PANTHER Trial Biospecimen Collection and Processing Guide

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2. Abbreviations

ARDS	Acute Respiratory Distress Syndrome
BAL	Bronchoalveolar Lavage
B-CRF	Biological sample Case Report Form
CTA	Clinical Trials Agreement
PBC	PANTHER Biology Committee
PK	Pharmacokinetic
PPIE	Patient Public Involvement Engagement
RCC	Regional Coordinating Center
SOP	Standard Operating Procedure
SST	Serum Separating Tube
VTM	Viral Transport Media

3. Introduction

Biospecimen collection and handling is an agreed upon process among PANTHER Trial sites.

3.1. Biological sampling objectives

This SOP has been designed to provide guidance on the collection of biospecimens within the PANTHER Trial. Sample collection within this study has been planned to allow several objectives to be met. These samples will allow a multicompartmental approach to exploring mechanisms of action of the investigational drugs and potential differential responses in different phenotypes. Collection of plasma and bronchoalveolar lavage (BAL) fluid will allow investigation of changes in transcriptome, epigenetics, inflammatory mediators, coagulation, and organ specific tissue damage in both the pulmonary and systemic compartments. For example, both baricitinib and simvastatin, the initial investigational therapies in the platform trial, are expected to be beneficial in ARDS due to their anti-inflammatory properties. Collection of plasma and bronchioalveolar lavage (BAL) fluid will allow us to examine the changes to inflammatory mediators such as TNF-α, IL-1β, IFNγ and IL-10 in these compartments to understand both systemic and local respiratory anti-inflammatory effects.

Sequential sample collection is important for understanding the evolution of ARDS phenotypes over time and how these may be modulated by trial treatments as well as for discovery of novel biomarkers that could rapidly identify treatment responders or act as proxies for clinical outcomes to enhance the efficiency of future trials.

Finally, although the inflammatory phenotypes are the best described ARDS sub-phenotypes, other critical illness phenotypes are known to exist, for example those based on gene expression (doi: 10.1016/S2213-2600(16)00046-1, doi: 10.1016/S2213-2600(17)30294-1). Collection of a broad range of specimens including RNA, DNA and plasma will facilitate investigation of whether other stratification approaches provide alternative or better ways to identify treatment responsive sub-groups of patients with ARDS by applying some of these other methodologies.

Although in the first instance bio-sampling has been planned to investigate mechanisms pertinent to the initial drugs in the platform trial, this SOP has been designed to be broad enough to be robust to the addition of subsequent drugs as the platform grows and be able to answer new mechanistic questions that arise throughout the lifespan of the platform without adding significant burden to recruiting sites and impacting study recruitment.

Objectives:

- To better understand the mechanisms of action of novel drugs in ARDS, especially regarding differential effects in the hyper- and hypo-inflammatory ARDS phenotypes
- To explore local (lung compartment) vs systemic (plasma) changes in response to therapies in ARDS that underpin mechanisms of action
- To understand the evolution of ARDS phenotypes over time and in response to therapy

- To discover biomarkers that identify treatment responders and that are surrogates for clinical outcomes
- To determine novel ARDS subphenotypes, e.g. transcriptomic phenotypes, and their potential to identify treatment responsive populations
- Create a biobank of samples that will allow for other mechanistic questions to be answered that arise as internal or external trial findings become available, new biomarkers or subphenotypes identified, or new therapies are added to the platform.

4. Biological sampling oversight and the Biology Committee

4.1. Governance

The PANTHER Biology Committee (PBC) will administer and have overall oversight of this PANTHER Trial Biospecimen Collection and Processing Guide. The PBC will oversee sample analysis prioritization. The PBC responsibilities are:

- Development and subsequent amendment of the protocol
- Development and approval of biological sample collection, processing and management guidelines
- Liaise with the local coordinating centers for regional sampling coordination
- Assist with obtaining funding for PANTHER biological sampling in participating regions
- Prioritize the best use of these finite samples (in terms of sample analysis)
- Consider internal and external proposals for sample analysis
- Evaluate feasibility, biological rationale and clinical relevance of such proposals
- Consider material and data access requests
- Liaise with the PANTHER Patient Public Involvement Engagement (PPIE) group to develop patient facing documentation related to biological sampling

Each regional coordinating center (RCC) will be responsible for the organization of the PANTHER biorepository in their region. They will identify the biorepository location for their region. Multiple locations can be established, where appropriate e.g., for feasibility. They will organize the biorepository according to local rules and regulations. Other responsibilities, in their region, include:

- Identification and management of biological sampling sites
- Consideration of national and site level sampling feasibility/suitability
- Obtain funding in their region
- Tailor sample collection, processing and management documents for their region in accordance with the guidelines set forth in this document

4.2. Working group members

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4.4. Conflicts of interest

All investigators of the PANTHER Trial maintain an updated and publicly available registry of conflicts of interests on the PANTHER Trial website. There will be a committee established to review conflicts of interest on an annual basis.

