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1 Purpose

This procedure describes how to process sputum specimens and store them until they are shipped to a reference laboratory or a repository. These sputum specimens will be used for future tuberculosis research investigations, e.g., development and validation of new biomarkers. Sputum specimens are to be collected according to the ACTG/IMPAACT approved "Collection, Clinic Storage and Transport of (Expectorated) Sputum Specimens SOP"(LTC-SOP-70 v1.0). The amount of specimen required, the time intervals at which specimens are to be collected, and specific shipping destination(s) are study-specific details, which will be indicated in the study's Laboratory Processing Charts (LPCs) and/or Manual of Operating Procedures (MOP).

Sputum processing for storage has one main purpose, to render the specimen to a homogenous state (evenly distributed suspension) for dispensing into multiple aliquots; thus, providing multiple specimens for research investigations with each aliquot being representative of the original specimen. Such a suspension can be prepared by either mechanical homogenization or chemical digestion. Once a homogenous suspension is prepared, the sputum is dispensed into cryovials in small amounts (1.5-1.8ml) and placed in an ultra-low freezer (-70°C or below) as soon as possible.

The processing method described in this SOP is different from common methods (usually NALC [N-acetyl L-cysteine]-NaOH [sodium hydroxide]) used in processing sputum for culture where NALC acts to digest the specimen, and NaOH, eliminates contaminants (bacteria or fungi) that would interfere with culture growth.

2 Scope

SOP is intended for ACTG and IMPAACT laboratories performing sputum processing. This SOP is part of the ACTG/IMPAACT Laboratory Manual:
https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx

3 Background

Sputum or phlegm is the discharge that is expectorated from the respiratory system. It is a combination of mucus produced by the airways combined with saliva from the mouth. Sputum can have different appearances, e.g., mucoid, purulent, mucopurulent, blood-streaked. It is a heterogeneous substance, which can be both watery and viscous. Consequently, the various host components and *M. tuberculosis* organisms in the sputum are not evenly distributed.

To provide samples for research it is critical that the sputum specimen be dispensed in volumes commensurate with the volumes required for testing, which is generally <2ml, and not undergo multiple freeze-thaw cycles that could possibly affect either the analyte being measured, or the viability of the *M. tuberculosis*. Furthermore, each cryotube prepared should contain an equivalent sample that is representative of the original sputum specimen. To this end, the specimen is processed by either a mechanical or chemical method to make it a homogenous suspension. Chemical digestion (Section 6 Procedure 1) is accomplished using dithiothreitol (DTT), which is highly effective in decreasing sputum viscosity. Its active component is a sulphydryl group, which cleaves disulfide bonds in the mucus. Studies have shown DTT to be more effective in liquefying sputum than NALC, which is used in sputum processing for culture. A commercial preparation (e.g., Sputasol) of DTT is available and commonly used. A small volume of DTT is added to the specimen, thus the sputum is not significantly diluted. A low concentration (final concentration of 0.65 mM DTT) is used thus the components (proteins, antigens, lipids, metabolites, nucleic acids) of the *M. tuberculosis* organism and host components (cytokines) are not altered or affected by the DTT[10.1-10.2].

Mechanical homogenization (Section 7 Procedure 2) is accomplished using a magnetic stirring bar and magnetic stirring plate or platform shaker. This is best accomplished in a wide-mouth collection container to provide sufficient space for stirring/agitation, and must be performed inside a biosafety cabinet. Stirring bars must be sterile. Use of glass beads and vortexing is not as effective and therefore is not suggested.

The laboratory should consult with the study protocol team to determine if there is a preference as to which method should be used, or if another method is required; for example the addition of a RNA stabilizer for transcriptional research. If there is no preference, the laboratory should decide which method works best in their sputum processing workflow.

4 Authority and Responsibility

4.1 The Network Laboratory Directors (or his/her designee), in conjunction with the ACTG CURE Transformative Science Group, have the authority to establish, review and update this procedure.

4.2 The MyLab Working Group of the ACTG TB Transformative Science Group (TBTSG) is responsible for maintenance and control of the scientific content of this SOP.

