Title:	Cross-Network PBMC Processing SOP		
Origination Date:	01 April 2009	**Total Pages:**	40
Effective Date:	01 May 2014	**SOP Number**	HANC-LAB-P0001v5
Written By:	Cross-Network PBMC SOP Working Group	**Supersedes SOP Dated:**	03 Oct 2011

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Revision History	For a complete revision history, see Appendix H.

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*Appendix A is also provided as a downloadable and editable form on the HANC public website at http://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx.

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

3 Background

3.1 Freshly collected or cryopreserved PBMC are used for the evaluation of vaccine or antiretroviral therapy-induced cellular immune responses, HIV-associated changes in immune response, and recovery of replication competent virus. These assays require PBMC that have been isolated and cryopreserved under strictly defined conditions that ensure optimal recovery, viability, and functionality. Some validation studies indicate that 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 Director (or his/her designee) has the authority to establish, review and update this procedure.

4.2 The HIV/AIDS Network Coordination (HANC) Office is responsible for the maintenance and control of SOP documentation.

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

- Include, *without procedural modification*, the portions of the current version of the Cross-Network PBMC Processing SOP that are used within the network site-affiliated laboratory
- Reference the current version of the Cross-Network PBMC Processing SOP

Note: For laboratories processing HVTN PBMCs, the laboratory must use the HANC PBMC SOP as written or the HVTN-specific version of the HANC SOP.

4.4 All technicians 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 the timing of processing, calculations and documentation of problems that arise during processing.

5.1.1 Requirements for all networks:

- Enter data into the LDMS for the generation of cryovial labels, storage location documentation and shipping manifest requirements. See the table below for requirement details.
- Report deviations according to laboratory protocol.

5.1.2 Requirements for HVTN

The use of the HVTN PBMC Processing Worksheet in its entirety is required. If a protocol-specific PBMC worksheet is not required in the "HVTN Protocol Specific Processing Instructions," the generic worksheet in Appendix A and at http://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx may be used.

5.1.3 Requirements for ACTG, IMPAACT, HPTN and MTN

- The lab may use the **HVTN PBMC Processing Worksheet**, or modify it as appropriate for that laboratory's procedures. If the lab chooses to develop its own PBMC Processing Worksheet and supplementary tracking materials (such as the LDMS, or a separate worksheet or log) the laboratory will use the guidelines below.
- Electronic versions of the editable PBMC Processing Worksheets and examples of supplementary tracking materials are provided at http://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx for download and modification.

Guidelines for Tracking PBMC Processing		
Field	**Worksheet Requirements for ACTG, HPTN, IMPAACT, MTN*** (HVTN: Use of the HVTN PBMC Processing Worksheet in its entirety is required.)	**LDMS Requirements for All Networks****
Specimen Processing Laboratory	W	L
Participant ID	W	L
Visit Number	W	L
Protocol	W	L
LDMS Global Specimen ID	W	[Automatic]
Processing Start Date/Time	W	N
Processed By (Tech)	W	N
Counting Method (name of instrument or manual count)	W	
Counting re-suspension volume WDR (V)	W	
Cell count average concentration (C)	W	
Total cell number (T) = C x V	W	N
Calculate the final CPS re-suspension volume (V_f)	W	
Freezing Date and Time	W	N
Comments and Protocol Deviations, including but not limited to: • All unexpected specimen conditions • If blood is clotted, number of tubes that contained clots, total number of tubes from the PTID batch and details of processing of clotted blood • Cell yield below expected range • Processing anomalies • Troubleshooting steps taken • Note if Total Time >8 hours	W	O
Collection Date/Time	S	L
Reagents (Manufacturers, Lot Numbers and Expiration Dates for DMSO, FBS, WDR, CSTFB, density gradient media)	S	
CPS (Volume of DMSO and FBS)	S	
Sample tube type (NaHep/ACD/EDTA/Other)	S	L
Blood condition (e.g. SAT/HEM/CLT)	S	L

Guidelines for Tracking PBMC Processing		
Field	**Worksheet Requirements for ACTG, HPTN, IMPAACT, MTN*** (HVTN: Use of the HVTN PBMC Processing Worksheet in its entirety is required.)	**LDMS Requirements for All Networks****
Usable whole blood volume	S	L ("Volume")
Cell Counts	S	
Actual number of cells per vial	S	L
Number of cryovials frozen	S	L
Freezer storage information (LDMS Storage Module)	O	N
Confirmation of visual QC of reagents (Tech)	O	
Cell yield/mL of whole blood	O	
Estimated CPS re-suspension vol. (V1)	O	
Confirmation of LDMS Label QC for content/barcodes (Tech)	O	
Confirmation of cryovial transfer to storage box locations assigned by LDMS (Tech)	O	
Date/time cryovials were transferred from slow-rate cooling device to storage box.	O	
Final Review Reviewer/Date	O	

* W = Tracking on a PBMC Processing Worksheet is required.
S = Tracking is required, but supplementary tracking materials (such as the LDMS or another worksheet or log) may be used
O = Tracking on a worksheet or supplementary tracking material is optional

** L = Required fields in LDMS for network specimens
N = Tracking in the LDMS is required by the networks
O = Tracking in the LDMS is optional

6 Specimen

6.1 Patient Preparation

None

6.2 Specimen Type

Anti-coagulated whole blood drawn in blood collection tubes

6.3 Optimum/Minimum Specimen Volume

Required blood volume determined by protocol

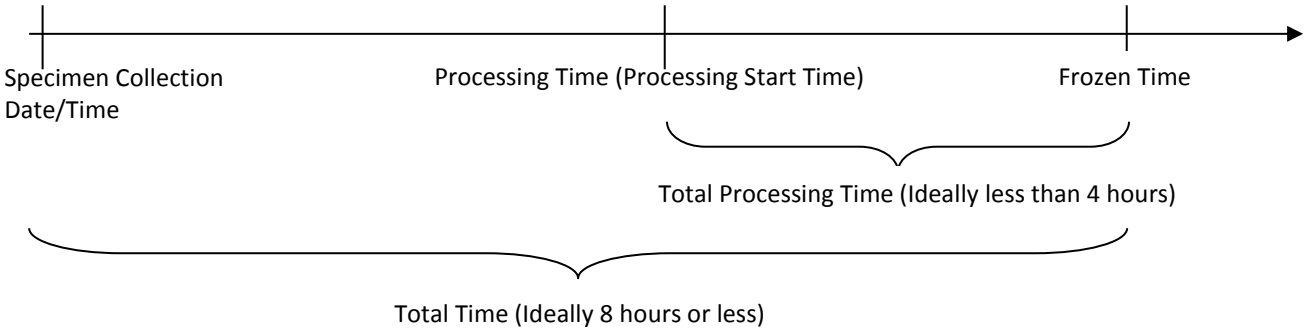
6.4 Handling Conditions

6.4.1 Fresh, anti-coagulated whole blood specimens should be stored at room temperature (15 to 30°C) from the time of collection until delivery to the laboratory/processing unit.

6.4.2 Fresh, anti-coagulated whole blood specimens should be delivered to the laboratory processing unit as soon as possible after collection to allow the processing laboratory ample time to complete the cryopreservation procedures.