5. Biological Sampling Design

Samples should be collected in accordance with informed consent rules and legislation described elsewhere in the PANTHER Trial protocols. The date of randomization may differ from the date of ICU admission. Here, all samples are described related to the date and time of randomization in the PANTHER Trial.

To minimize patient discomfort, blood should be collected from existing central lines or arterial lines, where possible. No lines should be placed as part of this study for blood collection. For patients who do not have any vascular access, blood collection should be done by venepuncture. It is important that the blood is NOT hemolyzed for the analysis to be valid. If the sample is hemolyzed, another tube of blood may be drawn. If repeat sampling is not possible, then the hemolyzed sample can be processed with a note written in the Specimen Collection Form to indicate that the sample is hemolyzed.

5.1. Biological sampling tiers

Staff will choose a sampling tier for each patient according to Table 1 and in accordance with the resources available at each site at that time. Enrollment of patients in the trial is not contingent upon sample collection beyond the **initial** required phenotype stratification sample. Thus, patients will be required to provide the initial blood sample for stratification. However, subsequent samples will be optional (although participants will be encouraged to provide subsequent samples to enhance the value of the trial).

Table 1. Sampling Tiers

Tier	Biological samples
0	Stratification sample only (Li heparin)
1	Stratification sample Baseline plasma/serum/Paxgene RNA Baseline nasopahyngeal swab (NPS)/ tracheal aspirate in patients on invasive mechanical ventilation
2	Tier 1 samples PLUS Longitudinal samples: plasma/serum/RNA plus Longitudinal tracheal aspirate sampling in patients on invasive mechanical ventilation
3	Tier 2 samples PLUS Baseline and longitudinal CPT samples
4	Tier 2 or 3 PLUS BAL where possible (selected sites only)

5.2. Biological specimen collection schedule

Table 2. Biological specimen collection schedule

	Timepoints			
Sample type	**Baseline**	**Day 2**	**Day 6**	**Notes**
Lithium heparin	✓	✓	✓	For stratification sample (Randox assay) plus plasma cytokines
Serum Separation Tube	✓	✓	✓	For biomarker and antibody analyses.
EDTA Tube	✓	✓	✓	For plasma
CPT heparin	✓	✓		For isolation of immune cells
PAXgene RNA	✓	✓	✓	For RNA and DNA extraction
Nasal swab (mid-turbinate)	✓			Identifying viral pathogens, pathogen studies
Tracheal aspirate	✓	✓	✓	For biomarker analyses
BAL	✓	✓		For biomarker, gene expression, microbiome and cellular analyses

Definition

Baseline – the 24-hour period prior to randomization

Day 0 – the remainder of the calendar day after randomization took place

Day 1 – the first calendar day following randomization

Day 2 – the second calendar day following randomization (essentially the 48-hour time samples)

Baseline samples including the phenotyping (stratification) specimen must be collected prior to randomization.

For all blood draws, PAXgene RNA needs to be drawn last.

Day 2 samples must be drawn at least 48 hours after the first dose of investigational agent. Day 2 samples may be collected +1 day and Day 6 specimens may be collected +/- 1 day to accommodate weekend staffing. For example, if Day 2 is Sunday, the Day 2 blood samples may be collected on Monday. If early discharge or death is anticipated Day 6 samples can be substituted with Day 5. Time of drug and blood specimen must be recorded in the Biological Case Report Form (B-CRF) for pharmacokinetic (PK) analysis use.

5.3. Biological specimen data collection

A Biological Specimen Case Report Form (B-CRF) will be used at each site to record basic information on the biological samples. Data collected on this B-CRF will include:

- Biological sample set ID
- PANTHER participant study number
- Timepoint
- Sample type
- Collection date and time
- Processing (Y/N)
- Storage date and time
- Number of aliquots
- Storage location
- Any comments (e.g. any issues with processing, hemolyzed specimen, etc.)

6. Required On-Site Equipment

All sites will be required to process the initial blood sample for centrifugation to separate plasma and be able to measure TNFr-1 and IL-6 for stratification.

All participating centers must be equipped with the following minimum equipment:

- Centrifuge with 2000g force capability to process Li heparin tube for stratification sample
- Evidence MultiSTAT point of-care analyzer (Randox laboratories Inc., Antrim, Northern Ireland) and assay kits for IL-6 and sTNFR1

For sites participating in biosampling beyond tier 0 (most sites)

- Freezer at -80°C for plasma, serum, RNA, and BAL storage
- Barcode scanner capable of QR barcode scanning

Additional equipment required based on participating Tier are outlined in Table 3.

