



Title:	PBMC Isolation from Leukapheresis Standard Operating Procedure		
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1 Purpose

The purpose of this SOP is to provide instruction for consistent processing of leukapheresis samples to obtain a large number of peripheral blood mononuclear cells (PBMC) for use in quantifying virus persistence in subjects with low viral loads.

2 Scope

Users of the ACTG/IMPAACT Lab Manual

3 Background

One of the limits to a comprehensive and systematic evaluation of multiple lymphocyte subsets for their contribution to viral persistence are practical limits on blood volumes that can safely be collected from patients at any one time. In order to routinely isolate and quantify virus persistence, large numbers of CD4+ T cells must be obtained, because the level of replication competent virus in long-term treated patients is very small (<1 per million of CD4 cells harbor HIV).

Leukapheresis is a safe, well-tolerated, outpatient procedure that has been used in clinical practice for over 25 years whereby leukocytes, or in some instances specific cell fractions (e.g. PBMCs) are selectively harvested but red cells and other blood components are returned to the subject. In a typical 1.5-3 hour leukapheresis procedure, 3-10 x10⁹ PBMCs can be isolated with only minimal loss of red blood cells [3, 4]. In addition, the amount of plasma obtained from the leukapheresis procedure can be adjusted as required. No sedation is required. The purpose of this document is to define the procedure for PBMC isolation from leukapheresis specimens.

This SOP has been adapted from an SOP formulated by the ACTG Leukapheresis Working Group and the ACTG Reservoirs Sampling Focus Group.

4 Authority and Responsibility

- 4.1 The Network Laboratory Directors (or his/her designee) have the authority to establish, review and update this procedure.
- 4.2 The ACTG/IMPAACT Laboratory Technologist Committee (LTC) is responsible for the maintenance and control of SOP documentation.
- 4.3 The Laboratory Director is responsible for the implementation of this LTC SOP or laboratory-specific SOP and for ensuring that all appropriate personnel are trained. A laboratory SOP must:
 - 4.3.1 Include, without procedural modification, the portions of the current version of the LTC SOP that are used within the network site-affiliated laboratory
 - 4.3.2 Reference the current version of the LTC SOP
- 4.4 All laboratory technicians are responsible for reading and understanding this SOP prior to performing the procedures described.
- 4.5 The site PI and designees are responsible for understanding and adhering to the patient preparation and specimen collection components.

5 Budgetary Considerations

- 5.1 Leukapheresis
- 5.2 Subject reimbursement
- 5.3 Sample analyses (will vary with the protocol)

6 Eligibility Requirements

- 6.1 Inclusion Criteria:
 - 6.1.1 Able to give informed consent
 - 6.1.2 Palpable antecubital veins in both arms, as determined by a clinician
- 6.2 Exclusion Criteria:
 - 6.2.1 Prior history of difficulty obtaining peripheral blood through phlebotomy
 - 6.2.2 Weight <120 lbs, unless otherwise specified by protocol
 - 6.2.3 High blood pressure > 160/100
 - 6.2.4 Low blood pressure < 100/70
 - 6.2.5 Known blood coagulation disorder
 - 6.2.6 Platelets < 50,000/mm³
 - 6.2.7 PTT > 2x ULN (PTT = partial thromboplastin time; ULN = upper limit of normal)
 - 6.2.8 INR > 1.3 (INR = international normalized ratio)
 - 6.2.9 Use of aspirin, NSAIDs, Plavix®, Coumadin, or other blood thinners that cannot be stopped for clinical reasons for 5 days before the procedure and 5 days after the procedure
 - 6.2.10 Albumin < 2.0 g/dL
 - 6.2.11 ALT > 5x ULN (alanine aminotransferase)
 - 6.2.12 AST > 5x ULN (aspartate aminotransferase)
 - 6.2.13 Biopsy-proven or clinical diagnosis of cirrhosis
 - 6.2.14 Pregnancy
- 6.3 Screening Laboratory Tests

Note: If phlebotomy for these screening laboratory tests will be performed within 1 week of the leukapheresis procedure, it is best to avoid the antecubital veins if possible.

 - 6.3.1 Complete blood count (CBC)
 - 6.3.2 PT/PTT (PT = Prothrombin time)
 - 6.3.3 Complete metabolic panel
 - 6.3.4 Urine/serum pregnancy test

- 6.4 Screening Vein Check Visit: Palpable antecubital veins in both arms, as determined by a clinician

7 Staff and Equipment Requirements for Leukapheresis Collection

7.1 Required staff

One Registered Nurse (most apheresis centers also have a Medical Doctor on-site)

7.2 Required equipment

In general, it is necessary to collaborate or contract with existing clinical/research apheresis centers that have existing apheresis machines, appropriately trained and experienced clinical staff and the expertise to conduct leukapheresis.

8 Procedure: Leukapheresis

8.1 Pre-leukapheresis procedures

8.1.1 No aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), clopidogrel (e.g. Plavix®), warfarin (e.g. Coumadin), or other blood thinners for 5 days before the procedure.

8.1.2 Leukapheresis should be conducted in the morning to allow for same-day processing or same-day shipping, as required by protocol instructions. Schedule coordination must include the processing laboratory.

8.1.3 In order to optimize the leukapheresis procedure:

8.1.3.1 Instruct subjects to hydrate for 72 hours prior to the scheduled procedure, and to avoid strenuous exercise, alcohol, or caffeinated drinks for 48 hours prior to the procedure.

8.1.3.2 On the morning of the procedure, subjects may eat breakfast but are asked to limit intake of fluids (especially caffeinated drinks), in order to minimize bathroom needs during the procedure. In addition, subjects are also instructed to not take blood pressure medicines but to bring these with them to the apheresis center. If protocol requires a fasting collection, that sample should be obtained and time allowed for a breakfast prior to initiating the leukapheresis procedure.

8.1.3.3 Subjects should wear loose-fitting clothing, especially clothing with sleeves that can be raised above the elbow.

8.2 Procedure description

8.2.1 The leukapheresis procedure will take approximately 1.5-3 hours. The duration of leukapheresis and desired number of leukocytes to be recovered must be specified in the parent protocol.

8.2.2 Subjects will be seated in a reclining position for the duration of the procedure, with both arms resting comfortably on cushioned armrests.

- 8.2.3 Vital signs (temperature, heart rate, blood pressure) will be obtained prior to initiation of the procedure.
- 8.2.4 A sterile needle will be placed into a vein in one arm and remain in the arm throughout the procedure.
- 8.2.5 A sterile plastic intravenous catheter will be placed into a vein in the other arm and remain in the arm throughout the procedure.

Note: Any extra blood vacutainer tube collections that are to be obtained should be collected prior to the initiation of the leukapheresis. These specimens will need to be labeled and transported to the local processing laboratory per protocol guidelines for local processing according to the LPC instructions.

- 8.2.6 The leukapheresis staff will then begin the leukapheresis procedure under constant nursing supervision. Blood will flow from the subject through the needle in one arm, through the sterilized apheresis machine, and return back to the subject through the catheter in the other arm. Blood flow will be adjusted according to blood pressure during the procedure.
- 8.2.7 A small amount of anticoagulant (citric acid/sodium citrate) is used as blood is pumped out to prevent clotting. Citric acid/sodium citrate removes free calcium, so oral calcium supplementation will be given if the subject experiences symptoms of numbness suggesting low calcium levels. The collection unit may be rocked gently during the procedure to prevent clumping.

Note: The use of heparin as an anticoagulant should be avoided if the cells will be used for molecular testing. Approval must be obtained from the protocol team if a site's blood center only uses heparin for this procedure.

- 8.2.8 At the completion of the procedure, the needle and catheter will be removed, pressure-dressing bandages will be applied, and the subject will be checked for bleeding and stable vital signs prior to discharge.

8.3 Post-leukapheresis procedures

- 8.3.1 No aspirin, NSAIDs, Plavix, Coumadin, or other blood thinners for 5 days after the procedure.
- 8.3.2 Instruct subjects to rest, drink extra fluids, and eat well.
- 8.3.3 Instruct subjects to keep the pressure dressing bandages in place for at least 3-4 hours after the procedure, to keep the sites dry and to avoid exercise or heavy lifting for the rest of the day.

8.4 Timing of leukapheresis

Although there are no known limits to the frequency of research-related leukapheresis procedures, the leukapheresis procedure should not be performed more than six times in any given 12 month period for subjects with CD4 > 300 cells/mm³. This procedure should not be performed on subjects with lower CD4 counts more than three times in any given 12-month period [2].

