RePORT International Laboratory Manual

Regional Prospective Observational Research for TB (RePORT)

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REVISION HISTORY

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VERSION	DESCRIPTION	REVISED BY	REVISION DATE
1.0	Initial Version	Fatima Jones	March 01, 2014
2.0	Section 1.0: Added saliva to list of specimens for collection and storage; revised collection timeframes for Month 1 and Month 2 specimens; removed collection of QuantiFERON®-TB Gold In-Tube (QFT-GIT) from Cohort A as per changes to the RePORT Common Protocol. Updated information for barcoded specimen and freezer box labels. Section 2.0: Revised to include updated procedures for pooling and processing sputum specimens (2.2.1, 2.7.2); new procedures for sputum induction (2.4); revised procedures for preparation of Sputasol/DTT for sputum storage (2.8); and updated instructions for sputum processing, inoculation of solid and liquid cultures, and reading/reporting of semi-quantitative solid cultures (2.10). Adjusted storage volume for sputum specimen aliquots from 1 mL to 0.5 mL. Updated to include new participant eligibility requirements for sputum storage at Baseline. Section 3.0: Updated procedures for collection of pediatric urine specimens (3.5). Section 4.0: Included new guidelines for handling participants who are apprehensive about blood draws (4.6); updated procedures for PBMC processing and storage using sodium heparin tubes and new PBMC SOP (4.12); updated details for processing and handling of QuantiFERON tubes, including links to	Fatima Jones	April 09, 2015

	Cellestis instructional videos for blood collection and processing (4.7, 4.13). Updated procedures to reflect use of the fourth generation QuantiFERON assay (QuantiFERON®-TB Gold Plus, QFT-Plus) in place of the third generation assay (QuantiFERON®-TB Gold In Tube). Adjusted storage volume for QuantiFERON specimen aliquots from 50 µL to 100 µL. Section 5.0: New procedures added for collection of saliva specimens from adults and pediatric participants. Section 8.0: Updated Appendices to include new Total Blood Volume (TBV) Collection Chart, and RePORT PBMC Processing and Storage SOP (Version 2.0).		

1.0 Biomarker Specimen Collection

1.1 Specimen Collection Schedule

This section addresses instructions and specimen collection procedures in support of the RePORT Common Protocol. Specimens from study eligible participants enrolled or provisionally enrolled in Cohort A (Active Pulmonary TB, Active TB) or Cohort B (Latent TB Infection, LTBI), who have provided consent to participate in the RePORT Common Protocol, will be collected at regularly scheduled visits.

Specimens from eligible participants enrolled in **Cohort A (Active TB)** will be collected at up to **4 scheduled visits** – Baseline, Month 1, Month 2, and at the End of Treatment (See Table 1). Additional samples will be collected if (1) relapse/treatment failure is suspected and evaluated at an unscheduled visit; or (2) the participant withdraws prematurely from the study.

- The **Month 1** (week 3-7) visit specimens are preferably collected at 4 weeks post enrollment; however may be collected at any time between 3 and 7 weeks after enrollment.
- The **Month 2** (week 8-12) visit specimens are preferably collected at 8 weeks post enrollment; however may be conducted any time between 8 and 12 weeks after enrollment.
- There must be at least 3 weeks in between the Month 1 and Month 2 visits.
- The **End of Treatment** (End of Tx) visit will take place when the participant completes his/her prescribed TB treatment regimen. For drug-sensitive (DS) TB participants on first-line multi-drug TB therapy, this will be approximately 6 months; however this is expected to be later for those with Multidrug-resistant (MDR) or Extremely drug-resistant (XDR) TB. The End of Treatment visit may be conducted up to 4 weeks before or after the target visit date (i.e., Month 5, 6, or 7 for DS TB participants).

Specimens for **Cohort B (LTBI)** will be collected at Baseline, and when/if the participant develops active pulmonary TB. Cohort B participants who develop active TB may be considered for enrollment in Cohort A.

Table 1. Common Protocol Specimen Collection Schedule

Specimen Type	Cohort A [Active TB]	Cohort B [LTBI]
Whole blood (PAXgene Blood RNA)	Baseline, Month 1, Month 2, End of Tx	Baseline
Whole blood (QuantiFERON®-TB Gold Plus)	-	Baseline
Whole blood (DNA)[1]	Baseline	-
Whole blood (PBMCs, Plasma)	Baseline, Month 1, Month 2, End of Tx	Baseline
Urine	Baseline, Month 1, Month 2, End of Tx	Baseline
Saliva (DNA)	Baseline, End of Tx	Baseline
Sputum (storage)[2]	Baseline, Month 1, Month 2	-
Sputum (AFB smear, culture)[2,3]	Baseline, Month 1, Month 2, End of TX	-
MTB Isolate (storage)	Baseline	-

[1] If blood volume is problematic, this specimen may be may be acquired at any time during the study follow-up through the End of Treatment or TX F/R/W Visits. Refer to blood collection weight chart (Appendix I) for maximum blood volume collection limits.
[2] A Baseline sputum specimen for both testing and storage is required for study eligibility in Cohort A.
[3] For children whose diagnosis was made on the basis of nasopharyngeal (NP), gastric aspirates (GA) or clinical criteria, subsequent GA/NP inductions will not be required.

Table 2. Biomarker Specimen Collection and Storage Chart (Adults and Children)

Specimen Type		Adults and Children	Aliquot Size	Expected Number of Aliquots	Storage Temperature
Whole blood (PAXgene Blood RNA)		2.5 mL	2.5 mL	1	-80°C
Whole blood (QuantiFERON®-TB Gold Plus)		4 mL (1 mL/tube)	100 µL plasma	8 (2 aliquots/ tube)	-80°C
Whole blood (DNA)		4 mL (EDTA)	1 mL	4	-80°C
Whole blood (PBMCs)		6 mL or 10 mL (sodium heparin tubes)	5 x 10^6 cells/mL	1-4	-150°C
Whole blood (Plasma)		Collected from sodium heparin tubes used for PBMCs	150 - 200 µL	8	-80°C
Saliva (DNA)		Collected using SalivaBio passive collection device	1-2 mL	4	-80°C
Urine		Spot urine (10 mL)	1 mL	8	-80°C
Sputum (storage)		Whatever volume is possible to collect	0.5 mL	1-4	-80°C
Extracted host RNA		Prepared from PAXgene Blood RNA tube	100 – 150 µg/ vial	-	-150°C
MTB Isolate	Cohort A	Subculture of original MTB isolate; and relapse or failure isolate	1 mL	4	-80°C
	Cohort B	Subculture of confirmatory MTB isolate from each participant who develops active MTB	1 mL	4	-80°C

1.2 Specimen Collection Kits

Specimen collection kits (for adults and children) and barcoded specimen labels will be provided to all sites through the Central Biorepository. Kit contents should be verified upon receipt and stored at room temperature (17-25°C), unless otherwise specified. The following items will be provided in each participant specimen collection kit:

- Barcoded specimen labels – for labeling specimen aliquots for storage
- Barcoded cryostorage box labels – for labeling cryostorage freezer boxes
- 21-gauge butterfly needle blood collection set, with 12" tubing and safety-lock for needle (23-gauge needle is preferred for pediatric participants)
- BD Vacutainer holder
- 6 mL or 10 mL BD Vacutainer tube with sodium heparin – for plasma and PBMCs
- QuantiFERON®-TB Gold Plus blood collection tubes (4) – Nil, Mitogen, TB1, TB2
- PAXgene Blood RNA collection tube – for blood RNA stabilization
- 4 mL BD Vacutainer tube with EDTA anticoagulant – for whole blood (DNA)
- Sterile, disposable, screw-cap, polypropylene urine specimen collection cup
- Urine specimen collection bag for pediatric urine collection
- Sterile, disposable, wide-mouth sputum collection container (or 50 mL conical tube)
- Saliva Collection Aid (SCA), SalivaBio Children's Swab (SCA) or SalivaBio Infants Swab (SIS)

In addition to the specimen collection kits provided by the Central Biorepository, the following materials and supplies will be required at the clinic site:

- Test tube rack (to hold blood collection tubes)
- Antiseptic towelettes for urine collection
- Ancillary phlebotomy supplies (paper tape, alcohol wipes, disposable gloves, etc.)
- Disposable tourniquet (latex free)
- Adhesive bandages to be applied to the venipuncture site
- Sharps disposal containers for used needles and other biohazardous materials
- Insulated coolers and ice packs for specimen transport
- Leak-proof biohazard plastic bags and absorbent material for specimen transport
- Transport cooler and ice packs
- Ambient temperature transport container (e.g. ORCATHERM)

1.3 Barcoded Specimen Labels

The barcoded specimen label will function as the unique identifier for each visit at which specimen collection occurs, and will link the specimens and related documents for one specific participant to each specific visit. Each label is intended for a specific purpose – either specimen identification or labeling of forms. It is imperative that each label be used as indicated to prevent misclassification or confusion concerning specimens and/or data.

Each specimen label will include the barcode, barcode number (in readable format), and label indicator (e.g. Sputum, see example in Figure 1 below). The barcode number will consist of the participant identification number (PID), which includes the site ID (e.g 101) and single letter designation for cohorts A or B; two letter designation for specimen type (e.g. "SP" for Sputum); and visit number (e.g. "M1" for Month 1). No personal identifiers will be collected; specimen aliquots, case report forms, and freezer logs will be labeled with the barcode labels only which will enhance maintaining participant anonymity and data confidentiality. Unused duplicate labels should be discarded immediately to prevent misuse.

In addition to the barcoded labels for specimen aliquots, barcoded labels for freezer storage boxes will aslo be generated. The barcode number for the freezer box labels will consist of the two letter designation for the specimen type (e.g. 'SP' for Sputum), site ID (e.g. 101), and box number (e.g. 001, 002, etc.). Examples of barcoded specimen and freezer box labels are shown below; the actual label may differ from the example provided.

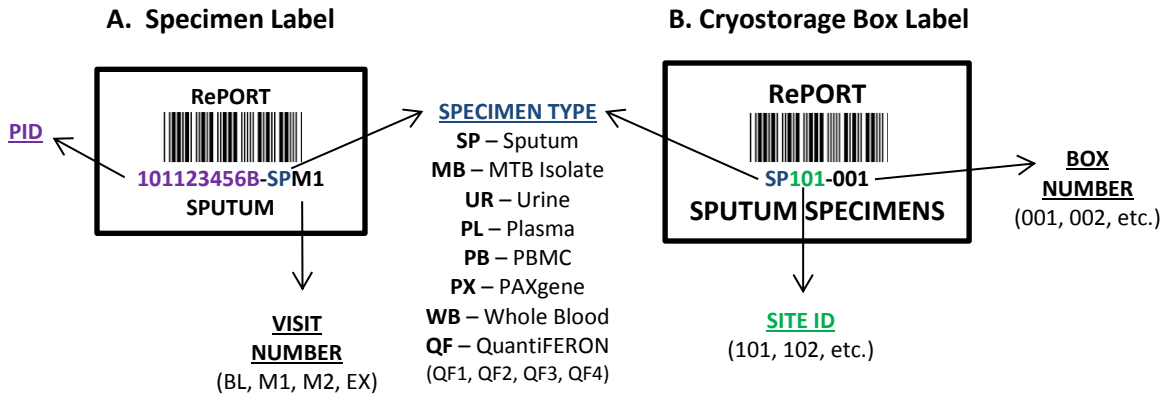


Figure 1. Examples of the barcoded specimen (A) and cryostorage box (B) labels.

2.0 Sputum Specimen Collection, Processing, and Storage

2.1 Sputum Collection Schedule

For the RePORT Common Protocol, up to two sputum specimens will be collected per visit (or on two consecutive days) for routine diagnosis of pulmonary TB and/or assessment of treatment response at the time of the scheduled visit; and for storage at the Central Biorepository.

For participants who are eligible for enrollment in **Cohort A** (Active Pulmonary TB), sputum specimens for routine acid-fast bacilli (AFB) smear and culture will be collected at 4 scheduled visits – Baseline, Month 1, Month 2, and End of Treatment (Refer to **Table 1**, Section 1.1). A sputum specimen will also be collected for storage in the Central Biorepository at 3 scheduled visits – Baseline, Month 1, and Month 2. Additional sputum specimens will be collected at the time of suspected treatment failure or relapse, or withdrawal from the study.

For participants enrolled in **Cohort B**, sputum specimens will only be collected when/if the enrolled participant develops active pulmonary TB.

2.2 Sputum Collection Guidelines

Where possible, sputum specimens should be collected in the early morning by the participant in their home, or at the hospital for in-participants. However, if the quality is unsatisfactory (saliva, spit), quantity is insufficient (< 2 mL), or transport from home to clinic has been delayed (> 3 hours), a specimen must be collected at the clinic under staff supervision. An attempt should be made to collect at least 3-5 mL of sputum at each scheduled visit; howewver, as treatment progresses successfully, deep-cough sputum production may become more difficult. Thus it is expected that sputum collection for storage in the Central Biorepository may not be possible for those participants who are successfully responding to treatment in later phases of the study. However a sputum specimen for testing and storage is required at Baseline to satisfy participant eligibility requirements (please refer to the Common Protocol for additional details).

Sputum collection procedures must only be carried out by trained, designated study/clinic staff. Universal precautions must be followed when performing the procedure, and handling and transport of the specimen. Mask and gloves must be worn by clinic staff and discarded appropriately in a waste collection container after use. The study staff member must wear a N95 mask or equivalent during the procedure.

Collection of a single-expectorated sputum specimen is preferred, however, for children under 5 years of age, or others who have difficulty producing an expectorated sputum specimen, **sputum induction** (see Section 2.6) or **nasopharyngeal** or **gastric aspirate** will be accepted.

- **Sputum Induction:** Sputum production may be induced by the inhalation of a warm aerosol of sterile 3-5% sodium chloride in water produced by a nebulizer. The specimen

should be clearly marked "INDUCED"; the induced sputum specimen is watery in consistency and could be mistaken for saliva. Additional instructions are provided in Section 2.6.

- **Gastric Lavage (Aspirate):** Gastric lavage is a technique used to collect gastric contents that can be used in the diagnosis of tuberculosis and is the procedure of choice for microbiologic confirmation of tuberculosis in children with pulmonary tuberculosis who are unable to produce sputum, though yield is frequently low. The following guidelines are recommended to ensure a quality specimen is collected.

    - The gastric lavage procedure requires medical attention and should only be performed in a hospital setting.

    - Gastric lavage should be performed early in the morning before eating and at least 8-10 hours after the participant has eaten or taken oral drugs.

    - Collection of 2-3 consecutive early morning specimens is recommended, as per standard protocol.

    - The gastric lavage specimen must be placed on ice and immediately transported to the laboratory for processing.

    - A 5-10 mL specimen is required per collection attempt and must be neutralized with 100 mg of sodium bicarbonate when there is delay of more than 4 hrs.

- **Nasopharyngeal Aspirate:** Nasopharyngeal aspiration (NPA) involves the insertion of a suction catheter (or tube) through the nostril into the nasopharynx. Respiratory secretions are then aspirated into a sterile container using an electrical suction device or hand-held aspirator. NPA is less invasive than collection of a gastric aspirate/lavage, and does not require fasting or hospitalization. Care should be taken to ensure that the catheter (or tube) is inserted a short distance into the child's nostril to avoid or minimize discomfort. Nasopharyngeal aspiration, like other sputum collection methods, is an aerosol-generating procedure which may pose infection transmission risks to healthcare workers as well as to nearby patients and other staff. Appropriate precautions should be taken to ensure the procedure is performed as far as possible from other patients, ideally in separate sputum collection room. Procedures should be performed in accordance with institutional guidelines.

2.2.1 Considerations for Pooled Sputum Specimens

The pooling of sputum specimens is not generally an accepted practice; however, if larger volumes are needed, pooling sputum can be effective if handled properly to prevent specimen mix-up and reduce introduction of contaminants. Sputum specimens intended to be pooled for testing and storage must be collected within no more than 24 hours of each other. Collected

specimens must be stored at 2-8°C immediately after collection to minimize introduction of contaminants. All specimens must be properly labeled, including Study ID, PID, date/time of collection, and specimen number (1 or 2). Specimen collection details for each specimen must be recorded on separate Sputum Specimen Transport Forms (Form 92) and a copy of the form must be included during transport of the specimens to the laboratory. Specimens will be pooled and processed upon receipt at the laboratory.

