Duke Human Vaccine Institute

IVQAC

Immunology & Virology Quality Assessment Center

Standard Operating Procedure
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SOP No.:	CRYO #015 Copy for IQA Sites – Do Not Distribute 28/AUG/15
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Effective Date:	

Title: **Peripheral Blood Mononuclear Cells (PBMC) Thawing Method**

By signing the "Approved By" section below, the person attest that he/she has personally conducted a review of the document for completeness and accuracy and approves the contents of the SOP document.

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1.0 Purpose and Introduction

This Standard Operating Procedure (SOP) describes the method of thawing cryopreserved human peripheral blood mononuclear cells (PBMC).

2.0 Scope and Application

This SOP applies to all IVQAC lab personnel working with incidence assays, who should be aware of and competent with these procedures.

3.0 Definitions

3.1 Not Applicable

4.0 Safety

4.1 All PBMC processing work is done inside a Biological Safety Cabinet (BSC). Refer to SOP IVQAC #089.

4.2 Appropriate safety precautions are utilized for biohazard materials including proper usage of Personal Protective Equipment (PPE) - seamless gowns, gloves, and face shields.

4.3 PPE and Safety precautions in connection with Universal Blood precautions should be used under BSL-2 conditions when handling specimens. Refer to SOPs DHVI SOP #001, DHVI SOP #011, DHVI SOP #003, and DHVI SOP #088.

4.4 Refer to Material Safety Data Sheets (MSDS) on each particular reagent for detailed information on the reagent.

4.5 Polypropylene cryovials are not recommended for storage in liquid nitrogen (LN_2).

NOTE 1: Cryogenic vials are intended for placement only in the vapor phase of LN_2, and should not be used for storage in the liquid phase of LN_2. Immersion of the vials in the liquid phase of LN_2 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 LN_2 storage units. Always examine vials before use to ensure no visible defects around the closure rims. Always use full face shields, heavy safety gloves and laboratory protective apparel when removing vials from cryogenic storage. Always permit vials to warm slowly in a BSC.

4.6 Never reuse cryogenic vials.

4.7 For Waste Generation, Handling and Disposal refer to the SOP IVQAC #004.

5.0 Specimens

5.1 PBMCs (Refer to SOPs IVQAC #007, Leukopak #060)

6.0 Reagents and Materials

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

6.1 Filter Flask Unit 0.22 µm, 500 mL
Manufacturer: Generic

6.2 Polypropylene Conical Cryovials 15 mL
Manufacturer: Generic

6.3 Filtered Pipet Tips 20 µl, 200 µl
Manufacturer: Generic

6.4 Dry Ice (Refer to SOP CRYO #093)
Manufacturer: Generic

6.5 Ethanol Resistant Marker
Manufacturer: Generic

6.6 Household bleach (10% hypochlorite)
Manufacturer: Generic

6.7 70% Ethanol
Manufacturer: Generic

6.8 Personal Protective Equipment (PPE)
Manufacturer: Generic

6.9 Biohazardous waste container
Manufacturer: Generic

6.10 Kim wipes or other lint-free disposable lab wipes
Manufacturer: Generic

6.11 Roswell Park Memorial Institute Medium (RPMI) 1640
Manufacturer: Sigma

6.12 Fetal bovine serum (FBS)
Manufacturer: Gemini

7.0 Equipment

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

7.1 BSC (Refer to SOP IVQAC #089)
Manufacturer: NuAire

7.2 Centrifuge (Refer to SOP IVQAC #081)
Manufacturer: Beckman Coulter

7.3 Water Bath (Refer to SOP IVQAC #085)
Manufacturer: Barnstead International

7.4 Pipettors: Manual 10 µl, 200 µl, 1000 µl, Serological 2 mL, 5 mL l, 10 mL (Refer to SOP IVQAC #091)
Manufacturer: Generic

8.0 Procedure

8.1 Preparation of Thawing Media

8.1.1 Wipe the BSC area with a 10% bleach solution followed by 70% Ethanol before use.

8.1.2 Make sure all appropriate information is documented according to network and laboratory requirements. If calculations are incorrect, the proper notations will need to be made and included in the documentation for that specimen and reviewed (Refer to SOP #QADVIP-M002).

8.1.3 Thawing media should be prepared prior to processing.

8.1.3.1 Filter FBS with a 0.22 µm filter flask.

8.1.3.2 Mix 9 parts RPMI + 1 part FBS (the thawing media contains 90% RPMI and 10% FBS).

8.1.3.3 Once the required amount of thawing media has been produced, it is labeled with technician's initials and expiration date and stored at 2°C-8°C until needed.

8.1.3.4 Thawing media should be protected from light and can be used for up to one month.

8.2 Preparation of Media Tubes

8.2.1 Use an ethanol resistant marker to label one 15 mL centrifuge conical tube for each PBMC sample to be thawed with the corresponding specimen ID. It is recommended that the lid of the centrifuge conical tube also be identified with the specimen ID to prevent cross-contamination.

8.2.2 Dispense 10 mL of the thawing media in each 15 mL centrifuge conical tube with a sterile serological pipette. For every vial thawed there will be an additional volume of 10-12 mL of thawing media needed in a separate centrifuge conical tube. If thawing several vials, this additional volume can be consolidated.

8.2.3 Check the temperature and cleanliness of the 37°C water bath prior to use.

8.2.4 Place the media filled centrifuge conical tube in the 37°C water bath for 10 min.

NOTE 2: Do not exceed 10 minutes. Do not allow centrifuge conical tubes to become submerged in the water bath. Utilize a plastic rack.

