into small pieces in a sterile petri dish.
2. Transfer the small pieces of tissue into a homogenizer or tissue grinder.
3. Add approximately 2 ml of sterile PBS.
4. Grind the solution of tissue and PBS with a tissue grinder (or homogenizer or mortar and pestle) until a homogeneous suspension has been obtained.
5. Transfer the suspension (approx. 2-5 ml) to a 50 ml falcon/conical tube.
6. Add an equal volume of 4% NaOH and tighten the screw-cap.
7. Vortex thoroughly to homogenize the suspension.
8. Let the tube stand for 15 minutes at room temperature.
9. Fill the tube with PBS upto 45ml.
10. Centrifuge at 3000rcf(g) for 15 minutes.
11. Carefully pour off the supernatant into a discard can containing mycobacterial disinfectant (chlorine tablet/ 1% Sodium Hypochlorite)
12. Add 1-2ml PBS into the pellet and vortex for 10-15 seconds.
13. Wait for 1-2 minutes to allow aerosol to settle down.
14. In case of culture
    a. For Liquid culture, add 800µl Growth Supplement and PANTA solution into the MGIT tube and then add 500µl of the decontaminated specimen.
    b. For solid culture, transfer 2-3 drops of the decontaminated specimen onto two slopes of egg-based L-J medium.
15. a. For GeneXpert, transfer approximately 0.7 ml of the decontaminated specimen to a screw-capped tube and add a double volume (1.4 ml) of the Xpert MTB/RIF Sample Reagent.
    b. Vigorously shake 10–20 times or vortex for at least 10 seconds.
    c. Incubate for 10 minutes at room temperature, and then shake the specimen again followed by

additional 5 minutes of incubation.
d. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.
e. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.
NOTE:
- Avoid transferring any clumps of tissue that have not been properly homogenized.
- Label the culture tubes/cartridge with appropriate ID.

1.3 Lymph nodes and other tissues (sterile collection for Xpert MTB/RIF and culture)

1. Using a sterile pair of forceps and surgical blade, cut the tissue sample into small pieces in a sterile petri dish.
2. Transfer the small pieces of tissue into a homogenizer or tissue grinder
3. Add approximately 2 ml of sterile PBS.
4. Grind the solution of tissue and PBS with a tissue grinder (or homogenizer or mortar and pestle) until a homogeneous suspension has been obtained.
5. Transfer the homogeneous suspension (1.5-2ml) into a fresh tube.

A. For Liquid culture, add 800µl Growth Supplement and PANTA solution into the MGIT tube and then add 500µl of the decontaminated specimen.

B. For solid culture, transfer 2-3 drops of the decontaminated specimen onto two slopes of egg-based L-J medium.

6. A. For GeneXpert, transfer approximately 0.7 ml of the decontaminated specimen to a screw-capped tube and add a double volume (1.4 ml) of the Xpert MTB/RIF Sample Reagent

b. Vigorously shake 10–20 times or vortex for at least 10 seconds.

c. Incubate for 10 minutes at room temperature, and then shake the specimen again followed by additional 5 minutes of incubation.

d. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.

e. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.

NOTE:

Avoid transferring any clumps of tissue that have not been properly homogenized.

- Label the culture tubes/cartridge with appropriate ID.

1.4 CSF (for Xpert MTB/RIF and culture)

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

NOTE: Blood-stained and xantho chromic CSF specimens may cause false-negative results from Xpert MTB/RIF.

If there is more than 5ml of CSF

1. Transfer all of the specimen to a conical centrifuge tube and concentrate the specimen at 3000 rcf(g) for 15 minutes.
2. Carefully pour off the supernatant into a discard can containing mycobacterial disinfectant (chlorine tablet/ 1% Sodium Hypochlorite)
3. A. In case of culture add 1-2ml PBS into the pellet and vortex for 10-15 seconds.
   B. For Liquid culture, add 800µl Growth Supplement and PANTA solution into the MGIT tube and then add 500µl of the decontaminated specimen.
   C. For solid culture, transfer 2-3 drops of the decontaminated specimen onto two slopes of

egg-based L-J medium.