Table 3. Required on-site equipment by tier of participation

Tier	Biological samples	Required equipment
0	Stratification sample	• Centrifuge with 2000g force capability • Randox MultiStat device
1	Stratification sample Baseline Li Heparin, EDTA, SST, PAXgene tubes, nasal swab*, tracheal aspirate*	• Centrifuge with 2000g force capability • Freezer at -70°C to -80°C for sample storage • Barcode printer and scanner capable of QR barcode scanning to anonymize patients' samples • Vortex (US sites for tracheal aspirates) Centrifugee (as above)
2	Tier 1 samples PLUS Longitudinal samples: Li heparin, EDTA, SST, PAXgene tubes Longitudinal tracheal aspirate*	
3	Tier 2 samples PLUS Baseline and longitudinal CPT samples	• Tier 1 & 2 equipment PLUS • Mr. Frosty™ freezing container • Liquid nitrogen tank storage (optional) • Microscope and haemocytometer or automated cell counter (optional)
4	Tier 2 or 3 PLUS BAL in select patients	

*where possible; if sites are unable to take tracheal aspirates or nasopharyngeal swabs the collection of plasma and serum at tier 1 or 2 is strongly encouraged.

7. Specimen collection kits

The PANTHER trial regional coordinating center will provide each site with sample collection kits and sample storage boxes. Kits are timepoint specific and labeled with the timepoint, e.g., baseline, Day 2, Day 6. The appropriate kit should be used for each day. One kit should be used per patient. Kits will differ across jurisdictions. Please check local jurisdiction. The UK kits contain the following:

	Volume	Baseline kit	Day 2 kit	Day 6 kit
Tier 0				
Screw-topped tubes	--	1		
Tier 1				
PAXgene RNA tube	2.5 ml	1		
Screw-topped tubes	--	17		
Barcodes	--	Strip		
Tier 2				
PAXgene RNA tube	2.5 ml	1	1	1
Screw-topped tubes	--	17	16	16
Barcodes	--	Strip	Strip	Strip
Tier 3				
PAXgene RNA tube	2.5 ml	1	1	1
CPT heparin tube	8 ml	2	2	
Cryovials	--	2	2	2
Screw-topped tubes	--	19	18	18
Barcodes	--	Strip	Strip	Strip
Tier 4				
PAXgene RNA tube	2.5 ml	1	1	1
CPT heparin tube	8 ml	2	2	2
Cryovials	--	5	5	3
Screw-topped tubes	--	32	31	18
Barcodes	--	Strip	Strip	Strip

Unused materials must not be used for another trial patient. After the last timepoint, the unused kit should be discarded. Kit expiration date is clearly marked on the kit and must be checked before using. Expired collection tubes should not be used. Extra blood collection tubes will be sent to each site (without pre-labelled participant-specific barcodes). These extra tubes can be used for specimen collection if tubes are lost or vacutainers do not function properly.

Each kit is labelled with the biological sample set ID (see Section 8 of this document for labelling information).

8. Biological sample labelling

Biological samples will be collected at the timepoints described in section 5.2. Each kit will be labelled with the biological sample set ID, and this ID should be used on the same collection forms. Pre-printed kits and/or labels will be provided by each RCC. These can be used to label study tracking forms and samples. One label set will contain all labels for a single subject. Every sample, aliquot and tracking form should be clearly labeled with the provided labels.

Biological samples	Baseline	Day 2	Day 6
EDTA	XXXXX-0001-EDTA-D0-A	XXXXX-0001-EDTA-D2-A	XXXXX-0001-EDTA-D7-A
Li heparin	XXXXX-0001-LiHp-D0-A	XXXXX-0001-LiHp-D2-A	XXXXX-0001-LiHp-D7-A
Serum	XXXXX-0001-SER-D0-A	XXXXX-0001-SER-D2-A	XXXXX-0001-SER-D7-A
PAXgene RNA	XXXXX-0001-PAX-D0-A	XXXXX-0001-PAX-D2-A	XXXXX-0001-PAX-D7-A
CPT Heparin	XXXXX-0001-PBMC-D0-A	XXXXX-0001-PBMC-D2-A	
VTM tube	XXXXX-0001-VTM-D0-A		
Tracheal Aspirate	XXXXX-0001-TA-D0-A	XXXXX-0001-TA-D2-A	XXXXX-0001-TA-D7-A
BAL	XXXXX-0001-BAL-D0-A	XXXXX-0001-BAL-D2-A	

The sample identifier has the following information in order:

- The biological sample set ID which consists of the unique 5-digit PANTHER site number
- The biological sample set patient number- this begins at 0001 for all sites indicating that this is the first patient that samples are collected from at that site i.e. the first set of samples, followed by 0002 for the second set of samples etc. This is different from the PANTHER study number for the participant. This will be linked to the PANTHER participant study number in two ways: 1. This biological sample set ID will be entered into the PANTHER core Biological sample CRF (B-CRF). 2. Likewise, the PANTHER participant study number will be entered onto the Biological Sample CRF (sample collection log).
- The type of biological sample
- Timepoint of collection
- Aliquot label indicated by a letter (e.g Aliquot A/B/C/D)

9. Biologic Case report forms (B-CRF)

An electronic biological sample case report form (B-CRF), part of the main PANTHER Trial CRF, must be completed for each scheduled collection based on each site's participation tier. If a collection is not made for any reason, the B-CRFs must still be completed to indicate the reason the collection was not made. Each B-CRF will contain the following:

Sampling tier

The sampling tier of the participant is collected as part of the basic information along with domains to participate in. The variation of sampling tiers among participants is also reflected by the informed consent form and consent withdrawal form.

