ITN PBMC Processing SOP

1000-1136 Isolation and Cryopreservation of PBMC from whole blood

Purpose:	
This document provides the procedure for the isolation and cryopreservation of peripheral blood mononuclear cells (PBMC) from whole blood samples using Ficoll-Paque PLUS™ density gradient centrifugation.	
Scope:	
This procedure applies to personnel isolating PBMC from up to 300mL whole blood with subsequent storage.	
A. Roles and Responsibilities:	
Role	**Responsibility**
Lab technician	Responsible for performing all steps of procedure and documentation as well as ensuring all equipment is up to date and functioning correctly.
Lab management	Same responsibilities as technicians and to ensure acceptance criteria are met. Report deviations. Assist with troubleshooting as required.
B. Definitions:	
Term	**Description**
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
RBC	Red blood cell
RCF(g)	Relative Centrifugal Force (g-force)
RT	Room Temperature (18-25°C)
HuAB	Human AB serum
D. Equipment/Materials Required:	
Number	**Equipment/Material Description**
N/A	Biosafety cabinet

Eppendorf, 5810 5810R, or equivalent	Centrifuge for tubes with horizontal rotor
N/A	Centrifuge buckets with adapters for 50mL conical tubes and 15mL conical tubes
ChemoMetec A/S	NucleoCounter NC-200
ChemoMetec A/S, 941-0012	Via1-Cassette
N/A	LN2-safe cryovials for PBMC aliquots
N/A	250mL plastic container, sterile
N/A	50mL conical tubes, sterile
N/A	15mL conical tubes, sterile
N/A	1.5mL microcentrifuge tubes
N/A	Serological pipettes graduated 5mL, 10mL, 25mL, 50mL
N/A	100-1000µL sterile, filtered, pipette tips, normal and wide bore
N/A	20-200µL sterile, filtered, pipette tips, normal and wide bore
Gilson, Pipetman, Rainin	Manual pipettes, suitable to volumes being transferred
N/A	Pipette aid
N/A	Ficoll-Paque PLUS
N/A	1X Dulbecco's PBS sterile, Ca++, Mg++ Free
N/A	1X RPMI 1640, with L-glutamine
N/A	DMSO, Sterile
N/A	HuAB
N/A	Mr Frosty freezing container or equivalent, or controlled rate freezer

Specific content in this procedure is identified as follows:

	**Safety – Precautions or safety issues associated with the entire SOP or with a particular step**
	**Radioactive – Radioactive samples**
	**Sample Integrity – Handle samples with care to avoid compromising the integrity.**
	**Scan – Scan the barcode.**
	**Verify – Verify a task per the stated attributes.**
	**If, then – Contingency instructions.**
	**Information – Information used for the execution of the associated step.**

General Information

	**Use caution when handling DMSO and media containing DMSO.**
	All reagents and consumables must be sterile and must only be opened in the Biosafety Cabinet to maintain sterility.
	Record lot number and expiration date of HuAB. Record freeze/thaw on aliquots of HuAB and do not thaw more than 5 times.

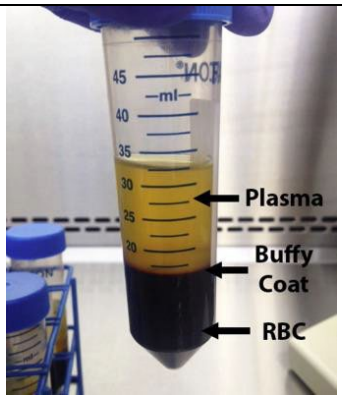
E. Procedure

1. Pre-Processing

Step	Instruction
1.1.	ℹ All steps MUST be performed in the Biosafety Cabinet.
1.2.	Label the first page with the processing date, the corresponding Registration numbers, Subject ID and Visit, ITN Study Name or ID, or any other unique identifiers on the sample. Ensure the Processing date and Subject ID are recorded on page 2. ℹ Use one Subject Record per subject received on a given day if pooling is performed. Record all Registration numbers.
1.3.	Record the expiration dates and lot numbers of all reagents being used.
1.4.	Record the processing start time on the first page.
1.5.	Visually inspect all whole blood tubes for a subject. Note any irregularities.

