FHCRC HVTN Endpoint Assay Laboratory
Standard Operating Procedure for:
Thawing PBMC Samples

SOP #: FH-HVTN-P0004 ***Version:*** 5.0

Name: Thawing PBMC Samples

Effective Date: October 13, 2010

Owner(s): **Donald Carter**, FHCRC HVTN Laboratory Manager

Approval: **Donald Carter**, FHCRC HVTN Laboratory Manager

Donald Carter — Signature — 10-7-10 — Date

Bridget Hahn, Laboratory QA/QC Manager

Bridget Hahn — Signature — 10/07/10 — Date

Revision History:

Version	Description	Revised by	Revision Date
1.0	Initial Version		4/7/06
2.0	Added Sorvall Centrifuge to Instrumentation. Updated format.	T. Stewart	4/24/07
3.0	Removed and or added minor details.	D. Harlan, C. Beckham, A. Hughes	5/29/08
4.0	Clarified 5.1.6 on the Storage of R10/BENZ	M. Pickett	6/16/10
5.0	Revised pepraration of media from 9 mLs to 19 mLs. Following centrifugation, add 20 mLs of warmed R10 instead of 5 mLs.	Nicole Frahm	8/31/10

Purpose

This standard operating procedure (**SOP**) describes how to thaw cryopreserved peripheral blood mononuclear cells (PBMC). These cells may then be used in the HVTN Endpoints Laboratory.

Scope

This SOP is applicable to all PBMC samples thawed for use in HVTN Clinical Trials.

Authority and Responsibilities

1. The FHCRC HVTN Laboratory using this process has the authority to establish this procedure.
2. Quality Assurance is responsible for the control of this SOP.
3. The Laboratory Manager is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.
4. All technicians working on HVTN studies are responsible for reading and understanding this SOP prior to performing the procedures described.

Personal Protective Equipment (PPE)

1. Appropriate personal protective equipment (PPE) must be used including lab coat, protective eyewear and gloves while thawing samples.

Reagents and Materials

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

1. L-glutamine

Vendor: Gibco BRL Life Technologies

200mM L-glutamine, 100x concentration, store at –20ºC

2. Penicillin-Streptomycin

Vendor: Gibco BRL Life Technologies

10,000 Units, Store at -5°C to -20°C

3. Fetal Bovine Serum (FBS) (Heat Inactivated)

Vendor: Gemini Bio-products

500ml, Gem Cell

4. Benzonase

Vendor: Novagen

25U/µl or 250U/µl

Store at -20°C.

5. RPMI 1640 media

Vendor: Gibco BRL Life Technologies

RPMI 1640 with 25mM HEPES buffer and L-glutamine

Store at 4ºC.

5.1 **Preparation of culture media referred to as R10**

5.1.1 Into 500ml of RPMI 1640 with 25mM HEPES buffer and L-glutamine add 55ml of FBS, 5ml of L-glutamine, 5ml of Penicillin-Streptomycin.

5.1.2 R10 can be stored for 2 weeks at 4°C.

5.2 **Preparation of thawing media, R10/Benz (R10 supplemented with benzonase at 50U/ml)**

5.2.1 For 25U/µl Benzonase: Into 565ml of **warmed** R10 add 1.13ml of benzonase (or 20µl/10ml if less media is needed)

5.2.2 For 250U/µl Benzonase: Into 565ml of R10 add 0.113ml of benzonase (or 2µl/10ml if less media is needed)

5.2.3 R10/Benz must be prepared fresh each time it is used. Filter with a 0.22um filter.

5.2.4 Place the R10/BENZ in a 37°C, 5% CO2 incubator until use to maintain temperature.

5.2.5 R10/BENZ expires the same day it was prepared.

6. 0.22µm Millipore Filter

Vendor: Fisher

Catalog Number: 5CGVUOIRE

Instrumentation

1. Biological Safety Cabinet

Manufacturer: NuAire

2. Water Bath (37°C)

3. Guava PCA for counting cells

Manufacturer: Guava Technologies Inc.

4. Incubator (37°C, 5% CO2)

5. Pipettors

Manufacturer: Eppendorf or Rainin

6. Centrifuge

Manufacturer: Sorvall or Beckman Coulter

Procedure

1. Prepare Media

1.1 Label the 50 ml conical tubes with the PTID number or abbreviated PTID number.

1.2 Prepare culture (R10) and thawing media (R10/Benz) as described above.

1.3 Pipet 19 ml of R10/Benz into a 50 ml conical tube, creating one tube of media per PTID to be thawed.

1.4 Place media-filled tubes in the incubator to warm the media. It takes an hour to warm up if the media is cold, and substantially less time if the R10 media was warmed in a water bath prior to making R10 Benz.

2. Remove cell vials from LN2 freezers

2.1 Prepare insulated lab pan for transfer of PBMC sample vials. Refer to SOP FH-HVTN-S0008 "Insulated Lab Pan for Cryogenic Sample Transfer."

