



SOS STOOLBOX

Simple One Step (SOS) stool processing method and Xpert MTB/RIF (Ultra) testing for the detection of *Mycobacterium tuberculosis* complex and rifampicin resistance

STANDARD OPERATING
PROCEDURE (SOP)

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ABBREVIATIONS USED IN THIS SOP

cfu	colony forming units
DNA	deoxyribonucleic acid
HIV	human immunodeficiency virus
IS	insertion sequence
LOD	limit of detection
MDR	multidrug resistance (resistance to rifampicin and isoniazid)
MSDS	material safety data sheet
MTB(C)	<i>Mycobacterium tuberculosis</i> (complex)
PCC	probe check control
PCR	polymerase chain reaction
PLHIV	people living with HIV
RIF	rifampicin
SOS	Simple One-Step
SPC	sample processing control
SR	sample reagent (provided by Cepheid)
TB	tuberculosis
WHO	World Health Organization

Standard operating procedure (SOP) for detection of *Mycobacterium tuberculosis* complex and rifampicin resistance in stool by using the Simple One-Step (SOS) stool processing method and the Xpert MTB/RIF (Ultra) assay

1: Introduction

The diagnosis of pulmonary tuberculosis (TB) in children and people living with the human immunodeficiency virus (HIV) (PLHIV) is hampered because they frequently have nonspecific signs and symptoms and laboratory confirmation of their disease is hardly possible due to its paucibacillary nature and the difficulty to obtain sputum. In 2020, the World Health Organization (WHO) published updated guidelines that include the use of Xpert MTB/RIF on stool as an initial diagnostic test for detection of TB and rifampicin (RIF) resistance in children with signs and symptoms of pulmonary TB.^{1,2} Furthermore, in case of a negative result on the initial test, repeated testing with the Xpert MTB/RIF assay (for a total of two tests) may be done in settings with a high TB prevalence (pre-test probability of $\geq 5\%$). In addition, the use of the Xpert MTB/RIF Ultra assay under operational research conditions is encouraged by WHO.^{1,2} Two recent systematic review studies have demonstrated that both the Xpert MTB/RIF and the more sensitive Xpert MTB/RIF Ultra assay can be used to detect *Mycobacterium tuberculosis* complex (MTBC) bacilli in stool with high specificity.^{3,4} Thus, Xpert and Xpert Ultra testing on stool can be used as a rule-in test near the point of care. Given the high sensitivity and specificity of stool as compared to sputum Xpert testing, stool can probably also be used as an alternative to sputum for the diagnosis of TB in adults, including PLHIV, who cannot expectorate sputum.⁵

Various methods to process stool for Xpert testing have been described.⁶⁻¹² However, some of these methods are complex, labor-intensive, time-consuming, include centrifugation and need well-equipped infrastructure.^{6,7,9} The centrifuge-

free methods^{8,10-12} include two or more specimen processing steps and all these methods require additional supplies, reagents and specific biosafety measures because the specimen needs manipulation before the MTBC bacilli are inactivated. De Haas et al.¹³ developed a method that uses the same supplies and equipment as used for Xpert testing of sputum, and constitutes of only one release-and-sedimentation step: the Simple One-Step (SOS) stool method for the detection of MTBC and RIF resistance in stool. This method has the potential of widespread application of Xpert testing for the detection of MTBC in stool at primary health care level.

2: Purpose

This SOP describes how to collect stool and process stool samples by using the SOS stool 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.

3: Principle

Stool is an alternative specimen type for the diagnosis of TB, as sputum containing MTBC is coughed up and, subsequently, swallowed and passed through the gastrointestinal system to finally end up in the stool.

By using the SOS stool method¹³, stool processing for use in the Xpert assay is as simple as sputum processing.¹⁴ Approximately 0.8 g of stool is added directly to the sample reagent (SR) that is provided with the Xpert MTB/RIF (Ultra) kit (Cepheid,

California, USA)^{14,15}, and this SR-stool mixture is shaken vigorously. During this step, the stool disintegrates, and the bacteria are released into the SR and MTBC bacilli are inactivated. Subsequently, sedimentation by gravity of the stool occurs during incubation of the SR-stool suspension, and the MTBC bacilli will remain in suspension. After the SR-stool suspension has been shaken vigorously again and incubated at room temperature, 2 mL of this suspension is transferred to the Xpert cartridge. Transfer should be done very carefully, avoiding transfer of any stool particles, as this may cause clogging of the filter in the cartridge. In addition, stool might contain more PCR inhibitors than sputum. For both these reasons, a higher rate of invalid/error results are expected than for sputum testing.