9 Shipping/Transport of the Leukopak

- 9.1 The leukopak may need to be sent to a centralized processing laboratory as defined in the protocol.
 - 9.1.1 Properly label the leukopak with the patient identification (PTID), protocol, visit identification (VID) and date of collection.
 - 9.1.2 Document the collection and transport of the leukopak on the corresponding Case Report Form (CRF).
 - 9.1.3 If processing the leukopak in real-time, transport the leukopak to the processing laboratory under ambient conditions (15 to 30°C). Use an insulated container to avoid temperature extremes, include absorbent material, and comply with local specimen transport requirements.
 - 9.1.4 If preparing the leukopak, or a fraction of the leukopak for shipment to a testing laboratory, package the leukopak material using ACTG/IMPAACT guidelines for shipping an ambient package under packing instructions 650 for Biological Substances, Category B (UN3373).
<https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx>
 - 9.1.4.1 Place the labeled leukopak in a zip-lock or sealable specimen bag with sufficient absorbent material to contain the specimen if it leaked during transit. Alternately, transfer a portion of the leukopak to a 50mL conical tube and seal the lid with parafilm or tape to prevent uncapping during shipment; place each 50mL conical tube in a sealable bag with padding to maintain separation and sufficient absorbent material to contain the specimen if leaked during transit. Roll the bag to minimize trapped air and seal closed.
 - 9.1.4.2 Place each specimen bag into a Tyvek® pouch or other equivalent secondary pressure vessel and include the appropriate documentation (i.e. CRF) making sure it is protected by placing in a separate zip-lock or sealable bag.
 - 9.1.4.3 It is advisable to package the Tyvek bag into an inner box and place it into a properly labeled over pack to protect the leukopak from extreme temperatures during transport. Fill the over pack with bubble wrap or other insulator to help secure the inner box. Shipping conditions (room temperature vs. cool) will vary with testing intent and will be detailed in the protocol support documents, LPC and/or MOP.
 - 9.1.4.4 Notify the receiving laboratory per ACTG/IMPAACT guidelines. Ship the leukopak by priority overnight courier. Track the shipment to ensure proper delivery of the specimen.

10 Requirements

- 10.1 Specimen Type
 - Anticoagulated leukapheresis product (leukopak).

10.2 Optimum/Minimum Specimen Volume

Required leukopak volume determined by protocol.

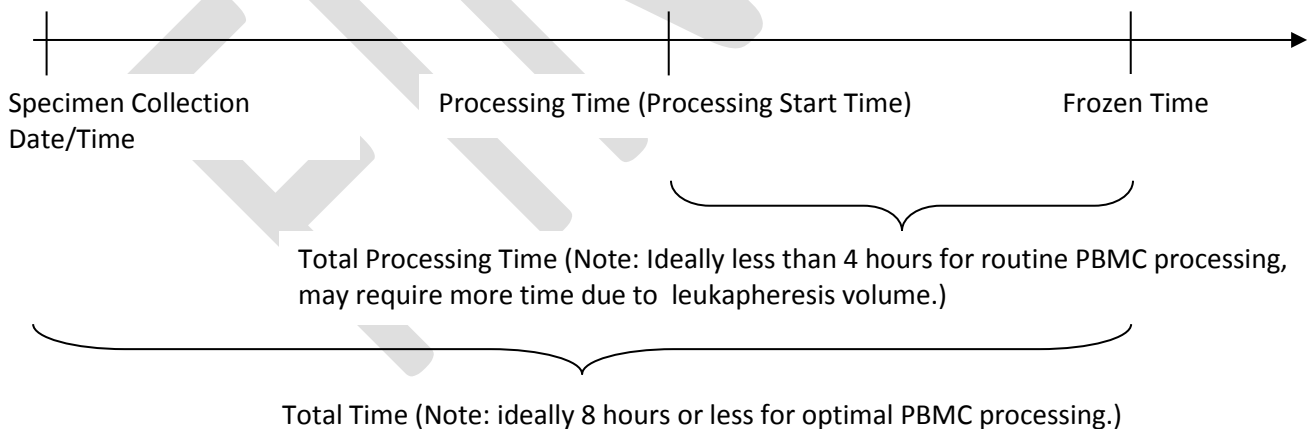
10.3 Handling Conditions

10.3.1 Fresh, anticoagulated leukopaks should be stored at room temperature (15 to 30°C) from the time of collection until delivery to the laboratory/processing unit. Leukopaks being sent to a testing laboratory via overnight shipping must be packaged properly to help maintain constant temperature conditions during transit (follow the instructions provided in the protocol support documents, LPC and/or MOP as the required conditions will vary with the analyte). The use of temperature monitors is recommended to record shipping conditions.

10.3.2 Fresh, anticoagulated leukopak specimens should be delivered to the laboratory-processing unit as early in the day as possible to allow the processing laboratory ample time to complete the cryopreservation procedures.

10.3.3 Fresh, anticoagulated leukopak specimens should be processed by the laboratory processing unit as soon as possible upon receipt:

- Processing Time (processing start time) is the time when the leukopak is first opened.
- Frozen Time is defined as the time when the StrataCooler® Cryo, NALGENE® Mr. Frosty or biocision® CoolCell is put into the -80°C freezer.
- Total Time is calculated from Specimen Collection Time and Frozen Time.
- Total Processing Time is calculated from the Processing Time and the Frozen Time



10.3.4 Do NOT freeze leukopak product. The leukopak must be at room temperature (15 - 30°C) for PBMC processing. Leukopaks may be refrigerated if required by the protocol to improve analyte stability but should be warmed to room temperature before processing.

10.4 Marginal Specimens

10.4.1 Document all unexpected specimen conditions and actions taken according to network and laboratory requirements. See Section 18 for details.

10.4.2 Clotted specimens

- 10.4.2.1 All blood should be processed regardless of whether or not it contains clots, unless otherwise directed by protocol.
- 10.4.2.2 Remove the clot and process as usual.
- 10.4.2.3 If the cell yield is insufficient to meet the needs of the protocol, contact the clinic and protocol team as soon as possible.

10.5 Unacceptable Specimens

- 10.5.1 Unlabeled or mislabeled leukopak must be reported immediately to the clinic and protocol team to determine the appropriate course of action.
- 10.5.2 Leaking specimens
Notify the clinic and protocol team if any of the leukopaks are leaking and determine whether or not the specimens are usable.

11 Equipment

11.1 Preparation & Processing

- 11.1.1 Class II biosafety cabinet (BSC) as set up by laboratory (BSL2, BSL2.5 or BSL3)
- 11.1.2 Centrifuge, low-speed (capable of 300 to 1000 x g), with swinging bucket rotor and sufficient capacity for twenty 50mL conical centrifuge tubes. A refrigerated unit may be used to create ambient temperatures (15 - 30°C) if laboratory conditions are extreme
- 11.1.3 Micropipettes, range 20, 200, 1000µL
- 11.1.4 Pipet-Aid (cordless preferred) for disposable, serological pipets
- 11.1.5 2 to 8°C refrigerator
- 11.1.6 -20°C (or lower) freezer without automatic defrost (for FBS storage)
- 11.1.7 -80°C freezer (-65 to -95°C); for short-term PBMC storage
- 11.1.8 -150°C mechanical freezer (if LN2 storage tank is not available for long-term storage)
- 11.1.9 Ice water bath (optional for preparation of CPS)
- 11.1.10 Bucket or beaker for bleach or other disinfectant, for rinsing pipets if required by local safety practice
- 11.1.11 Scissors (wrapped and sterilized or clean and sprayed with alcohol prior to use)

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

- 11.2.1 LN2 storage tank (\leq -140°C)

11.3 Cell Counting (select one of following options)

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

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

Note: If an automated cell counter that is not capable of distinguishing viable cells is used, viability must be determined with a manual cell counting chamber.

- 11.3.3 Manual cell counting chamber (hemacytometer, disposable or re-useable) and light-field microscope. Note: non-disposable hemacytometers must be used with hemacytometer grade coverslips to ensure accurate volume in the counting chamber. Regular coverslips are not adequate for this function.

11.4 Cryopreservation

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

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

- 11.4.1 Make sure sufficient capacity is available to accommodate the large number of aliquots that will be generated.

$$\text{Estimated number of aliquots} = N_c / (C_c \times V_a)$$

N_c = expected number of cryopreserved cells, as specified by the protocol, plus an additional 10%

C_c = expected concentration of cryopreserved cells (cell/mL), as specified by the protocol

V_a = the aliquot volume (mL) as specified by the protocol

Example: If the protocol expects 1×10^9 cryopreserved PBMC at 10×10^6 cells/mL in 1.0 mL aliquots, the estimated number of aliquots is $(1.1 \times 10^9 \text{ cells}) / (10 \times 10^6 \text{ cells/mL} \times 1.0 \text{ mL})$, or approximately 110 aliquots. Note that the LDMS accommodates a maximum of 99 aliquots per primary. If more than 99 aliquots are necessary to meet protocol expectations, multiple primaries will need to be defined in the LDMS for the same leukopak.

- 11.4.2 Stratagene StrataCooler® Cryo

The StrataCooler® Cryo unit must be pre-chilled to 2 to 8°C overnight (16 to 18 hours) as recommended by the manufacturer prior to using for cryopreservation. Do not place cryovials in a StrataCooler® Cryo that is below an initial temperature of 2°C.

