Institut für HIV Forschung
SOP#13-00 **(Feb 2017 - JS)**

PBMC Isolation Using Ficoll – HIV$^-$/Buffy Coat

I. Purpose: The purpose of this procedure is to isolate plasma and peripheral blood mononuclear cells (PBMC) from buffy coats.

II. 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	

III. Personal Protective Clothing and Equipment:

a. Fluid-resistant disposable lab coat must be worn during all steps.
b. Two pair of exam gloves must be worn for all steps performed in the tissue culture hood. A single pair of gloves for all other steps.
c. Protective eyewear is optional.

IV. Notes:

a. Before starting the procedure, take the R+ out of the refrigerator and let warm to room temperature.
b. When separating PBMC from a buffy coat, the PBMC are far more concentrated, so the goal is a ratio of ~20mL buffy coat: ~10mL media:~15mL Ficoll.
c. Be sure to dispose of all pipettes that come into contact with blood/PBMCs in the **black** waste bucket. The red one can be used for pipettes that only came into contact with media
d. Note the number of the buffy coat!

V. Protocol

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

2. Spray the buffy coat bag with 70% EtOH and place inside the hood.

3. Transfer the blood from the buffy coat into 2 - 3 50 ml falcons, be careful when filling the conicals!

4. Spin all conical tubes on Program 1 (600xg, RT, 10 min, 9 acceleration, 6 brakes). Note that spinning will take slightly longer due to decreased brake. Make sure to balance the centrifuge and put covers on the containers.

5. If the ordered buffy coat has the blood group AB, please prepare 1x 50 ml falcon per buffy coat to collect the plasma for cell culture. Label it with "Human Plasma", Buffy Coat ID, date and your initials. If the blood group is A, B, or 0 skip this step.

6. After the spinning, **carefully** move the conical tubes to the hood and collect plasma with a 25ml pipette, take care not to take any of the PBMCs or red blood cells, and fill the plasma into the prepared 50 ml falcon (for blood group AB only!). Freeze the plasma at-20°C.
   For blood groups A, B or 0: Only remove the plasma and discard it into the liquid waste.

7. Mix the leftover blood with medium (10ml media per 20 ml blood). After adding appropriate volume of media, carefully but thoroughly mix the suspension. Label appropriate amount of Falcon tubes per buffy coat (number + Ficoll; 30ml/tube) and place in blue tube rack [for example: a buffy coat with 40 ml of blood will require 20 ml media and will need 2 Falcon tubes].

8. Aliquot 15ml of Ficoll into each 50ml Falcon tube you labeled with "Ficoll"

9. Turn off the blow out setting on the pipetter. Remove the cap from the 50ml conicals of Ficoll you are layering. This will help you layer the Ficoll.

10. Using a 25ml pipette, aspirate 30ml of the blood/media mixture. Picking up the Ficoll conical and turning it to a 45° angle, carefully layer the blood/media on top of the Ficoll by **slowly** dripping the mixture down the inside of the conical allowing it to slowly form a layer. After ~8ml, slowly rotate the Ficoll conical to a more vertical angle (70°) and finishing layering at this angle, always allowing the mixture to drip down the inside of the conical. See Figure 1 for what the final product should look like.

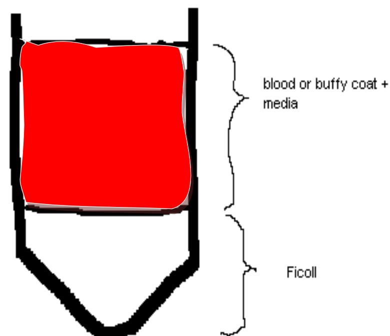

Figure 1. PBMC Isolation on Ficoll prior to centrifugation.

11. Spin all conical tubes on Program 2 (900xg, RT, 35min, 9 acceleration, 2 brakes). Note that spinning will take closer to 45 min than 30 min. Make sure to balance the centrifuge and put covers on the containers.

12. Label 1x new 50 ml falcon per buffy coat for collection of PBMCs after spinning and label it with the appropriate ID number.

13. Tube will look like Figure 2 after centrifugation.

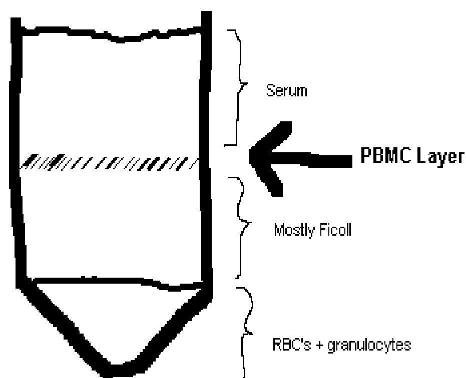

Figure 2. PBMC Isolation on Ficoll prior after centrifugation.

14. Harvest the PBMC layer (layer between the media and the Ficoll) using a 10 ml pipet or a transfer pipet and place it into the before prepared 50 ml conical (labeled with buffy coat number!). 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. Combine PBMCs from the same buffy coat into one 50ml tube (or 2, if the PBMC layer is very thick).

15. Fill all conicals up to 45 ml with media and spin down using Program 3 (600xg, RT, 10min, 9 acceleration, 9 brakes).

16. During the spin, take out 2 x 1.7ml Eppy tubes per buffy coat from the sterile box inside the hood. These will be used to count the cells. Pipette 190ul of R+ into half of the Eppy tubes and 10ul of Trypan Blue into the other half.

17. After the first wash, aspirate all except for a 1-2mL of media. Make sure not to take any of the pellet. (If you had more than 1 50ml Falcon with PBMCs for 1 buffy coat, combine the PBMCs into 1 of the Falcons).

18. Add 40 ml of media to each tube. Resuspend the pellet with a 10ml serological pipette.

19. Now we will count the cells. As the cell counter is more accurate at lower concentrations, we will perform a 1:20 dilution first by taking 10ul from resuspended cell solution and mixing it in a tube containing 190ul of R+. Take 10ul from this thoroughly mixed dilution and mix it with an aliquot (10µl) of Trypan Blue.

20. Retrieve 1 slide per buffy coat from near the cell counter and remove them from their packages. Label 1 slide per buffy coat. Pipette 10 µl of the Trypan Blue/cell mixture into each side of the slide for every buffy coat.

21. Begin spinning the cells again on Program 4 (same as Program 3, except at 4°C to begin chilling cells). During the spin, count the cells by averaging the "Live" measurements of both sides of each slide and multiplying by 800 (40ml media x 1:20 dilution. The cell counter already factors in the 1:2 dilution of Trypan Blue. So if your average live count is 2.3×10^5, your final cell count is $[2.3 \times 10^5] \times 800 = 184 \times 10^6$ PBMCs).

22. After second wash, wash a third time (aspirate media down to 1-2mL, then add 40mL media and resuspend pellet.) using Program 4.

23. For freezing cells, please refer to Protocol 08-01 Freezing.

24. Be sure to log the patient, number of cells isolated, and all other relevant information in the "PBMCs aus Buffy Coats" excel file on the lab server.