MGH COVID-19 Effort Blood Processing Protocol for PBMC, Neutrophils and Plasma Isolation

For blood processing protocol questions, please contact:
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OVERVIEW

- This protocol is designed to have two people processing samples: one person should focus on PBMC isolation while the other person on neutrophil isolation and plasma aliquots.
- This protocol will require the use of **two separate biosafety cabinets** to allow required "social distancing" between individuals processing samples.
- This protocol will require the use of **two separate centrifuges: one for blood and one for neutrophil processing. Neutrophil processing is optional.**
- The number of patient samples that can be processed in a single batch is limited by centrifuge access. Currently plan to be able to do up to 28 patient samples in a batch.
- ~8 mLs of blood per patient will be split into two parts:
  - 0.25-0.5 mL of the ~8mL will be used for neutrophil isolation and lysis (details on p. 7-9),
  - remainder will be used to isolate and cryo-preserve PBMCs, and freeze plasma aliquots (details on p. 3-6)

BIOSAFETY

- Sample hand-off: Upon arriving at the lab entrance (doors are locked), the CRC bringing the sample(s) should call the lab. There will be a box in front of the locked door. The CRC with the blood will put the blood sample in the box and once they see one of the researchers through the window, they will walk away. This will ensure that samples are never left unattended. Once the CRC is gone, the researcher will open the door with proper PPE, and wipe down the box containing the samples with 10% bleach before entering any lab space.
- Perform all procedures in a BL2+ lab space, inside a sterile hood
- All plastics (tubes, tips, pipets) should be disposed in a large beaker filled with 10% bleach.
- PPE: Disposable lab gown, face shield, face mask, double gloves, sleeve covers, shoe covers.
- Use a sealed container (e.g. covered centrifuged buckets, plastic box, or ziploc bag) when transferring samples in and out of the hood. Spray down and wipe the secondary container with 10% bleach followed by 70% EtOH before putting inside hood, after opening the bag/box, spray down the tube inside with 10% bleach followed by 70% EtOH.
- When using covered centrifuged buckets, spray with 70% EtOH before putting inside the hood. Upon closing the buckets, spray again the covered buckets with 70% EtOH before moving the buckets outside the biosafety cabinet (prior to moving the buckets back to the centrifuge).
- When moving the centrifuge buckets to the centrifuge with samples in them, make sure to use a cart.
- After finishing your work and/or before a new person starts working in the hood, make sure to clean surfaces first with 10% bleach, wait ~10 min, and then spray with 70% EtOH.

PBMC Isolation

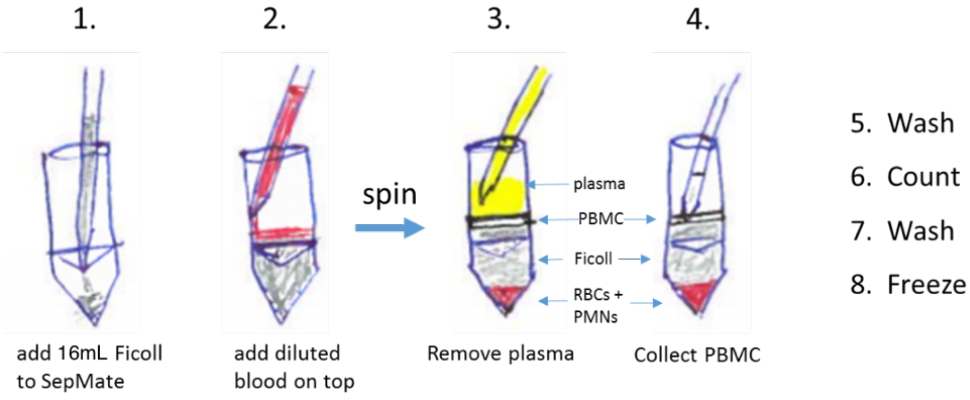
Reagents and materials required per draw

- 1 50 mL SepMate tube
- 1 50 mL conical tube
- 2 15 mL conical tubes
- 1 5 mL transfer pipet
- Serological pipets: aspirating, 10 mL
- 1.5mL cryopreservation tubes
- trypan blue at room temperature
- 15 mL Ficoll at room temperature (Note, Ficoll will remain at room temperature in the hood in aluminium foil)
- 16 mL RPMI at room temperature
- 50 mL RPMI refrigerated
- ~2.5mL Cryostor (CS10) refrigerated