Status of sampling

The B-CRF clarifies the participant's specimen collection schedule by presenting an interactive summary table. For each date/time for biospecimen collection, one of the following options is selected: "(already) collected", and "missed". If "collected" is selected, status of each specific specimen, e.g., type of blood container, is recorded. If "missed" is selected, the reason is recorded with a combination of categorical and text items.

Additional items

To facilitate logistics and ensure consistent handling of biological specimens, additional items may be added to capture such things as date of specimen transportation and the lab to which the specimen is transported. Such additional items may be tailored to the needs of regions/sites.

10. Biological sample handling

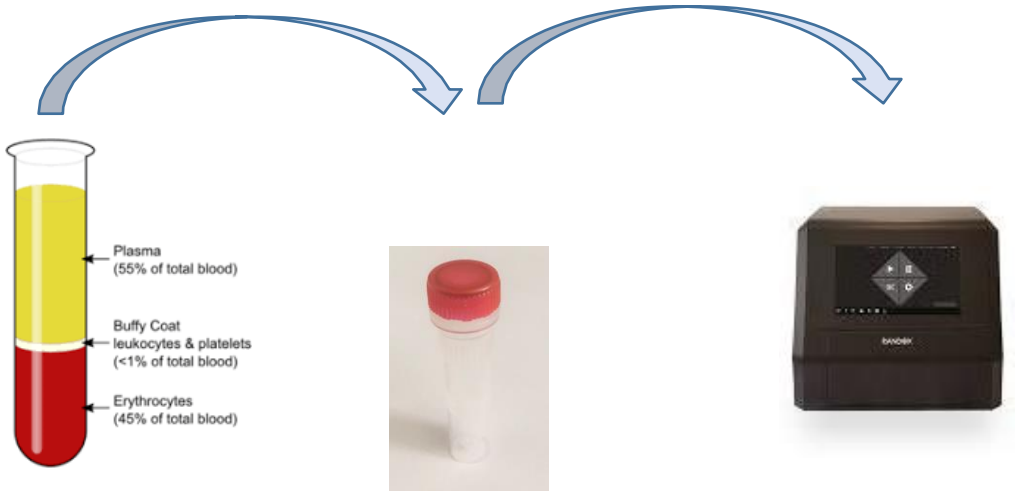
10.1. General

Personnel must follow 'Universal Precautions' when handling blood products as well as site-specific requirements for handling each type of specimen. Processing of blood specimens should be initiated as soon as possible (~ 30 minutes) following collection with all plasma and serum samples ideally processed and frozen within one hour. If centrifugation of the tubes is not immediate, the BAL, EDTA and lithium heparin tubes should remain on ice. The SST tube should be held at room temperature for up to an hour until processing.

Plasma will be separated and stored in 500-1000 microlitre aliquots according to the local jurisdiction's specification. In the UK this will be in 500 microlitre aliquots. Sites should clarify their local jurisdiction's arrangements and incorporate them into their local handling manual.

10.2. Preparation of the stratification sample

4ml blood will be collected in a Lithium heparin tube for use in the point of care assay. Invert the tube several times and place on ice. The Multistat device requires the preparation of a plasma sample from the blood. Samples should be processed within 30 minutes of blood draw. To prepare plasma, centrifuge blood at 2000g in a pre-chilled (if possible) centrifuge for 10 minutes. Using a Pasteur pipette, take the supernatant (plasma- see diagram below) and put into 1ml screw topped tube (supplied unlabelled). Keep on ice until sample analysed in Multistat. Keep any residual plasma in the red-topped tube on ice, until assay is complete (see below).



Please refer to Randox Instructions for Use Manual for operation of the Multistat device. After sample has been successfully analyzed by the Multistat, discard this sample only.