2.3 Sputum Collection (Expectorated)

2.3.1 Materials Needed for Sputum Collection

The following materials and supplies are needed to collect each participant sputum specimen:

- Specimen labels
- Sterile or bottled drinking water
- Clean disposable cups
- Sterile, wide-mouth, specimen collection containers or 50 mL conical tube
- Disposable gloves
- Refrigerator
- Respirator (N95 or equivalent)
- Insulated cooler and ice packs
- Leak-proof biohazard specimen bags
- Absorbent material for transportation of specimen
- Participant instructions for at home collection
- Permanent marker
- Sputum Specimen Transport Form (Form 92)

2.3.2 Instructions for Sputum Collection (Expectorated)

The ideal sputum specimen is produced by repeated deep inhalation and exhalation of breath followed by a cough as deep within the chest cavity as is possible for the participant. Sputum should consist of thick, mucoid, white-yellow, sometimes blood-tinged, material from the lower airways and lungs (not saliva or oral/nasal secretion). Collection of early morning specimens is preferred because of the overnight accumulation of secretions; however, you may collect specimens at any time for participants who have a deep cough that is readily productive. Direct observation of specimen collection should be practiced to insure the quality and integrity of the sputum collection.

2.3.2.1 Supervised Collection by Clinic or Study Staff

When collected in the clinic, collection staff should remain within viewing distance of the participant during the procedure to provide assistance as needed, and to ensure that he/she is isolated from others until sputum collection is complete. Specimens should be collected in a well-ventilated area, or sputum collection booth. Clinic staff collecting the sputum, regardless

of the setting, must observe the appropriate infection control precautions (i.e., wear a N95 mask and wear gloves when hand contact with blood or other potentially infectious materials is anticipated). The procedural details below must be followed:

1. Collect sputum specimen in a disposable, wide-mouth, leak-proof container or 50 mL conical tube.
2. Prior to collection, label the specimen container with the appropriate identifying label including the study ID, participant ID (PID), visit number, specimen number (1 or 2), and date and time of collection.
3. Positively identify the participant by his/her name and PID number.
4. Inform the participant that saliva and upper respiratory/nasal secretions are not sputum and are not acceptable specimens.
5. Demonstrate to the participant how to properly rinse his or her mouth and how to collect a sputum specimen using a demonstrator bottle/cup of water and container/tube.
6. *Instruct the participant to*:
    a) Thoroughly clean his/her hands with soap and water. Provide the participant clean disposable paper towels to dry his/her hands.
    b) Rinse his/her mouth with bottled water prior to collection of sputum. Provide a new, clean, disposable cup for each participant
    c) Breathe deeply a number of times and then cough from deep down within the lungs.
    d) Lean forward, breathe in and out slowly twice, hold breath for 2-3 seconds each time, and on third time forcefully cough to bring up the sputum.
    e) Collect the sputum into the sterile container provided and avoid touching the inside or edge of the specimen container or lid.
    f) Once collection has been completed, thoroughly clean his/her hands with soap and water. Provide the participant clean disposable paper towels to dry his/her hands.
7. Repeat the above sequence until an adequate amount of sputum is collected. This may take up to 1 hour. If the participant is unable to produce enough sputum within 1 hour, decide if the participant is “unable to expectorate”, requires rescheduling for another attempt at collection, or needs to undergo sputum induction.
8. Tighten the lid/cap on the container/tube to avoid leakage.
9. Estimate the volume of sputum collected by comparison with container/tube with appropriate markings. A minimum volume of 2 mL of sputum must be collected; however a target volume of 3-5 mL is preferred.

10. After the specimen is collected, wrap the container in cotton wool or other absorbent material (enough to sufficiently absorb the entire contents in case of leakage), and place specimen container in a leak-proof biohazard bag (or clear zip-lock bag).
11. Store the sputum specimen in a refrigerator or cool box with ice packs unless it is being transported to the laboratory within 1 hour.
12. Complete all the relevant fields on the Sputum Specimen Transport Form (Form 92).

2.3.2.2 Instructions for At-Home Participant Collection

At screening, participants are expected to provide a spot sputum specimen while in the clinic. At this time, the participants will be instructed how to properly collect the specimen and are directly observed during the collection. Participants may also be instructed to collect another specimen (early morning preferred) at home. Participants will be instructed again by the collection staff on how to obtain sputum with attention to details regarding a first morning collection, storing the specimen in a cool place, and bringing the specimen to the clinic as soon as possible. The procedural details below must be followed:

1. Provide the participant with a labeled specimen container including the appropriate identifying information (e.g. Study ID, PID, specimen number, and visit number), and zip-lock bag with absorbent material for temporary storage and transportation.
2. Mark container/tube 'HOME' to indicate the specimen was collected at home.
3. Advise the participant to attempt to collect 3-5 mL of sputum.
4. Inform the participant that saliva and upper respiratory/nasal secretions are not sputum and are not acceptable specimens.
5. *Instruct the participant to:*
    a) Collect the sputum after getting out of bed, before the morning meal, and prior to taking any medications.
    b) Thoroughly clean his/her hands with soap and water.
    c) Rinse his/her mouth with bottled water prior to collection of sputum.
    d) Breathe deeply a number of times and then cough from deep down within the lungs.
    e) Lean forward, breathe in and out slowly twice, hold breath for 2-3 seconds each time, and on third time forcefully cough to bring up the sputum.
    f) Collect the sputum into the sterile container provided and avoid touching the inside or edge of the specimen container or lid with their fingers.
    g) Replace the lid/cap after collection and close tightly to avoid leakage.
    h) Wrap the collection container in sufficient absorbent material (e.g. cotton wool), and place in a leak proof biohazard bag (or clear zip-lock bag).

i) Once collection has been completed, thoroughly clean his/her hands with soap and water.

j) Store the container in the refrigerator or cool box with ice packs, if provided.

k) Bring the specimen container to the clinic as soon as possible.

l) If refrigeration is not available, advise the participant to return the sputum to the laboratory within 2-3 hours of collection. The specimen must be refrigerated and processed immediately upon receipt in the laboratory.

6. Suggest placing the bottled water and specimen container in a place that will remind the participant to collect the specimen first thing in the morning upon rising.
7. Inform the participant that they will be asked to provide the date and time of collection when he/she brings back the specimen. This information should be recorded on the specimen label.

2.4 Sputum Collection (Induced)

Sputum induction is the preferred method for collection of sputum in children as it is non-invasive, does not require overnight hospitalization, and can be performed in an out-patient setting. The technique involves inducing sputum production by the inhalation of a warm aerosol of sterile 3-5% hypertonic saline produced by a nebulizer.

2.4.1 Sputum Induction Precautions

The use of hypertonic saline should provoke deep coughing, however may also trigger bronchospasm (e.g. wheezing, shortness of breath, or other difficulties in breathing) in some participants. **Participants should be monitored for signs of intolerance or respiratory distress throughout the procedure.** The following precautions should be noted:

- If bronchospasm occurs, stop the induction and follow your local institutional guidelines for handling such an event and/or initiate emergency response procedures as necessary.
- Improper deep breathing may cause hyperventilation, producing symptoms of tingling fingers, light-headedness, and dizziness.
- If the participant hyperventilates, stop the induction, encourage the participant to relax, and reinstruct the participant in proper breathing techniques.
- Vomiting may be precipitated by excessive coughing in susceptible participants. If vomiting occurs, stop the induction and assist participant using appropriate measures.
- When the participant recovers, if appropriate, resume the induction if the sputum specimen has not been obtained.

- In infants, caution should be used when using an ultrasonic nebulizer for sputum induction due to the high output of mist which may result in fluid overload if used for a prolonged period of time.

- Pediatric participants are required to be NPO (fasting, nothing by mouth) for at least four hours prior to induction to reduce risk of vomiting and aspiration.

2.4.2 Equipment and Materials Needed for Sputum Induction

- Hypertonic saline (3-5%)
- Sterile or bottled water
- Clean drinking cups for gargling/rinsing mouth
- Ultrasonic Nebulizer
- Disposable corrugated aerosol tubing (102 cm)
- Sterile, leak-proof, specimen collection container
- Specimen label
- Permanent marker (indelible ink)
- Clear, leak-proof biohazard specimen bag and absorbent material
- Disposable gloves
- Respiratory Mask (e.g. N95)
- 100% oxygen setup (for pediatric participants)
- Sputum Specimen Transport Form (Form 92)

2.4.3 Preparation for Sputum Induction

The sputum induction procedure must only be carried out by trained, designated study/clinic staff (e.g. nurse, doctor or physiotherapist). Universal precautions must be followed when performing the procedure, and handling and transport of the specimen. Mask and gloves must be worn and discarded appropriately in waste collection container after use. The study staff member must wear a N95 mask or equivalent during the procedure.

Below is a general procedure for Sputum Induction; however additional considerations may need to be included based on institutional guidelines and type of instrument available at the clinic/hospital.

Prior to initiating the sputum induction procedure, clinic staff must:

1. Clean hands with soap and water before setting up the nebulizer, after completing the procedure, and in between each participant collection.
2. Collect all materials and prepare the ultrasonic nebulizer (with 3-5% hypertonic saline) before starting procedure.
    a) Follow all operating instructions for the ultrasonic nebulizer.

b) Prepare the nebulizer and inspect for cleanliness and integrity of all plastic parts, and disinfect the surface of the nebulizer with an appropriate disinfectant.

c) Place sodium chloride solution in the cup portion of the nebulizer tubing.

d) Hook the tubing set to the nebulizer and test to ensure it is functional.

The participant should be instructed to take nothing by mouth after midnight on the days when sputum induction will be performed. Sputum induction should be performed in an area with adequate ventilation and no recirculation of exhausted air; or in a separate negative flow room to minimize risk of potentially pathogenic aerosols.

2.4.4 Procedure for Sputum Induction

1. Identify the participant (e.g. PID, name).
2. Identify yourself to the participant or caregiver and explain the purpose of the procedure and answer any questions they may have.
3. Instruct the participant to gently brush the tongue and buccal surfaces, teeth and gingival margins with water or normal saline (DO NOT USE toothpaste, mouthwash, or other solutions containing alcohol, oil or antimicrobials).
4. Have the participant rinse their mouth and throat several times with clean water or saline to remove oral contaminants.
5. Instruct participant to sit in a chair maintaining good posture. The participant should not be lying down during the procedure.
6. Explain to the participant that coughing is normal and a desired effect of the procedure, which will increase mucous production.
7. Turn on the nebulizer and verify good aerosol output.
8. Instruct the participant how to use the nebulizer, and coach him/her to inhale aerosol deeply.
9. The participant should be instructed to do the following:
    a) Remain in the booth or room until the procedure is complete.
    b) Breathe normally through the mouth and take occasional deep breaths.
    c) Hold the sputum collection container upright and loosen the lid.
10. Begin the ultrasonic nebulizer treatment and adjust the output control accordingly so that a “cloud” is formed. Ensure that the participant is not overwhelmed.
11. Place the aerosol mask over the participants face, or alternatively, instruct the participant to inhale the aerosolized saline through the end of the disposable 102cm tubing and then exhale through the nose or around the tube.

a) Pediatric participants may not tolerate wearing a mask. With small children, the face mask may need to be removed from the aerosol tubing, and the mist directed towards the child's face.

12. After 15-20 minutes of saline inhalation, the participant should be encouraged to cough forcefully and expectorate into the specimen collection container.

    a) Nasotracheal or oropharyngeal suction may be required in pediatric participants. If required, explain the procedure to the participant, parent or guardian to ease child anxiety.

    b) Assess the participant's respiratory rate, breath sounds, heart rate, and oxygen saturation before and after suctioning.

    c) Supplemental oxygen should be supplied during the suctioning procedure.

13. The nebulization is continued for up to 30 minutes if necessary to obtain the needed samples.

14. Remain near the sputum induction room or within viewing distance of the participant during the procedure to provide assistance if needed. Watch carefully for signs of respiratory distress and ensure participant does not leave room until coughing has stopped.

15. Instruct the participant to collect the specimen in the sterile specimen collection cup.

16. Label the sputum collection container with identifying label, including PID, sputum sample type, and date and time of collection. The specimen label and form should be clearly marked as "Induced", as the watery consistency could be mistaken for saliva.

17. Complete the collection details on the Sputum Specimen Transport Form (Form 92)

18. Specimens should be placed in a cooler or cold box and delivered to the laboratory soon after collection.

19. Samples should be refrigerated at 2-8°C if transport is delayed for more than 1 hour.

2.5 Receipt of Specimen Container at the Clinic

Upon receipt of the specimen container at the clinic, ask the participant at what time he/she collected the specimen and record their response on the container. The following steps should also be completed:

1. Estimate the volume of sputum collected by comparison with container/tube with markings. If specimen volume is inadequate (< 2 mL), try to collect another specimen from the participant while in the clinic.
2. Refrigerate the specimen until transported to the laboratory.

3. Complete all the relevant fields on the Sputum Specimen Transport Form (Form 92).

2.6 Transporting Sputum Specimens to the Processing Laboratory

Each study site has been assigned a testing laboratory, and all study related sputum specimens must be forwarded to the designated laboratory for testing. Consistent attention to participant identification on the specimen collection container and specimen tracking/request form significantly reduces error.

Proper transport of sputum specimens to the processing laboratory is critical to ensure successful isolation of MTB. The following guidelines should be implemented for proper sputum collection, transport and storage.

- Each sputum specimen must be labeled with appropriate identifying information including study ID, PID, specimen number, visit number, and date and time of collection.
- Sputum specimens should be placed in a leak-proof biohazard bag with sufficient absorbent material and transported to the laboratory in a 2-8°C cooler as soon as possible after collection. If refrigeration or cooler is not available, the specimen should be kept as cool as possible, and delivered to the laboratory and refrigerated within 2-3 hours of collection.
- The Sputum Specimen Transport Form (one for each sputum specimen) must be included in an appropriate envelope and transported with the specimen to the laboratory.
- If delay is unavoidable, the specimens should be refrigerated at 2-8°C to inhibit growth of undesired microorganisms. Sputum should NEVER be frozen.
- Sputum specimens must be delivered to the laboratory as soon as possible and within 24 hours of collection; however, delays up to 3 days in transport from clinic to laboratory are allowable if the transport distance is long and agreed upon by the study/protocol team.
- Notify the testing laboratory of all shipments in advance of transport. Provide the date and time specimens are expected to be delivered. This ensures that laboratory personnel are prepared to receive and process the specimens.

Sputum and other specimens suspected to contain Mycobacteria are classified as "**Infectious substance, Category B**". All sputum specimens must be transported in compliance with local and national regulations governing the transport of potentially infectious materials. These rules must be followed, no matter how short the transport distance. Staff handling the packaging and transport of potentially infectious materials must be International Air Transport Association (IATA) trained and certified; certification must be renewed every 2 years, or per local regulations.

2.7 Receipt of Sputum Specimens at the Processing Laboratory

Sputum specimens should be processed as soon as possible upon receipt at the laboratory. All sputum processing must be done in a biological safety cabinet (BSC), and performed according to containment Biosafety Level 3 (BSL3) practices. To minimize aerosol production, open specimen containers slowly, allowing the tubes to stand for a few minutes before opening, and avoid expulsion of the last drop from the pipette during transfer. Centrifugation must be carried out in sealed buckets which are subsequently opened in a microbiological safety cabinet. Proper aseptic technique is critical to avoid contamination by bacteria other than MTB, and potential cross contamination with other MTB specimens.

2.7.1 Immediate Specimen Handling Procedures

Always wear disposable gloves during receipt and inspection of incoming specimens. Immediately upon arrival in the laboratory, remove specimen containers from the cool box and place in the refrigerator.

Before specimens are processed, designated laboratory staff must compare the information (PID number, study ID, specimen number, visit number, and date/time of collection) on the specimen container labels with that on the respective specimen tracking/request form to make sure they match. If they do not match or information is missing, the laboratory must contact the clinical site to obtain any outstanding/missing information before the specimen is processed. In addition, the laboratory staff must record the date and time the specimens are received, carry out a visual check of the specimens to confirm they are in good condition (e.g., the specimen does not contain only saliva or excessive blood quantity) and are of appropriate volume (at least 2 mL), and record information on the accompanying specimen transport form.

Sputum specimens should be rejected for processing, and the clinic notified, in the event of the following:

- The specimen is unlabeled or mislabeled
- The participant name or PID does not match the participant requisition form
- The specimen container is broken or leaking
- The specimen was not collected in an appropriate container

NOTE: To satisfy participant eligibility requirements, a sputum specimen must be collected at Baseline for both testing (AFB, culture, DST, etc.) and storage of sputum and original MTB isolate. If sufficient sputum is not collected (< 2 mL), please notify the clinic immediately to arrange to collect another participant specimen.

2.7.2 Pooling Sputum Specimens

The pooling of sputum specimens is not generally an accepted practice; however, where larger volumes are needed, pooling sputum can be effective if handled properly to prevent specimen mix-up and reduce introduction of contaminants. Sputum specimens intended for both testing and storage must be collected within 24 hours of each other, and stored at 2-8°C immediately after collection to minimize introduction of contaminants. All specimens must be properly labeled; including Study ID, PID, date/time of collection, and specimen number (1 or 2). Specimen collection details for each specimen must be recorded on separate Sputum Specimen Transport Forms (Form 92) and included with the specimens during transport to the laboratory.

