ACTG IMPAACT
Laboratory Center

Standard Operating Procedure		
Title	PBMC Processing for ACTG and IMPAACT Networks	
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1. Purpose

1.1. This Standard Operating Procedure (SOP) describes procedures for the isolation and cryopreservation of Peripheral Blood Mononuclear Cells (PBMC) from whole blood.

2. Scope

2.1. This procedure is to be utilized for processing blood samples for the isolation, cryopreservation, and storage of PBMC samples.

2.2. Network protocol-specific instructions supersede those in this SOP.

***Note for IMPAACT*: IMPAACT Network specific notes are indicated in this SOP; highlighted in yellow, bolded, and in red text.**

3. Background

3.1. Freshly collected or cryopreserved PBMC are used for the evaluation of study objectives. These assays require PBMC that have been isolated and cryopreserved under strictly defined conditions that ensure optimal recovery, viability, and functionality. It is optimal for blood to be processed and frozen within 8 hours from the time of blood draw to maintain maximum function of the cells in immune- monitoring assays.

4. Authority and Responsibility

4.1. The Network Laboratory Center Directors/PIs (or designee(s)) have the authority to establish, review and update this procedure.

4.2. All site and laboratory personnel involved in collecting, processing and/or management of PBMCs are responsible for reading and understanding this SOP prior to performing the procedures described.

5. Reporting Results

5.1. Use of a PBMC Processing Worksheet and the Laboratory Data Management System (LDMS) is required for all networks to track key processing details including the timing of processing, calculations and documentation of problems that arise during processing.

5.1.1. For ACTG/**IMPAACT** protocols, all components indicated in the ACTG/**IMPAACT** PBMC Processing Worksheet in Appendix B must be captured.

5.2. Use of LDMS is Required to capture participant and processing information, generate unique identifiers, create cryovial labels, capture storage locations and generate shipping manifests.

5.3. Key elements required for the tracking of PBMC processing include:

Key Elements Table	
Key elements for tracking PBMC processing	**Where captured**
Specimen Processing Laboratory	W L
Participant ID	W L
Visit Number	W L
Protocol	W L
LDMS Global Specimen ID	Auto-generated by LDMS
Processing Start Date/Time	W L
Processing Tech Initials (Tech)	W L
Counting Method (name of instrument or manual count)	W
Counting resuspension volume WDR (V)	W
Cell count average concentration (C)	W
Target number of cells per vial (N1)	W
Total cell number (T) = C x V	W L
Calculate the final CPS resuspension volume (V_f)	W
Frozen Date and Time	W L
Comments and Protocol Deviations, including but not limited to:	W

Key Elements Table	
• All unexpected specimen conditions • Clotted blood (number of tubes with clots, total number of tubes from the PTID batch, and processing details) • Cell yield below expected range • Processing anomalies • Troubleshooting steps taken • Note if Total Time >8 hours • Processing Time >4 hours	
Collection Date/Time	W L
Reagents (Manufacturers, Lot Numbers and Expiration Dates for DMSO, FBS, WDR, CSTFB, density gradient media)	W
ACTG/IMPAACT Only: FBS (Manufacturers, Lot Numbers, and Expiration Dates)	L
CPS (Volume of DMSO and FBS)	W
Sample tube type (HEP/ACD/EDTA/Other)	W L
Blood condition (e.g. SAT/HEM/CLT)	W L
Measured usable whole blood volume	W L
Cell Counts	W
Actual number of cells per vial (N2)	W L
Number of cryovials frozen	W L
Freezer storage information (LDMS Storage Module)	O L
Confirmation of visual QC of reagents (Tech)	O
Cell yield/mL of whole blood	W
Estimated CPS resuspension vol. (V1)	W
Confirmation of LDMS Label QC for content/barcodes (Tech)	W
Confirmation of cryovial transfer to storage box locations assigned by LDMS (Tech)	W
Date/time cryovials were transferred from controlled-rate freezing unit to storage box.	W
Final Reviews, Reviewers/Dates	W

W= Tracking on a PBMC worksheet is required

L = Entry/tracking in LDMS is required

O = Tracking on a worksheet or supplementary tracking material is optional

6. Specimen

6.1. Fresh anti-coagulated whole blood collected per the protocol requirements.

6.2. Handling Conditions

6.2.1. Specimens should be stored at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) from the time of collection until delivery to the processing laboratory.

6.2.2. Specimens should be delivered to the processing laboratory as soon as possible (best practices would be within 30 minutes to 4 hours of collection) to allow the processing laboratory ample time to complete the cryopreservation procedures. Clinic should discuss specific requirements with processing laboratory before protocol enrollment.

6.2.3. Specimens should be processed by the processing laboratory as soon as possible upon receipt (best practices would be to begin processing within 30 minutes of receipt of sample):

- Processing Time (processing start time) is the time when the tube is first opened or placed in the centrifuge, whichever comes first.
- Frozen Time is defined as the time when:
  - The Agilent Technologies StrataCooler®, Nalgene® Mr. Frosty™ or Corning® CoolCell® is put into a freezer set at - 80°C. See LPC for acceptable temperature ranges to account for routine fluctuations in freezer temperatures.
  - The cooling program of the controlled-rate freezer, such as CryoMed®, has started.

- Total Time is calculated from Specimen Collection Time and Frozen Time; ideally, this is 8 hours or less, but all specimens should be processed regardless of the Total Time.
- Total Processing Time is calculated from the Processing Time and the Frozen Time; less than four hours is recommended and processing under 3 hours ideal.

6.2.4. Do not refrigerate or freeze whole blood. Do not place blood in direct contact with cold packs if you are using them during extreme heat conditions.

6.3. Marginal Specimens

6.3.1. Clotted specimens

6.3.1.1. All blood should be processed regardless of whether it is clotted, unless otherwise directed by protocol.

6.3.1.2. Remove the clot(s) and continue processing as usual.

6.3.1.3. Mark condition as CLT on the worksheet and in LDMS. Include details in the comments section of the processing worksheet.

6.3.2. Hemolyzed specimens

6.3.2.1. Hemolysis may affect the quality of the PBMCs.

6.3.2.2. Process as usual.

6.3.2.3. Mark condition as HEM on the worksheet and in LDMS. Include details in the comments section of the processing worksheet.

6.3.3. Low cell yield

6.3.3.1. If, after performing the cell counts, the cell yield is insufficient to meet the needs of the protocol, contact the clinic for possible specimen replacement. If the cell yield is $\leq 0.4 \times 10^6$ cells/mL, contact the clinic for possible sample replacement and notify the network protocol team.

6.3.3.2. Capture troubleshooting or data verification steps taken in the comment section of the processing worksheet.

6.4. Unacceptable Specimens

6.4.1. Unlabeled or mislabeled specimens will be rejected.

6.4.2. Follow network guidance for rejection of delayed specimens.

6.4.3. Leaking samples: Notify the clinic if any of the samples are leaking and determine whether the samples are usable. Sterility of the sample and safety of the laboratory personnel handling the samples are of particular importance.

7. Equipment

7.1. Preparation & Processing

7.1.1. Class II biosafety cabinet (BSC) level 2 or higher

7.1.2. Centrifuge, low speed (capable of 200 to 1000 x g), with swinging bucket rotor, refrigerated preferred, ambient acceptable. Buckets with caps/lids are required.

7.1.3. Micropipettes, range 20, 200, 1000µL

7.1.4. Pipet-Aid (cordless preferred) for use with disposable, serological pipets

7.1.5. 2 to 8°C refrigerator

7.1.6. -20°C (or lower) freezer without automatic defrost (for FBS storage)

7.1.7. -80°C freezer (-65 to -95°C); for short-term PBMC storage.

7.1.8. 37 to 56°C water bath (for heat inactivating FBS, if necessary)

7.1.9. Bucket or beaker for bleach or other disinfectant

7.1.10. Racks suitable for holding blood collection tubes, 15mL, and 50mL conical tubes upright during processing and transport steps

7.1.11. Cryovial rack to allow for one handed opening/closing of cryovials during aliquoting steps (Nunc specific rack preferred)

7.2. LDMS required equipment. (Refer to LDMS website and network specific requirements for details).

7.2.1. Computer meeting specifications defined by Frontier Science for Web LDMS

7.2.2. Printer for LDMS generated labels

7.2.3. 2D barcode scanner

7.2.4. LN_2 compatible labels with 1x1 inch printable area

7.2.5. LN_2 compatible ink ribbon, printer specific, abrasion and chemical resistant

7.3. Liquid Nitrogen (LN_2) equipment (if required by network)

7.3.1. LN_2 storage tank (≤ -140°C)

7.3.2. IATA-approved LN_2 dry shipper

7.4. Cell Counting:

Note: Counting methods may need network approval. Follow the applicable manufacturer's calibration procedures if using an automated cell counter.

7.4.1. Automated cell counter capable of enumerating viable cells (Beckman-Coulter Vi-Cell, Muse® or equivalent).

7.4.2. Automated cell counter not capable of distinguishing viable cells (Coulter Counter, Abbott Cell-Dyn®, Sysmex® or equivalent).

Note: An automated cell counter not capable of identifying viable cells may be used to obtain a total cell count without distinguishing viable cells. However, specimens that are being prepared for the IQA PBMC Cryopreservation Proficiency Testing Program must include a viable cell count.

7.4.3. Manual cell counting chamber (hemacytometer) and light-field microscope.

Note: If a manual cell counting chamber is used with trypan blue, viable cells must be enumerated and used for cell calculations. If crystal violet is used, total cell count can be used for cell calculations.

7.5. Cryopreservation

Note: One of the following controlled-rate freezing units (CRFUs) may be used per the manufacturer's instructions; the Agilent Technologies StrataCooler® and Corning® CoolCell® are preferred.

Note: If manufacturer's instructions aren't followed, a validation study must be completed.

7.5.1. Agilent Technologies StrataCooler® Cryo Preservation Module – 400005

7.5.1.1. StrataCooler® must be at 2 to 8°C before starting the cool down of the cryovials. Do not place cryovials in a StrataCooler® that is below an initial temperature of 2°C.

7.5.2. Corning®(formerly BioCision®) CoolCell®

7.5.2.1. Make sure that all parts of the CoolCell®, including the central ring, return to room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) between uses.

7.5.3. Nalgene® Mr. Frosty™, 1°C/minute cryo-freezing container

7.5.3.1. Mr. Frosty™ should be stored at ambient temperature (15 to 25°C or up to 30°C, depending on climatic conditions) between uses.

7.5.3.2. The isopropanol level must be correct, and the isopropanol must be completely replaced after the fifth freeze-thaw cycle. A log must be used to track freeze/thaw cycles and reagent changes. See Appendix C.

7.5.4. Control-rate freezer, such as CryoMed® Freezing Chamber (Gordinier).

8. Disposables

8.1. Plastics

8.1.1. Serological pipets, disposable, 1, 5, 10, 25, 50mL, sterile

8.1.2. Micropipette tips, 20, 100, 200, 1000µL, sterile

8.1.3. 15 and 50mL disposable centrifuge tubes, sterile, conical bottom, graduated, polypropylene.

8.1.4. 50mL cell separation tubes with frit barriers (CSTFB), dry (not purchased as pre-filled with cell separation media).

8.1.4.1. Review LPC for protocol specific requirements for all networks.

8.1.5. Cryogenic vials (cryovials) with internal threads, 1.8mL to 2mL, screw cap with o-ring, sterile, polypropylene only, self-standing, graduated, leak-proof, formulated for vapor-phase LN_2 preservation (approximately -140°C). Confirm acceptability of any substitutions for cryogenic vials with the network(s) before purchase (See Appendix F).

Note: Snap cap tubes *must not* be used. Additionally, cryovials must not be filled beyond the capacity specified by the manufacturer or to the top of the tube.