- 11.4.3 BioCision® CoolCell

Make sure that all parts of the CoolCell, including the central ring, return to room temperature between uses. The larger capacity CoolCell FTS30 units hold up to 30 cryovials. Make sure when using these units that the central vent ports and pathway are not blocked; do not stack FTS30 units on top of each other in the freezer.

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

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

The isopropanol level must be correct and the isopropanol must be completely replaced after the fifth freeze-thaw cycle. A labeling and log system must be used to track freeze/thaw cycles and reagent changes. An example log can be found in the appendices of the Cross-Network PBMC Processing SOP at <https://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx>.

While Mr. Frosty units are acceptable, they add to the complexity of the processing because they hold so few aliquots and require tracking of the freeze/thaw cycles.

12 Disposables

12.1 Plastics

12.1.1 Serological pipets, disposable, 1, 5, 10, 25, 50mL, sterile

12.1.2 Precision pipet tips, 20, 100, 200, 1000 μ L, sterile

12.1.3 50mL disposable centrifuge tubes, sterile, conical bottom, graduated polypropylene

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

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

12.1.5 Sterile disposable bottles (250mL and 500mL) or flasks, 45mm neck, 175cm² for pooling and diluting leukapheresis specimen and/or making cryopreservation solution (CPS). (See Appendix A for bottle examples.)

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

12.2 Markers

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

12.3 Labels

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

13 Personal Protective Equipment

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

13.1 Laboratory coat

13.2 Eye protection

13.3 Non-powdered, nitrile or equivalent gloves

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

14 Reagents

- 14.1 The use of sterile reagents and aseptic technique are required.
 - 14.1.1 Store opened bottles at the temperature recommended by the manufacturer until used or until manufacturer's expiration date.
 - 14.1.2 Discard if visible signs of contamination, such as a cloudy appearance, develop.
- 14.2 1.077g/ml Density Gradient Media (DGM)
- 14.3 Wash Diluent Reagents (WDR)
 - Hanks' Balanced Salt Solution (HBSS*) without calcium or magnesium, ready-to-use.
 - *Alternative: 1X Phosphate-Buffered Saline (PBS) without calcium or magnesium, ready-to-use or RPMI Medium without FBS or antibiotics.
- 14.4 Freezing Reagents
 - 14.4.1 Heat-Inactivated Fetal Bovine Serum (HI-FBS),
 - 14.4.1.1 An Immunology Quality Assessment (IQA) approved lot must be used. Ordering information is posted at <https://www.hanc.info/labs/labresources/procedures/Pages/ActgImpactFbsOrdering.aspx>
 - 14.4.1.2 Obtain a certificate of analysis from the vendor for local laboratory quality control records.
 - Note:* A copy of the FBS certificate of analysis may be required to export (or import) PBMC aliquots between countries.
 - 14.4.2 Dimethylsulfoxide (DMSO), cell-culture grade
 - 14.4.2.1 Use cell-culture grade DMSO. See Appendix A for examples that meet these requirements.
 - 14.4.2.2 Store unopened bottles at room temperature (15 to 30°C). Check bottle for expiration date and discard if expired.
 - 14.4.2.3 After opening, undiluted DMSO is stable at room temperature (15 to 30°C) for 6 months when protected from light and moisture.
 - 14.4.2.4 Use aseptic technique when removing DMSO from the bottle to avoid possible contamination.
 - 14.4.2.5 Discard open bottle if visible signs of contamination are noted.
 - 14.4.2.6 Reagent may be aliquoted in small amounts to help preserve sterility. Label aliquots with "DMSO," the date opened/aliquoted, the expiration date (six months from opening) and tech initials. Protect aliquots from light.
 - 14.4.3 Disinfectant
 - 14.4.3.1 70% v/v ethanol disinfectant, spray bottle
 - 14.4.3.2 10% v/v bleach, bucket or beaker and spray bottle

14.4.3.3 Other disinfectant as specified by local laboratory policy

14.5 Cell Counting Reagents

14.5.1 0.4% trypan blue solution

14.5.2 *Optional:* 0.05% crystal violet solution can be used to stain the cell nucleus so mononuclear cells can be identified and counted using a hemacytometer. If crystal violet is used, a second manual count using trypan blue must be performed.

0.05% Crystal Violet Solution:

0.05-g crystal violet

2mL glacial acetic acid

98mL distilled or deionized H₂O

15 Reagent Preparation

15.1 Heat-Inactivated FBS (HI-FBS)

15.1.1 To thaw, aliquot and use:

15.1.1.1 Remove the HI-FBS from the freezer.

15.1.1.2 Thaw in the refrigerator (2 to 8°C) overnight, 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.

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

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

15.1.1.5 Aliquot into sterile, labeled 50mL conical centrifuge tubes, or other aliquot sizes as appropriate for the anticipated workload.

15.1.1.6 Label each tube as "HI-FBS" and include the lot number, the aliquot date, the expiration date, and the technician's initials.

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

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

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. Discard any FBS that has visual signs of contamination.

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

15.1.3 The volume of FBS that will be needed will depend on the number of cryopreserved PBMCs (N_c) and the final cell concentration (C_c) required by the protocol.

15.1.3.1 Refer to the protocol or LPC to determine the expected numbers of PBMC for cryopreservation (N_c). Plan to process an additional 10%.

Number of cells to be cryopreserved (N_c) = protocol expectations + 10%

15.1.3.2 Refer to the protocol or LPC to determine the final cell concentration for cryopreservation (C_c).

15.1.3.3 Determine the volume of FBS (V_{FBS}) needed using the expected number of PBMC for cryopreservation (N_c) and the final cell concentration (C_c) plus approximately 10%.

$$V_{FBS} = N_c / C_c$$

Note: Cryopreservation solution (CPS) is only 90% FBS, so this formula will yield the extra FBS needed.

Example: If the protocol expects 1×10^9 cryopreserved PBMC at 10×10^6 cells/mL, $V_{FBS} = (1.1 \times 10^9 \text{ cells}) / (10 \times 10^6 \text{ cells/mL}) = 110\text{mL}$.

15.2 Fresh Cryopreservation Solution (CPS)

Note: CPS is a mixture of FBS and DMSO. When prepared, an exothermic reaction occurs. Therefore, it is important to prepare the CPS and equilibrate to 2 to 8°C before adding it to the cells.

15.2.1 Determine the volume of DMSO (V_{DMSO}) that will be needed.

$$V_{DMSO} = V_{FBS} / 9, \text{ rounded to the nearest } 0.1 \text{ mL}$$

Example: If $V_{FBS} = 110 \text{ mL}$, $V_{DMSO} = 110 / 9 = 12.2 \text{ mL}$.

15.2.2 Combine the DMSO and FBS in a sterile container and mix well.

15.2.3 Equilibrate to 2 to 8°C before adding the CPS to the cells.

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

16 Calibration

16.1 Centrifuges and pipettors must have current acceptable calibration status.

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

17 Quality Control

17.1 Cell Yields

17.1.1 The number of cells obtained from a leukapheresis procedure will depend upon vein size, blood pressure, duration of the procedure, and CD4+ T cell count.

17.1.2 For example, Wong and colleagues at the University of California San Diego performed 5 leukapheresis procedures in a pilot study of 4 HIV-infected subjects

who had suppression of plasma viremia for 2 to 5 years and CD4+ T-cell counts ranging from 400 to 900cells/mm³. The procedures were well tolerated. Subjects experienced mild, transient (<20%) reductions in total white blood cell (WBC) count post-leukapheresis and no significant alteration in hemoglobin. No subject required premature termination of the procedure and no adverse events were encountered. The lymphocyte yields approached or exceeded all the lymphocytes calculated to be present in the entire circulating blood volume for these subjects: in cells/mm³, Patient A: 4.8 x 10⁹; Patient B1: 1.2 x 10¹⁰; Patient C: 1.3 x 10¹⁰; Patient D: 3.8 x 10¹⁰; Patient B2: 1.4 x 10¹⁰. Surprisingly, despite yields of such magnitude that suggested mobilization of cells from lymphoid, bone marrow or thymic sources of lymphocytes, phenotypic analysis by flow cytometry showed very little difference between lymphocyte populations in whole blood before leukapheresis, the leukapheresis product and post-leukapheresis blood based on expression of T-cell markers (CD4, CD8, CD3), memory, naive and tissue homing subset markers (CD45Ro, CD45Ra, CCR7, CD27, CD103), or proliferation, activation and co-stimulatory markers (62L, HLA-DR, CD25, CD38 or CD28). This observation suggests that, at least in treated subjects with prolonged suppression of viremia, a large pool of lymphocytes with phenotypic characteristics nearly identical to circulating lymphocytes can be instantaneously translocated into peripheral blood [2].

17.2 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. Calculate and record the % viable cells according to laboratory and network requirements.