Required equipment

- Pipet-aid and serological pipets (10mL, 25mL)
- P200 pipet and tips
- Microscope
- Hemocytometer
- Tally counter (hand clicker)
- Calculator
- Centrifuge capable of accommodating at least two 15mL conical tubes.
- Alcohol-resistant markers
- Mr. Frosty freezing container containing isopropanol, refrigerated at 4deg

OVERVIEW



DETAILED PBMC ISOLATION PROTOCOL

1. **First thing EVERY morning:**
   - RPMI at room temperature: Note that before starting work on blood samples, RPMI (20ml per sample) will need to be brought at room temperature. Every morning, bring out at least 2 bottles of RPMI out. It may take up to 30min to bring it to room temperature. Note that only the 2:1 blood dilution step requires **RT RPMI**, the remainder of the protocol uses **cold** RPMI to maintain cell viability.
   - **Ficoll at room temperature:** Ficoll should be used at room temperature. We will keep a ficoll bottom in the biosafety cabinet in aluminium foil. Every morning, make sure you have at least half a bottle full in the hood. If not, bring in a new bottle wrap with aluminium foil
   - Prepare 1mL TCL + 1% BME in a 1.5 mL eppendorf tube that should be enough for the day. This may need to be adjusted based on number of samples received. Make sure to RNaseZap the surface of the chemical hood and pipettes before transferring.
   - Prepare 2 trash buckets with 10% bleach solution (one for each hood)
   - Make sure there are enough labels in the label printer
2. **Receiving batches of blood and transport to biosafety cabinet:** Blood samples will be processed every 3 hours. Upon receiving a batch of blood samples, in a team of 2 people, bring the bag(s) containing the vacutainer in the BL2+ cell culture space. Spray down and wipe the bag(s) containing the vacutainers with 10% bleach followed by 70% EtOH before putting inside the hood, after opening the bag/box, spray down the tube inside with 10% bleach followed by 70% EtOH.
3. **Sample organizing and recording:** In team of two, one person will organize the vacutainers in the hood in holder, taking care of grouping the 2 vacutainers collected for each individual together. Person #1 will read the information on the vacutainers to person #2 that will take note in a spreadsheet of:
   - (i) sample ID (will be already encoded)
   - (ii) date
   - (iii) time of blood collection,
   - (iv) blood volume received or at least whether 1 or 2 vacutainers was received
   - (v) time at which blood processing started
   - **Labeling tubes:** Upon compiling information for EACH patient, person #1 labels the following sets of tubes with patient code for all collected samples
   - one 50ml falcon tubes (for RPMI mixing),
   - one 50ml SepMate tube (if receive < 3ml of blood, use 15mL sepmate tube)
   - one 15mL falcon tube to keep empty (for neutrophil step)

   Organize the tubes in the hood in racks for blood transfer

50ml Rack 2nd row	50ml Sepmate with 16ml Ficol #1	50ml Sepmate with 16ml Ficol #2	50ml Sepmate with 16ml Ficol #3
50ml Rack 1st row	50ml tube Blood+RPMI #1	50ml tube Blood+ RPMI #2	50ml tubeBlood+ RPMI #3

15mL rack 1st row	15ml empty sample #1	15ml empty sample #2	15ml empty sample #3
Rack that holds vacutainer	2X vacutainers sample #1	2X vacutainers sample #2	2X vacutainers sample #3

4. Invert vacutainer 5x to mix blood. Don't shake
5. For transferring blood to the different tubes, proceed with ONLY one patient sample set at a time and avoid opening and closing vacutainers.
6. Gently open one vacutainer and move whole blood to a separate 15 mL conical tube for neutrophil isolation, based on the amount of blood received. **Do NOT put on ice. Note that this step could be skipped if neutrophil isolation is not needed**:
   - 4-8ml blood- take 500ul for neutrophil isolation
   - 2-3ml blood- take 250ul for neutrophil isolation
   - Don't take blood for neutrophil isolation if have <2ml of blood

