

Owner	HIV/AIDS Network Coordination (HANC)
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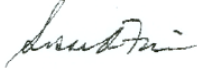

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*Appendix A is also provided as a downloadable and editable form on the HANC public website at <http://www.hanc.info/labs/Pages/PBMC SOP.aspx>.

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.

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. HVTN requires that the total time from collection to freezing is less than or equal to 8 hours. For other networks, the time limit may vary; check the appropriate protocol documents.

4 Authority and Responsibility

- 4.1 Network Laboratory Director's (or his/her designee) have 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 Principal Investigator/Laboratory Manager is responsible for the implementation of this procedure or an equivalent network version and for ensuring that all appropriate personnel are trained.
- 4.4 All technicians are responsible for reading and understanding this SOP prior to performing the procedures described.

5 Specimen

5.1 Patient Preparation

None

5.2 Specimen Type

Anti-coagulated whole blood drawn in blood collection tubes

5.3 Optimum/Minimum Specimen Volume

Required blood volume determined by protocol

5.4 Handling Conditions

5.4.1 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.

5.4.2 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 cryo-preservation procedures.

5.4.3 Fresh, anti-coagulated whole blood specimens should be processed by the laboratory processing unit as soon as possible upon receipt. HVTN requires that the total time from collection to freezing is less than or equal to 8 hours. For other networks, the time limit may vary; check the appropriate protocol documents. Record the collection time on the **PBMC Processing Worksheet** (Appendix A) and/or in LDMS.

5.4.4 Do not refrigerate or freeze whole blood.

5.4.5 Record the time that processing began on the **PBMC Processing Worksheet** or equivalent record document.

Note: The use of the **PBMC Processing Worksheet** is *required* for HVTN to track the timing of processing and document problems that may arise during processing. The **PBMC Processing Worksheet** is available in the appendices and at <http://www.hanc.info/labs/Pages/PBMCSOP.aspx>.

Note: The use of the **PBMC Processing Worksheet** is *recommended* for ACTG, HPTN, IMPAACT and MTN, but these networks may also use an equivalent record document, such as the Laboratory Data Management System (LDMS), for this purpose.

5.4.6 If some of the specimen blood tubes contain small clots, try to remove the clots prior to processing. Record the total number of tubes that contained small clots in the comments section on the **PBMC Processing Worksheet** or in LDMS.

5.4.7 Hemolysis may affect the quality of the PBMCs. Note any hemolysis on the **PBMC Processing Worksheet** using the hemolysis definitions found in the Glossary of Terms section. If specimens are grossly hemolyzed (3+ to 4+), attempt PBMC isolation and cryopreservation. If the cell yield is significantly below the expected range, store the PBMC with appropriate notations and contact the clinic for possible specimen replacement.

5.5 Unacceptable Specimens

- 5.5.1 Unlabeled or mislabeled specimens will be rejected.
- 5.5.2 If only a few Vacutainer® tubes of blood from a PTID batch are grossly clotted (see glossary), these tubes may be discarded if acceptable practice for the network (see notes below), and the unclotted ones processed.

Note for HVTN: Process the tubes of clotted blood with appropriate comments in the **PBMC Processing Worksheet**. If the cell yield is $<0.4 \times 10^6$ cells/mL, contact clinic for possible specimen replacement.

Note for ACTG, IMPAACT, HPTN, and MTN: All blood should be processed and complications should be noted on the **PBMC Processing Worksheet** and/or in LDMS, unless otherwise directed by the LPC (Lab Processing Chart), SSP (Site-Specific Protocol) or SOP (Standard Operating Procedure). If all of the Vacutainer® tubes of blood from a PTID batch are clotted, remove the clot and continue to process the remaining fraction.

Note: In the “Comments and Protocol Deviations” section of the **PBMC Processing Worksheet**, note the number of clotted tubes and the total number of tubes from the PTID batch. If applicable, record the details of processing of clotted blood. If clotted blood is processed, enter “from clotted blood” in the comments section of the LDMS entry for both the PBMC and the plasma specimens.

Record the total number of tubes that were discarded due to gross clots or gross hemolysis in the comments section on the **PBMC Processing Worksheet**.

- 5.5.3 Document all unexpected specimen conditions on the **PBMC Processing Worksheet** and enter the information into a laboratory data management system, such as in the comment section of the Laboratory Data Management System (LDMS).

6 Equipment

Recommended vendors and equipment are listed. Unless otherwise specified, equipment of equal or better quality than those recommended can be used.

6.1 Preparation & Processing

- 6.1.1 Biological laminar flow safety cabinet, as set up by laboratory (P2, P2.5 or P3)
- 6.1.2 Centrifuge, low-speed (capable of 300 to 1000 x g), with swinging bucket rotor, refrigerated preferred, ambient acceptable
- 6.1.3 Micropipettes, range 20, 200, 1000µL
- 6.1.4 Pipet-Aid (cordless preferred) for disposable, serological pipets
- 6.1.5 2 to 8°C refrigerator
- 6.1.6 -20°C (or lower) freezer **without** automatic defrost (for FBS storage)
- 6.1.7 -80°C freezer (-65 to -95°C); for short-term PBMC storage
- 6.1.8 37 to 56°C water bath

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

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

- 6.2.1 LN2 storage tank ($\leq -140^{\circ}\text{C}$)
- 6.2.2 IATA-approved LN2 dry shipper

6.3 Cell Counting (select one of following options)

- 6.3.1 Automated cell counter capable of enumerating viable cells (Beckman-Coulter Vi-Cell, Guava PCA[®] or equivalent)
- 6.3.2 Manual cell counting chamber (Neubauer hemacytometer) and light-field microscope
- 6.3.3 Automated cell counter not capable of enumerating viable cells (Coulter Counter, Abbott CelDyne[™], Sysmex[®] or equivalent) used in parallel with a hemacytometer to enumerate viable cells.

Note for HVTN: An automated cell counter not capable of identifying viable cells may be used to obtain a total cell count without enumerating viable cells.

Note for labs participating in the IQA PBMC Cryopreservation Proficiency Testing Program: Viability evaluation is required.

6.4 Cryopreservation (use one of following options)

- 6.4.1 StrataCooler[®] Cryo (Stratagene). 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.
- 6.4.2 NALGENE[®] Mr. Frosty, 1°C/minute cryo-freezing container. Mr. Frosty should be stored at ambient temperature (15-30°C).

Note: Replace isopropanol every fifth freeze/thaw cycle. A log must be used to track freeze/thaw cycles and reagent changes. See Appendix B.

- 6.4.3 Control-rate freezer, such as CryoMed[®] Freezing Chamber (Gordinier)

7 Disposables

7.1 Plastics

- 7.1.1 Serological pipets, disposable, 1, 5, 10, 25, 50mL, sterile
- 7.1.2 Precision pipet tips, 20, 100, 200, 1000 μL , sterile
- 7.1.3 15 and 50mL disposable centrifuge tubes, sterile, conical bottom, graduated polypropylene.

- 7.1.4 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).

NALGENE® NUNC® Brand Products, catalog #377267; Wheaton, catalog #985742; Fisher Scientific, catalog #05-669-57; Corning, catalog #430659 (12.7x49mm), SARSTEDT, #72.694.006.

Note: Not all cryovial brands are suitable for long-term storage in LN2. Check with applicable network(s) or the manufacturer prior to substitution of this item.

- 7.1.5 Optional: Sterile bottles/flasks, disposable, 45mm neck, 250 to 500 mL
- 7.1.6 Optional: 5mL sterile, individually wrapped plastic transfer pipets
- 7.1.7 Optional: If pre-filled cell separation tubes with frit barriers (CSTFB) are not used, empty CSTFB tubes (see 9.2 for more details) or 15 and 50mL disposable centrifuge tubes as in 7.1.3 will be required.

7.2 Markers

Markers for writing on processing tubes and vials should have a fine point, and contain fast drying, indelible ink. (Example: Fisher Scientific, fine line, felt tip marking pen).

Optional: Markers of various colors can be useful for color-coding different PTIDs.

7.3 Labels

Cryogenic labels suitable for -80°C and LN2 temperatures.