4.3 The ACTG/IMPAACT Laboratory Technologist Committee (LTC) is responsible for the maintenance and control of SOP documentation.

4.4 The Laboratory Director is responsible for the implementation of this LTC SOP or laboratory-specific SOP and for ensuring that all appropriate personnel are trained. A laboratory SOP must:

4.4.1 Include, without procedural modification, the portions of the current version of the LTC SOP that are used within the network site-affiliated laboratory.

4.4.2 Reference the current version of the LTC SOP.

4.5 All laboratory technicians are responsible for reading and understanding this SOP prior to performing the specimen processing procedures described herein.

4.6 The site PI and designees are responsible for understanding and adhering to the participant preparation and specimen collection components.

5 Specimen Requirements

For routine TB testing a minimum volume of 1mL of sputum must be collected. However, for sputum storage the minimum collection volume is 2mL. The target volumes for time intervals (e.g. 5mL sputum at screening, baseline, and at all other visits through week 4; 3mL at all subsequent visits thereafter) should be collected wherever possible. These specimens are collected solely for freezer storage and are not concurrently used for routine TB testing. Thus specimens for freezer storage are collected specifically for this purpose, although the collection procedure is the same as for routine TB testing.

If the collected volume of sputum is less than 2mL, it is not practical to homogenize or digest this specimen. The entire specimen can be transferred to the cryovial and the Specimen Request form/CRF is annotated to state that the specimen was not homogenized/digested because of insufficient volume. Alternatively, the specimen is reported as being unsatisfactory.

Complete all appropriate site-specific and protocol-specific documentation.

For any given sample, proceed to Section 6 Procedure 1 or Section 7 Procedure 2, as appropriate.

6 Procedure 1 – Chemical Digestion with Dithiothreitol (DTT)

6.1. Equipment, Consumables, and PPE

6.1.1 Biological safety cabinet (BSC), Class II

6.1.2 Mycobactericidal disinfectant (e.g. STERIS Vesphene)

6.1.3 Test tube rack for 50mL centrifuge tubes

6.1.4 4mm glass beads (sterile) (optional)

6.1.5 Liquid DTT (e.g. Sputasol) or powder DTT (Sigma-Aldrich, Appendix A)

6.1.6 Nitrocellulose or polycarbonate membrane filters (22 µm) and sterile syringe (for powder DTT)

6.1.7 Calibrated balance (for powder DTT)

6.1.8 Sterile Phosphate Buffered Saline (PBS, pH 7.4) (for powder DTT)

6.1.9 Sterile distilled/deionized water

6.1.10 Sterile 125mL flask or bottle

6.1.11 Sterile 100mL graduated cylinder

6.1.12 Sterile serological pipettes (3mL, 10mL)

6.1.13 Micropipettor and sterile, aerosol-resistant tips (1000µL)

6.1.14 Sterile transfer pipettes (3mL)

6.1.15 Cryotubes (2mL) or specific storage tubes as defined in LPC. Tubes with external threading are preferred. (See Appendix A for examples)

6.1.16 Ice bucket

6.1.17 Vortex mixer

6.1.18 Storage boxes 5”x5”x2” or 5”x5”x3” (2 or 3 inch cryostorage boxes, 81-cell)

6.2 Preparation of Sputasol or 0.1% DTT working solutions

Liquid DTT can be procured from a commercial source (e.g. Sputasol, Sputolysin) or prepared fresh from powder form (e.g. Sigma-Aldrich). These can be prepared as per the following:

6.2.1. Sputasol working solution

6.2.1.1 The Sputasol reagent contains 0.1 g of DTT in solution, which when dissolved in a final volume of 100mL of distilled water yields a final concentration of 0.1% DTT (6.5mM).

6.2.1.2 Aseptically add the contents of one Sputasol vial (7.5mL) to 92.5 mL of sterile distilled water using a graduated cylinder and flask/bottle.

6.2.1.3 Mix and label the flask with the contents, date of preparation, and date of expiration.

The Sputasol working solution should be used immediately or stored at 2-8°C for up to 48 hours.