2.2 Retrieve cells from LN2 freezer(s) and transfer immediately into the lab pan.

2.3 Record acceptance of samples on the Thaw List to maintain chain of custody documentation.

3. Thaw cells

3.1 Thaw no more than 2 PTIDs or no more than 4 vials of PBMC per technician at one time.

3.2 Check and record the equipment number and temperature of the water bath (just prior to thawing) on the HVTN Assay worksheet.

3.3 For each PTID, remove the labeled 50 ml conical tube from the incubator, and place it in the biosafety cabinet.

3.4 Take the cryovial(s) from the lab pan and partially submerge the vial in the water bath, flicking the vial gently. Keep the vial cap above water.

Note: Do not leave the cryovial unattended during the thawing process. It is important for cell viability that the cells are thawed and processed quickly - thawing takes a matter of minutes.

3.5 When a small (pea-sized) bit of ice remains in the cryovial, transfer the cryovial to the biosafety cabinet. Dry off the outside of the cryovials and wipe with an alcohol solution before opening to prevent contamination.

3.6 Using a pipet, remove 1 ml of R10/Benz from the 50 mL conical tube for every vial thawed for that sample. When 2 different samples are thawed at the same time, remove media from both 50ml conicals.

3.6.1. For example, if you are thawing 2 vials from each of two different samples, then remove 2ml of media from each of 50ml conical for each sample, for a total of 4ml.

3.7 Add several drops of R10/Benz at a time to each vial dropwise. For example, add several drops of R10/Benz to Vial 1, then Vial 2, then Vial 3, and Vial 4. Repeat until each vial has approximately 1 mL of R10/Benz added.

3.8 Transfer cells from each vial into the remaining R10 Benz in the 50ml conical tube, combining vials from the same sample into the same 50ml tube. After transfer, rinse the vial or vials once using approximately 1 mL of the cell suspension per vial. Total volume of cells and media in the 50mL conical tube is approximately 20 mL (or 21 ml if two vials for one PTID were combined).

3.9 Centrifuge the conical tubes at 250 x g for 10 minutes.

3.9.1. Following centrifugation of the thawed, diluted cells, decant the supernatant. Resuspend the cells in the media that remains after decanting by using a micropipettor (for example, P-200 set to 150-175 ul), and then add 5mL of pre-warmed R10 to each tube.

3.9.1.1. It is important that the cells do not sit in a pellet for more than 5 minutes after centrifugation is complete. Once they are resuspended in 5ml media in step 3.9.1, it is ok to leave them in a hood while thawing additional samples if there is a big thaw.

3.9.2. After removing 20ul of cell suspension as described in 4.3, add an additional 20 mL of pre-warmed R10 to each tube. Total volume of the cells and media in the 50mL conical tube is now approximately 25mL.

3.10 If more vials need to be thawed, repeat steps 3.1 to 3.9. While thawing additional vials remember that the other thawed samples that are being spun down in the centrifuge should not stay in a pellet for long (see 3.9.1.1).

4. Count cells on the Guava

4.1 Refer to SOP FH-HVTN-E0018 “Guava Counter” for use of the Guava. Some additional details are provided below.

4.2 Prepare the Guava counting tubes in advance; 380ul of cold Guava counting solution (Via Count) per 1.5 mL eppendorf tube. Prepare 1 extra tube to be used to establish Guava settings. Store the tubes in the dark (in a drawer) until the tubes come to room temperature (a minimum of 10 minutes).

4.3 Add 20ul of cell suspension to a Guava counting tube, refer to 3.11.2.

4.4 Incubate tubes at room temperature for at least 5 minutes (but no more than 20 minutes) before counting.

4.5 When counting on Guava, load previous settings in the computer (in Sluf50\Vaccine\Guava Settings\settings for thaw) and setup gates with any vial.

Note: When performing daily QC of the Guava, make certain that the correct settings are entered for the specific lot of QC beads used.

4.6 Record total number of viable cells and viability for each sample on the Thaw List.

5. Preparation for Overnight Incubation.

5.1 Centrifuge conical tubes at 250 x g for 10 minutes.

5.2 Following centrifugation, decant the supernatant. Resuspend the cells in the media that remains after decanting by using a micropipettor (for example, P-200 set to 150-175 ul), and then add pre-warmed R10 for a final cell concentration of $2x10^6$ cells/mL in each tube. Do not exceed 25mL/tube. If there are more than 50M cells, split into extra tubes so that volume of resuspended cells does not exceed 25mL/tube.

5.3 Incubate overnight at 37°C **with the cap of the 50 mL conical tubes loosened**.

Definitions

Term	Definition
LN_2	Liquid Nitrogen
PBMC	Peripheral blood mononuclear cells
Cryovial	A Polypropylene ~1.8ml vial for storing PBMC.
PTID	Patient Identification; Refers to one sample ID per visit.

References

1. FH-HVTN-E0018, Guava Counter
2. FH-HVTN-S0008, Insulated Lab Pan for Cryogenic Sample Transfer
3. FH-HVTN-S0005, Placing PBMC Samples into Storage and Retrieving Samples