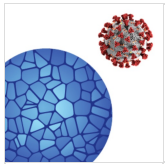

Oct 30, 2020

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

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1 Works for me dx.doi.org/10.17504/protocols.io.bjhnkj5e

Human Cell Atlas Method Development Community | Coronavirus Method Development Community

Megan Freund

ABSTRACT

This protocol outlines the steps and methods for blood processing for PBMC to assist the MGH COVID-19 effort.

ATTACHMENTS

Villani_AC_MGH_COVID_Blood_Processing_Protocol_04-23-2020_SUBMITTED.pdf

DOI

dx.doi.org/10.17504/protocols.io.bjhnkj5e

PROTOCOL CITATION

Moshe Sade-Feldman, Miguel Reyes, Alexandra-Chloe Villani 2020. MGH COVID-19 Effort Blood Processing Protocol for PBMC, Neutrophils and Plasma Isolation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bjhnkj5e

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COVID-19, blood processing, PBMC, neutrophils, plasma isolation

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Villani_AC_MGH_COVID_Blood_Processing_Protocol_04-23-2020_SUBMITTED.pdf

GUIDELINES

1. The protocol is designed to have two people processing samples: one person should focus on PBMC isloation while the other person on neutrophil isolation and plasma aliquots.
2. This protocol will require the use of two separate BSCs to allow required "social distancing" between individuals

processing samples.

3. This protocol will require the use of two separate centrifuges: one for blood and one for neutrophil processing. Neutrophil processing is optional.
4. The number of patient samples that can be processed in a single bacth is limited by centrifuge access. Currently plan to be able to do up to 28 patient samples in a batch.
5. Approximately 8 mLs of blood per patient will be split into two parts: 0.25-0.5 mL of the 8 mL will be used for neutrophil isolation and lysis while the remainder will be used to isolate and cryo-preserve PBMCs and freeze plasma aliquots.

MATERIALS TEXT

Materials and equipment required for PBMC Isolation

PPE

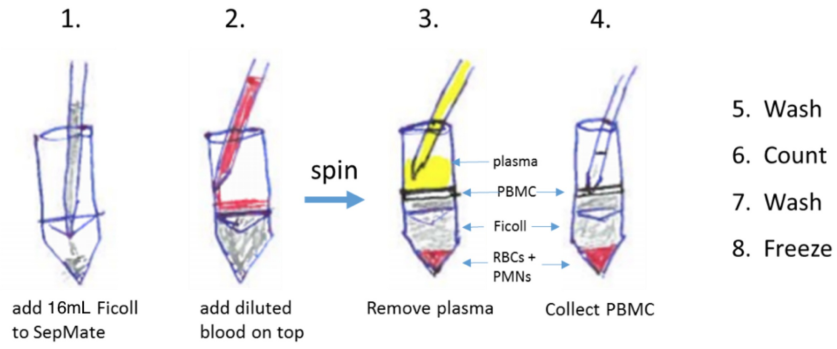
- Disposable lab gown
- Face shield
- Face mask
- Double gloves
- Sleeve covers
- Shoe covers

Required Equipment

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

Reagents and materials required per draw

- One 50mL SepMate tube
- One 50mL conical tube
- Two 15mL conical tubes
- One 5mL transfer pipet
- Serological pipets: aspirating, 10mL
- 1.5mL cryopreservation tubes
- Trypan blue at room temperature
- 15mL Ficoll at room temperature (NOTE: Ficoll will remain at room temperature in the hood in aluminium foil)
- 16mL RPMI at room temperature
- 50mL RPMI refrigerated
- ~2.5mL Cryostart (CS10) refrigerated



PBMC Isolation OVERVIEW

Citation: Moshe Sade-Feldman, Miguel Reyes, Alexandra-Chloe Villani (10/30/2020). MGH COVID-19 Effort Blood Processing Protocol for PBMC, Neutrophils and Plasma Isolation. https://dx.doi.org/10.17504/protocols.io.bjhnkj5e

