**Blood Processing**

**Note: For blood draws use at least a 21G needle to minimize hemolysis. Also avoid aggressive suction during phlebotomy for this same reason.**

**PEth Card**

**Notes:**

* **DO NOT USE ETHANOL NEAR THE PEth CARDS.**
* **Use a purple top blood tube.**
* **When storing and shipping, do not use a plasticized envelope or airtight plastic specimen bag.**

1. Label a PEth card (USDTL) with the patient ID, the collection day (if applicable), and the date. Place a corresponding barcode label on a drying box.
2. Open the PEth card and lay on a new kimwipe.
3. PRIOR TO CENTRIFUGATION, load 0.5mL of whole blood from a purple top (EDTA) blood tube into a pipet tip.
4. Slowly pipet a large drop of blood at the end of the pipet tip. The drop should be big enough to fill a collection circle of the PEth card in a single attempt. Allow the drop of blood to come into contact with the center of a collection card and wick to fill the circle. DO NOT touch the card with the pipet tip or layer successive drops.
5. Repeat to fill the remaining collection circles.
6. Pipet any remaining whole blood back into the purple top tube and re-cap.
7. Close the PEth card to cover the blood spots and place the specimen in a barcode labeled drying box and close the ends. Spray a couple of kimwipes with hydrogen peroxide disinfectant, wipe off the PEth cardboard box and place it to the left “clean” side.
8. Once PEth blood spot card is dry, place it in the box and place box in the drawer for room temperature storage until shipment.

**Blood Draw for Serum, Plasma, and Buffy Coat Collection**

**Notes:**

* **Centrifuge tubes at 1000X g for 10 min at room temperature.**
* **After aliquoting, store all cryotubes at -80°C.**

**Green top (sodium heparin; AUDs, Controls, and Cannabis Users only)**(BD Vacutainer 4mL #367871, 6mL #367878) **-** aliquot 0.25-0.5mL aliquots of plasma off the top phase into 4 of the 1.8mL cryotubes (Nunc #375418) with green cap inserts (Nunc #355018), then collect red blood cells (RBCs) from the bottom phase and divide them into two 1.8mL cryotubes with yellow cap inserts (Nunc #355077). A 4mL green top will typically yield between 1 and 3mL of plasma.

**Red top** (BD Vacutainer 4ml #367812, 6mL #367815, 10mL #367820) **–** aliquot 0.25-0.5mL aliquots of serum off of the top phase into 8 of the 1.8mL cryotubes with red cap inserts (Nunc #354968). A 6mL red top will typically yield between 1 and 4mL of serum.

**Purple top (EDTA)**(BD Vacutainer 4mL #367861, 6mL #367863) **–** aliquot 0.25-0.5mL aliquots of plasma off the top phase into 8 of the 1.8mL cryotubes with purple cap inserts (Nunc #375922). A 4mL purple top will typically yield between 1 and 3mL of plasma. Then collect the buffy coat by pipetting the white layer of cells between the top phase and the red blood cell (RBC) bottom layer and pipetting it into a 1.8mL cryotube with a pink cap insert (Nunc #375884). Collect the RBC layer and pipette it into a 1.8mL cryotube with a grey cap insert (Nunc #375906).

**Blood draw for PBMC collection (AUDs, Controls, Cannabis Users)**

1. Centrifuge CPT tubes (BD #362761) at 1800X g for 30 min at room temperature.
2. Pipet off the top half of the supernatant. Collect the bottom half of the supernatant containing the PBMC layer and place into a 15mL conical tube. Bring the volume up to 15mL with 1XDPBS and centrifuge at 450 X g for 5 minutes.
3. Discard the liquid and resuspend the PBMC pellet in 2 mL of 1XDPBS.
4. Count cells and check viability:
5. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 40µm. Print the count.
6. Divide up the cells according to need below.