Examples: Cryo-Tags® and Cryo-Babies® from Diversified Biotech, Brady B461 or B490, Shamrock freezer labels.

8 Personal Protective Equipment

Personal protective equipment suitable for use with Blood Borne Pathogens is required. Follow local laboratory guidelines and practices for the handling of blood products.

8.1 Laboratory coat

8.2 Eye protection

8.3 Non-powdered, nitrile or equivalent gloves

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

9 Reagents

9.1 Wash Diluent Reagents (WDR)

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

*Alternative: 1X Phosphate-Buffered Saline without calcium or magnesium, ready-to-use.

**Alternative for ACTG and IMPAACT: RPMI Medium without FBS or antibiotics.

Note: Store opened bottles at the temperature recommended by the manufacturer until used or until manufacturer's expiration date. Discard if visible signs of contamination, such as a cloudy appearance, develop.

9.2 Pre-filled Cell Separation Tube with Frit Barrier (CSTFB)

The capacity of the tube required will depend on the total blood volume

Total blood volume (mL)	Tube capacity (mL)
≥ 15	50
< 15	12 or 14

Pre-filled CSTFB, 12 to 14mL or 50mL tubes with 1.077 density gradient media (Examples: Accuspin™ System Histopaque®-1077 by Sigma or Ficoll-Paque™ PLUS by Greiner Bio-One):

Storage conditions:

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

Alternatives to pre-filled CSTFB System:

Combine a dry CSTFB with 1.077 density gradient media

Tube capacity (mL)	Density gradient media volume (mL)
50mL	15mL
12 to 14mL	3mL

Examples listed below:

Dry CSTFB (tubes)

- Dry Accuspin™ separation tubes (12mL or 50mL)
- Dry Leucosep® separation tubes (14mL or 50mL)

1.077 Density Gradient Media

- Sigma Histopaque®
- Amersham Biosciences Ficoll-Hypaque™
- Axis-Shield Lymphoprep™ media (Greiner Bio-One)

Note: If using CSTFB, use [Chapter 14](#). If using Manual Overlay or Underlay (without frit barriers), use [Chapter 15](#).

9.3 Freezing Reagents

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

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.

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.

FBS stored frozen ($\leq -20^{\circ}\text{C}$) is good until the manufacturer's expiration date.

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

9.3.2 Dimethylsulfoxide (DMSO), cell-culture grade

Be sure to use a cell-culture grade DMSO, for example: Hybrimax, Sigma-Aldrich cat# D2650, endotoxin tested, hybridoma tested.

Store unopened bottles at room temperature (15 to 30°C). Check bottle for expiration date.

DMSO must be fresh and sterility maintained. (Reagent may be aliquoted in small amounts to help preserve sterility.) Label with the date upon opening.

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

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

Discard open bottle if visible signs of contamination are noted.

9.3.3 Disinfectant

9.3.3.1 70% v/v ethanol disinfectant, spray bottle

9.3.3.2 10% v/v bleach, bucket or beaker and spray bottle

9.3.3.3 Other disinfectant as specified by local laboratory policy

9.4 Cell Counting Reagents

If performing manual cell counts, the following reagents are needed:

9.4.1 0.4% trypan blue solution

9.4.2 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 H₂O

10 Reagent Preparation

10.1 Heat-Inactivated FBS (HI-FBS)

HI-FBS can be ordered from the manufacturer. If you receive HI-FBS, follow these instructions for thawing, aliquoting and use.

10.1.1 Remove HI-FBS from the -20°C freezer.

10.1.2 Thaw in the refrigerator (2 to 8°C), preferred, or for several hours at room temperature. Do not allow HI-FBS to sit at room temperature any longer than necessary to complete the thawing process.

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

10.1.4 Once thawed, mix the HI-FBS gently but thoroughly using aseptic technique. Aliquot into sterile, labeled 50mL conical 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.

10.1.5 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 -20°C 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.

10.1.6 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.

10.2 FBS Heat Inactivation Preparation

If FBS is not heat-inactivated by the manufacturer, heat inactivate it using the instructions below.

10.2.1 Remove FBS from the -20°C freezer.

10.2.2 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.

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

10.2.4 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.

10.2.5 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: If the top of the bottle comes in contact with the water bath then swab the top of the bottle with 70% v/v ethanol before opening.

10.2.6 Mix the FBS gently but thoroughly using aseptic technique. Aliquot into sterile, labeled 50mL conical tubes.

Note: Labels should identify these tubes as “HI-FBS” (heat inactivated 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 until the original manufacturer’s expiration date if stored at -20°C.

Refrigerate (2 to 8°C) the number of aliquot tubes needed for the expected workload. Mix well before use.

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.

10.2.7 The remaining aliquot tubes can be returned to the -20°C freezer and are stable until the original manufacture’s expiration date.

10.2.8 When ready 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.

10.3 Fresh Cryopreservation Solution (CPS)

10.3.1

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

10.3.2 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 for HPTN, HVTN, MTN: CPS can be stored at 2 to 8°C for 1 working day (<18 hours).

Note for ACTG, IMPAACT: CPS can be made up in larger batches and stored at -20°C for no more than one working week, and thawed to 2 to 8°C before use.

10.3.3 Use the formula below to **estimate** the volume of CPS to prepare. Examples are also shown.

$$\text{Usable Whole Blood (mL)} \times \text{Cell Yield (cells/mL)} \times \text{Freeze-down Concentration (mL/cells)} = \text{Estimated CPS (mL)}$$

Round up to the nearest whole mL.

Examples: Adult Blood—Large Volume Blood Collection

Usable Whole Blood x	Cell Yield x	Freeze-down Concentration =	Estimated CPS to Prepare
(10mL) x	$(1.5 \times 10^6 \text{ cells/1mL}) \times$	$(1\text{mL}/15 \times 10^6 \text{ cells}) =$	1mL
(85mL) x	$(1.0 \times 10^6 \text{ cells/1mL}) \times$	$(1\text{mL}/15 \times 10^6 \text{ cells}) =$	6mL
(140mL) x	$(1.5 \times 10^6 \text{ cells/1mL}) \times$	$(1\text{mL}/15 \times 10^6 \text{ cells}) =$	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	$(1.5 \times 10^6 \text{ cells/1mL}) \times$	$(0.5\text{mL}/5 \times 10^6 \text{ cells}) =$	2mL
(2mL) x	$(1.0 \times 10^6 \text{ cells/1mL}) \times$	$(0.5\text{mL}/2.5 \times 10^6 \text{ cells}) =$	1mL

10.3.4 Use the following formulas to calculate the amount of DMSO and FBS needed.

Estimated CPS Volume	DMSO Volume = (.1)(CPS volume)	HI-FBS Volume = CPS volume – DMSO volume	Total CPS Volume = DMSO volume + FBS volume
1mL	0.1mL	0.9mL	1mL
9mL	0.9mL	8.1mL	9mL
50mL	5mL	45mL	50mL

10.3.5 Record the CPS, DMSO and FBS volumes on the **PBMC Processing Worksheet**.

10.4 Counting Reagents

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

10.4.1 0.4% Trypan Blue Solution

10.4.2 Optional: 0.05% Crystal Violet Solution

11 Calibration

11.1 No calibration is required for the processing steps.

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

12 Quality Control

12.1 Cell Yields

Cell yields are fairly consistent within 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 that should be obtained** for the population of participants for which the processing is being done. 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.

12.1.1 Expected Cell Yields

Population	Mononuclear Cell Yield Range (cells/mL)
Adult	0.7×10^6 to 3×10^6
Pediatric—less than 6 months	3×10^6 to 10×10^6
Pediatric—6 mo. to 2 years	2×10^6 to 9×10^6
Pediatric—2 to 5 years	1×10^6 to 6×10^6
Pediatric—more than 5 years	0.8×10^6 to 4×10^6
Pediatric—Unknown age	1×10^6 to 10×10^6

12.1.2 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.

Record all results and any problems that occur during processing on the **PBMC Processing Worksheet**.

Note for HVTN: Record any problems and actions in the cell yield comments section of the Internal Quality Control records.