Sputum specimens intended for both testing and storage must be pooled and processed immediately upon receipt at the laboratory. Sputum specimens will be treated with Sputasol (final 0.01% DTT) as described in Section 2.8 below, and aliquots prepared for storage prior to decontamination and processing for AFB smear and culture. To avoid potential mix ups, pool specimens from only one participant at a time.

1. Assemble sputum specimens #1 and #2 in the BSC.
2. Confirm PID to verify that specimens #1 and #2 were collected from the same participant.
3. Aseptically transfer the contents of each sputum container into a sterile 50 mL conical tube pre-labeled with the appropriate PID.
4. The pooled sputum volume must be ≥ 2 mL in order to ensure sufficient material is available for testing (at least 1 mL) and storage.
5. Treat the pooled specimen with 10% Sputasol (0. 1% DTT) as outlined in Section 2.8.
6. Prepare up to 4 aliquots of sputum (0.5 mL each) in the pre-labeled cryovials and store at -80°C as outlined in Section 2.8.
7. Process the remaining sputum volume (minimum 1-2 mL) using the NALC-NaOH procedure according to approved laboratory SOPs.
8. Prepare AFB smears and inoculate cultures using guidelines specified in Section 2.10.
9. Record the date and time of processing, final pooled volume, and number of aliquots stored on the Sputum Specimen Processing and Storage Form (Form 93).

2.8 Preparation of Sputum Aliquots for Long-Term Storage

Sputum specimens intended for long-term storage must not be decontaminated or treated in any way that may impact future testing or diagnostic applications. All sputum specimens collected for storage in the Central Biorepository will be treated with **Sputasol** (dithiothreitol, DTT; Oxoid Ltd) to liquefy the sample and aid in aliquoting for long-term storage. Sputasol is highly effective in decreasing sputum viscosity. The concentrated Sputasol solution contains 0.1g DTT, which when re-suspended in 100 mL of deionized water will yield a concentration of

0.1% (6.5mM) DTT. A low concentration of DTT (final 0.01%) is used in the sample in order to enable specimens to be analyzed for cell wall components and cytokines; this concentration of DTT and has not been shown to inhibit other downstream processing activities.

2.8.1 Materials Needed for Processing and Storage of Sputum Specimens

- Barcoded specimen labels
- Barcoded cryostorage box identification labels
- Preprinted cryostorage box specimen orientation labels
- Sputasol (dithiothreitol, DTT; Oxoid Ltd)
- 2 mL self-standing, graduated cryovials
- Sterile, serological pipettes, 1 mL or 5 mL
- Pipette aid
- Sputum Specimen Storage Form
- Biomarker Freezer Log
- Freezers, -80^{o}C
- 2-inch cryostorage box
- Cryomarker Pen

2.8.2 Prepare a working stock of 10% Sputasol (0.1% DTT)

1. Aseptically add the contents of one Sputasol vial (7.5mL) to 92.5 mL of sterile distilled water.
2. Mix and label the flask with the contents, date of preparation, and date of expiration.
3. The stock solution (10% Sputasol) should be used immediately or **stored at 2-8°C for up to 48 hours**.
4. **NOTE:** Alternatively, a fresh 0.1% DTT stock solution in phosphate buffered saline (PBS) can be prepared powdered DTT (Sigma, Cat #43815). DTT solutions are prone to oxidation and loss of activity, and must be prepared fresh daily. Prepare a solution of 0.1% DTT, filter sterilize, and store at 2-8°C for up to 48 hours.
    a) Dissolve 10 mg of DTT in 10 mL PBS (sterile, cell grade, pH 7.4 ± 0.2)
    b) Filter sterilize and prepare 1mL aliquots
    c) Store at 2-8°C for up to 48 hours

2.8.3 Procedures for Treatment of Sputum Specimens with Sputasol (DTT)

1. Prepare and label up to 4 cryovials with the appropriate identifying barcoded specimen label (depending on the quantity of sputum available for storage).
2. Determine the amount of the stock solution (10% Sputasol or 0.1% DTT) to add to the sputum specimen to yield a final concentration of 1% Sputasol (0.01% DTT).

a) Divide the total sputum volume by 10 and add this volume in mL of 10% Sputasol (0.1% DTT)

   - For example, add 100 µL of 10% Sputasol (0.1% DTT) per 1 mL of sputum; or 500 µL 10% Sputasol (0.1% DTT) for 5 mL of sputum; etc.

3. Using a sterile serological pipette, add the appropriate volume of 10% Sputasol to the sputum specimen. **Both Sputasol/DTT solution and sputum specimens must be at room temperature for the digestion procedure to be effective.**
4. Vortex the specimen for 20 seconds.
5. Place the specimen on a platform shaker to shake mechanically at 60 rpm for 20 minutes; (while shaking, prepare and label the cryostorage boxes as indicated in Section 2.9 below).
6. Using a sterile transfer pipette, transfer 0.5 mL of the DTT homogenized sputum to each of the pre-labeled 2 mL cryovials.
7. Prepare up to 4 aliquots. [**Be sure to reserve at least 1-2 mL of the DTT treated sputum for decontamination by NALC-NaOH and preparation of AFB smears and inoculation of liquid and solid cultures**]
8. Tightly cap and seal each cryovial.
9. Store sputum aliquots at -80°C (see instructions below).
10. Record specimen processing and storage details on the Sputum Specimen Processing and Storage Form (Form 93).

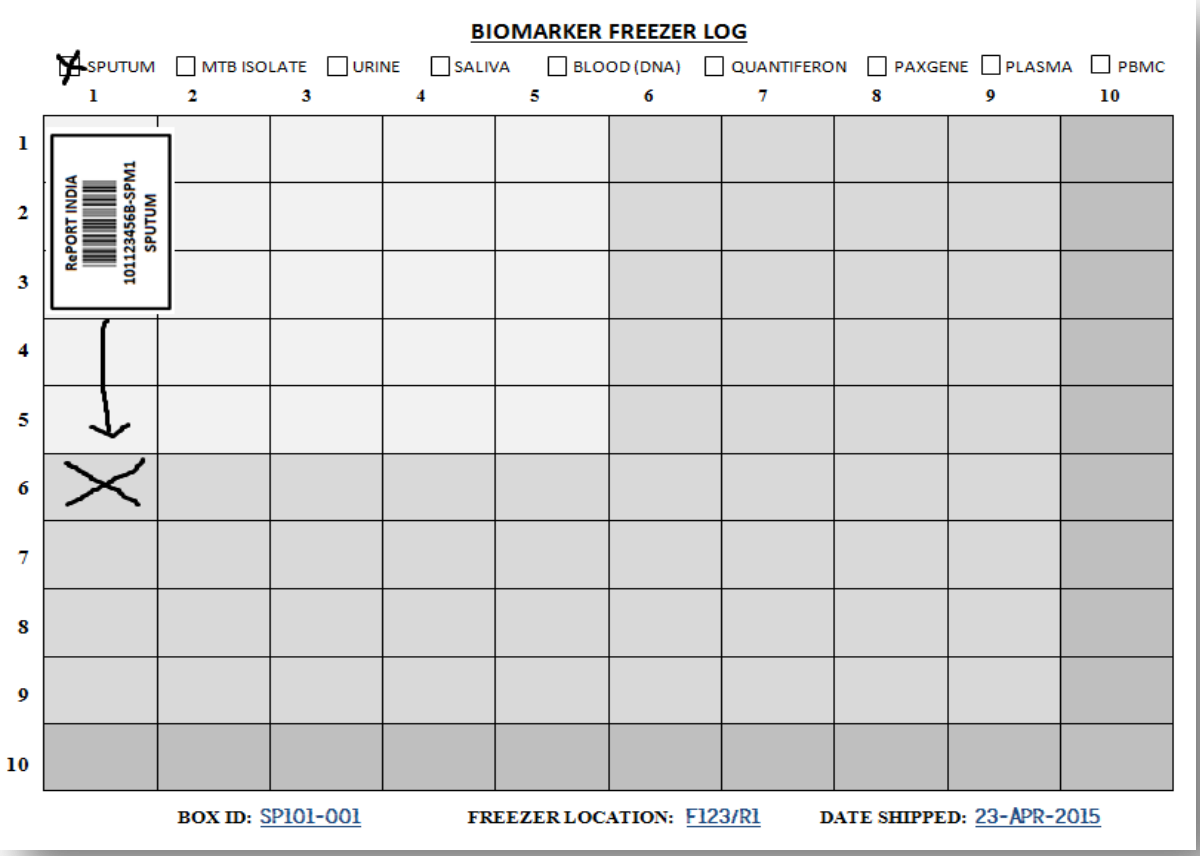
2.9 Storage of Sputum Aliquots

Sputum aliquots should be stored in 2-inch cryostorage boxes, with 9x9 (81 cell) or 10x10 (100 cell) sectioned inserts. Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number designation (see Figure 1). Two labels will be used to identify each cryostorage box.

1. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.
2. Label the outside of the cryostorage box. Affix one copy of the cryostorage box label to the side of the cryostorage box lid, and the other label to one side of the cryostorage box bottom.

3. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
4. Begin storing cryovials from the top left hand corner of the box, moving vertically.
5. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
6. After storing an entire collection of specimens from one participants visit, begin storing the next set of participant specimens adjacent to the previous collection.
7. Place the cryostorage box in the -80^{o}C freezer.
8. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'Sputum' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    d) Indicate the Box ID (e.g. SP101-001) on the Biomarker Freezer Log, and note the freezer storage location (freezer, shelf, rack) for future reference.
    e) See example completed Biomarker Freezer Log in Figure 2 below.
9. The person responsible for processing the specimens will record the time the specimen was received in the laboratory and the number of cryovials stored on the Sputum Specimen Processing and Storage Form (Form 93). A comment documenting any problems with processing the specimens should be added.

Figure 2. Partially Completed Biomarker Freezer Log (Example)



2.10 Processing Sputum Specimens for AFB Smear and Culture

Sputum specimens received for AFB smear and culture should be processed as soon as possible upon receipt at the laboratory. All processing must be done in a biological safety cabinet (BSC), and performed according to containment Biosafety Level 3 (BSL3) practices.

All relevant information regarding the sputum specimen and its collection (date and time collected; condition and volume of sputum; visit number; date and time of receipt) will be recorded on the appropriate laboratory request form. The same form will be used to collect all the test results as well as important parameters related to the testing (e.g., AFB smear and culture results).

Each sputum specimen will be processed by AFB microscopy (ZN or fluorescence), Lowenstein Jensen (LJ) solid culture or liquid Mycobacterial Growth Indicator Tube (MGIT) liquid culture, and culture identification using standard laboratory procedures in accordance with national guidelines. Inoculation on both solid and liquid media is recommended; however either solid or liquid media may be used.

To promote standardization, quality results, and comparison of data across all laboratory sites, the guidelines outlined in Sections 2.10.1, 2.10.2, and 2.10.3 must be incorporated into all standard laboratory procedures as appropriate. All laboratory procedures must be reviewed to ensure incorporation of these guidelines prior to initiating the Common Protocol. In addition a copy of the TB Laboratory Information Form documenting general laboratory practices and acknowledging the guidelines outlined below must be completed by each TB laboratory; and reviewed and updated annually.

All microbiological laboratory data related to the sputum specimen must be documented on the Mycobacteriology Laboratory Form (Form 3).

2.10.1 Guidelines for Sputum Specimen Receipt and Processing

Table 3. Guidelines for Sputum Specimen Receipt and Processing
1. The specimen must be refrigerated at 2-8^{o}C unless it is processed within 1 hour of receipt by the laboratory.
2. Sputum specimens must be digested and decontaminated with NALC/NaOH at a final sodium hydroxide (NaOH) concentration of 1-1.5%.
3. Sputum specimens must be processed in a centrifuge capable of generating a relative centrifugal force (RCF) of 3000-3500g *(centrifuge must be calibrated annually per manufacturer's instructions).* Use of a refrigerated centrifuge is preferred.
4. During specimen processing, the sputum is decontaminated in NaOH for 15-20 minutes before adding buffer. Do not exceed 20 minutes decontamination time.
5. The digested and decontaminated sputum must be washed and re-suspended in 1.5 mL Phosphate Buffered Saline (PBS) pH 6.8.
6. Positive and negative controls must be included in each processing batch.
7. AFB smear results must be reported according to WHO/IUATLD reporting scheme (Table 4).

Updates to WHO/IUATLD reporting scheme for AFB smears: Due to historical inaccuracy, the fluorescent microscopy reporting scale for positive smears has been revised. The actual field observed is larger than previously calculated, and therefore more AFB are visible per field. See Table 4 below for updated 2013 guidelines for reporting positive AFB smears.

Table 4. WHO/IUATLD Grading Scales for AFB Smears (2013)			
REPORT	**ZN (1000X)** 1 length = 100 fields	**Fluorescence (200x)** 1 length = 30 fields	**Fluorescence (400x)** 1 length = 40 fields
No AFB	No AFB/100 fields	No AFB/length	No AFB/length
Confirmation required **	-	1-4 AFB/length	1-2 AFB/length
Scanty	1 – 9 AFB/100 fields (Record actual number)	5-49 AFB/length	3-24 AFB/length
1+	10 – 99 AFB/100 fields	3-24 AFB/field	1-6 AFB/field
2+	1 – 10 AFB/field (check 50 fields)	25-250 AFB/field	7-60 AFB/field
3+	> 10 AFB/field (check 20 fields)	> 250 AFB/field	> 60 AFB/field

2.10.2 Guidelines for Solid Lowenstein-Jensen (LJ) Culture

Lowenstein-Jensen (LJ) slope/plates should be processed according to national guidelines, with consideration of the following:

Table 4. Guidelines for Solid Culture (LJ)
1. Inoculate each Lowenstein-Jensen (LJ) slope/plate with 200 µL of the decontaminated sputum sediment.
2. Check colony formation every week; preferably twice within the first week to allow detection of early contamination and timely request of another specimen if necessary.
3. Solid media must be incubated for at least 8 weeks before being reported as negative.
4. Appropriate controls (e.g. containing low amounts of MTB) must be tested before each batch of media is used, regardless if purchased commercially or prepared in-house.
5. All positive solid media must be examined by ZN staining and subcultured on blood agar plates (BAP) to confirm purity.
6. Semi-quantitative colony counts must be reported according to the WHO/IUATLD reporting scheme for solid cultures. See Table 5.
7. Growth of MTB complex must be confirmed using an appropriate identification test method (e.g., PNB or MPT 64 TB Antigen Test).

Table 5. WHO/IUATLD Reporting Scheme – Solid Culture (2007)	
No colonies	Negative
<10 colonies	Record actual number
10-100 colonies	1+
101-200 colonies	2+
>200 colonies	3+

2.10.3 Guidelines for Mycobacterial Growth Indicator Tube (MGIT) Cultures

All MGIT cultures must be processed according to the guidelines of the FIND BD MGIT manual. A copy of the manual can also be found at (*click to follow link*): FIND BD MGIT Manual (2006)

Table 6. Guidelines for MGIT Culture
1. All MGIT cultures (positive and negative) are worked up according to the FIND MGIT Manual using appropriate algorithms.
2. MGIT cultures must be inoculated with 0.5 mL of the decontaminated sputum sediment.
3. All positive MGIT cultures must be examined by ZN staining and subcultured on blood agar plates (BAP) to confirm purity.
4. The machine generated time-to-positivity (TTP) must be recorded for all positive MGIT cultures.
5. Growth of MTB complex must be confirmed using an appropriate identification test method (e.g. PNB or MPT 64 TB Antigen Test).

2.11 Drug Susceptibility Testing (DST)

Susceptibility testing of first-line (INH, rifampin, ethambutol, pyrazinamide) and second-line drugs (if applicable) will be done on all MTB positive cultures for specimens collected at Baseline, and at the time of treatment failure, relapse, or withdrawal from the study. Procedures should be performed according to the national guidelines. Record DST results on the Mycobacteriology Laboratory Form (Form 3). If a referral laboratory is used for DST, the name and contact information must be recorded on the TB Laboratory Information Form, which must be reviewed and updated annually.

2.12 Long Term Storage of MTB Culture Isolates

A subculture of each MTB isolate collected at Baseline or at the time of treatment failure, relapse or withdrawal, will be processed for long-term storage. Subcultures should be prepared from well-grown (non-contaminated) solid or liquid cultures of each of the MTB isolates to be stored. Use of solid media for subculture is preferred as it is easier to determine whether or not the culture is contaminated prior to preparing aliquots for long-term storage.