**SNAP FROZEN ALIQUOTS:**

**PBMCs for RNA Extraction or Protein:** Pipette 1x10 6 or 2x10 6 aliquots of cells into labelled microcentrifuge tubes. The number of aliquots will be determined by the total number of cells available. Centrifuge at 14000 X g for 2 minutes to pellet the cells. Aspirate off and discard the supernatant, and then immediately freeze and store the cell pellets at -80°C.

**CRYOPRESERVED ALIQUOTS:**

**Cryopreservation of PBMCs for Flow Cytometry and other applications:**

1. After cells have been counted, make an aliquot of 1x106 - 4x106.
2. Pellet cells at 400 X g for 5 minutes and remove supernatant.
3. Resuspend in freezing media at a final concentration of 10 x 106 cells/mL (Freezing Media: 10% DMSO - Sigma #D2650 + 90% FBS - Atlanta Biologicals #S11550H, filter sterilized -Corning #430320). For 1 x 106 cells, resuspend in 100ul of freezing media, for 4 x 106 cells, resuspend in 400ul of freezing media.
4. Place them in a freezing container (Nalgene Cryo 1°C Freezing Container or Biocision Cool Cell) and store at -80°C for 24 hours. Store the cells in a liquid nitrogen storage tank long term.

**STIMULATED CELL CULTURE**

**Secretion of cytokines/growth factors by LPS treated PBMCs:**

Make **Complete Media** for PBMCs: RPMI 1640 medium +10% FBS + 5% Pencillin/Streptomycin

Cell Medium- Corning RPMI 1640 modified w/L-Glutamine (#MT10040CV)

Fetal Bovine Serum- (Sigma #12306C-50mL)

100X Penicillin (10,000 IU)-Streptomycin (10,000 ug/mL) Solution- Corning (#30002CI)

Remove 55 mL of RPMI media and replace with 50 mL FBS and 5 mL of 100 X Pen/Strep Sol’n.

Store at 4◦C for no more than 1 month.

Take 1 mg stock Lipopolysaccharides from Escherich (LPS- Sigma #L6529-1MG) and reconstitute with 1 mL 1X PBS. Add 2 uL of reconstituted stock to 10 ml of RPMI+FBS+Pen/Strep. (Complete PBMC Media) for an LPS concentration of 200 ng/mL.

1. Will need 2 x 10 6 PBMCs. Plate 500,000 PBMCs in 1mL Complete PBMC media per well in 4 wells of a 12-well plate (Costar #3513)
2. To 2 of these wells, add an additional 1mL of Complete RPMI media
3. To the other 2 wells, add 1mL of RPMI with FBS, antibiotics, and LPS for a final LPS concentration of 100ng/mL.
4. Incubate the plate for 18 hours at 5% CO2 and 37°C.
5. The next day at the 18 hour time point, collect the media and cells in a 2mL microcentrifuge tube. Centrifuge for 2 minutes at max speed and collect the media in 1mL aliquots. Store at -80°C.
6. Save the cells for RNA extraction by adding 700 uL Buffer RLT from an RNeasy Plus Mini Kit (Qiagen #7414) with β-mercaptoethanol (Sigma #63689) to each pellet in order to lyse cells.
7. Load the collected sample onto a QiaShredder column (Qiagen #79654) placed inside a 2 mL collection tube from the kit. Centrifuge at max speed for 2 minutes. Discard QiaShredder column and cap the tube with sample with the provided caps and store the homogenized sample at -80◦C for up to 3 months.

**Bronchial Brushings Processing (AUDs, Controls, Cannabis Users)**

**Cytology Brush – DNA/RNA Shield**

1. One cytology (ConMed, #129R) brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 0.7mL DNA/RNA Shield (Zymo Research R1100-50).
2. Vortex the tube and store without removing brush at -80°C.