12.2 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. For networks requiring cell viability (see section 6.3), calculate and record the % viable cells on the worksheet.

12.2.1 Freshly isolated PBMC viability should be >95%.

12.2.2 If the fresh PBMC viability is <95%, review the results with the supervisor and document on the PBMC Processing Worksheet.

12.3 Handling Times

Handling times can adversely affect cell recovery and viability. The collection, handling and processing times are recorded on the **PBMC Processing Worksheet** and/or LDMS.

12.3.1 Expected Times

- HVTN requires that the total time from collection to freezing is less than or equal to 8 hours. For other networks, the time limit may vary.
- HVTN requires that the actual processing time from introduction of fresh blood into the density gradient tubes until initiation of controlled-rate freezing be completed within 2 to 3 hours to maximize cell integrity. For other networks, the time limit may vary.

12.3.2 Unexpected (Long) Processing times

Review any long processing times with the supervisor and document on the **PBMC Processing Worksheet** and/or LDMS.

12.4 PBMC Processing Worksheet

For HVTN: The use of the **PBMC Processing Worksheet** is *required* to track the timing of processing, calculations and documentation of problems that arise during processing. The **PBMC Processing Worksheet** is provided in Appendix A. This worksheet is also available as a downloadable and editable form at <http://www.hanc.info/labs/Pages/PBMCSOP.aspx>.

For other networks: The use of the **PBMC Processing Worksheet** is recommended.

13 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
<p><i>Cell Separation and Blood Treatment</i></p> <p>Chapter 14: Cell Separation and Blood Dilution with Optional Plasma Replacement by Cell Separation Tube with Frit Barrier (CSTFB)</p> <p style="text-align: center;">OR</p> <p>Chapter 15: Cell Separation by Manual Ficoll® Overlay or Underlay and Blood Dilution with Optional Plasma Replacement by Manual Density Gradient Cell Separation</p>	<p>Can be used for all networks; check protocol requirements and available materials</p> <p>Can be used for all networks; check protocol requirements and available materials</p>
<p><i>Washing, Counting, Resuspension, Concentration, and Overnight Controlled-Rate Freezing</i></p> <p>Chapter 16</p>	<p>All networks</p>
<p><i>Onsite Storage</i></p> <p>Chapter 17: Onsite Storage at -70/-80°C</p> <p style="text-align: center;">OR</p> <p>Chapter 18: Onsite Storage in Liquid Nitrogen (LN2)</p>	<p>ACTG and HVTN</p> <p>IMPAACT, HPTN and MTN</p>

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

Chapter 14 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Chapter 14 or Chapter 15, but not both.

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

- 14.1.1 All pipetting and mixing takes place in a biological safety cabinet (BSC), level 2 or greater.
- 14.1.2 Spray down all surfaces, racks, and reagent bottles with 70% v/v ethanol or equivalent disinfectant prior to entering and using the BSC.
- 14.1.3 Unless otherwise noted, the procedure is carried out at room temperature (15 to 30°C).
- 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 Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see Chapter 10 Reagent Preparation).
- 14.2.2 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 before processing.
- 14.2.3 Record on the **PBMC Processing Worksheet** (and/or equivalent): the PTID, visit number, protocol, collection date/time, processing start date/time, lot numbers and expiration dates of all reagents, CPS, DMSO, and FBS volumes.
- 14.2.4 Before adding the blood, visually check the CSTFB tubes to see if there is liquid above the frit. If there is liquid above the frit, centrifuge the CSTFB tubes at 1000 x g for 30 seconds. If any density gradient solution remains above the frit after centrifuging, it should be aspirated.
- 14.2.5 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.

14.3 Optional 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 14.4.

Note for IMPAACT: Plasma replacement is required.

- 14.3.1 Blood collection tubes from the same PTID and same anticoagulant may be processed individually or pooled in 50mL conical tubes.
- 14.3.2 Mark the whole blood volume at the meniscus.
- 14.3.3 Centrifuge the whole blood at 200 to 400 x g for 10 minutes.

- 14.3.4 Transfer plasma to a 15 or 50mL centrifuge tube for second centrifugation to remove any cellular debris.
- 14.3.5 Add sufficient quantity of WDR (see section 9.1) 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. This may be done at a later time when the centrifuge is not in use for PBMC processing.
- 14.3.7 Aliquot spun plasma into labeled aliquot tubes as specified by protocol and discard any cellular debris in spun plasma tube.

14.4 Blood Dilution for CSTFB separation

Note: An accurate measurement of the usable blood volume must be determined and recorded on the **PBMC Processing Worksheet** and LDMS where applicable. This can be accomplished by using a sterile pipet to transfer the whole blood to the CSTFB tubes and keeping track of the blood volume as it is pipetted, or by pooling the blood in a sterile graduated container before transferring to the CSTFB tubes and taking the measurement from the container.

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

Note: Ficoll® is toxic to cells; work quickly and efficiently during the separation steps.

- 14.4.1 Label each CSTFB with the PTID.
- 14.4.2 If a tube is grossly clotted (see glossary), discard it.
- 14.4.3 Document the type of specimen received, the total number of tubes discarded and the blood condition on the **PBMC Processing Worksheet**.
- 14.4.4 Using a sterile pipet, add 5mL (for adults) or 2mL (for pediatrics) of WDR to each CSTFB.
- 14.4.5 Mix whole blood gently then use a sterile pipet to transfer 10 to 20mL (for adults) or 4 to 5mL (for pediatrics) of blood into the labeled CSTFBs.
- 14.4.6 Using a sterile pipet, rinse each original anti-coagulated blood tube with WDR, add rinse volumes to the CSTFB tubes making sure not to exceed the 30mL (for adults) or 7.5mL (for pediatrics) total tube volume (WDR + Whole Blood).
- 14.4.7 Record the volume of usable blood on the **PBMC Processing Worksheet**.
 - Note:** Do not estimate the volume of usable blood based on the tube size.
 - Note:** Do not include the volume of WDR used to dilute the blood sample.
- 14.4.8 Carefully cap the CSTFB tubes.

14.5 CSTFB density centrifugation and collection

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

14.5.2 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. Refer to Chapter 20 Calculations to convert g to rpm for your rotor length.

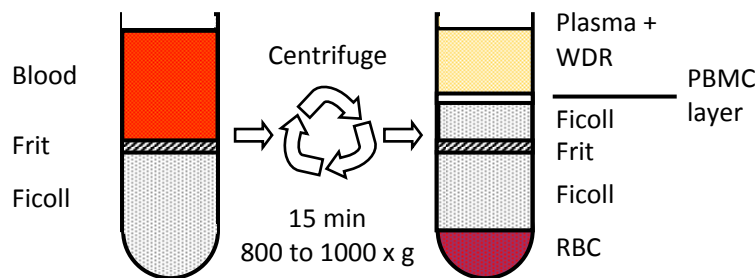
14.5.3 Prepare the same number of new sterile 50mL (for adults) or 15mL (for pediatrics) conical tubes as CSTFB tubes used in the separation centrifugation step.

14.5.4 Label each tube with the PTID. Use these new tubes for the next wash.

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

14.5.6 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
- Ficoll®
- Frit
- Ficoll®
- Packed red blood cells (RBC) and granulocytes



14.5.7 Inspect the tubes for the following possible problems:

Hemolysis in the Plasma layer + WDR. If present, grade hemolysis +1 through +4 based on the description given in 29 Glossary of Terms.

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.

Record observations in the comments section of the **PBMC Processing Worksheet** and/or LDMS. Document any follow-up actions taken.

14.5.8 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.

14.5.9 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.

14.5.10 Transfer the collected cells from one CSTFB tube to a single corresponding, pre-labeled, sterile 50mL (for adults) or 15mL (for pediatrics) conical tube. Tubes can be pre-filled to 25mL (for adults) or 5mL (for pediatrics) with WDR to save time.

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

Skip Chapter 15 and proceed to Chapter 16.

15 Cell Separation by Manual Ficoll® Overlay or Underlay and Blood Dilution with Optional Plasma Replacement by Manual Density Gradient Cell Separation

Chapter 15 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Chapter 14 or Chapter 15, but not both.