17.2.1 Freshly isolated PBMC viability should be >95%.

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

18 Reporting Results

18.1 Use of a “PBMC Isolation from Leukapheresis – Processing Record” and the Laboratory Data Management System (LDMS) is required to track the timing of processing, calculations and document problems that arise during processing.

18.1.1 Enter data into the LDMS for the generation of cryovial labels, storage location documentation and shipping manifest requirements. See the table below for requirement details.

18.1.2 Report deviations according to laboratory protocol.

18.1.3 The laboratory may use the “PBMC Isolation from Leukapheresis – Processing Record” provided in Appendix B, or the Leukapheresis Processing Worksheet (Excel) (example in Appendix C), or a modification appropriate for the laboratory’s procedures. If the laboratory chooses to develop its own document and supplementary tracking materials (such as the LDMS, or a separate worksheet or log) the laboratory will comply with the guidelines below.

18.1.4 Electronic and editable versions of these documents are provided at <https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx> for download and utilization or modification.

Guidelines for Documentation of PBMC Processing		
Field	Documentation Requirements*	LDMS Requirements**
Specimen Processing Laboratory	W	[Automatic]
Participant ID	W	L
Visit Number	W	L
Protocol	W	L
LDMS Specimen Number	W	[Automatic]
Processing Start Date/Time	W	N
Processed By (Tech)	W	N
Counting Method (name of instrument or manual count)	W	
Harvested cell concentration (C_h)	W	
Total cell number (N)	W	N
CPS re-suspension volume for each whole batch (V_{fw})	W	
CPS re-suspension volume for each partial batch (V_{fp})	W	
Freezing Date and Time	W	N
Comments and Protocol Deviations, including but not limited to: 1. All unexpected specimen conditions 2. Blood is clotted 3. Cell yield below expected range 4. Processing anomalies 5. Troubleshooting steps taken 6. Note if Total Processing Time >8 hours	W	O
Collection Date/Time	S	L
Number of cells to be tested real-time (N_r)	S	
Number of cells to be cryopreserved (N_c)	S	
Aliquot volume of cryopreserved cells (V_a)	S	
Final concentration of cryopreserved cells (C_c)	S	
Reagents (Manufacturers, Lot Numbers and Expiration Dates for DMSO, FBS, WDR, density gradient media)	S	
CPS (Volume of DMSO and FBS)	S	
Leukapheresis product volume (primary volume)	S	L
Cell Counts	S	
Volume of harvested cells to be tested real-time (V_r)	S	
Volume of harvested cells to be cryopreserved (V_c)	S	
Number of aliquots per whole batch (B)	S	
Number of whole batches (N_{Bw})	S	
Number of partial batches (N_{Bp})	S	
Volume of harvested cells to distribute to each whole batch tube for final centrifugation (V_w)	S	
Volume of harvested cells to distribute to partial batch tube for final centrifugation (V_p)	S	

Guidelines for Documentation of PBMC Processing		
Field	Documentation Requirements*	LDMS Requirements**
Number of cells per cryovial	S	L
Number of cryovials frozen	S	L
Freezer storage information (LDMS Storage Module)	O	N
Confirmation of visual QC of reagents (Tech)	O	
Cell yield/mL of leukapheresis product	O	
Confirmation of LDMS Label QC for content/barcodes (Tech)	O	
Confirmation of cryovial transfer to storage box locations assigned by LDMS (Tech)	O	
Date/time cryovials were transferred from slow-rate cooling vessel to storage box.	O	
Final Review Reviewer/Date	O	

* W = Tracking on a PBMC Isolation from Leukapheresis-Processing Record or equivalent worksheet is required.
 S = Tracking is required, but supplementary tracking materials (such as the LDMS or another worksheet or log) may be used
 O = Tracking on a worksheet or supplementary tracking material is optional
 ** L = Tracking in the LDMS is required by the LDMS
 N = Tracking in the LDMS is required by the networks
 O = Tracking in the LDMS is optional

19 Procedure: PBMC Isolation from Leukapheresis using Manual Density Gradient Media Overlay/Underlay Method

19.1 Review protocol requirements for final cell yield, concentration and aliquot volume.

19.1.1 Refer to the protocol to determine the expected numbers of PBMC for cryopreservation (N_c) and for real-time testing (N_r). Plan to process an additional 10%.

Number of cells to be cryopreserved (N_c) = protocol expectations + 10%

Number of cells to be tested real-time (N_r) = protocol expectations + 10%

Example: If the protocol expects 2.0×10^9 cryopreserved cells and 20×10^6 fresh cells,

$N_c = 2.2 \times 10^9$ cells and $N_r = 20 \times 10^6$ cells.

19.1.2 Refer to the protocol to determine the final cell concentration for cryopreservation (C_c).

19.1.3 Refer to the protocol to determine the final aliquot volume for cryopreservation (V_a).

19.2 Separation of lymphocytes from Leukopak using Manual Density Gradient Media (DGM) Overlay/Underlay Method

19.2.1 Perform all pouring, pipetting and mixing in a biological safety cabinet (BSC), in a BSL-2 or greater facility.

19.2.2 Use sterile reagents and work aseptically.

- 19.2.3 Spray down all surfaces, racks, and reagent bottles with 70% v/v ethanol prior to entering and using the BSC.
- 19.2.4 Unless otherwise noted, the procedure is carried out at room temperature (15 to 30°C).
- 19.3 Prepare leukapheresis product samples, reagents, and supplies. Advance preparation is strongly recommended to facilitate timely processing of the leukopak. Several steps can be completed prior to receipt of the leukopak in the processing laboratory. Once the leukapheresis procedure is started, the clinic should FAX a copy of the CRF providing the PID, protocol, date and start time of the collection. Based on the CRF information, the processing laboratory can log the leukapacks into the LDMS, generate the expected number of aliquot labels, affix the labels to the cryovials and QC the label process. Freeze media should be prepared and refrigerated at this time as well. DGM should have been equilibrated to room temperature and be added to the labeled 20 x 50mL conical centrifuge tubes. Verify that -80°C space is available for the anticipated step-down freezer units that will be used later in the process. If the laboratory uses Mr. Frosty units, verify the isopropanol status. Conical tubes (20) for washing the cells should also be labeled prior to receiving the leukopak.
- 19.3.1 Prior to processing or sufficiently in advance of mixing with PBMC, prepare and chill the CPS (see section 15 Reagent Preparation).
- 19.3.2 Allow the DGM to come to room temperature (15 to 30°C). See Section 14 Reagents for more information.
- 19.3.3 Prepare twenty (20) 50mL conical tubes by labeling each tube with the PTID and adding 15mL of DGM in each tube.
- 19.3.4 Based on the CRF information, log leukopak into the LDMS, generate labels, affix labels to cryovials and QC the label process.
- 19.3.5 Label twenty (20) new sterile conical 50 mL centrifuge tubes with the PTID. These tubes will be used for Wash 1 (section 20.1).
- 19.3.6 Set up BSC and work areas with needed supplies to initiate the processing.
- 19.4 Upon receipt, carefully check the PTID and other information on the leukopak and accompanying paperwork. It is not advisable to process more than one leukopak at a time. If the leukopak is cold to the touch (due to cold ambient conditions such as transport in cooler months), allow it to reach room temperature (15 to 30°C) before processing.
- 19.4.1 Spray the leukapheresis pouch and scissors with 70% ethanol. Hold the pouch in one hand, and cut one of the tubing ports with the clean scissors.
- 19.4.2 Pour the blood through the tubing into a sterile flask or bottle. Determine and record the approximate blood volume (typically 150 – 200mL).
- Note:* If the leukopak needs to be aliquoted to permit shipping to a testing laboratory, pour the leukopak into the sterile flask or bottle and measure and transfer half into labeled 50mL conical tubes. Secure the lids on the 50mL conical tubes with parafilm or tape to prevent uncapping during transit.

- 19.5 Leukopak Dilution and Manual Density Gradient Cell Separation: See flow diagram in Appendix D.
- 19.5.1 Add sufficient Wash Diluent Reagent (WDR) to dilute the cells to 600mL total. Mix the blood well with a serologic pipet 3-4 times; avoid creating bubbles.
- 19.5.2 Carefully and slowly pipet (overlay) 30mL of the diluted leukopak on top of each tube containing density gradient medium. Cap each tube after the diluted leukopak is added.
- Suggestion:* Gently allow the WDR-diluted leukopak mixture to flow down the side of the tube and pool on top of the density gradient media surface without breaking surface plane. Tilting the conical tube to approximately 45° angle often helps this process.
- 19.6 Lymphocyte density centrifugation and collection
- 19.6.1 Hold the tubes in an upright position and gently transfer them to the centrifuge.
- 19.6.2 Centrifuge at 400 x g for 30 minutes at 15 to 30°C with the Brake OFF, or as outlined in the package insert that accompanies the gradient medium.
- Note:* Leaving the brake is on will disrupt the layers. The centrifuge brake must be turned OFF to maintain a sharp interface between the gradient media and the PBMC layer. This will maximize retrieval of the PBMCs with minimal contamination by unwanted cell species (i.e., granulocytes).
- Note:* Refer to Section 20 in the Cross-Network PBMC Processing SOP for calculations to convert g to rpm for your rotor length.
- 19.6.3 This is a good opportunity to take a short break and to verify that you have all of the supplies pulled for the rest of the procedure.
- 19.6.4 Carefully remove the tubes from the centrifuge.
- If the cell layers are not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuge the tube(s). Document the problem and actions taken according to network and laboratory requirements.
- Note:* If the “plasma”-WDR is very cloudy, it may be difficult to see the interface with the density gradient medium. It is possible to improve the collection of lymphocytes by removing most of the liquid above the interface with a 10mL pipet, leaving only a few cm remaining. This allows for better positioning of the tip of the pipet for collection of cells.
- 19.6.5 Inspect the tubes for small clots visible at the cell interface that were not previously noted and document them. Record your observations in LDMS or other documentation.
- 19.6.6 Use a sterile pipet (serological or transfer pipet), to remove the upper, “plasma”-WDR fraction in each tube down to within (above) approximately 2 cm of the cloudy white PBMC band located at the interface between the “plasma”-WDR 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.