8.1.6. Optional: Sterile bottles/flasks, disposable, 45mm neck, 250 to 500 mL for pooling large volume whole blood draws before PBMC separation.

8.1.7. Optional: 5mL sterile, individually wrapped plastic transfer pipets

8.1.8. Optional: Pre-filled 50mL cell separation tubes with frit barriers (CSTFB).

8.2. Markers

Note: Markers for writing on processing tubes and vials should have a fine point, and contain fast drying, indelible ink.

8.3. Labels

Note: Cryogenic labels and ink must be suitable for -80°C or LN_2 vapor phase storage temperatures.

9. Personal Protective Equipment

Note: Personal protective equipment suitable for use with bloodborne pathogens is required. Follow local laboratory guidelines and practices for the handling of blood products.

9.1. Laboratory coat or gown

9.2. Eye protection

9.3. Non-powdered, nitrile gloves or equivalent

9.4. Cryogloves

9.5. Face shields (with chin cap if preferred or required by local biosafety regulations), required when working with LN_2.

10. Reagents

10.1. The purchase of sterile reagents and use of aseptic techniques are required.

10.1.1. Store opened bottles at the temperature recommended by the manufacturer until fully consumed, until instructed to discard below, or until manufacturer's expiration date whichever comes first.

10.1.2. See Appendix F for recommended products

10.1.3. Discard if visible signs of contamination, such as a cloudy appearance, develop.

10.2. Wash Diluent Reagents (WDR)

10.2.1. 1X Hanks' Balanced Salt Solution (HBSS) without calcium or magnesium, ready-to-use

10.2.2. 1X Phosphate-Buffered Saline (PBS) without calcium or magnesium, ready-to-use.

10.3. Density Gradient Media (density 1.077 g/mL)

10.3.1. Ready to use sterile medium for high yield isolation of human lymphocytes from peripheral blood

10.3.2. See Appendix F for recommended products

10.4. Cell Separation Tube with Frit Barrier (CSTFB, if used).

10.4.1. Non-filled CSTFB System (combine a dry CSTFB with 1.077 density gradient media)

10.4.1.1. Bring density gradient media (DGM) to room temperature (15 to 25°C or up to 30°C, depending on climatic conditions). Protect from light.

10.4.1.2. Work in the BSC following aceptic technique

10.4.1.3. Record related CSTFB and DGM information directly from the package or bottle used onto the PBMC worksheet. CSTFBs prepared in advance must have labels including all required information, date of preparation and initials of person who prepared the tubes.

10.4.1.4. Prepare tubes by pipetting the volume of DGM appropriate for the size of CSTFB tube being used (as noted below).

Tube capacity (mL)	Density gradient media volume (mL)
50mL	15mL

10.4.1.5. Cap the CSTFBs with DGM added and centrifuge at 800 x g for 30 seconds (or lowest time setting above 30 seconds) at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions).

10.4.1.6. The DGM should now be below the frit barrier. Inspect tubes for gaps or large bubbles between the DGM layer and the frit barrier or for DGM remaining above the frit.

10.4.1.7. If there are gaps or large bubbles below the frit, add additional media, centrifuge the CSTFB again at 800-1000 x g for 30 seconds to 1 minute (select lowest centrifuge setting) and re-inspect.

10.4.1.8. If there is liquid (DGM) above the frit, centrifuge the CSTFB again at 800-1000 x g for 30 seconds to 1 minute (select lowest centrifuge setting). If any density gradient solution remains above the frit after re-centrifuging, remove it following aseptic technique.

10.4.1.9. Follow density gradient media manufacturer's storage recommendations.

10.4.2. Pre-filled CSTFB (1.077 density gradient media)

Note: The capacity of the tube required will depend on the whole blood volume. Store in the refrigerator (2 to 8°C).

- Protect from light.
- A cloudy appearance indicates deterioration of the product. Discard if visible signs of contamination are noted.
- Allow pre-filled CSTFB to come to room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) prior to use.

10.5. Freezing Reagents

10.5.1. Fetal Bovine Serum (FBS), heat-inactivated required. (See Section 11.1 Heat-Inactivated FBS for handling and management details).

*Note for ACTG/**IMPAACT** studies:* There are limited approval FBS lots. Refer to https://www.actg-impaact-lc.org under resources > documents for current FBS ordering information and list of approved lots. Please ensure that the FBS manufacturer, lot number, and expiration date is entered into LDMS.

10.5.1.1. Check with applicable network(s) for preferred vendors.

10.5.1.2. Obtain a certificate of analysis from the vendor for local laboratory quality control records.

Note: A copy of the FBS certificate of analysis may be required to export (or import) PBMC aliquots between countries.

10.5.1.3. FBS stored frozen (≤ -20°C/according to manufacturer's recommendations) is good until the manufacturer's expiration date.

10.5.1.4. FBS thawed and stored at 2 to 8°C is stable for one calendar month.

10.5.2. Dimethylsulfoxide (DMSO), cell-culture grade

10.5.2.1. Use cell-culture grade DMSO.

10.5.2.2. Store unopened bottles at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions). Check bottle for expiration date and discard if expired.

10.5.2.3. Once opened, undiluted DMSO, when protected from light and moisture, is stable at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) for 6 months (or until the manufacturer's expiration date if that date is < 6 months from the date of opening). Correct the label on the bottle to reflect the new expiration date.

10.5.2.4. Use aseptic technique when removing DMSO from the bottle to avoid possible contamination.

10.5.2.5. Discard contents of open bottle if visible signs of contamination are noted.

10.5.2.6. Reagents may be aliquoted in small amounts to help preserve sterility. Label aliquots with "DMSO," manufacturers name, lot number, the date opened/aliquoted, the expiration date (six months from opening or expiration date from original bottle whichever is sooner) and tech initials. Protect aliquots from light.

10.5.3. Disinfectant

10.5.3.1. 70% v/v ethanol disinfectant, spray bottle

10.5.3.2. 10% v/v bleach, bucket or beaker and spray bottle (must be made daily)

10.5.3.3. Other disinfectant as specified by local laboratory policy

10.6. Cell Counting Reagents

Note: The requirements for counting reagents will vary depending on the method used. Refer to the network approved SOP/instructions for the method being used.

10.6.1. 0.4% Trypan blue solution

10.6.2. Optional: 0.05% crystal violet solution can be used to stain the cell nucleus so mononuclear cells can be identified and counted using a hemacytometer. If viability is required, a second manual count using trypan blue must be performed. 0.05% Crystal Violet Solution contains: 0.05 g crystal violet, 2mL glacial acetic acid, and 98mL distilled or deionized H_2O.

11. Reagent Preparation

11.1. Heat-Inactivated FBS (HI-FBS)

Note: HI-FBS can be ordered from the manufacturer, or FBS can be ordered from the manufacturer and heat inactivated in the lab. Follow these instructions for thawing, aliquoting and use.

11.1.1. Remove the FBS from the freezer.

11.1.2. Thaw in the refrigerator (2 to 8°C), preferred, or for several hours at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions). Do not allow FBS to sit at room temperature any longer than necessary to complete the thawing process.

11.1.3. Gently swirl two or three times over the course of the thaw.

11.1.4. Follow these additional instructions if the FBS was not heat inactivated. If the FBS was heat inactivated by the manufacturer, skip to 11.1.5.

11.1.4.1. Place FBS in a 56°C (55 to 57°C) water bath. Carefully monitor the water bath temperature. Higher temperatures can degrade components of the FBS.

Note: The water level in the water bath should cover the level of the FBS in the bottle, but not touch the cap of the bottle. This will help ensure even heating of the FBS and avoid contamination.

11.1.4.2. Once the water bath has returned to 56°C (55 to 57°C), heat the FBS for 30 minutes, mixing every 5 to 10 minutes. Heating for longer periods of time can degrade components of the FBS.

Note: Clean the bottle with 70% v/v ethanol before opening.

11.1.5. Mix the HI-FBS gently but thoroughly using aseptic technique.

11.1.6. Aliquot into sterile, labeled, 50mL sterile, conical bottom, graduated polypropylene centrifuge tubes, or other size aliquots appropriate for the anticipated workload.

Note: Labels should identify these tubes as "HI-FBS" and include the manufacturer's name, lot number, the aliquot date, storage conditions, the original manufacturer's expiration date, and the technician's initials. FBS is stable for 1 month (if 1 month period does not exceed original manufacturer's expiration

date) at 2 to 8°C, or until the original manufacturer's expiration date if kept at -20°C. Remember to update the expiration date and storage conditions on aliquots/bottles removed from -20°C storage for use.

11.1.7. Refrigerate (2 to 8°C) the number of aliquot tubes needed for the expected workload. Mix well before use. The aliquot tubes that are not immediately needed should be frozen and are stable until the original manufacturer's expiration date.

Note: Repeated freeze/thaw cycles will have an adverse effect on the quality of the FBS. Do not refreeze aliquots that have been stored at refrigerated temperatures.

11.1.7.1. To use the frozen aliquots, thaw in advance in the refrigerator overnight, preferred, or at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) for several hours. Do not allow FBS to sit at room temperature any longer than necessary to complete the thawing process.

11.1.7.2. Once thawed, FBS is stable for 1 month at 2 to 8°C, or expiration date from original bottle, whichever is sooner. Remember to update the expiration date and storage conditions on aliquots/bottles removed from -20°C storage for use. Mix well before use.

11.2. Fresh Cryopreservation Solution (CPS)

11.2.1. CPS Components

Components	Percent (v/v)
DMSO	10%
FBS (heat-inactivated)	90%

11.2.2. For CPS preparation, Method 1 is the traditional HANC method, and Method 2 is for ACTG.

Note for ACTG: When using Method 2 for ACTG, proceed directly to Section 11.2.7.

***Note for IMPAACT*: Use Method 2 when maximum number of aliquots for each visit and final aliquot volume (V2) are defined.**

11.2.3. **Method 1: Traditional HANC CPS Preparation**

Note: This is the traditional HANC method of estimating CPS volume needed in advance of processing. Use when target cells per vial (N1) and final aliquot volume (V2) are defined, but number of aliquots varies depending on the total number of cells harvested.

11.2.3.1. Use sterile, labeled, disposable 15mL or 50mL conical centrifuge tube(s) to hold prepared CPS.

11.2.3.2. Prepare CPS in advance, ideally prior to the start of processing, and chill in the refrigerator (2 to 8°C). CPS can be stored at 2 to 8°C for up to 1 working day (<18 hours).

Note: Mixing DMSO and FBS is an exothermix reaction. Record the time of CPS batch placement in the refrigerator and *ensure a minimum of 30 minutes* of chilling at 2 to 8°C has passed before using. If CPS is urgently needed, chill in an ice bath (wet ice and water) for a *minimum of 15 minutes prior to use*.

11.2.3.3. CPS must be maintained at 2 to 8°C to avoid damage to cells. Do not use CPS that has not met the minimum requirement for chilling noted above

11.2.3.4. Use the formula below to estimate the volume of CPS to prepare in advance for final resuspension of PBMC when number of aliquots is variable. Examples are shown below.

Maximum Whole Blood Volume (mL) x Cell Yield (cells/mL) x Freeze-down Concentration (mL/cells) = Estimated CPS (mL)

Round this result up to the next whole mL

Note: For Maximum whole blood volume: Use the maximum possible total volume of whole blood to be processed. Multiply the number of tubes scheduled for collection by the total volume capacity, blood + liquid anticoagulant, of each tube to get this total. (i.e. consider each 10 mL NaHep tube, 10mL EDTA tube and 8.5mL ACD tube (8.5mL whole blood capacity + 1.5mL liquid anticoagulant) to have a 10mL volume capacity).

For Cell Yield: Use an average adult yield of 1.5 million cells/mL as a default. Exceptions may be made when warranted, (i.e. participant known to have cell yield above 3.0 million cells/mL).