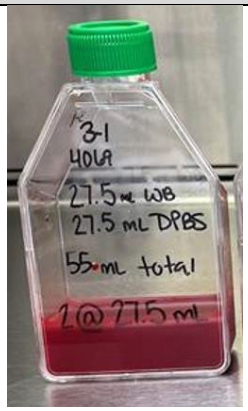
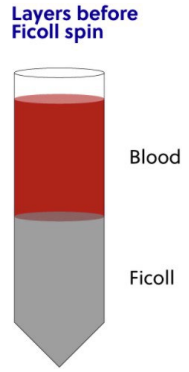
2. Undiluted Plasma Collection

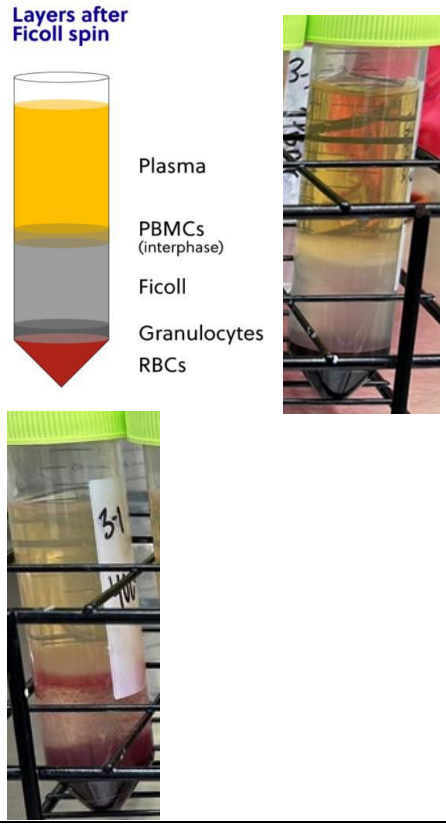
Step	Instruction	Example
2.1.	✓ Verify that plasma isolation is required. ◆ If plasma isolation is **not** required, proceed to section 3.	
2.2.	Confirm all data is identical for tubes to be pooled together.	
2.3.	Prepare a 50mL conical tube with the subject ID or identifying information for every 50mL of blood received.	
2.4.	Pool up to 50mL of blood in each prepared conical tube. ◆ If only 1 vacutainer was received for a subject, do not transfer blood to a 50mL tube.	
2.5.	Record the total blood volume for the subject.	

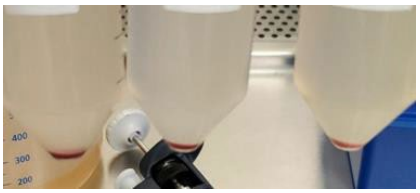
Step	Instruction	Example
2.6.	Place conical tubes (or vacutainer if only 1 blood tube was received) in appropriate centrifuge buckets and balance them. Centrifuge at **650 x g for 10 minutes** with brake OFF.	
2.7.	Remove the tubes carefully without disturbing the separation layers. ◆ If the sample does not fractionate, notify Lab Management immediately.	
2.8.	Record plasma observations. Plasma observations may include color and clarity/turbidity.	
2.9.	◆ If multiple conical tubes were prepared in 2.4., pool all plasma into 1 container before creating plasma aliquots.	
2.10.	Record the total plasma volume.	
2.11.	Create plasma aliquots at desired volume. Store at -80°C.	
2.12.	Add enough 1X DPBS to replace the volume of plasma that was removed in the conical tube containing the buffy coat and RBCs. Gently pipette up and down 3 times to mix the blood.	

3. PBMC Isolation from Whole Blood Using Ficoll-Paque PLUS density gradient centrifugation

Step	Instruction	Example
3.1.	Label a 250 mL plastic container with the subject ID or identifying information. Use 1 container per subject. ◆ If total blood volume is <25mL, use a 50mL conical tube. ◆ If total blood volume is >120mL, use multiple 250mL plastic containers.	
3.2.	Combine all vacutainers from one subject into the appropriately labeled plastic container using a serological pipette. Record the whole blood pooled volume. Do not exceed 125mL of whole blood in a single container. ◆ If plasma isolation was performed, pool the 50mL conical tubes from step 2.12. ◆ If only 1 vacutainer was received, transfer the blood to a conical tube.	