- 19.6.7 Use a sterile serological or transfer pipet to collect all cells at the cloudy white interface. Take care not to aspirate any more separation medium solution than necessary. Do NOT scrape cells that are adhering to the plastic tube; these adherent cells are generally not PBMCs.
- 19.6.8 Transfer the collected cells from one conical gradient tube into one 50 mL conical wash tube. Repeat these steps for the remaining tubes.
- 19.6.9 After removing the cell layer, re-cap the conical centrifuge tubes containing the residual DGM and discard according to laboratory policy.
- 19.7 Make sure all appropriate information is documented according to network and laboratory requirements (See Section 18, Reporting Results).

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

- 20.1 Wash 1:
 - 20.1.1 Q.S. each 50 mL centrifuge tube containing the PBMC fraction to approximately 45mL by adding WDR. Mix gently to resuspend the cells.
 - 20.1.2 Re-cap all of the harvested cell tubes and place the 20 tubes in the centrifuge.
 - 20.1.3 Centrifuge the 20 tubes containing diluted cells at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).
 - 20.1.4 Remove the 20 tubes from the centrifuge and check for the cell pellets.
 - 20.1.5 If the cell pellets are not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuge the tubes. Document the problem and actions taken according to network and laboratory requirements. If the cell pellets are still not visible after re-centrifuging the tube, document.
 - 20.1.6 Carefully remove and discard the supernatants without disturbing the cell pellets. Note: these pellets will be quite large and relatively “loose” compared to routine PBMC pellets.
- 20.2 Wash 2:
 - 20.2.1 Resuspend each pellet in 5mL of WDR, mixing gently but thoroughly, into a homogenous cell suspension.
 - 20.2.2 Combine the pellet suspensions from four (4) 50mL conical tubes into one tube. Rinse the 4 “empty” tubes with 5 mL WDR and transfer to the collective tube. Discard the empty tubes after this rinse. This step condenses the cells from 20 tubes to five (5) tubes.
 - 20.2.3 In each 50 mL centrifuge tube, QS the PBMC fraction to approximately 45mL by adding WDR and mix gently.

20.2.4 Re-cap the tubes and place the five (5) tubes in the centrifuge. Balance the centrifuge with dummy tube.

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

20.2.6 Remove the tubes from the centrifuge and check for the cell pellets.

Note: If the cell pellets are not visible, confirm that the centrifuge is operating properly. Correct any problems and re-centrifuge the tubes. Document the problem and actions taken according to network and laboratory requirements. If the cell pellet is still not visible after re-centrifuging the tubes, document and proceed.

20.2.7 Remove and discard the supernatants without disturbing the cell pellets.

20.2.8 Resuspend each of the 5 cell pellets in 10mL of WDR and combine into one sterile 175cm² flask or sterile 250 or 500mL bottle. Rinse each of the 50 mL conical tubes with 10 mL WDR and transfer to the collection flask or bottle.

20.2.9 Q.S. to 200mL with WDR and mix gently. It is important to be as accurate as possible because the cell count will be based on a resuspension volume of 200mL. This is the harvested cell flask or bottle.

20.3 PBMC Cell Count

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

20.3.2 Transfer a small volume (<100 µL) of the re-suspension to a small tube for counting.

Note: It is likely this aliquot will require an additional 1:100 dilution if performing manual cell counts. If repeated counts are necessary, minimize the sampling volume needed. If applicable, document any additional dilution volumes used for counting the cells.

20.3.3 Follow the SOP for the cell counting method approved at the processing laboratory to determine the total cell number (N) and the harvested cell concentration (C_h). Viability counts are required.

Note: Cells at 10³/µL = cells at 10⁶/mL.

Note: Automated counts may be run once, though duplicate counts are preferred. Manual counts using a hemacytometer should count the four large corner squares (1mm²).

Note: The cell suspension is very concentrated. A serial dilution may be required to bring the suspension into a countable range. Be sure to document the dilutions and multiply the cell counts by the appropriate dilution factor.

Example manual counting procedure using a microtiter plate or microcentrifuge tubes for dilutions:

Note: Be sure to keep dilution ratios comparable to this example

20.3.3.1 Add 160µL of WDR each to 2 tubes/wells and 50µL of trypan blue in a third tube/well.

- 20.3.3.2 Transfer 40 μL of the cell suspension to the first tube/well with 160 μL of WDR. Mix well.
- 20.3.3.3 Transfer 40 μL of this cell suspension to the second tube/well with 160 μL of WDR. Mix well.
- 20.3.3.4 Transfer 50 μL of this diluted suspension to the tube/well with 50 μL of 0.4% trypan blue. Mix well. Within 2 to 3 minutes, immediately proceed to the next step.
- 20.3.3.5 Transfer enough of the cell/trypan blue suspension to completely fill the hemacytometer counting chamber. Fill by capillary action; do not over or under fill the chamber. Count cells in the 4 corner squares using a light field microscope and determine the average (= n). Note: cell counts should range between 50 and 200 per large square and be consistent (+/- 20%). If inconsistent or out of range, repeat starting with step 20.3.3.2.
- 20.3.3.6 Total number of cells (N) = average count/square x dilution factor x 10^4 x number of mLs of suspension
 $n \times 5 \times 5$ (dilution WDR) x 2 (dilution trypan blue) x 10,000 (hemacytometer factor) x 200 mL (total volume) i.e. $N = n \times 10^8$
Example: $n = 89 \Rightarrow N = 89 \times 5 \times 5 \times 2 \times 10,000 \times 200 = 89 \times 10^8 = 8.9 \times 10^9$ PBMCs.

20.3.4 Determine the total number of cells (N) and the percent viability.

20.3.5 Determine the concentration of the harvested cells.

$$(C_h) = N/200\text{mL.}$$

Example: If $N = 8.9 \times 10^9$ cells, $C_h = 8.9 \times 10^9/200 = 44.5 \times 10^6$ cells/mL.

Determine the volume of cells currently in the flask that will be tested in real-time (V_r) and cryopreserved (V_c).

$$V_r = N_r/C_h$$

$$V_c = N_c/C_h$$

Example: If $C_h = 44.5 \times 10^6$ cells/mL, $N_r = 5.5 \times 10^6$ cells and $N_c = 1.1 \times 10^9$ cells, then $V_r = (5.5 \times 10^6)/(44.5 \times 10^6) = .12\text{mL}$. $V_c = (1.1 \times 10^9)/(44.5 \times 10^6) = 24.7\text{mL}$.

Note: If N is insufficient to meet the protocol expectations for the number of fresh PBMC and the number of cryopreserved PBMC, record any deviations from the protocol expectations on the PBMC Isolation from Leukapheresis-Processing Record or equivalent documentation.

20.4 Labeling

Note: Steps 20.4.1 through 20.4.4 should be completed early in the process. Since this procedure will be scheduled in advance, calculations and preparations can be made when the sample is expected and labels printed and affixed prior to receipt of the leukopak or during the first centrifugation step.

- 20.4.1 Estimate the number of labels and cryovials that will be needed using the volume of CPS (V_{CPS}) and V_a , the aliquot size (mL) required by the protocol.

Number of labels and cryovials = V_{CPS}/V_a rounded down to the nearest whole number.

Example: If $V_{CPS} = 122.2\text{mL}$ and $A = 1.5\text{mL}$, the number of labels and cryovials needed will be $122.2/1.5 = 81.5$, rounded down to 81.

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

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

- 20.4.3 Generate cryovial labels using the LDMS.

20.4.3.1 Follow network laboratory practice for completing the data entry.

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

20.4.3.3 For all labels, visually inspect the label barcode and print area for alignment, and print quality.

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

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

- 20.5 Transfer the cells that will be tested fresh (V_r) to a separate, sterile tube of appropriate capacity. Label the tube with the PTID, N_r , V_r , and “fresh” and process according to protocol instructions.