For freeze-down concentration: Use the final aliquot volume (V2) and the target number of cells per aliquot (N1) defined in the protocol specific LPC. Record as V2/N1.

Monitor CPS daily usage to maximize efficiency and minimize waste and adjust as needed. For example, if the calculation yields CPS volumes that are regularly not sufficient, i.e., multiple batches must be made to complete processing, consider increasing the final volume by a fixed percentage such as 20% and round **up** to the next whole mL.

Examples: Adult Blood—Daily CPS batch - multiple visits scheduled:

- Target Freeze-down Concentration (mL/cells) for all is (1.0mL/15 x 10^6 cells) or 15 million cells (N1) frozen in 1mL (V2) of CPS.
- Calculate total maximum expected whole blood volume:
  Note: Remember to include the liquid anticoagulant when calculating maximum total volumes from ACD tubes. NaHep and EDTA tubes do not have liquid anticoagulants so the tube volume as recorded on the laboratory requisition is the maximum possible volume.
  - First scheduled visit includes 18 x 8.5mL ACD tubes for PBMC processing
  - Second scheduled visit includes 8 x 8.5mL ACD tubes for PBMC processing
  - Third scheduled visit includes 8 x 10.0mL NaHep tubes for PBMC processing
  - Total tubes expected (18+8+8) = 34, 34 tubes x 10.0mL = 340.0mL maximum expected volume

Usable Whole Blood x	Cell Yield x	Freeze-down Concentration =	Estimated CPS to Prepare
(340.0mL) x	(1.5 x 10^6 cells/1mL) x	(1.0mL/15 x 10^6 cells) =	34.0mL

Example: Adult Blood—One Large Volume Blood Collection. Target Freeze-down Concentration (mL/cells) is (1.0mL/15 x 10^6 cells) or 15 million cells frozen in 1mL (V2) of CPS. CPS batch created after UWBV was measured.

Usable Whole Blood x	Cell Yield x	Freeze-down Concentration =	Estimated CPS to Prepare
(135.0mL) x	(1.5 x 10^6 cells/1mL) x	(1.0mL/15 x 10^6 cells) =	14.0mL

Example: Adult Blood—8 x 10.0mL NaHep Blood Collection. Target Freeze-down Concentration (mL/cells) is (1.0mL/**10 x 10^6** cells) or **10 million cells** (N1) frozen in 1mL (V2) of CPS. CPS batch created before UWBV was measured.

Usable Whole Blood x	Cell Yield x	Freeze-down Concentration =	Estimated CPS to Prepare
(80.0mL) x	(1.5 x 10^6 cells/1mL) x	(1.0mL/**10 x 10^6** cells) =	12.0mL

11.2.4. Use the following formula to calculate the amount of DMSO and FBS needed.

CPS = 1 part DMSO + 9 parts FBS

Examples:

Estimated CPS Volume	DMSO Volume = (.1)(CPS volume)	HI-FBS Volume = CPS volume – DMSO volume	Total CPS Volume = DMSO volume + FBS volume
10.0ml	1.0mL	9.0mL	10.0mL
5.0ml	0.5mL	4.5mL	5.0mL

11.2.5. Record the CPS, DMSO and FBS volumes on the PBMC worksheet. It is recommended that the date and time of creation and initials of person creating the batch be captured on the worksheet and conical tube as well.

11.2.6. If secondary batches are created to complete processing, remember to capture the same details for the additional batch, including the method/time of chilling, on the worksheet.

11.2.7. **Method 2: CPS Preparation for ACTG**

Note: This is the ACTG method of estimating CPS volume. Use when maximum number of aliquots for each visit and final aliquot volume (V2) are defined.

11.2.7.1. Use sterile, labeled, disposable 15mL or 50mL conical centrifuge tube(s) to hold prepared CPS.

11.2.7.2. Prepare CPS in advance, ideally prior to the start of processing, and chill in the refrigerator (2 to 8°C). CPS can be stored at 2 to 8°C for up to 1 working day (<18 hours)

Note: Mixing DMSO and FBS is an exothermic reaction. Record the time of CPS batch placement in the refrigerator and *ensure a minimum of 30 minutes* of chilling at 2 to 8°C has passed before using. If CPS is urgently needed, chill in an ice bath (wet ice and water) for a *minimum of 15 minutes prior to use*.

11.2.7.3. CPS must be maintained at 2 to 8°C to avoid damage to cells. Do not use CPS that has not met the minimum requirement for chilling noted above.

11.2.7.4. Determine the volume required based on protocol mandated maximum number of aliquots per visit or additional batch volume required to meet current V_f needed.

11.2.7.5. Add additional 10-20% to CPS volume required to account for potential loss during mixing/pipetting steps and round the result UP to the next whole number.

11.2.7.6. Scenario A: Fixed maximum number of aliquots per visit.

Example: Lab schedule has two PBMC visits requiring a maximum allowable number of aliquots of four per visit. The protocol specific volume per aliquot (V2) is 1.0mL. Total of eight aliquots maximum for the day. Multiply number of aliquots by V2 to determine required FBS. Add additional volume, such as 10%, to account for loss during mixing/pipetting. Round UP to next whole number for final CPS volume to make.

(8 x 1.0mL = 8.0mL, 8.0mL x 1.1 = 8.8mL. Round up to final volume of 9.0mL)

11.2.8. Use the formula below to calculate the amount of DMSO and FBS needed.

CPS = 1 part DMSO + 9 parts FBS

Examples:

Estimated CPS Volume	DMSO Volume = (.1)(CPS volume)	HI-FBS Volume = CPS volume – DMSO volume	Total CPS Volume = DMSO volume + FBS volume
10.0ml	1.0mL	9.0mL	10.0mL
5.0ml	0.5mL	4.5mL	5.0mL

11.2.9. Record the CPS, DMSO and FBS volumes on the PBMC worksheet. It is recommended that the date and time of creation and initials of person creating the batch be captured on the worksheet and conical tube as well.

11.2.10. If secondary batches are created to complete processing, remember to capture the same details for the additional batch including the method/time of chilling on the worksheet.

12. PBMC Processing Introduction and Guidelines

There are standard principles and steps common to all PBMC processing procedures. Variations occur with the choice of separation techniques (CSTFB versus manual overlay), the treatment of the blood (dilution with or without plasma replacement versus direct plasma harvest), final cell concentration, and freezing/storage. Select the appropriate procedure sections for cell separation and blood treatment, and freezing and storage based on network and protocol requirements.

13. Cell Separation and Blood Dilution by Cell Separation Tube with Frit Barrier (CSTFB) with Plasma Replacement

Section 13 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Section 13 or Section 14, but not both.

***Note for IMPAACT*: IMPAACT does not use CSTFB.**

13.1. Separation of lymphocytes from peripheral blood using CSTFB separation tubes with room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) density gradient media (DGM) added.

13.1.1. Perform all pipetting and mixing in a Class II biosafety cabinet (BSC) level 2 or higher.

13.1.2. Spray down all surfaces, racks, and reagent bottles with 70% v/v ethanol or equivalent disinfectant each time prior to entering and using the BSC.

13.1.3. Unless otherwise noted, the procedure is carried out at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions).

13.1.4. Use a new pipet for each participant identification number (PTID) and additive.

13.2. Prepare whole blood samples, reagents, and supplies.

13.2.1. Prepare and chill the CPS (see Section 11 Reagent Preparation) prior to processing or sufficiently in advance of mixing with PBMC if additional CPS is needed.

Note: CPS batches should be prepared in advance of beginning specimen processing steps. Batch volumes must be monitored and creation of additional batches, when needed, captured in full detail on the PBMC worksheet.

13.2.2. Prepare enough CSTFB tubes to manage the maximum amount of blood expected.

13.2.2.1. For 50mL CSTFB tubes, plan for a maximum volume of 20mL, or one CSTFB for every two 8.5-10.0mL blood collection tubes. Tip: Divide the total number of 8.5-10.0mL collection tubes to be received by 2. If the result is a fraction, (an odd number of blood collection tubes), round up to the nearest whole number (i.e. add an additional CSTFB tube).

13.2.2.2. Examples:

- Visit 1 has 3 x 10.0mL NaHep tubes. Prepare 2 CSTFB tubes.

- Visit 10 has 10 x 8.5mL ACD tubes. Prepare 5 CSTFB tubes

13.2.3. Ensure that the prepared CSTFB tubes containing DGM are at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) before starting processing steps.

13.2.4. Visually inspect the filled CSTFB tubes to confirm no large bubbles, gaps exist between the DGM and frit barrier and that no DGM is present above the frit before adding the WDR or blood to the tubes. (See CSTFB preparation instructions in Section 10.4 for instructions on managing DGM volume issues when noted).

13.2.5. Gather the same number of new 50mL sterile, conical bottom, graduated polypropylene centrifuge tubes as CSTFB prepared (see Section 13.2.2) for use. These "wash" tubes will be used for the harvested cells and subsequent wash steps.

CSTFB Size (mL)	Conical Centrifuge Tube Size (mL)
50	50

13.2.6. If plasma replacement is required, gather a 15 or 50mL sterile, conical bottom, graduated polypropylene centrifuge tube appropriate for the required volume of plasma to be harvested. Label with PTID, anticoagulant, and derivative.

13.2.7. If the specimen tubes are cold to the touch (due to cold ambient conditions such as transport in cooler months), allow the tubes to reach room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) before processing

13.2.8. Carefully check the PTID on all tubes of blood received. Organize primary tubes such that there is no possibility of mixing tubes between PTID/PIDs or anticoagulants.

Suggestion: Place all tubes for each PTID/anticoagulant in one rack. Different racks can be used to separate PTIDs or tube types and use different colored markers for each PTID to avoid confusion.

13.3. Plasma Replacement

Note: Perform this plasma replacement step only if plasma aliquots are required from the PBMC collection tubes per the protocol or LPC; proceed to step 13.4 if plasma aliquots are not required.

13.3.1. Blood collection tubes from the same PTID and same anticoagulant must be processed individually (not pooled in 50mL conical centrifuge tubes) unless otherwise indicated in the protocol specific LPC.

13.3.2. Mark the whole blood volume on each collection tube at the meniscus.

13.3.3. Centrifuge the whole blood at 200 to 400 x g for 10 minutes. Record processing start time on the PBMC worksheet.

13.3.4. Transfer plasma to a labeled 15 or 50mL conical centrifuge tube for second centrifugation to remove any cellular debris.

13.3.5. Add a quantity of WDR sufficient to bring blood back to its original whole blood volume, mix gently and continue PBMC processing at step 13.4.

13.3.6. Complete the plasma processing by centrifuging the collected plasma at 800 to 1200 x g for 10 minutes to obtain PL2 aliquots or according to protocol or LPC. This step may occur later when the centrifuge is not in use for PBMC processing.

13.3.7. Aliquot double spun plasma into labeled aliquot tubes as specified by protocol specific LPC and discard remaining cellular debris.

13.4. Blood Dilution for CSTFB separation

Note: Use one 50mL tube for each 12 to 20mL of whole blood. Use as many CSTFB tubes as required to distribute all the blood for each PID/PTID.

Note: Density gradient media is toxic to cells; work quickly and efficiently during the separation steps.

13.4.1. Label each CSTFB and corresponding sterile conical centrifuge "wash" tube with the PTID (and anticoagulant if appropriate)

13.4.2. Using a sterile serological pipet, add WDR to each labeled, prepared CSTFB:

CSTFB Size (mL)	Approximate Volume of WDR (mL)
50	5

13.4.3. Using a sterile serological pipet, add WDR to each pre-labeled, sterile conical centrifuge "wash" tube:

CSTFB Size (mL)	Conical Centrifuge Tube Size (mL)	WDR Pre-Fill Volume (mL)
50	50	25

13.4.4. Uncap the tubes of anti-coagulated blood. If plasma replacement steps were not required/performed, record the time of cap removal as processing start time on the PBMC worksheet.