10.3. PAXGene RNA Tubes

Action	Procedure
Preparation	• Label each PAXgene RNA tube with kit barcodes per labeling scheme (section 8). • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimens.
Collection	• Obtain the designated 2.5 ml of blood from the participant in the prepared tube. • **Tube must be filled to line** for reagent ratios to be correct. • Gently invert tube 8-10 times immediately after collection to disperse anticoagulant into the entire blood sample. • Record time of each collection on CRF
Processing	• Store tubes upright at room temperature for at least 2 hours. ○ Tubes may be held at room temperature for up to 2 days before freezing. • After 2 hours at room temp, freeze tubes first at -20°C for 24 hours, then transfer them to -70°C or -80°C. • Freeze vials upright in a wire rack. Do not freeze tubes in a Styrofoam™ tray as this may cause the tubes to crack. • Store at -70 to -80°C until shipping.
Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated time point.
Shipping	• Ship on dry ice (shipping instructions per each RCC). • Samples should be batch shipped.
Notes	• All specimens should remain at -70ºC or colder prior to shipping. • The samples should not be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

10.4. EDTA Tubes

Action	Procedure
Preparation	• Label 1 EDTA tube with sample ID per sampling scheme (section 8). • Ensure that centrifuge is pre-chilled to 4oC • Label five 1ml screw-topped tubes for plasma with kit barcodes • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimens. • Note: Biomarker analysis may be affected by environmental ethanol. After cleaning with disinfectant please allow safety hood surfaces to fully dry prior to starting work to avoid cross-contamination. If possible, use isopropanol or a hydrogen peroxide-based disinfectant.
Collection	• Obtain the designated amount of blood from the participant in the prepared tube. • **Tube must be filled to line** for reagent ratios to be correct. • Gently invert EDTA tubes 8-10 times immediately after collection to disperse anticoagulant into the entire blood sample. • Place on ice or at 2-8ºC until centrifugation • Record time of each collection on CRF.

Processing	• Centrifuge at 2000 x g (RCF) for 10 minutes with brake ON in pre-chilled (4ºC) swinging bucket rotor at temperature.
	• Ensure that any volatile cleaning agents (i.e. ethanol) used to sterilize biosafety cabinets/working area have evaporated completely before aliquoting. Vapors will contaminate samples.
	• Consider processing in Biosafety Cabinet (BSL2+) conditions depending on institutional guidelines.
	• Keeping the tubes upright, gently remove vacutainer tube cap and transfer plasma (upper layer) in 1ml aliquots to the 5 labeled screw-topped tubes using a P1000 micropipette (or sterile plastic transfer pipet) being careful to avoid disturbing the interface between plasma and blood pellet.
	  ○ The last aliquot may be 0.1 to 0.99 ml.
	  ○ If the last aliquot is less than 0.1 ml, combine with previous aliquot.
	• Cap the screw-topped tubes, place in freezer box, and place at -80°C within 30 minutes of processing.
	• Store at -80°C until shipping.

Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated timepoint.
Shipping	• Ship on dry ice to the central lab. • Samples should be batch shipped.
Notes	• All specimens should remain at -80ºC or colder prior to shipping. • The samples should not be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

10.5. Li heparin Tubes

Action	Procedure
Preparation	• Label 1 Li heparin tube with sample ID per sampling scheme (section 8). • Ensure that centrifuge pre-chilled to 4oC. • Label 5 1ml screw-topped tubes for plasma with kit barcodes • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimens. • Note: Biomarker analysis may be affected by environmental ethanol. After cleaning with disinfectant please allow safety hood surfaces to fully dry prior to starting work to avoid cross-contamination. If possible, use isopropanol or a hydrogen peroxide-based disinfectant.
Collection	• Obtain the designated amount of blood from the participant in the prepared tube. • **Tube must be filled to line** for reagent ratios to be correct. • Gently invert Li-heparin tubes 8-10 times immediately after collection to disperse anticoagulant into the entire blood sample. • Place on ice or at 2-8°C until centrifugation • Record time of each collection on CRF.

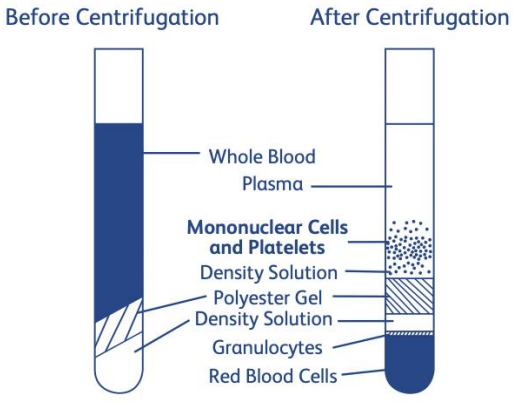
Processing	• Centrifuge at 2000 x g (RCF) for 10 minutes with brake ON in pre-chilled (4°C) swinging bucket rotor
	• Ensure that any volatile cleaning agents (i.e. ethanol) used to sterilize biosafety cabinets/working area have evaporated completely before aliquoting. Vapors will contaminate samples.
	• Consider processing in Biosafety Cabinet (BSL2+) conditions depending on institutional guidelines.
	• Keeping the tubes upright, gently remove vacutainer tube cap and transfer plasma (upper layer) in 1ml aliquots to the 5 labeled screw-topped tubes using a P1000 micropipette (or sterile plastic transfer pipet) being careful to avoid disturbing the interface between plasma and blood pellet.
	  ○ The last aliquot may be 0.1 to 0.99 ml.
	  ○ If the last aliquot is less than 0.1 ml, combine with previous aliquot.
	• Cap the screw-topped tubes, place in freezer box, and place at -80°C within 30 minutes of processing.
	• Store at -80°C until shipping.

Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated timepoint.
Shipping	• Ship on dry ice to the central lab. • Samples should be batch shipped.
Notes	• All specimens should remain at -80ºC or colder prior to shipping. • The samples should not be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

10.6. Serum Separating Tubes

Action	Procedure
Preparation	• Label one SST tube with barcode per sampling scheme (section 8). • Label 4 1ml screw-topped tubes with kit barcodes. • Ensure that centrifuge is at ambient temperature. • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimens.
Collection	• Obtain the designated amount of blood from the participant in the prepared tube. • Allow the tube to clot for at least 30 minutes in a vertical position at room temperature. Try not to allow the tube to stand more than one hour before centrifuging. SST tubes need to be processed within **6 hours** of collection. • Record time of each collection on CRF.
Processing	• Centrifuge at 2000 x g (RCF) for 10 minutes at ambient temperature (swinging-out rotor and brake ON). • After centrifugation, the gel should be intact and the cells and serum completely separated. Do not re-centrifuge the tube if the barrier is incomplete. • Aliquot 500ul into screw-topped tubes. Prepare up to 4 tubes, or as many as possible. Store at -80°C until shipping.
Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated time point.
Shipping	• Ship in dry ice to central lab. • Must be batch shipped.
Notes	• All specimens should remain at -80ºC or colder prior to shipping. • The samples should not be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

10.7. CPT heparin tubes

Action	Procedure
Preparation	• Label two CPT heparin tubes with sample ID per labeling scheme (section 8). • Label four (4) 15ml conical tubes and 2 screw-topped tubes plus 2 cryovials per labeling scheme (section 8). • Thaw freezing medium to room temperature. • Scan barcodes into electronic specimen CRF. • Print Specimen CRF to accompany specimens
Collection	• Obtain the designated amount of blood from the participant in the prepared tube. i.e., vacuum is exhausted and blood ceases to flow. • Tube must be filled for heparin ratio to be correct. • Gently invert collection tubes 8-10 times immediately after collection to disperse preservative into the entire blood sample. • Store upright at room temperature • Record time of each collection on CRF.
Processing	• Remix the blood samples immediately prior to centrifugation by • Verify that the screw-topped tubes and cryovial IDs match ID on collection tubes. ○ If labeling does not match STOP. Verify identity of collected samples and match up kits.. **NOTE**: This centrifuge step of PBMC isolation process must be completed within 2 hours of blood collection. • Remix the blood samples immediately prior to centrifugation by gently inverting tubes 8 to 10 times. • Centrifuge at 1500g for 25 minutes at room temperature. Ensure the centrifuge brake is OFF. • After centrifugation, mononuclear cells and platelets will be in a whitish layer just underthe plasma layer (see figure below)  • Prepare and label one new 15ml tube per sample • Add 15ml HBSS/RPMI medium to the new labelled Falcon tubes or conical centrifugetubes with cap and place on ice.

Action	Procedure
	• Bring the sample tube to eye level and very gently use an 1ml pipette to slowly removethe mononuclear cells and platelets (PBMC) layer of white blood cells – the white fluffylayer of cells above the polymer gel. **NOTE**: you will need to go through the layer of plasma with the pipette button down inorder to avoid disturbing the layers • Collect the cell layer with an 1ml pipette and transfer to a 15 mL conical centrifuge tube with cap. **AVOID**: Avoid taking up any polymer gel during the pipetting • Wash cells: o Add PBS or HBSS to the 15mL conical tube to bring the volume to **15mL**. Cap tube, then mix cells by inverting the tube 5 times. o Centrifuge for 10mins at 400g with brake ON. o Aspirate as much supernatant as possible without disturbing the cell pellet. ▪ Quickly pour out the supernatant and carefully pipette out any remaining supernatant around the pellet, if applicable. o Add PBS or HBSS to bring volume to **10mL**. Cap tube, then mix cells by inverting the tube at least 5 times and tapping the tube. ▪ Resuspend the cell pellet by gently vortexing or tapping the tube. o **Split the 10mL** resuspended pellet volume into two 5mL aliquots in two 15mL conical tubes. One of these will be used for freezing PBMCs and the other will be lysed PBMCs in Shield. o Centrifuge the two 15mL conical tubes for **10** minutes at 400g with brake ON. Aspirate as much supernatant as possible without disturbing cell pellet. • Resuspend cell pellet in the desired medium o Take the PBMC in Shield aliquot and add 500uL of 1x DNA/RNA Shield ▪ Aliquot two 250uL aliquots into 1mL screw-topped tubes ▪ Label as SHIELD1 and SHIELD2 o For the other cell pellet add 500uL of freezing media (10% DMSO in FBS) ▪ Aliquot two 250uL aliquots into 1mL cryovials ▪ Label as PBMC1 and PBMC2 ▪ These PBMCs will need to be stored in Mr. Frosty in -80 freezer for 24 hours, then can be moved into a long-term storage box in -80 freezer, once box is full, move to LN tank storage.
Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated time point.
Shipping	• Ship in liquid nitrogen to central lab. • Must be batch shipped.
Notes	• Please note any variations or deviations from these instructions, problems, or issues