D. Re-suspend the rest of the decontaminated specimen to a final volume of 2 ml by adding the Xpert MTB/RIF sample reagent.

E. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.

F. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.

4. A. In case of direct GeneXpert use the concentrated CSF directly (without adding any PBS)
   B. Re-suspend the concentrated CSF to a final volume of 2 ml by adding the Xpert MTB/RIF sample reagent.
   C. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.
   D. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.

If there is 1–5ml of CSF

1. For culture inoculate specimen directly at appropriate volume into MGIT/L-J tube.
2. For GeneXpert add an equal volume of sample reagent to the CSF.
3. Add 2 ml of the sample mixture directly to the Xpert MTB/RIF cartridge.
4. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.

If there is 0.1–1 ml of CSF

5. For culture add 1ml PBS and inoculate the specimen directly at appropriate volume into MGIT/L-J tube.
   A. For GeneXpert followed by culture re-suspend the rest of the specimen to a final volume of 2 ml by adding the Xpert MTB/RIF sample reagent. (For direct GeneXpert use the CSF without adding any PBS)
6. A. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.
   B. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions

If there is less than 0.1 ml

1. This is an insufficient sample for testing using the Xpert MTB/RIF assay.

1.5 Urine Sample processing for MTB/RIF and Culture

1. Collect first morning midstream 40ml urine sample into a 50ml conical falcon tube.
2. Centrifuge the specimen to concentrate the specimen at 3000 g for 15 minutes.
3. Carefully pour off the supernatant into a discard can containing mycobacterial disinfectant (chlorine tablet/ 1% Sodium Hypochlorite)
4. In case of culture and then GeneXpert
   A. Add an equal volume of 4% NaOH and tighten the screw-cap.
   B. Vortex thoroughly to homogenize the suspension.
   C. Let the tube stand for 15 minutes at room temperature.
   D. Fill the tube with PBS up to 45ml.
   E. Centrifuge at 3000 g for 15 minutes.
   F. Carefully pour off the supernatant into a discard can containing mycobacterial

disinfectant (chlorine tablet/ 1% Sodium Hypochlorite)

G. Add 1-2ml PBS into the pellet and vortex for 10-15 seconds.
H. Wait for 1-2 minutes to allow aerosol to settle down.
I. For Liquid culture, add 800µl Growth Supplement and PANTA solution into the MGIT tube and then add 500µl of the decontaminated specimen.
J. For solid culture, transfer 2-3 drops of the decontaminated specimen onto two slopes of egg-based L-J medium.
K. For GeneXpert Add double volume of Xpert MTB/RIF sample reagent into processed urine specimen
L. Vigorously shake 10–20 times or vortex for at least 10 seconds.
M. Incubate for 10 minutes at room temperature, and then shake the specimen again followed by additional 5 minutes of incubation.
N. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.
O. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.

5. In case of direct GeneXpert add double volume of Xpert MTB/RIF into urine pellet tube
   A. Vigorously shake 10–20 times or vortex for at least 10 seconds.
   B. Incubate for 10 minutes at room temperature, and then shake the specimen again followed by additional 5 minutes of incubation.
   C. Using a fresh transfer pipette, transfer 2ml of the processed specimen to the Xpert MTB/RIF cartridge.
   D. Load the cartridge into the GeneXpert instrument following the manufacturer's instructions.

Note:
Ensure the balance during centrifugation.

2.0 Stool sample processing for Child TB case detection

Purpose

This SOP describes how to collect stool and process stool samples by using simple one step (SOS) method for use in the Xpert MTB/RIF (Ultra) assay for the detection of MTBC and RIF resistance. It does not provide guidance on the handling of the GeneXpert instrument, performance of the Xpert testing, and the interpretation of Xpert results.