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.^{14,16} Because the cartridges are self-contained, the chance of cross-contamination between samples is minimized. In the Xpert MTB/RIF assay cartridge a nested real-time PCR takes place with primers directed at the *rpoB* gene, containing the 81 base pair hot-spot region. The *rpoB* gene is probed with five molecular beacons that enable differentiation between the wild-type sequence and mutations in the core region that are associated with RIF resistance. RIF is one of the most potent first line anti-TB drugs and is a surrogate marker for multi-drug resistant TB (MDR-TB). A sample processing control (SPC), consisting of spores from *Bacillus globigii*, is included in the assay as an internal control to ensure adequate processing of the sample as well as to monitor the presence of PCR inhibitors. A probe check control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity and dye stability.^{14,16,17}

The Xpert MTB/RIF Ultra assay uses the same GeneXpert system as Xpert MTB/RIF assay, and has shown to be non-inferior to latter assay¹⁸, but has

been adjusted to increase the sensitivity of the assay to detect MTBC. The Ultra assay detects MTBC by amplification of the multi-copy insertion sequences (IS) IS6110 and IS1081 and uses a larger PCR reaction volume than Xpert MTB/RIF (50 µL versus 25 µL PCR reaction chamber). The Ultra assay has a lower limit of detection (LOD) than Xpert MTB/RIF (16 versus 113 bacterial colony forming units (cfu) per mL¹⁹) because it uses nested nucleic acid amplification, more rapid thermal cycling, and improved microfluids. The Xpert Ultra assay incorporates melting temperature-based analysis instead of real-time PCR for the detection of RIF resistance. Specifically, four probes identify RIF resistance conferring mutations in the *rpoB* gene by shifting the melting temperature away from the wild type reference value.¹⁹

4: Responsible Personnel

Laboratory staff trained on stool processing by using the SOS stool method, and the Xpert MTB/RIF (Ultra) assay.

5: Safety

- Treat all specimens as potentially infectious biological material.
- Always wear a laboratory coat, protective eyewear and disposable gloves.
- The sample reagent buffer of the Xpert kit contains the irritant substances NaOH and isopropanol. Refer to the material safety data sheet information for details.²⁰
- Specimen processing should take place under a ventilated hood or in a well-ventilated room.
- If any spillage of stool occurs, then the affected area should be cleaned with 0.5% sodium hypochlorite solution (bleach) and, subsequently, with 70% alcohol.
- Dispose of all waste in a biohazard medical waste bin.
- Refer to your county-specific national health laboratory safety and waste management regulations for further considerations.

6: Materials

Note: This list of materials should be customized to country specific needs.

- 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)
- Printer, if a standard Xpert test report should be issued

7: Procedure

Important notes before you start specimen processing:

- Prior to starting the procedure, clean the work desk with 0.5% sodium hypochlorite solution (bleach) and subsequently with 70% alcohol.
- Process only as many specimens at one time as there are GeneXpert modules available to run the tests
- Avoid simultaneous processing of multiple samples, process samples one by one in a series of three

samples maximum to avoid competing processing steps between samples.

- All samples, SR bottles and cartridges should be carefully labeled with a unique patient identifier (ID).
- The procedure below details the steps for stool specimen collection, stool sample preparation and loading of the sample on the Xpert MTB/RIF (Ultra) cartridge specific for Xpert testing of stool. It does not provide guidance on the handling of the GeneXpert instrument, performance of the Xpert testing, and the interpretation of Xpert results. For these steps, the manufacturer's instructions for sputum processing or the existing instructions in the national SOP for Xpert MTB/RIF testing can be used.