10.8. Nasal swabs and viral transport media (VTM) tubes

Action	Procedure
Preparation	• Label nasal swab with kit barcodes per labeling scheme (section 8). • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimen.
Collection	• Obtain a mid-turbinate nasal swab sample from participant. • Remove cap from VTM tube and insert flocked tip of nasal swab into VTM tube so that it is fully immersed in the viral transport fluid. • Bend the handle of the swab until the tip portion breaks off into the tube and cap. • Record time of collection on CRF.
Processing	• Store at -80°C until shipping.
Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated time point.
Shipping	• Ship on dry ice (shipping instructions per each RCC). • Samples should be batch shipped.
Notes	• All specimens should remain at -80ºC prior to shipping. • The samples should not be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

10.9. Tracheal aspirate

Action	Procedure
Preparation	• Label tracheal aspirate samples with sample number (section 8). • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimen.
Collection	• Obtain tracheal aspirate (~2-5 mL) if patient is ventilated. • Record time of collection on CRF.
Processing	• Aliquot 0.5 mL tracheal aspirate to two 1mL screw-topped tubes. These aliquots contain no reagent and are stored neat. • Immediately transfer to -^{80oC} freezer • (US sites only) Use a micropipette to remove 100microlitres of buffer from each of three ZYMO RNA Shield with bashing beads tubes without disturbing the beads • Add 0.5ml tracheal aspirate from the specimen trap into each of the three tubes • Vortex tubes for 10 seconds • Immediately transfer to -80^{o}C freezer
Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated time point.
Shipping	• Ship on dry ice (shipping instructions per each RCC). • Samples should be batch shipped.
Notes	• All specimens should remain at -80ºC prior to shipping. • The samples should <u>not</u> be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

10.10. Bronchoalveolar lavage

Action	Procedure
Preparation	• Label 10x1ml screw topped tubes for cell-free BAL aliquots, 1x1ml screw-topped tube for microbiome, 2x1ml screw-topped tubes for Tri-Reagent/RNA-later/Trizol lysates, 3 cryovials for cell pellets, and up to 6x15ml conical centrifuge tubes for cell-free BAL (depending on BAL return volume) with kit barcodes per labelling scheme (section 8) Store tubes in the refrigerator at 4°C. • Prepare: ice, ice container, 100 mcl and 1000 mcl pipettes and corresponding tips, cryovial rack, thawed freezing media, Mr. Frosty™ (it provides a rate of cooling very close to -1°C/minute, the optimal rate for cell preservation), and storage box. • Prepare: protective personal equipment (laboratory coat, gloves, eye protection and face mask) and further protection equipment if required by the known potential presence of a specific biological hazard. • Place screw-topped tubes, cryovials and 15ml centrifuge tubes in their racks on ice • Ensure freezing media is thawed from -20°C to 4°C in adequate amount.

Action	Procedure
	• Cool a centrifuge to 4°C. • Scan barcodes into electronic specimen CRF. • Print specimen CRF to accompany specimen.

Collection	• Obtain the bronchoalveolar lavage fluid sample from the participant into one or two 50 ml conical centrifuge tubes and place it on ice.
	  ○ Samples must be obtained from wedged position in the right middle lobe or lingula.
	  ○ Three separate 50mL aliquots of 0.9% saline should be instilled and recovered by suction.
	  ○ Samples must be placed on ice and directly transferred to lab for processing.
	• Record time of collection on CRF.