6.4.3 Fresh, anti-coagulated whole blood specimens should be processed by the laboratory processing unit as soon as possible upon receipt:

- 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 StrataCooler® Cryo, NALGENE® Mr. Frosty or biocision® CoolCell is put into the -80°C freezer.
  - The cooling program of the controlled-rate freezer, such as CryoMed®, is 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.



6.4.4 Do not refrigerate or freeze whole blood.

6.5 Marginal Specimens

Document all unexpected specimen conditions and actions taken according to network and laboratory requirements. See Chapter 5 for details.

6.5.1 Clotted specimens

6.5.1.1 All blood should be processed regardless of whether or not it is clotted, unless otherwise directed by protocol.

6.5.1.2 Remove the clot and process as usual.

6.5.1.3 If the cell yield is insufficient to meet the needs of the protocol, contact the clinic for possible specimen replacement. For HVTN, if the cell yield is ≤0.4 x 10^6 cells/mL, contact the clinic for possible specimen replacement.

6.5.2 Hemolyzed specimens

6.5.2.1 Hemolysis may affect the quality of the PBMCs.

6.5.2.2 Process as usual.

6.5.2.3 If the cell yield is significantly below the expected range, store the PBMC with appropriate notations and contact the clinic for possible specimen replacement. For HVTN, if the cell yield is ≤0.4 x 10^6 cells/mL, contact the clinic for possible specimen replacement

6.6 Unacceptable Specimens

6.6.1 Unlabeled or mislabeled specimens will be rejected.

6.6.2 Leaking specimens

Notify the clinic if any of the specimens are leaking and determine whether or not the specimens are usable.

7 Equipment

7.1 Preparation & Processing

7.1.1 Class II biosafety cabinet (BSC) as set up by laboratory (P2, P2.5 or P3)

7.1.2 Centrifuge, low-speed (capable of 300 to 1000 x g), with swinging bucket rotor, refrigerated preferred, ambient acceptable

7.1.3 Micropipettes, range 20, 200, 1000µL

7.1.4 Pipet-Aid (cordless preferred) for 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 -150°C mechanical freezer (for IMPAACT, HPTN, and MTN, if LN2 freezer is not available for long-term storage)

7.1.9 37 to 56°C water bath (for heat inactivating FBS, if necessary)

7.1.10 Bucket or beaker for bleach or other disinfectant, for rinsing pipets if required by local safety practice

7.2 Liquid Nitrogen (LN2) equipment (if required by network)

7.2.1 LN2 storage tank (≤ -140°C)

7.2.2 IATA-approved LN2 dry shipper

7.3 Cell Counting (select one of following options)

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

Note for HVTN: Counting methods must be reviewed and pre-approved by the HVTN.

7.3.2 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, unless the samples are being prepared for the IQA PBMC Cryopreservation Proficiency Testing Program. If samples are being prepared for the IQA PBMC Cryopreservation Proficiency Testing Program, trypan blue must be used to obtain a viable cell count.

7.3.3 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. If samples are being prepared for the IQA PBMC Cryopreservation Proficiency Testing Program, trypan blue must be used to obtain a viable cell count.

7.4 Cryopreservation

Use one of following options according to manufacturer's instructions. The Stratagene StrataCooler® and biocision® CoolCell are preferred.

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

7.4.1 Stratagene StrataCooler® Cryo

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

7.4.2 biocision® CoolCell

Make sure that all parts of the CoolCell, including the central ring, return to room temperature between uses.

7.4.3 NALGENE® Mr. Frosty, 1°C/minute cryo-freezing container

Mr. Frosty should be stored at ambient temperature (15-30°C) between uses.

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 B.

7.4.4 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 Precision pipet tips, 20, 100, 200, 1000 µL, sterile

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

8.1.4 Cryogenic vials (cryovials), 1.8 to 2mL, screw cap with o-ring, sterile, polypropylene only, self-standing, graduated, leak-proof, formulated for vapor-phase LN2 preservation (approximately -140°C).

Note: Not all cryovial brands are suitable for long-term storage in LN2. See Appendix G for examples that meet the requirements.

Note: If a protocol requires harvesting of plasma, then externally-threaded tubes are preferred for plasma storage.

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

8.1.6 ***Optional:*** 5mL sterile, individually wrapped plastic transfer pipets

8.1.7 ***Optional:*** If pre-filled cell separation tubes with frit barriers (CSTFB) are not used, empty CSTFB (see 9.2 for more details) or 15 and 50mL disposable conical centrifuge tubes as in 8.1.3 will be required. See Appendix G for examples that meet the requirements.

8.2 Markers

Markers for writing on processing tubes and vials should have a fine point, and contain fast drying, indelible ink. (See Appendix G for examples.)

8.3 Labels

Cryogenic labels suitable for -80°C and LN2 temperatures. See Appendix G for examples that meet the requirements.

9 Personal Protective Equipment

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

9.2 Eye protection

9.3 Non-powdered, nitrile or equivalent gloves

9.4 Cryogloves and face shields (with chin cap optional), are necessary if you are using LN2

10 Reagents

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

10.1.1 Store opened bottles at the temperature recommended by the manufacturer until used or until manufacturer's expiration date.

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

10.2 Wash Diluent Reagents (WDR)

Hanks' Balanced Salt Solution (HBSS*) without calcium or magnesium, ready-to-use.
*Alternative: 1X Phosphate-Buffered Saline (PBS) without calcium or magnesium, ready-to-use.

10.3 Cell Separation Tube with Frit Barrier (CSTFB)

***Note**:* The laboratory may use a CSTFB or it can use a manual overlay/underlay with a conical centrifuge tube.

***Note**:* If using CSTFB, use Chapter 15. If using Manual Overlay or Underlay (without frit barriers), use Chapter 16.

10.3.1 Pre-filled CSTFB (1.077 density gradient media)

The capacity of the tube required will depend on the whole blood volume (see 15.4). See Appendix G for examples pre-filled CSTFB.

Storage conditions:

- Store in the refrigerator (2 to 8°C)
- Protect from light
- A cloudy appearance indicates deterioration of the product.
- Allow CSTFB to come to room temperature (15 to 30°C) prior to use

10.3.2 Alternatives to pre-filled CSTFB System:

Combine a dry CSTFB with 1.077 density gradient media. See Appendix G for examples that meet the requirements.

Tube capacity (mL)	Density gradient media volume (mL)
50mL	15mL
15mL	6mL

Follow density gradient media manufacturer's storage recommendations.

10.4 Freezing Reagents

10.4.1 Fetal Bovine Serum (FBS), heat-inactivated preferred

10.4.1.1 *Check with applicable network(s) for preferred vendors.* Not all brands of FBS are equivalent. Issues regarding quality control, toxicity, background, and shipping/importation must be addressed before changing vendors.

10.4.1.2 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.

10.4.1.3 FBS stored frozen (≤ -20°C) is good until the manufacturer's expiration date.

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

10.4.2 Dimethylsulfoxide (DMSO), cell-culture grade

10.4.2.1 Be sure to use a cell-culture grade DMSO. See Appendix G for examples that meet the requirements.

10.4.2.2 Store unopened bottles at room temperature (15 to 30°C). Check bottle for expiration date and discard if expired.