15.1 Separation of lymphocytes from peripheral blood using Manual Ficoll® Overlay Method

- 15.1.1 All pipetting and mixing takes place in a biological safety cabinet, level 2 or greater.
- 15.1.2 Spray down all surfaces, racks, and reagent bottles with 70% v/v ethanol prior to entering and using the BSC Hood.
- 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 (utilize the PBMC Processing Worksheet or other tracking tool as defined by the protocol or network to document and track specimen processing).

- 15.2.1 Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see Chapter 10 Reagent Preparation).
- 15.2.2 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.
- 15.2.3 Allow the Ficoll-Hypaque™ (or equivalent density gradient media) to come to room temperature (15 to 30°C). See the Reagent section of this document for more information.
- 15.2.4 Complete the sample and reagent sections of the **PBMC Processing Worksheet**.
- 15.2.5 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.

15.3 Optional 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.

Note for IMPAACT: Plasma replacement is required.

- 15.3.1 Blood collection tubes may be processed individually or pooled in 50mL conical tubes.
- 15.3.2 Mark the whole blood volume in each tube 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 15 or 50mL centrifuge tube for second centrifugation to remove any cellular debris.
- 15.3.5 Add sufficient quantity of WDR (see section 9.1) 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. 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 and Manual Density Gradient Cell Separation

Note for ACTG, IMPAACT and HPTN: For larger blood volume collections, pooling buffy coats is allowed (see Appendix D: Pooling Buffy Coat Layers for Ficoll PBMC Isolation).

Note for HVTN: An accurate measurement of the usable blood volume must be determined and recorded.

Note: Dilution of blood with WDR can help improve separation (see Appendix D).

- 15.4.1 Label each 15 or 50mL centrifuge tube with the PTID. Use one 50mL tube for each 15 to 20mL of adult whole blood (or one 15mL tube for each 4 to 5mL of pediatric whole blood).
- 15.4.2 Uncap the tubes of anti-coagulated blood.
- 15.4.3 If a tube is grossly clotted (see glossary), discard it.
- 15.4.4 Document the type of blood received on the PBMC Worksheet.
- 15.4.5 Record the total number of tubes discarded or any other problems noted with the blood.
- 15.4.6 Record the blood condition.
- 15.4.7 Record the total volume of undiluted whole blood.

Note: Do not estimate the volume of usable blood based on the tube size.

Note: Do not include the volume of WDR used to dilute the blood sample.

- 15.4.8 Transfer the blood to a sterile, labeled 15 or 50mL centrifuge tube and add sufficient volume of WDR to dilute the blood according to the lymphocyte separation medium 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.

15.4.9 For Density Gradient Cell Separation:

On any given sample, use either the Overlay Method (15.4.9.1) or Underlay Method (15.4.9.2), but not both.

15.4.9.1 Ficoll®-Hypaque **Overlay** Method: Based on the volume of WDR-diluted blood, determine the number and size of sterile centrifuge tubes required for the density gradient separation.

Aseptically add the required Ficoll® or LSM to the sterile centrifuge tubes.

Note: The ratio of Ficoll® to whole blood may vary according to the manufacturer's recommendation and laboratory experience. For example, some manufacturers recommend 4 parts diluted blood to 3 parts Ficoll® reagent; however practical experience has shown good results using 3 parts blood to 1 part gradient medium.)

Carefully and slowly pipet diluted blood on top of gradient medium.

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

15.4.9.2 Ficoll®-Hypaque **Underlay** Method:

If plasma was removed for storage, add a volume of WDR equal to volume of plasma removed.

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. Carefully and slowly pipet Ficoll®-hypaque solution UNDER blood-WDR in sterile 15mL or 50mL centrifuge tubes.

Note: The ratio of Ficoll® to whole blood may vary according to manufacturer's recommendations and laboratory experience. For example, some manufacturers recommend 4 parts diluted blood to 3 parts Ficoll® reagent, however practical experience in some labs has shown good results using 3 parts blood to 1 part Ficoll®.

15.4.10 Carefully cap the tubes.

15.5 Lymphocyte density centrifugation and collection

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

15.5.2 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 20 Calculations to convert g to rpm for your rotor length.

15.5.3 Prepare the same number of new sterile 15 or 50mL conical tubes as centrifuge tubes used in the separation centrifugation step.

- 15.5.4 Label each tube with the PTID. Use these new tubes for the next wash.
- 15.5.5 Remove the tubes from the centrifuge.
- 15.5.6 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.
- Note:** If the cell layer is still not visible after re-centrifuging, document, remove and discard the WDR supernatant and proceed.
- 15.5.7 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.
- 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. Take care not to aspirate any more separation medium solution than necessary.
- 15.5.10 Transfer the collected cells from one centrifuge tube to a single corresponding, pre-labeled, sterile 15 or 50mL conical tube. Tubes can be pre-filled to 5mL (for 15mL tube) or 25mL (for 50mL tube) with WDR to save time.
- 15.5.11 Re-cap the centrifuge tube containing the remaining red blood cells/separation medium and discard the tube as biohazard waste following laboratory policy.

Proceed to Chapter 16.

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

Use Chapter 16 for all networks.

16.1 Wash 1:

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

16.1.2 Re-cap all of the harvested cell tubes.

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

16.1.4 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 in the comments section of the **PBMC Processing Worksheet**. If the cell pellet is still not visible after re-centrifuging the tube, document.

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

16.2 Wash 2:

16.2.1 For 15mL and 50mL conical tubes, re-suspend each pellet in a small volume (no more than 10mL total) of WDR mixing gently but thoroughly into a homogenous cell suspension.

16.2.2 For 50mL conical tubes, combine up to four pellet suspensions (<20mL total) from the same donor. For 15mL conical tubes, combine up to two pellet suspensions (<10 mL total) from the same donor. This is the harvested cell tube. QS the PBMC fraction to approximately 10mL (for 15mL conical tubes) or approximately 45mL (for 50mL conical tubes) by adding WDR. Mix gently.

16.2.3 Use a small volume of WDR to rinse the tubes from which the pellets were transferred.

Note: The combined rinse and harvested volume should not exceed 45mL (for 50mL tube) or 10mL (for 15mL tube).

16.2.4 Collect the WDR rinse in the harvested cell tube.

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

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

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. Re-centrifuge the tube. Document the problem and actions taken in the comments section of the **PBMC Processing Worksheet**. If the cell pellet is still not visible after re-centrifuging the tube, document.

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

16.3 PBMC Cell Count

- 16.3.1 Record the counting method used on the **PBMC Processing Worksheet**,
- 16.3.2 Calculate and record on the processing worksheet, the WDR counting re-suspension volume (V). This is the volume on which the cell count is based.

Note: The re-suspension volume should be approximately 20% of the usable whole blood volume rounded to the nearest mL. Depending on the size of the cell pellet, the resuspension volume generally ranges from 10% to 50% of the usable whole blood volume.

- 16.3.3 If there is more than one pellet, 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.

- 16.3.4 Complete the cell count using the SOP for the cell counting method approved at the laboratory.

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

- 16.3.6 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.

- 16.3.7 Follow the SOP for the cell counting method approved at the processing laboratory and the **PBMC Processing Worksheet** to determine the cell concentration $\times 10^6$ per mL.

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

- 16.3.8 Use the appropriate Cell Counts section on the **PBMC Processing Worksheet** to record the cell count concentrations for each PTID (cells $\times 10^6$ per mL).

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

- 16.3.9 On the **PBMC Processing Worksheet**, record the automated or manual cell count.

- 16.3.10 Calculate the total number of cells using the following formula:

$$T = C \times V$$

T = Total number of cells

C = Concentration ($10^6/\text{mL}$) determined in counting method

V = Count re-suspension volume of WDR in mL

- 16.3.11 Record the total number of cells (T) on the **PBMC Processing Worksheet**.

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

$$\text{Cell Yield } (10^6 \text{ cells/mL}) = T / \text{Usable Whole Blood Volume}$$

- 16.3.13 Record the cell yield on the **PBMC Processing Worksheet**.

Note: The cell yield is calculated for quality purposes only. Refer to section 12 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 Quality Control section. Re-dilute and re-count if necessary.