2.12.1 Materials and Supplies for Processing and Storage of MTB Isolates

- BSC, Class I or II
- Freezer, -80°C
- Micropipette (1000 µL) and Pipet Tips
- 2 mL self-standing, graduated cryovials with external thread
- Middlebrook 7H9 Medium (with 0.5% glycerol)
- 2-inch cryostorage box
- Barcoded specimen labels
- Barcoded cryostorage box identification labels

- Preprinted cryostorage box specimen orientation labels
- Cryomarker
- Biomarker Freezer Log
- MTB Isolate Storage Form (Form 94)

2.12.2 Preparation of MTB Isolates

The following are instructions for preparation and storage of MTB isolates:

1. Select a well-grown (non-contaminated) solid or liquid culture of MTB. Fresh growth is important; slopes or plates should be less than 2 months old at the time of inoculation.
2. Prepare a subculture on egg-based medium slant or plate (e.g. LJ, 7H10, or 7H11).
3. Incubate at 37±1°C in an aerobic atmosphere supplemented with carbon dioxide (5%) for 3-5 weeks, or until well grown.
4. Prepare and label 4 cryovials with the appropriate identifying barcoded specimen label.
5. Gently scrape as many colonies as possible from the surface of the egg-based medium; being careful not to collect any culture medium.
6. Suspend the colonies in a tube containing 5mL of Middlebrook 7H9 Medium (supplemented with 0.5% glycerol).
7. Homogenize the solution by carefully pipetting up and down.
8. Pipette 1 mL of the bacterial suspension into each 2 mL labeled cryovial tube; prepare up to 4 aliquots.
9. If available, immediately transfer to a dry-ice bath, or store immediately at -80°C.
10. When frozen, store at -80°C (see instructions below).
11. Document storage details on the MTB Isolate Storage Form (Form 94)

2.12.3 Storage of MTB Isolates

All MTB isolates will be temporarily stored on-site at the laboratory, and then batch shipped to the Central Biorepository with other participant specimens. The following outlines procedures for local storage of MTB Isolates.

MTB isolates should be stored in 2-inch cryostorage boxes, with 5x5 (25 cell) sectioned inserts – these smaller boxes are used to facilitate shipment of the isolates (Category A specimens are limited to 50 mL per shipment). Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number (see Figure 1). Two labels will be used to identify each cryostorage box.

Two labels will be used to identify each cryostorage box.

1. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.
2. Label the outside of the cryostorage box. Affix one copy of the cryostorage box label to the side of the cryostorage box lid, and the other label to one side of the cryostorage box bottom.

3. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
4. Begin storing cryovials from the top left hand corner of the box, moving vertically.
5. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
6. After storing an entire collection of specimens from one participants visit, begin storing the next set of participant specimens adjacent to the previous collection.
7. Place the cryostorage box in the -80^{o}C freezer.
8. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'MTB Isolate' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    d) Indicate the Box ID (e.g. MB101-001) on the Biomarker Freezer Log, and note the freezer storage location (freezer, shelf, rack) for future reference.
    e) See example completed Biomarker Freezer Log in Figure 2.
9. The person responsible for processing the sputum will record the time the specimen was received in the laboratory and the number of cryovials stored on the MTB Isolate Storage Form. A comment documenting any problems with processing the sputum specimen should be added.

3.0 Urine Specimen Collection, Processing, and Storage

3.1 Urine Specimen Collection Schedule

Urine specimens will be collected in all participants enrolled in the Common Protocol (**Cohort A**) at four scheduled time points – Baseline, Month 1, Month 2, and End of Treatment; and at Baseline for participants enrolled in **Cohort B** (Refer to **Table 1**, Section 1.1). At each scheduled urine specimen time point, 10 mL of urine will be collected, providing up to 8 cryovials, each containing 1 mL of urine.

3.2 Urine Specimen Collection and Transport Guidelines

Urine is a highly useful source of protein for biomarker discovery and assessment. It is easily accessible, can be collected noninvasively, and enables monitoring of numerous physiological processes and diseases. Urine is a source of numerous potential biomarkers, including metabolites, cells, proteins, and nucleic acids. As urine specimens demonstrate a high degree of variability, collection methods and processing procedures must be harmonized to ensure suitability for biomarker research.

As with any type of laboratory specimen, there are certain criteria that need to be met for proper collection and transportation of urine specimens. The following will ensure proper stability of the specimen and more accurate downstream testing results:

- All urine collection and transport containers should be clean and free of particles or other interfering substances.
- The collection and transport container should have a secure leak-resistant lid, and secure closures to prevent specimen loss and to protect the specimen from contaminants.
- For safety, containers should be made of durable break-resistant plastic (not glass).
- Specimen containers should not be reused.
- Use of a primary collection container that holds at least 120 mL, with a wide base and opening to prevents spillage and ensure adequate urine collection.
- Proper labeling should be applied to all collection container or tubes.
- Refrigerate the urine specimen immediately after collection, or store in cooler with ice-packs.
- The urine specimen should be received at the laboratory within 24 hours of collection and immediately processed (same day delivery is preferred).

As with other specimens suspected to contain potentially infectious agents, urine specimens must be transported in compliance with local and national regulations governing the transport of potentially infectious materials. The primary specimen container (i.e., urine collection cup)

must be leak-proof, tightly closed, and placed in zip-lock bag with sufficient absorbent material to cushion the specimen and absorb any potential leakage during transport. Staff handling the packaging and transport of potentially infectious materials must be IATA trained and certified; certification must be renewed every 2 years, or per local regulations.

3.3 Materials and Supplies for Urine Specimen Collection

The following materials are needed to collect the participant's urine specimen:

- Specimen labels
- Sterile, wide-mouth, polypropylene urine collection cup (120 mL)
- Urine specimen collection bag (for pediatric specimen collections)
- Antiseptic wipes and paper towels
- Refrigerator
- Insulated cooler and ice packs
- Leak-proof biohazard specimen bags with absorbent material
- Clean (boiled or filtered) water for drinking to stimulate urine production, if necessary/available
- Specimen Transport Form – Blood, Urine, Saliva (Form 95)
- Permanent marker

3.4 Procedures for Urine Specimen Collection (Adults and Older Children)

A spot (random, clean-catch, mid-stream) urine specimen is preferred as it reduces the incidence of cellular and microbial contamination. Avoiding the introduction of contaminants is critical to the specimen collection process, thus specific instructions should be provided to the participant.

Approximately 10 mL of urine will be collected at each scheduled participant visit. No preservatives or other chemicals will be added to the specimens, so it is very important to place the urine specimens in the transport cooler and immediately transport to the laboratory processing center.

3.4.1 Clean-Catch Urine Collection

The following collection technique will minimize external contamination and will allow the urine specimen to be thawed and used for laboratory tests years after it is collected:

1. Collect urine specimen in a disposable, wide-mouth container.
2. Prior to collection, label the specimen container with the appropriate identifying label, including study/screening number, participant ID number, visit number, and date and time of collection.
3. Positively identify the participant by his/her name and PID number.

4. Instruct the participant to provide at least 10 mL of urine, if possible.
5. For the clean-catch procedure, instruct the participant to:
    a) Wash his/her hands with soap and water; if available, follow with alcohol-based hand sanitizer. Give the participant clean disposable paper towels to dry his/her hands.
    b) Cleanse the genital area with an antiseptic towelette.
    c) Open the urine collection container.
    d) Do not touch the inside of the cup or cup lid.
    e) Void the first portion of the urine stream into the toilet.
    f) Collect the middle (mid-stream) portion of the urine specimen in the container.
    g) Tightly secure the lid on the container.
    h) Wash his/her hands with soap and water.
    i) Return the collected urine to the clinic nurse.
6. Immediately after collection, place the urine specimen in the refrigerator or in a cooler with ice packs, and transport to the laboratory for processing within 24 hours of collection (same day delivery is preferred).
7. Refer to Specimen Transport Guidelines in Section 3.2 above.

3.5 Collection of Pediatric Urine Specimens

The most popular non-invasive method used for non-toilet-trained children is the clean-catch procedure described above; however, for children who are too young to collect a urine specimen, pediatric urine specimen collection bags can be used. This can be done at the hospital or in the participant's home. As bacteria are often present in the genital area it is very important to clean this area properly with soap/water or towelettes prior to attempting to collect a urine specimen.

1. Clinic staff must thoroughly cleanse and dry hands prior to initiating the urine collection procedure. Gloves must be worn during the procedure, and changed in between participant collections.

2. Explain the urine collection procedures to the parent/guardian.

3. Thoroughly cleanse the genital area with pre-saturated towelettes; being careful to avoid contamination from the rectal area. Clean from the front to the back on a female infant, and from the tip of the penis down on a male infant. Repeat 3 times. Discard towelette after each use.

4. Ensure the area is completely dry prior to applying the specimen collection bag. Once the cleansing procedure is completed do not touch the area again.

5. Apply and adhere the specimen collection bag per instructions provided. For infant males, place the entire penis in the bag and attach the adhesive to the skin. For female infants, place the bag over the labia. Once in place, a diaper may be applied securely over the specimen collection bag.

6. Check the specimen collection bag every 15 minutes to confirm if the child has urinated.

7. To prevent skin irritation, remove the bag as soon as a urine specimen is available or within 30 minutes, if no specimen is collected.

8. Once the child has urinated, gently remove the bag from the child's body.

9. Pour the contents of the specimen collection bag into a sterile urine collection container. Do not allow the outer part of the bag to touch the urine, and do not touch the inside of the cup or cup lid.

10. Tightly secure the lid on the container.

11. Apply the appropriate specimen label to the urine collection container (including Study ID, PID, and date/time of collection).

12. Remove your gloves and thoroughly wash/dry your hands.

13. Immediately after collection, place the urine specimen in the refrigerator or in a cooler with ice packs, and transport to the laboratory for processing within 8-24 hours of collection (same day delivery is preferred).

14. Refer to Specimen Transport Guidelines in Section 3.2 above.

15. Record collection details on the Specimen Transport Form – Blood, Urine, Saliva (Form 95).

3.6 Receipt of Urine Specimens at the Processing Laboratory

Immediately upon arrival at the laboratory, urine containers are removed from the cooler and placed in refrigerator until processed. Before specimens are processed, designated laboratory staff must compare the information (PID, study/screening number, and date/time of collection) on the specimen container labels with that on the accompanying specimen transport form to make sure they match. If they do not match or information is missing, the laboratory must contact the clinical site to obtain any outstanding/missing information before the specimen is processed. In addition, the laboratory staff must record the date and time the specimens are received, carry out a visual check of the specimens to confirm they are of appropriate volume

and the container is not leaking, and record information on the accompanying Specimen Transport Form - Blood, Urine, Saliva (Form 95).

3.7 Materials Needed for Processing and Storage of Urine Specimens

- Barcoded specimen labels
- Barcoded cryostorage box identification labels
- Preprinted cryostorage box specimen orientation
- 2 mL self-standing, graduated cryovials, with external thread
- Sterile, serological pipettes, 5 mL or 10 mL
- Pipette aid
- Specimen Storage Form – Blood, Urine, Saliva (Form 96)
- Biomarker Freezer Log
- Freezers, -20°C, -80°C
- 2-inch cryostorage box
- Cryomarker Pen

3.8 Urine Specimen Processing and Storage Procedures

Urine specimens should be stored in 2-inch cryostorage boxes, with 9x9 (81 cell) or 10x10 (100 cell) sectioned inserts. Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number designation (see Figure 1).

1. Label 8 cryovials with the appropriate identifying barcoded label.
2. Thoroughly mix the urine before aliquoting.
3. Using a sterile serological pipette, aliquot 1 mL of urine into each cryovial. Prepare up to 8 aliquots and appropriately discard any remaining urine. Tightly seal each cap.
4. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.
5. Label the outside of the cryostorage box. Affix one copy of the cryostorage box label to the side of the cryostorage box lid, and the other label to one side of the cryostorage box bottom.

6. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
7. Begin storing cryovials from the top left hand corner of the box, moving vertically.

8. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
9. After storing an entire collection of specimens from one participants visit, begin storing the next set of participant specimens adjacent to the previous collection.
10. Place the cryostorage box in the -80°C freezer.
11. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'Urine' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    d) Indicate the Box ID (e.g. UR101-001) on the Biomarker Freezer Log; also note the local freezer storage location for future reference (freezer, shelf, rack).
    e) See example completed Biomarker Freezer Log in Figure 2 below.
12. The person responsible for processing the urine specimens will record the time the specimen was received in the laboratory and the number of cryovials stored on the Specimen Storage Form – Blood, Urine, Saliva (Form 96).
13. A comment documenting any problems with processing the urine specimens should be added.

4.0 Blood Specimen Collection, Processing, and Storage

4.1 Blood Specimen Collection Schedule

Whole blood for the Common Protocol will be collected at 4 scheduled visits for **Cohort A**– Baseline, Month 1, Month 2, and End of Treatment; and Baseline for **Cohort B** (Refer to Table 1, Section 1.1). During each visit, a total volume of up to 16.5 mL of blood is expected to be drawn for each adult participant; and up to 12.5 mL for pediatric participants under five years of age. Additional blood (up to 8 mL) may also be collected for other diagnostic testing (HIV, CD4, Hemoglobin A1C, etc.).

4.2 Blood Specimen Collection Tubes

- **BD Vacutainer Sodium Heparin Tubes:** Whole blood for preparation of plasma and peripheral blood mononuclear cells (PBMCs) will be collected using the BD Vacutainer® sodium heparin tubes. For adults and children ≥ 5 years old, 10 mL of whole blood will be collected for PBMC processing and storage; while for blood collection for children < 5 years of age, a 6.0 mL blood collection tube will be used.

- **BD Vacutainer EDTA Tubes:** For genotyping and other genetic analysis, a one-time collection of 4 mL of whole blood will be collected using BD Vacutainer® EDTA tubes at Baseline or at any scheduled visit, for both adults and children. EDTA (Ethylenediaminetetraacetic acid) functions by binding calcium in the blood and keeping the blood from clotting.

- **PAXgene Blood RNA Tubes:** PAXgene Blood RNA tubes will be used for the collection and stabilization of blood RNA. The PAXgene Blood RNA system consists of a blood collection tube and nucleic acid purification kit. It is intended for the collection, storage, and transport of blood, and facilitates stabilization of intracellular RNA for subsequent isolation and purification. The PAXgene Blood RNA tubes must be at room temperature (17-25°C) prior to use, and should be kept upright at all times.

- **QuantiFERON®-TB Gold Plus (QFT-Plus, 4th Generation):** The QuantiFERON assay is a blood-based interference-gamma release assay (IGRA) used in diagnosing latent MTB infection (and active TB disease). The QFT-Plus assay is based on the measurement of cell-mediated immune response to a cocktail of mycobacterial proteins (ESAT-6 and CFP-10), which are specific to MTB, and absent from all Bacille-Calmette-Guerin (BCG) strains and most non-tuberculosis mycobacteria (NTM). The test is interpreted using quantitative cutoff points to determine positive, negative, and indeterminate results; results are based on measurement of gamma interferon (INF-γ) which is produced in vitro by patient T-lymphocytes upon recognition of specific MTB antigens. For each of the four collection tubes (nil control, mitogen control, and TB1 and TB2 antigens), 1 mL

of whole blood will be collected. The blood collection tubes must be at room temperature (17-25°C) prior to use, and during blood collection.

4.3 Blood Collection Limits

In accordance with ethical considerations for biomedical research on human participants, blood collection volumes from adults and children (where the age, weight and health of the participants has been considered); **must not exceed 50 mL of blood or 3.5 mL/kg (whichever is lesser) in an 8 week period**, and collected at a frequency of no more than two times per week. For healthy participants, blood collection volumes of up to 500 mL (3.5 mL/kg) in an 8-week period are acceptable. Please consult your local IRB for applicable standards.

Refer to the **Blood Volume Weight Chart** (see Appendix I) for maximum blood volume collection limits. Please note that the Blood Volume Weight Chart is based on collection of blood from otherwise healthy participants at the limitation of 3.5 mL/kg per 24 hour collection. If blood volume is problematic, specimens will be prioritized as follows:

1. Whole blood for plasma and PBMC storage
2. Whole blood for QuantiFERON®-TB Gold Plus (QFT-Plus)
3. Whole blood for PAXgene Blood RNA storage
4. Whole blood for DNA

4.4 Order of Blood Draw

Blood collection tubes must be drawn in a specific order to avoid cross-contamination of additives between tubes. To ensure proper blood collection, specimens should be collected in the following order:

1. BD Vacutainer sodium heparin tube – 6 mL or 10 mL (for plasma and PBMCs)
2. BD Vacutainer EDTA tube – 4 mL (for whole blood DNA)
3. QuantiFERON®-TB Gold Plus – 4 x 1 mL (Nil, Mitogen, TB1 and TB2 antigen)
4. PAXgene Blood RNA tube – 2.5 mL

Technicians must be familiar with the arrangement of blood collection tubes, order in which tubes should be drawn, and possible sources of error in handling each tube, prior to initiating the phlebotomy process.