13.4.5. If the blood in a collection tube is clotted or grossly hemolyzed, see Section 6.3.

13.4.6. Use a sterile serological pipet to mix the whole blood gently then transfer the blood into the labeled CSTFBs.

Note: Blood must be transferred using a 10mL serological pipet, in measured quantities of 10.0mL until less than 10.0mL remains. Distribute the blood across CSTFB tubes in fixed increments to ensure accurate tracking of volumes for UWBV measurement throughout the distribution of whole blood.

13.4.7. Target transfer of 15 – 20mL total amount of blood to each labeled CSTFB. (The expanded range below is allowable only in certain situations where the target range is not possible). Do not start to fill a new 50mL CSTFB unless a minimum of 12mL of blood is available/remaining.

CSTFB Size (mL)	Approximate Volume of Blood (mL)*
50	15-20 (12 to 22 allowable in certain scenarios)

*Lower blood volumes, especially in the presence of low hematocrits, may cause the buffy coat to drop close to/onto the frit, making it difficult to harvest. Higher blood volumes may contribute to increased background/debris in specimens. Refer to protocol-specific guidelines for lower blood draw volumes.

13.4.8. Determine and record an accurate measurement of the usable whole blood volume within 0.1mL.

Note: the volume of usable whole blood is not necessarily equal to the tube size.

13.4.9. Using a sterile pipet, rinse each original anti-coagulated blood tube with WDR, add rinse volumes to the CSTFB making sure not to exceed the total tube volume (WDR + Whole Blood) limit.

CSTFB Size (mL)	Total Tube Volume Limit (mL) (Whole Blood +WDR)
50	30

13.4.10. Carefully cap the CSTFB.

13.5. CSTFB density centrifugation and collection

13.5.1. Hold the tubes in an upright position, place them in a rack and transfer them to the centrifuge.

13.5.2. Centrifuge at 800 to 1000 x g for 15 minutes at 15 to 25°C (or up to 30°C, depending on climatic conditions) with the Brake OFF/set to zero.

Note: If the brake is on, it will disrupt the layers.

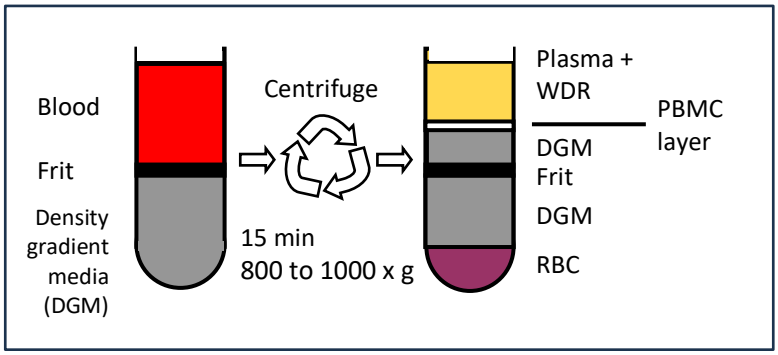
13.5.3. While the CSTFB tubes are spinning, discard all emptied collection tubes following laboratory practices, clean the BSC surface and organize for the next steps.

13.5.4. Confirm the correct number of labeled, sterile conical centrifuge tubes pre-filled with WDR ("wash" tubes) are ready in the BSC for the harvesting/wash steps.

13.5.5. Once the centrifuge comes to a complete stop, gently remove each CSTFB from the centrifuge so as not to disturb the layers. Use a rack to support the tubes and carefully transfer to the BSC.

13.5.6. Centrifugation results in the tube contents dividing into six distinct layers including the frit. From the top of the tube, these are:

- Plasma + WDR
- PBMC layer
- Density gradient media (DGM)
- Frit
- Density gradient media (DGM)
- Packed red blood cells (RBC) and granulocytes



13.5.7. Inspect the tubes for the following potential problems. Document observations and any follow-up actions taken according to network and laboratory requirements.

- Hemolysis in the Plasma + WDR layer
- Clots visible on the frit after centrifugation.
- Poor PBMC layer due to errors in centrifugation such as speed time or braking. PBMC layer will appear small and indistinct while Plasma + WDR layer may be slightly cloudy. Refer to Appendix D for troubleshooting.
- PBMC layer formed on frit due to low RBC count or hematocrit volume.
- RBC layer immediately below and touching the PBMC layer.

13.5.8. Using a new sterile serological pipet for each PTID, remove the upper yellowish, plasma-WDR fraction down to within 1 to 2 cm of the cloudy white PBMC band located at the interface between the plasma-WDR (yellowish) fraction and the clear separation medium solution. Be careful not to disturb the cell layer during this process. Discard the plasma-WDR fraction per laboratory policy.

Note: Alternatively, the cloudy white PBMC band may be removed by carefully inserting the pipet through the upper plasma-WDR layer.

13.5.9. Using a sterile serological pipet, collect all cells at the cloudy white interface above the frit. Take care not to aspirate any more density gradient medium than necessary.

13.5.9.1. Transfer the collected cells from one CSTFB to a single corresponding, pre-labeled, sterile conical centrifuge "wash" tube (prepared as noted in Section 13.4). Tubes are pre-filled with WDR to save time.

13.5.10. Re-cap the CSTFB containing the remaining red blood cells and separation media. Discard the CSTFB as biohazardous waste following laboratory policy.

13.6. Ensure that all key elements are recorded according to network and laboratory requirements. Proceed to Section 15.

14. Cell Separation by Manual Density Gradient Media Overlay or Underlay and Blood Dilution by Manual Density Gradient Cell Separation with Plasma Replacement

Section 14 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Section 13 or Section 14, but not both.

***Note for IMPAACT*: The use of the underlay method is not recommended.**

14.1. Separation of lymphocytes from peripheral blood using Manual Density Gradient Media Overlay Method

14.1.1. Perform all pipetting and mixing in a Class II biosafety cabinet (BSC) level 2 or higher.

14.1.2. Spray down all surfaces, racks, and reagent bottles with 70% v/v ethanol each time prior to entering and

using the BSC.

14.1.3. Unless otherwise noted, the procedure is conducted at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions).

14.1.4. Use a new pipet for each participant identification number (PTID) and additive.

14.2. Prepare whole blood samples, reagents, and supplies

14.2.1. Prepare and chill the CPS (see Section 11 Reagent Preparation) prior to processing or sufficiently in advance of mixing with PBMC if additional CPS is needed.

Note: CPS batches should be prepared in advance of beginning specimen processing steps. Batch volumes must be monitored and creation of additional batches, when needed, captured in full detail on the PBMC worksheet.

14.2.2. Allow the density gradient media to come to room temperature (15 to 25°C or up to 30°C, depending on climatic conditions). See Section 10 Reagents for more information.

14.2.3. Gather enough 15 or 50mL [**15mL for IMPAACT**, 50mL for ACTG] sterile, conical bottom, graduated polypropylene centrifuge tubes to manage all dilution, overlay/underlay and wash steps.

14.2.4. If plasma replacement is required, gather a 15 or 50mL [**15mL for IMPAACT**, 50mL for ACTG] sterile, conical bottom, graduated polypropylene centrifuge tube appropriate for the required volume of plasma to be harvested. Label with PTID, anticoagulant, and derivative.

14.2.5. If the specimen tubes are cold to the touch (due to cold ambient conditions such as transport in cooler months), allow the tubes to reach room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) before processing.

14.2.6. Carefully check the PTID on all tubes of blood received. Organize primary tubes such that there is no possibility of mixing tubes between PTIDs or anticoagulants within a PTID collection.

Suggestion: Place all tubes for each PTID/anticoagulant in one rack. Different racks can be used to separate PTIDs or tube types, and a different color of marker can be used for each PTID to avoid confusion.

14.2.7. Determine and record an accurate measurement of the usable whole blood volume within 0.1mL using a 10mL serological pipet (or other network approved method). As a reminder, the volume of usable whole blood is not necessarily equal to the tube size.

14.3. Plasma Replacement

Note: Perform this plasma replacement step only if plasma aliquots are required from the PBMC collection tubes per the protocol or LPC; proceed to step 14.4 if plasma aliquots are not required.

14.3.1. Blood collection tubes from the same PTID and same anticoagulant may be processed individually or pooled in 15 or 50mL [**15mL for IMPAACT**, 50mL for ACTG] conical centrifuge tubes.

14.3.2. Mark the whole blood volume on each collection tube at the meniscus.

14.3.3. Centrifuge the whole blood at 200 to 400 x g for 10 minutes. Record processing start time on the PBMC worksheet.

14.3.4. Transfer plasma to a labeled 15 or 50mL [**15mL for IMPAACT**, 50mL for ACTG] conical centrifuge tube for second centrifugation to remove any cellular debris.

14.3.5. Add a quantity of WDR sufficient to bring blood back to its original whole blood volume, mix gently and continue PBMC processing at step 14.4.

14.3.6. Complete the plasma processing by centrifuging the collected plasma at **800** to 1200 x g for 10 minutes to obtain PL2 aliquots or according to protocol specific LPC . This step may occur later when the centrifuge is not in use for PBMC processing.

***Note for IMPAACT*: Spin plasma at 800 g for 10 minutes.**

14.3.7. Aliquot double spun plasma into labeled aliquot tubes as specified by protocol specific LPC and discard remaining cellular debris.

14.4. Blood Dilution for Manual Density Gradient Cell Separation

14.4.1. Uncap the tubes of anti-coagulated blood. If plasma replacement steps were not required/performed,

record the time of cap removal as processing start time on the PBMC worksheet.

14.4.2. If a tube is grossly clotted or grossly hemolyzed, see Section 6.3.

Note: The pooling of buffy coats is allowed according to the guidelines in Appendix E: Pooling Buffy Coat Layers for Density Gradient Media PBMC Isolation. To pool buffy coats, replace steps 14.4.3 and 14.4.4 with the instructions in Appendix E.

14.4.3. Label each conical centrifuge tube with the PTID and anticoagulant.

Conical Centrifuge Tube Size (mL)	Approximate Blood volume (mL)
50	12 to 22
15	**4 to 5**

14.4.4. Transfer the blood to a sterile, labeled 15 or 50mL [**15mL for IMPAACT**, 50mL for ACTG] conical centrifuge tube and add sufficient volume of WDR to dilute the blood according to the density gradient media package insert (The ratio of WDR to whole blood should ideally be one part WDR and one part whole blood).

14.5. For Density Gradient Cell Separation:

Note: Use either the Overlay Method (Section 14.5.1) or Underlay Method (Section 14.5.2), but not both.

14.5.1. Overlay Method:

14.5.1.1. Prepare a labeled sterile conical centrifuge tube for each tube containing diluted blood.

14.5.1.2. Add the appropriate volume of density gradient media to the empty sterile conical centrifuge tubes.

Note: The volume of density gradient media will depend on the ratio of density gradient media to diluted blood recommended by the manufacturer.

14.5.1.3. Carefully and slowly pipet diluted blood on top of the density gradient media.

Suggestion: Gently allow the WDR-diluted blood mixture to flow down the side of the tube and pool on top of the density gradient media surface without breaking surface plane.

***Note for IMPAACT*: Rinse the primary blood tube with WDR and add to the overlay to maximize recovery.**

14.5.1.4. Carefully cap the tubes. Proceed to Section 14.6.

14.5.2. Underlay Method:

***Note for IMPAACT*: The use of the underlay method is not recommended.**

14.5.2.1. Mix gently and thoroughly to decrease clumping of the cells during separation.

Optional: To either whole blood or blood-WDR, add another volume of WDR equal to the total blood volume.

14.5.2.2. Based on the volume of WDR-diluted blood, determine the volume of density gradient medium required for each tube.