Processing	• Process samples inside a biosafety cabinet, wearing the appropriate protective personal equipment. • Gently and thoroughly mix the sample. • Withdraw 100microlitres of the well-mixed sample and perform cell count using haemocytometer and trypan blue under microscope or automated cell counter (such as the Countess). • Record the BAL white cell count in the B-CRF. Include total cell count and percentage live and dead. • Collect 1ml aliquot of raw BAL fluid sample for microbiome analysis in a screw-topped tube, and store at -80°C. • Decant 10ml of BAL and spin at 1000g for 5mins at 4°C with brake ON • Pipette off the supernatant and aliquot into 10x1ml aliquots in 1ml screw-topped tubes. Freeze at -80°C • Resuspend the cell pellet in 2ml Trireagent or RNAlater for mRNA/DNA analysis. Aliquot into 2x1ml screw-topped tubes and store at -80°C • Centrifuge the rest of the sample at 500 RCF for 5 minutes at 4oC. • Decant the supernatant in the 50 ml conical tube, leaving the cell pellet intact. • Resuspend the cell pellet in 1.5 ml of freezing media and place 3 cell pellet aliquots of 0.5 ml into 3 cryovials. • Immediately place the cell pellet aliquots in Mr. Frosty and then transfer it, within 5 minutes to prevent degradation, in the -80°C freezer for at 24-72 hours. After this point the cell pellets should be stored in liquid nitrogen or at -150°C. • Record the number of cell pellet aliquots. • Centrifuge the decanted sample at 1000 RCF for 5 minutes at 4oC.. • Aliquot the supernatant in 10-15ml amounts in 15ml centrifuge tubes 10x1 ml tubes. Store in -80°C freezer. • Store at -80°C until shipping.
Case Report Form Completion	• Complete, print out, and include the form specific for the specimens collected at the designated time point.
Shipping	• Ship on dry ice to the central lab. • Samples should be batch shipped.
Notes	• All specimens should remain at -80ºC prior to shipping. • The samples should not be thawed prior to shipping. • Please note any variations or deviations from these instructions, problems, or issues.

11. Biological sample storage

11.1. Frozen specimens

Specimens should be stored at -80C until shipment. Do not allow to thaw.

If the sample does thaw, or exceeds temperatures of -60C, please indicate this on the specimen form. Storage freezers should have temperature monitored and recorded 24/7.

11.2. CPT heparin tubes

Heparin tubes may be stored, temporarily, at room temperature and shipped on day of collection or overnight to a central processing facility as determined by each RCC.

12. Specimen shipment

All frozen specimens are shipped to the central lab designated by each RCC. Specimens should be batch shipped quarterly or more frequently depending on volume. At the time of shipment, shipment question portion of the electronic Specimen CRF must be completed. Sender must verify that specimens match those listed on the electronic Specimen CRF. Frozen material should not be shipped on Friday's or the day prior to national or institutional holidays. The receiving laboratory must be notified of any shipments prior to shipment so they can anticipate arrival. Each site must be aware of local shipping conditions, e.g., weather, that could delay transportation or delivery. Shipment box must be filled with dry ice in the event that there is a delay in receiving the shipment.

12.1. Shipping materials

Primary container: the blood collection tube, screw-topped tube or cryovial will be the primary container. Secondary container storage boxes will be provided for screw-topped tubes and blood tubes.

The rigid outer container and other shipping materials are to be procured by the site or as determined by the RCC.

- Insulated shipping boxes
- Biohazard bags for shipping
- Absorbent material for packaging and shipping
- Shipping documents
- IATA dry ice label (UN1845)
- UN3373 Category B label
- Dry ice

12.2. Frozen specimen shipment preparation

A Styrofoam container suitable for the number of samples being shipped must be used with adequate room for at least 3kg of dry ice. More dry ice will be required in summer when transport temperatures can be high.

All previous labels on the cardboard outer box must be removed or covered up prior to shipment.

- Separate individual patients in separate bags or sorted together in a 5x5 inch freezer box.
    - Boxes should be packed in a sealed bag.
- Check the sample ID numbers against the specimen form.
- Include the specimen form(s) in the shipment, enclose in the biospecimen bag pocket of each set of specimens. Each sample set should be in its own bag with its matching specimen form or sorted together in a freezer box.
- Include absorbent material in the bag or box per IATA shipping regulations.
- Place a layer of dry ice at the bottom of the frozen shipper.
- Place bags or boxes into the bottom of the shipping box
    - Note: Frozen PAXgene tubes are fragile; handle and pack like glass.
- Fill the remainder of the box with dry ice. Include at least 3 kg (6.6lb) of dry ice
- Place the Styrofoam cover on the inner Styrofoam container.
- Enclose an itemized shipping list. A manifest template is provided to each site.
    - Complete a row for each set of specimens, filling in the kit barcode, timepoint and number of each type of vial.
- Close box flaps and seal the exterior box with tape. Do not tape the interior Styrofoam box.
- Include an IATA required Dry Ice UN1845. Label with recipient and sender information
- Ship **Priority** Overnight - See local RCC instructions for detailed label preparation instructions
- Notify designated receiving lab of planned shipment the week prior and also notify again on day of shipment, including tracking number.

13. Discontinuation of sampling

A study participant may revoke their consent to provide samples for the trial, even if their participation in the trial is continued. This withdrawal of consent for specimen collection will be reflected in the patient information forms. Patients (or their legally authorized representatives) may request to have already collected samples destroyed. These requests will be logged in the PANTHER Trial CRF.