8.2.5 Example: The technician is thawing a total of 4 PBMC samples. Four 15 mL centrifuge conical tubes are labeled with the specimen ID. Each centrifuge conical tube contains 10 mL of thawing media and is placed in the 37°C water bath. An additional volume of 48 mL of thawing media is also placed in the 37°C water bath to come up to temperature.

8.3 Removing Cryovials from LN_2 Freezer

8.3.1 Retrieve samples from LN_2 freezer (Refer to SOP CRYO #097) and place immediately on dry ice (Refer to SOP CRYO #093) until the thawing process begins.

NOTE 3: Some cryovials have been reported to explode during the thawing process. To minimize this risk, use only unbreakable polyethylene cryovials for storage in LN_2. If polypropylene cryovials are used, they should have a polyethylene-internal threaded screw cap with an O-ring.

8.4 Thawing Procedure

8.4.1 No more than 8 cryovial samples of PBMCs should be processed per technician and only one vial thawed at a time.

8.4.2 Have the BSC set up before the thawing process begins. This will include, but is not limited to: a waste beaker, sterile transfer pipettes, and a rack for the 15 mL centrifuge conical tubes.

8.4.3 Transfer the media filled 15 mL centrifuge conical tubes from the water bath, into the BSC. Lightly spray with 70% Ethanol and wipe with a lint free disposable

wipe to remove excess moisture.

8.4.3.1 Before thawing check all specimen IDs.

8.4.4 Gently turn the cap of the selected cryovial without opening and close again to release pressure.

8.4.5 Transfer the selected cryovial from the dry ice directly into the 37°C water bath.

8.4.6 Hold the cryovial just below the surface of the water bath with an occasional gentle “flick” during thawing.

8.4.7 Do not leave cryovial unattended or completely submerge during the thawing process. Thawing in a timely manner is imperative to accurately access the viability and viable recovery of a specimen.

8.4.8 When a small bit of ice remains (~1-2 minutes) in the cryovial, transfer the cryovial to the BSC. To prevent contamination, lightly spray the cryovial with 70% Ethanol and dry with a lab wipe.

8.4.9 Using a 2 mL sterile transfer pipet, remove 1mL of ambient or warmed media from the corresponding 15 mL conical tube for the sample.

8.4.10 Uncap the cryovial and dispense the 1 mL of media into the cryovial: add in a slow, drop wise manner. Once the 1 mL has been dispensed, mix contents, by slowly pipetting up/down 3 to 5 times.

8.4.11 With the 2 mL sterile pipette, transfer the entire cell suspension to the sterile 15 mL conical tube containing the remainder of the media.

8.4.12 Continue with any remaining cryovials using steps 8.4.3.1 to 8.4.11.

8.4.13 Wash #1: Centrifuge all conical tubes at 400 xg for 10 minutes (brake optional).

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

8.4.14.1 If the cell pellet is not visible, confirm that the centrifuge is operating properly. Correct any problems you find. Re-centrifuge the tube. Document the problem and actions taken according to network and laboratory requirements. If the cell pellet is still not visible after re-centrifuging the tube, document and continue the process.

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

8.4.16 Gently resuspend cell pellet by flicking.

8.4.17 Using a 10 mL sterile serological pipette, Quantity Sufficient (QS) to 8.0 mL with prepared thawing media. Use the same pipette to gently mix the cell suspension up/down 3 to 5 times.

8.4.18 Wash #2: Centrifuge all conical tubes at 400 xg for 10 minutes (brake optional).

8.4.19 Repeat steps 8.4.14 to 8.4.16.

8.4.20 Using a 5mL sterile serological pipette, QS to appropriate volume with prepared thawing media. The final volume of the cell suspension will be dependent on the cell concentration of the cryovial. Refer to the table below for final cell suspension volumes.

Cell Concentration per cryovial (cells/mL)	**Final Cell Suspension Volume (mLs)**
5.0 -10.0 x10^6 cells/mL	2 mL
10.0 – 20.0x10^6cells/mL	4 mL

8.4.21 Follow the cell counting method required by specific protocol to determine the total viable cell count, viable recovery, and viability percentage. Document all observations and calculations on the appropriate worksheets. Refer to SOPs CRYO #010, Vi-Cell #011.

9.0 References

9.1 HANC Cross- Network PBMC Processing Procedure

9.2 Guide to Pipetting by AccuTek Laboratories

9.3 IQA Cryopreservation Consensus protocol Version 3.0 ACTG NIAID NIH 2002

9.4 VI-Cell #011 “Determination of In-Suspension Cell Viability and Cell Count Using the Beckman Coulter Vi-Cell and Vi-Cell XR Viability Analyzer”

9.5 CRYO #010 “Manual Total Viable Cell Count using Trypan Blue”

9.6 CRYO #097 “Liquid Nitrogen Freezers”

9.7 IVQAC #093 “Dry Ice”

9.8 IVQAC #081 “Centrifuges”

9.9 IVQAC #085 “Water Bath”

9.10 IVQAC #089 “Biological Safety Cabinet”

9.11 IVQAC #004 “Access, Safety, and Waste Management within the IVQAC Laboratory”

9.12 IVQAC #007 “Separating PBMCs from Whole Blood Using Ficoll Hypaque”

9.13 Leukopak #060 “Separating PBMCs from Leukopak Using Ficoll Hypaque”

9.14 QADVIP-M002 “Good Documentation Practices”

9.15 DHVI SOP #001 “General Biosafety Procedures”

9.16 DHVI SOP #011 “Acceptable Handwashing Practices”

9.17 DHVI SOP #003 “Personal Protective Equipment”

9.18 DHVI SOP #088 “Biosafety Level 2”

10.0 Attachments

10.1 Not Applicable