Please Note: The determination for which blood collection tubes will be drawn should be determined prior to initiating the blood collection process. For example, if blood collection limits are likely to be exceeded, or if problems with blood draw are anticipated, omit collection of the whole blood for DNA (BD EDTA tubes) and proceed with the order of draw as indicated.

4.5 Blood Collection Materials and Supplies

Many of the blood collection materials and supplies can be found in the specimen collection kit provided by the Central Biorepository; while other ancillary phlebotomy supplies listed must be provided by the clinic. The following materials are needed for collection of participant blood specimens.

- 21-gauge Becton-Dickinson (BD) butterfly needle collection set with safety-lock
- 6 mL or 10 mL BD CPT™ Vacutainer tube (for plasma and PBMCs) – 4 mL tubes will be used for pediatric participants under the age of 5 years; 8 mL tubes will be used for older children and adults
- 4 x 1 mL in QuantiFERON®-TB Gold Plus ('nil', 'mitogen', 'TB1 and 'TB2'')
- PAXgene Blood RNA collection tube (for RNA stabilization and extraction)
- 4 mL BD EDTA Vacutainer tube (for whole blood DNA)
- Specimen barcoded label(s)
- Insulated cooler with cold packs to transport participant samples
- Ambient temperature transport box
- Alcohol wipes and cotton wool balls
- Leak-proof biohazard plastic bags and absorbent sheets
- Sharps disposal containers for used needles and other biohazardous materials
- Calibrated volumetric pipette(s) (10µL to 1000µL) with disposable tips.
- Disposable gloves
- Specimen Transport Form – Blood, Urine, Saliva (Form 95)

4.6 Handling Participants Who Are Apprehensive About Blood Draws

Some participants may be apprehensive about having blood drawn. Under no circumstances should a participant be forced to have blood drawn; however below are suggestions for alleviating their concerns if needed:

1. Ensure the participant is as comfortable as possible.
2. Explain to the participant that blood drawing is designed to be as painless and safe as possible.
3. Briefly describe the procedure, e.g. *"I am going to take about 2 tablespoons of blood in about 4-5 separate tubes. We hope to be able to use the test results to better understand the progression and treatment of Tuberculosis in adults and children"*.
4. If necessary, allow the participant to move onto another part of the visit, and revisit blood collection later in the process.
5. Allow the participant to relax in the blood drawing chair so the phlebotomist can check the veins in the participant's arms, without actually drawing blood.

6. Use the **Total Blood Volume (TBV) Collection Chart** (Figure 3, Appendix II) to demonstrate that the relative volume of blood being collected is less than 1-2% of their TBV, and less than what is typically recommended for blood draws, thus minimizing risk.

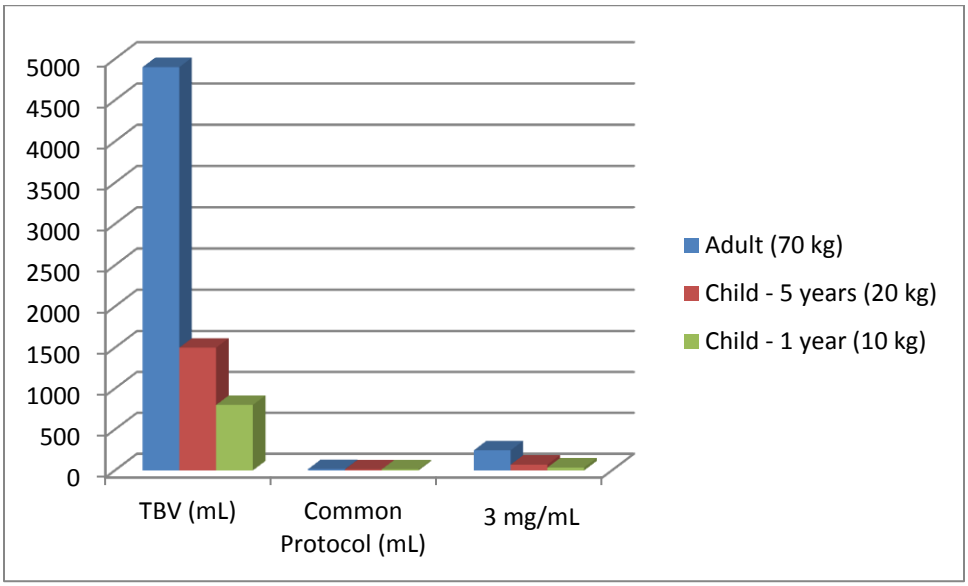


Figure 3: Total Blood Volume (TBV) Collection Chart.

The TBV Collection Chart indicates the average TBV in a healthy adult male (70 mL/kg) and a 5 year old (75 mL/kg) or 1 year old child (80 mL/kg) weighing 20 kg and 10 kg respectively; with respect to blood collections for the Common Protocol and established 3.5 mg/mL blood collection limits. Based on review of related studies for blood collection in children, the defined range for minimal risk is 1-5% of the TBV within 24 hours; and up to 10% TBV over 8 weeks ("*Blood sample volumes in child health research: review of safe limit", Bulletin of the World Health Organization 2011, Stephen RC Howie*). The maximum amount of blood to be collected (3.5 mL/kg) is within this range at less than 5% of the TBV. Respectively, blood collections for the Common Protocol (up to 16.5 mL for adults and older children, and up to 12.5 mL for children under 5 years old) represent less than 1-2% of the TBV. Please note however, TBV may differ in otherwise unhealthy participants, and caution should be taken when assessing these individuals.

4.7 Blood Collection Procedure (Venipuncture)

Prior to initiating the phlebotomy process, the clinic nurse must attach the provided barcoded specimen labels to all collection and transport tubes for each study visit. All tubes must be appropriately labeled. Specimen collection tubes must be appropriately labeled with the study ID, PID, visit number, and date and time of collection. **Each phlebotomist should be permitted only two unsuccessful venipuncture's per participant.**

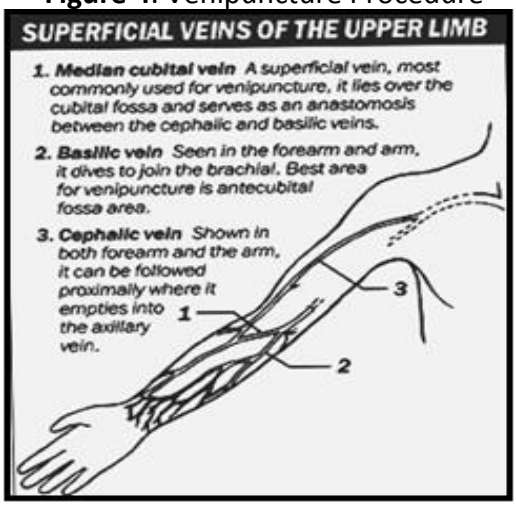
Blood and Body Fluid Precautions designed to reduce the risk of transmission of blood borne pathogens applies to all procedures in the health care setting regardless of the diagnosis or presumed infection status. Clinic staff should observe universal (standard) safety precautions with regard to potentially infectious specimens

The following are recommended guidelines for blood specimen collection. These are outlined in accordance with the order of blood draw outlined in Section 4.4; however can be adapted accordingly to accommodate collection of other blood collection tubes. If your site/institution has specific guidelines for blood drawing, incorporate those as necessary.

Trained personnel at each site must adhere to all local guidelines for blood collection. Gloves and appropriate PPE (laboratory coat, goggles, etc.) must be worn at all times when handling specimens.

1. Confirm that the participant has signed the informed consent agreement.
2. Confirm the participant's identity (e.g., name/date of birth) and verify the PID.
3. Prepare the blood collection supplies and equipment to be used for venipuncture. Assemble blood collection tubes according to the order of blood draw.
4. **Ensure that all blood collection assemblies are fitted securely, to avoid frothing; and inspect tubes for damage and expiration dates.**
5. Position the participant to ensure they are comfortable.

Figure 4. Venipuncture Procedure



6. Gloves should be worn for venipuncture procedure; a new pair of disposable gloves should be used for each participant.
7. Decontaminate and/or wash hands after glove removal and before donning a new pair in between participants.
8. Inspect both arms to select the venipuncture site. Specimens must be collected from the median cubital vein in the antecubital fossa (See Figure 4, the side of the arm within the fold of the elbow). Take your time to locate a good vein before sticking the participant. Each phlebotomist should be permitted only two unsuccessful venipuncture's per participant.
9. Position the arm so that it is resting on a table or on the bed alongside the participant. The arm should be supported firmly and should not be bent at the elbow. A pillow placed under the arm to provide additional support may be used if necessary.
10. Prepare the participant for venipuncture using aseptic technique. Cleanse the venipuncture site with alcohol or antiseptic wipe using a circular motion from the center

to the periphery. Allow the area to dry completely before performing the venipuncture. Not allowing the site to dry may cause hemolysis.

11. Apply the tourniquet. The tourniquet should not be applied for longer than 1-2 minutes at a time. Prolonged tourniquet time causes the interstitial fluid to leak into the tissue and may cause hemolysis.
12. Anchor the vein and smoothly insert the needle bevel up (to minimize the possibility of hemolysis).
13. During the collection procedure hold the participant's arm in the downward position below the level of the heart, as gravity will improve the blood flow.
14. Push the first Vacutainer tube (**BD sodium heparin**) gently into the holder, puncturing the diaphragm of stopper, and initiating blood flow. *A reduced draw of approximately 0.5 mL is expected on the first tube due to trapped air in the blood collection set tubing*.
15. Release the tourniquet as soon as the blood starts to flow into the tube, or within 1-2 minutes of application. If blood flow ceases after the tourniquet is removed, it may be reapplied for another 2 minutes. Because so many tubes are being collected sequentially, it may be necessary to have the participant periodically make a fist and release his hand several times to maintain good blood flow.
16. Once the Vacutainer tube is filled, remove it and **invert** it gently **8-10 times** to thoroughly mix the anticoagulant with the blood.
17. Next, insert the next Vacutainer tube (**BD EDTA**) gently into the Vacutainer holder, again puncturing the diaphragm of the stopper.
18. Once the Vacutainer tube is filled, remove it and gently **invert 8-10 times** to thoroughly mix the EDTA anticoagulant with the blood.

NOTE: The following steps must be followed precisely

19. Insert the first of the four **QFT-Plus** tubes directly into the cap of the butterfly needle. If this is the first tube of blood collected, and the butterfly needle is used; ensure an empty "purge tube" is used prior to filling the QFT-Plus tubes.

    NOTE: If desired, blood may be collected in a single generic tube and later transferred to the 4 QFT-Plus tubes. If so, a minimum of 5 mL of blood should be collected in a littium heparin tube, and maintained at 17-25°C before transferring to the QFT-Plus tubes for incubation. Regardless, incubation must be initiated within 16 hours of blood collection.

    a) Tubes should be between 17-25°C at the time of blood draw.

    b) Hold the tube vertically below the participants arm while collecting the blood.

    c) Each tube is designed to draw exactly 1 mL of blood – the black mark on the side of the tubes indicates the validated range (0.8 – 1.2 mL). If the level of blood is outside or under this range, collect another sample.

d) As 1 mL tubes draw relatively slowly, keep the tube on the needle for 2-3 seconds once the tube appears to have completed filling to ensure that the correct volume is drawn. The blood collection tubes are designed to draw between 0.8 and 1.2 mL of blood. Under or overfilling the tube may lead to erroneous results.

e) Remove the needle from the tube, and then repeat steps to collect **QFT-Plus** tubes 2, 3, and 4.

f) Gently, but firmly, shake all 4 filled tubes **10 times** (up and down) to ensure that the entire inner surface of the tube is coated with blood to solubilize antigens on the side walls. **Over-energetic or overly vigorous shaking may cause gel disruption and could lead to aberrant results.**

g) Place the tubes in a rack. QFT-Plus tubes may be stored at room temperature (17-25°C) for up to 16 hours post collection, or immediately incubated at 37±1°C for 16-24 hours (See Section 4.13 for additional details); **do not refrigerate or freeze**.

20. Next, insert the **PAXgene Blood RNA** tube into the Vacutainer holder. The PAXgene Blood RNA tubes should be the last tube in your order of draw.

    a) Tubes should be between 17-25°C at the time of blood draw.

    b) Hold the butterfly collection line and the attached PAXgene Blood RNA tube vertically below the participant's arm while collecting the blood.

    c) Allow the PAXgene Blood RNA tube to stay in place at least **10 seconds** to ensure that the tube fills completely.

    d) Confirm that blood has stopped flowing into the PAXgene Blood RNA tube before removing it from the holder.

    e) Immediately **invert** the PAXgene Blood RNA tube gently **10 times**, and then store it upright in the tube rack.

21. Once completed with the blood draw process, quickly remove the needle from the vein, pressing the safety button on the butterfly to completely retract the needle. Lightly place clean gauze over venipuncture site, remove the needle quickly, and immediately apply gentle pressure to the site with a gauze pad. Apply a clean cotton ball to the puncture site, and have the participant elevate his or her arm and press gently on the puncture site with the cotton ball for several minutes to stop any bleeding, and to prevent a hematoma from forming.

22. Lower the participant's arm, remove the gauze, and inspect the puncture site to make sure that the bleeding has stopped.

23. If bleeding continues, reapply firm pressure on the puncture site for several more minutes. If you are unable to stop the bleeding, call for assistance from the site investigator or physician.

24. When all bleeding has stopped, apply an adhesive bandage to the puncture site.
25. Apply the appropriate participant identifier labels to the tubes containing the collected blood.
26. Dispose of all needles in the Sharps container and used collection supplies in the container for biohazardous waste. DO NOT ATTEMPT TO RECAP NEEDLES.
27. Remove and discard gloves, and wash your hands thoroughly after you finish collecting blood from each participant.
28. Place all collected and labeled tubes in the labeled leak-proof bag for this participant in the cooler. Repeat this process for each participant visit.
29. Record the exact time of collection and visit details. If it takes several minutes to fill the tube, record the midpoint time. A comment documenting any problems with the blood collection should also be noted.

NOTE: If problems arise after blood begins to flow (e.g., the vein collapses), stop the procedure and try the other arm. If the other arm also fails to yield enough blood, stop trying to collect blood, and record "failed" on the participant's visit form.

4.8 Steps to Avoid Hemolysis

Hemolysis is the breakage of the red blood cell membrane, causing the release of the hemoglobin and other internal components into the surrounding fluid. Hemolysis may be due to pathological conditions, but more often hemolysis is caused by improper specimen collection, specimen processing, or specimen transport. Hemolyzed specimens will have a pinkish/reddish color to the serum rather than pale yellow color. The following steps should be taken to avoid hemolysis:

1. Avoid using a needle that is too small or too large to minimize mechanical damage of red blood cells
2. Draw blood from the veins in the antecubital fossa rather than the distal arm if possible
3. Excessive fist clenching and prolonged tourniquet time should be avoided
4. Avoid a traumatic venipuncture, do not probe for a vein
5. If using a needle and syringe, avoid drawing the syringe plunger back too forcefully
6. If using a needle and syringe, avoid pushing the plunger too forcefully when transferring blood to the Vacutainer tube
7. Avoid shaking the specimen once drawn
8. Centrifuge at the recommended RPM for the recommended time

4.9 Packaging and Transport of Blood Specimens to the Processing Laboratory

Staff handling the packaging and transport of potentially infectious materials must be IATA trained and certified; certification must be renewed every 2 years, or per local regulations. Blood specimens must be placed in a leak-proof biohazard bag and delivered to the laboratory as soon as possible after collection. All specimens must be transported in compliance with local and national regulations governing the transport of potentially infectious materials.

- **PAXgene Blood RNA** and **BD EDTA** tubes must be properly sealed, placed in a leak-proof biohazard bags (with absorbent sheets); and transported to the processing laboratory in the 2-8°C transport cooler. Maintain tubes in upright position. If needed, PAXgene Blood RNA tubes may also be transported at 17-25°C for up to 3 days.
- **QFT-Plus** tubes must be transported in an ambient temperature (17-25°C) transport container. **Note: The optimal transport temperature for transportation of the tubes is 17-27°C; temperatures above or below this range may lead to aberrant results; care should be taken to ensure proper transport temperature.**
- **BD sodium heparin tubes** must be properly sealed, placed in a leak-proof biohazard bags (with absorbent sheets); and transported in an ambient temperature (17-25°C) transport container.
- **All blood collection tubes must be received at the laboratory within 8 hours of collection.**
- Complete the Specimen Transport Form - Blood, Urine, Saliva (Form 95), seal in a separate zip-lock bag, and submit along with the participant samples to the laboratory for processing.