Principle

By using the SOS stool method, stool processing for use in the Xpert assay is as simple as sputum processing. Approximately 0.8 g of stool is added directly to the sample reagent (SR) that is provided with the Xpert MTB/RIF (Ultra) kit. The Xpert MTB/RIF assay system enables the rapid detection of MTBC and RIF resistance by combining automated sample purification, nucleic acid amplification and detection of the target DNA sequences in a self-contained cartridge that is run on a GeneXpert system.

Procedure

Equipment and materials

- Disposable, screw-capped stool containers with a spoon (or, alternatively, screw-capped universal sputum or urine containers/cups)
- Toilet paper or plastic sheet for stool collection (in case of onsite collection of the stool)
- Plastic bag with absorbent material
- Disposable gloves
- Laboratory coat
- Protective eyewear
- Wooden sticks
- Timer
- Permanent marker pen
- 0.5% sodium hypochlorite solution and 70% alcohol or other tuberculocidal disinfectant
- Xpert MTB/RIF (Ultra) kit, including:
  - single-use, disposable, Xpert MTB/RIF (Ultra) cartridges
  - sterile disposable transfer pipettes
  - bottles with sample reagent (SR)
- Spare sterile transfer pipettes with 2 mL marking (in case of many liquid stool samples)
- GeneXpert instrument with appropriate infrastructure, equipped with a computer, GX 4.7b software and barcode reader (Cepheid Inc. Sunnyvale, USA)

Stool specimen Collection

A simple method for stool collection is described, alternatively, dedicated stool collection kits may be used.

1. Supply the patient or caretaker with a stool container and a plastic bag with absorbent material.
2. Provide the patient or caretaker with the following instructions on how to collect the stool Sample:
   a. Ideally, collect the stool sample during the first daily bowel movement. Try to avoid mixing of the stool sample with urine by first emptying the bladder.
   b. Put some toilet paper or a clean plastic sheet on the spot where the stool will be dropped to ensure the collection of a clean sample. Avoid that the stool comes into contact with soil, detergent, or disinfectant from the toilet.

c. If stool needs to be collected from a child that uses a diaper, then collect the stool directly from the diaper, as soon as possible after defecation. Avoid prolonged contact with the surface of the diaper, as some diapers may contain unknown substances that may inhibit the test.
d. Fill maximum half of the stool container with stool by using e.g. the spoon provided with the container, a clean plastic bag, a clean piece of cardboard or a clean spoon. Do not fill the container to the brim because it is difficult for the laboratory personnel to handle full containers. Only a small amount of stool is required for testing.
e. Close the container tightly and seal the container in the plastic bag provided. Leave the absorbent material in the plastic bag, so that this material can absorb any substances that may leak out of the container.
f. Directly after the stool collection, store the stool container in a clean, cool place (e.g. in a fridge, if available), avoiding exposure to direct sunlight. Do not freeze the sample.
g. Bring the stool sample to the laboratory, preferably on the same day the stool is collected.