16.3.14 Record any anomalies in processing in the comments section on the **PBMC Processing Worksheet**.

16.4 Calculation of final re-suspension volume

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

The target final cell concentration varies by network and protocol. Use the table below to determine the usual appropriate target concentration and acceptable range.

Network	Target Concentration (cells/mL)	Acceptable Range (cells/mL)
HVTN	15 x 10 ⁶	10 to 20 x 10 ⁶
ACTG, HPTN and MTN	10 x 10 ⁶	5 to 10 x 10 ⁶
IMPAACT	10 x 10 ⁶	10 x 10 ⁶

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

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

T = Total number of cells

N1 = Desired final cell concentration

V2 = final aliquot volume in mL

Record the estimated volume (V1) on the **PBMC Processing Worksheet**.

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 LPC or protocol.

Record the Actual CPS re-suspension volume (V_f) on the **PBMC Processing Worksheet**.

16.4.2 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

16.4.3 Record the final number of cells per vial (N2) on the **PBMC Processing Worksheet**.

16.4.4 Confirm that the **PBMC Processing Worksheet** is complete and that the calculations are correct.

16.5 Labeling

16.5.1 Complete the printing and labeling of the cryovials PRIOR to the final centrifugation.

Note: This is important to ensure that cells do not sit in a pellet for an extended period of time.

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

Follow network lab practice for completing the data entry.

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

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

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

16.5.3 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.

16.6 Final Centrifugation

16.6.1 Place the harvested cell tube in the centrifuge.

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

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

16.6.3 Verify that all cryovials are labeled and easily accessible.

16.7 Aliquoting for cryopreservation

Note: The following steps should be performed quickly to preserve cell integrity. It is recommended that vials 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.

16.7.1 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.

16.7.2 Re-suspend the pellet using the volume of cold CPS (V_f) that you determined in 16.4.

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

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

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

16.7.3 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.

16.7.4 Aliquot 0.5 to 1mL per tube, depending on network and protocol requirements. Prepare a final partial aliquot with any excess volume that may be present due to the cell pellet size.

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

16.8 Overnight controlled-rate freezing

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

16.8.2 Select the freezing method to be used: StrataCooler® Cryo, NALGENE® Mr. Frosty or CryoMed®.

Note: StrataCooler® Cryo should be stored at to 2 to 8°C prior to each use.

Note: Ideally, allow NALGENE® Mr. Frosty to equilibrate to 2 to 8°C prior to each use in an explosion-proof refrigerator. If an explosion-proof refrigerator is not available, allow NALGENE® Mr. Frosty to equilibrate to ambient room temperature prior to each use. The isopropanol level must be correct and the isopropanol must be completely replaced after the fifth freeze-thaw cycle.

Follow the appropriate on-site SOP for a controlled-rate freezer, such as CryoMed®.

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

For NALGENE® Mr. Frosty 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 overnight for StrataCooler® Cryo.

For CryoMed®, start the cooling program.

16.8.4 Record the date and time at which the cryovials were moved into the -80°C (-65 to -95°C) freezer on the **PBMC Processing Worksheet**.

Note: This is the completion time of processing.

16.8.5 Record the actual number of cryovials frozen on the **PBMC Processing Worksheet**.

Proceed to Chapter 17 (for ACTG and HVTN) or Chapter 18 (for HPTN, IMPAACT and MTN).

17 Onsite Storage at -70/-80°C

Use Chapter 17 only PBMC are being processed for ACTG or HVTN.

17.1 Transfer PBMC cryovials to -70/-80°C freezer

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

Note: Do not store in liquid nitrogen (LN2).

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

17.1.2 Record the initials of the person making the transfer and the date/time of the transfer on the **PBMC Processing Worksheet**.

17.1.3 Record the appropriate storage information on the **PBMC Processing Worksheet** based on your laboratory's defined storage program.

17.1.4 Store at -65 to -95°C until shipped.

Note for HVTN: Ship on dry ice to the central specimen repository within 2 weeks of collection.

Note for ACTG: Ship on dry ice within 3 to 5 weeks from the date of freezing.

17.1.5 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.

17.1.6 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.

17.2 Final Worksheet Review

17.2.1 The technician should confirm that the **PBMC Processing Worksheet** is complete and that the calculations are correct.

17.2.2 A second reviewer checks the worksheet for completeness and accuracy and then initials and dates the **PBMC Processing Worksheet**.

Note: HVTN requires that all reviews are completed within two days of processing.

17.2.3 Store the **PBMC Processing Worksheet** according to laboratory policy.

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

18 PBMC Processing Section 6B: Onsite Storage in Liquid Nitrogen (LN2)

Use Chapter 18 only if PBMC are being processed for HPTN, IMPAACT or MTN.

18.1 Transfer of PBMC cryovials to LN2

- 18.1.1 The next working day, transfer the cryovials from the controlled-rate cooling system to the designated storage location in the LN2 storage system.
- 18.1.2 Record the initials of the person making the transfer along with the date/time of the transfer on the **PBMC Processing Worksheet**.
- 18.1.3 Record the appropriate storage information on the **PBMC Processing Worksheet** based on your laboratory's defined storage program.
- 18.1.4 Frozen PBMC samples can be stored safely in LN2 indefinitely (vapor phase preferred).
- 18.1.5 Once samples have been stored in LN2, all transfers or shipments must be maintained in LN2 ($\leq -140^{\circ}\text{C}$) and samples cannot be shipped on dry ice.

***Note for IMPAACT:** Samples may be shipped on dry ice after storage in LN2.*
- 18.1.6 Do not temporarily store samples in LN2.
- 18.1.7 Do NOT transfer samples from LN2 back to -70°C or -80°C freezers unless directed to by network or protocol team.

18.2 Final Worksheet Review

- 18.2.1 The technician should confirm that the **PBMC Processing Worksheet** is complete and that the calculations are correct.
- 18.2.2 A second reviewer checks the worksheet for completeness and accuracy and then initials and dates the **PBMC Processing Worksheet**.

***Note:** Reviews should be completed within 2 working days of processing.*
- 18.2.3 Store the **PBMC Processing Worksheet** according to laboratory policy

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

19 Reporting Results

19.1 A completed PBMC Processing Worksheet is required for HVTN only.

19.2 Requirements for all networks:

19.2.1 Data are entered into the Laboratory Data Management System for the generation of cryovial labels, storage location documentation and shipping manifest requirements.

19.2.2 Deviations are reported according to laboratory protocol.

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 Procedural Notes

- 22.1 If the plasma is very cloudy, it may be difficult to see the interface of the Ficoll® gradient. 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.
- 22.2 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.

23 Glossary of Terms

Term	Definition
<i>ACTG</i>	AIDS Clinical Trials Group
<i>Centrifuge Temperature</i>	15 to 30°C
<i>Clotted, Grossly</i>	More than ¾ of the whole blood mass is clotted and there is very little free whole blood remaining.
<i>Clot, Small</i>	Small clots will not usually be seen in the whole blood tube, but can be seen on the separation tube frit after centrifugation.
<i>CPS</i>	Cryopreservation Solution
<i>CSR</i>	Central Specimen Repository
<i>CSTFB</i>	Cell Separation Tube with Frit Barrier
<i>DG Media</i>	Density Gradient Media

Term	Definition
<i>FBS</i>	Fetal Bovine Serum
<i>HBSS</i>	Hanks' Balanced Salt Solution
<i>Hemolysis</i>	<p>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:</p> <p>1+ Pale pinkish-red color in serum or plasma, able to clearly read news print placed behind the blood tube</p> <p>2+ Pinkish-red color in serum or plasma, news print is readable but not as sharp</p> <p>3+ Dark pinkish-red color in serum or plasma, news print appears obscured.</p> <p>4+ Dark red mahogany color in serum or plasma, unable to read news print</p> <p>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.</p>
<i>HI-FBS</i>	Heat Inactivated Fetal Bovine Serum
<i>HPTN</i>	HIV Prevention Trials Network
<i>HVTN</i>	HIV Vaccine Trials Network
<i>Icteric</i>	A green or orange tinted plasma suggesting the presence of increased bilirubin.
<i>IMPAACT</i>	International Maternal Pediatric Adolescent AIDS Clinical Trials Network
<i>LDMS</i>	Laboratory Data Management System
<i>MTN</i>	Microbicides Trials Network
<i>PBMC</i>	Peripheral Blood Mononuclear Cells
<i>PBS</i>	Phosphate-buffered saline
<i>PTID/PID</i>	Participant Identification Number
<i>QS</i>	Quantity Sufficient—add sufficient quantity of liquid to bring to specified volume
<i>Room Temperature (RT)</i>	15 to 30°C
<i>WDR</i>	Wash Diluent Reagent (HBSS, PBS or RPMI; RPMI can be used for ACTG/IMPAACT only)

