1.0	**Title**
	Isolation and Cryopreservation of PBMCs - Ficoll Method without plasma collection

2.0	**Purpose**
	To Describe the Procedure for Isolation and Cryopreservation of PBMC
	PBMC are isolated from whole blood by Ficoll density gradient centrifugation and cryopreserved for subsequent testing.

3.0	**Definitions and Abbreviations**	
	DMSO	Dimethyl Sulfoxide
	PBMC	Peripheral Blood Mononuclear Cells
	PBS	Phosphate Buffered Saline
	RT	Room Temperature (18 - 25˚C)
	RCF	Relative Centrifugal Force
	RPM	Revolutions Per Minute
	HuAB	Human AB Serum

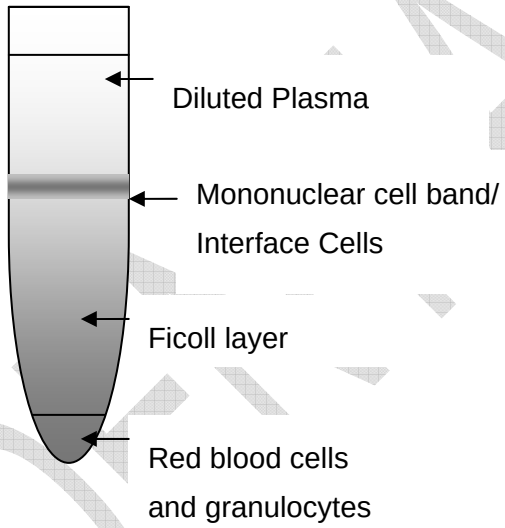
4.0	**Equipment and Reagents**				
	4.1	Equipment			
		4.1.1	Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
		4.1.2	Conical Tubes, 50ml, sterile, Falcon	Falcon 352074	Fisher#14-432-22
		4.1.3	Guava Personal Cytometer	Guava Technologies	0100-1430
		4.1.4	Hemacytometer	Reichert Bright-Line	Fisher# 02-671-5
		4.1.5	Ficoll-Paque Premium	GE Healthcare	Cat#17-5442-03 Lot#310345
		4.1.6	Hematology Mixer Model M26125	Barnstead/Thermolyne	Fisher#12-814-2
		4.1.7	Liquid nitrogen storage tank	Thermo Forma	8030

		4.1.8	Nalgene Cryo Freezing Container filled with 2-propanol	Nalgene #5100-0001	Fisher#15350-50
		4.1.9	Serological Pipette, 2 ml	Fisher	13-678-11C
		4.1.10	Pipette Aids	Drummond 4-000-100	Fisher#13-681-19
		4.1.11	Vials, cryogenic, 1.8ml, sterile	Nalgene/Nunc# 368632	Fisher#12-565-171N
		4.1.12	Biological Safety Cabinet	Forma Scientific	Model 1286
		4.1.13	250ml Plastic Storage Bottle	Corning Life Sciences	Fisher cat# 09-761-4
	4.2	Reagents			
		4.2.1	PBS sterile, Ca^{++}, Mg^{++} free	Cellgro 21-031-CM	VWR# 45000-436
		4.2.2	Whole blood	Subject Source	
		4.2.3	Freezing Medium A, HuAB serum. Tissue culture tested, heat inactivated, filtered	Provided by ITN	
		4.2.4	Freezing Medium B, 20%DMSO, HuAB serum. Tissue culture tested, heat inactivated, filtered.	Provided by ITN	
		4.2.5	Trypan Blue	Fisher Biotech	BP1302-10

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.

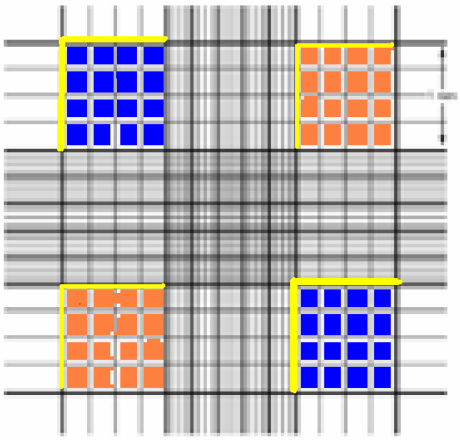
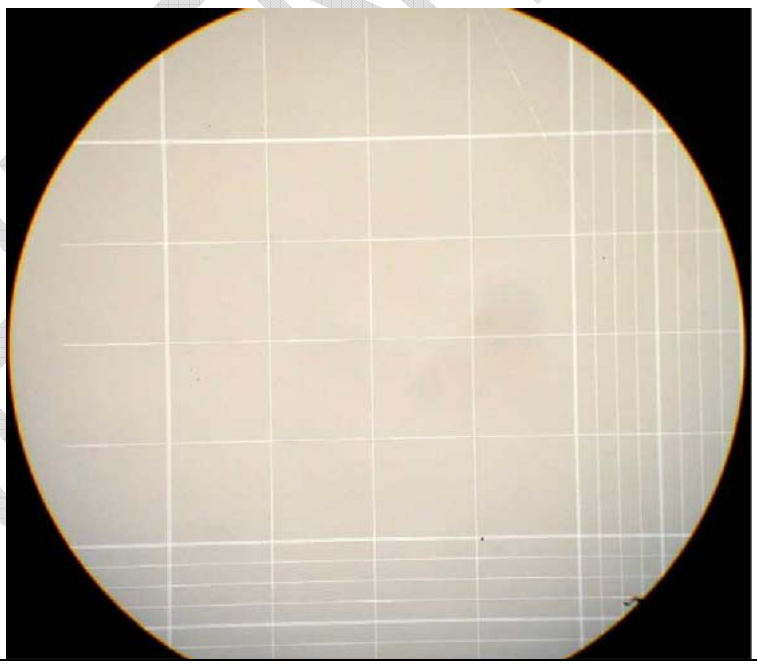
5.0	**Procedures**		
	CAUTION All work needs to be performed under the biological safety cabinet observing biosafety regulations and using the sterile technique. Take precautions while handling the glass tubes as they have the potential for breakage. Handle all biological samples and blood collection "sharps" in accordance with the policies and procedures of your facility.		
	5.1	Dilution of blood	
		5.1.1	Obtain blood samples from subjects according to protocol.
		5.1.2	Pool blood of each patient from Vacutainers into 250 ml Corning Plastic Storage Bottle.
		5.1.3	Dilute blood from Step 5.1.2 with an equal amount of 1X PBS (room temperature). Mix gently. Note: Make sure to use cell culture grade Mg++ and Ca++ free PBS.
	5.2	Isolation of PBMC's by Ficoll density gradient centrifugation	
		5.2.1	Add 15 ml room temperature Ficoll to a sterile 50 ml conical tube.
		5.2.2	Gently overlay the Ficoll with 30 ml of diluted blood (from Step 5.1.3) using a sterile serological pipette. Minimize the mixing of the two phases. Alternatively to overlaying Ficoll with blood, you can also underlay the blood with Ficoll. If this method is used, use a 10 ml serological pipette to underlay the blood with Ficoll. Again, underlay carefully as to avoid mixing.
		5.2.3	Centrifuge at 591 RCF (use formula in Appendix A to calculate RPM for a given RCF) for 30 minutes at room temperature with <u>brake off</u> to ensure that deceleration does not disrupt the density gradient.