4.10 Equipment and Materials Needed for Processing Blood Specimens

At minimum, all processing laboratories should be equipped with the following:

- Class II BSC
- Micropipettes (20 µL, 200 µL, 1000 µL)
- Disposable, graduated, fine-tipped bulb transfer pipettes
- Sterile precision pipet tips, 20-1000 µL
- 2 mL self-standing graduated cryovials with external thread screw cap and silicone washer seal, suitable for long term vapor-phase LN2 storage
- Pipet-Aid and disposable, serological pipets
- Preprinted barcoded labels for specimen aliquots
- Bench-top centrifuge with a sealed swinging bucket rotor; capable of speeds up to 1800g
- Tube racks to use during sample processing and for sample storage
- Refrigerator (2 – 8°C)
- Freezer (-20°C) for temporary storage up to 24 hours post-processing

- Freezer (-80°C) for specimen storage prior to shipping to the central Biorepository.
- NALGENE® "Mr. Frosty", Biocision® "CoolCell", or Stratagene StrataCooler® for PBMC processing
- Automated or manual cell counter (e.g., Hemocytometer)
- Permanent markers for marking processing tubes and vials (fine point, fast-drying indelible ink)
- Specimen Storage Form - Blood, Urine, Saliva (Form 96)

Each laboratory freezer should be equipped with an audible alarm system to monitor temperature fluctuations or power failures; a chart recorder system or system of internal temperature monitoring on a monthly basis; and a back-up power supply (diesel or gasoline-powered generator) with at least 72 hours' worth of fuel on hand at all times. Routine temperature monitoring and documentation must be available for all units.

4.11 Immediate Blood Specimen Handling Procedures

The quality of the assay results depends upon the proper handling and storage of the clinical material prior to testing. Delays in processing should be avoided and care taken to expedite final disposition of materials. The following steps should be taken immediately upon receipt of participant samples from the clinic site:

1. Review the Specimen Transport Form – Blood, Urine, Saliva (Form 95) and other information received from the clinic.
2. Inspect contents of the cooler to ensure that samples are intact and sealed in plastic leak-proof bags.
3. Confirm all blood samples are present per participant (as indicated on the transport form), and labeled as appropriate.
4. Compare specimen ID labels to the information recorded on the Specimen Transport Form – Blood, Urine, Saliva (Form 95); note any discrepancies and notify the clinic.
5. Unlabeled specimens should not be processed.

In preparation for processing blood specimen, assemble all processing supplies (including pipets, tips, cryovials, labels, and racks) under the biosafety class II hood. To minimize the chance of confusing specimen sets with each other, label the racks appropriately. In addition, adhere to the following guidelines:

- Ensure that the centrifuge is in good condition and that the tubes are properly closed and balanced to avoid breakage and spilling.
- Refer to the Centrifuge Operating Manual for specific operating and balancing instructions.
- **Always use the Relative Centrifugal Force (RCF) or g values** when centrifuging blood samples; not Revolutions Per Minute (RPM). If your centrifuge does not display RCF values, consult the manual for a conversion chart or use the following formula:

$$RCF = 1.12r\ (RPM/1000)^2$$

r = radius in millimeters (distance from center of rotor pin to the middle of the swinging bucket or center of fixed angle rotor)

- Use a disposable, plastic-backed, absorbent countertop liner for each specimen processed to avoid cross-contamination.
- No more than 4 to 5 participant specimens should be processed at any given time.

4.12 Processing and Storage of Plasma and PBMCs

PBMC processing and storage must be performed in accordance with the **RePORT PBMC SOP (**Appendix V**)**: *Whole Blood Separation of PBMCs Using Manual Density Gradient Media Overlay Method.*

To minimize cell degradation and possible cell loss, all blood collection tubes must be received at the laboratory and centrifuged within 8 hours of collection.

- All pipetting and mixing procedures must be performed in a BSC, class II, or greater.
- All surfaces, racks, and reagent bottles, must be sprayed with 70% ethanol or equivalent disinfectant prior to entering the BSC.
- Unless otherwise noted, the PBMC processing procedure is carried out at room temperature.
- Ensure all tubes are at room temperature prior to processing.
- Prepare and chill the PBMC freezing medium (see instructions below).
- Prepare Mr. Frosty or CoolCell freezing container per manufacturer's instructions.

4.12.1 Materials and Supplies Needed for PBMC and Plasma Processing

- Barcoded specimen labels
- Barcoded cryostorage box identification labels
- Trypan Blue Stain (0.4%)
- Hank's Balanced Salt Solution (HBSS) or Phosphate-Buffered Saline (PBS), without calcium or magnesium
- Fetal Bovine Serum (FBS, heat inactivated, see instructions below)
- 70% Ethanol, or other appropriate disinfectant (e.g. 10% bleach)
- Dimethylsulfoxide (DMSO), cell-culture grade
- Disposable, graduated, fine-tipped bulb transfer pipettes
- 2 mL self-standing graduated cryovials with external thread screw cap and silicone washer seal, suitable for long term vapor-phase LN2 storage
- Micropipettes (20 µL, 200 µL, 1000 µL) and pipet tips
- Pipet-Aid and disposable, serological pipets (2 mL, 5 mL, 15 mL)
- NALGENE® "Mr. Frosty", Biocision® "CoolCell", or Stratagene StrataCooler® for PBMC processing

- Automated or manual cell counter (e.g. Hemocytometer)
- 15 mL polypropylene centrifuge tubes, sterile, conical bottom
- Permanent markers for marking processing tubes and vials (fine point, indelible ink)
- **RePORT PBMC SOP #001**: Whole Blood Separation of PBMCs Using Manual Density Gradient Media Overlay Method (Appendix V)
- **PBMC Processing Worksheet** (Appendix VI)
- Specimen Storage Form – Blood, Urine, Saliva (Form 96)

4.12.2 Preparation of Heat-Inactivated Fetal Bovine Serum (FBS)

Heat inactivated FBS is preferred for long-term storage of PBMCs. Heat inactivated FBS may be purchased commercially; alternatively FBS can be purchased and heat inactivated in the laboratory using the following instructions:

1. Thaw frozen FBS overnight in a refrigerator (2-8°C), or for several hours at room temperature. Caution, do not allow FBS to sit at room temperature longer than necessary to allow complete thawing.
2. Place FBS in a 56°C water bath for 30 minutes, with gentle mixing every 5-10 minutes. Carefully monitor the water temperature; high temperatures can degrade components of the FBS. The water level of the bath should not exceed the level of cap on the bottle.
3. Wipe the top of the FBS bottle with 70% ethanol prior to opening.
4. In a BSC, aliquot the heat-inactivated FBS into 50 mL conical centrifuge tubes, or other size appropriate for anticipated workload.
5. Label each tube with the contents (FBS, heat inactivated), preparation and expiration date, and initials of technician.
6. The heat-inactivated FBS is stable for 1 month at 2-8°C or the original manufacturer's expiration date when stored at -20°C. Avoid repeated freeze thaws.
7. To use frozen aliquots, thaw overnight at 2-8°C or at room temperature for several hours. The expiration date should be adjusted to 1 month.

4.12.3 Preparation of PBMC Cryopreservation Solution (CPS)

PBMC CPS/freezing medium (90% FBS, 10% DMSO) must be prepared in advance and chilled on ice or place in a refrigerator (2-8°C) for at least 30 minutes prior to use. Follow the instructions below:

1. To prepare a 10 mL solution of PBMC CPS, mix 9 mL of heat-inactivated FBS with 1 mL DMSO. Adjust accordingly as needed to prepare larger or smaller volumes.
2. Prepare only the amount of CPS you expect to use within one working day (18 hours), depending on expected workload.
3. Freezing medium may be **stored at 2-8°C for up to 18 hours**.

4.12.4 Instructions for Processing PBMC and Plasma Specimens

PBMC processing and storage must be performed in accordance with the **RePORT PBMC SOP (Appendix V)**: *Whole Blood Separation of PBMCs Using Manual Density Gradient Media Overlay Method.*

1. PBMC cell pellets must be re-suspended in freshly prepared PBMC CPS at 5 x 10^6 viable cells per mL.
2. Prepare 1-4 aliquot of 1.0-1.5 mL each in pre-labeled 2 mL cryovials.
3. Transfer the cryovials to the "Mr. Frosty" or "Cool Cell" box and store at -80°C (Prepared according to manufacturer's instructions).
4. Once frozen, PBMCs should be transferred to a 2-inch cryostorage box and stored at -80°C until shipment to the Central Biorepository. See storage instructions in Section 4.12.5.
5. Do not store PBMC's in liquid nitrogen (LN2) unless resources are available for transportation in LN2 to the Central Biorepository.
6. **PBMCs should be stored at -80°C for no longer than 3-5 weeks prior to shipping to the Central Biorepository; where they will be transferred to LN2 for long term storage. See local storage instructions in Section 4.12.5 below.**
7. Aliquot the collected **plasma** in aliquots of 150 µL and **store at -80°C** until shipment.
    a) Prepare and label up to 8 cryovials with the appropriate barcoded specimen label
    b) Aliquot 150-200 µL of plasma into each cryovial.
    c) Store at -80°C as described in Section 4.12.5 below.
8. Complete the **PBMC Processing Worksheet** (Appendix IV).
9. Complete all sections of the Specimen Storage Form – Blood, Urine, Saliva (Form 96)

4.12.5 Storage of PBMC and Plasma Aliquots

PBMC and plasma aliquots should be stored separately in 2-inch cryostorage boxes, with 9x9 (81 cell) or 10x10 (100 cell) sectioned inserts. Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number designation (see Figure 1). Two labels will be used to identify each cryostorage box.

1. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.

2. Label the outside of the cryostorage box. Affix one copy of the cryostorage box label to the side of the cryostorage box lid, and the other label to one side of the cryostorage box bottom.

1. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
2. Begin storing cryovials from the top left hand corner of the box, moving vertically.
3. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
4. After storing an entire collection of specimens from one participants visit, begin storing the next set of participant specimens adjacent to the previous collection.
5. Place the cryostorage box in the -80^{o}C freezer.
6. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'Plasma' or 'PBMC' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    f) Indicate the Box ID (e.g. SP101-001) on the Biomarker Freezer Log, and note the freezer storage location (freezer, shelf, rack) for future reference.
    d) See example completed Biomarker Freezer Log in Figure 2 below.
7. The person responsible for processing the specimens will record the time the specimens were received in the laboratory and the number of cryovials stored on the Specimen Storage Form – Blood, Urine, Saliva (Form 96). A comment documenting any problems with processing the specimens should be added.

4.13 Processing and Storage of QuantiFERON®-TB Gold Plus (QFT-Plus)

The blood specimens from the four **QFT-Plus** tubes will be used for unstimulated (Nil), TB antigen-stimulated (TB1, TB2) and mitogen-stimulated (M) assays. These assays may not be done immediately, but the specimens will be initially incubated, processed, and subsequently frozen and stored to preserve their integrity. It is critical that the tubes be maintained at room temperature (17-27°C) during transport, and prior to initial processing. All processing should be performed while working under the Class II BSC.

The following should be performed immediately upon receipt of the QuantiFERON tubes at the laboratory:

1. As soon as possible after specimens are received from the clinic (or within 16 hours of collection), incubate the tubes upright in a rack in a **37°C incubator** (CO_2 is not required) for **16 to 24 hours**; if the blood was not incubated immediately after collection, remix tubes by gently inverting 10 times prior to incubation.
2. After incubating at 37±1°C for 16-24 hours, centrifuge the tubes at **2000–3000g** (RCF) for **15 minutes**.
    a) **NOTE**: The blood collection tubes contain a gel plug that separates the plasma from the cells when centrifuged; if the gel plug does not move to separate the cells from the plasma (as may be evident with refrigerated tubes), the tubes should be re-centrifuged at a higher speed.
3. Prepare and label 2 microfuge tubes for each of the 4 QFT-Plus tubes (TB1, TB2, Nil, Mitogen) with the appropriate specimen barcoded label (total 8 tubes per participant).
4. Harvest **150-200 µL** of **plasma** from each tube and **aliquot at 100µL** per 0.5 mL microfuge tube.
5. Place filled vials upright in a rack at −80°C (see instructions below).
6. Complete the Specimen Storage Form – Blood, Urine, Saliva (Form 96)

If required, after incubation the concentration of IFN-γ in the separated plasma can be determined using the QuantiFERON®-TB Gold enzyme-linked immunosorbent assay (ELISA) kit. Performance of the ELISA should be done in accordance with the QuantiFERON®-TB Gold Plus (QFT-Plus) package insert.

4.13.1 Storage of Stimulated Plasma from QuantiFERON®-TB Gold Plus tubes

Stimulated plasma aliquots from the **QFT-plus** should be stored in 2-inch cryostorage box, with 10x10 (100 cell) sectioned inserts. Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number designation (see Figure 1). One set of labels should be used for each cryostorage box.

1. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.
2. Label the outside of the cryostorage box. Affix each label to one side of the cryostorage box lid, and to one side of the cryostorage box bottom.

3. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
4. Begin storing cryovials from the top left hand corner of the box, moving vertically.
5. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
6. Place the cryostorage box in the -80^{o}C freezer.
7. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'QuantiFERON' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    d) Continue storing the next set of participant specimens as described above.
    e) Indicate the Box ID (e.g. QF101-001) on the Biomarker Freezer Log; also note the local freezer storage location for future reference (freezer, shelf, rack).
    f) See example completed Biomarker Freezer Log in Figure 2 below.
8. The person responsible for processing the specimens will record the time the specimens were received in the laboratory and the number of cryovials stored on the Specimen Storage Form – Blood, Urine, Saliva (Form 96). A comment documenting any problems with processing the specimens should be added.

4.13.2 Considerations for Use of QuantiFERON®-TB Gold In-Tube (3rd Generation)

The 3rd generation version of the QuantiFERON assay (QuantiFERON®-TB Gold In Tube, QFT-GIT) consists of 3 blood collection tubes – nil control, mitogen control, and TB antigen tube containaing a cocktail of 3 mycobacterial antigens (ESAT-6, CFP-10, and TB 7.7). With the exception of an additional antigen tube used to measure CD8+ T cell responses, and removal of the TB 7.7 antigen, blood collection and processing procedures for the QFT-GIT assay are identical to that of the current 4th generation QFT-Plus assay. QFT-Plus was recently CE-IVD (In Vitro Diagnostic) marked, and is now being made available in Europe and other markets. As use of the QFT-Plus assay expands over the next few years, it is expected to gradually replace the QFT-GIT assay; and as such the QFT-Plus assay will be used exclusively for the RePORT Common Protocol. However, for additional reference, the QFT-GIT package insert and video instructions for blood collection, incubation, processing, and ELISA and data analysis, can be accessed by clicking the links below. With the exception of the additional antigen tube, and other notations listed above, the blood collection, transport and incubation instructions referenced in the instructional videos (Part I) are also relevant for the QFT-Plus assay.

- QuantiFERON®-TB Gold In-tube (QFT-GIT) Package Insert

- QuantiFERON (QFT-GIT) Instruction Video Part I: Blood Collection
- QuantiFERON (QFT-GIT) Instruction Video Part II: Blood Incubation, ELISA, Data Analysis
- QuantiFERON (QFT-GIT) Instruction Video Part III: ELISA Procedure

4.14 Processing and Storage of PAXgene Blood RNA Tubes

1. **Immediately upon arrival at the laboratory**, remove the glass PAXgene Blood RNA tube from the cooler, affix barcoded specimen label provided, and place the tube in a wire rack at **-20°C for 24 hours** (freezing in a styrofoam tray may cause tubes to crack).
2. After the tube contents are frozen, move the rack to a -80°C freezer.
3. After the material is frozen (at least one day at -80°C), place each tube in an IATA-approved participant-labeled bag, and keep it in freezer until shipment to the Central Biorepository. Alternatively, PAXgene Blood RNA tubes can be stored upright in wire-rack at -80°C until prepared for shipment to the Central Biorepository.
4. If the PAXgene Blood RNA tube is not carefully handled in this manner, the tube can shatter. Tilting the tube slightly allows for expansion during freezing without pushing out the tube's stopper.
5. Complete the Specimen Storage Form – Blood, Urine, Saliva (Form 96)

4.15 Processing and Storage of Whole Blood for DNA

The whole blood EDTA tubes will be aliquoted and stored for future genomic DNA isolation and related genetic analysis. The whole blood specimen should be received in the laboratory and processed within 24 hours of collection.

4.15.1 Materials Needed for Processing and Storage of Whole Blood for DNA

- Barcoded specimen labels
- Preprinted cryostorage box identification labels
- Preprinted cryostorage box specimen orientation
- 2 mL self-standing, graduated cryovials, with external thread
- Sterile, serological pipettes, 1 mL or 5 mL
- Pipette aid
- Specimen Storage Form – Blood, Urine, Saliva (Form 96)
- Biomarker Freezer Log
- Freezers -80^{o}C
- 2-inch cryostorage box
- Cryomarker Pen

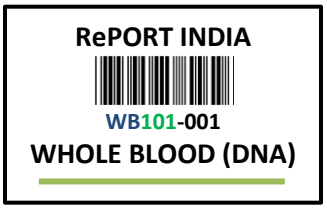
4.15.2 Procedure for Processing and Storage of Whole Blood for DNA

Upon receipt in the laboratory, remove the BD EDTA tube (lavender cap) from the cooler.