24 References

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- 24.2 CHAVI SOP for PBMC Isolation and Cryopreservation, CHAVI-A0001, v5, Nov 3 2008.
- 24.3 Cox J.H., DeSouza M., Ratto-Kim S., Ferrari G., Weinhold K.J., and Birx B.L. Cellular Immune assays for evaluation of Vaccine efficacy in developing countries. *Manual of Clinical laboratory Immunology*. Rose N.R., Hamilton R.G., Detrick B. Eds. (6th ed.) p.301-315 (2002).
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- 24.5 Immunovirology Research Network (IVRN) Laboratory Manual: Separation and storage of serum, plasma and PBMCs. IVRN. Dec 12 1007.
- 24.6 Kierstead L.S., Dubey S., Meyer B., Tobery T.W., Mogg R., Fernandez V.R., Long R., Guan L., Gaunt C., Collins K., Sykes K.J., Mehrotra B.V., Chirmule N., Shiver J.W., and Casimiro B.R. Enhanced rates and magnitude of immune responses detected against an HIV vaccine: effect of using an optimized process for isolating PBMC. *AIDS Res Hum Retroviruses* 23:86-92 (2007).
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- 24.8 Sigma-Aldrich Accuspin™ System-Histopaque®-1077, procedure number A6929/A7054/A0561, Dated 2003-09.
- 24.9 Weinberg A., Betensky R., Zhang L., and Ray G. Effect of shipment, storage, anticoagulant, and cell separation on lymphocyte proliferation assays for human immunodeficiency virus-infected patients. *Clin. Diagn. Lab. Immunol.* 5:804-807 (1998).

25 Additional Documents (To be maintained by the laboratory)

- 25.1 FBS Package Insert (and Certificate of Analysis)
- 25.2 WDR (HBSS, PBS or RPMI) package insert
- 25.3 Density Gradient Medium package insert
- 25.4 Cell separation tube with frit barrier package insert

26 Appendices

26.1 Appendix A: 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>.

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

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

26.4 Appendix D: Pooling Buffy Coat Layers for Ficoll PBMC isolation

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

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

26.7 Appendix G: Revision History

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Appendix A: PBMC Processing Worksheet (Required for HVTN)

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
Usable whole blood volume				mL
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)				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 = (Aliquot volume * T)/(V _f); HVTN aliquot volume is equal to 1mL.				x 10 ⁶ cells/vial
Date and time processing completed (Note in comments if not within 8 hours of processing start time)				hrs:mins
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.				
For HVTN, complete LDMS entries including freeze time.				

Appendix A: PBMC Processing Worksheet (Required for HVTN)

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/mm ²)(DF)(10 ⁴); convert to 10 ⁶ cells/mL	x 10 ⁶ cells/ml	x 10 ⁶ cells/ml	x 10 ⁶ cells/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 ⁶ cells/ml			

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

Guava Counter Cell Counts (10 ³ /μl=10 ⁶ /ml)	Count #1			
Cell Count (C) as cells x 10 ⁶ /mL				
Total Cells (T) as cells x 10 ⁶				
% viability				

Comments and Protocol Deviations:

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

C.1 Background:

- C.1.1 If something has gone wrong during the density gradient centrifugation of the blood, the Ficoll® and plasma layer will mix and there will be no visible PBMC layer. Do not panic. PBMC can be retrieved 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 the sample became mixed. Possible causes are listed below:
- C.2.2.1 The tube was dropped.
 - 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.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 Ficoll®
 - C.4.1.2 50 mL tubes
 - C.4.1.3 Pipets
- C.4.2 Method:
- Note:** Ficoll® is toxic to cells so work efficiently
- C.4.2.1 Add 15 mL of Ficoll® to sterile 50 mL tubes (not CSTFB tubes).
 - C.4.2.2 Allow the Ficoll® 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 tube. (Typically, the CSTFB frit will have dislodged.)

- C.4.2.4 Transfer up to 30 mL of the mixed sample to the tube containing Ficoll®.
- 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 Ficoll®, 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 Ficoll® 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 centrifuge tube labeled with the PTID identifier. Use one new tube for every Ficoll® tube.
- C.4.2.11 Re-cap the Ficoll® tube.
- C.4.2.12 Return to Chapter 16 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 Ficoll®. Also, note that the plasma sample recovered was potentially contaminated with Ficoll® 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 Ficoll PBMC isolation

The procedure can be used when isolating PBMCs from multiple tubes of blood. This procedure allows one to consolidate the buffy coat layers to reduce Ficoll consumption. Done well, the pooled buffy coat layers yield large cell numbers that are typically very clean.

Generally, the buffy coats from two 10mL tubes can be layered onto 6mL of Ficoll® or equivalent density gradient separation solution in a 15mL conical tube. By pooling buffy coats, a single 50mL conical tube with Ficoll® can be used to process up to six 10mL EDTA tubes.

Procedure:

- D1. Centrifuge EDTA (or Heparin or ACD) blood at 400 x g for 10 minutes.
- D2. Harvest plasma from each tube to about 5mm from the white buffy coat layer.
- D3. Add 2mL of WDR to a sterile 10mL polypropylene tube and leave in the laminar flow hood.
- D4. Hold the plasma depleted tube (which now contains only packed cells) at about a 30° angle. Use a sterile, wide bore, disposable polypropylene pipet to harvest the buffy coat. Aspirate the buffy coat by moving down the low end of the tube. The buffy coat will “slide” down the packed red cell layer with most white cells coming in the first 1mL of aspirate. Transfer the buffy coat to the WDR-containing tube, rinsing the pipet 2 to 3 times with WDR/cell suspension. Harvest and pool the buffy coats from the remaining tubes. Depending on the number of original blood collection tubes, the buffy coat suspension will be about 5mL.
- D5. To capture the cells clinging to the disposable pipet, dispense 3mL of WDR to a sterile disposable 5mL tube and rinse the pipet contents into the 3mL of fresh WDR. Pool the rinsed cell with the primary pooled buffy coats.
- D6. Gently mix the buffy coat pool 3 to 4 times with a standard 10mL pipet and layer the Buffy coat suspension onto Ficoll.
- D7. Proceed with the standard Ficoll isolation SOP.

Extra Materials Needed:

- One sterile 10mL polypropylene tube for 15mL Ficoll harvest
OR
One 50mL tube for a 50mL Ficoll harvest (with experience Ficoll can be layered under the 50mL tube pool)
- One sterile 5mL tube for pipet rinse
- One sterile 2.5mL, sterile, wide bore, polypropylene pipet.

Note: Try to layer the buffy coat pools in such a way that the pooled material is diluted about 1:2 (buffy coat: diluent), in the same fashion as for whole blood.