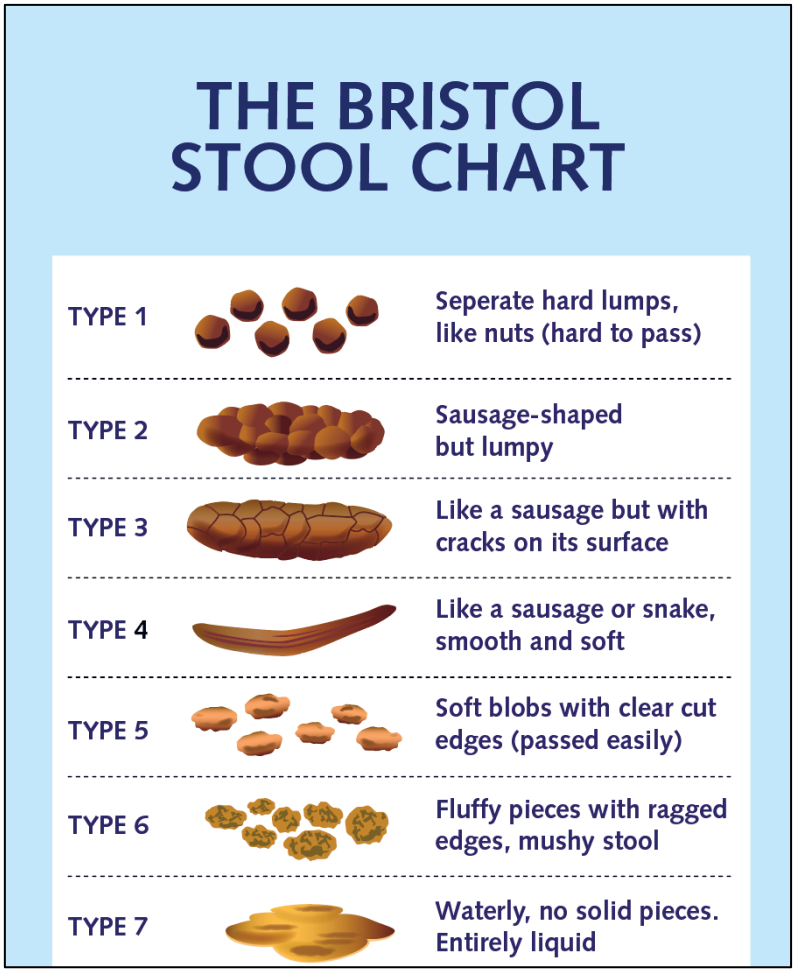
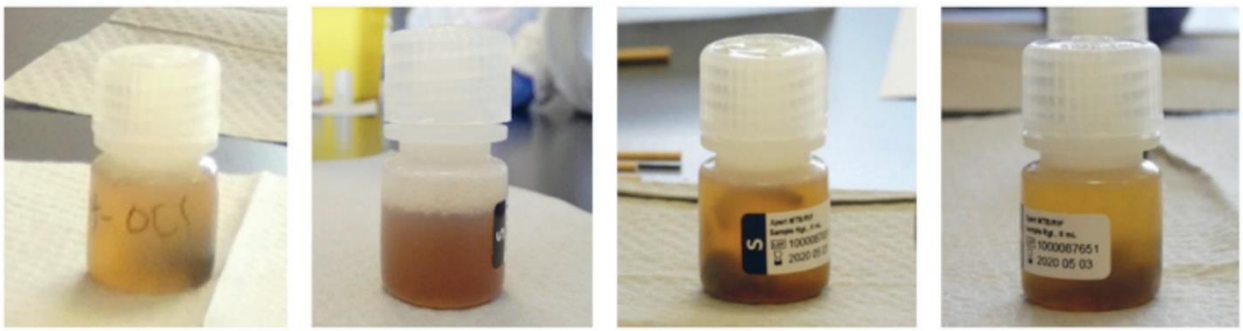


Figure 1: The Bristol Stool Scale, named after the University of Bristol

2.1 Sample preparation

1. Upon arrival at the laboratory, record the date and time of the stool collection and the date and time the stool arrived at the laboratory.
2. Store the stool sample containers in the refrigerator (2-8^{o}C) until testing can be performed. Ideally, sample preparation and testing should start as soon as possible, and stool samples should not be stored for longer than 5 days in the refrigerator or 48 hours at room temperature.