1. Prepare and label 4 cryovials with the appropriate barcoded label.
2. Using a sterile serological pipette, aliquot 1 mL of whole blood into each of the pre-labeled cryovials and store at -80°C as described below.

Whole blood aliquots should be stored in 2-inch cryostorage boxes, with 9x9 (81 cell) or 10x10 (100 cell) sectioned inserts. Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number designation (see Figure 1). One set of labels should be used for each cryostorage box.

1. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.
2. Label the outside of the cryostorage box. Affix one copy of the cryostorage box label to the side of the cryostorage box lid, and the other label to one side of the cryostorage box bottom.



1. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
2. Begin storing cryovials from the top left hand corner of the box, moving vertically.
3. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
4. After storing an entire collection of specimens from one participants visit, begin storing the next set of participant specimens adjacent to the previous collection.
5. Place the cryostorage box in the -80^{o}C freezer.
6. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'BLOOD (DNA)' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    d) Indicate the Box ID (e.g. WB101-001) on the Biomarker Freezer Log; also note the local freezer storage location for future reference (freezer, shelf, rack).
    e) See example completed Biomarker Freezer Log in Figure 2.

7. The person responsible for processing and storing the whole blood specimens will record the time the specimen was received in the laboratory and the number of cryovials stored on the Specimen Storage Form – Blood, Urine, Saliva(Form 96). A comment documenting any problems with processing the sputum specimen should be added.
8. Complete the form Specimen Storage Form – Blood, Urine, Saliva (Form 96).

5.0 Collection and Storage of Saliva Specimens

5.1 Saliva Specimen Collection Schedule

Saliva specimens will be collected for storage, DNA, and future genetic analyses. Up to 4 aliquots of 1 – 1.5 mL each will be collected per scheduled visit.

For participants who are eligible for enrollment in **Cohort A** (Active Pulmonary TB), saliva specimens will be collected at 2 scheduled visits – Baseline and End of Treatment (Refer to **Table 1**, Section 1.1). An additional specimen will be collected at the time of suspected treatment failure or relapse, or withdrawal from the study.

For participants enrolled in **Cohort B**, saliva specimens will be collected at Baseline and when/if the enrolled participant develops active TB.

5.2 Saliva Specimen Collection Guidelines

Following proper saliva collection and handling procedures will ensure a quality sample for future research purposes. To prevent contamination of saliva samples for DNA analysis, the following precautions are recommended.

- Use only single-use/disposable materials to prevent contamination between research participants.
- Clinic staff assisting with collections should wear gloves throughout the procedure, and avoid touching collection device materials and samples.
- Participants should not brush their teeth within 45 minutes prior to sample collection.
- Participants should be advised to rinse mouth with water to remove food residue prior to sample collection (**wait at least 10 minutes after rinsing to avoid sample dilution**).
- Where possible, avoid foods with high sugar or acidity (or high caffeine content), immediately before sample collection, as these may lower saliva pH and increase potential for bacterial growth.

- To minimize bacterial growth, collected saliva samples should be refrigerated or placed on ice bricks immediately after collection.
- Saliva samples visibly contaminated with blood should be discarded and recollected.

5.3 Procedure for Collection of Saliva Specimens

Saliva samples will be collected using the passive drool technique; which involves the collection of unstimulated whole saliva that pools on the floor of the mouth. Collection of saliva by passive drool technique will be facilitated using the SalivaBio Saliva Collection Aid (SCA) from SalivaBio (www.salivabio.com). The SCA is approved for use in adults and children ≥ 6 years old. For infants and children > 6 years old, collection of saliva will be facilitated through the use of either the SalivaBio Children's Swab (SCS) or SalivaBio Infant's Swab (SIS). Detailed instructions for the use of these saliva collection aides can be found in the Salimetrics Saliva Collection Handbook (*click to follow link*), and are summarized below for quick reference.

5.4 Collection of Saliva Specimens from Adults and Children ≥ 6 years old

The following procedures are for collection of saliva from adults and children ≥ 6 years old using the SalivaBio Saliva Collection Aid (SCA). Prior to initiating the procedure, assemble all required collection materials and provide detailed instructions to the participant.

- 2 mL cryovials
- Saliva Collection Aid (SCA)
- Barcoded Specimen Labels
- Cryostorage box

Clinic staff assisting with the procedure should thoroughly wash hands before and after the collection procedure, and in between each participant.

1. Apply barcoded specimen labels to cryovials
2. Remove the cap from the cryovial
3. Remove the SCA from packaging and place securely into the cryovial
4. Instruct participant to allow saliva to pool in the mouth. Some may find it helpful to imagine eating their favorite food
5. With head tilted forward, participants should drool through the SCA to collect the saliva in the cryovial
6. Repeat until sufficient sample is collected
7. Replace the cap onto cryovial.
8. Place filled/labeled cryovials in a leak-proof specimen collection bag with absorbent material

9. Transport specimens to the processing laboratory in a cooler with ice packs.

5.5 Collection of Saliva Specimens from Infants and Children < 6 years old

Although some younger children are able to provide saliva samples using the passive drool method, the use of an absorbent device is more commonly used for small children. With the increased potential for choking when collection devices are placed in the mouth, collecting saliva from infants and small children requires special consideration.

The following procedures are for collection of saliva from infants and children < 6 years old. The **SalivaBio Children's Swab (SCS)** should be used for saliva collection in children under 6 years old; while for infants less than 6 months of age, use the **SalivaBio Infant's Swab (SIS)**.
Prior to initiating the procedure, assemble all required collection materials and provide detailed instructions to the participant.

- SalivaBio Children's Swab (SCS) **OR** SalivaBio Infant's Swab (SIS)
- Saliva Storage Tube
- Barcoded Specimen Labels
- Cryostorage box (4")

Clinic staff assisting with the procedure should thoroughly wash hands before and after the collection procedure, and in between each participant.

1. Peel open the outer package of the SCS or SIS, and remove the device.
2. Securely hold one end of the device and try to place the other end under the child's tongue.
3. With infants it may only be possible to collect pooling saliva (often at the corners of the mouth or under the tongue).
4. Rest the swab against the inside of the child's mouth, or collect in intervals by re-inserting the swab into the mouth as needed until the lower third of the swab is saturated (60-90 seconds total).
5. Place the saturated SIS or SCS into the Swab Storage Tube for recovery by centrifugation, or use a 3-5 mL syringe for immediate compression

5.6 Receipt and Storage of Saliva Specimens

Upon receipt in the laboratory, remove the saliva specimens from the cooler. Saliva aliquots should be stored in 2-inch cryostorage boxes, with 9x9 (81 cell) or 10x10 (100 cell) sectioned inserts. Boxes should be maintained on dry-ice during transfer of tubes to boxes. All cryostorage boxes should be pre-labeled with the appropriate barcoded cryostorage box label indicating the specimen type, site ID, and box number designation (see Figure 1). One set of labels should be used for each cryostorage box.

1. Confirm that each cryovial has the appropriate preprinted barcode label and sample designation attached.
2. Label the outside of the cryostorage box. Affix each label to one side of the cryostorage box lid, and to one side of the cryostorage box bottom.

3. Affix the cryostorage box specimen orientation label (e.g. ↓Start) at the top of the left-hand corner of the cryostorage box to designate the starting position.
4. Begin storing cryovials from the top left hand corner of the box, moving vertically.
5. When finished with one column of the box, begin again at the top of the next column of the box directly to the right.
6. Place the cryostorage box in the -80^{o}C freezer.
7. All samples placed in the freezer should be noted on the **Biomarker Freezer Log** (Appendix II) to aid in rapid retrieval of specimens.
    a) Check off the box corresponding to 'SALIVA' at the top of the form.
    b) Use a barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that participant specimen.
    c) If necessary, draw an "X" to designate an empty box.
    d) Continue storing the next set of participant specimens as described above.
    e) Indicate the Box ID (e.g. SA101-001) on the Biomarker Freezer Log; also note the local freezer storage location for future reference (freezer, shelf, rack).
    f) See example completed Biomarker Freezer Log in Figure 2.
8. The person responsible for processing and storing the whole blood specimens will record the time the specimen was received in the laboratory and the number of cryovials stored on the Specimen Storage Form – Blood, Urine, Saliva (Form 96). A comment documenting any problems with processing the specimen should be added.

6.0 Packaging and Shipping Biomarker Specimens

All collected biomarker specimen aliquots (sputum, urine, PBMCs, plasma), MTB isolates, PAXgene Blood RNA, and whole blood for DNA, will be batch shipped to the Central Biorepository. Specimen shipping will occur at pre-specified times, to be arranged directly with those sites.

6.1 Procedure for Shipping Biomarker Specimens

When specimens are ready to be shipped, the **Central Biorepository** must be notified in advance and be prepared to accept the shipments. Notification should be sent to the Central Biorepository in two steps:

1. **24 to 48 hours** before the planned shipment, send an email to **[INSERT CONTACT INFORMATION HERE]**

2. The **Shipment Notification Fax** must be faxed or emailed to the Central Biorepository the day the specimens are scheduled for shipping. The Shipment Notification Fax should include the tracking number and the number of cryovials or boxes to be shipped.

Shipments should occur ONLY on Monday, Tuesday, or Wednesday. Specimens should NEVER be shipped on a holiday or the day before a holiday. Because these specimens are perishable, it is critical that this schedule be followed.

When shipping specimens to the Central Biorepository a collection of related documents will be sent with the specimens and will serve as the "packing slip"; these include:

- The Biomarker Freezer Log used to itemize the contents of the shipment. Each cryostorage box within the shipment will be accompanied by the original Biomarker Freezer Log corresponding to the specimens contained in the cryostorage box (a copy of the Biomarker Freezer Log should be maintained at the original laboratory for documentation purposes).
- A copy of the Shipment Notification Fax must be placed in an envelope or plastic sleeve and taped on top of the polystyrene cooler lid within the shipper.
- A copy of the Shipment Manifest printed from the FreezerPro LDMS detailing the contents of the shipment.
- All packages will be shipped to the Central Biorepository at the following address:

  The Director
  RePORT Central Biorepository
  [INSERT ADDRESS HERE]

Upon receipt of the specimens, the Central Biorepository will check the samples against the Biomarker Freezer Log to identify missing or damaged specimens. Sites will be notified of receipt of the shipment and any problems identified.

6.2 IATA Packaging and Shipping Guidelines

Packing and shipping of all specimens should be done according to local and national regulations, and in accordance with IATA shipping regulations. IATA approved shipping boxes and related shipping supplies will be provided to each site for packing and shipping specimens to the Central Biorepository.

All specimens must be tripled packaged in compliance with IATA packaging instructions for Category A or Category B infectious substances.

The **triple packaging system** consists of (1) primary specimen container, (2) leak-proof secondary container, and (3) a rigid and durable outer container. The primary container (e.g., specimen vial) holds the biological material and must be leak-proof and watertight. The secondary container (e.g., leak-proof biohazard bag) must be durable, watertight, and leak-proof; and capable of protecting the primary receptacle. If the primary container contains any liquid, sufficient absorbent material must also be used to absorb fluid in the event of breakage. The outer container (e.g., transport box) must be rigid and durable, and capable of housing the secondary container as well as withstanding physical damage during transit.

For samples of human origin, an international biohazard symbol must be displayed on the secondary container. All dry-ice shipments must also display the UN 1845 or STP-804 label as appropriate. An itemized packaging list should be placed in a leak-proof zip-lock bag, and be included inside the final package.

Below are instructions for packaging shipping **Category A and Category B infectious substances**. All MTB isolates must be packaged and shipped according to the instructions specified for Category A infectious materials; while all other specimens (urine, plasma, PBMC, sputum, PAXgene Blood RNA tubes, whole blood) must be packaged and shipped according to instructions for shipment of Category B infectious substances.

6.2.1 Category A Infectious Substances (MTB Isolates)

Category A infectious substances, specifically MTB isolates, must be tripled packaged and compliant with IATA Packing Instruction 620.

- Triple packaging must consist of leak proof primary receptacles, leak proof secondary packaging, and an outer package of sufficient strength to meet the design type test (1.2 meter drop test).
- Packages must be of good quality and strong enough to withstand the rigors of transport.

- The maximum quantity of a Category A infectious substance allowable on passenger aircraft is 50mL or 50g.
- The outer container of all Category A infectious substance packages must be appropriately labeled with the following:
  - Sender's name and address
  - Recipient's name and address
  - Infectious substance label
  - Proper shipping name and net quantity of infectious substance
  - Name and telephone number of person responsible for shipment
  - Cargo Aircraft Only label when shipping over 50mL or 50g
  - UN 2814 label
  - Class 9 label, including UN 1845, and net weight if packaged with dry ice
- Shipments must be prepared so they arrive in good condition and pose no hazard during transport.
- The primary receptacle or secondary packaging must be capable of withstanding a 95Kpa internal pressure differential.
- Packaging must contain absorbent material sufficient enough to absorb the entire contents of the shipment.
- An itemized list of contents must be included between the secondary and outer packaging.

6.2.2 Category B Infectious Substances (Blood, Urine, Saliva)

Category B infectious substances must be tripled packaged and compliant with IATA Packing Instruction 650. <u>These include all other specimens: sputum, urine, plasma, PBMCs, whole blood, and PAXgene Blood RNA collection tubes:</u>

- Triple packaging consisting of leak-proof primary containers, leak-proof secondary packaging, and outer packaging of sufficient strength to meet the design type test (1.2 meter drop test).
- Packages must be of good quality and strong enough to withstand the rigors of transport.
- The outer container of all Category B infectious substance packages must be appropriately labeled with the following:
  - Sender's name and address
  - Recipient's name and address
  - "Biological Substance, Category B" label
  - UN 3373 label
  - Class 9 label, including UN 1845, and net weight if packaged with dry ice

- Shipments must be prepared so they arrive in good condition and pose no hazard during transport.
- The primary receptacle or secondary packaging must be capable of withstanding a 95Kpa internal pressure differential.
- Packaging must contain absorbent material sufficient enough to absorb the entire contents of the shipment.
- An itemized list of contents must be included between the secondary and outer packaging.

6.3 Packaging and Shipment of Specimens to the Central Biorepository

Packing and shipping of all specimens must be done according to local and national regulations, and in accordance with IATA shipping regulations. Packaging systems for Category A and B insulated shipping containers can be purchased commercially and are equipped with required preprinted shipping labels (e.g., Biological Substance Category B, UN3373, etc.), outer insulated shipping box, disposable secondary container, and absorbent strips. However, provided below are general packaging instructions to assure a safe package for shipment, and compliance with IATA requirements.

1. Place the polystyrene insulated chest into the outer carton.
2. Place approximately 3-5 pounds dry ice in the bottom of the insulated shipping container.
3. Place a sheet of absorbent material (e.g., laboratory mat) on top of the dry ice so that it will be between the dry ice and the freezer boxes.
4. Collect all freezer boxes containing samples to be shipped.
5. Place a rubber band around each box to secure the lid.
6. Wrap an absorbent sheet around each box and secure lid with another rubber band.
7. Place each freezer box in a leak-proof biohazard bag and seal tightly.
8. Place each zip-locked freezer box in the insulated shipping container (plastic bags should not come in contact with dry-ice).
9. Add another layer of absorbent material (e.g., laboratory mat) on top of the freezer boxes in the shipping container.
10. Add an additional 3-5 pounds of dry-ice to the shipping container.
11. Close and tape the Styrofoam lid.
12. Enclose a copy of the Shipment Notification Fax and copies of all associated forms (Specimen Storage Forms, Biomarker Freezer Log, etc.) corresponding to the

specimens contained in the shipment in a plastic bag, and tape to the lid of the insulated chest.

13. Close the top of the outer cardboard sleeve of the shipping container with packing tape.
14. Label as appropriate for the shipment (See Section 6.3.1 below).

6.3.1 Labeling the Shippers

Label placement may be preprinted on the outer carton for convenience. Affix the labels in the appropriate designated location on the box (see example below):

- Affix the recipient and return address labels (including telephone numbers) in the designated places on the left upper corner of the shipper.
- Affix the Class 9 label in the designated place on the shipper.
- Print the net quantity of dry ice in kilograms on the Dry Ice UN1845 marking.
- Affix any other handling labels as appropriate on the shipper.

