

PBMC Isolation Using Ficoll

- I. **Purpose:** The purpose of this procedure is to isolate peripheral blood mononuclear cells (PBMC) from whole blood or from buffy coats.

II. **Reagents:**

	Item	Vendor	Catalogue #	Lot #
1	Biocoll (Ficoll)	Biochrom	L 6115	
2	RPMI-1640	Sigma	R0883	
3	Pen/Strep (5000 IU Pen/ 5000ug/mL Strep)	Mediatech	30-001-CI	
4	Fetal Bovine Serum (must first be Heat Inactivated by our lab)	Sigma	F-6178	
5	Hanks Balanced Salt Solution	Sigma	H-9394	

III. **Buffers**

a. **R+**

RPMI 1640 500ml

Pen/Strep 5.5ml

IV. **Personal Protective Clothing and Equipment:**

- Fluid-resistant disposable lab coat must be worn during all steps.
- 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.
- Protective eyewear is optional.

V. **Notes:**

- Before starting the procedure, take the R+ out of the refrigerator and warm to 37C.
- All centrifugations are performed at room temperature.
- When separating PBMC from whole blood, the goal is to centrifuge with a 1:1:1 ratio of whole:media: Ficoll (e.g. ~15mL whole blood:~15mL media:~15mL Ficoll)
- When separating PBMC from a buffy coat, the PBMC are far more concentrated, so the goal is a ratio of ~3-4mL buffy coat: ~26-27mL media:~15mL Ficoll.
- 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

VI. Protocol

1. Wipe down tissue culture hood with 70% ethanol. Keep everything inside the hood completely sterile.
2. Spray the bag containing the vials of blood with 70% EtOH and place inside the hood. Using the red rack, organize the vials of blood into rows. The red caps on the vials indicate whole blood (usually 4-5 per patient), while the brown cap vial (there is only 1) contains coagulated blood and serum.
3. Place all the serum vials in the centrifuge (using the brown adapters) and place the lids on the buckets to seal them. Use program 1 to spin them (Program 1 = 600xg, 10min, 9 acceleration, 6 brake).
4. Using the gauze located in the hood, remove the caps from vials containing whole blood and discard both the gauze and cap in the garbage bag inside the hood. Carefully transfer the blood using a 10ml serological pipet into a 50 ml conical tube for a final volume of ~15mL blood/50 mL conical tube (pipette 2 vials worth of blood into each Falcon tube and split the 5th vial into both). Add 3% Incidin from the squeeze bottle to all empty blood vials and allow them to sit for at least 15 minutes in the back of the tissue culture hood prior to aspirating.
5. We collect serum and plasma from every patient, but we need to first assign the patient an identification number. Login to the computer in the lab and on the server open “HPSTD-PBMC Log MASTER”
6. Enter as much patients’ names, birthdays, and case numbers (usually begins with 36) from the printed label on the blood vials into the appropriate columns. Assign the patient an ID number by doing 1 of 2 things:

If this is a repeat patient (you will know this because excel will try to autofill when you begin to type their name in) then find the patient from earlier in the list and copy their ID number to the new entry.

If this is a new patient, click on the tab labelled “Free ID Numbers” at the bottom. Choose any number and click the cell. Press “strg+X” to cut the cell and paste it into the ID number space for the new patient entry. This will delete that ID from the list of available numbers. Repeat for every new patient.

7. Using the template located on the server (“Cryo Template”), create 6 labels per patient using this format:

Patient: #####

Case: #####
Substance (see below)
Today's Date

We collect 5 x 1.5ml of Plasma and 1 x 1.5ml of Serum. On 5 of the labels, replace "Substance" with "Plasma", and on the last one replace it with "Serum". Use the cryo label sheets located next to the printer and print the labels. When inserting the sheet into the printer, make sure the labels are face-up.

8. You will collect the whole blood into 2 x 50ml Falcon tubes and, later, layer each tube of blood onto its own Ficoll. Label 4 x 50ml Falcon tubes per patient (Last name + blood or Ficoll) and place in blue tube rack.
9. Place 6 external threaded cryo tubes for each patient in the small, blue Eppendorf rack. Place the printed labels from Step 6 on each tube. (Pro tip: loosen the caps on the cryo tubes because they are annoying to unscrew with one hand.)
10. After the serum has completed spinning, **carefully** move the serum vials to the hood and aspirate ~1.5ml of serum using a 5ml pipette from the vial and aliquot 1.5ml serum into the properly labeled external threaded cryotube for freezing. If you are able to aspirate 3ml of serum (very rare) then get another cryo tube and label/aliquot the additional serum.
11. Repeat Step #3 with the 50ml Falcon tubes containing the whole blood, but using the blue adapters in the centrifuge.
12. While the blood is spinning, Aliquot 15ml of Ficoll into each 50ml Falcon tube you labeled with "Ficoll"
13. We keep a hard record of all samples we have processed. Remove the label on the serum vial and place in the logbook located near the cell counter. Please also record the number of 1.5ml vials of serum and plasma collected when possible.
14. After the blood has finished spinning, **carefully** move the blood tubes to the hood. Use a 10ml serological pipette and aspirate 4ml of plasma from one tube and 3.5ml from the second tube for each patient. Aliquot 1.5ml of the collected plasma into 5 labeled external threaded cryotubes. Take care not to aspirate the blood.
15. Add 15ml R+ to each conical tube, to a final volume of 30mL. This is best done by aspirating 30ml of R+ with a 25ml serological pipette and aliquoting 15ml into each tube of a patient. Mix the blood/R+ thoroughly and keep the pipette!
16. 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.