7.1 Stool specimen Collection

Stool collection is usually done by the patients themselves, or in case of pediatric patients, by their caretakers. Collection can take place either in the health care facility or at the patient's home. Following the procedures at the local setting, the patient or caretaker should be instructed by a nurse, a doctor or other medical- or laboratory staff on how to collect the stool.

Below we describe a simple method for stool collection. 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

THE BRISTOL STOOL CHART

TYPE 1		Seperate hard lumps, like nuts (hard to pass)
TYPE 2		Sausage-shaped but lumpy
TYPE 3		Like a sausage but with cracks on its surface
TYPE 4		Like a sausage or snake, smooth and soft
TYPE 5		Soft blobs with clear cut edges (passed easily)
TYPE 6		Fluffy pieces with ragged edges, mushy stool
TYPE 7		Waterly, no solid pieces. Entirely liquid



Figure 1.

The Bristol Stool Scale, named after the University of Bristol where it was first described by Lewis and Heaton ²¹; from type 1, being the most solid, to type 7, being the most liquid.

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.

7.2 Stool 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°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 ²¹(see figure 1) and record it on the laboratory form. The stool type determines how to manipulate the stool; go to the next step for stool types 1 till 5 or to step 6 for stool types 6 or 7.
5. If the stool is of **type 1 till 5** (solid stool types from most solid to soft blobs) (figure 1):

- 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 1 by 1.5 cm), as shown in figures 2A and 3A, and transfer it to the SR bottle.
 - b. Use a wooden stick to remove the stool from the spoon or stick if needed (see figure 3A).
 - c. 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 of **type 6 or 7** (fluffy pieces with ragged edges (mushy stool) and watery (entirely liquid) stool) (figure 1), 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 (see figures 2B and 3B).
 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 in step 7.3.4.
 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 (see figure 4).