10.4.2.3 After opening, undiluted DMSO is stable at room temperature (15 to 30°C) when protected from light and moisture, for 6 months.

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

10.4.2.5 Discard open bottle if visible signs of contamination are noted.

10.4.2.6 Reagent may be aliquoted in small amounts to help preserve sterility. Label aliquots with "DMSO," the date opened/aliquoted, the expiration date (six months from opening) and tech initials. Protect aliquots from light.

10.4.3 Disinfectant

10.4.3.1 70% v/v ethanol disinfectant, spray bottle

10.4.3.2 10% v/v bleach, bucket or beaker and spray bottle

10.4.3.3 Other disinfectant as specified by local laboratory policy

10.5 Cell Counting Reagents

The requirements for counting reagents will vary depending on the method used. See the instructions for the method being used.

10.5.1 0.4% Trypan blue solution

10.5.2 ***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 can be performed.
0.05% Crystal Violet Solution contains:
0.05 g crystal violet
2mL glacial acetic acid
98mL distilled or deionized H_20

11 Reagent Preparation

11.1 Heat-Inactivated FBS (HI-FBS)

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. 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 If the FBS was not heat-inactivated by the manufacturer, follow these additional instructions. 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.**

11.1.4.2 ***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.

11.1.4.3 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.**

11.1.4.4 ***Note***: If the top of the bottle comes into contact with the water bath, swab the top of 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 conical centrifuge tubes, or other size aliquots appropriate for the anticipated workload.

Note: Labels should identify these tubes as “HI-FBS” and include the lot number, the aliquot date, the expiration date, and the technician’s initials. FBS is stable for 1 month at 2 to 8°C or the original manufacturer’s expiration date at -20°C.

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 aren’t immediately needed can be put in the freezer 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.

11.1.8 To use the frozen aliquots, thaw in the refrigerator overnight, preferred, or for several hours at room temperature. Change the expiration date to one month. Mix well before use.

11.2 Fresh Cryopreservation Solution (CPS)

11.2.1

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

11.2.2 Preparation of CPS

Use a sterile, disposable 15mL or 50mL container to prepare CPS. Mixing of DMSO and FBS is an exothermic reaction. CPS must be prepared in advance and chilled in the refrigerator (2 to 8°C) for at least 30 minutes or in an ice bath for at least 15 minutes prior to use.

Note: CPS can be stored at 2 to 8°C for 1 working day (<18 hours).

11.2.3 Use the formula below to **estimate** the volume of CPS to prepare for resuspension of PBMC. Examples are also shown.

Usable Whole Blood (mL) x Cell Yield (cells/mL) x Freeze-down Concentration (mL/cells) = Estimated CPS (mL)
Round up to the nearest whole mL.

***Note*:** The usable whole blood volume is the volume of whole blood that is actually processed. (The usable whole blood volume may not be equal to the capacity of the tube.)

Examples: Adult Blood—Large Volume Blood Collection

Usable Whole Blood x	Cell Yield x	Freeze-down Concentration =	Estimated CPS to Prepare
(140mL) x	(1.5 x 10^6 cells/1mL) x	(1mL/15 x 10^6 cells) =	14mL

Examples: Adolescent/Pediatric Blood—Small Volume Blood Collection

Usable Whole Blood x	Cell Yield x	Freeze-down Concentration =	Estimated CPS to Prepare
(10mL) x	(1.5 x 10^6 cells/1mL) x	(0.5mL/5 x 10^6 cells) =	1.5mL

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
9mL	0.9mL	8.1mL	9mL

11.2.5 Record the CPS, DMSO and FBS volumes according to network and laboratory requirements. See Chapter 5 for details.

12 Calibration

12.1 No calibration is required for the processing steps.

12.2 Follow the applicable laboratory calibration procedures if using an automated cell counter.

13 Quality Control

13.1 Cell Yields

Cell yields are fairly consistent within healthy populations. Infant populations typically generate higher lymphocyte yields than adult populations. Similarly, patients with AIDS or advanced HIV disease may be lymphopenic. It is important to be aware of the expected recovery for the population of participants for which the processing is performed. Based on this consistency, the 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. 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.

13.1.1 **Expected** Cell Yields for healthy populations:

Population	Mononuclear Cell Yield Range (cells/mL)
Adult	(0.8 to 3.2) x 10^6
Pediatric—less than 6 months	(3 to 10) x 10^6
Pediatric—6 mo. to 2 years	(2 to 9) x 10^6
Pediatric—2 to 5 years	(1 to 6) x 10^6
Pediatric—more than 5 years	(0.8 to 4) x 10^6
Pediatric—Unknown age	(1 to 10) x 10^6

13.1.2 Unexpected Cell Yields

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. Cell yields from HIV-infected patients may be lower than those shown in the above table. If cell dilution or counting errors are suspected, make a fresh dilution and recount.

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

Note for HVTN: Record any problems and actions on the HVTN PBMC Processing Worksheet and in the cell yield comments section of the Atlas HVTN PBMC Program.

13.2 Cell Viability

Fresh PBMC cell viability is fairly consistent. Long processing time, poor technique and occasionally a specific participant specimen may adversely affect the viability. If counting viable cells, calculate and record the % viable cells according to laboratory requirements.

13.2.1 Freshly isolated PBMC viability should be >95%.

13.2.2 If the fresh PBMC viability is <95%, review the results with the supervisor and document according to network and laboratory requirements. See Chapter 5 for details.

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

14 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 (pre-filled 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.

PBMC Processing Chapter	Use for these Networks
Cell Separation and Blood Treatment	
Chapter 15: Cell Separation and Blood Dilution with Plasma Replacement by Cell Separation Tube with Frit Barrier (CSTFB) **OR** Chapter 16: Cell Separation by Manual Density Gradient Media Overlay or Underlay and Blood Dilution with Plasma Replacement by Manual Density Gradient Cell Separation	Can be used for all networks; check protocol requirements and available materials Can be used for all networks; check protocol requirements and available materials
Washing, Counting, Resuspension, Concentration, and Overnight Controlled-Rate Freezing	
Chapter 17	All networks
On site Storage	
Chapter 18.2: Onsite Temporary Storage at -70/-80°C **OR** Chapter 18.3: Onsite Storage in Liquid Nitrogen (LN2)/150°C Mechanical Freezer	ACTG and HVTN IMPAACT, HPTN and MTN

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

Chapter 15 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Chapter 15 or Chapter 16, but not both.

15.1 Separation of lymphocytes from peripheral blood using pre-filled CSTFB separation tubes

15.1.1 Perform all pipetting and mixing in a biological safety cabinet (BSC), level 2 or greater.

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

15.1.3 Unless otherwise noted, the procedure is carried out at room temperature (15 to 30°C).

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

15.2 Prepare whole blood samples, reagents, and supplies.

15.2.1 Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see Chapter 11 Reagent Preparation).

15.2.2 Ensure that the tubes are at room temperature before processing.

15.2.3 Before adding the blood, visually check the CSTFB to see if there is liquid above the frit. If there is liquid above the frit, centrifuge the CSTFB at 1000 x g for 30 seconds. If any density gradient solution remains above the frit after centrifuging, it should be aspirated.

15.2.4 Carefully check the PTID on all tubes of blood received. Organize primary tubes such that there is no possibility of mixing tubes between PTID 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.