   After completing blood transfer for neutrophil, disinfect the 15ml conical tubes with 10% bleach solution and handoff the tubes to person #2 who can move the tubes to another biosafety cabinet to proceed with RBC lysis and neutrophil isolation (see p.7)

7. Using a 10 mL serological pipet, transfer the remaining blood into the **EMPTY 50 mL tube**. Take note of the total volume of blood transfer (you need the number to calculate RPMI volume). After finishing the blood transfer, move serological pipet to the bleach bucket
8. For each of the tubes listed in step 7, make sure to fill with the correct buffer:
   - Fill 50 mL conical with (2x volume of blood) RPMI at room temperature (e.g. if received 8ml of blood, should fill the falcon tube with 16mL of RPMI)
   - Fill one 50 mL SepMate tube with 16 mL room temp Ficoll, add through the hole in the middle of the cone inside the tube
     i. If blood volume is < 3 mL, use a 15 mL SepMate tube and fill with 4.5 mL Ficoll
9. Repeat steps 4-8 until all blood samples have been transferred to the empty 15mL falcon tube and the 50mL falcon tube containing blood
10. Mix all the 50 mL tube by inverting 5x
11. Using a different 25 mL serological pipet for each patient, transfer the diluted blood into the SepMate tube. Slowly pipet down the side of the tube to avoid blood mixing with Ficoll that may come out of the hole in the insert (<1 mL/s . After finishing the blood transfer, move serological pipet to the bleach bucket
12. Centrifuge at 1200 rcf 20 min at **20 deg C** with maximum acceleration and the brake on.
13. During centrifugation, prepare:
    - Grab Mr. Frosty from -80 freezer if not already in lab refrigerator
    - An ice bucket
    - Pre-fill round-bottom well 96-well plate with 10uL trypan blue. Prepare one well per sample (for counting)
    - Label one 15 mL conical for plasma per sample, leave empty
    - Label another 15 mL conical for PBMC per sample, leave empty
    - Grab RPMI bottle from 4C fridge and keep it on ice
14. After centrifugation, carefully remove the SepMate tube, verify the PBMC layer is visible, and place in a rack.
    - If no layer is visible, centrifuge again at 1200 rcf for **10 min** at 20 deg C with the brake on
15. Change centrifuge temp to 4C and close the lid.
16. Using a 10mL serological pipet collect 5 ml plasma from the tube and transfer into the empty 15 mL conical tube. **Place on ice until later.**

17. Remove remaining plasma by aspirating, leave ~3 mL above the layer
18. With a transfer pipet**,** collect and transfer PBMC layer from the SepMate tube and move to the 15 mL conical
19. Top up the conical tube to 15 mL with **COLD** RPMI
20. Centrifuge 300 rcf 5 min a**t 4 deg C using max acceleration and max break**.
21. Remove the supernatant by aspirating, avoid disturbing the pellet, and place tube on ice.
22. Resuspend the pellet in **1 mL COLD** RPMI. **Place on ice.** If the pellet is not visible, resuspend in 500 ul or less cold RPMI.
23. Add 10uL of the cell suspension to the round bottom 96-well plate containing 10uL trypan blue. Count the cells to determine the yield and number of aliquots to be frozen. *See "Counting PBMCs" protocol.*
24. Top up the tube to 10 mL with RPMI, centrifuge the cell suspension 300 rcf 8 mins at 4 deg C with max acceleration and max brake
25. During centrifugation
    - Gather cell freezing materials: cold Crystor (CS10), refrigerated Mr. Frosty, cryo vials.
    - Print cryotube labels and stick on tubes: label with "**Blood PBMC**", subject ID and timepoint "**PXX-DY**", **aliquot number, number of cells,** and **date**.
    - For each patient, we will freeze aliquots of 0.5M cells each and freeze the extra leftover as a separate aliquot (e.g. if get 4.3M cells, will freeze 8 aliquots of 0.5M cell each and freeze 300K cells separately). Note that if the leftover cell is less than 200K cells (e.g. having 4.2M cells), then distribute the 200K cells across all aliquots.
26. Remove supernatant through aspiration,
27. Resuspend pellet in cold Cryostor (0.5mL per 0.5M cell aliquot) using the total volume to be used to cover all aliquots accounted for per sample (e.g. If obtained 4.6M cells, will use 4.6mL to resuspend pellet). Resuspend pellet by gently pipetting up and down 10X
    - Note that cryostor solution is viscous, so should pipette up and down slowly.
28. Pipet 0.5 mL cell suspension into cryotubes, transfer to Mr. Frosty, **IMMEDIATELY** place in -80 freezer (minimum 12 hrs). **Record time blood sample was frozen.**
29. The next day, cells can be moved from Mr. Frosty to a cardboard box (labeled PBMC-1...). Note down on the spreadsheet which box and position the samples are placed.
30. Frozen cells can be kept between 1-7 days in -80, before being sent to the Broad on dry ice.