Materials and equipment required for optional Neutrophil Isolation and Lysis

- 4 x 15mL conical tubes
- Serological pipets: aspirating, 10mL
- 2 x 1.5mL cryopreservation tubes
- 250u: 10X RBC Lysis Buffer
- EasyStep 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 corrosives chemical cabinet)
- Pipet-aid and serological pipets
- P1000 and tips
- P200 and tips
- P20 and tips
- EasySep Magnet for 5Ml round-bottom tube
- Microscope
- Hemocytometer
- Tally conter (hand clicker)
- Calculator
- Centrifuge capable of accommodating at least two 15mL conical tubes
- Alcohol-resistent markers

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

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

1. For 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.
2. Perform all procedures in a BL2+ lab space inside a sterile hood.
3. All plastics (tubes, tips, pipets) should be disposed of in a large beaker filled with 10% bleach.
4. 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 the hood. After opening the bag/box, spray down the tube inside with 10% bleach

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followed by 70% EtOH.

5. 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 BSC (prior to moving the uckets back to the centrifuge).
6. When moving the centrifuge buckets to the centrifuge with samples in them, make sure to use a cart.
7. 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 approximately 10 minutes, then spray with 70% EtOH.

ABSTRACT

This protocol outlines the steps and methods for blood processing for PBMC to assist the MGH COVID-19 effort.

BEFORE STARTING

First thing EVERY morning:

- RPMI at room temperature: Note that before starting work on blood samples, RPMI (20mL per sample) will ned to be brought at room temperature. Every morning, bring out at least 2 bottles of RPMI. It may take up to 30 minutes to bring it to room temperature. Also note that only the 2:1 blood dilution step requires **RT RPMI**, the remainer 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 BSC in aluminium foil. Every morning, makre sure you have at least half a bottle full in the hood. If not, bring a new bottle wrapped with aluminium foil.
- Prepare 1mL TCL + 1% BME in a 1.5mL eppendorf tube that should be enough for the day. This may need to be adjusted based on number of samples received. Makre sure to RNaseZap the surface of the chemical hood and pipettes before transferring.
- Prepare two trash buckets with 10% bleach solution (one for each hood).
- Make sure there are enough labels in the label printer.

Receiving batches of blood and transport to the BSC: 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.

Sample organizing and recording: In a 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 teh vacutainers to per #2 that will take note in a spreadsheet of:

- sample ID (will be already encoded)
- date
- time of blood collection
- blood volume received or at least whether 1 or 2 vacutainers was received
- 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 tube (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 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

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1

Follow the steps detailed in the "Before Start" section prior to moving forward in this protocol.

2

Invert vacutainer 5x to mix blood.

Do not shake.

3 For transferring blood to the different tubes, proceed ONLY one patient sample set at a time and avoid opening and closing vacutainers.

4

Gently open one vacutainer and move whole blood to a separate 15mL conical tube for neutrophil isolation based on the amount of blood received.

- 4-8ml blood - take 5000ul for neutrophil isolation
- 2-3ml blood - take 250ul for neutrophil isolation
- don't take blood for neutrophil isolation if there is < 2ml of blood

Do not put on ice.

This step could be skipped if neutrophil isolation is not needed.

4.1 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 BSC to proceed with RBC lysis and neutrophil isolation.

5

Using a 10mL serological pipet, transfer the remaining blood in the EMPTY 50mL tube.

Take note of the total colume of blood transfer (you need the number to calculate RPMI volume).

6 After finishing the blood transfer, move serological pipet to the bleach bucket.

7 For each of the tubes listed in Step 5, make sure to fill with the correct buffer:

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- Fill 50mL conical with (2x volume of blood) RPMI at **Room temperature**
- Fill one 50mL SepMate tube with **16 mL room temperature Ficoll** and add through the hole in the middle of the cone inside the tube.
- If the blood volume is < 3mL, use a 15mL SepMate tube and fill with **4.5 mL Ficoll**

8 Repeat Steps 2-7 until all blood samples have been transferred to the empty 15mL falcon tube and 50mL falcon tube containing blood.