15.2.5 Determine and record an accurate measurement of the usable whole blood volume within 0.5mL. The volume of usable whole blood is not necessarily equal to the tube size.

15.3 Plasma Replacement

Perform this plasma replacement step *only* if plasma aliquots are required per protocol instructions. If plasma aliquots are not required, skip this step and proceed to step 15.4.

15.3.1 Blood collection tubes from the same PTID and same anticoagulant may be processed individually or pooled in 50mL conical centrifuge tubes.

15.3.2 Mark the whole blood volume at the meniscus.

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

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

15.3.5 Add sufficient quantity of WDR (see section 10.2) to bring blood back to its original whole blood volume, mix gently and continue PBMC processing at step 15.4.

15.3.6 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 instructions to obtain the

derivative required for the protocol. This may be done at a later time when the centrifuge is not in use for PBMC processing.

15.3.7 Aliquot spun plasma into labeled aliquot tubes as specified by protocol and discard any cellular debris in spun plasma tube.

15.4 Blood Dilution for CSTFB separation

***Note**:* The maximum ratio of blood to WDR should be approximately 2:1. Use one 50mL tube for each 10 to 20mL of whole blood (or one 12-to-14mL tube for each 4 to 5mL of whole blood). Use as many CSTFB as required to distribute all of the blood for each PTID.

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

15.4.1 Label each CSTFB with the PTID.

15.4.2 If a tube is clotted, see section 6.5 (Unacceptable Specimens).

15.4.3 Using a sterile pipet, add WDR to each CSTFB:

CSTFB Size (mL)	Approximate Volume of WDR (mL)
50	5
15	2

15.4.4 Mix whole blood gently then use a sterile pipet to transfer blood into the labeled CSTFB.

CSTFB Size (mL)	Approximate Volume of Blood (mL)*
50	12 to 22
15	4 to 5

15.4.5 ****Note:*** 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.

15.4.6 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
15	7.5

15.4.7 Carefully cap the CSTFB.

15.5 CSTFB density centrifugation and collection

15.5.1 Hold the tubes in an upright position and gently transfer them to the centrifuge. Refer to Chapter 20 Calculations to convert g to rpm for your rotor length.

15.5.2 Centrifuge at 800 to 1000 x g for 15 minutes at 15 to 30°C with the Brake OFF.

Note: PBMC separation may be improved for some specimens by centrifuging at 1000 x g.

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

15.5.3 Prepare the same number of new sterile conical centrifuge tubes as CSTFB used in the separation centrifugation step.

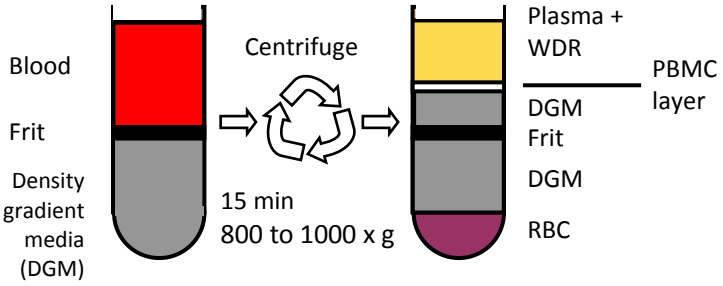
CSTFB Size (mL)	Conical Centrifuge Tube Size (mL)
50	50
15	15

15.5.4 Label each tube with the PTID. Use these new tubes for the following wash.

15.5.5 Gently remove the CSTFB from the centrifuge so as not to disturb the layers.

15.5.6 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
- Frit
- Density gradient media
- Packed red blood cells (RBC) and granulocytes



15.5.7 Inspect the tubes for the following possible 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 error 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 C for troubleshooting.
- PBMC layer formed on frit due to low RBC count or hematocrit volume.

15.5.8 Using a new sterile pipet (serological or transfer pipet) for each PTID, remove the upper yellowish, plasma-WDR fraction down to within approximately 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. Discard the plasma-WDR fraction per laboratory policy.

Note: Alternatively, the upper plasma-WDR fraction may be left in place and the cloudy white PBMC band may be removed by carefully inserting the pipet through the upper layer to the PBMC band.

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

15.5.10 Transfer the collected cells from one CSTFB to a single corresponding, pre-labeled, sterile conical centrifuge tube. Tubes can be pre-filled with WDR to save time.

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

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

15.6 Make sure all appropriate information is documented according to network and laboratory requirements. See Chapter 5 for details.

Skip Chapter 16 and proceed to Chapter 17.

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

Chapter 16 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Chapter 15 or Chapter 16, but not both.

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

16.1.1 Perform all pipetting and mixing in a biological safety cabinet (BSC), level 2 or greater.

16.1.2 Spray down all surfaces, racks, and reagent bottles with 70% v/v ethanol prior to entering and using the BSC.

16.1.3 Unless otherwise noted, the procedure is carried out at room temperature (15 to 30°C).

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

16.2 Prepare whole blood samples, reagents, and supplies

16.2.1 Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see Chapter 11 Reagent Preparation).

16.2.2 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 30°C) before processing.

16.2.3 Allow the density gradient media to come to room temperature (15 to 30°C). See Chapter 10 Reagents for more information.

16.2.4 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.

16.2.5 Determine and record an accurate measurement of the usable whole blood volume within 0.5mL. The volume of usable whole blood is not necessarily equal to the tube size.

16.3 Plasma Replacement

Perform this plasma replacement step only if plasma aliquots are required per protocol instructions. If plasma aliquots are not required, skip this step and proceed to step 16.4.

16.3.1 Blood collection tubes may be processed individually per instructions listed below or buffy coat pooling technique may be used as outlined in Appendix D.

16.3.2 Mark the whole blood volume in each tube at the meniscus.

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

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

16.3.5 Add sufficient quantity of WDR (see section 10.2) to bring blood back to its original whole blood volume, mix gently and continue PBMC processing at step 16.4.

16.3.6 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 instructions to obtain the derivative required for the protocol. This may be done at a later time when the centrifuge is not in use for PBMC processing.

16.3.7 Aliquot spun plasma into labeled aliquot tubes as specified by protocol and discard any cellular debris in spun plasma tube.

16.4 Blood Dilution and Manual Density Gradient Cell Separation

16.4.1 Uncap the tubes of anti-coagulated blood.

16.4.2 If a tube is grossly clotted, see section 6.5 (Marginal Specimens).

16.4.3 ***Note***: For larger blood volume collections, pooling buffy coats is allowed according to the guidelines in Appendix D: Pooling Buffy Coat Layers for Density Gradient Media PBMC Isolation. To pool buffy coats, replace steps 16.4.4 and 16.4.5 with the instructions in Appendix D.

16.4.4 Label each conical centrifuge tube with the PTID.

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

16.4.5 ****Note:*** 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.

16.4.6 Transfer the blood to a sterile, labeled 15 or 50mL conical centrifuge tube and add sufficient volume of WDR to dilute the blood according to the density gradient media package insert (maximum ratio of blood to diluent should be 2:1).

Optional: Addition of WDR and mixing can occur in the initial blood tube, if sufficient volume is available.

16.4.7 For Density Gradient Cell Separation:

On any given sample, use either the Overlay Method (16.4.7.1) or Underlay Method (16.4.7.2), but not both.