6.2.2 0.1% DTT working solution

6.2.2.1 Measure 0.1 g DTT powder on a calibrated balance.

6.2.2.2. Re-suspend in a final volume of 100mL PBS, pH 7.4.

6.2.2.3. Filter sterilize with Millipore filter and syringe and prepare aliquots of 10mL each.

Use fresh and/or store at 2-8°C for up to 48 hours.

6.2.2.4 Alternatively for smaller volumes, measure 10 mg DTT and resuspend in a final volume of 10mL PBS and filter sterilize. Use as 10mL or aliquot if smaller volumes are desired, e.g., 1-2 mL aliquots. Use fresh and/or store at 2-8°C for up to 48 hours.

6.3 Digestion of sputum specimens with 0.1% DTT/Sputasol

6.3.1 This procedure must be performed inside a biosafety cabinet.

6.3.2 Prepare and label cryovials with the appropriate identifying LDMS barcoded specimen label. The number of cryovials will be study-specific. (Study may require any number up to a specific number, e.g., 1-4. Alternatively, the study may allow for as many tubes that can be prepared with the volume of sputum collected.)

6.3.3 Allow sputum specimens and 0.1% DTT/Sputasol to come to room temperature. Effective digestion requires that both 0.1% DTT/Sputasol solutions and specimens must be at room temperature.

6.3.4 Determine the volume of the sputum specimen by comparison with graduated markings on container/tubes.

6.3.5 Calculate the amount of the 0.1% DTT/Sputasol to add to the sputum by dividing the total sputum volume by 10 (i.e., 1/10 volume of sputum).

Example: for 5mL of sputum, add 500µL of 0.1% DTT/Sputasol.

6.3.6 Using a sterile serological pipette or micropipettor with tip, add the calculated volume of 0.1% DTT/Sputasol to the specimen. The final concentration of DTT in the specimen is 0.01%.

6.3.7 Vortex the specimen for 20 seconds.

6.3.8 Place the specimen on a platform shaker to shake mechanically at 60 rpm for 20 minutes.

Note: if specimen appears to not be completely liquefied after 20 minutes, aseptically add about 10 sterile glass beads and continue to shake another 10 minutes.

6.3.9 Using a sterile transfer pipette, aliquot 1.5mL of the DTT homogenized sputum to the pre-labeled 2.0mL cryovials. Use any remaining sputum to top off vials up to 1.8mL. Aliquot larger volumes of the homogenized sputum into larger cryovials if specified in the LPC.

6.3.10 Prepare as many aliquots as possible or the specific number of aliquots required for the study as indicated in the LPC, and tightly seal each cap.

6.3.11 Place cryovials in ice bucket until they can be placed in freezer. Store cryovials of digested sputum at -70°C or below.

Skip Chapter 7 and proceed to Chapter 8

7 Procedure 2 – Mechanical Homogenization with stirring bar

7.1 Equipment, Consumables, and PPE

7.1.1 Biological safety cabinet (BSC), Class II

7.1.2 Mycobactericidal disinfectant (e.g. STERIS Vesphene)

7.1.3 Test tube rack for 50mL centrifuge tubes

7.1.4 Sterile, wide-mouth, universal specimen containers

7.1.5 Sterile, 3mm cylindrical magnet with pivotal ring or 3cm stirring bar

7.1.6 Magnetic stirring plate or platform shaker

7.1.7 Cryotubes (2mL) or specific storage tubes as defined in the LPC (see Appendix A for examples)

7.1.8 Ice bucket

7.1.9 Storage boxes 5”x 5” x 2”/3” (2 or 3 inch cryostorage boxes, 81-cell)

7.2 Homogenization of sputum specimens with stirring bar

7.2.1 This procedure must be performed inside a biosafety cabinet.

7.2.2 If sputum specimen is in a tube, transfer sputum to a wide-mouth, sterile disposable container (universal collection container) and label with subject ID number, visit interval, specimen accession number, and date.

7.2.3 Add a sterile magnetic stirring bar to the sputum and close lid tightly.

7.2.4 Place on magnetic stirrer or platform shaker. Stir magnetically or shake for 30 minutes until the specimen is thoroughly homogenized.