9 

Mix all the 50mL tubes by inverting 5x.

10 

Using a different 25mL serological pipet for each patient, transfer the diluted blood into the SepMate tube.

11 

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 bleach bucket).

12 

Centrifuge at **1200 rpm, 20°C, 00:20:00** with maximum acceleration and the brake on.

13 During centrifugation, prepare the following:
- Grab Mr. Frosty from **-80 °C** freezer if not already in lab refrigerator
- An ice bucket
- Pre-fill round-bottom well 96-well plate with **10 µl Trypan Blue** and prepare one well per sample (for counting)
- Label one 15mL conical for PBMC per sample, leave empty
- Grab RPMI bottle from **4 °C** fridge and keep **On ice**

14 After centrifugation, carefully remove the SepMate tube and verify the PBMC layer is visible.

If no layer is visible, centrifuge again at **1200 rpm, 20°C, 00:10:00** with the brake on.

15 Place the SepMate tube into a rack.

16 Change the centrifuge temperature to **4 °C** and close the lid.

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17 Using a 10mL serological pipet, collect **5 mL plasma** from the tube and transfer into the empty 15mL conical tube.

18 Place **On ice** until later.

19 Remove remaining plasma by aspirating and leave approximately **3 mL** above the layer.

20 With a transfer pipet, collect and transfer PBMC layer from the SepMate tube and move to the 15mL conical tube.

21 Top up the conical tube to 15mL with **cold** RPMI.

22 

Centrifuge **300 rpm, 4°C, 00:05:00** using max acceleration and max break.

23 Remove the supernatant by aspirating, making sure to avoid disturbing the pellet, and place tube **On ice** .

24 Resuspend the pellet in **1 mL cold RPMI** .

25 Place **On ice** .

If pellet is not visible, resuspend in **500 µl or less cold RPMI.**

26 Add **10 µl cell suspension** to the roung bottom 96-well plate containing **10 µl Trypan Blue** .

27 

Count the cells to determine the yield and number of aliquots to be frozen.

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See the "Counting PBMCs" protocol.

28 Top up the tube to 10mL with RPMI.

29 

Centrifuge the cell suspension at **300 rpm, 4°C, 00:08:00** with max acceleration and max break.

30 During centrifugation:

- gather cell freezing materials: cold Crystor (CS10), refrigerated Mr. Frosty, cryo vials
- print cryotube labels and stick on tubes: lable with "Blood PBMC", subject ID, and timepoint "PXX-DY", aliquot number, number of cells, and date
- for each patient, we will freeze aliquots of **0.5 Molarity (M)** cells each and freeze the extra leftover as a spearate aliquot.

If the leftover cell is less than 200K cells, then distribute the 200K cells across all aliquots.

31 Remove supernatant through aspiration.

32 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.

33 

Resuspend pellet by gently pipetting up and down 10x.

The Cryostor solution is viscous so be sure to pipette up and down slowly.

34 

Pipet **0.5 mL cell suspension** intro cryotubes.

35 

Transfer to Mr. Frosty and immediately place in **-80 °C** freezer for a maximum of **12:00:00** .

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35.1 Record the time the blood sample was frozen.

36 The next day, cells can be moved from Mr. Frosty to a cardboard box (labeled PBMC-1...so on).

Note down on the spreadsheet which box and position the samples are placed.

Frozen cells can be kept between **24:00:00** and **168:00:00** in **-80 °C** before being send to the Broad Institute on dry ice.

Counting PBMCs and Determining # of aliquots of PBMCs

37 

Make sure the cell suspension is well mixed by pietting up and down.

38 

Transfer **10 µl cell suspension** into cryovial containing **10 µl Trypan Blue** and mix with a pipet slowly, up and down 5x.

39 Load **10 µl diluted cells** into one of the hemocytometer reservoirs.

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 into the machine and press the "Count Cells" button. 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 the lower gate is on 7um to avoid counting red blood cells.