Note: The volume of density gradient media will depend on the ratio of density gradient media to diluted blood recommended by the manufacturer.

***Note for IMPAACT*: Rinse the primary blood tube with WDR and add to the overlay to maximize recovery.**

14.5.2.3. Carefully and slowly pipet density gradient media solution UNDER blood-WDR.

14.5.2.4. Carefully cap the tubes. Proceed to Section 14.6.

14.6. Lymphocyte density centrifugation and collection:

14.6.1. Hold the tubes in an upright position, place them in a rack and gently transfer them to the centrifuge.

14.6.2. Centrifuge at 400 x g for 30 minutes at 15 to 25°C (or up to 30°C, depending on climatic conditions) with the Brake OFF/set to zero, or as outlined in the package insert that accompanies the gradient medium.

Note: If the brake is on, it will disrupt the layers. The centrifuge brake must be OFF for the separation to be clean and to maximize retrieval of the PBMCs.

14.6.3. While the conical tubes are spinning, discard all emptied collection tubes following laboratory practices,

clean the BSC surface and organize for the next steps.

14.6.4. Label (with the PTID/anticoagulant) the same number and size of new sterile conical centrifuge tubes as conical centrifuge tubes used in the separation centrifugation step. Use these new tubes for the following cell harvest and wash steps.

14.6.5. Using a sterile pipet, add WDR to each pre-labeled, sterile conical centrifuge "wash" tube:

Conical Centrifuge Tube Size (mL)	WDR Pre-Fill Volume (mL)
50	25
15	**5**

14.6.6. Once the centrifuge comes to a complete stop, gently remove each conical tube from the centrifuge so as not to disturb the layers. Use a rack to support the tubes and carefully transfer to the BSC.

14.6.7. If the cell layer is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuge the tube. Document the problem and actions taken according to network and laboratory requirements.

14.6.8. Document hemolysis or small clots visible at the cell interface.

Note: Look for hemolysis, or clots after centrifugation. Grade hemolysis +1 through +4 based on the description given in the glossary. Record your observations.

14.6.9. Using a new sterile pipet (serological or transfer pipet) for each PTID, remove the upper, yellowish, plasma-WDR fraction down to within 1 to 2 cm of the cloudy white PBMC band located at the interface between the plasma-WDR (yellowish) fraction and the clear separation medium solution. Be carefull not to disturb the cell layer during this process. Discard the plasma-WDR fraction per laboratory policy.

Note: Alternatively, the cloudy white PBMC band may be removed by carefully inserting the pipet through the upper plasma-WDR layer.

14.6.10. Using a sterile serological or transfer pipet, collect all cells at the cloudy white interface. Take care not to aspirate any more separation medium solution than necessary.

14.6.11. Transfer the collected cells from one conical centrifuge tube to a single corresponding, pre- labeled, sterile conical centrifuge tube. Tubes can be pre-filled with WDR to save time (Section 14.6.5).

14.6.12. Re-cap the conical centrifuge tube containing the remaining red blood cells/separation medium and discard the tube as biohazardous waste following laboratory policy.

14.7. Ensure that all key elements are recorded according to network and laboratory requirements. Proceed to section 15.

15. Washing, Counting, Resuspension, Concentration, and Overnight Controlled- Rate Freezing

15.1. Wash 1:

15.1.1. QS (bring the volume of the PBMC fraction up) to approximately 45mL (for 50mL conical centrifuge tubes for ACTG) or **10mL (for 15mL conical centrifuge tubes for IMPAACT)** by adding WDR. Mix gently.

15.1.2. Re-cap all the conical tubes now containing harvested cells diluted in WDR.

15.1.3. Centrifuge diluted cells at 200 to **400** x g for 10 minutes at 15 to 25°C (or up to 30°C, depending on climatic conditions) low brake optional.

***Note for IMPAACT*: Centrifuge diluted cells at 400 g for 10 minutes.**

15.1.4. Remove the tubes from the centrifuge and check for the cell pellet.

15.1.4.1. If the cell pellet is not visible, confirm that the centrifuge is operating properly and the correct settings were used. Correct any problems you find. Re-centrifuge the tubes. Document the problem and actions taken according to network and laboratory requirements. If the cell pellet is still not visible after re-centrifuging the tube, continue processing steps and document details on processing worksheet.

15.1.5. Remove and discard the WDR supernatant carefully without disturbing the cell pellet by quickly decanting into the designated liquid waste container in the BSC. Alternative methods such as removal with serological pipet or by aspirating may be used for pellets that are loose or contain large quantities of red blood cells.

15.2. Wash 2:

15.2.1. Resuspend each pellet in a small volume of WDR mixing, gently but thoroughly into a homogenous cell suspension.

Tube Size (mL)	WDR Resuspension Volume (mL)
50	≤ 5
15	**≤ 3**

15.2.2. Combine the pellet suspensions from the same PTID/anticoagulant. This is the harvested cell tube.

Tube Size (mL)	Number of Pellets Suspensions to Combine	Total Volume (mL)
50	≤ 4	≤ 20
15	**≤ 2**	**≤ 6**

15.2.3. Use a small volume of WDR to rinse the tubes from which the pellets were transferred. Collect the WDR rinse in the harvested cell tube.

15.2.4. QS the PBMC fraction by adding WDR and mix gently.

Tube Size (mL)	QS Volume (mL)
50	≤ 45
15	**≤10**

15.2.5. Re-cap the tubes and place the tubes in the centrifuge.

15.2.6. Centrifuge diluted cells at 200 to **400** x g for 10 minutes at 15 to 25°C (or up to 30°C, depending on climatic conditions) low brake optional.

***Note for IMPAACT*: Centrifuge diluted cells at 400 g for 10 minutes.**

15.2.7. Remove the tubes from the centrifuge and check for the cell pellet.

Note: If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems and re-centrifuge the tube. Document the problem and actions taken according to network and laboratory requirements. If the cell pellet is still not visible after re-centrifuging the tube, continue processing steps and document details on processing worksheet.

15.2.8. Remove and discard the WDR supernatant carefully without disturbing the cell pellet by quickly decanting into the designated liquid waste container in the BSC. Alternative methods such as removal with serological pipet or by aspirating may be used for pellets that are loose or contain large quantities of red blood cells.

15.3. PBMC Cell Count

15.3.1. Determine and record the WDR counting resuspension volume (V) accurate to within 0.1mL. V is important because this is the volume on which the cell count is based.

***Note for IMPAACT*: The counting re-suspension volume (V) is 1mL for blood volumes ≤3mL or 2mL for blood volumes >3mL. For blood volumes >10mL, V is 20% of the measured usable whole blood volume rounded to the nearest whole mL (recorded X.0).**

***Example*: Measured usable whole blood volume recorded as 3.0mL. The counting re-suspension volume would be 1.0mL.**

Note for ACTG: The counting resuspension volume (V) is 20% of the measured usable whole blood volume, rounded to the nearest whole mL (recorded X.0) in almost all cases.

Example: Measured usable whole blood volume recorded as 78.6mL. The counting resuspension volume would be 16.0mL (78.6 x 20% = 15.72. 15.72 rounded to the nearest whole number is 16.0mL)

Examples of scenarios where V may be modified:

- Calculated V slightly exceeds capacity of the conical tube. UWBV measured and recorded as 255.2mL. 20% rounded to the nearest whole number is 51.0mL. In this scenario, V may be reduced to 45.0mL to allow for safe mixing and subsequent centrifugation in a 50mL conical tube.
- Participant sample results in very faint cell bands and subsequent significantly small cell pellets. In this scenario, V may be reduced to prevent out of range cell counts.

15.3.2. If there is more than one pellet from the same PTID/anticoagulant, use a small measured/tracked

amount of WDR to gently resuspend and combine cell pellets into one tube. Using the remaining resuspension volume, rinse the tubes from which the cells were transferred. Add the rinse to the tube containing the cell suspension.

15.3.3. Mix cells gently, but thoroughly, immediately prior to sampling for the cell count.

15.3.4. Transfer a small volume of the resuspension to a small tube for counting. Follow instrument or counting SOP guidance for appropriate volumes.

Note: If repeated counts are necessary, minimize the sampling volume needed.

15.3.5. Follow the SOP for the cell counting method approved at the processing laboratory to determine the cell concentration x 10^6 per mL.

Note: Cells at $10^3/\mu L$ = cells at 10^6/mL.

Note: Automated counts may be run once. Manual counts must count at least the four large corner squares ($1mm^2$).

15.3.6. Calculate the total number of cells using the following formula:

T = C x V

T = Total number of cells

C = Concentration (10^6/mL) determined in counting method

V = Counting resuspension volume of WDR in mL

15.3.7. Calculate the cell yield in cells/mL of usable whole blood using the formula below.

Cell Yield (10^6 cells/mL) = T /Usable Whole Blood Volume

Note: Calculate the cell yield for quality purposes. Refer to Section 16 Quality Control for the expected range of cell yields and troubleshooting tips.

15.4. Calculation of final resuspension volume

15.4.1. Calculate the CPS freeze-down resuspension volume required by completing the steps below for the target number of cells per vial.

Note: The target number of cells per vialvaries by network and protocol. Refer to the protocol or protocol specific LPC for target final cell concentration information.

15.4.2. Calculate the estimated CPS freeze-down resuspension volume (V1) required by using the target number of cells per vial.

Note: Confirm adequate volume of CPS is prepared and has been chilled in the refrigerator (2 to 8°C) for a minimum of 30 minutes. Preparation in an ice bath (wet ice with water) for a minimum of 15 minutes prior to use is allowed in emergency situations only.

V1 = (T/N1) x V2

T = Total number of cells

N1 = Target number of cells per vial

V2 = Final aliquot volume in mL

Round V1 down to the nearest whole (1.0) mL to determine V_f.

15.4.3. Calculate the actual number of cells per vial (N2) using the actual CPS freeze- down volume (V_f) determined in the previous calculation.

N2 = (T/ V_f) x V2

N2 = Actual number of cells per vial

T = Total number of cells

V2= Final aliquot volume in mL (1.0mL unless otherwise directed in protocol-specific documentation).

15.5. Labeling

15.5.1. Complete the printing, quality control and labeling of the cryovials PRIOR to the final centrifugation.

Note: It is important to minimize the time that the cells remain in a pellet.

15.5.2. Generate cryovial labels using the LDMS.

15.5.2.1. Follow network laboratory practice for completing the data entry.

15.5.2.2. Proof each derivative type of cryovial label for data entry errors against the laboratory requisition and processing worksheet PRIOR to labeling cryovial.

15.5.2.3. Visually inspect the label barcode and print area for alignment and print quality.

15.5.2.4. Correct any data entry errors in LDMS and re-print labels as needed.

15.5.3. Apply the labels on the cryovials so that the contents of the tube are visible.

15.5.4. Scan the empty, labeled cryovials in sequential GSID order into the LDMS storage module to ensure that the barcode is scannable; the verification of ability to scan and the assignment of storage locations are combined at this step.

Note: Use of Nunc racks to allow for one-handed opening/closing of vials is highly recommended. Cryovial caps may not be placed on any surfaces.

15.6. Final Centrifugation

Note: If cells are to be frozen as nonviable PBMC Pellets (PEL) and as viable cells, remove the volume of PBMCs needed for creating nonviable cell pellets prior to the final centrifugation step and complete processing of nonviable cell pellets and follow protocol specific instructions for non-viable PBMC pellets.

15.6.1. QS (bring up the volume of the cell suspension) to 45mL (for 50mL conical centrifuge tubes for ACTG) or **10mL (for 15mL conical centrifuge tubes for IMPAACT)** with WDR and place the conical tube(s) containing the diluted harvested cells in the centrifuge.

15.6.2. Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 25°C (or up to 30°C, depending on climatic conditions) brake optional.

