Title:	Peripheral Blood Mononuclear Cell (PBMC) Thawing Standard Operating Procedure		
Origination Date:	26 Nov 2012	**Total Pages:**	9
Effective Date:	26 Nov 2012	**SOP Number**	LTC-SOP-65-v1.0
Prepared By:	ACTG/IMPAACT Lab Tech Committee	**Supersedes SOP Dated:**	04 July 2004 (PACTG PBMC Processing Cryopreservation, and Thawing Method)

	Network	Name, Title	Signature	Date
Approved By (Network):	ACTG	Robert W. Coombs, MD, PhD, FRCPC ACTG Network Laboratory Principal Investigator		15 Nov 2012
	IMPAACT	Susan Fiscus, PhD IMPAACT Network Laboratory Principal Investigator		07 Nov 2012

	Name, Title	Signature	Date
Reviewed By (Laboratory):			

	Version Effective Date (dd/mmm/yy)	Comments
Revision History		

Table of Contents

1 Purpose

The purpose of this SOP is to provide a procedure for thawing cryopreserved human peripheral blood mononuclear cells (PBMC).

2 Scope

Users of the ACTG/IMPAACT Laboratory Manual.

3 Background

Most protocols undertaken by the AIDS Clinical Trials Network and IMPAACT Network are clinical in nature, with both real-time and post-study requirements. Real-time assay results may be used to determine individual patient responses to therapies and determine when changes in protocol steps (i.e. changes in patient management) are warranted. Post study analyses are used to make recommendations for future treatment and management models for patient populations. As such, all specimens should be processed appropriately to ensure the best results for both individual patient management and completion of the collective protocol objectives.

The success of a protocol depends upon the adequate collection, processing, preservation, storage, transport, and retrieval of specimens. Guidelines for sample collection and storage need to anticipate the requirements of future studies that are yet to be designed or technological advances which are in the early stages of development. While this is not always possible, certain basic tenets exist. For example, all specimens should be collected and processed using aseptic techniques and Universal Safety Precautions. This includes the use of sterile tubes, pipette tips and reagents, and a work environment that is designed to prevent contamination of samples and provide adequate safety measures for everyone in the lab.

The proper separation, freezing and thawing of PBMCs is critical for the downstream use of cells-based assays and other applications. Without the proper thawing, the subsequent use of PBMCs in immunology and other assays may be compromised.

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 Requirements

5.1 Peripheral Blood Mononuclear Cells (PBMCs) cryopreserved.

6 Equipment, Consumables and Personal Protective Equipment (PPE)

6.1 Equipment

6.1.1 Biological safety cabinet

6.1.2 Centrifuge

6.1.3 Pipette aid

6.1.4 Single channel pipettor - 20 µL, 200 µL

6.1.5 Water bath, set at 37°C ± 2°C

6.1.6 -20°C (or lower) freezer ***without*** automatic defrost (for FBS storage)

6.2 Consumables

6.2.1 Serological pipettes, sterile - 2 mL, 5 mL, 10 mL

6.2.2 Filter flask unit, with a 0.22µm filter Sterile - 500 mL

6.2.3 Polypropylene conical tubes, sterile - 15 mL and 50 mL

6.2.4 Filtered pipette tips, sterile - 20 µL, 200 µL

6.2.5 If overnight reset of cells is required for assay: 15 mL tissue culture tubes

6.2.6 Dry ice in styrofoam box or LN2 pan

Note: Labs that have limited access to dry ice or an LN2 Pan may use a NALGENE® Mr. Frosty, Stratagene StrataCooler® Cryo, or biocision® CoolCell that has been equilibrated to -80°C according to manufacturer's instructions.

6.2.7 Lab marker

6.2.8 Bleach

6.2.9 70% isopropyl alcohol

6.2.10 Delicate task wipes (e.g. Kimwipes®)

6.3 PPE

6.3.1 Seamless gown

6.3.2 Disposable gloves (for example, latex, nitrile, vinyl or PVC)

6.3.3 Cryogenic gloves

6.3.4 Face shield

7 Reagents and Reagent Preparation

7.1 The use of sterile reagents and aseptic technique are required.

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

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

7.2 RPMI 1640 Medium without L-glutamine, store at 2- 8°C

7.3 Penicillin-Streptomycin (10,000 IU Penicillin, 10,000 µg/mL Streptomycin), store at -20°C

7.4 L-Glutamine (200 mM), store at -20°C

7.5 Heat-Inactivated Fetal Bovine Serum (HI-FBS),

7.5.1 An Immunology Quality Assessment (IQA) approved lot must be used. Ordering information is posted at https://www.hanc.info/labs/labresources/procedures/Pages/ActgImpaactFbsOrdering.aspx

7.5.2 To thaw, aliquot and use:

7.5.2.1 Remove the HI-FBS from the freezer.

7.5.2.2 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.