- 20.6 Distribution of Cells for Final Centrifugation

- 20.6.1 Determine the appropriate batch size (B) for centrifuging and aliquoting the cells.

IMPORTANT: To preserve cell viability, it is necessary to minimize the time that the cells are in pellet form and in DMSO before cryopreservation. Therefore, the cells should be centrifuged and aliquoted in batches. The appropriate number of aliquots per batch will depend on the capacity of the controlled-rate freezing vessel used, with a maximum batch size of 32.

- 20.6.2 Determine the number of whole (N_{Bw}) and partial batches (N_{Bp}) that will be needed.

$$N_B = N_c / (C_c \times V_a \times B)$$

The whole number portion of N_B is N_{Bw} ; the decimal portion of N_B is N_{Bp} .

Example: If $N_c = 1.1 \times 10^9$ cells, $C_c = 10 \times 10^6$ cells/mL, $V_a = 1.5$ mL, and $B = 24$, then $N_B = 1.1 \times 10^9 / (10 \times 10^6 \times 1.5 \times 24) = 3.06$, $N_{Bw} = 3$, $N_{Bp} = .06$, or three whole batches and a partial .06 batch.

Note: If the whole batches will yield the number of cells required by the protocol plus an additional 5%, there is no need to process the partial batch.

Example: If N_{Bp} is .06 batches, and $B = 24$, the partial batch will yield only $.06 \times 24 = 1.4$ aliquots. If the protocol requires 1.0×10^9 cells there should be a total of at least 1.05×10^9 cells at 15×10^6 cells/aliquot, or 70 aliquots. The whole batches would yield 3×24 or 72 aliquots, so it would be unnecessary to process the partial batch.

20.6.3 Prepare the conical centrifuge tubes for final centrifugation. There will be one conical centrifuge tube for each whole batch and each partial batch.

20.6.3.1 For each whole batch (V_w) and each partial batch (V_p), calculate the volume of harvested cells that will be distributed to each conical centrifuge tube for final centrifugation.

$$V_w = V_a \times C_c \times B / C_h, \text{ rounded to the nearest } 0.1 \text{ mL}$$

Example: If $V_a = 1.5\text{mL}$, $C_c = 10 \times 10^6$ cells/mL, $B = 24$ and $C_h = 44.5 \times 10^6$ mL, $V_w = 1.5 \times 10 \times 10^6 \times 24 / (44.5 \times 10^6) = 8.08$ mL, rounded to 8.1mL.

$$V_p = N_{Bp} \times V_w, \text{ rounded to the nearest } 0.1 \text{ mL}$$

Example: If $V_w = 8.1\text{mL}$ and $N_{Bp} = .73$, $V_p = .73 \times 8.1 = 5.91$ mL, rounded to 5.9 mL.

Note: The maximum capacity of each conical centrifuge tube is 45mL. If the calculated V_w or V_p is greater than 45mL, then distribute the cells among two or more conical centrifuge tubes such that all the cells in a given batch can be processed at the same time.

20.6.3.2 Determine the final resuspension volume of each whole batch tube (V_{fw}) and each partial batch tube (V_{fp}).

$$V_{fw} = V_a \times B$$

Example: If $V_a = 1.5\text{mL}$ and $B = 24$, then $V_{fw} = 1.5 \times 24 = 36$ mL.

$$V_{pw} = N_{Bp} \times V_{fw}, \text{ rounded to the nearest } 0.1 \text{ mL}$$

Example: If $N_{Bp} = .73$ and $V_{fw} = 36\text{mL}$, then $V_{fp} = .73 \times 36 = 26.28\text{mL}$, or 26.3mL.

20.6.3.3 Label each conical centrifuge tube with the PTID, the volume of harvested cells that will be distributed to the tube (V_w/V_p), and the final resuspension volume (V_{fw}/V_{fp}).

20.6.4 Gently mix the flask of harvested cells prior to each distribution.

20.6.5 Distribute the harvested cells among the prepared conical centrifuge tubes as was determined in 20.6.3.1 (V_w/V_p). Carefully cap the tubes.

20.6.6 Set aside the flask with the remaining cells, in case more aliquots need to be cryopreserved to meet protocol expectations.

20.7 Final Centrifugation, Resuspension in CPS, Aliquoting for Cryopreservation, and Overnight Controlled-Rate Freezing

Note: While one batch of cells is being resuspended in CPS, aliquoted, and transferred to the controlled-rate freezing vessel, the next batch of cells can be in the centrifuge. Work *quickly* once the CPS has been added. Do not allow the cells to be in contact with DMSO for longer than 10 minutes before placing in the freezer. Do not allow cells to sit in the centrifuge once spinning has stopped. For each batch of cells, follow 20.7.1 through 20.7.6.

20.7.1 Place the conical centrifuge tube(s) containing one batch of cells in the centrifuge and spin at 200 to 400 x g for 10 minutes at 15 to 30°C (brake optional).

20.7.2 Verify that all cryovials are labeled and easily accessible.

Note: Pre-chilling vials and/or working on wet ice are allowed. If working on wet ice, make sure the vial caps are above the ice/water level. Don't allow moisture near the caps of the vials. Disinfect exterior of cryovials with 70% ethanol if the vials become submerged.

20.7.3 Resuspension in CPS

20.7.3.1 Remove and discard the WDR supernatant. Keep the pellet(s).

20.7.3.2 *Gently* resuspend the cell pellet(s) by flicking or pipetting.

20.7.3.3 *Gently* resuspend each pellet(s) in the volume of prepared CPS (V_{fw}/V_{fp}) that was determined in 20.6.3.2; add the CPS to the resuspended cells with continuous swirling.

Note: If the resuspension volume (V_{fw}/V_{fp}) is greater than 45mL, the capacity of the conical centrifuge tube will not be sufficient for resuspension. If this is the case, resuspend the cells in 20mL of CPS, transfer half of the cells to another conical centrifuge tube, and then add half of the remaining resuspension volume to each tube with continuous swirling.

20.7.4 Aliquot the PBMC into the cryovials at the aliquot volume required by the protocol (V_a) per cryovial. Discard any leftover cells according to laboratory and institutional requirements; do not create any partial aliquots.

20.7.4.1 Laboratories may opt to use an Eppendorf repeater pipettor to expedite the aliquoting of the cells into the cryovials.

20.7.4.2 Be sure to mix the cells well before refilling the repeater pipettor or in between every 10 aliquots.

20.7.5 Immediately transfer all cryovials to the controlled-rate freezing vessel.

20.7.6 Close the container and place it in a -80°C (-65 to -95°C) freezer, in a location that is not disturbed by repeated freezer access (i.e. away from the front or top of the freezer near the opening door/lid) for a minimum of 4 hours for Mr. Frosty and CoolCell or overnight for StrataCooler® Cryo.

Note: The time that the last batch is placed in the -80°C freezer is the Frozen Time. Record the Frozen Time in the LDMS and on the PBMC isolation from Leukapheresis- Processing Record or equivalent.

- 20.8 Make sure all appropriate information is documented according to network and laboratory requirements.
- 20.9 Make sure that protocol expectations for the number of cryopreserved cells have been met. If not, cryopreserve any extra cells that were set aside, if possible, or document on the PBMC Isolation from Leukapheresis-Processing Record or equivalent.
- 20.10 Discard extra cells according to laboratory and institutional requirements.

21 Onsite Storage

Requirements for onsite storage of cryopreserved PBMC depend on the network. ACTG requires temporary onsite storage in a -70/-80°C freezer with shipment to testing laboratory or repository for long term storage.

21.1 Onsite Temporary Storage in a -70/-80°C Freezer (ACTG)

- 21.1.1 The cold-chain must be maintained during all transfer steps to avoid damage to the cells.
- 21.1.2 Transfer the cryovials from the controlled-rate cooling system to the designated storage location in a -70/-80°C freezer the cryovials after a minimum of 4 hours for NALGENE® Mr. Frosty and biocision® CoolCell or overnight for StrataCooler® Cryo.

Recommendation: Use a dry ice transfer pan. Make sure the cryovial freezer box is deeply covered on all sides with dry ice. Work rapidly and efficiently to minimize cryovial exposure to ambient temperature.
- 21.1.3 Ship on dry ice within 4 weeks of cryopreservation. Maintain the cold-chain during preparation for shipping by pre-chilling the dry ice shipper and using a dry ice transfer pan during the packing steps. Make sure the dry ice shipper is completely full of dry ice.
- 21.1.4 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.
- 21.1.5 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

22 Forms

See attached: Example Leukapheresis Informed Consent Form Text (Appendix E)

23 Limitations of the Procedure

- 23.1 The optimum 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.