40 

Count the number of cells in one corner 4x4 grid (count all cells in the squares).

- PBMC cells should have a light blue hue (due to nucleus staining)
- count the cells in contact with the borders of only two sides of the squares

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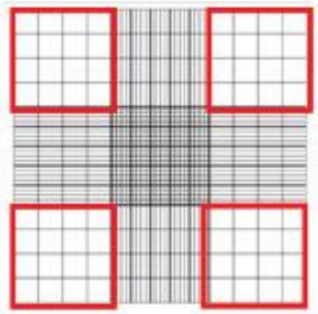

Figure 1: Hemacytometer grid

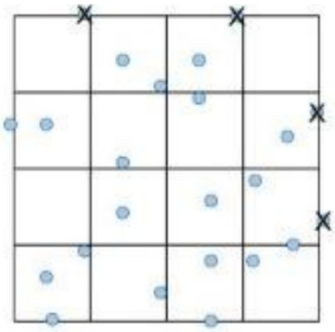

Figure 2: Cell counting guidelines

Plasma Protocol

41

Once samples are in the freezer, centrifuge the plasma at **1000 rpm, 4°C, 00:05:00** with max acceleration and max brake.

42 During centrifugation, print cryotube labels an stick on 3 cryotubes: label with "Plasma", subject ID, timepoint "PXX-DY", aliquot number, and date.

43 Without disturbing the pellet, aliquot **1.5 mL supernatant** into 3 cryovials (4.5mL total).

The remaining can be discarded.

44 Freeze the aliquoted plasma at **-80 °C** in a normal cardboard box or plastic box (labeled Plasma-1...so on).

44.1 Take note on the spreadsheet which box and position the samples are placed.

44.2 Take note of the time of when the samples were frozen.

Plasma samples can be shipped to the Broad Institute with the PBMCs.

Neutrophil Isolation (OPTIONAL)

45

Before starting the upcoming sections:

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

RBS Lysis

46

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

Organize the 15mL conical tubes containing **500 µl whole blood** in the hood.

47

Add **8 mL ACK lysis buffer** to blood using a 10Ml serological pipette and mix the sample 5 times.

48 When done, move the pipette to the bleach bucket.

49

Spin **300 x g, Room temperature , 00:05:00** with max acceleration and max deceleration.

50 Aspirate supernatant.

Be careful not to disturb the pellet.

51

Resuspend cells in **250 µl PBS+EDTA buffer** at **Room temperature** by pipetting up and down gently 5x.

52 Continue to EasySep isolation.

Neutrophil EasySep Isolation

53

Aft anytime, **DO NOT** cap the 15mL tube.

Vortex RapidSpheres for **00:00:30** .

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54 

Mix the isolation cocktail with 200uL pipette up and down 10x.

	RapidSphere volume	Isolation Cocktail volume
500-1mL blood	50uL	50uL
250uL blood	25uL	25uL

55 

Add isolation cocktail to sample.

56 

Add RapidSpheres to sample.

57 

Mix gently by pipetting up and down 10x.

58 

Incubate for **00:05:00** at **Room temperature** .

59 After **00:05:00** , top up to **4 mL** with PBS+EDTA buffer at **Room temperature** .

60 

Close the tube tightly and then mix gently by tilting the tube back and forth 5x.

61 Place tube into magnet.

Make sure each 15mL tube sits well at the bottm of it without lid.

62 

Incubate at **Room temperature** for **00:05:00** on magnet.

While on the magnet, label a second set of 15mL tubes.

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63 

After **00:05:00** , using a 5mL serological pipet, transfer supernatant into new 15mL tube (tube 2).

Angle the pipet such that the tip is opporsite the magnet to avoid toughing the beads.

Procedure is negative selection so neutrophils will be in supernatant.