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Appendix E: PBMC SOP Quick Guide—CSTFB Tubes

Use of the **PBMC Processing Worksheet** (Appendix A) is **required for HVTN**. 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 <i>italicized</i> .)	Reference to SOP
1. Prepare and chill the CPS.	10.3
2. Prepare whole blood samples, reagents, and supplies.	14.2
3. If plasma aliquots are required per protocol instructions: <ol style="list-style-type: none"> Centrifuge the whole blood at 200 to 400 x g for 10 minutes. Mark the total blood volume at the meniscus then transfer plasma to a 15 or 50mL centrifuge tube for further processing (800 to 1200 x g for 10 minutes, brake optional) Add sufficient quantity of WDR to bring blood back to its original whole blood volume, mix gently and continue PBMC processing. 	14.3
4. Add 5mL (<i>2mL</i>) of WDR to each CSTFB.	14.4
5. Transfer 10 to 20mL (<i>4 to 5mL</i>) of blood into the labeled CSTFBs.	
6. Add WDR tube rinse and final WDR to CSTFB tubes up to 30 mL (<i>7.5mL</i>) (WDR + Whole Blood).	
7. Centrifuge at 800 to 1000 x g for 15 minutes at 15 to 30°C with the <u>Brake OFF</u> .	14.5
8. Inspect the tubes for possible problems.	
9. Harvest each CSTFB buffy coat into a corresponding single 50mL (<i>15mL</i>) conical tube.	
10. Add WDR to QS to a total volume of 45 mL (<i>10mL</i>) and mix gently.	16.1
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.	16.2
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 tube (<i>2 into 15mL tube</i>).	
16. Add WDR tube rinse and final WDR of 45 mL (<i>10mL</i>) 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.	
20. Calculate the WDR counting re-suspension volume (V).	16.3
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.	16.4
24. Calculate final CPS re-suspension volume. Check calculations.	
25. Complete the printing, labeling and QC of cryovials PRIOR to the final centrifugation.	16.5
26. Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).	16.6
27. Gently remove the supernatant without disturbing the cell pellet.	16.7
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.	
30. Immediately (\leq 10 minutes) transfer all cryovials to the controlled rate freezing equipment and begin freezing.	16.8
31. After the appropriate time period, transfer cryovials to the onsite storage equipment and ship within the time period designated by the network.	17 or 18
32. For HVTN, review the PBMC Processing Worksheet for completeness and accuracy.	17.2

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Appendix F: PBMC SOP Quick Guide—Manual Overlay

Use of the **PBMC Processing Worksheet** (Appendix A) is **required for HVTN**. 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 <i>italicized</i> .)	Reference to SOP
1. Prepare and chill the CPS.	10.3
2. Prepare whole blood samples, reagents, and supplies.	14.2
3. If plasma aliquots are required per protocol instructions: <ol style="list-style-type: none"> Centrifuge the whole blood at 200 to 400 x g for 10 minutes. Mark the total blood volume at the meniscus then transfer plasma to a 15 or 50 mL centrifuge tube for further processing (800 to 1200 x g for 10 minutes, brake optional). Add sufficient quantity of WDR to bring blood back to its original whole blood volume, mix gently and continue PBMC processing. 	15.3
4. Transfer whole blood to sterile, 50 mL (<i>15mL</i>) centrifuge tube and dilute with WDR as needed.	15.4
5. Carefully and slowly overlay blood on top of the density gradient medium. (Underlay Method is an approved alternative.)	
6. Centrifuge at 400 x g for 30 minutes with the <u>Brake OFF</u> .	15.5
7. Check each centrifuge tube for possible problems.	
8. Harvest each buffy coat into a corresponding single, 50mL (<i>15mL</i>) conical centrifuge tube.	
9. Add WDR to QS to a total volume of 45 mL (<i>10mL</i>) and mix gently.	16.1
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.	
13. Re-suspend the cell pellet in small amount of WDR making a homogenous cell suspension.	16.2
14. Combine up to 4 pellet suspensions into one 50mL conical tube (<i>2 into 15mL tube</i>).	
15. Add WDR tube rinse and final WDR of 45 mL (<i>10mL</i>) 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.	
19. Calculate the WDR counting re-suspension volume (V).	16.3
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.	
23. Calculate final CPS re-suspension volume. Check calculations.	16.4
24. Complete the printing, labeling and QC of cryovials PRIOR to the final centrifugation.	16.5
25. Centrifuge at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).	16.6
26. Gently remove the supernatant without disturbing the cell pellet.	16.7
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.	
29. Immediately (\leq 10 minutes) transfer all cryovials to the controlled rate freezing equipment and begin freezing.	16.8
30. After the appropriate time period, transfer cryovials to the onsite storage equipment and ship within the time period designated by the network.	17 or 18
31. For HVTN, review the PBMC Processing Worksheet for completeness and accuracy.	17.2

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Appendix G: Revision History

Version Effective Date (dd/mmm/yy)	Comments	
1.0 01/Apr/09	Initial	
2.0 01/Jul/09	Section	Change
	Throughout SOP	“HBSS” and “PBS” changed to “WDR” (Wash Diluent Reagent).
	Throughout SOP	Replaced “18 to 26°C” with “15 to 30°C”
	Cover page	Removed Annual SOP Review section.
	3.1	Changed to: 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. HVTN requires that the total time from collection to freezing is less than or equal to 8 hours. For other networks, the time limit may vary; check the appropriate protocol documents.
	5.4.3	Changed to: HVTN requires that the total time from collection to freezing is less than or equal to 8 hours. For other networks, the time limit may vary; check the appropriate protocol documents. Record the collection time on the PBMC Processing Worksheet and/or in LDMS.
	6.1.9	Changed to: Bucket or beaker for bleach or other disinfectant for rinsing pipets, if required by local safety practice.
	6.3.3	Changed to: Note for HVTN: An automated cell counter not capable of identifying viable cells may be used to obtain a total cell count without enumerating viable cells.
	6.3.3	Changed to: Note for labs participating in the IQA PBMC Cryopreservation Proficiency Testing Program: Viability evaluation is required.
	6.4.1 and throughout SOP	“StrataCooler®” changed to “StrataCooler® Cryo”.
	6.4.2	Changed to: NALGENE® Mr. Frosty, 1°C/minute cryo-freezing container. Mr. Frosty should be stored at ambient temperature (15-30°C). Note: Replace isopropanol every fifth freeze/thaw cycle. A log must be used to track freeze/thaw cycles and reagent changes. See Appendix B.
	7.1.4	Cryogenic vials can be internally or externally threaded
	7.1.7	Changed to: Optional: If pre-filled cell separation tubes with frit barriers (CSTFB) are not used, empty CSTFB tubes (see 9.2 for more details) or 15 and 50mL disposable centrifuge tubes as in 7.1.3 will be required.
	8.3	Powdered gloves are not acceptable; all gloves should be non-powdered.
	9	No vendors are recommended.
	9.1	“Blood Separation Reagents” changed to “Wash Diluent Reagents”
9.1	An alternative wash solution for ACTG and IMPAACT is RPMI Medium without FBS or antibiotics.	

Version Effective Date (dd/mmm/yy)	Comments	
	9.2	"Check the manufacturer's expiration date" changed to "Use before the manufacturer's expiration date".
	9.2	Note changed to: If using CSTFB, use <u>Chapter 14</u> . If using Manual Overlay or Underlay (without frit barriers), use <u>Chapter 15</u> .
	9.3.3.2	Changed to: 10% v/v bleach, bucket or beaker and spray bottle
	10.1.2	Changed to: Thaw in the refrigerator (2 to 8°C), preferred, or for several hours at room temperature. Do not allow HI-FBS to sit at room temperature any longer than necessary to complete the thawing process.
	10.1.4	10.1.4 and 10.1.5 combined and reordered for clarity.
	10.2.2	Changed to: Thaw in the refrigerator (2 to 8°C), preferred, or for several hours at room temperature. Do not allow HI-FBS to sit at room temperature any longer than necessary to complete the thawing process.
	12.1.1	"Cell Yield Range" changed to "Mononuclear Cell Yield Range"
	12.3	Changed to: Handling times can adversely affect cell recovery and viability. The collection, handling and processing times are recorded on the PBMC worksheet and/or LDMS.
	12.3.1	Changed to: <ul style="list-style-type: none"> • HVTN requires that the total time from collection to freezing is less than or equal to 8 hours. For other networks, the time limit may vary. • HVTN requires that the actual processing time from introduction of fresh blood into the density gradient tubes until initiation of controlled-rate freezing be completed within 2 to 3 hours to maximize cell integrity. For other networks, the time limit may vary.
	12.3.2	Changed to: Review any long processing times with your supervisor and document on the PBMC Processing Worksheet and/or LDMS.
	14 through 16	Included alternative quantities for use with smaller (pediatric) samples.
	14	Changed instructions to: Chapter 14 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Chapter 14 or Chapter 15, but not both.
	14.1.4	Changed to: Use a new pipet for each participant identification number (PTID) and additive.
	14.2.1	Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see Chapter 10 Reagent Preparation).
	14.2.3	Changed to: Record on the PBMC Processing Worksheet (or equivalent): the PTID, visit number, protocol, collection date/time, processing start date/time, lot numbers and expiration dates of all reagents, CPS, DMSO, and FBS volumes.