16.4.7.1 **Overlay** Method:

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

Aseptically add the appropriate volume of density gradient media to the empty sterile conical centrifuge tubes. The volume of density gradient media will depend on the ratio of density gradient media to diluted blood recommended by the manufacturer.

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.

16.4.7.2 **Underlay** Method:

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.

Based on the volume of WDR-diluted blood, determine the volume of density gradient medium required for each tube. The volume of density gradient media will depend on the ratio of density gradient media to diluted blood recommended by the manufacturer.

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

16.4.7.3 Carefully cap the tubes.

16.5 Lymphocyte density centrifugation and collection

16.5.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.

16.5.2 Centrifuge at 400 x g for 30 minutes at 15 to 30°C with the Brake OFF, 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 turned OFF for the separation to be clean and to maximize retrieval of the PBMCs. Refer to Chapter 20 Calculations to convert g to rpm for your rotor length.

16.5.3 Prepare the same number and size of new sterile conical centrifuge tubes as conical centrifuge tubes used in the separation centrifugation step.

16.5.4 Label each tube with the PTID/anticoagulant. Use these new tubes for the following wash.

16.5.5 Remove the tubes from the centrifuge.

16.5.6 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.

Note: If the cell layer is still not visible after re-centrifuging, document, remove and discard the WDR supernatant and proceed.

Note: If the plasma is very cloudy, it may be difficult to see the interface with the density gradient medium. It is possible to improve the collection of lymphocytes by removing most of the plasma above the interface with a 10mL pipet, leaving only 0.5 cm remaining. This allows for better positioning of the tip of the pipet for collection of cells.

16.5.7 Inspect the tubes for hemolysis or small clots visible at the cell interface that were not previously noted and document them.

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

16.5.8 Using a new sterile pipet (serological or transfer pipet) for each PTID, remove the upper, yellowish, plasma-WDR fraction down to within approximately 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. Discard the plasma-WDR fraction per laboratory policy.

Note: Alternatively, the upper plasma-WDR fraction may be left in place and the cloudy white PBMC band may be removed by carefully inserting the pipet through the upper layer to the PBMC band.

16.5.9 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.

16.5.10 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.

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

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

16.6 Make sure all appropriate information is documented according to network and laboratory requirements. See Chapter 5 for details.

Proceed to Chapter 17.

17 Washing, Counting, Resuspension, Concentration, and Overnight Controlled-Rate Freezing

Use Chapter 17 for all networks.

17.1 Wash 1:

17.1.1 QS the PBMC fraction to approximately 10mL (for 15mL conical centrifuge tubes) or 45mL (for 50mL conical centrifuge tubes) by adding WDR. Mix gently.

17.1.2 Re-cap all of the harvested cell tubes.

17.1.3 Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).

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

If the cell pellet 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. If the cell pellet is still not visible after re-centrifuging the tube, document.

17.1.5 Remove and discard the supernatant without disturbing the cell pellet.

17.2 Wash 2:

17.2.1 Re-suspend 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

17.2.2 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

17.2.3 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.

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

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

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

17.2.6 Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).

17.2.7 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, document.

17.2.8 Remove and discard the supernatant without disturbing the cell pellet.

17.3 PBMC Cell Count

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

Note: Usually, V is approximately 20% of the usable whole blood volume rounded to the nearest mL. However, V may vary depending on the size of the cell pellet and the cell counting method. It can also be adjusted to allow for convenient measuring or resuspension. Therefore, V may range from 10% to 50% of the usable whole blood volume. Refer to the cell counting method approved in the laboratory for guidance.

17.3.2 If there is more than one pellet from the same PTID/anticoagulant, use a small amount of WDR to gently re-suspend and combine the cell pellets into one tube. Using the remaining volume, rinse the tubes from which the cells were transferred. Add the rinse to the harvested cell tube.

17.3.3 Complete the cell count using the SOP for the cell counting method approved at the laboratory. For an example manual cell counting SOP, refer to http://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx.

17.3.4 Mix cells gently, but thoroughly, before sampling for the cell count.

17.3.5 Transfer a small volume of the re-suspension to a small tube for counting.

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

17.3.6 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 should count at least the four large corner squares ($1mm^2$).

17.3.7 Calculate the total number of cells using the following formula:

$$T = C \times V$$

T = Total number of cells

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

V = Count re-suspension volume of WDR in mL

17.3.8 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: The cell yield is calculated for quality purposes only. Refer to Chapter 13 Quality Control for the expected range of cell yields. If the cell yield is outside of the expected range, follow the trouble-shooting guidelines in the Chapter 13 Quality Control. Re-dilute and re-count if necessary.

17.4 Calculation of final re-suspension volume

17.4.1 Calculate the CPS freeze-down re-suspension volume required by completing the steps below for the target final cell concentration.

Note: The target final cell concentration varies by network and protocol. Refer to the protocol for target final cell concentration information.

17.4.2 Calculate the estimated CPS freeze-down re-suspension volume (V1) required by using the target final cell concentration.

$$V1 = (T/N1) \times V2$$

T = Total number of cells

N1 = Target final cell concentration

V2 = final aliquot volume in mL

Round V1 down to the nearest 0.1 mL to determine the actual CPS re-suspension Volume (V_f).

Note for HVTN: Round V1 down to the nearest whole (1.0) mL to determine V_f.

Note: For some networks, V2 will be 1 mL/cryovial so the number of vials required will equal the milliliters of CPS. For ACTG and IMPAACT, adjust the volume per cryovial according to the lab processing chart or protocol.

Note for IMPAACT: Most IMPAACT protocols will require viable PBMCs at 10^7/mL and 5.0 x 10^6/vial and request that all PBMCs recovered be stored. In general, when PBMC recoveries are less than a targeted number:

- Add residual PBMC numbers less than 2 x 10^6 to a tube with 5.0 x 10^6 cells
- Aliquot residual cell numbers greater than 2 x 10^6 but less than 5 x 10^6 into a separate vial.

Examples:

IMPAACT Protocol Requirement	Recovery	Aliquot Preparation
10^7 PBMCs in 2 vials	9.3 x 106 PBMCs	Prepare one vial with 5.0 x10^6 PBMCs and another vial with 4.3 x10^6 PBMCs
20 million PBMCs	16.5 x 106 PBMCs	Prepare two vials with 5 x 10^6 PBMCs each and one vial with 6.5 x 10^6 PBMCs

Remember to record actual PBMC number/vial in the LDMS.

17.4.3 ***For HVTN only***: Calculate the actual number of cells per vial (N2) using the actual CPS freeze-down volume (V_f) determined in the previous calculation.

$$N2 = (T/V_f) \times V2$$

N2 = Actual number of cell per vial

T = Total number of cells

V2= final aliquot volume in mL

17.5 Labeling

17.5.1 Complete the printing 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.

17.5.2 Cryovial labels will be generated using the Laboratory Data Management System (LDMS).

17.5.2.1 Follow network lab practice for completing the data entry.

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

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

17.5.2.4 Correct any data entry errors in LDMS and re-print labels as needed (making sure the appropriate global ID's are selected).

17.5.3 Apply the labels on the cryovials so that the information can be easily read and the contents of the tube can be clearly seen.