***Note*: For IMPAACT centrifuge diluted cells at 400 g for 10 minutes.**

15.6.3. Verify that all cryovials are labeled and easily accessible.

Note: Pre-chilling vials and/or working on wet ice is allowed.

15.6.4. Select the controlled-rate freezing unit: StrataCooler®, Mr. Frosty™, CoolCell®. Label freezing unit(s) appropriately to allow laboratory staff to determine contents. Ensure unit is at correct temperature for use at time of vialing and easily accessible just prior to removal of final cell pellets from centrifuge. See section 7.5 for storage and maintenance information.

15.7. Aliquoting for cryopreservation

Note: The following steps must be performed quickly to preserve cell integrity.

15.7.1. Remove and discard the WDR supernatant carefully without disturbing the cell pellet by quickly decanting into the designated liquid waste container in the BSC. Alternative methods such as removal with serological pipets or by aspirating may be used for pellets that are loose or contain large quantities of red blood cells.

15.7.2. Gently resuspend the cell pellet prior to adding the CPS by flicking, tapping, or pipetting as noted below.

15.7.2.1. Gently add the volume of cold CPS (V_f) determined in section 15.4 (minus any resuspension volume used to break up the pellet) to the resuspended cells with continuous swirling.

15.7.3. Work quickly once the CPS has been added. Do not allow the cells to sit in the cryopreservation solution for longer than 10 minutes before placing them in the freezer.

15.7.4. Mix the entire suspension with a serological pipet gently, but thoroughly and quickly, immediately prior to aliquoting. Take care not to create bubbles during these steps. Maintain the suspension throughout the vialing process.

15.7.4.1. Aliquot **0.5**-1.0mL per cryovial, unless directed otherwise by protocol specific LPC.

Note: **Wait about 30 seconds for the CPS to pool at the bottom of the conical tube** and then evenly distribute any excess volume among all the cryovials for that PTID.

15.8. Overnight controlled rate freezing

15.8.1. Following processing and counting, complete the required steps to freeze cells immediately.

15.8.2. Position the selected controlled-rate freezing unit for transfers: Agilent Technologies StrataCooler®, Nalgene® Mr. Frosty™, Corning® CoolCell®. See Section 7.5 for storage and maintenance information.

15.8.3. Immediately transfer all cryovials in sequential global specimen ID order to the controlled-rate freezing unit.

For all CRFUs, Nalgene® Mr. Frosty™, Corning® CoolCell® and Agilent Technologies StrataCooler®, close the container and place it in a -80°C (-65 to -95°C for ACTG/**IMPAACT**) freezer, in a location that is not disturbed by repeated freezer access (i.e., away from the front or top of the freezer near the opening door/lid). Do not stack. It is recommended that all CRFUs remain in the freezer overnight.

For CryoMed® or other mechanical controlled-rate freezer, start the cooling program according to the appropriate on-site SOP.

15.8.4. Record the frozen date/time immediately on the PBMC worksheet. Enter the frozen date/time recorded on the worksheet into LDMS.

15.9. Document all the key elements according to network and laboratory requirements.

16. Quality Control

16.1. Cell Yields

It is important to be aware of the expected recovery for the population of participants for which the processing is performed. Cell yields can serve as internal quality control markers for each run. Yields outside the expected ranges may indicate a procedural error, reagent deterioration, cell count error, or calculation error.

Note: The recommendations provided below are meant to provide guidelines to help identify egregious technical errors prior to cryopreservation. These values may vary depending on the anti-coagulant used.

16.1.1. Expected Cell Yields for adult populations:

Population	Mononuclear Cell Yield Range (cells/mL)
Adult	(0.8 to 3.2) x 10^6
Pediatric – birth to 2 years	**(2.5 to 7) x 10^6**
Pediatric - >2 years	**(1.5 to 5) 10^6**

16.1.2. Unexpected Cell Yields

16.1.2.1. If cell yields are outside the expected range, review dilution schemas, calculations, processing technique (especially adequate mixing of cell counting suspensions) and PTID history if available for possible causes.

16.1.2.2. Cell yields from participants living with HIV may be lower than those shown in the above table.

16.1.2.3. If cell dilution or counting errors are suspected, make a fresh dilution and recount.

16.1.3. Record all results and any problems that occur during processing and actions according to network and laboratory requirements. See Section 5 for details.

16.2. Cell Viability

16.2.1. Freshly isolated PBMC viability should be >95%.

16.2.2. If the fresh PBMC viability is <95%, review the results with the supervisor and document according to network and laboratory requirements.

Note: If samples are being prepared for the IQA PBMC Cryopreservation Proficiency Testing Program, a viable cell count is required.

17. PBMC Storage (Interim or On-Site)

17.1. Maintain cold chain during all transfer steps to avoid damage to the cells.

***Note for IMPAACT*: Transfer PBMCs to LN_2 dewar or -150°C mechanical freezer within 72 hours of transferring the cryovials to the controlled-rate freezing unit.**

Note for ACTG: Ship on dry ice within 4 weeks of the date of freezing unless instructed otherwise in the LPC.

Continue to 17.2

17.2. Transfer PBMCs to temporary storage in a -80°C freezer.

17.2.1. Transfer the cryovials from the controlled-rate cooling system to the designated storage location at -65 to -95°C for ACTG.

17.2.2. Transfer the cryovials after a minimum of 4 hours for Nalgene® Mr. Frosty™ and Corning® CoolCell® and overnight for StrataCooler® (overnight storage for all CRFUs is recommended as a standard practice to reduce risk to specimens). For CryoMed®, transfer the cryovials upon completion of the program to the -80°C freezer.

Note: **Recommended for ACTG:** Use of a dry ice transfer pan is allowed for controlled-rate freezing units only; pre-chilled tall-sided coolers are required for PBMC freezer/storage boxes. See Cross-Network Cold Chain Guidelines (Section 7, Specimen Transfer Procedures) for additional details. Make sure the cryovial freezer box and lid is covered on all sides with dry ice. Work rapidly and efficiently to minimize cryovial exposure to ambient temperatures.

Note for ACTG: Do not store in liquid nitrogen (LN_2). Store at -65 to -95°C until shipped.

Note: Pre-chill the dry ice shipper and use a pre-chilled tall-sided cooler during the QC and packing steps. Make sure the dry ice shipper is completely full of dry ice before sealing.

17.2.3. Contact network laboratory center personnel if samples cannot reach their destination within the network allotted temporary storage time. The network laboratory center will determine if transfer to LN_2 storage and shipment in LN_2 shippers is appropriate.

17.3. Transfer PBMCs to a LN_2 dewar or -150°C mechanical freezer.

17.3.1. Within 72 hours of freezing the cryovials in the controlled-rate cooling system, transfer on dry ice to the designated storage location in the LN_2 dewar or -150°C storage system.

17.3.2. Frozen PBMC samples are viable in LN_2 vapor phase indefinitely. Do NOT transfer samples from LN_2 or -150°C back to -80°C freezers unless directed to by network or protocol team.

17.3.3. Once samples have been stored in LN_2, all transfers or shipments must be maintained in LN_2 (≤ -140°C) and shipped in an IATA-approved LN_2 dry shipper.

18. Completing Processing Documents

18.1. Make sure all appropriate information is documented, following good documentation practices, according to network and laboratory requirements and that all calculations are correct. See Section 5 for details.

18.2. Store the laboratory requisitions, PBMC Processing Worksheets and any other tracking documents according to laboratory policy.

19. Definitions and Acronyms

Term	Definition
ACTG	Advancing Clinical Therapeutics Globally
IMPAACT	**International Maternal Pediatric Adolescent AIDS Clinical Trials Network**
Centrifuge Temperature	15 to 25°C or up to 30°C, depending on climatic conditions
Clotted, Grossly	More than ¾ of the whole blood mass is clotted.
Clot, Small	Small clots noted after PBMC processing in the whole blood tube but noted on the separation tube frit after centrifugation.
CPS	Cryopreservation Solution
CSTFB	Cell Separation Tube with Frit Barrier
DGM	Density Gradient Media
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution

Term	Definition
Hemolysis	A pink to red coloration of serum or plasma due to the lysis of red blood cells. Grade hemolysis according to the following scale: 1+ Pale pinkish-red color in serum or plasma, able to clearly read newsprint placed behind the blood tube 2+ Pinkish-red color in serum or plasma, newsprint is readable but not as sharp 3+ Dark pinkish-red color in serum or plasma, newsprint appears obscured. 4+ Dark red mahogany color in serum or plasma, unable to read news print *Note*: Lysed red blood cells give serum or plasma a colored but clear quality where red blood cell contamination gives the serum or plasma a cloudy quality.
HI-FBS	Heat Inactivated Fetal Bovine Serum
Icteric	A green or orange tinted plasma suggesting the presence of increased bilirubin.
LDMS	Laboratory Data Management System
LPC	Laboratory Processing Chart (ACTG/**IMPAACT**)
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered saline
PI	Principal Investigator
PTID/PID	Participant Identification Number
QS	Quantity Sufficient—add sufficient quantity of liquid to bring to specified volume
Room Temperature (RT)	15 to 25°C or up to 30°C, depending on climatic conditions
Usable whole blood volume (UWBV)	The measured volume of whole blood that is actually processed (The usable whole blood volume may not be equal to the capacity of the tube)
Vapor phase storage	Liquid nitrogen (LN_2) vapor-phase storage is the space in the storage tank that is above the LN_2 liquid at the bottom of the tank.
WDR	Wash Diluent Reagent (HBSS, PBS)

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21. Appendices

21.1. Appendix A: Generic PBMC Processing Worksheet

Note: Appendix A is also provided as downloadable forms on the HANC public website at https://www.hanc.info/resources/sops-guidelines-resources/laboratory/cross-network-pbmc-processing-sop.html

21.2. Appendix B: ACTG/IMPAACT PBMC Processing Worksheet

21.3. Appendix C: Example Nalgene® Mr. Frosty Isopropanol Change Log

21.4. Appendix D: Troubleshooting: Recovery of PBMC in the Absence of a Defined PBMC Band after Density Gradient Centrifugation

21.5. Appendix E: Pooling Buffy Coat Layers for Density Gradient Media PBMC isolation

21.6. Appendix F: Cross-Network PBMC SOP Quick Guide—CSTFB

21.7. Appendix G: Cross-Network PBMC SOP Quick Guide—Manual Overlay Method

21.8. Appendix H: Example Reagents

21.9. Appendix I: Revision History

22. SOP Approval

APPROVING BODY	DATE OF APPROVAL
UCLA-LC PI: Grace Aldrovandi 	2026AUG31
Process Owner: Kathie Ferbas, PhD 	2026AUG31

23. Revision History

VERSION #	EFFECTIVE DATE	REPLACES	DATE OF REVISION	RATIONALE FOR REVISION/RETIREMENT
1.0	2024Aug30			
2.0	2026Sept4	1.0	2026Aug31	Cross-Network PBMC SOP updated to include changes to FBS tracking in LDMS, processing improvements, CPS calculations, and the update of the ACTG/IMPAACT PBMC Processing Worksheet.

Appendix A: Generic PBMC Processing Worksheet - NOT TO BE USED FOR ACTG/IMPAACT PROTOCOLS

Note: Complete all fields by hand, using a pen.