17. Using a 25ml pipette, aspirate the full volume 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.

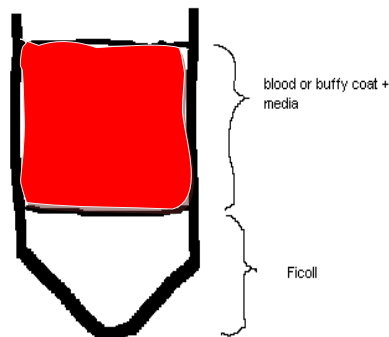


Figure 1. PBMC Isolation on Ficoll prior to centrifugation.

18. Spin all conical tubes on Program 2 (900xg, 35min, 2 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.
19. During the spin, take the collected plasma/serum to the -80C freezer and place in the appropriate box located on the 3rd shelf from the top. Make note of which box, each patient's samples went into.
20. Tubes will look like Figure 2 after centrifugation.

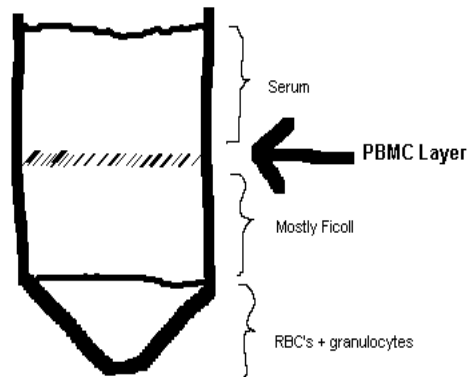


Figure 2. PBMC Isolation on Ficoll prior after centrifugation.

21. Harvest the PBMC layer (layer between the media and the Ficoll) using a 10 ml pipet and placing into a new 50 ml conical. 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 buffy coats from the same patient into one 50ml tube.
22. Fill all conicals up to 45 ml with media (either R+ or Hank's depending on when the blood was drawn) and spin down using Program 3 (600xg, 10min, 9 acceleration, 9 brakes).
23. During the spin, take out 2 x 1.7ml Eppy tubes per patient from the sterile box inside the hood. These will be used to prepare the cells for being counted. Pipette 90ul of R+ into half of the Eppy tubes and 10ul of Trypan Blue into the other half.
24. After the first wash, aspirate all except for a 1-2mL of media. Make sure not to take any of the pellet.
25. Add 30 ml of media to each tube. Resuspend the pellet with a 10ml serological pipette.
26. Now we will count the cells. Aliquot 10ul Trypan Blue into 1 x 1.7ml Eppy tube for every patient. Pipette 10ul of the cell suspension from Step #25 into one of the Eppy tubes containing Trypan Blue and mix thoroughly.

27. Retrieve 1 slide per patient from near the cell counter and remove them from their packages. Label 1 slide per patient. Pipette 10ul of the Trypan Blue/cell mixture into each side of the slide for every patient.
28. Begin spinning the cells again on Program 3. During the spin, count the cells by averaging the “Live” measurements of both sides of each slide and multiplying by 300 (30ml media x 1:10 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 $[2.3 \times 10^5] \times 300 = 69 \times 10^6$ PBMCs).

If the cell counter gives an error message (“above accurate range”) then they must be recounted. The cell counter is more accurate at lower concentrations, you can perform a 1:10 dilution first by taking 10ul from resuspended cell solution and mixing it in a tube containing 90ul of R+.
29. Record the number of live cells and viability of each patient in the log book.
30. After second wash, wash a third time (aspirate media down to 1-2mL, then add 30mL media and resuspend pellet.) If using cells immediately, spin using Program 3, if freezing cells use Program 4 (same as Program 3, except at 4C to begin chilling cells).
31. Place PBMCs in a 37° C, 5% CO₂ incubator with cap slightly unscrewed, or transfer into a T25 to work with the cells at a later time.
32. If freezing cells, please refer to Protocol 08-01 Freezing