Note for HVTN: Scan the empty, labeled cryovials following current HVTN guidelines to ensure that the barcode can be scanned.

17.6 Final Centrifugation

17.6.1 Place the harvested cell tube in the centrifuge.

Note for HVTN: QS cell suspension to 45mL with WDR prior to centrifugation.

17.6.2 Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).

17.6.3 Verify that all cryovials are labeled and easily accessible.

17.7 Aliquoting for cryopreservation

Note*:* The following steps should be performed quickly to preserve cell integrity. It is recommended that the cryovials be kept chilled on wet ice. Don't allow the vials to become submerged in the wet ice. Don't allow moisture near the caps of the vials.

17.7.1 Remove and discard the WDR supernatant. Keep the pellet.

Note for ACTG and IMPAACT: If cells are to be frozen as nonviable PBMC Pellets (PEL), resuspension of cells in freezing medium (CPS) it is not recommended because DMSO is a potent PCR inhibitor. If the PBMCs have been in contact with DMSO (e.g. freezing medium), wash the PEL twice with WDR prior to storage. Refer to the Specimen Processing SOP at http://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx.

17.7.2 Re-suspend the pellet using the volume of cold CPS (V_f) that you determined in 17.4.

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

17.7.2.1 ***Gently*** resuspend the cell pellet prior to adding the CPS by flicking, racking or pipetting.

17.7.2.2 ***Gently*** add the CPS to the re-suspended cells with continuous swirling.

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

17.7.4 Aliquot 0.5 to 1mL per cryovial, depending on network and protocol requirements. If required by network or protocol, prepare a final partial aliquot with any excess cell suspension volume that may be present.

Note for HVTN: Instead of creating a final partial aliquot, evenly distribute any excess volume among all of the cryovials for that PTID.

17.8 Overnight controlled-rate freezing

17.8.1 Following processing and counting, cells should be frozen immediately.

17.8.2 Select the freezing method to be used: StrataCooler® Cryo, NALGENE® Mr. Frosty, biocision® CoolCell or CryoMed®. See section 7.4 for storage and maintenance information.

17.8.3 Immediately transfer all cryovials to the controlled-rate freezing container.

For NALGENE® Mr. Frosty, biocision® CoolCell and StrataCooler® Cryo, close the container and place it in a -80°C (-65 to -95°C) 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) for a minimum of 4 hours for Mr. Frosty and CoolCell and overnight for StrataCooler ® Cryo.

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

Note: This is the Frozen Time.

17.9 Make sure all appropriate information is documented according to network and laboratory requirements. See Chapter 5 for details.

18 PBMC Storage (Interim or On-Site)

18.1 The cold-chain must be maintained during all transfer steps to avoid damage to the cells.

Note for HVTN: Ship on dry ice to the central specimen repository within **1 week of collection** unless otherwise directed by the HVTN.

Note for ACTG: Ship on dry ice within 4 weeks of the date of freezing.

18.2 Transfer PBMCs that will be temporarily stored on site at -70/-80°C freezer.

18.2.1 Transfer the cryovials from the controlled-rate cooling system to the designated storage location at -70/-80°C.

Transfer the cryovials after a minimum of 4 hours for NALGENE® Mr. Frosty and biocision® CoolCell and overnight for StrataCooler ® Cryo. If CryoMed® is being used, transfer the cryovials upon completion of the program to the -70/-80°C freezer.

Note: Required for HVTN, recommended for ACTG: Use a dry ice transfer pan. Make sure the cryovial freezer box is deeply covered on all sizes with dry ice. Work rapidly and efficiently to minimize cryovial exposure to ambient temperatures.

18.2.2 ***Note:*** Do not store in liquid nitrogen (LN2). Store at -65 to -95°C until shipped.

18.2.3 ***Note: Required for HVTN, recommended for ACTG:*** Maintain the cold-chain during preparation for shipping by pre-chilling the dry ice shipper and using a dry ice transfer pan during the packing steps. Make sure the dry ice shipper is completely full of dry ice.Do NOT temporarily store samples in LN2 unless instructed to do so by network or protocol. Do NOT transfer samples from LN2 back to -70/-80°C freezers, unless directed to do so by network or protocol team.

18.2.4 Contact network laboratory operations personnel if samples cannot reach their final destination within the network allotted temporary storage time. Permission to move samples to LN2 storage and ship in LN2 shippers is needed if the temporary storage and shipping conditions cannot be met.

18.3 Samples that will be stored on site longer than 4 weeks must be transferred into LN2/-150°C mechanical Freezer.

Note for HVTN: -150°C mechanical freezers are not acceptable for HVTN PBMC storage.

18.3.1 Transfer/storage of PBMC cryovials into LN2/-150°C mechanical freezer

18.3.2 The next working day, transfer the cryovials from the controlled-rate cooling system on dry ice to the designated storage location in the LN2/-150°C storage system.

18.3.3 Frozen PBMC samples can be stored safely in vapor phase LN2 indefinitely. Do NOT transfer samples from LN2/-150°C back to -70°C or -80°C freezers unless directed to by network or protocol team.

18.3.4 Once samples have been stored in LN2, all transfers or shipments must be maintained in LN2 (≤ -140°C) and samples cannot be shipped on dry ice.

All infectious material samples must be shipped in IATA approved LN2 shippers; check protocol requirements for exceptions.

19 Completing Processing Documents

19.1 Make sure all appropriate information is documented according to network and laboratory requirements and that all calculations are correct. See Chapter 5 for details.

19.2 Store the PBMC Processing Worksheet and any other tracking documents according to laboratory policy. See Chapter 5 for details.

This marks the end of processing and storage.
Follow the appropriate laboratory procedures for preparation and processing of shipments.

20 Calculations

20.1 RPM is usually read off a nomogram chart. Nomogram charts are often included in the centrifuge maintenance manual. Be sure to use centrifuge and rotor specific charts.

20.2 It is recommended that the appropriate g-to-RPM conversion be posted on your centrifuge for easy reference.

20.3 If a nomogram chart is not available, g forces can be converted to RPMs using the following formula.

$$RPM = \sqrt{\frac{g}{1.18r \times 10^{-5}}}$$

r = radius of rotor in centimeters

g = relative centrifugal force expressed in units of gravity

RPM = revolutions per minute

21 Limitations of the Procedure

21.1 Optimum processing time from collection to freezing of fresh blood for PBMC is <8 hours from the time of collection. Cell function may drop for older specimens.

21.2 Optimum processing time for PBMC is <3 hours from the time of adding blood to the cell separation tubes (Accuspin™ or equivalent) to the initiation of the controlled rate freezing cycle.

21.3 Studies indicate that specimens collected in an EDTA anti-coagulant give lower yields over time.

21.4 Avoid removing excess amounts of the separation media with the PBMC band as that can increase granulocyte contamination.

