PBMC CPT v.009

Isolation and Cryopreservation of PBMC – CPT without Plasma Collection

Author: Immune Tolerance Network
Approved by:

1.0 Title

Isolation and Cryopreservation of PBMC – CPT without Plasma Collection

2.0 Purpose

To describe the procedure for isolation and cryopreservation of PBMC isolated from CPT for subsequent testing

3.0 Definitions and Abbreviations

DMSO	Dimethyl Sulfoxide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
CPT	Cell Preparation Tube
RT	Room Temperature (18-25˚ C)
RCF	Relative Centrifugal Force
RPM	Revolutions Per Minute
HuAB	Human serum

4.0 Equipment and Reagents

4.1 Equipment

4.1.1	Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
4.1.2	Conical tubes, 15 ml, sterile	Falcon 352097	Fisher# 14-959-70C
4.1.3	Hematology Mixer Model M26125	Barnstead/Thermolyne	Fisher#12-814-2
4.1.4	Nalgene Cryo Freezing Container filled with 2-propanol	Nalgene #5100-0001	Fisher#15350-50
4.1.5	Pasteur pipettes	Fisher	13-678-20
4.1.6	Pipette aids	Drummond 4-000-100	Fisher#13-681-19
4.1.7	Vials, cryogenic, 1.8ml, sterile	Nalgene/Nunc# 368632	Fisher#12-565-171N
4.1.8	Biological safety cabinet	Forma Scientific	Model 1286
4.1.9	Alcohol swabs	Fisher	S17032
4.1.10	Gloves	Fisher	N/A
4.1.11	Sharps disposable system	Fisher	N/A

	4.1.12	Guava Personal Cytometer	Guava Technologies	0100-1430
4.2	Reagents			
	4.2.1	BD Vacutainer™ CPT™ Preparation Tubes with Sodium Citrate	BD REF 362761	
	4.2.2	PBS sterile, Ca^{++}, Mg^{++} free	Cellgro 21-031-CM	VWR# 45000-436
	4.2.3	Whole blood	Subject Source	
	4.2.4	Freezing Medium A • HuAB serum • Tissue culture tested • Heat inactivated • Filtered	Provided by ITN	
	4.2.5	Freezing Medium B • 20%DMSO • HuAB serum • Tissue culture tested • Heat inactivated • Filtered	Provided by ITN	
	4.2.6	Trypan Blue	Any Vendor	

Note:
Keep all reagents at room temperature. Pay particular attention to the "HOT SPOT" steps. These steps are crucial to optimizing cell yield and viability. Please contact the TAG representative indicated on the Lab Manual Contact Page if you have any SOP related questions.

5.0 Procedures

CAUTION

All work needs to be performed under the biological safety cabinet observing biosafety regulations and using sterile technique.

Take precautions while handling the glass tubes as they have the potential for breakage. Handle all biological samples and blood collection "sharps" in accordance with the policies and procedures of your facility.

5.1 *Blood Collection*

5.1.1 The BD Vacutainer™ CPT™ Tube with Sodium Citrate should be at room temperature (18 -25˚ C) and properly labeled for patient identification.

5.1.2 Collect blood into the tube using the standard technique for BD Vacutainer™ Evacuated Blood Collection Tubes. Invert tubes approximately 10 times.

5.1.3 Complete visit-specific requisitions (3-part NCR forms included in the kit), which have fields corresponding to the tube labels.

5.1.4 After collection, store the tube upright at room temperature until centrifugation. For best results, do not store the tube for more than 2 hours after blood collection.

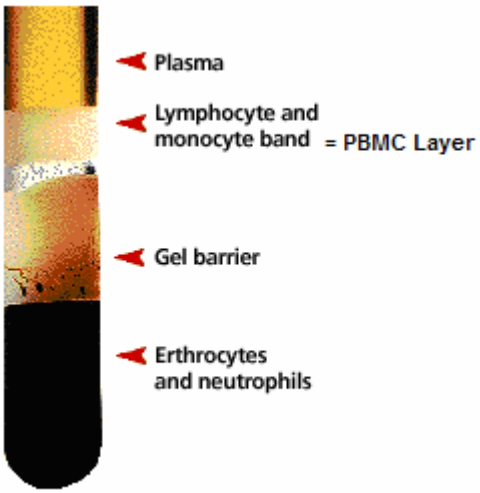
5.2 *PBMC Isolation*

Hot Spot

5.2.1 Before centrifugation, remix the blood by gently inverting the tube 8-10 times.

5.2.2 Use a temperature-controlled centrifuge and set the temperature to 18-25°C. Centrifuge tube with blood sample at 1,800 RCF (use the formula in Appendix A to calculate RPM for a given RCF) for 30 minutes at RT. Keep the brake OFF.
Note: Use centrifuge with horizontal rotor (swinging bucket) and an adaptor that can accommodate a 16x125 mm size tube.

