Monocyte Isolation Protocol

Materials Needed:

- At Least 1x 10 mL sodium heparin vacuum tube filled with whole blood (provided via blood draw)
- 10 mL of 1-Step Polymorphs for every 10mL of whole blood
- 3x 8 mL tubes for every 10 mL of whole blood.
- Many 5 mL flow tubes. At least 2 per 10 mL of whole blood plus 4 for general processing.
- Aspirating mechanism (e.g. Glass Pipettes + Vacuum Flask, or Cell Culture Hood Aspirator)
- **Wash Buffer** (Stored in fridge, ingredients below, mix then sterile filter (0.22µm filter) in hood)
  - 500 mL $HBSS^{--}$
  - 1.19g HEPES sodium salt for 10 mM concentration

    **We have 1 M HEPES buffer solution in Awad Lab. So, you can add 5mL of 1 M HEPES solution into the 495 mL of $HBSS^{--}$ for the correct concentration of 10mM (0.01M)*

  - 2.5g of lyophilized BSA for a 5 mg/mL concentration
- PBS at 1/6thx and 4x concentrations
- Hemocytometer, Microscope, and Centrifuge capable of holding 8 mL tubes and 5 mL flow tubes.
- Two 50 mL tubes to store wash and isolation buffer respectively to keep the source sterile. This should be enough to process 20 mL of whole blood
- 15 mL conicals (at least one for every 8 mL tube).
- **Isolation Buffer** (Stored in fridge, ingredients below, mix then sterile filter in hood)
  - 500 mL $DPBS^{--}$
  - 500 mg of lyophilized BSA
  - 2 mL of 0.5M EDTA
- Electronic Pipettor and many Serological Pipettes (10 mL works well)
- Regular Pipettor (P1000 w/ tips, and P200 w/ tips)
- Monocyte Isolation Kit (Dynabeads FlowComp Human CD14 Kit)
  - Dynabeads $CD14^{+}$ (Invitrogen Cat. # 11149D)
  - Human CD14 Monoclonal Antibody (Invitrogen Cat. # MHCD1404)
  - Magnet for collecting CD14+ cells
    - (https://www.thermofisher.com/us/en/home/brands/product-brand/dynal/magnets.html?cid=fl-bid-magnets)
  - Mixer allowing mixing and rotation of tubes
- Preferred Media (usually MCDB-131, *or RPMI 1640 with 10% FBS, 1% anti-anti, 1% P/S*)

A note on centrifuges for wash steps: McGrath Lab protocol is to go 350g for 10 minutes at room temp, Ma Rie uses 350g for 7 minutes at 15 degrees C. For neutrophils our way of doing it is sufficient, for monocytes Ma Rie suggests her way.

Protocol:

1. Perform a blood draw and acquire at least 10 mL of whole blood from a donor.

2. Cool the blood samples to room temperature, takes about 30-40 minutes. The blood will be in sodium heparin tubes as provided by the phlebotomist.

3. Aliquot 3 mL of 1-Step Polymorphs into 8 mL tubes. Fill one tube per 3 mL of whole blood that needs to be processed. For example, if you get 10 mL of blood -> 9 mL blood usable -> 3 tubes needed each with 3 mL 1-Step Polymorphs.

4. Using an electronic pipettor, take 3 mL of whole blood and GENTLY layer it on top of the 1-Step Polymorphs inside the 8 mL tube. The best way to do this is slowly let the blood drip down the side wall of the tube. You will end up with a 3 mL layer blood on top of a 3 mL layer of 1-Step Polymorphs. Do not aggressively pipette or else the two fluids will mix, ruining the sample.

5. Centrifuge at 500g for 30 mins. Prepare the wash buffer in the meantime. It's best to aliquot something like 45 mL in a 50 mL conical so you can keep your source sterile. If the wash buffer is cold, warm it up in a water bath (2-3 mins) so that it comes to room temperature. Also begin to warm up your preferred media.

6. Post centrifugation 6 layers are present:

-Plasma

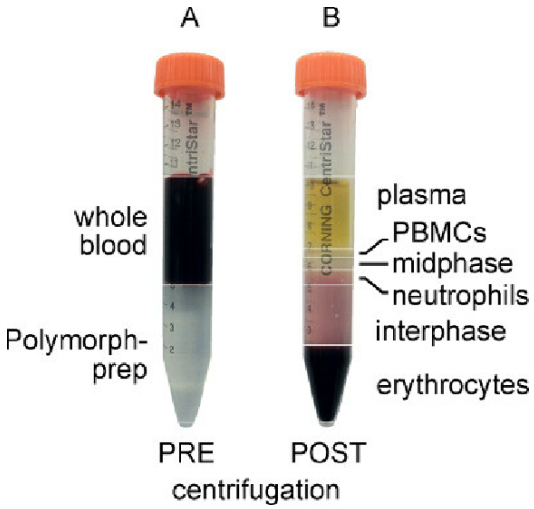
-Monocytes (PBMCs)

-Buffer/Midphase

-Neutrophils

-Buffer/Interphase

-RBC's



For each 8 mL tube, take the monocyte layer with a P1000. Set it to 500 microliters, carefully extract the layer, and deposit it in a 15 mL conical. Note, if you need neutrophils, you can grab the neutrophil layer instead. The washing and RBC lysing steps are the same for both neutrophil and monocyte isolation.