Specimen Processing Laboratory: Protocol:

Participant ID (PTID/PID): Visit Number: Visit Type:

Collection Date: Collection Time:

Processing Start Date: Processing Start Time: Processed By (Initials):

Reagents	Manufacturer	Lot Number	Expiration Date
DMSO			
FBS			
WDR: HBSS or PBS (circle one)			
Cell Separation Tube (frit)			
Density Gradient Media			

	Volume in mL (record as X.Y)			Expiration Date
	CPS	DMSO	FBS	
CPS				1 working day (<18hrs)

Data to be Captured During Processing	Sample
Sample tube type (circle one or record "other" tube type)	ACD / HEP / EDT Other: _____
Blood condition (circle one or more; add comments on reverse as needed)	SAT/ HEM / CLT
Measured usable whole blood volume (to the nearest 0.1mL)	mL
Indicate processing method (circle one)	CSTFB / overlay / underlay
Counting Method: Name of specific instrument or manual count (record in field to right)	
Counting resuspension volume of HBSS (or other WDR) (**V**) (record as X.Y)	mL
Cell count average concentration (**C**)	x 10^6 cells/mL
Total cell number (**T) = C x V**	x 10^6 cells
Calculate cell yield/mL of whole blood (QC check)= (T/Usable Whole Blood Volume)	x 10^6 cells/mL
Calculate estimated CPS resuspension vol. ***(V1)=(T/15x10^6 cells)(1mL)***	mL
Calculate final CPS resuspension volume (V_f), (V1 rounded DOWN to nearest whole (X.0) mL)	mL
Calculate actual number of cells per vial ***N2*** *= (T/V_f)* x V2; (V2=1 mL).	x 10^6 cells/vial
Print and QC LDMS Label content/barcodes (initials of person (s) performing QC)	
Frozen Date and Time (ddMMMyyyy /HH:MM) (Explain in comments section if not within **4 hours** of processing start time)	
Number of Cryovials actually frozen ***Note:*** Should be equal to final CPS resuspension volume for 1mL aliquots (V_f).	
Complete remaining LDMS entries including total cell count & frozen time (Initials)	

Appendix A: Generic PBMC Processing Worksheet - NOT TO BE USED FOR ACTG/IMPAACT PROTOCOLS

Note: Complete all fields by hand, using a pen.

Specimen Processing Laboratory:

PTID/PID:

Transfer of Cryovials to Freezer Storage Box	
Person who transferred cryovials to storage box locations assigned by LDMS	
Date (ddMMMyyyy)/time cryovials were transferred from controlled-rate freezing device to storage box. (Sample must be maintained at -80°C during transfer)	
Initial (Primary) Review (Initials/Date)	
Final (Secondary) Review (Initials/Date)	

Hemacytometer Counts	Total Count	Viable Cells	Non-Viable
Square #1 (cells/mm^2)			
Square #2 (cells/mm^2)			
Square #3 (cells/mm^2)			
Square #4 (cells/mm^2)			
Average Cell Count per Square (cells/mm^2)			
PBMC Dilution Factor (1:DF*)			
Hemacytometer Factor for cells/mL	10^4	10^4	10^4
Cell count concentration (C) = (Average Cells/mm^2)(DF)(10^4); convert to 10^6 cells/mL	Not applicable	x 10^6cells/mL	Not applicable
% viability = (Viable cells 4 squares/total cells 4 squares) (100)	Not applicable		Not applicable

**Note:* Dilution Factor (DF) = (parts cells + parts dilution fluid)/ parts cells

Automated Cell Counts (10^3/µl=10^6/mL)	Count #1
Cell Count (C) as cells x 10^6/mL	
PBMC Dilution Factor (1:DF**)	
Cell Concentration = (C)(DF)	x 10^6cells/mL
% viability (if applicable)	

***Note:* Dilutions for automated counters are extremely rare. If performing direct counts, enter a 1 in the DF box and complete the column.

Comments, protocol deviations, and additional information not captured elsewhere in this worksheet:

Appendix B: ACTG/IMPAACT PBMC Processing Worksheet v8.0 (Page 1 of 2)

Note: The fields in this worksheet must be filled out by hand, using a pen.
Refer to Protocol LPC for N1 and A (for IQA certification/testing refer to current program description).

Specimen Processing Laboratory: Submission type (circle one): IQA or Protocol

Protocol Number (N/A if IQA): N1: A*:

Participant ID (PTID/PID): Visit Number: Visit Type:

Collection Date: Collection Time:

Processing Start Date: Processing Start Time: Processed By (Initials):

Reagents	Visual QC (yes/no)	Manufacturer	Lot Number	Expiration Date
DMSO				
FBS				
WDR: HBSS or PBS (circle one)				
Cell Separation Tube (frit)				
Density Gradient Media				

	Volume in mL (record as X.Y)			Expiration Date
	CPS	DMSO	FBS	
CPS				1 working day (<18hrs)

Data to be Captured During Processing	Sample
Sample tube type (circle one or record "other" tube type)	ACD / HEP / EDT Other: _____
Blood condition (circle one or more; add comments on reverse as needed)	SAT/ HEM / CLT Other:_____
Measured usable whole blood volume **(WBV)** (to the nearest 0.1mL)	mL
Measured plasma volume removed & replaced with equal volume of WDR (to the nearest 0.1mL)	
Indicate processing method (circle one)	CSTFB / overlay / underlay
Counting Method: Name of specific instrument or manual count (record in field to right)	
Counting re-suspension volume of WDR) (**V**) = WBV X 0.20 (rounded to the nearest whole (X.0)mL) ***For IMPAACT: V is 1mL for WBV ≤3mL, 2mL for WBV >3mL, or 20% for WBV >10mL***	mL
Cell count average concentration (**C**)	x 10^6 cells/mL
Total cell number (**T) = C x V**	x 10^6 cells
Calculate cell yield/mL of whole blood. (QC check)= (T/Usable Whole Blood Volume)	x 10^6 cells/mL
If ***T/A ≥ N1***; then CPS re-suspension vol ***(V1) = A**** If ***T/A < N1***; then calculate estimated CPS re-suspension vol. ***(V1)=(T/N1x10^6 cells)(1mL)*** ***For IMPAACT: Please follow protocol defined requirements.***	mL
Calculate final CPS re-suspension volume (V_f), (V1 rounded DOWN to nearest whole (X.0) mL)	mL
Calculate actual number of cells per vial***. N2*** = ***(T/V_f)*** **x V2**; (V2=1 mL). ***Note:*** Do not store more than 50M cells per vial	x 10^6 cells/vial
Number of Cryovials actually frozen ***Note:*** Should be equal to final CPS re-suspension volume for 1mL aliquots (V_f) and **≤ (A)***	
Print and QC LDMS Label content/barcodes (initials of person (s) performing QC)	
Frozen Date and Time (ddMMMyyyy /HH:MM) (Explain in comments section if not within **4 hours** of processing start time)	
Complete remaining LDMS entries including total cell count & frozen time (Initials)	

Note: **A*** = The maximum number of aliquots required according to the protocol-specific Laboratory Processing Chart (LPC). Do not store more than this number of aliquots. For IMPAACT please follow protocol defined requirements.

Appendix B: ACTG/IMPAACT PBMC Processing Worksheet v8.0 (Page 2 of 2)

Note: The fields in this worksheet must be filled out by hand, using a pen.

Specimen Processing Laboratory:

PTID/PID:

Transfer of Cryovials to Freezer Storage Box	
Person who transferred cryovials to storage box locations assigned by LDMS	
Date (ddMMMyyyy)/time cryovials were transferred from controlled-rate freezing device to storage box. (Sample must be maintained at -80°C during transfer)	
Initial (Primary) Review (Initials/Date)	
Final (Secondary) Review (Initials/Date)	

Hemacytometer Counts	Total Count	Viable Cells	Non-Viable
Square #1 (cells/mm^2)			
Square #2 (cells/mm^2)			
Square #3 (cells/mm^2)			
Square #4 (cells/mm^2)			
Average Cell Count per Square (cells/mm^2)			
PBMC Dilution Factor (1:DF*)			
Hemacytometer Factor for cells/mL	10^4	10^4	10^4
Cell count concentration (C) = (Average Cells/mm^2)(DF)(10^4); convert to 10^6 cells/mL	Not applicable	x 10^6cells/mL	Not applicable
% viability = (Viable cells 4 squares/total cells 4 squares) (100)	Not applicable		Not applicable

Automated Cell Counts (10^3/µl=10^6/mL)	Count #1
Cell Count (C) as cells x 10^6/mL	
PBMC Dilution Factor (1:DF**)	
Cell Concentration = (C)(DF)	x 10^6cells/mL
% viability (if applicable)	

***Note:* Dilution Factor (DF) = (parts cells + parts dilution fluid)/ parts cells

*****Note:* Dilutions for automated counters are extremely rare. If performing direct counts, enter a 1 in the DF box and complete the column.

Comments, protocol deviations, and additional information not captured elsewhere in this worksheet:

Appendix C: Example Nalgene® Mr. Frosty™ Isopropanol Change Log

Note: Isopropanol must be changed every fifth freeze/thaw cycle.

Date of Use	Isopropanol change (Y/N)	Initials

Date of Use	Isopropanol change (Y/N)	Initials

Reviewed by: ______________________________ Date: ______________________

Appendix D: Troubleshooting: Recovery of PBMC in the Absence of a Defined PBMC Band after Density Gradient Centrifugation

D.1. Background: If something has gone wrong during the density gradient centrifugation of the blood, the density gradient media and plasma layer will not separate cleanly and you may not see a PBMC layer. Do not panic. Partial recovery of the PBMC can be achieved with additional steps.

D.2 Identify the problem:

D.2.1 Remove tubes from centrifuge and transfer to a rack.

D.2.2 Try to identify why there isn't a clear PBMC layer. Possible causes are listed below:

- The tube was dropped or bumped.
- The brake was left on.
- The centrifugation speed was too high. Verify that the rpm setting was correct for the procedure used (CSTFB or manual density gradient cell separation) by checking the RCF/rpm chart for the rotor. Some centrifuges require that the settings on the centrifuge match the type of bucket used. If the settings are not correct then the centrifuge may miscalculate its speed.
- The centrifuge stopped due to a discontinuity in the electricity supply.
- The frit dislodged. (This is often due to a centrifugation speed that was too high, but occasionally there is a defective tube in the batch.)
- The centrifuge was misbalanced.
- The donor has low lymphocyte, white blood cell or hematocrit counts.

D.3 Of the above causes, the first five causes are easily fixed. If the cause is due to a misbalanced centrifuge, determine why the centrifuge was misbalanced. Check the following:

D.3.1 Check that the tubes were balanced.

D.3.2 Check that the centrifuge buckets were balanced.

D.3.3 Check that the centrifuge arms and buckets were properly greased and oiled

Note: If ever in doubt about a centrifuge, use another one.

D.4 Assuming the problem is fixed, re-centrifuge the samples as follows:

D.4.1 Reagents:

- Density gradient media
- 50mL conical centrifuge tubes
- Pipets

D.4.2 Method:

Note: Density gradient media is toxic to cells so work efficiently

D.4.2.1 Add 15mL of density gradient media to sterile 50 mL tubes (not CSTFB).

D.4.2.2 Allow the density gradient media to warm to room temperature (15 to 25°C or up to 30°C, depending on climatic conditions) while working with the sample.

D.4.2.3 For each mixed tube, label 50mL tubes with subject PTID. Use a pipet to slowly remove the contents of the mixed sample from the separation or CSTFB. (Typically, the CSTFB frit will have dislodged.)

D.4.2.4 Transfer up to 30mL of the mixed sample to the tube containing density gradient media.

D.4.2.5 Repeat this for all mixed samples.

D.4.2.6 Place the tubes into the centrifuge, checking that the tubes are balanced.

D.4.2.7 Centrifuge for 30 to 40 minutes at 400 x g with the Brake OFF at room temperature (15 to 25°C or up to 30°C, depending on climatic conditions).

D.4.2.8 A PBMC layer should now be visible. (Often some cells will have been lost, so the layer could be thin.)

D.4.2.9 Carefully transfer the PBMC layer to a 50mL conical centrifuge tube labeled with the PTID identifier. Use one new tube for every density gradient media tube.

D.4.2.10 Re-cap the density gradient media tube.