5.2.3 After centrifugation you should be able to see the following layers:



Hot Spot

5.2.4 Using a serological pipette, carefully transfer top half of plasma layer into a 50 ml tube (can later discard). DO NOT DISTURB the lymphocyte and monocyte band (PBMC layer).

Hot Spot

5.2.5 From an individual CPT tube, collect remaining plasma with the PBMC layer by serological pipette. Try to aspirate the remaining volume of plasma on the gel by tilting the tube and having the pipette tip touch the side of the tube, not the gel barrier. Combine PBMC layers from 3-6 CPT tubes into one 50 ml tube.

5.2.6 Add 1x PBS to bring the volume up to 45 ml.
Note: Make sure to use cell culture grade Mg++ and Ca++ free PBS.

5.2.7 Cap 50 ml tube and invert 5 times to mix cell solution.

5.2.8 Centrifugate at 330 RCF for 10 minutes at RT. Keep the break ON.

5.2.9 After the centrifuge stops, carefully remove the tube without disturbing the pellet.

5.2.10 Using a serological pipette, carefully remove and discard the supernatant without touching the pellet.
Note: After most of the supernatant is aspirated, remove remaining supernatant by progressively tilting the tube while the pipette tip touches the side of the tube. Do not lower pipette tip close to the cell pellet.

5.2.11 Loosen the pellet by tapping the tube with your finger. Then add 1X PBS+1% HuAB serum (Freezing Media A) to bring volume up to 10 ml. If you are processing multiple 50 ml tubes, refer to 5.2.11.1 for combining cells.
Example: To make 10 ml of 1X PBS+1% HuAB serum,
mix 100 µl of HuAB serum (Freezing Medium A)
with 10 ml of 1X PBS.

Hot Spot

5.2.11.1 If you are processing multiple 50 ml tubes, combine contents from each 50 ml tube into ONE 50 ml tube.
Note: When calculating cell count, the "volume of original cell suspension" will depend on the volume of cell suspension after combining cells from multiple 50 mls tubes.

5.2.12 Mix the cells well by pipetting. Take out a 20 µl aliquot from the cell suspension to count cells [SEE SECTION 6].

5.2.13 Centrifugate the cell suspension from Step 5.2.12 at 330 RCF for 10 minutes at RT.

5.2.14 Based on the cell count, label the appropriate number of 1.8 ml cryotubes per patient (see 5.3.3.1). All cells should be cryopreserved. For example, a total of 28 million cells should be aliquoted into 5 cryovials at 5 million cells (500 µl), and 3 million cells (300 µl). Write cell number on tube.

5.2.15 As soon as centrifugation is complete, carefully remove and discard supernatant without disturbing the cell pellet (again, this can be achieved by progressively tilting the tube while having the pipette tip touch the side of the tube. Do not lower pipette tip close to the cell pellet). Re-suspend the cell pellets by tapping the tube with your finger until no clumps are visible. Do not pipette or vortex, as this will damage the cells. Pelleted cells will start dying if not re-suspended promptly.

5.3 *Cryopreservation of PBMC*

5.3.1 After re-suspending the cell pellet by tapping, add the appropriate amount of room temperature Freezing Medium A to adjust the cell concentration to 20 x 10^6/ml. Mix cells by gently tapping the tube; do not use a pipette.

Hot Spot

5.3.2 Slowly, drop by drop, add to the side of the tube an equal volume of Freezing Medium B (20%DMSO) to Freezing Medium A containing the PBMCs. To mix the cells, GENTLY pipette up and down 3 times. Avoid bubbles. The final concentration should be 10x10^6/ml.

5.3.3 Once mixing is complete, aliquot the appropriate volume of PBMC suspension (based on 5.3.3.1 into the pre-labeled 1.8 ml cryovials (~10 million cells per vial; do not freeze more than 10 million cells per vial). Again, all cells should be cryopreserved. **Pipette gently (using serological pipette) to minimize shear force.**

IMPORTANT: Write cell number/vial in field provided on the label. Suffixes that correspond to the PBMC specimen are -21 through -29. Please make sure that you start from -21 and move towards -29 while aliquoting your PBMC sample.