21.5 Avoid removing excess supernatant with the PBMC band to limit contamination from plasma proteins.

22 Glossary of Terms

Term	Definition
ACTG	AIDS Clinical Trials Group
Centrifuge Temperature	15 to 30°C
Clotted, Grossly	More than ¾ of the whole blood mass is clotted and there is very little free whole blood remaining.
Clot, Small	Small clots will not usually be seen in the whole blood tube, but can be seen on the separation tube frit after centrifugation.
CPS	Cryopreservation Solution
CSR	Central Specimen Repository
CSTFB	Cell Separation Tube with Frit Barrier
DG Media	Density Gradient Media
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution
Hemolysis	A pink to red coloration of serum or plasma due to the lysis of red blood cells. Hemolysis is graded and reported according to the following scale:

Term	Definition
	1+ Pale pinkish-red color in serum or plasma, able to clearly read news print placed behind the blood tube 2+ Pinkish-red color in serum or plasma, news print is readable but not as sharp 3+ Dark pinkish-red color in serum or plasma, news print 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
HPTN	HIV Prevention Trials Network
HVTN	HIV Vaccine Trials Network
Icteric	A green or orange tinted plasma suggesting the presence of increased bilirubin.
IMPAACT	International Maternal Pediatric Adolescent AIDS Clinical Trials Network
LDMS	Laboratory Data Management System
MTN	Microbicides Trials Network
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered saline
PTID/PID	Participant Identification Number
QS	Quantity Sufficient—add sufficient quantity of liquid to bring to specified volume
Room Temperature (RT)	15 to 30°C
Usable whole blood volume	The 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 (LN2) vapor-phase storage is the space in the storage tank that is above the LN2 liquid at the bottom of the tank.
WDR	Wash Diluent Reagent (HBSS, PBS or RPMI; RPMI can be used for ACTG/IMPAACT/HPTN only)

23 References

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24 Additional Documents (To be maintained by the laboratory)

24.1 FBS Package Insert and Certificate of Analysis

24.2 WDR (HBSS, PBS or RPMI) package insert

24.3 Density Gradient Medium package insert

24.4 Cell separation tube with frit barrier package insert

25 Appendices

25.1 Appendix A: HVTN-Required PBMC Processing Worksheet

Appendix A is also provided as downloadable and editable forms on the HANC public website at http://www.hanc.info/labs/Pages/PBMCSOP.aspx.

25.2 Appendix B: Example NALGENE® Mr. Frosty Isopropanol Change Log

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

25.4 Appendix D: Pooling Buffy Coat Layers for Density Gradient Media PBMC isolation

25.5 Appendix E: Cross-Network PBMC SOP Quick Guide—CSTFB

25.6 Appendix F: Cross-Network PBMC SOP Quick Guide—Manual Overlay Method

25.7 Appendix G: Example Reagents

25.8 Appendix H: Revision History

Appendix A: HVTN PBMC Processing Worksheet

Specimen Processing Laboratory:

Participant ID (PTID): Visit: Protocol:

Collection Date: Time:

Processing Start Date: Time: Processed By:

Reagents/Manufacturer	Lot Number			Expiration Date
DMSO (Manuf.:_____)				
FBS (Manuf.:_____)				
HBSS or other WDR (Manuf.:_____)				
Cell Separation Tube (Manuf.:_____)				
Density Gradient Media (Manuf.:_____)				
	Volume in mL			
CPS	CPS	DMSO	FBS	1 working day

Data to be Captured During Processing	Sample
Sample tube type (circle one)	NaHep / ACD / EDTA Other: _____
Blood condition (circle one or more)	NORM / HEMO/ CLOTTED
If indicated in protocol or processing instructions, harvest plasma prior to PBMC processing. Replace plasma volume with HBSS/WDR. Indicate harvesting.	Yes No
Usable whole blood volume	mL
Indicate processing method: Frit Barrier, Manual O/U-Lay or Buffy Coat Pooling	
Counting Method (name of instrument or manual count)	
Counting re-suspension volume of HBSS (or other WDR) (**V**)	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 re-suspension vol. ***(V1)=(T/15x10^6 cells/ml)(1mL)***	mL
Calculate the final CPS re-suspension volume (V_f), rounded DOWN to the nearest whole mL	mL
Calculate actual number of cells per vial ***N2*** *= (T/V_f)* x V2; (v2=1mL for most HVTN protocols).	x 10^6 cells/vial
Freezing Date and Time (Explain in comments section if not within 4 hours of processing start time)	
Print and QC LDMS Label content/barcodes (initials of person performing QC)	
Number of cryovials actually frozen ***Note:*** Should be equal to freeze-down re-suspension volume for 1mL aliquots.	
Complete remaining LDMS entries including total cell count & freeze time.	

Appendix A: HVTN PBMC Processing Worksheet

Specimen Processing Laboratory:

PTID:

Transfer of Cryovials to Freezer Storage	
Person who transferred cryovials to storage box locations assigned by LDMS	
Date (ddmmyyyy)/time cryovials were transferred from slow-rate cooling device to storage box. (Sample must be maintained at -70/-80°C during transfer)	
Final Review (Reviewer/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/mm2)(DF)(10^4); convert to 10^6 cells/mL	x 10^6cells/ml	x 10^6cells/ml	x 10^6cells/ml	
% viability = (viable cells/total cells)(100)	Not applicable		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			

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

Comments and Protocol Deviations:

Appendix B: Example NALGENE® Mr. Frosty Isopropanol Change Log

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

C.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.

C.2 Identify the problem:

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

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

C.2.2.1 The tube was dropped or bumped.
C.2.2.2 The brake was left on.
C.2.2.3 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.
C.2.2.4 The centrifuge stopped due to a discontinuity in the electricity supply.
C.2.2.5 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.)
C.2.2.6 The centrifuge was misbalanced.
C.2.2.7 The donor has low lymphocyte, white blood cell or hematocrit counts.

C.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:

C.3.1 Check that the tubes were balanced.

C.3.2 Check that the centrifuge buckets were balanced.

C.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.

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

C.4.1 Reagents:

C.4.1.1 Density gradient media
C.4.1.2 50 mL tubes
C.4.1.3 Pipets

C.4.2 Method:

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

C.4.2.1 Add 15 mL of density gradient media to sterile 50 mL tubes (not CSTFB).
C.4.2.2 Allow the density gradient media to warm to room temperature while working with the sample.
C.4.2.3 For each mixed tube, label 50 mL 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.)

C.4.2.4 Transfer up to 30 mL of the mixed sample to the tube containing density gradient media.
C.4.2.5 Repeat this for all mixed samples.
C.4.2.6 Place the tubes into the centrifuge, checking that the tubes are balanced.
C.4.2.7 Centrifuge for 30 to 40 minutes at 400 x g with the Brake OFF at 15 to 30°C
C.4.2.8 A PBMC layer should now be visible. (Often some cells will have been lost, so the layer could be thin).
C.4.2.9 The top layer, which is plasma potentially contaminated with density gradient media, may be collected at this stage and processed as in Sections 'PBMC and Plasma Isolation' and 'Plasma Storage' of the main protocol. However, the information that this plasma sample is potentially contaminated with density gradient media must be entered in the comments section for this sample in the LDMS.
C.4.2.10 Carefully transfer the PBMC layer to a 50 mL conical centrifuge tube labeled with the PTID identifier. Use one new tube for every density gradient media tube.
C.4.2.11 Re-cap the density gradient media tube.
C.4.2.12 Return to Chapter 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 B" 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. Also, note that the plasma sample recovered was potentially contaminated with density gradient media on the **PBMC Processing Worksheet** and in the comments section of the LDMS entry for the plasma specimens.