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	14.2.5	<p>Changed to: 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.</p> <p>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.</p>
	14.3	<p>Plasma replacement is required for IMPAACT.</p>
	14.3.1	<p>Changed to: Blood collection tubes from the same PTID and same anticoagulant may be processed individually or pooled in 50mL conical tubes.</p>
	14.4	<p>Changed to: Note: The maximum ratio of blood to WDR should be approximately 2:1. Use one 50mL tube for each 10 to 20mL of adult whole blood (or one 12 to 14mL tube for each 4 to 5mL of pediatric whole blood). Use as many CSTFB tubes as required to distribute all of the blood for each PTID.</p>
	14.4.6	<p>Changed to: Using a sterile pipet, rinse each original anti-coagulated blood tube with WDR, add rinse volumes to the CSTFB tubes making sure not to exceed the 30mL total tube volume (WDR + Whole Blood).</p>
	14.5.2	<p>Changed to: Centrifuge at 800 to 1000 x g for 15 minutes at 15 to 30°C with the Brake OFF.</p>
	14.5.7	<p>Changed to: Poor PBMC layer due to error in centrifugation such as speed, time or braking. PBMC layer will appear small and indistinct while WDR + Plasma layer may be slightly cloudy. Refer to Appendix C for troubleshooting.</p>
	14.5.9	<p>Changed to: 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.</p>
	15	<p>Changed instructions to: Chapter 15 can be used for all networks; check protocol requirements and available materials. For any given sample, use either Chapter 14 or Chapter 15, but not both.</p>
	15.1.4	<p>Changed to: Use a new pipet for each participant identification number (PTID) and additive.</p>
	15.2.1	<p>Changed to: Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see Chapter 10 Reagent Preparation).</p>
	15.2.5	<p>Changed to: 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.</p> <p>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.</p>
	15.3	<p>Plasma replacement is required for IMPAACT.</p>
	15.4	<p>Changed to: Note for HVTN: An accurate measurement of the usable blood volume must be determined and recorded.</p>

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	15.4	Changed to: Note for ACTG, IMPAACT and HPTN: For larger blood volume collections, pooling buffy coats is allowed (see Appendix D: Pooling Buffy Coat Layers for Ficoll PBMC Isolation).
	15.4	Changed to: Note: Dilution of blood with WDR can help improve separation (see Appendix D).
	15.4.1	Changed to: Label each 15 or 50mL centrifuge tube with the PTID. Use one 50mL tube for each 15 to 20mL of adult whole blood (or one 15mL tube for each 4 to 5mL of pediatric whole blood).
	15.4.9	Added instructions for adding Ficoll® to tubes and reworded/reformatted for clarity.
	15.5.2	Changed to: Centrifuge at 400 x g for 30 minutes at 15-30°.
	15.5.9	Changed to: 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.
	15.5.10	Changed to: Tubes can be pre-filled to 5mL or 25mL with WDR to save time.
	16.1.3	Changed to: Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).
	16.2.1	Changed to: For 15mL and 50mL conical tubes, re-suspend each pellet in a small volume (no more than 10mL total) of WDR mixing gently but thoroughly into a homogenous cell suspension.
	16.2.2	Changed to: For 50mL conical tubes, combine up to four pellet suspensions (<20mL total) from the same donor. For 15mL conical tubes, combine up to two pellet suspensions (<10 mL total) from the same donor. This is the harvested cell tube. QS the PBMC fraction to approximately 10mL (for 15mL conical tubes) or 45mL (for 50mL conical tubes) by adding WDR. Mix gently.
	16.2.6	Changed to: Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).
	16.3.3	Changed to: If there is more than one pellet, 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.
	16.4.1	Changed note to: 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 LPC or protocol.
	16.5.3	Changed to: Note for HVTN: Scan the empty, labeled cryovials following current HVTN guidelines.
	16.6.1	Optional for HVTN: QS cell suspension to 45mL with WDR prior to centrifugation.
	16.6.2	Changed to: Centrifuge diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).
	16.6.3	Changed to: Verify that all cryovials are labeled and easily accessible.

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	16.7	Changed Note: The following steps should be performed quickly to preserve cell integrity. It is recommended that vials 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.
	16.7.2	Changed to: Gently resuspend the cell pellet prior to adding the CPS by flicking, racking or pipetting.
	16.7.2	Changed to: Pre-chilling vials and/or working on wet ice are allowed.
	16.8.2	Changed to: Select the freezing method to be used: StrataCooler® Cryo, NALGENE® Mr. Frosty or CryoMed®. Note: StrataCooler® Cryo should be stored at to 2 to 8°C prior to each use. Note: Ideally, allow NALGENE® Mr. Frosty to equilibrate to 2 to 8°C prior to each use in an explosion-proof refrigerator. If an explosion-proof refrigerator is not available, allow NALGENE® Mr. Frosty to equilibrate to ambient room temperature prior to each use. The isopropanol level must be correct and the isopropanol must be completely replaced after the fifth freeze-thaw cycle. Follow the appropriate on-site SOP for a controlled-rate freezer, such as CryoMed®.
	16.8.3	Changed to: Immediately transfer all cryovials to the controlled-rate freezing container. For NALGENE® Mr. Frosty 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 overnight for StrataCooler® Cryo. For CryoMed®, start the cooling program.
	17.1.1	Changed to: Transfer the cryovials after a minimum of 4 hours for Mr. Frosty and overnight for StrataCooler® Cryo.
	17.1.1	Deleted note about equilibrating StrataCooler® Cryo and NALGENE® Mr. Frosty.
	17.1.5	Changed to: 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.
	17.2.2	Changed note to: HVTN requires that all reviews are completed within two days of processing.
	18.1.7	Changed to: Do NOT transfer samples from LN2 back to -70°C or -80°C freezers unless directed to by network or protocol team.
	19	Changed to: 19.1 A completed PBMC Processing Worksheet is required for HVTN only. 19.2 Requirements for all networks: 19.2.1 Data is entered into the Laboratory Data Management System for the generation of cryovial labels, storage location documentation and shipping manifest requirements. 19.2.2 Deviations are reported according to laboratory protocol.

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	21.1	Changed to: 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.
	22.3	Deleted "Modification of the centrifuge speed..."
	23	Changed to: Clotted, Grossly = More than ¾ of the whole blood mass is clotted and there is very little free whole blood remaining.
	23	Changed to: Centrifuge Temperature = 15 to 30°C
	23	Changed to: PBS = Phosphate-buffered saline; WDR = Wash Diluent Reagent (HBSS, PBS, or RPMI; RPMI can be used for ACTG/IMPAACT only.)
	25.2	Changed to: WDR (HBSS, PBS or RPMI) Package Insert
	Appendices	Inserted Appendix B: Example NALGENE® Mr. Frosty Isopropanol Change Log and Appendix D: Pooling Buffy Coat Layers for Ficoll PBMC Isolation and re-lettered other appendices as necessary.
	Appendices	Replaced network-specific quick guides with quick guides for CSTFB tubes and Manual Overlay Method that are not specific to network.
	Appendix A	Added space to write in reagent manufacturers
	Appendix A	Changed "HBSS" to "HBSS (or other WDR)"
	Appendix A	Changed to: Calculate actual number of cells per vial N₂ = (Aliquot volume * T)/(V _f); HVTN aliquot volume is equal to 1mL.
	Appendix A	Changed to: Number of cryovials actually frozen Note: Should be equal to freeze-down re-suspension volume for 1mL aliquots.
	C.2	Renumbered
	C.2.5	Changed to: The centrifugation speed was too high. Verify that the rpm setting was correct for CSTFB 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.