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

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

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

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

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

7.5.2.8 HI-FBS thawed and stored at 2 -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.

7.5.2.9 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.

7.6 ***Optional***: Benzonase®

7.6.1 Clumping of PBMC has been observed in some laboratories. Addition of Benzonase® to the thawing media has been shown to reduce clumping of PBMC.

7.6.2 Only use Benzonase®during the initial wash.

7.6.3 Benzonase®should not be used for thawing PBMCs that will later be used for PCR.

7.7 Thawing Media: RPMI 10 (Complete RPMI with HI FBS with 200 IU Penicillin, 200 µg/mL Streptomycin), and 2 mM L-Glutamine)

7.7.1 Media used to thaw cells MUST be sterile filtered.

7.7.2 Thaw reagents then combine and sterile filter in a 500mL filter flask unit.

435.0 mL of RPMI 1640 Medium

10.0 mL of 10,000 IU Penicillin, 10,000 µg/mL Streptomycin

50.0 mL HI-FBS

5.0 mL of 200 mM L-Glutamine

7.7.3 Label the flask with the date, your initials, and media type.

7.7.4 Tightly cap and keep media at room temperature while working with cells.

7.7.5 Keep at 2-8°C after use.

7.7.6 Use within 1 month.

7.8 Thawing Media with Benzonase®

7.8.1 For each vial of cryopreserved PBMC, add 500 units of Benzonase® to 10 mL of Thawing Media in a 50mL conical tube. (Final concentration of Benzonase®will be 50 U/mL.)

7.8.2 Mix well

8 Procedure

Warning: Appropriate safety precautions (Universal Bloodborne Pathogens-BSL2) must be utilized for biohazard materials including proper usage of seamless gowns, gloves, face shields, certified biological safety cabinets and certified chemical fume hoods.

Warning: Cryogenic vials are intended for placement only in the vapor phase of LN2, and should not be used for storage in the liquid phase of LN2. Immersion of the vials in the liquid phase could result in penetration of the liquefied gas into the vial, resulting in rapid vaporization of the liquid upon removal and possible violent explosion or leakage from the vial/closure perimeter. To prevent cryogenic vials from exploding:

- Never overfill LN2 storage units.
- Always examine vials before use to ensure no visible defects around the closure rims. DO NOT THAW a vial that is visibly compromised.
- Always use full faceshields, heavy safety gloves and laboratory protective apparel when removing vials from cryogenic storage and thawing.

8.1 Prepare Media Tubes

8.1.1 Prepare one 15 mL conical tube for each cryovial of PBMC to be thawed.

8.1.2 Label each tube with 3 unique identifiers, which will include at least the sample PID and sample date.

8.2 Removing cryovials from LN2 Freezer

8.2.1 Remove no more than four cryovials for thawing from the LN2/-150°C freezer at a time.

8.2.2 Retrieve cryo samples from LN2/-150°C freezer and place immediately on dry ice or in an LN2 pan (or Stratagene StrataCooler® Cryo, biocision® CoolCell, or NALGENE® Mr. Frosty equilibrated to -80°C).

8.2.3 Keep on dry ice or in an LN2 pan until ready to thaw.

8.2.4 ***Warning***: Some cryovials have been reported to explode during the thawing process. The following precautions should be taken to minimize this risk.

8.2.4.1 If vials were inadvertently stored in the liquid phase, LN2 may have seeped into the cryovial, in this case place the vials in a separate box and place the vials in the vapor phase for a minimum of 2 hours.

8.2.4.2 Upon thawing these vials, loosen the cap slightly to allow the nitrogen to escape.

8.3 Thawing PBMC

8.3.1 Thaw each of the four cryovials one at a time:

8.3.1.1 Check temperature of 37°C (± 2°C) water bath prior to use.

8.3.1.2 Transfer the cryovial from the dry ice/LN2 pan directly into the 37°C water bath.

8.3.1.3 Hold the cryovial in the surface of the water bath with an occasional gentle “flick” during thawing.

8.3.1.4 Do not leave cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly).

8.3.1.5 When a small bit of ice remains (approximately 1 minute) in the cryovial, transfer the cryovial to the biosafety hood.