3. Take a bottle containing 8 mL of sample reagent (SR bottle) from the Xpert MTB/RIF (Ultra) kit and label it with the unique patient ID.
4. Before testing, determine the consistency type of the stool sample by using the Bristol Stool Scale (Figure: 1) and record it on the laboratory form. The stool type determines how to manipulate the stool
   a) Use the spoon connected to the lid of the stool container, or a wooden stick, to take a portion of the stool of approximately the size of a thumb nail (this corresponds to 0.8 g or a clump of approximately 1X1.5 cm) and transfer it to the SR bottle.
   b) Use a wooden stick to remove the stool from the spoon or stick if needed.
5. In case the stool is of type 1 or 2 (very hard), then, once transferred into the SR bottle, cautiously cut the stool into small pieces by using a wooden stick to ensure a better suspension in the SR buffer. Make sure that the stool sample does not emerge from the SR during the procedure!
6. If the stool is like fluffy pieces (Type 6 and & 7) with ragged edges (mushy stool) and watery (entirely liquid) stool, then use a transfer pipette to remove 2 mL of SR from the SR bottle and dispose it. Subsequently, use the same pipette to transfer 2 mL of the stool sample into the SR bottle.
7. Close the lid of the SR bottle tightly and shake the bottle vigorously for 30 seconds. Do not vortex as this may lead to the formation of a stable suspension of fine particles which may not sediment well.
8. Incubate the bottle for 10 minutes at room temperature.
9. Shake the bottle vigorously again for 30 seconds (do not vortex).
10. Slightly untighten the screw cap of the SR bottle and put bottle in such position that the supernatant can easily be aspirated.
11. Let the bottle stand for 10 minutes at room temperature to allow the solid particles and debris to settle.
12. If the stool debris has not fully sedimented, the incubation time can be prolonged with an additional 10 min.
13. If there are still solid parts visible in the supernatant (upper layer) after the prolonged incubation time, then repeat steps 7 and 8.



A B C D

Figure 2: Appearance after mixing with sample reagent

An example of stool mixed with sample reagent in a SR bottle; A) before shaking, B) after shaking, C) after incomplete sedimentation, i.e. with still some solid particles in the supernatant D) after sedimentation, with a clear supernatant

Loading of the specimen into the Xpert MTB/RIF (ultra) assay cartridge

Note: In principle, samples should be loaded into the Xpert MTB/RIF (ultra) cartridges immediately after sample processing and, unlike with sputum processing, it is not recommended to re-use the SR- stool suspension in the SR bottle for repeat testing, to avoid the risk of aspirating particles from the sediment layer. Usually, there will be enough stool sample left to process another portion of the same specimen.