Step	Instruction	Example
3.3.	Dilute the pooled blood using an equal amount of 1XDPBS (RT) using a serological pipette. Mix gently by pipetting. Record the diluted blood total volume.	
3.4.	Calculate the number of Ficoll-Paque PLUS™ tubes required to layer all diluted blood. Divide the total volume of diluted blood by 30 and round up to the nearest whole number. Label each Ficoll-Paque PLUS™ tube with the sample identifier. ℹ Up to 35mL of diluted blood can be layered into 1 Ficoll-Paque PLUS™ tube. ◆ If the total diluted blood volume from step 3.3. is less than 10mL, then only 1 15mL conical tube will be required.	
3.5.	Prepare Ficoll-Paque PLUS™ tubes by pipetting 15mL Ficoll-Paque PLUS™ at RT into each 50 mL conical tubes prepared in 3.4. ◆ If the total diluted blood volume from step 3.3. is less than 10mL, dispense 5mL of Ficoll-Paque PLUS™ into 15mL conical tube.	
3.6.	Gently layer up to 35 mL of diluted blood onto the dispensed Ficoll-Paque PLUS™ tube. ★ Layer blood by placing the serological tip to the inside of the conical tube and <u>slowly</u> dispense to minimize the mixing of the 2 phases. ℹ RBC may start to settle prior to centrifugation. This is expected.	 
3.7.	Centrifuge 50 mL conical tube(s) at **650 x g for 30 minutes** with the brake OFF.	

<table>
<tr><th>Step</th><th>Instruction</th><th>Example</th></tr>
<tr><td>3.8.</td><td>Carefully remove the tubes from the centrifuge without disturbing the separation layers. Record observations.
RBC contamination should be noted at this step.
If the tubes do not have clearly defined separation layers, then improper separation may have occurred. Notify Lab Management immediately.</td><td> 
</td></tr>
<tr><td>3.9.</td><td colspan="2">Prepare new, labeled 50mL conical tubes with subject ID or identifying information. Prepare 1 new tube per 2 Ficoll-Paque PLUS™ tubes per subject.</td></tr>
<tr><td>3.10.</td><td colspan="2">Use a pipette to carefully remove and discard the top half of the diluted plasma layer.</td></tr>
<tr><td>3.11.</td><td colspan="2">Collect the PBMCs from the diluted plasma/Ficoll-Paque PLUS™ interface using a sterile serological pipette. Dispense into the labeled 50 mL conical tube. Interface cells from two 50 mL tubes can be combined into one wash tube.
While collecting the cells, be sure to aspirate as little Ficoll-Paque PLUS™ as possible (<5 mL). Lower cell numbers will pellet if the proportion of Ficoll-Paque PLUS™ is too high in the wash tube.</td></tr>
<tr><td>3.12.</td><td colspan="2">Add 1X DPBS (RT) to bring the total volume of each tube to 45 mL. Gently invert to mix. Record the total number of 50mL conical tubes after pooling PBMCs.</td></tr>
<tr><td>3.13.</td><td colspan="2">Centrifuge conical tube(s) at 360 x g 10 minutes with the brake on low.</td></tr>
<tr><td>3.14.</td><td>Prepare 50 mL HuAB washing solution in a new, sterile 50 mL conical tube. Prepare 1 50mL conical tube per sample.</td><td><table><tr><th colspan="2">HuAB Washing Solution</th></tr><tr><td>HuAB, RT</td><td>1X dPBS, RT</td></tr><tr><td>500 µL</td><td>49.5 mL</td></tr></table></td></tr>
</table>

