STANDARD OPERATING PROCEDURE				
Ragon Institute of MGH, MIT and Harvard	**PBMC and Plasma Separation from Leukapheresis Blood Products for the Processing Laboratory**		**SOP #**	PL-SOP-002
	Originated by:	Ildiko Toth	Date:	25 April 17
	Laboratory:	Processing Laboratory	Pages:	1 of 3
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I. PURPOSE:

The purpose of this procedure is to establish and outline the process for PBMC and Plasma Separation from Leukapheresis Blood products.

II. SAFETY:

This protocol needs to be carried out in a biosafety cabinet in BSL 2+ Lab. Personnel are required to wear safety glasses, gloves and a disposable gown before initiating any work and a secondary pair of gloves over the first before entering the biosafety cabinet. Secondary pair of gloves MUST be removed every time while exiting from the hood even if it is for a short time.

III. REAGENTS:

	Item	Vendor	Catalogue #	Lot #
1	Histopaque 1077 (Ficoll)	Sigma	H8889	
2	RPMI-1640	Sigma	R0883	
3	Pen/Strep (5000 IU Pen/ 5000ug/mL Strep)	Mediatech	30-001-Cl	
4	L-glutamine (200mM; 29.2 mg/mL)	Mediatech	25-002-Cl	
5	HEPES (1M; 238.3mg/mL)	Mediatech	25-060-Cl	
6	Fetal Bovine Serum (must first be Heat Inactivated by our lab)	Sigma	F-6178	
7	Hanks Balanced Salt Solution	Sigma	H-9394	
	DMSO	Fisher	EC200-664-3	
	Brady printer labels	Fisher	22500854	
	Internal cryotubes	Fisher	12565167 N	
8	Vesphene	Steris	14-415-11	

IV. PROCEDURE:

1. Warm up media (Hanks') and Histopaque 1077 (Ficoll).

2. Using sterile technique transfer the content of plasma bag into two 50ml conicals. Spin for 15 minutes at 2600 RPM and **collect plasma** (1.5 ml for each cryo-preserve tube). Store in –80 ASAP. **Sorval program #3**
3. Using sterile technique transfer blood product to a T75 flask and record the volume.
4. **Transfer 10 ml** of the blood product to 50 ml conical. Add Hanks' media up to 45 ml and **spin for 5 minutes at 1500 RPM – SLOW START AND SLOW STOP.**
5. Remove and discard supernatant, but do not get too close to the pellet (those steps are to remove platelets) and add 45 ml of Hanks' again, mix and **repeat spin**.
6. Remove and discard supernatant leaving 15 ml of volume in the tube. Add 15 ml of Hanks' and re-suspend by gently pipetting up and down several times.
7. **Layer** 15ml of Histopaque under the cells/Hanks' mix in each tube.
8. **Spin** for 30 minutes 1500 R+ - SLOW START/STOP. **Sorval program #1**
9. Gently remove tubes from centrifuge and discard supernatant leaving *at least* 10 ml above cell layer.
10. Using 10 ml pipette, gently collect the buffy coat layer, which is the middle small cloudy layer. Transfer to a new 50 ml conical tube, and discard the residual blood product.
11. Add up to 45 ml of Hanks' and mix well. PBMC layer will be very thick but try and dissolve it the best you can. **Spin** 1500 RPM 10 minutes **(first wash)**. **Sorval program #2.**
12. After first wash – remove supernatant leaving approximately 10 ml of media and the pellet of PBMC. Resuspend by mixing cells with 10 ml pipette and pool contents of 2 tubes into 1 x 50 ml conical. Add up to 45 ml of Hanks' and mix gently. **Spin** 10 minutes at 1500 RPM (second wash).
13. **Repeat** step 15 (third wash).
14. **Resuspend cells** in 300 ml R10 media (*added with pipette so that counting calculations are accurate)* in a T175 flask. *Now that cells are in a large volume/container it's extremely important to mix very well before taking any aliquots!!*
15. Take 50 ul of the suspension and add to 10 ml of R10 (200 fold dilution). After load the nucleocasette with cells, wipe the end of it with diluted Vesphene and insert the casette into the nucleocounter immediately. Multiply the reading by 200 and you get the PBMC/ml results (in general between 19-50 M/ml). Multiply by another 300 and you will get the final yield (in general in 3-10 billion). Repeat the counting twice.

16. **Transfer** 25 ml cell suspension into each of the 12 conicals, Repeat the washing step.
17. During spin **start the freezing machine**.
18. Aspirate media leaving about 200-500 ul above the pellet. Re-suspend the pellet by **scraping** the conicals on the perforated surface of the hood and add the freezing solution to the suspension. **Mix** the cells and solution carefully and aliquot it into the cold cryovials. Place any conicals not being used for freezing on ice. Freezing needs three people 4 x 25 ml aliquots/each person. Note that concentration is irrelevant for freezing calculations since all we want to do is split up the volume of cells we have into 300 equal vials.
19. After you completed aliquoting 25 cryotubes put the rack on ice before you take out the next rack with the pre-cooled cryotubes.
20. When all 300 vials are ready, load the FM. When the freezing is completed, **transfer** the vials into Liquid Nitrogen tanks and enter the location into CIDB.

V. REFERENCES/ ADDITIONAL/ NOTES:

1. Before receiving the blood product/s, **print** 300 PBMC vials (1 M PBMC/vial) and 25 plasma labels (1.5 ml) using the Brady printer and CIDB. Ensure that the visit has been entered and the labels show pheresis. Store the vials in -20C freezer before use.

2. **Prepare freezing solution**: In a T175, mix 291.6 ml of Fetal Bovine Serum (FCS) and 32.4 ml of DMSO. Aliquot 27 ml solution into 12 conicals: We always make 2 ml more freezing solution than the actual volume (25 ml)