COUNTING PBMCS AND DETERMINING # OF ALIQUOTS OF PBMCs

1. Make sure the cell suspension is well mixed by pipetting up and down.
2. Transfer 10uL of cell suspension into cryovial containing 10uL of trypan blue and mix with pipet. Pipet up and down slowly, at least 5 times.
3. Load 10uL of the diluted cells into one of the hemocytometer reservoirs.
4. If using an automated cell counter (Countess- Invitrogen or BioRad), make sure to load 10ul of the diluted cells into the right plastic cell counter chamber. Insert to machine and press button- “count cells”. Cells will be counted automatically and concentration per ml will be given at the end, including the viability of cells. When using the BioRad counting machine make sure that lower gate is on 7um to avoid counting red blood cells.
5. Count the number of cells in one corner 4x4 grid (count all cells in the squares).
    a. PBMC cells should have a light blue hue (due to nucleus staining)
    b. Count the cells in contact with the borders of only two sides of the squares.

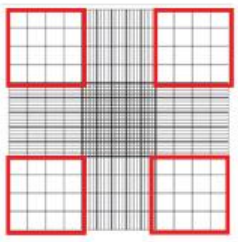


Figure 1: Hemacytometer grid

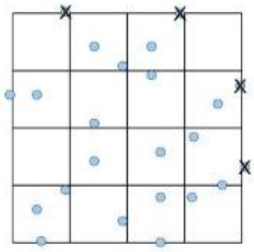


Figure 2: Cell counting guidelines

PLASMA PROTOCOL

1. Once samples are in the freezer, centrifuge the plasma 1000 rcf 5 mins at 4 deg C with max acceleration and max brake.
2. During centrifugation, print cryotube labels and stick on 3 cryotubes: label with “**Plasma**”, subject ID and timepoint “**PXX-DY**”, **aliquot number,** and **date**.
3. Without disturbing the pellet, aliquot 1.5 mL of supernatant into 3 cryovials (4.5 mL total). The remainder can be discarded.
4. Freeze the aliquoted plasma at -80 in a normal cardboard or plastic box (labeled Plasma-1...). Note down on the spreadhsset which box and position the samples are placed. Take note of the time when samples were frozen.
5. Plasma samples can be shipped to Broad along with the PBMCs.

NEUTROPHIL ISOLATION AND LYSIS (*OPTIONAL*)

** Note that the neutrophil isolation and lysis steps are not essential to the PBMC isolation protocol. The steps detailed below allow to isolate neutrophil cell fractions from fresh blood in parallel to isolating PBMC from the same blood sample.*

Reagents and materials required per draw

- 4 X 15 mL conical tubes
- Serological pipets: aspirating, 10 mL
- 2 X 1.5mL cryopreservation tubes
- 250uL 10X RBC lysis buffer
- EasySep Direct Human Neutrophil Isolation Kit (includes Isolation Cocktail and RapidSpheres)
- Trypan Blue
- 1X PBS
- UltraPure 0.5M EDTA, pH 8
- 200 uL TCL
- 2ul 2-Mercaptoethanol, bME (store in a Corrosives chemical cabinet)

Required equipment

- Pipet-aid and serological pipets
- P1000 and tips
- P200 and tips
- P20 and tips
- EasySep Magnet for 5mL round-bottom tube
- Microscope
- Hemocytometer
- Tally counter (hand clicker)
- Calculator
- Centrifuge capable of accommodating at least two 15mL conical tubes
- Alcohol-resistant markers

Before starting:

- *Set centrifuge to room temp (RT) for RBC lysis*
- *Have EasySep magnet on hand*
- *If the anticoagulant in the vacutainer is not EDTA, add 1 uL of 0.5 M EDTA to the blood*
- *Make PBS+EDTA buffer: 500mL PBS + 1mL of 0.5M EDTA, keep at room temperature*
- *Label three round-bottom tubes and one 15 mL conical tube with patient ID*

RBC Lysis

This step should be performed while other person is preparing for Ficoll isolation.