5.3.3.1 If total cell yield is **30 million cells or more**, aliquot 10 million cells per aliquot (1 ml).

If total cell yield **is less than 30 million cells**, aliquot 5 million cells per aliquot (500 µl).

Write cell number on cryovial. Do not aliquot less than 3 million cells (300 µl) per cryovial. If you have less than 3 million remaining cells to aliquot, please add it to an already existing aliquot and make sure you write the cell number on the cryovial. **Do not discard any cells.**

5.3.4 In case you run out of the pre-labeled cryovials provided in the kit, please use the tubes from the bulk supply. Indicate in the comments field of the requisition form that the bulk supply of tubes was used for aliquoting PBMCs.

5.3.5 Freeze the cryovials using one of the following processes.

5.3.5.1 Add 2-propanol at RT into Nalgene Cryofreezing Container. Place the cryovials into the container. Put the freezing container into -70°C freezer for a minimum of 12 hours. Then remove cryovials and store in - 70° C freezer until shipment. During the freezing time avoid opening the freezer in order to avoid shaking the cryovials or raising the freezer's temperature.

5.3.5.2 If Nalgene Cryofreezing Containers are not available, the following "low technology" method works well.
Place the cryovials in a Styrofoam tube container (they are supplied with the 15ml conical tubes) to reduce direct contact with cold surfaces and to slow the rate of freezing. Place a second Styrofoam container of the same type over the first one, tape the two containers together, wrap in a plastic bag (leave some air in the plastic bag) and tie the bag shut. Place the bagged container with the cells in -70°C freezer for at least 12 hours. During the freezing time avoid opening the freezer in order to avoid shaking the cryovials or raising the freezer's temperature. Transfer the cryovials to - 70° C until shipment.

5.3.6 Cryovials must be completely frozen prior to shipping to Fisher.

5.3.7 When samples are ready to be shipped from the investigator's lab to Fisher, the Lab Coordinator must log ALL samples in a shipment log provided by ITN.

5.3.8 The Lab Coordinator must document any deviations to the protocol, or SOP, specified aliquoting or freezing process in the comment field.

5.3.9 Follow your lab's procedures to decontaminate biohazardous material.

6.0 COUNTING CELLS

6.1 To get an equal cell distribution mix cell suspension prior to adding the stain and again just before loading the hemacytometer.

6.2 To prepare the hemacytometer, first clean the hemacytometer with H_2O and then with 70% ethanol. Dry it off with a Kimwipe.

6.3 Staining cells with Trypan Blue: On a piece of parafilm (parafilm can be replaced by Eppendorf tube or well from 96-well plate) combine 20 µl of cell suspension that was set aside in 5.2.12 with 20 µl of 0.4% Trypan Blue (1:1). Mix well with pipette.
Note: After mixing cells with Trypan Blue, count cells immediately. Your goal is to achieve an accurate cell distribution with cell clumping kept to a minimum.

6.4 *Loading the Hemacytometer*

6.4.1 Place hemacytometer on the counter. Center a cover glass over the hemacytometer chambers.

. 6.4.2 Fill one chamber with 10 µl of the cell dilution using a 20 µl pipette. The solution will pass under the cover glass by capillary action. Do not over fill. Allow the cell suspension to settle in the hemacytometer for at least 10 seconds before counting. If the solution spreads into the two lateral grooves adjoining the grid table, clean the hemacytometer and repeat the application. If there are any bubbles in the solution covering the grid table clean the hemacytometer and repeat the application.

6.5 *Observing and Counting Cells*

6.5.1 Place the hemacytometer on the stage of a microscope and adjust focus using 10X magnification, then change to 20X and refocus if necessary.

NOTE:

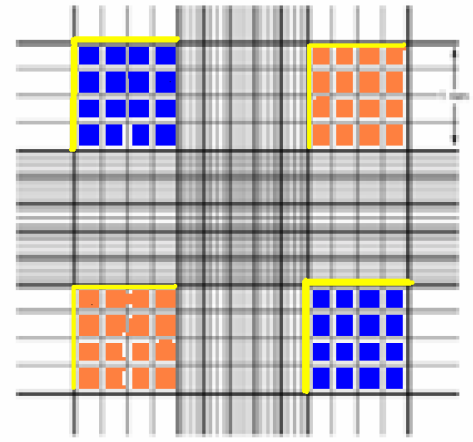
- Assess if the cells are evenly distributed among the squares.
- PBMCs may contain population of erythrocytes. Use caution when counting cells to distinguish lymphocytes from erythrocytes.