**Cytology Brush – Cytospins**

1. One cytology brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 1mL 1XDPBS (Cellgro #21-031-CV).
2. Vortex the cytology brush in 1XDPBS
3. Create 2 cytospins using 200ul of the cells in 1XDPBS for each slide.
4. Fix slides in methanol fixing solution from the Protocol Hema 3 kit (Fisher Scientific #22-122-911) for 2 minutes at room temperature.
5. Store one cytospin in 1XDPBS at 4°C.
6. Use the 2nd cytospin to do the differential staining from the Protocol Hema 3 kit, dry, and store in a slide box at room temperature for subsequent tabulation of differentials.

**Cytology Brush—Cryopreservation**

Freezing medium: DMEM/F12 Cell Media (Sigma-Aldrich #D0697) + 10% FBS + 10 % DMSO

1. Add 1 ml freezing medium in 2 ml vial

2. Place brush into medium, cut brush handle and tightly close vial

3. Gently invert**, do not vortex**

4. Place in Mr. Frosty or similar cryochamber

5. Store at -80C for at least 24h but no more than 1 wk

6. Transfer to liquid nitrogen

**Nasal Brushings Processing (AUDs, Controls, Cannabis Users)**

**Nasal Brush—Cryopreservation**

Freezing medium: DMEM/F12 Cell Media (Sigma-Aldrich #D0697) + 10% FBS + 10 % DMSO

1. Add 1 ml freezing medium in 2 ml vial

2. Place brush into medium, cut brush handle and tightly close vial

3. Gently invert**, do not vortex**

4. Place in Mr. Frosty or similar cryochamber

5. Store at -80C for at least 24h but no more than 1 wk

6. Transfer to liquid nitrogen

**Nasal Cytology Brush – Cytospins-**

1. One cytology brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 1mL 1XDPBS (Cellgro #21-031-CV).
2. Vortex the cytology brush in 1XDPBS.
3. Create 2 cytospins using 200ul of the cells in 1XDPBS for each slide.
4. Fix slides in methanol fixing solution from the Protocol Hema 3 kit (Fisher Scientific #22-122-911) for 2 minutes at room temperature.
5. Store one cytospin in 1XDPBS at 4°C.
6. Use the 2nd cytospin to do the differential staining from the Protocol Hema 3 kit, dry, and store in a slide box at room temperature for subsequent tabulation of differentials.

**BAL Processing (AUDs, Controls, Cannabis Users)**

**Notes:**

* **In general, 150-200mL sterile isotonic saline will be used for bronchoalveolar lavage, instilled in 50mL aliquots. We have used hand aspiration to more carefully control suction amount in the lung.**
* **The first 50mL aliquot instilled/aspirated is reflective of the airways, and is kept separate from the subsequent aliquots and should be marked ‘Upper’. The volume aspirated from this first aliquot (assuming 50mL instilled) is generally only 10-20mL.**
* **The second, third, and any subsequent aliquots reflect distal airway/alveolar sampling and should be marked ‘Lower’.**
* **Aliquot cell free BAL in a biosafety cabinet (hood).**

1. Record volume received for the Upper BAL and the Lower BAL.
2. Centrifuge at 1000 X g for 10 minutes at room temperature to pellet the cells.
3. Aliquot 2 mL of Upper BAL into 4mL cryotubes (Simport #T310-4A). Add Halt proteinase inhibitor (100X ThermoScientific #78429) to half the tubes at 10uL/mL
4. Aliquot 3 mL of the Lower BAL into 4mL cryotubes. Add Halt proteinase to half the tubes at 10uL/mL
5. Store Upper and Lower BAL aliquots at -80°C

**BAL Cell Processing (AUDs, Controls, Cannabis Users)**

1. In a biosafety cabinet (hood), resuspend the cells from the above aliquots (‘Upper’ and ‘Lower’) in a total of 5 to 6 mL 1XDPBS. Place 1-2mL of 1XDPBS in each 50mL conical tube, gently resuspend the cells, and then combine all of the resuspended cells into one tube.
2. Count cells and check viability:
3. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 15 µm and 30µm. Print the count.
4. Divide up the cells according to assay need below.