Hot Spot		5.2.4	Using a Pasteur or serological pipette, carefully aspirate and discard top half of the diluted plasma layer. DO NOT DISTURB the lymphocyte and monocyte band (PBMC layer). Collect the PBMCs (cloudy layer) from the diluted plasma/Ficoll interface using a serological pipette and place the cells into a sterile 50 ml conical tube. Interface cells from a maximum of two 50 ml tubes can be combined into one wash tube.  NOTE: While collecting the cells, be sure to aspirate as little Ficoll as possible. Lower cell numbers will pellet if the proportion of Ficoll is too high in the wash tube.
		5.2.5	Add 1x PBS to bring the volume up to 45 ml. Gently pipette up and down to mix cell solution with PBS.
		5.2.6	Centrifugate at 330 RCF for 10 minutes at RT. Keep brake ON.
		5.2.7	After the centrifuge stops, carefully remove the tube without disturbing the pellet. 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.

		5.2.8	Loosen the pellet by tapping the tube with your finger. Then add 5 ml of sterile 1X PBS+1% HuAB serum (Freezing Media A) and carefully mix the cells by gently pipetting up and down. Example: To make 50ml of 1X PBS+1% HuAB serum Mix 500µl of HuAB serum (Freezing Medium A) with 50.0 ml of 1X PBS.
		5.2.9	Pool all the individual cell suspensions into a single sterile 50 ml tube.
		5.2.10	Bring the total volume of the cell suspension to 40 ml with 1X PBS + 1% HuAB (for preparation use example in Step 5.2.8)
		5.2.11	Mix cells well by pipetting. Take out 20µl of the cell suspension to count cells in Step 6.0.

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 5.2.11 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 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.
Hot Spot		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 of left vertical perimeter line of any corner square (indicated with yellow lines). Do not count any cells that touch either the bottomline or right vertical perimeter line of any corner square. Use the formula in Section 6.6, to determine the number of cells per ml. 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 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 5.2.10 Example: Total viable cells = 94.5 x 10^4 x 40 ml = 378 x 10^5 = 3.78 x 10^7

…in continuation from 5.2.11			
		5.2.12	Centrifuge cell suspension at 330 RCF from Step 5.2.11 for 10 minutes at RT. Keep the break on.
		5.2.13	Using the formula in Section 6.6, estimate the number of cells. Based on the cell count, label the appropriate number of cryotubes (1.8ml) 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.
		5.2.14	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). Resuspend 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 resuspended promptly
	5.3	Cryopreservation of PBMC	
		5.3.1	After resuspending 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 gently by 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 10 x 10^6/ml.	
Hot Spot		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 forces!** IMPORTANT: Write cell number 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 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 (300µl) 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 bulk supply and additional labels found inside the kit. Indicate in the comments field of the Requisition Form that 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 do not open the freezer in order to avoid shaking 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 (that is 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 (let 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 do not open 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 laboratory 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.	

Reagent Preparation

Dulbecco's PBS Ca++, Mg++ free, 1X
Catalog # 21-031-CM Cellgro Vendor: VWR Standard
Also can use standard PBS (Ca++Mg++ free) or HBSS (Ca++Mg++free)

Freezing Medium A: HuAB serum (GeminiBiotech), tested for assay compatibility, heat inactivated and filtered. It can be thawed and refrozen up to 3 times or can be thawed and kept at 4°C for up to one month.

Freezing Medium B: 20% DMSO (Sigma D2650) in heat inactivated HuAB serum, filtered. It can only be thawed one time and must be used the same day.

DSMO Hybri-Max tm Sigma D2650, graded for sensitive cells.
It is not primarily the toxicity of DMSO itself, but the osmotic shock caused by DMSO rapidly that harms the cells most. Also, the viability and functionality of the cells is much better if DMSO is added at room temperature.

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

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