1. Organize the 15mL tubes containing 500ul whole blood in the hood
2. Add 8 mL of ACK lysis buffer to blood using a 10mL serological pipette and mix the sample (x5 times) . When done, move the pipette to the bleach bucket.
3. Spin 300g for 5 min at **room temperature** with max acceleration and max deceleration
4. Aspirate supernatant, be careful not to disturb the pellet.
5. Resuspend cells in 250ul of PBS+EDTA buffer at RT by pipetting gently up and down gently 5X.
6. Continue to EasySep isolation

Neutrophil EasySep Isolation

- *At anytime, do not cap the 15mL tube*

1. Vortex RapidSpheres for 30 seconds
2. Mix the isolation cocktail with 200ul pipette by pipetting up and down 10X

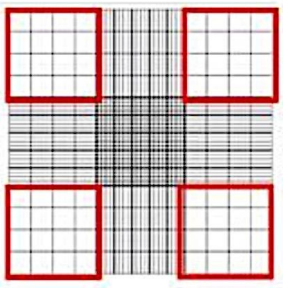
	RapidSphere volume	Isolation cocktail volume
500-1ml blood	50ul	50ul
250ul blood	25ul	25ul

3. Add Isolation Cocktail to sample
4. Add RapidSpheres to sample
5. Mix gently by pipetting up and down 10X, and incubate for 5 min at RT
6. After 5 min, top up to 4 mL with PBS+EDTA buffer at RT
7. Close the tube tightly and then mix gently by tilting the tube back and forth 5X Place tube into magnet. Make sure that each 15mL tube sit well at the bottom of it without lid
8. Incubate for 5 min at RT on magnet
    - While on the magnet, label a second set of 15ml tube
9. After 5 min, using a 5 mL serological pipet, transfer supernatant into new 15mL tube (**tube 2**)
    - Angle the pipet such that the tip is opposite the magnet to avoid touching the beads
    - **Note, procedure is negative selection so neutrophils will be in supernatant**
10. Add 25ul of RapidSpheres to the new 15mLtube (**tube 2**) if started with 500ul whole blood or 12.5ul if started with 250ul whole blood.
11. Close the tube tightly and mix gently by tilting the tube back and forth 5X (avoid bubbles)
12. Incubate at RT for 5 min
13. After 5 min, place tube on magnet (without lid)
14. Incubate 5 min at RT on magnet
    - While on the magnet, label a third set of 15mL tube
15. After 5 min, using a 5 mL serological pipet, transfer supernatant into new 15mL tube (**tube 3**)

16. Place the new tube (tube 3) with the supernatant back on the magnet
17. Incubate for 5 min at RT on magnet
    - While on the magnet, label a 15ml Falcon tube (**tube 4**)
18. After 5 min, using a 5 mL serological pipet, transfer supernatant (~3.5mL) into a 15 mL tube (**tube 4**)
19. Spin 300g at RT for 5min with full acceleration and full deceleration
20. Aspirate supernatant, be careful not to disturb the pellet
21. Resuspend in 1 mL PBS+EDTA and mix well. Count the cells to determine the yield and number of aliquots to be frozen. *See "Cell counting" protocol.*

Cell counting

1. Add 10 ul of resuspended cells to 10 ul of trypan blue
2. Mix well by pipetting up and down, then add 10ul to hemocytometer
3. If using an automated cell counter (Countess- Invitrogen or BioRad), make sure to load 10ul of the diluted cells into the right plastic cell counter chamber. Insert to machine and press button- "count cells". Cells will be counted automatically and concentration per ml will be given at the end, including the viability of cells. When using the BioRad counting machine make sure that lower gate is on **4um** to avoid counting red blood cells.
4. Counting cells with a hemocytometer:
    i. There are four sections of 4x4 grids (outlined in red below)
    ii. Count cells in two of the 4x4 grids (take average between the two grid counts)
    iii. When counting, include cells on the edges of 2 sides of each grid, and exclude cells on the edges of the other 2 sides of each grid (as shown below on the right)
    iv. Note: if there are not many cells, count number of cells in all four grids and take the average of all four (will be more accurate)
    v. Take average of cells counted in 4x4 grids to use as "# cells" for the calculation below