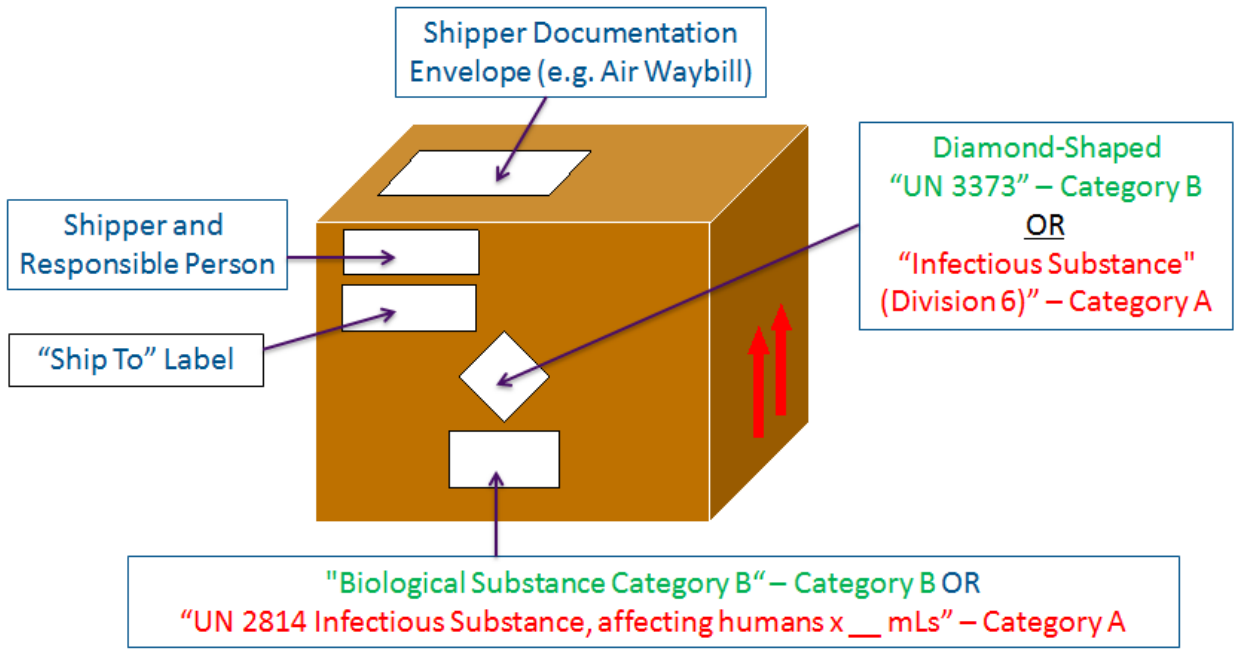


Figure 5. Labeling boxes for specimen shipment

6.4 Preparations for the Day of Shipment

The following tasks must be completed when shipping specimens to the Central Biorepository:

1. Select the specimens to be shipped and pack the specimens as described in Section 6.3.
2. Complete the specimen Shipment Notification Fax and email or fax to the Central Biorepository.
3. Include a copy of the Shipment Notification Fax, along with a copy of the Shipment Manifest, in the shipping container as described in Section 6.3 above.
4. Label the shipping container(s) with the appropriate label (See Section 6.4)

6.5 Reporting Problems with Shipments

If problems occur with a shipment at either end, tracking procedures should be initiated. The site should retain copies of the air bills and fax cover sheets.

7.0 Central Biorepository Storage Instructions

7.1 Receipt of Specimens

The Central Biorepository will track scheduled shipments and verify that shipments are received on schedule. If delivery of a shipment is delayed, the Central Biorepository will work with the transporter to expedite delivery.

- Shippers will be notified of receipt of shipments. The Central Biorepository will notify shippers if packages are not received on the expected day and will provide updates of the shipment status until arrival.
- The Central Biorepository will inventory shipments upon receipt. Shippers will be notified regarding specimens that are damaged or thawed during shipment.
- The Principal Investigator/Repository Director overseeing the Central Biorepository will notify the principal investigator at the shipping site to determine the final disposition of damaged or thawed specimens.
- The Central Biorepository will verify that specimens are shipped at the appropriate temperature and that shipments are appropriately packaged, labeled and accompanied by required documentation.
- The Central Biorepository will notify shippers of issues to minimize shipping delays and damage of specimens in future shipments.

7.2 Inventory of Specimens

Upon receipt of the shipment the biomarker specimens will be taken to the Central Biorepository for inventory, scanning, and storage.

- The Central Biorepository will check specimens against the corresponding freezer log to confirm receipt and will notify the collection site of any discrepancies, such as missing specimens, missing labels, incorrectly applied labels, empty vials, invalid dates, etc.
- The condition of specimens received will be noted on the Biomarker Specimen Tracking Form, section noted FOR CENTRAL BIOREPOSITORY USE ONLY.
- No personal identifiers should be written on specimen containers or associated documentation. If personal identifiers are found, the Central Biorepository will notify the shipper.
    - Personal information may be removed from the specimen containers depending on the extent of the problem.
    - Documentation with personal identifiers will be returned to the collection site.

- Each cryostorage box will be labeled with an identification code upon receipt at the Central Biorepository.
- The barcode label affixed to each cryovial will be scanned and stored in the Central Biorepository upon receipt. Specimens will be maintained at the appropriate temperature during inventory and the scanning process.

Immediately following scanning, each cryostorage box will be stored in the Central Biorepository freezer.

- All paper records accompanying each shipment received including the original freezer log will be filed together and maintained in a secure location.
- Following the specimen verification procedure, the PID number from the corresponding Form will be entered into the system.
- A distribution log will be maintained to document the dissemination of each specimen in storage; i.e., when specimens are shipped from the Central Biorepository to the central laboratory performing analyses.

NOTE: A document containing specific information regarding to whom and how specimens will be transferred, including delineation of issues such as intellectual property and publication rights (if applicable) may be required prior to initiating shipment of specimens from the Central Biorepository to an outside entity.

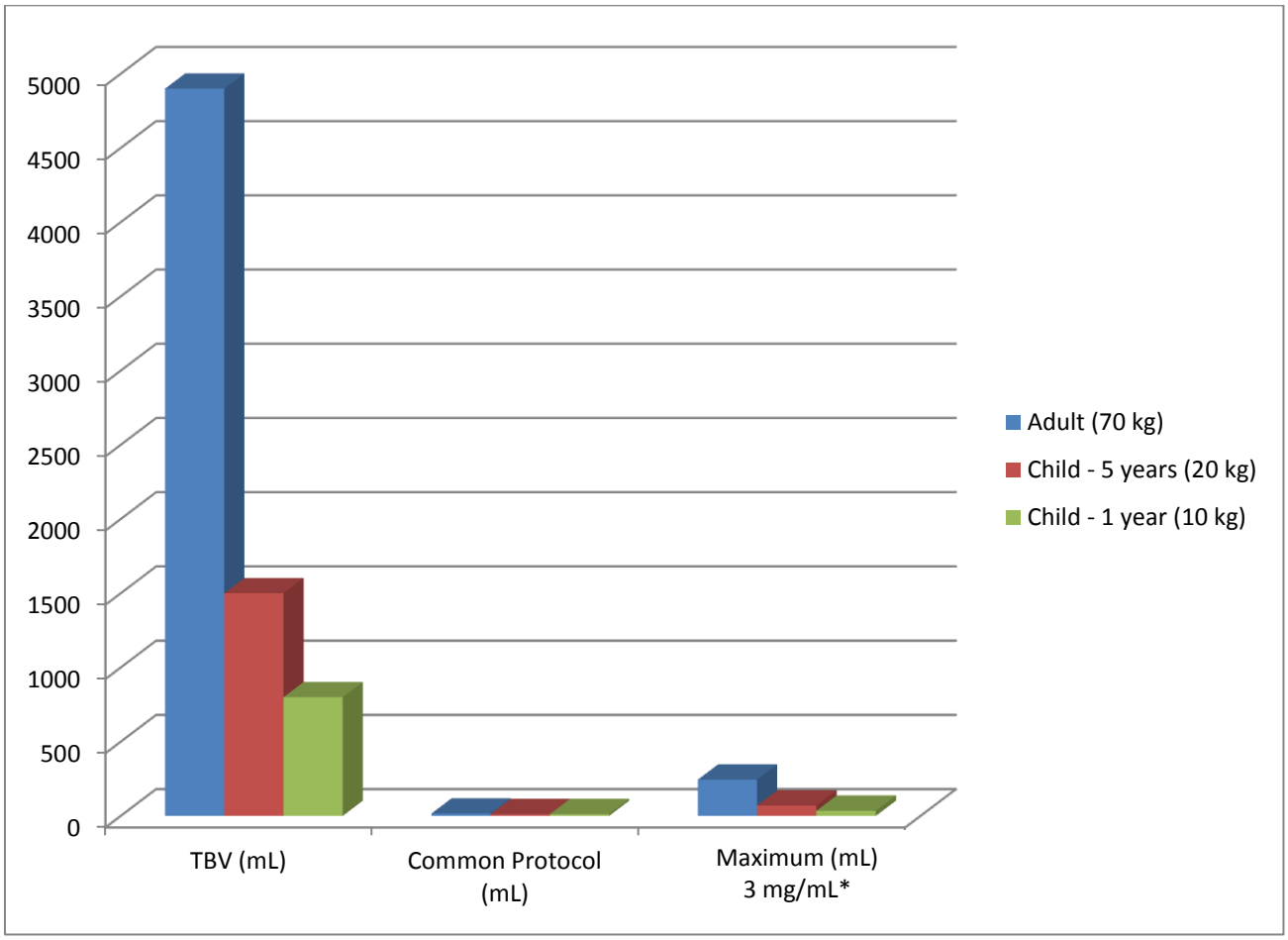
8.0 Appendices

Appendix I. Blood Collection Weight Chart

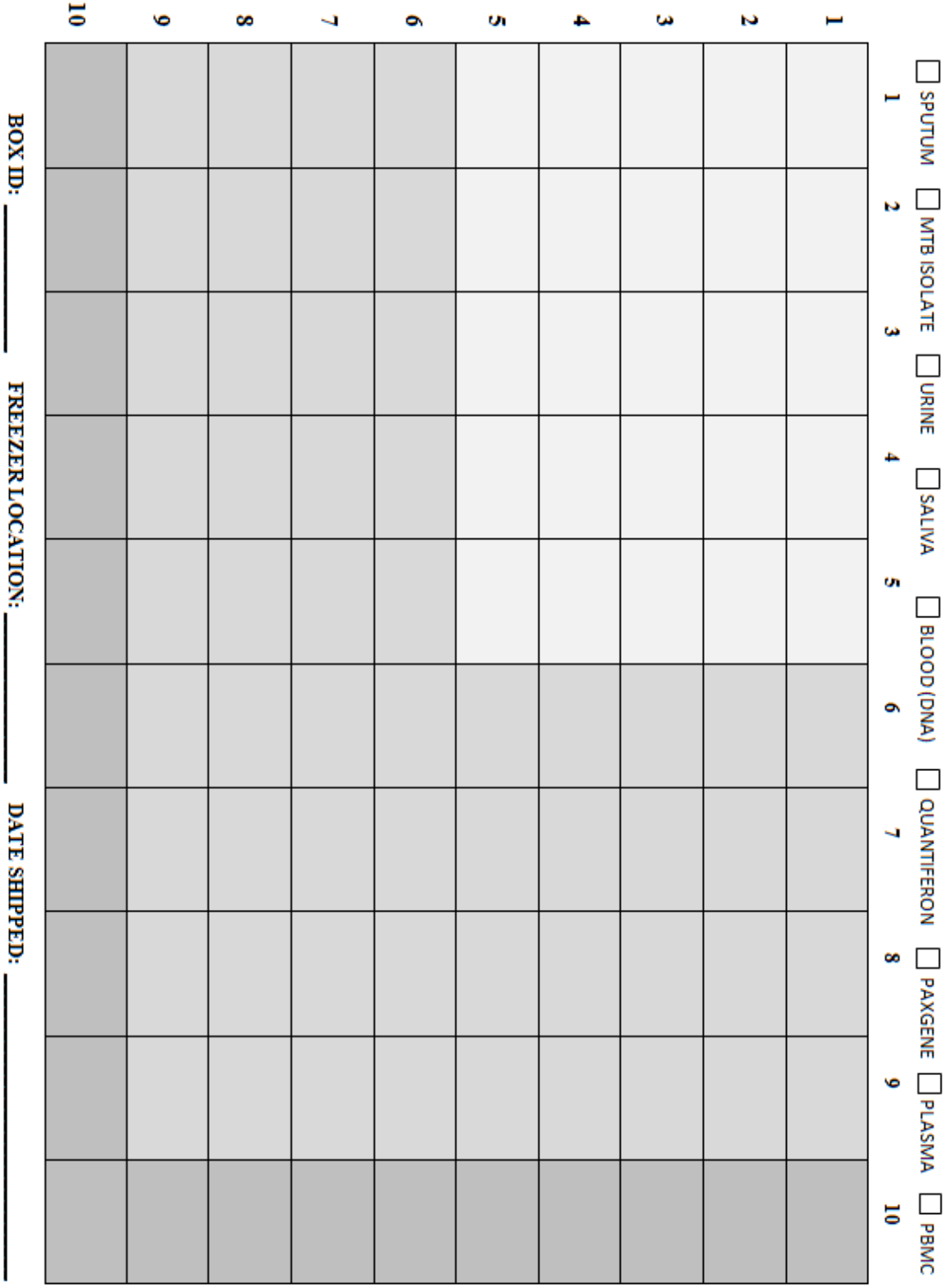
Maximum Blood Draw Volume Limit - Adults and Children*			
Weight (kg)	**Weight (lb)**	**Single Draw (24 hrs) 3.5 mL/kg (units, mL)**	**Cumulative Draw (8 wks) 5 mL/kg (units, mL)**
1	2.2	3.5	5
2	4.4	7	10
3	6.6	10.5	15
4	8.8	14	20
5	11	17.5	25
6	13.2	21	30
7	15.4	24.5	35
8	17.6	28	40
9	19.8	31.5	45
10	22	35	50*
11	24.2	38.5	55
12	26.4	42	60
13	28.6	45.5	65
14	30.8	49*	70
15	33	52.5	75
16	35.2	56	80
17	37.4	59.5	85
18	39.6	63	90
19	41.8	66.5	95
20	44	70	100
25	55	87.5	125
30	66	105	150
35	77	122.5	175
40	88	140	200
45	99	157.5	225
50	110	175	250
55	121	192.5	275
60	132	210	300
65	143	227.5	325
70	154	245	350
75	165	262.5	375
80	176	280	400
85	187	297.5	425
90	198	315	450
95	209	332.5	475
100	220	350	500

*Blood collection limits above are for otherwise healthy participants; however, blood collection volumes from adults and children (*where the age, weight and health of the participants has been considered*); **must not exceed 50 mL of blood or 3.5 mL/kg (whichever is lesser) in an 8 week period**, and collected at a frequency of no more than two times per week. Please consult your local IRB for additional applicable standards.

Appendix II. Total Blood Volume (TBV) Collection Chart



Appendix III. Biomarker Freezer Log



Appendix IV. PBMC Processing Worksheet

PBMC Processing Worksheet			
Processing Laboratory:			
Protocol/Study:			
Processing Start Date [MM/DD/YY]:		Participant ID (PTID):	
Processing End Date [MM/DD/YY]:		Time Received in the Lab (hh(24):mm):	
Processing Start Time (hh(24):mm):		Visual Condition [Norm, Hemolyzed, Clotted]:	
Processing End Time (hh(24):mm):		Sample Tube Type:	
Reagents	**Manufacture**	**Lot #**	**Expiration Date**
HBSS or other WDR:			
FBS :			
DMSO :			
Ficoll:			
Processing Information			
Useable Whole Blood Volume (mL):			mL
Volume of Plasma Isolation (mL or µL)			
Volume of Final Cell Suspension (mL):			mL
Viable Cell Count (cell/mL)			x 10^6 cells/mL
Total Viable Cell Count (cells 10^6)=(Viable Cell Count cells/mL)) x [10^4x Volume of Final Cell Suspension x Dilution Factor]			x 10^6 cells
% Viability: [#Viable cells/total cells x100]			%
QC Check: (Total viable cells/Processed whole blood volume) [Expected cell yields for Normal adults: 1X10^6 cell/mL to 3X10^6 cell/mL			cell/mL
Estimated Volume of CPS (mL)= (Total Viable Cell Count (cells))/(Target Final Concentration (cells/mL))			mL
Freezing Media Date Made:		Volume DMSO/FBS:	mL / mL
Freezing Time (hh(24):mm): and Date [MM/DD/YY]:		Number of vials frozen at ___X10^6/___mL:	
Technician (s) Responsible for Processing Whole Blood (Signature/Time/Dated):	Signature	Date	Time (hh(24):mm)
Technician (s) Responsible for Transferring to -80°C (Signature/Time/Dated):	Signature	Date	Time (hh(24):mm)
Comments/Deviations:			
Reviewed By:		**Date Reviewed:**	

Appendix V. RePORT PBMC Processing and Storage SOP