Panel A

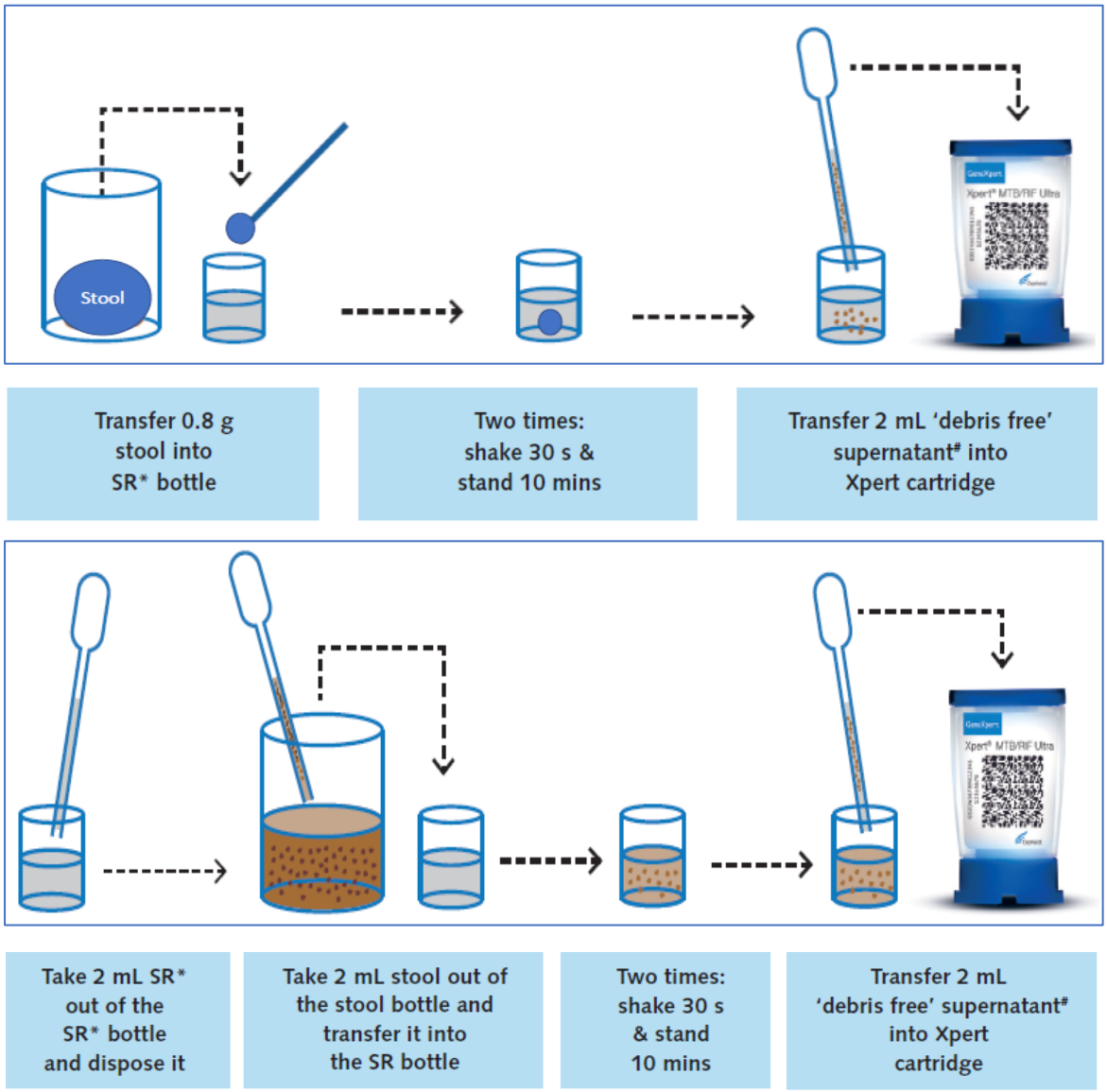


Figure 3: Schematic overview of simple one-step stool processing

Panel A shows the procedure for Bristol type 1 to 5 stool (solid stool) and panel B for Bristol type 6 and 7 stool (liquid stool).

* SR sample reagent (Cepheid), 8 mL mixture of sodium hydroxide (pH>12.5) with isopropanol provided with every Xpert cartridge.

After sedimentation by gravitation of the organic debris, carefully - without lifting the bottle and without disturbing the sedimentation - transfer 2 mL of the upper layer of the 'debris free' supernatant to the Xpert cartridge (KNCV SOS Stoolbox)

1. Label an Xpert MTB/RIF (ultra) cartridge with the unique patient ID.
2. Open the lid of the cartridge.
3. Open the SR bottle containing the SR-stool suspension. To avoid any debris from whirling up, do not move or lift the SR bottle, but carefully hold the bottle between your fingers while leaving it on the table.
4. By using a new pipette, carefully aspirate 2 mL from the supernatant in the SR bottle and transfer it to the Xpert MTB/RIF cartridge by slowly dispensing it into the open port of the cartridge.

Important: be careful not to take any debris. Avoid touching the debris at the bottom of the SR bottle and do not move or lift the bottle while aspirating the supernatant. Aspirate the supernatant from the upper layer of the supernatant and avoid air bubbles. You can do this as follows:

a) Press the transfer pipette hard enough to be able to aspirate the 2 mL in one go.
b) Place the pipette tip just under the surface of the solution against the wall of the bottle, and slowly move down with the surface while aspirating the sample into the transfer pipette.
c) If, accidentally, air bubbles are aspirated into the pipette or if the balloon was not pressed hard enough to take up 2 mL in one go, then slowly transfer the solution back into the SR bottle, by keeping the pipette tip against the wall and without lifting the bottle from the table. Let the SR bottle stand for 10 minutes to ensure sedimentation is re-established before trying again. If it is not possible to take 2 mL from the upper layer without including any debris, then repeat steps 7 and 8 of the previous section.

5. Close the lid of the cartridge and the lid of the SR bottle.
6. Place the cartridge in the GeneXpert instrument and follow the instructions of the manufacturer in the package insert for sputum processing or the existing instructions in the national SOP for Xpert MTB/RIF testing.
7. Recording and Reporting formats

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