6.5.2 If available use a hand-held counter to record cell counts. Count live cells in the four large corner squares. Squares are represented in the image as blue and orange. Include cells that touch either the top line or left vertical perimeter line of any corner square (indicated with yellow lines). Do not count any cells that touch either the bottom line or right vertical perimeter line of any corner square. Use the formula in Section 6.6, to determine the number of cells per ml. Blue cells are dead and clear are alive. Count only live cells.

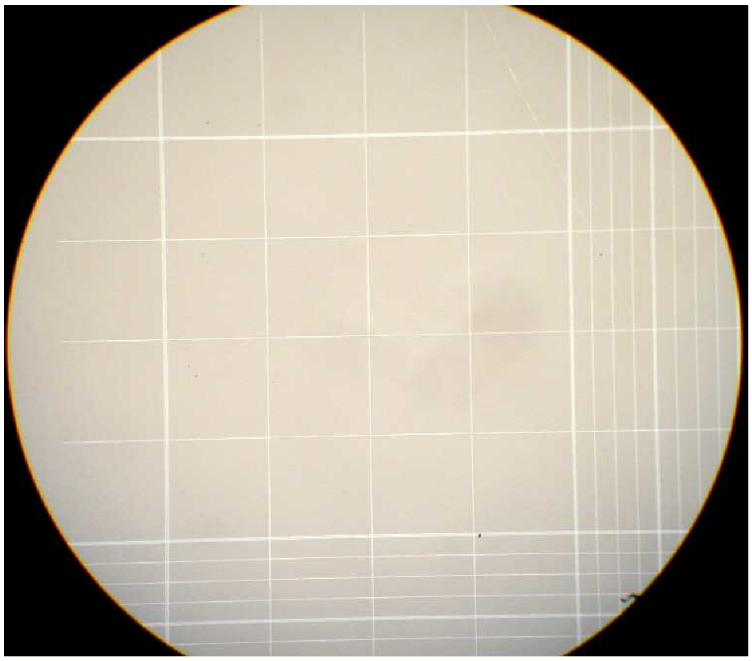
Note: If using hemacytometer other than Neubauer, follow manufacturer's instructions.

Hot Spot

6.5.2.1 This is the grid that you see under the microscope:



The image below is a magnified view of one of the squares represented in either blue or orange:



6.6 *Formula to Determine Cell Counts*

6.6.1 To calculate the cell number per ml, follow the formula below:
Viable cells/ml =(total# of viable cells/squares counted) x 10^4 x dilution factor (in this case, the dilution factor is 2 since we added 20µl trypan blue to the 20µl cell aliquot in step 6.3)

Example

Square 1: counted 55 cells

Square 2: counted 40 cells

Square 3: counted 45 cells

Square 4: counted 49 cells

Viable cells/ml = [(55 + 40 + 45 + 49) / 4] x 10^4 x 2 = 94.5 x 10^4

6.6.2 To calculate the total viable cells, follow the formula below:
Total viable cells = viable cells/ml x volume of original cell suspension in 5.2.11 (or 5.2.11.1 if processing multiple 50ml tubes)
Example: Total viable cells = 94.5 x 10^4 x 10 ml = 94.5 x 10^5 = 9.45 x 10^6

APPENDIX A

Tube description summary:
BD Vacutainer™ CPT™ Preparation Tubes with Sodium Citrate
8 ml Draw Capacity

Contains: 0.45 ml Sodium Citrate Solution (top fluid layer)
3.0 g of polyester gel (middle layer)
2.0 ml Ficoll Hypaque solution (bottom fluid layer)
Silicon coated glass tube
Silicon lubricated rubber stopper

To calculate the correct centrifuge speed for a given RCF, use the following formula:

$$\text{RPM Speed Setting} = \sqrt{\left((\text{RCF}) \times (100{,}000) / (1.12) \times (r)\right)}$$

REVISION HISTORY:			
Rev	**Section**	**Type**	**Initials/Dates**