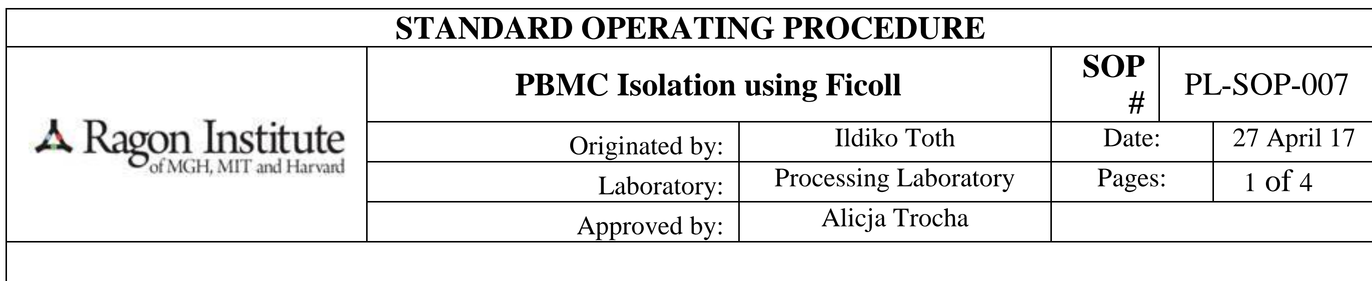


STANDARD OPERATING PROCEDURE				
Ragon Institute of MGH, MIT and Harvard	PBMC Isolation using Ficoll		SOP #	PL-SOP-007
	Originated by:	Ildiko Toth	Date:	27 April 17
	Laboratory:	Processing Laboratory	Pages:	1 of 4
	Approved by:	Alicja Trocha		

I. PURPOSE:

The purpose of this procedure is to isolate peripheral blood mononuclear cells (PBMC) from blood collected in vacutainers (ACD, EDTA or Heparin), whole blood collected in a bag, or from buffy coats.

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/Strp (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	
8	Vesphene D-125	Steris Microgen, Inc	14-415-11	

IV. PROCEDURE:

1. Wipe down tissue culture hood with 70% ethanol. Keep everything inside the hood completely sterile.

2. If the starting material is blood in vacutainers, add 70% ethanol to a gauze sponge and holding the blood tube in the tissue culture hood, open the rubber stopper away from you and discard the rubber stopper in the small bucket containing diluted Vesphene (or D-125) already in the hood. Carefully transfer the blood of two vacutainers using a serological pipet into a 50 ml conical tube. Add Expose from the squeeze bottle to all empty vacutainers and allow setting at least 15 minutes in the back of the tissue culture hood prior to disposing the liquid into the Erlenmeyer flask and the vacutainers into the sharp containers. Skip to step #5.

3. If the starting material is a buffy coat, wipe scissors with alcohol and place inside the hood on a gauze sponge. Working over the waste bucket in the hood, carefully cut open the buffy tubing. Let the blood pour into a 50 ml conical and later aliquot 3-4 ml buffy coat/50 ml conical tube. Avoid touching the tube with the buffy. Do not squeeze the buffy—just let gravity do its work.

4. If the starting material is whole blood, wipe scissors and the bag with alcohol and place inside the hood on a gauze sponge. Working over the waste bucket in the hood, carefully cut open the tubing of the bag. Let the blood pour into a T225 flask. Aliquot 20 ml of blood into 50 ml conicals.

5. To collect plasma (no plasma is collected from the buffy coat) spin the 50 ml conicals for 15 min at 2600 rpm. Carefully removed the conicals from the centrifuge and harvest the plasma using a 5 ml pipette. In general we save 4 vials of 1.5 ml plasma from any HIV-, 5 from any chronic treated, 10 from chronic untreated, 15 from viremic controller and the maximum number (15 from 6 ACD and 25 from 10) from elite controller. From the whole blood large blood donation we save 50 x 1.5 vials of plasma (you need to spin 10x 50 ml conicals to be able to harvest that amount of plasma).

6. Whether the starting material is blood in vacutainers, whole blood, or buffy coat, adds Hank's solution + PLGH media to the conical tube(s) to a final volume (blood or buffy coat + media) of 30ml. Buffy coat is concentrated , so the tube can only contain about 6-8 ml of buffy and rest would be Hanks .

7. Mix the media and blood thoroughly with a 10 ml pipet taking care not to introduce air bubbles.

8. Put the pipettor on the slowest down setting. Tear off the side of one of the holes in the Styrofoam conical holder so that you can see down to the bottom of the conical tube. This will help you layer the Ficoll.

9. Layer a full 10 ml pipet (14 ml) of Ficoll **underneath** the blood/media mixture by putting the filled pipet all the way to the bottom of the conical and then raising up a tiny bit. Let the Ficoll come out very slowly to create the sharp layer. See Figure 1.