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

FINAL

24 Glossary of Terms

Term	Definition
<i>ACTG</i>	AIDS Clinical Trials Group
<i>ALT</i>	Alanine aminotransferase
<i>AST</i>	Aspartate aminotransferase
<i>BSC</i>	Biosafety cabinet
<i>Clotted, Grossly</i>	More than ½ of the blood mass is clotted; very little free blood
<i>CPS</i>	Cryopreservation solution
<i>CRF</i>	Case Report Form
<i>DGM</i>	Density Gradient Medium
<i>DMSO</i>	Dimethyl sulfoxide
<i>FBS</i>	Fetal Bovine Serum
<i>HBSS</i>	Hanks' Balanced Salt Solution
<i>HI-FBS</i>	Heat Inactivated Fetal Bovine Serum
<i>IMPAACT</i>	International Maternal Pediatric Adolescent AIDS Clinical Trials Group
<i>INR</i>	International normalized ratio
<i>IQA</i>	Immunology Quality Assessment
<i>LDMS</i>	Laboratory Data Management System
<i>Leukopak</i>	The product obtained from the leukapheresis
<i>LN2</i>	Liquid nitrogen
<i>NSAID</i>	Non-steroidal anti-inflammatory drug
<i>PBMC</i>	Peripheral Blood Mononuclear Cells
<i>PBS</i>	Phosphate-buffered saline
<i>PTID/PID</i>	Participant Identification Number
<i>PT</i>	Prothrombin time
<i>PTT</i>	Partial thromboplastin time
<i>QS</i>	Add sufficient quantity of liquid to bring to specified volume
<i>Room Temperature (RT)</i>	15 to 30°C
<i>ULN</i>	Upper limit of normal
<i>Vapor phase storage</i>	The space in the LN2 storage tank that is above the LN2 liquid.
<i>WDR</i>	Wash Diluent Reagent (HBSS, PBS or RPMI)

25 Literature References

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- 25.4 Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* 1997,94:13193-13197.
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<https://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx>

26 Acknowledgments

- 26.1 ACTG HIV Reservoirs Sampling Focus Group
- 26.2 ACTG Leukapheresis Working Group:
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27 Appendices

- 27.1 Appendix A: Example Reagents and Supplies
- 27.2 Appendix B: PBMC Isolation from Leukapheresis – Processing Record
- 27.3 Appendix C: Leukapheresis Processing Worksheet
- 27.4 Appendix D: Processing Flow Diagram
- 27.5 Appendix E: Example Informed Consent Form

Appendix A: Example Reagents and Supplies

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

Reagent/Supply	Example(s)
1.077g/ml Density Gradient Media	<ol style="list-style-type: none"> 1. Ficoll-Paque PLUS and PREMIUM 2. Lymphoprep™ 3. Lymphocyte Separation Media – LSM™
Dimethyl sulfoxide (DMSO), cell culture grade	Hybrimax, Sigma-Aldrich cat# D2650, endotoxin tested, hybridoma tested
Fetal Bovine Serum (FBS)	Refer to information about current IQA-validated lots at https://www.hanc.info/labs/labresources/procedures/Pages/ActgImpaactFbsOrdering.aspx
Cryovials	<ol style="list-style-type: none"> 4. Corning® 2mL external thread polypropylene cryogenic vial, self-standing with round bottom #430659 5. Nunc CryoTubes™, internal thread, polypropylene (PP) tubes and screw cap #377267 6. WHEATON Cryule® Plastic Cryogenic Vials, external thread, #985742 7. SARSTEDT Screw cap micro tube, external thread #72.694.006
Cryogenic labels	<ol style="list-style-type: none"> 8. Cryo-Tags® and Cryo-Babies® Brady B461 or B490 9. Shamrock freezer labels.
Marking pens	<ol style="list-style-type: none"> 10. Fisher brand* Marking Pens #13-379 11. Nalgene® Lab Pen/Lab Marker #6310/#6311
Disposable bottles	<ol style="list-style-type: none"> 12. Bottles <p>250 ml bottles</p> <ul style="list-style-type: none"> • Corning # 430281 • 250 ml storage bottles, plug seal cap, polystyrene, nonpyrogenic • http://catalog2.corning.com/Lifesciences/en-US/Shopping/ProductDetails.aspx?productid=430281%28Lifesciences%29&categoryname <p>500 ml bottles</p> <ul style="list-style-type: none"> • Corning #430282 • 500 ml storage bottles, plug seal cap, polystyrene, nonpyrogenic • http://catalog2.corning.com/Lifesciences/en-US/Shopping/ProductDetails.aspx?productid=430282%28Lifesciences%29&categoryname

Appendix B: PBMC Isolation from Leukapheresis – Processing Record

Specimen Processing Laboratory:

Participant ID (PTID):	Visit:	Protocol:
Collection Date:	Time:	
Processing Start Date:	Time:	Processed By:
Data to be Captured from Protocol Requirements		
Number of cells to be tested real-time (N_r) = protocol expectations + 10%	cells	
Number of cells to be cryopreserved (N_c) = protocol expectations + 10%	cells	
Final concentration of cryopreserved cells (C_c)	cells/mL	
Aliquot volume of cryopreserved cells (V_a)	mL	
Reagents/Manufacturer	Lot Number	Expiration Date
DMSO (Manuf.: _____)		
FBS (Manuf.: _____)		
HBSS or other WDR (Manuf.: _____)		
Density Gradient Media (Manuf.: _____)		
	Volume in mL	
CPS	V_{FBS}	V_{DMSO}
	V_{CPS}	1 working day
Data to be Captured During Processing		
Leukopak type (circle one)	SCI/HEP	
Leukopak condition	NORM/HEMO/CLOTTED	
Leukapheresis product volume	mL	
Counting method (name of instrument or manual count)		
Counting re-suspension volume of WDR	200mL	
Total cell number (N)	cells	
Harvested cell concentration (C_h) = $N/200\text{mL}$	cells/mL	
Volume of harvested cells to be tested real-time (V_r) = N_r/C	mL	
Volume of harvested cells to be cryopreserved (V_c) = N_c/C , rounded up to the nearest 0.1 mL	mL	
Number of aliquots per whole batch (B)	aliquots/batch	
Number of whole (N_{Bw}) batches	batches	
Number of partial (N_{Bp}) batch	partial batch	
Volume of harvested cells to distribute to each whole batch tube for final centrifugation (V_w) = $V_a \times C_c \times B/C_h$, rounded to nearest 0.1 mL	mL	
Volume of harvested cells to distribute to partial batch tube for final centrifugation (V_p) = $N_{Bp} \times V_w$, rounded to nearest 0.1 mL	mL	
Resuspension volume of each whole batch tube (V_{fw}) = $V_a \times B$	mL	
Resuspension volume of partial batch tube (V_{pw}) = $N_{Bp} \times V_{fw} \times B$, rounded to nearest 0.1 mL	mL	
Freezing Date and <u>Time</u> (Place note in comments if not within 4 hours of processing start time)		
Number of cryovials actually frozen		

Appendix B: PBMC Isolation from Leukapheresis – Processing Record

Specimen Processing Laboratory:

PTID:

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				
% viability				
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

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 vessel to storage box. (Sample must be maintained at -70/-80°C during transfer)	
Final Review	
Reviewer/date	

Comments and Protocol Deviations:

Appendix C: Leukapheresis Processing Worksheet (Excel File)

File Source: <https://www.hanc.info/labs/labresources/procedures/Pages/actgImpaactLabManual.aspx>

Following is an image of the Excel spreadsheet

Appendix C: Leukapheresis Processing Worksheet (To be used with LTC-SOP-61 v1.0)

Processing Laboratory: _____
 Participant ID (PTID): _____ Visit: _____ Protocol: _____
 Collection Date: _____ Time: _____
 Processing Start Date: _____ Processing Start Time: _____ Processed by: _____

Data Captured from Protocol Defined Requirements:

Number of cells to be tested real-time (N _t) = protocol expectations + 10%	Cells
Number of cells to be cryopreserved (N _c) = protocol expectations + 10%	Cells
Final concentration of cryopreserved cells (C _c)	Cells/mL
Aliquot volume of cryopreserved cells (V _c)	mL
Number of Aliquots expected (vials and lables)	#DIV/0!