Step	Instruction	Example
3.15.	Carefully remove and discard the supernatant from each tube without touching the pellet. This can be accomplished with a serological pipette or by gently pouring the supernatant off. Record observations. ★ Do not let cells sit in the pellet for a prolonged time (more than a minute) as pelleted cells start dying if not resuspended immediately.	
3.16.	Add 5 mL of sterile HuAB washing solution from step 3.14. Mix by gently pipetting up and down.	
3.17.	Pool all the individual cell suspensions for one subject into a single sterile, empty-labeled 50 mL tube.	
3.18.	Bring the total volume in the tube to the nearest 10mL (10mL, 20mL, 30mL, or 40mL) with HuAB washing solution. Mix well by inverting tube. Record the cell suspension final volume.	
3.19.	Transfer 50µL of each sample to a clean, labeled 1.5mL microcentrifuge tube for automated cell counting. ◆ If improper separation is noted at step 3.8. the sample should also be analyzed at a dilution factor of 20. ◆ If sample is low volume and was layered on 5 mL of Ficoll-Paque PLUS™ at step 3.5., the sample should also be analyzed at a dilution factor of 2. This will be 100µL of cells to 100µL of cell counting media.	
3.20.	Store cell suspension at 4°C until counting is completed.	

4. Cell Counting

Step	Instruction	Example
4.1.	Calculate the total viable cells in the cell suspension. Use the volume of cell suspension from step 3.18. in the calculation.	_____ x _____ = _____ Viable cells/mL; Volume of cell suspension; Total Viable Cells
4.2.	Determine the volume of Freezing Media required for each cell suspension to meet the requested concentration. ⓘ Recommended concentrations: Viable PBMC yield ≤ $30x10^6$ = $5x10^6$ cells/vial. Viable PBMC yield > $30x10^6$ = $10x10^6$ cells/vial.	_____ / _____ = _____ Total Viable Cells; Requested Concentration; Total volume freezing media
4.3.	HuAB will be used to resuspend the cell pellet. Calculate the volume of HuAB to be used.	_____ x 0.5 = _____ Total volume freezing media; HuAB to add to cell pellet

<table>
<tr><th>Step</th><th>Instruction</th><th>Example</th></tr>
<tr><td>4.4.</td><td>Prepare 20% DMSO Freezing Media. Prepare enough Freezing Media to equal the volume calculated in 4.3.
ℹ Freezing Media may be prepared in a larger batch to accommodate for all samples received in a single day.</td><td><table><tr><td></td><td></td><td></td></tr><tr><td>1 mL</td><td>800 µL</td><td>200 µL</td></tr><tr><td>5 mL</td><td>1 mL</td><td>4 mL</td></tr><tr><td>15mL</td><td>3 mL</td><td>12 mL</td></tr><tr><td>20 mL</td><td>4 mL</td><td>16 mL</td></tr></table></td></tr>
<tr><td>4.5.</td><td colspan="2">Centrifuge 50mL conical tube(s) at 300 x g for 10 min with low brake (brake=3).
★Do not let cells sit in the pellet for a prolonged time (more than a minute) as pelleted cells start dying if not resuspended immediately.</td></tr>
<tr><td>4.6.</td><td colspan="2">Carefully remove and discard the supernatant from each tube without touching the pellet. This can be accomplished with a serological pipette or by gently pouring the supernatant off.</td></tr>
<tr><td>4.7.</td><td colspan="2">Resuspend the cell pellet in the volume of HuAB calculated in 4.3.</td></tr>
<tr><td>4.8.</td><td colspan="2">Add 20% DMSO Freezing Media prepared in 4.4. to the cell suspension. Freezing Media must be added slowly, drop by drop. Gently mix the contents by pipetting. Avoid producing bubbles.
ℹ The final Freezing Media concentration is a 10% DMSO solution.</td></tr>
<tr><td>4.9.</td><td colspan="2">Record the number and volume of PBMC aliquots.</td></tr>
<tr><td>4.10.</td><td colspan="2">Observe that there is liquid biomaterial in each aliquot. Place aliquots in Mr. Frosty at RT (or equivalent controlled freezing container).</td></tr>
<tr><td>4.11.</td><td colspan="2">Move Mr. Frosty to -80°C storage environment for at least 24 hours but not more than 48 hours. Aliquots must be moved to LN2 (-190°C) within the 24-48 hour timeframe. Record the time the Mr Frosty (or equivalent) was placed into the -80°C storage environment.</td></tr>
</table>

5. Post-Processing

Step	Instruction	Example
5.1.	Add any additional processing comments if necessary.	
5.2.	Ensure all PBMC aliquots are stored in LN2 (-190°C) long-term. Dispose consumed parent tubes using standard lab disposal process.	
5.3.	For any deviations from procedure or problems with sample integrity, the manager or supervisor must be notified.	