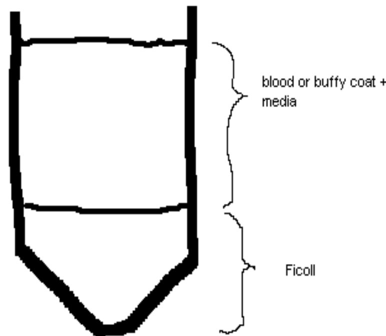


Figure 1. PBMC Isolation on Ficoll prior to centrifugation.

10. Spin all conical tubes at 1500 rpm for 30 min at room temperature with a slow start (#2) and no brakes. (Spinning will take closer to 45 min than 30 min.) Make sure to balance the centrifuge and put covers on the centrifuge buckets.

11. Tube will look like Figure 2 after centrifugation.

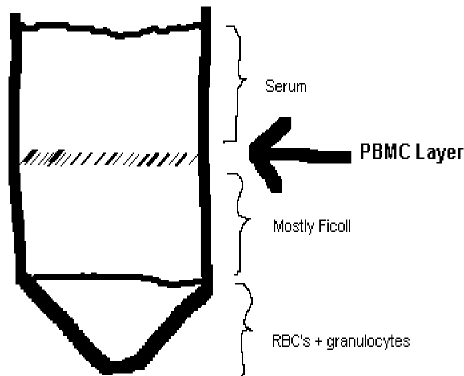
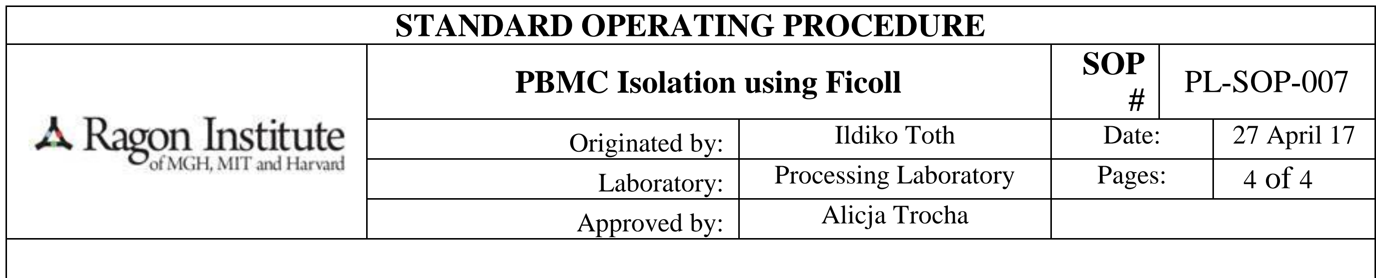


Figure 2. PBMC Isolation on Ficoll prior after centrifugation.

12. Aspirate most of the serum layer from each 50 ml conical tube, using caution not to get too close to the PBMC layer.

13. Harvest the PBMC layer (layer between the media and the Ficoll) using a 10 ml pipet and placing into a new 50 ml conical. (Note: If the starting material was a buffy coat, the PBMC layer from 4 conicals can be consolidated into 2 at this point.) Don't worry if you take some of the media and/or Ficoll with you, because that's what the washes are for.



Avoid taking the red blood cells on the bottom. It's better to leave a little of the mononuclear layer if it would cause you to take lots of red blood cells with it.

14. Fill all conicals up to 45 ml with media Hank's media, mix the media and cells gently and spin for 1500 rpm for 10 min.

15. After the first wash, aspirate all except for a 1-2 ml of media. Make sure not to take any of the pellet.

16. Add 10 ml of media and resuspend the pellet. Fill conicals up to 45 ml with media and then spin again 1500 rpm for 10 min.

17. After second wash, wash a third time by repeating the procedure as above.

18. After the third wash, aspirate the media and resuspend the pellet in 40 ml R10..) Take a representative aliquot of 200 ul into an Eppendorf tube and load the nucleocasette for counting.

19. If the starting material was buffy most probably you need to make a dilution with R10.

20. Place PBMCs in a 37° C, 5% CO_2 incubator with cap slightly unscrewed, or transfer into a T25 to work with the cells at a later time.

V. REFERENCES/ ADDITIONS/ NOTES:

a. Before starting the procedure, take the Ficoll and media out of the refrigerator and put into 37C water bath.
b. All centrifugations are performed at room temperature.
c. When separating PBMC from blood in vacutainers or whole blood, use 20 ml blood, 10 ml media and 14 ml Ficoll.
d. When separating PBMC from a buffy coat, the PBMC are far more concentrated, use ~3-4ml buffy coat: ~26-27mL media:~15ml Ficoll.