Washing Steps/RBC Lyse

7. Fill each 15 mL conical containing a monocyte layer with wash buffer, up to the 10 mL mark. Do a gentle inversion, and then spin at 350g (1000 rpm in the McGrath lab cell culture room centrifuge) for 7 minutes at 15 deg C.

8. After the spin, aspirate the wash buffer until you have about 500 microliters of fluid and a pellet left in each 15 mL conical. Resuspend each pellet with a P1000 (don't add any additional wash buffer), then

combine three of these volumes into one 15 mL conical. Every 10 mL of whole blood leads to three 0.5 mL suspensions, you should now have 1.5 mL in combined cell suspensions per 10 mL of whole blood.

9. Add wash buffer (up to the 10 mL mark) in the single 15 mL conical. Spin at 350g for 7 minutes at 15 deg C.

10. After this wash, aspirate to about 500 microliters, resuspend the pellet with a P1000 (don't add any additional fluid), and add 4.5 mL of 1/6thx PBS. Wait for 1 minute, then add 1.5 mL of 4x PBS. Spin at 350g for 7 minutes at 15 deg C.

> 10.1 (Alternative): Use 4.5 mL of Ultrapure H20, then immediately add 1.5 mL of 4x PBS. Spin at 350g for 7 minutes at 15 deg C.

11. Aspirate to ~500 microliters. Resuspend the pellet with a P1000 (adding no additional fluid), fill the conical with wash buffer up to the 10 mL mark, and spin at 350g for 7 mins at 15 deg C.

12. After this last wash, aspirate as much wash buffer as possible. Resuspend the monocyte pellet in 2 mL of ***isolation*** buffer. Count the monocytes using a hemocytometer (use 10 microliters). There should be around 10^7 PBMCs if all went well (for ~10mL of whole blood processed).

Monocyte Isolation from PBMC Layer

13. Add enough isolation buffer to the ~ 2 mL of suspended pellet to hit the 14 mL mark, then spin it down (350g, 7 mins, 15 deg C), aspirate the isolation buffer, and fill the tube again to 0.5 mL of fresh isolation buffer.

14. Prep the bead solution in the monocyte isolation kit. Vortex the bead solution to suspend it (30 seconds). Put 75 µL of bead solution in a 5 mL flow tube, do one for each sample (i.e. per 10 mL whole blood). Add 1 mL of isolation buffer, then place on the magnet for two minutes. Once the beads are magnetically attached to the side wall, aspirate the fluid, and resuspend the beads in 75 µL isolation buffer.

15. Add antibodies from the monocyte isolation kit (for each 500 µL suspension add 25 µL of antibodies) into the monocyte suspension and mix well. Store this at 2-8 deg C for 10 minutes, no tilting yet!

17. Retrieve the cells/antibodies mix after 10 minutes, now add 2 mL of isolation buffer to it. Spin at 350g for ***8 minutes*** at ***4 deg C***.

18. Remove the supernatant from the cells/antibodies mix, resuspend the pellet with 1 mL of isolation buffer.

19. Mix the beads in and mix well via pipetting. Put on tilt for 15 minutes at 2-8 deg C. This can go for a little longer and serves as a great time for a lunch break.

20. Add 1 mL of isolation buffer, put the monocyte isolation sample on a magnet for 2 minutes.

21. Aspirate the fluid, this removes anything that's CD14 negative. Add 1 mL of isolation buffer and wash with the magnet for 2 minutes. Aspirate this fluid once it's done.

22. Resuspend everything with 1 mL of ***release buffer*** (found in the monocyte isolation kit). Mix this well and incubate for 10 minutes at 2-8 deg C on a tilt table.

23. Pipette the sample ten times to release the beads, put the sample back on the magnet for two minutes.

24. Transfer this supernatant to a new tube, put it back on the magnet for 1 minute. After one minute, put the fluid containing our purified monocytes into a new tube.

25. Add 2 mL of isolation buffer, spin at 350g for 8 mins at 4 deg C. Discard the supernatant and resuspend the pellet in the media of choice. Keep monocytes at 2-8 deg C until ready to use. The cells should also be counted with a hemocytometer.