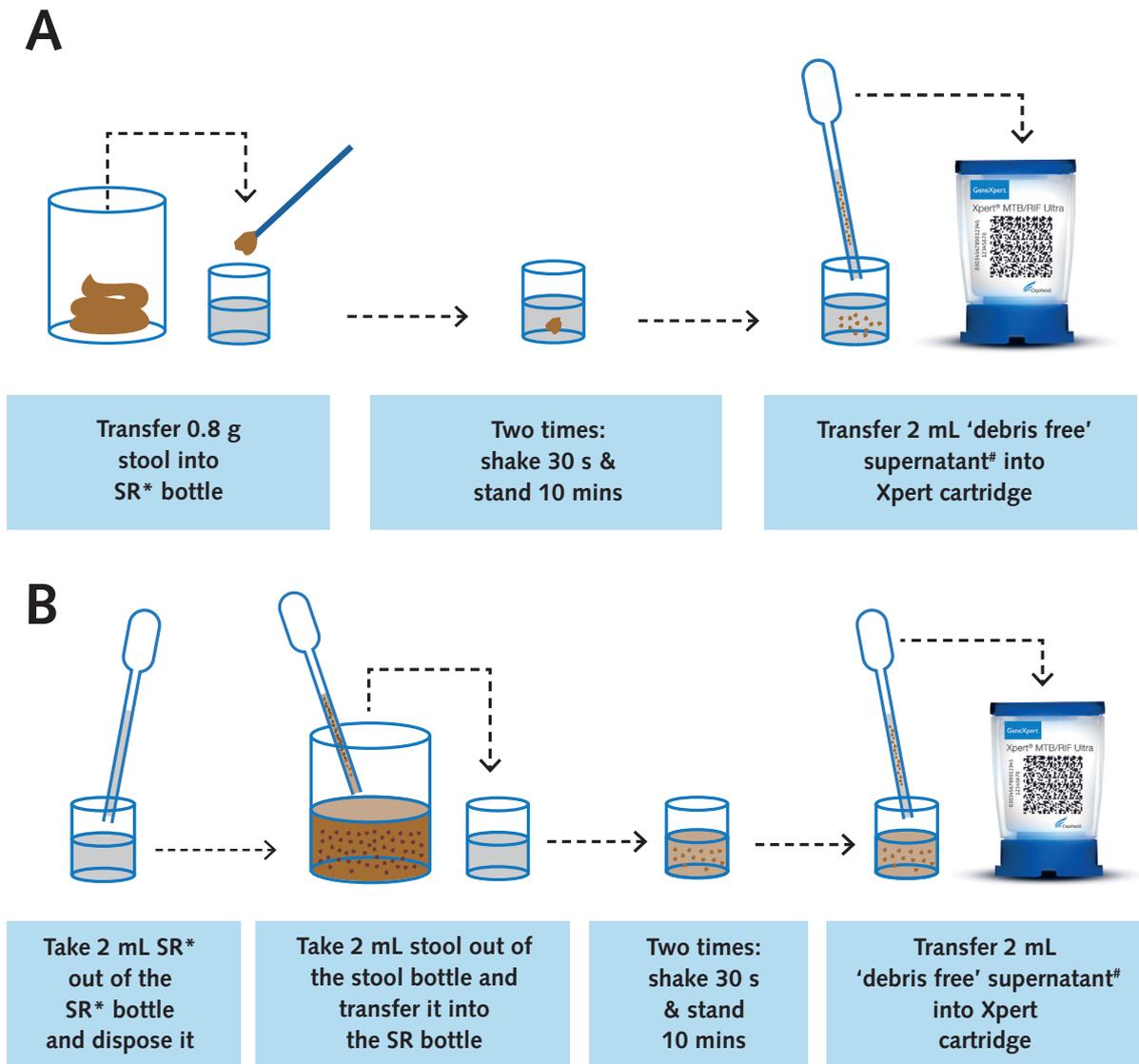


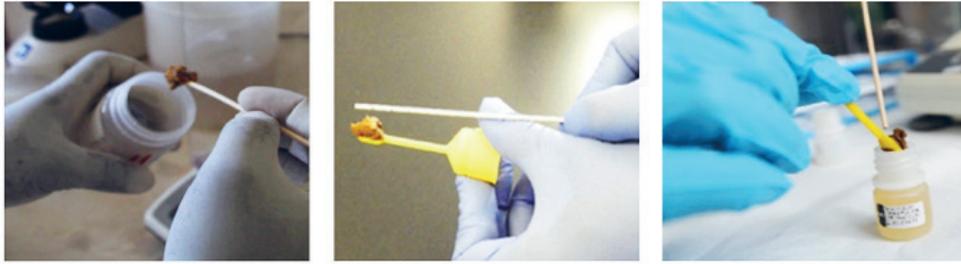
Figure 2.

Schematic overview of the SOP for the detection of *M. tuberculosis* complex and rifampicin resistance in stool by using the SOS stool processing method and the Xpert MTB/RIF (Ultra) assay for different types of stool. Panel A shows the procedure for Bristol type 1 till 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.

A



B

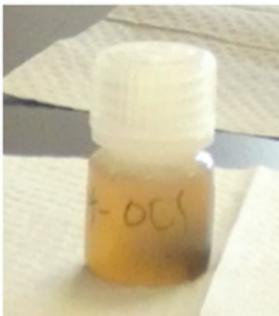


Figure 3.

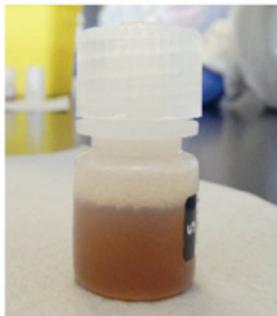
Photographs of typical examples of stool handling for the SOS stool processing method for Xpert MTB/RIF testing.

Panel A shows pictures for Bristol type 1 till 5 (solid) stool; picking of 0.8 g (approximately 1 x 1.5 cm) by either a wooden stick (left picture) or a spoon of a stool container (middle picture) and adding of the stool to the SR bottle with aid of a wooden stick (right picture). Panel B shows pictures for Bristol type 6 and 7 stool (liquid stool); a typical example (left picture), aspiration of liquid stool from the stool bottle (middle picture) and addition of the stool to the SR bottle (right picture).

A



B



C



D



Figure 4.

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.

7.3 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 (starting from step 7.2.4).

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 (Ultra) 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^{14,15,22,23} or the existing instructions in the national SOP for Xpert MTB/RIF testing.

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