8.3.1.6 Dry off the outside of the cryovials with a delicate task wipe before opening to prevent contamination.

Optional: Apply ethanol to delicate task wipe.

8.3.1.7 Remove 1mL of Thawing Media from the stock solution using a 2 mL sterile pipette.

8.3.1.8 Uncap the cryovial and dispense the 1mL of Thawing Media or Thawing Media with Benzonase into the cryovials

8.3.1.8.1 Add the Thawing Media slowly, in a drop wise manner.

8.3.1.8.2 Mix the cryovials contents by slowly pipetting up/down 3-5 times.

8.3.1.9 With the same 2 mL sterile pipette, transfer the entire cell suspension to the corresponding, pre-labeled, sterile 15 mL conical tube.

8.3.1.10 Very slowly add an additional 10 mL of Thawing Media or Thawing Media with Benzonase®.

8.4 Washing PBMC

8.4.1 Centrifuge all conical tubes at 400 x g for 10 minutes.

8.4.2 After centrifugation, uncap and decant wash into appropriate waste bottle for disposal according to institutional policy.

8.4.3 Gently re-suspend pellet in residual media using by finger flicking the tube.

8.4.4 Using a 10 mL sterile pipette, add 10 to 12 mL Thawing Media

8.4.5 Use the same pipette to gently mix the cell suspension up/down 3-5 times.

8.4.6 Centrifuge all conical tubes at 400 x g for 10 minutes.

8.4.7 After centrifugation, uncap and decant wash into appropriate waste bottle for proper disposal.

8.4.8 Re-suspend PBMC in 1 mL of sterile filtered media per 5 million PBMC thawed. The type of media will depend on the assay to be performed.

8.4.9 Use the SOP for the cell counting method approved at the laboratory to determine cell number and viability. For an example manual cell counting SOP, refer to http://www.hanc.info/labs/labresources/procedures/Pages/pbmcSop.aspx.

8.5 Optional Overnight Rest

Note: For some immunological or virologic studies it is necessary to allow the PBMCs to "rest" in culture for 14-18 hours. This rest allows the PBMC to recover functionality and is essential for allowing cells that may appear to be alive but after initial thaw to die.

Note: Only rest the cells overnight if it is necessary for the assay to be performed.

8.5.1 Resuspend the PBMC in 2-5 mL of Thawing Media.

8.5.2 Transfer PBMC into a 15 mL tissue culture tube.

8.5.3 Loosen the caps slightly and place in a 37°C humidity controlled incubator with 5% CO_2 for 14-18 hours.

8.5.4 After the incubation period repeat steps 8.4.4 through 8.4.9.

9 Helpful Links

9.1 HANC website for http://www.hanc.info/labs/labresources/Pages/informationActgImpaactLabs.aspx for the following resources:

9.1.1 ACTG-IMPAACT Laboratory Manual

9.1.2 Cross-network PBMC Processing SOP

9.2 DAIDS Guidelines for Good Clinical Laboratory Practice Standards http://www.niaid.nih.gov/LabsAndResources/resources/DAIDSClinRsrch/Documents/gclp.pdf

9.3 LDMS website: https://www.fstrf.org/apps/cfmx/apps/ldms/index.html

10 References

Immunology Quality Assessment (IQA) Standard Operating Procedure for PBMC Thawing Method, SOP CRYO #015, v2.1. 2011

Weinberg, A. Pediatric AIDS Clinical Trials Group (PACTG) PBMC Processing Cryopreservation, and Thawing Method, July 4, 2004.

Weinberg A, Song LY, Wilkening C, Sevin A, Blais B, Louzao R, Stein D, Defechereux P, Durand D, Riedel E, Raftery N, Jesser R, Brown B, Keller MF, Dickover R, McFarland E, Fenton T; Pediatric ACTG Cryopreservation Working Group. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. Clin Vaccine Immunol. 2009. 16(8):1176-86.

DAIDS Good Clinical Laboratory Practices (GCLP) Standards. 2011. http://www.niaid.nih.gov/LabsAndResources/resources/DAIDSClinRsrch/Documents/gclp.pdf