64 Add **25 µl RapidSpheres** to the new 15mL tube (tube 2) if started with **500 µl whole blood** or **12.5 µl** if started with **250 µl whole blood.**

65 

Close the tube tightly and mix gentle by tilting the tube back and forth 5x (avoid bubbles.

66 

Incubate **00:05:00** at **Room temperature** .

67 After **00:05:00** , place tube on magnet (without lid).

68 

Incubate **00:05:00** at **Room temperature** on magnet.

While on magnet, label a third set of 15mL tubes.

69 

After **00:05:00** , using a 5mL serological pipet, transfer supernatant into new 15mL tube (tube 3).

70 Place the new tube (tube 3) with the supernatant back on th magnet.

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71 

Incubate for **00:05:00** at **Room temperature** on magnet.

While on magnet, label a 15mL flacon tube (tube 4).

72 

After **00:05:00** , using a 5mL serological pipet, transfer supernatant (~3.5mL) into 15mL tube (tube 4).

73 

Spin **300 rpm, Room temperature , 00:05:00** with full acceleration and full deceleration.

74 Aspirate supernatant, being careful not to disturb the pellet.

75 

Resuspend in **1 mL PBS+EDTA** and mix well.

76 

Count the cells to determine the yield and number of aliquots to be frozen.

See "Cell Counting" in the next section.

Cell Counting

77 Add **10 µl resuspended cells** to **10 µl Trypan Blue** .

78 

Mix well by pipeting up and down.

79 Add **10 µl** to a hemocytometer.

If using an automated cell counter(Countess, Invitrogen or BioRad), make sure to load 10µl of the diluted cells into

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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 into the machine and press the "Count Cells" button. 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 the lower gate is on 4um to avoid counting red blood cells.

80

Count the number of cells with a hemocytometer.

- There are four section of 4x4 grids (outlined in red below)
- Count cells in two of the 4x4 grids (take average between the two grid counts)
- 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 right)
- 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)
- Take average of cells counted in 4x4 grids to use as "# cells" for the calculation below.

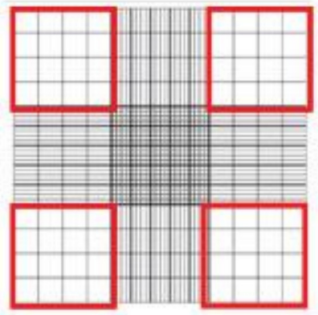

Figure 1: Hemacytometer grid

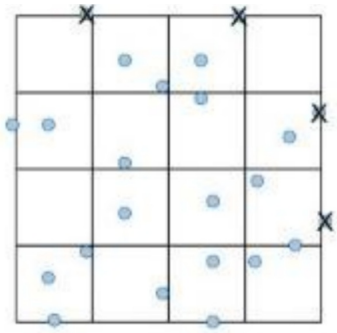

Figure 2: Cell counting guidelines

Cell Lysis

81 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

82

Mix 200K cells to a 1.5mL Eppendorf tube by transferring an appropriate volume of the cell suspension.

82.1 Determine fraction of cells to transfer to new tube: 20/('# cells").

83 Top up tube of cells to **1.5 mL** with PBS+EDTA.

84

Spin **300 rpm, Room temperature , 00:05:00** .

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84.1 Make fresh RNA lysis buffer (TCL + 1% bME) according to number of samples received.

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.

84.2 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.

85 

Pipe out supernatant and remove excess with P200 (be cautious).

86 Resuspend in **200 µl Lysis Buffer** .

If lower number of cells are received, resuspend cells in lower volumes to reach 1000 cell/1mL.

86.1 If less than 10,000 cells are recovered, resuspend in **10 µl** and note cell concentration on the cryotube. Freeze single cryotube.

87 

After resuspending cells in **200 µl** , mix well and transfer **100 µl** to two cryotubes.

88 Place tubes on dry ice immediately.

89 After **00:05:00** , the aliquoted lysates can be moved to the **-80 °C** in a normal cardboard or plastic box.

Note down on the spreadsheet which box and position the samples are placed and take note of the time when the samples were frozen.

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