LEGEND

- Live Entry
- Returned Value
- Locked Cell

Reagent:	Manufacturer:	Lot Number:	Expiration Date:
FBS			
DMSO			
HBSS			
FICOLL			
CPS (Cryopreservation Solution)			1 working day

Data Captured During Processing:

Leukopak type (circle one)	HEP / SCI
Leukopak condition	NORM/HEMO/CLOTTED
Leukapheresis product volume	mL
Counting method (name of instrument or manual count)	
Counting re-suspension volume of WDR	200 mL
Value from Automated or Manual Cell Count (C)	C = _____ x 10 ⁶ Cells/mL
Dilution Factor	
Total cell number (N = cell count x dilution factor ⁻¹ x 200 mL)	N = _____ x 10 ⁶ Cells
Harvested cell concentration (C _h) = N/200mL	_____ x 10 ⁶ Cells
Volume of harvested cells to be tested real-time (V _t) = N _t /C _h	V _t = #DIV/0! mL
Volume of harvested cells to be cryopreserved (V _c) = N _c /C _h [rounded up to the nearest 0.1 mL]	V _c = #DIV/0! mL
Batch Size (number of slots per cryounit) (B)	B = _____ Batch Size
Batch size according to cooler type: Stratacooler, B=32; CoolCell (large), B=30; Mr. Frosty, B=18; CoolCell (small), B=12.	
Number of Whole Batches (N _{wb} = N _c /C _h x V _c x B)	N _{wb} = #DIV/0! Whole Batches
Volume of Whole Batch (V _{wb})	V _{wb} = #DIV/0! mL
CPS Resuspension Volume per Whole Batch (V _{rbw})	V _{rbw} = 0.0 mL
Decimal Portion of Batch (N _{dp})	N _{dp} = #DIV/0!
Volume of Partial Batch (V _p)	V _p = #DIV/0! mL
CPS Resuspension Volume per Partial Batch (V _{rbp})	V _{rbp} = #DIV/0! mL
Freezing Date and Time (Place note in comments if not within 4 hours of processing start time)	
Number of cryovials actually frozen	Cryovials

PBMC Isolation from Leukapheresis

Processing Laboratory: _____
 Participant ID (PTID): _____

Hemocytometer Counting Method:

	Live	Dead	Total
Square 1 (cells/mm ²)			0
Square 2 (cells/mm ²)			0
Square 3 (cells/mm ²)			0
Square 4 (cells/mm ²)			0
Sum (sq 1+sq2+sq3+sq4)	0		0
Mean cell count (sum/4)	0		0
PBMC Dilution Factor			
Conversion Factor	1x10 ⁶ cells/mL		
Cell Concentration (b* ^a *c*d)	0.0		x 10 ⁶ Cells/mL
% Cell Viability (a/*100)	#DIV/0!		

Automated Counting Method:

Cell Count (C) as cells x 10 ⁶ /mL	
% Viability	
PBMC Dilution Factor (1/DF*)	
Cell Concentration = (C/DF)	

Transfer of Cryovials to Freezer Storage

Person who transferred cryovials to storage box location: assigned by LDMS _____

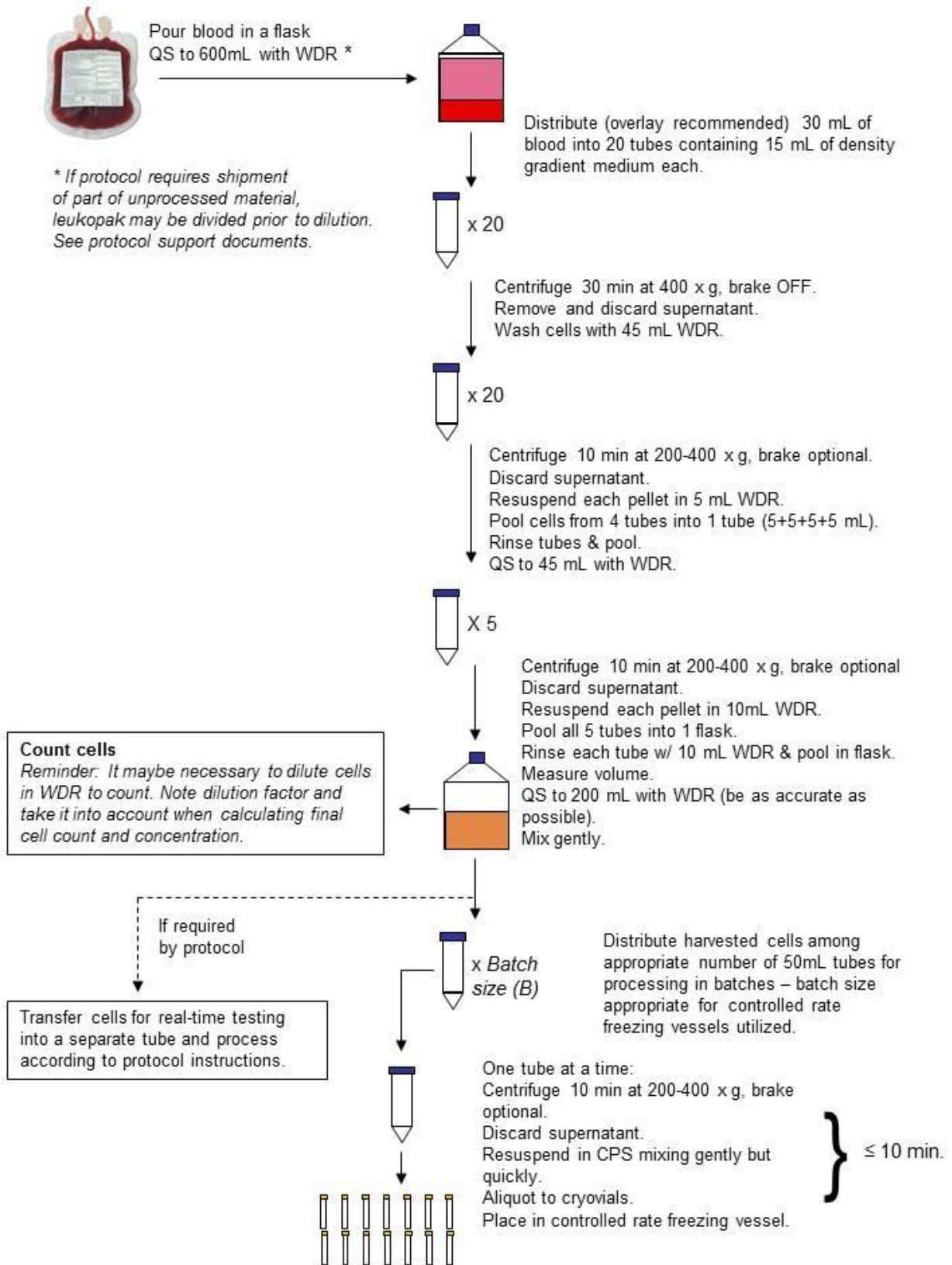
Date (ddmmyyy)time cryovials were transferred from slow-rate cooling vessel to storage box. (Sample must be maintained at -70/-80 °C during transfer) _____

Final Review

Reviewer/date _____

Comments and Protocol Deviations:

Appendix D: Processing Flow Diagram



Appendix E: Example Informed Consent Form Text

RESEARCHERS' STATEMENT

We are asking you to participate in an additional procedure for this study. The purpose of this consent form is to give you the information you will need to help you decide whether or not to participate. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When we have answered all your questions, you can decide if you want to be in the study or not. This process is called 'informed consent'. We will give you a copy of this form for your records.

PURPOSE OF THE STUDY

You are participating in a research study to learn more about [insert relevant study information here]. Leukapheresis is a procedure that allows the collection of a large volume of white blood cells. During leukapheresis white blood cells are separated from the blood and collected. White blood cells are the cells in the blood that fight infection and produce an immune response. [If you decide not to participate in this procedure, you may still participate in the main study.]

STUDY PROCEDURES

If you agree to participate in this [part of the] study, you will have a leukapheresis [insert timing and frequency of leukapheresis]. The leukapheresis procedure will be performed at [insert details of location here]. Leukapheresis involves withdrawing blood by inserting a needle attached to a small plastic tube in one arm and carrying the blood through sterile tubing to a machine called a blood cell separator. The machine spins the blood to separate it into red blood cells (cells which carry oxygen), white blood cells (cells which fight infection), platelets (pieces of a certain type of cell which help to form clots) and plasma (the fluid left after all the cells have been separated out). The white blood cells will be kept and the remaining parts of the blood (except for about 3 ½ tablespoons of plasma and a small number of red blood cells) will be recombined and returned to the body through another needle and tube in the other arm. A sterile salt solution or solution of 5% human serum albumin (a sterile solution which contains the protein albumin which is normally found in human blood) may be given to you during leukapheresis to replace the volume of plasma removed during the procedure. The time required for this visit is approximately [insert time here].

RISKS, STRESS, OR DISCOMFORT

There are risks associated with leukapheresis. The potential risks include nausea, vomiting, fainting or dizziness, bruising or swelling where the needles are put in, low blood pressure, increased pulse rate, seizures, blood loss and infection. About one half of the people who have leukapheresis feel weak or tired for the rest of the day. Rarely, albumin may cause an allergic reaction. The leukapheresis procedure might have to be stopped early and could result in the loss of as much as a 1/2 pint of blood.

During the procedure you will receive a compound called ACD-A, which prevents blood from clotting. ACD-A leaves the body in 15-30 minutes after the procedure is complete. Side effects of ACD-A include muscle cramping, tingling sensations around the mouth, chilliness, numbness or a vibrating feeling

If you notice any symptoms while undergoing leukapheresis please let the nurse know immediately since the symptoms will usually go away quickly if fluid is added or if the procedure is slowed down.

Insertion of the leukapheresis needles may cause temporary pain and a bruise may form.