**BAL Cell/AM Cytospins:**

1. Resuspend 80,000 macrophages in 0.8 ml of 1XDPBS (Cellgro #21-031-CV).
2. Create 2 cytospin slides at 40,000 cells per slide, 400 ul each .
3. Fix slides in methanol fixing solution from the Protocol Hema 3 kit (Fisher Scientific #22-122-911) for 2 minutes at room temperature.
4. Store one cytospin in 1XDPBS at 4°C.
5. Use the 2nd cytospin to do the differential staining from the Protocol Hema 3 kit, dry, and store in a slide box at room temperature for subsequent tabulation of differentials.

**SNAP FROZEN ALIQUOTS:**

**Cells for RNA Extraction or Protein:** Pipette 1x10 6 or 2x10 6 aliquots of cells into labelled microcentrifuge tubes. The number of aliquots will be determined by the total number of cells available. Centrifuge at 14000 X g for 2 minutes to pellet the cells. Aspirate off and discard the supernatant, and then immediately freeze and store the cell pellets at -80°C.

**CRYOPRESERVED ALIQUOTS:**

**Cryopreservation of BAL Cells/AMs for Flow Cytometry:**

1. After cells have been counted, make an aliquot of 2x106 - 4x106.
2. Pellet cells at 400 X g for 5 minutes and remove supernatant.
3. Resuspend in freezing media at a final concentration of 10 x 106 cells/mL (Freezing Media: 10% DMSO - Sigma #D2650 + 90% FBS - Atlanta Biologicals #S11550H, filter sterilized -Corning #430320). For 2 x 106 cells, resuspend in 200ul of freezing media, for 4 x 106 cells, resuspend in 400ul of freezing media.
4. Place them in a freezing container (Nalgene Cryo 1°C Freezing Container or Biocision Cool Cell) and store at -80°C for 24 hours. Store the cells in a liquid nitrogen storage tank long term.

**STIMULATED CELL CULTURE**

**Secretion of cytokines/growth factors by LPS treated AMs:**

Make **Complete Media** for AMs: RPMI 1640 medium + 5% Pencillin/Streptomycin **(NO FBS)**

Cell Medium- Corning RPMI 1640 modified w/L-Glutamine (#MT10040CV)

100X Penicillin (10,000 IU)-Streptomycin (10,000 ug/mL) Solution- Corning (#30002CI)

Remove 5 mL of RPMI media and replace with 5 mL of 100 X Pen/Strep Sol’n.

Store at 4◦C for no more than 1 month.

Take 1 mg stock Lipopolysaccharides from Escherich (LPS- Sigma #L6529-1MG) and reconstitute with 1 mL 1X PBS. Add 2 uL of reconstituted stock to 10 ml of RPMI+ Pen/Strep. (Complete AM Media) for an LPS concentration of 200 ng/mL.

1. Will need 2 x 10 6 AMs. Plate 500,000 AMs in 1mL Complete AM media per well in 4 wells of a 12-well plate (Costar #3513)
2. To 2 of these wells, add an additional 1mL of Complete AM media
3. To the other 2 wells, add 1mL of RPMI with antibiotics, and LPS for a final LPS concentration of 100ng/mL.
4. Incubate the plate for 18 hours at 10% CO2 and 37°C.
5. The next day at the 18 hour time point, collect the media and cells in a 2mL microcentrifuge tube. Centrifuge for 2 minutes at max speed and collect the media in 1mL aliquots. Store at -80°C.
6. Save the cells for RNA extraction by adding 700 uL Buffer RLT from an RNeasy Plus Mini Kit (Qiagen #7414) with β-mercaptoethanol (Sigma #63689) to each pellet in order to lyse cells.
7. Load the collected sample onto a QiaShredder column (Qiagen #79654) placed inside a 2 mL collection tube from the kit. Centrifuge at max speed for 2 minutes. Discard QiaShredder column and cap the tube with sample with the provided caps and store the homogenized sample at -80◦C for up to 3 months.