D.4.2.11 Return to Section 15 of the main protocol.

Note: In the "Comments and Protocol Deviations" section of the **PBMC Processing Worksheet**, record the details of the deviation from the SOP (i.e. that steps from "Appendix D" were taken to recover PBMC due to the absence of a defined PBMC band after density gradient centrifugation). In addition, note how long the re-centrifugation took in order to provide an estimate of how long cells were in density gradient media.

Appendix E: Pooling Buffy Coat Layers for Density Gradient Media PBMC Isolation

This procedure can be used when isolating PBMCs from multiple tubes of blood of the same PTID- anticoagulant combination. This procedure allows one to consolidate the buffy coat layers to reduce the use of reagents and consumables, increase recovery and decrease contamination.

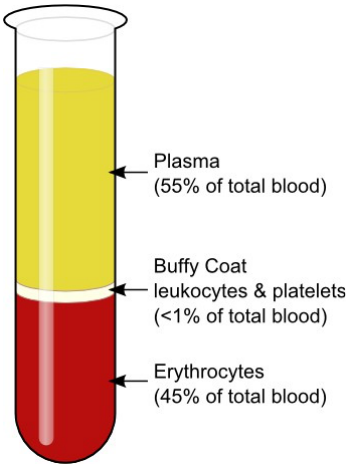
The buffy coat is the fraction of whole anticoagulated blood that contains white blood cells (WBCs) and platelets and occurs after centrifugation at the interface of plasma and red blood cell layers. Most WBCs are found in the buffy coat layer with only a very small fraction (<1 million total) remain in the red cell pack after buffy coat harvest. The buffy coat layer is harvested with a small fraction of plasma and red blood cells (approximately 1.5mL) and then diluted prior to overlaying on a gradient medium for lymphocyte separation.

Procedure:

E1. Make sure that you have completed the steps in Sections 14.4.1 through 14.4.2.

E2. Centrifuge whole blood at 200 to 400 x g for 10 minutes.

E3. Harvest plasma (and save as needed - see Section 14.3.4 through 14.3.7) from each tube to within about 5mm from the buffy coat layer (which is obvious in most cases unless the patient is severely neutropenic/lymphopenic).



E4. Determine the capacity and number of conical centrifuge tubes that will be needed for each PTID- anticoagulant combination. Do not pool samples from different PTIDs/anticoagulants. In general:

- Buffy coats from two 10mL blood collection tubes can be combined into one 15mL conical centrifuge tube.
- Buffy coats from up to six 10mL blood collection tubes can be combined into one 50mL conical centrifuge tube.

E5. Label each conical centrifuge tube with the PTID.

E6. Add WDR to each sterile conical centrifuge tube.

Conical Centrifuge Tube Capacity (mL)	WDR volume (mL)
15	3
50	10-15

E7. Hold the plasma depleted tube (which now contains a small amount of residual plasma and packed cells) at about a 30° angle.

E8. Use a sterile, 2.5mL, wide bore, disposable polypropylene pipet to harvest the buffy coat. Aspirate the buffy coat by moving down the low end of the tube. Slowly draw in the plasma followed by the buffy coat which will "slide" down the packed red cell layer (about 1.5mL of aspirate). Transfer the buffy coat to the WDR-containing tube, rinsing the pipet 2 to 3 times with WDR/cell suspension.

E9. Harvest and pool the buffy coats from the remaining tube(s) for that PTID-anticoagulant combination.

E10. QS the WDR/cell suspension with additional WDR to the desired volume for performing the density gradient cell separation. Gently mix the buffy coat pool 3 to 4 times with a pipet.

E11. Continue with density gradient cell separation at step in Section 14.5. For 14.5, "diluted blood" means "diluted buffy coat."

Appendix F: Example Reagents and Supplies

Note: All reagents must be purchased sterile, and the use of aseptic technique is required.

Reagent/Supply	Example(s)	Optional/Required
Dry CSTFB	• Accuspin™ separation tubes • Leucosep®separation tubes	Optional
Pre-filled cell separation tubes with frit barriers (CSTFB) with 1.077 density gradient media	• Accuspin™ System Histopaque®-1077	Optional
1.077 Density Gradient Media	• Ficoll-Paque PLUS and PREMIUM • Lymphoprep™ • Lymphocyte Separation Media – LSM ™	Optional
Dimethyl sulfoxide (DMSO), cell culture grade	• Hybrimax, Sigma-Aldrich cat# D2650, endotoxin tested, hybridoma tested • Or equivalent product	Required
Fetal Bovine Serum (FBS)		***ACTG/IMPAACT***: Refer to www.actg-impaact-lc.org under resources > documents for current FBS ordering inforamtion and required lots.
Cryovials	• Nunc CryoTubes™, internal thread, polypropylene (PP) tubes and screw cap #377267 • Greiner Cryotubes, internal thread, natural screw cap, polypropylene (PP), round bottomed, writing area, starfoot, sterile #122263 • Corning® 2mL internal thread polypropylene cryogenic vial, self-standing with round bottom #430488	Nunc Preferred / internal thread required for all networks.
Cryogenic labels	• Cryo-Tags® and Cryo-Babies® Brady B461 or B490 • Shamrock freezer labels.	Optional
Marking pens	• Fisherband* Marking Pens #13-379 • Nalgene® Lab Pen/Lab Marker #6310/#6311	Optional

Appendix G: PBMC SOP Quick Guide—CSTFB

Use of a **PBMC Processing Worksheet** and the LDMS is ***required for all networks*** (see Section 5 for details). Before using this quick guide for the first time, be sure to review the complete PBMC SOP for important notes and details, and network-specific guidelines.

Steps	Reference to SOP
1. Prepare and chill the CPS.	11.2
2. Prepare whole blood samples, reagents, and supplies.	13.2
3. **If** plasma aliquots **are** required per protocol instructions: a. Centrifuge the whole blood at 200 to 400 x g for 10 minutes. b. Mark the total blood volume at the meniscus then transfer plasma to a labeled 15 or 50mL conical centrifuge tube for further processing (800 to 1200 x g for 10 minutes, brake optional) c. Add sufficient quantity of WDR to bring blood back to its original whole blood volume, mix gently and continue PBMC processing.	13.3
4. Add 5mL of WDR to each labeled CSTFB and 25mL of WDR to each labeled corresponding 50mL conical wash tube. (***Note:*** Can be performed earlier as part of setup [step 2]). 5. Transfer 15 to 20mL (12-22 allowed) of blood into the labeled CSTFBs. a. Track measured volumes carefully. Record measured UWBV accurate to nearest 0.1mL. 6. Add WDR tube rinse and final WDR to CSTFB up to 30mL (WDR + Whole Blood).	13.4
7. Centrifuge at 800 to 1000 x g for 15 minutes at 15 to 30°C with the Brake OFF/set to zero. 8. Inspect the tubes for possible problems. 9. Harvest each CSTFB buffy coat into a labeled corresponding single labeled 50mL conical centrifuge tube pre-filled with 25mL of WDR.	13.5
10. Add WDR to QS to a total volume of 45mL and mix gently. 11. Wash #1—centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C, brake optional. 12. Check for the cell pellets! Document unexpected observations. 13. Gently remove the supernatant without disturbing the cell pellet.	15.1
14. Resuspend the cell pellet in small amount of WDR making a homogenous cell suspension. 15. Combine up to 4 pellet suspensions into one 50mL conical centrifuge tube. 16. Add WDR tube rinse and final WDR of 45mL to cell tube. 17. Wash #2—Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C, brake optional. 18. Check for the cell pellets! Document unexpected observations. 19. Gently remove the supernatant without disturbing the cell pellet.	15.2
20. Calculate the WDR counting resuspension volume (V). 21. Combine cell pellets into one tube using resuspension volume WDR. This is the volume on which the cell count is based. 22. Count and calculate the total number of cells 23. Calculate the cell yield in cells/mL of usable whole blood.	15.3
24. Calculate final CPS resuspension volume. Check calculations.	15.4
25. Complete the printing, labeling and QC of cryovials PRIOR to the final centrifugation.	15.5
26. Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C, brake optional.	15.6
27. Gently remove the supernatant without disturbing the cell pellet. 28. Gently resuspend the pellet in **cold** CPS (V_f) while swirling the tube for even distribution. Gently make CPS-cell aliquots.	15.7
29. Immediately (≤ 10 minutes from addition of CPS to sample) transfer all cryovials to the controlled rate freezing unit and place in freezer.	15.8
30. After the appropriate time period, transfer cryovials to the onsite storage equipment and ship within the time period designated by the network.	17
31. Complete and review the **PBMC Processing Worksheet** and data entries as directed by network.	18.1

Appendix H: PBMC SOP Quick Guide—Manual Overlay

Use of a **PBMC Processing Worksheet** and the LDMS is ***required for all networks*** (see Section 5 for details). Before using this quick guide for the first time, be sure to review the complete PBMC SOP for important notes and details, and network-specific guidelines.

Steps (Quantities for smaller sample volumes are *italicized.*)	**Reference to SOP**
1. Prepare and chill the CPS.	11.2
2. Prepare whole blood samples, reagents, and supplies.	14.2
3. If plasma aliquots **are** required per protocol instructions: a. Centrifuge the whole blood at 200 to 400 x g for 10 minutes. b. Mark the total blood volume at the meniscus then transfer plasma to a 15 or 50mL conical centrifuge tube for further processing (800 to 1200 x g for 10 minutes, brake optional). c. Add sufficient quantity of WDR to bring blood back to its original whole blood volume, mix gently and continue PBMC processing.	14.3
4. Transfer whole blood to sterile, 50mL (*15mL*) conical centrifuge tube and dilute with WDR as needed. 5. Carefully and slowly overlay blood on top of the density gradient medium. (Underlay Method is an approved alternative, with the exception of **IMPAACT** protocols.)	14.4
6. Centrifuge at 400-800 x g for 15-30 minutes with the Brake OFF/set to zero. 7. Check each conical centrifuge tube for possible problems. 8. Harvest each buffy coat into a corresponding single, 50mL (*15mL*) conical centrifuge tube.	14.5
9. Add WDR to QS to a total volume of 45mL (*10mL*) and mix gently. 10. Wash #1—centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C, brake optional. 11. Check for the cell pellets! 12. Gently remove the supernatant without disturbing the cell pellet.	15.1
13. Resuspend the cell pellet in small amount of WDR making a homogenous cell suspension. 14. Combine up to 4 pellet suspensions into one 50mL conical centrifuge tube (*2 into 15mL tube*). 15. Add WDR tube rinse and final WDR up to 45mL (*10mL*) to cell tube. 16. Wash #2—Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C, brake optional. 17. Check for the cell pellets! 18. Gently remove the supernatant without disturbing the cell pellet.	15.2
19. Calculate the WDR counting resuspension volume (V). 20. Combine cell pellets into one tube using resuspension volume WDR. This is the volume on which the cell count is based. 21. Count and calculate the total number of cells 22. Calculate the cell yield in cells/mL of usable whole blood.	15.3
23. Calculate final CPS resuspension volume. Check calculations.	15.4
24. Complete the printing, labeling and QC of cryovials PRIOR to the final centrifugation.	15.5
25. Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C, brake optional.	15.6
26. Gently remove the supernatant without disturbing the cell pellet. 27. Gently resuspend the pellet in **cold** CPS (V_f) while swirling the tube for even distribution. Working on wet ice is recommended. 28. Gently make CPS-cell aliquots.	15.7
29. Immediately (≤ 10 minutes from addition of CPS to sample) transfer all cryovials to the controlled rate freezing unit and place in freezer.	15.8
30. After the appropriate time period, transfer cryovials to the onsite storage equipment and ship within the time period designated by the network.	17
31. Complete and review the **PBMC Processing Worksheet** and data entries as directed by network.	18.1