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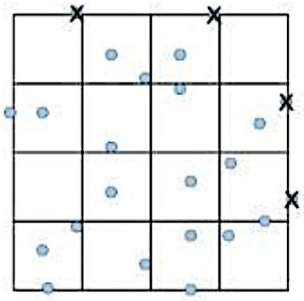

Figure 2: Cell counting guidelines

Cell Lysis

Desired outcome

- *2 cryotubes per patient each tube with 100ul of neutrophil lysate in each*
- *Final concentration of cells in RNA lysis buffer = 1000 cells per ul*
- *A total of 200k cells to split between two cryotubes*

1. Move 200k cells to a **1.5ml Eppendorf tube** by transferring an appropriate volume of the cell suspension
    a. Determine fraction of cells to transfer to new tube: 20/('# cells')
    b. Example: if '# cells' is 20, use 500 ul
    c. 100,000 / (20 x 10,000) = 0.5, transfer 0.5 x 1 mL
2. Top up tube of cells to 1.5 mL with PBS+EDTA
3. Spin 300g at RT for 5 min
    a. Make fresh RNA lysis buffer (TCL + 1% bME) according to number of samples received. For example: for 1ml of lysis buffer, take 990 uL TCL + 10 uL bME (perform this step in a chemical hood). Make sure to RNAZap the surface of the hood where the lysis buffer will be used and pipettes to be used and use Rnase free tips for pipetting.
    b. Print cryotube labels and stick on tubes: label with "**Neu Lysate**", subject ID and timepoint "**PXX-DY**", **aliquot number, concentration of cells ('1k/uL'),** and **date**.
4. Pipet out supernatant and remove excess with P200 (be cautious)
5. Resuspend in 200 uL RNA lysis buffer. if lower number of cells are retrieved, resuspend cells in lower volumes to reach a 1000 cell/ 1ul.
    a. If less than 10,000 cells are recovered, resuspend in 10ul and note cell concentration on the cryotube. Freeze a single cryotube.
6. After resuspending cells in 200ul, mix well and transfer 100ul to two cryotubes
7. Place tubes on dry ice immediately
8. After 5 mins, the aliquoted lysates can be moved to the -80 in a normal cardboard or plastic box. Note down on the spreadsheet which box and position the samples are placed. Take note of the time when samples were frozen.

Reagent Ordering Information

15 mL SepMate tubes: Stemcell Technologies, 85415; 100 tubes

50 mL SepMate tubes: Stemcell Technologies, 85450; 100 tubes

15 mL conical tubes: VWR, 21008-918; 50 tubes

Ficoll: VWR cat no. 95021-205; 6x100mL bottles.

Cryostor CS10: Stemcell Technologies cat no. 7956; 100mL bottle

PBS: ThermoFisher cat no. 10010023; 500mL bottle

RPMI: ThermoFisher cat no. 11835055; 10x500mL bottle

“Mr. Frosty” freezing container: VWR cat no. 55710-200

VWR lab marker, alcohol-resistant: VWR cat no. 52877-310

1.5 mL Cryovials: VWR cat no. 66008-710

5mL polystyrene round-bottom tube: VWR International Catalog #60819-820

UltraPure 0.5M EDTA, pH8: Life Technologies (Thermo Fisher) Catalog # 15575020

10X RBC lysis buffer: Life Technologies (Thermo Fisher) Catalog # 00-4333-54

EasySep Direct Human Neutrophil Isolation Kit: Stemcell Technologies Catalog # 19666

3% Acetic acid with Methylene Blue: Stemcell Technologies Catalog # 07060

PBS: Life Technologies (Thermo Fisher) Catalog #10010049 (10 bottles)

Buffer TCL: Qiagen Catalog # 1031576

2-Mercaptoethanol, bME: Thermo Fisher Catalog #21985023