7.2.5 Prepare and label cryovials with the LDMS identifying barcoded specimen label.

7.2.6 Using a sterile transfer pipette, place 1.5-1.8ml (or other volume as specified by the LPC) of homogenized sputum into the appropriate number of cryovials or the maximum number of cryovials that can be prepared with the sputum volume as detailed in the LPC. Aliquot larger volumes of the homogenized sputum into larger cryovials if specified by the study.

7.2.7 Place cryovials in an ice bucket until they can be placed in the freezer. Store cryovials of homogenized sputum at -70°C or below.

Proceed to Chapter 8

8 Sputum storage procedures

8.1 Sputum aliquots should be stored in an 81-cell 2-inch cryostorage box, with 9x9 sectioned inserts or in storage boxes designated in the LPC. Boxes should not exceed 5”x5”x 3” inches (127mmx127mmx76mm). Boxes should be maintained on dry-ice during transfer of tubes to boxes. All freezer boxes should be pre-labeled with Site ID, LDMS lab ID number, and box number designation (i.e., Box 1 of 1).

8.2 Confirm that each cryovial containing the sputum specimen has the appropriate preprinted barcode label with accession number and sample designation attached. Note: If sending to BRI or other repository, storage boxes and labels should conform to the specific repository regulations.

8.3 Place the cryostorage box in the -70/80°C freezer.

8.4 All samples placed in the freezer should be noted on the LDMS Freezer Log to aid in rapid retrieval of specimens. Use an LDMS barcoded specimen label to designate the first storage location box and then draw an arrow to the last aliquot for that specimen.

8.5 The person responsible for processing the sputum will record the time the specimen was received in the lab and the number of cryovials stored in the LDMS and on the specimen tracking/request form if requested. A comment documenting any problems with processing the sputum specimen should be added.

8.6 Sputum specimens do not have to be stored sequentially per participant. Store sputum specimens as they are collected.

9 Literature and References

9.1 Ribeiro-Rodrigues R, Resende Co T, Johnson JL, Ribeiro F, Palaci M, Sa RT, Maciel EL, Pereira Lima FE, Dettoni V, Toossi Z, Boom WH, Dietze R, Ellner JJ, Hirsch CS. Sputum cytokine levels in patients with pulmonary tuberculosis as early markers of mycobacterial clearance. Clinical and diagnostic laboratory immunology. 2002; 9(4): 818-23. PMCID: 120011.

9.2 Li L, Mahan CS, Palaci M, Horter L, Loeffelholz L, Johnson JL, Dietze R, Debanne SM, Joloba ML, Okwera A, Boom WH, Eisenach KD. Sputum Mycobacterium tuberculosis mRNA as a marker of bacteriologic clearance in response to antituberculosis therapy. Journal of clinical microbiology. 2010; 48(1): 46-51. PMCID: 2812283.

10 Acknowledgments/ Collaborators

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Appendix A: Example Supplies

Reagent/Supply	Example(s)
Marking pens	Fisher Scientific Fisherbrand Marking Pens cat#13-379, or Nalgene® Lab Pen/Lab Marker #6310/#6311, or equivalent
Absorbent Material	Saf-T-Pak STP-151, or equivalent
Dithiothreitol (DTT)	Liquid DTT (e.g. Sputasol) or powder DTT (Sigma-Aldrich) SDS/MSDS located on http://www.sigmaaldrich.com/safety-center.html Enter D06732 or CAS-No. 3483-12-3
Cryotube externally threaded with O-ring	Corning® 2mL external thread polypropylene cryogenic vial, self standing with round bottom #430659 Nunc CryoTubes™, external thread, polypropylene (PP) tubes and screwcap #377267 WHEATON Cryule® Plastic Cryogenic Vials, external thread, #985742 SARSTEDT Screw cap micro tube, external thread, #72.694.006
Platform Shaker	Cole-Parmer Economical Orbital Shaker; 16" x 16" Platform, 120 VAC, EW-51820-40