Appendix D: 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. The majority of 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:

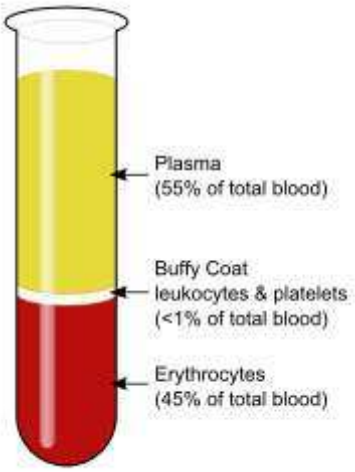
D1. Make sure that you have completed steps 16.4.1 through 16.4.3.

D2. Centrifuge whole blood at 200 to 400 x g for 10 minutes.

D3. Harvest plasma (and save as needed - see 16.3.4 to 16.3.7) 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).

D4. 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 10 mL blood collection tubes can be combined into one 50mL conical centrifuge tube.



D5. Label each conical centrifuge tube with the PTID.

D6. Add WDR to each sterile conical centrifuge tube.

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

D7. Hold the plasma depleted tube (which now contains a small amount of residual plasma and packed cells) at about a 30° angle.

D8. 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.

D9. Harvest and pool the buffy coats from the remaining tube(s) for that PTID-anticoagulant combination.

D10. Q.S. 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.

D11. Continue with density gradient cell separation at step 16.4.6 of the SOP. For 16.4.6, "diluted blood" means "buffy coat."

Extra Materials Needed: Sterile 2.5mL, wide bore, polypropylene pipet.

Appendix E: PBMC SOP Quick Guide—CSTFB

Use of a **PBMC Processing Worksheet** and the LDMS is ***required for all networks*** (see Chapter 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.	11.3
2. Prepare whole blood samples, reagents, and supplies.	15.2
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.	15.3
4. Add 5mL (*2mL)* of WDR to each CSTFB. 5. Transfer 12 to 22 mL (*4 to 5mL*) of blood into the labeled CSTFBs. 6. Add WDR tube rinse and final WDR to CSTFB up to 30 mL (*7.5mL*) (WDR + Whole Blood).	15.4
7. Centrifuge at 800 to 1000 x g for 15 minutes at 15 to 30°C with the Brake OFF. 8. Inspect the tubes for possible problems. 9. Harvest each CSTFB buffy coat into a corresponding single 50mL (*15mL*) conical centrifuge tube.	15.5
10. Add WDR to QS to a total volume of 45 mL (*10mL*) 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! 13. Gently remove the supernatant without disturbing the cell pellet.	17.1
14. Re-suspend 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 (*2 into 15mL tube*). 16. Add WDR tube rinse and final WDR of 45 mL (*10mL*) 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! 19. Gently remove the supernatant without disturbing the cell pellet.	17.2
20. Calculate the WDR counting re-suspension volume (V). 21. Combine cell pellets into one tube using re-suspension 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.	17.3
24. Calculate final CPS re-suspension volume. Check calculations.	17.4
25. Complete the printing, labeling and QC of cryovials PRIOR to the final centrifugation.	17.5
26. Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).	17.6
27. Gently remove the supernatant without disturbing the cell pellet. 28. Gently re-suspend the pellet in **cold** CPS (V_f) while swirling the tube for even distribution. Working on wet ice is recommended. 29. Gently make CPS-cell aliquots.	17.7
30. Immediately (≤ 10 minutes) transfer all cryovials to the controlled rate freezing equipment and begin freezing.	17.8
31. After the appropriate time period, transfer cryovials to the onsite storage equipment and ship within the time period designated by the network.	18 or 19
32. For HVTN, review the **PBMC Processing Worksheet** for completeness and accuracy.	19.2

Appendix F: PBMC SOP Quick Guide—Manual Overlay

Use of a **PBMC Processing Worksheet** and the LDMS is ***required for all networks*** (see Chapter 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.	11.3
2. Prepare whole blood samples, reagents, and supplies.	16.2
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 50 mL 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.	16.3
4. Transfer whole blood to sterile, 50 mL (*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.)	16.4
6. Centrifuge at 400 x g for 30 minutes with the Brake OFF. 7. Check each conical centrifuge tube for possible problems. 8. Harvest each buffy coat into a corresponding single, 50mL (*15mL*) conical centrifuge tube.	16.5
9. Add WDR to QS to a total volume of 45 mL (*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.	16.1
13. Re-suspend 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 of 45 mL (*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.	16.2
19. Calculate the WDR counting re-suspension volume (V). 20. Combine cell pellets into one tube using re-suspension 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.	16.3
23. Calculate final CPS re-suspension volume. Check calculations.	17.4
24. Complete the printing, labeling and QC of cryovials PRIOR to the final centrifugation.	17.5
25. Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).	17.6
26. Gently remove the supernatant without disturbing the cell pellet. 27. Gently re-suspend 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.	17.7
29. Immediately (≤ 10 minutes) transfer all cryovials to the controlled rate freezing equipment and begin freezing.	17.8
30. After the appropriate time period, transfer cryovials to the onsite storage equipment and ship within the time period designated by the network.	18 or 19
31. For HVTN, review the **PBMC Processing Worksheet** for completeness and accuracy.	19.2

Appendix G: 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
Pre-filled cell separation tubes with frit barriers (CSTFB) with 1.077 density gradient media	• Accuspin™ System Histopaque®-1077 • Ficoll-Paque™ PLUS	Optional
Dry CSTFB	• Accuspin™ separation tubes • Leucosep® separation tubes	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	Optional
Fetal Bovine Serum (FBS)		***ACTG, IMPAACT and HPTN:*** Refer to information about current IQA-validated lots. ***HVTN and MTN:*** Use HVTN validated lots; if unable to access or import HVTN specified lots, check with HVTN/MTN for other options.
Cryovials	• Corning® 2mL external thread polypropylene cryogenic vial, self-standing with round bottom #430659 • Nunc CryoTubes™, internal thread, polypropylene (PP) tubes and screw cap #377267 • WHEATON Cryule® Plastic Cryogenic Vials, external thread, #985742 • SARSTEDT Screw cap micro tube, external thread #72.694.006	Optional
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 H: Revision History from Version 4.0 to 5.0

Version Effective Date (dd/mmm/yy)	Comments	
5.0 01 May 2014	Approvals	Grace Aldrovandi replaced Susan Fiscus for IMPAACT Authorization
	5.1.3	Guidelines for Tracking PBMC Processing Chart : Instructions: "L = Tracking in the LDMS is required by the LDMS" was changed to "L= Required field in LDMS for network specimens"
	5.1.3	Guidelines for tracking PBMC processing table-: "LDMS Specimen Number" was changed to "LDMS Global Specimen ID"
	5.1.3	Cells that were filled in Gray to indicate that information is not needed is changed to black.
	7.1.8	Added HPTN and MTN
	10.2	Alternative for using RMPI as a substitute is omitted
	14	Formatting changes
	18 and 19	Combined interim and on-site storage instructions from two sections into one, Section 18.
	19	Completing processing documents language that was repeated in Section 18 and Section 19 is separated and separated into its own section.
	Appendix H	Deleted other Version history changes, maintaining only the current version changes appropriate to this document.