COVID Airway Processsing for scRNAseq v4 01JUL20
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1. Objective

The isolation of lymphocytes and pan-mononuclear cells from airway samples.

2. Materials

50mL Centrifuge Tube (Fisher Scientific, Cat. No.: 12-565-271)

5mL Falcon™ Round-Bottom Polypropylene Tubes (Fisher Scientific, Cat. No.: 14-959-11A)

Biotin anti-human CD235ab Antibody (100µg) (Biolegend, Cat. No.: 306618)

Biotin anti-human CD66b Antibody (100µg) (Biolegend, Cat. No.: 305120)

BioMag®Plus Streptavidin (Bangs Laboratories, Cat. No.: BP628)

Cryogenic Vials (Fisher Scientific, Cat. No.: 09-761-71)

Cryostor CS10 (Fisher Scientific, Cat. No.: NC9930384)

Dulbecco's Phosphate Buffered Saline (DPBS) (Fisher Scientific, Cat. No.: 14-190-144)

EDTA 0.5M pH 8.0 (Fisher Scientific, Cat. No.: 15-575-020)

Fetal Bovine Serum (FBS) (Fisher Scientific, Cat. No.: 10-099-14)

Ficoll-Paque™ PLUS Media (Fisher Scientific, Cat. No.: 45-001-749)

Human TruStain FcX (Biolegend, Cat. No.: 422302)

Iscove's Modified Dulbecco's Medium (IMDM) (Fisher Scientific, Cat. No.: 12-440-053)

NC-Slide A8 (Chemometec, Cat. No.: 942-0003)

Penicillin-Streptomycin-Glutamine (100X) (Fisher Scientific, Cat. No.: 10-378-016)

Solution 13 AO/DAPI (Chemometec, Cat. No.: 910-3013)

Dead Cell Removal Kit (Miltenyi, Cat. No.: 130-090-101)

- Dead Cell Removal Microbeads
- 20x Binding Buffer Stock Solution

MS Columns (Miltenyi, Cat. No.: 130-042-101)

3. Equipment

Centrifuge

Cell Counter - NC-3000

EasyEights™ EasySep™ Magnet (Stemcell Technologies, Cat. No.: 18103)

MACS Multistand (Miltenyi, Cat. No.: 130-108-934)

4. Protocol

4.1. Biosafety Notes

- All materials required for sample processing are to be prepared in the biosafety cabinet before handling of Airway samples.
- All Airway sample manipulation takes place in a biosafety cabinet unless specificially stated.
- All centrifugation steps must take place in capped containers. Upon completion of a centrifugation step, return the capped container to the biosafety cabinet remove all tubes, and spray and wipe them with >70% ethanol before continuing to the next step.

4.2. Preparing Buffer and Media

4.2.1. Create the following **DPBS Solution-EDTA** in a bottle of DPBS by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.*
DPBS	474	-	-
FBS	25	100%	5%
EDTA	1	0.5M	1mM

Table 1.

4.2.2. Create **IMDM Layering Media** in a bottle of IMDM.

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.*
IMDM	470	-	-
FBS	25	100%	5%
Pen-Strep-Glutamine	5	100%	1%

Table 2.

4.3. Preparation of Sample

4.3.1. Place sample box into biosafety cabinet.

4.3.2. Remove samples from box and from the containment bags, discard bags, spray sample containers with >70% ethanol and wipe down.

NOTE: If sputum traps come with tubing attached flush tubing with 1-2mL (or more) before proceeding to optimize yield.

4.3.3. Record the total volume of Airway sample to be processed below:

_____mL

4.3.4. Transfer the Airway Sample to a 50mL tube, add 4mL of DPBS to the airway sample

4.3.5. Spin the Airway for 400 x g for 10 minutes, 20°C, remove and save four aliquots of the supernatant in cryovials. **Add the same volume to all cryovials.**

Record number of vials: _____ and the volume per vial _____ mL.

NOTE: Ensure that cryovials are decomtaminated prior to removal from the biosafety cabinet.

4.3.6. Add **IMDM Layering Media** to the Airway sample to bring the total volume to 25mL.

4.3.7. Add 40µL of Benzonase to the sample, pipette up and down vigorously to mix and dissocate airway pellet, incubate at room temperature for 30 minutes.

4.3.8. Add 750µL of 0.5M EDTA, and filter sample through a 100µM filter.

4.4. Ficoll-Paque

4.4.1. Aliquot 15mL of Ficoll-Paque Media PLUS to a 50mL tube.

4.4.2. Using the **slow** setting on the pipette gun, **gently** layer the airway/IMDM mixture on top of the 15mL of Ficoll-Paque Media PLUS. **Take extra care not to disturb the interface while layering. Disturbing the interface excessively prevents the mononuclear cells from becoming a clean layer.**

4.4.3. Spin for 20 minutes, 1200 x g at 20°C with **no brake**, 4 acceleration.

Note: Centrifuge should be pre-warmed to 20°C.

4.4.4. Remove the mononuclear cell layer from each tube and transfer to a new 50mL tube. **Take extra to care to avoid pulling cells from the ficoll layer (underneath the mononuclear cell layer) as this typically contains a lot of granulocytes.** Pulling from the plasma layer is not an issue.

4.4.5. Top MNC with cold DPBS Solution-EDTA to 40mL (ensure at least 2-3 volumes are added) and centrifuge the cell suspension(s) for 10 minutes at 400 × g, 4°C.

4.4.6. (Platelet Spin) Discard the supernatant, top tube to 40mL with cold DPBS Solution-EDTA, and centrifuge the cell suspension for 10 minutes at 120 × g, 4°C.

4.4.7. Remove the supernatant (**caution: pellet may be loose**), and re-suspend the cell pellet in 4.5mL **Dulbecco's Phosphate Buffered Saline (DPBS)** (final volume should be about 5mL).

4.5. Cell Counting of COVID Samples

4.5.1. Add 0.050mL of sample, 0.050mL of DPBS, and 0.005mL of Solution 13 to a 1.5mL centrifuge tube, incubate for 2 minutes at room temperature.

4.5.2. Add 0.100mL of BD Cytofix Fixation Buffer to the samples and incubate 30 minutes, room temperature, and protect from light.

4.5.3. Aliquot 0.010mL of sample to the well of a NC-Slide A8 and count on the NC-3000.

4.5.4. Record number and viability below, calculate total cells:

cell number: _____cells/mL,_____% viable

4.6. Division of Sample for scRNAseq Analysis and Freeze-down

4.6.1. Aliquot up to 2 x 10^7 cells to a 5mL Falcon Round-Bottom tube and place on ice for subsequent sample clean-up (Step 4.7).

4.6.2. Freeze down up to 1 x 10^8 cells in approximately 1 x 10^7 aliquots (1mL each) using Cryostor CS10 Medium, a Mr. Frosty, and a -80°C freezer. Record the number of vials frozen: _____ and the cells per cryovial frozen: _____.

4.7. Sample Clean Up for scRNAseq – CD66b and CD235ab removal

4.7.1. Centrifuge the single cell suspension for 5 minutes at 400 × g, 4°C.

4.7.2. Discard the supernatant and resuspend the cell pellet in 50L of DBPS Solution-EDTA; add 10µL of Human TruStain FcX to the single cell suspension and incubate for 10 minutes, 4°C.

4.7.3. Add 10µL of biotinylated anti-CD66b and biotinylated anti-CD235ab to the sample and incubate for 30 minutes, 4°C.

4.7.4. While the single cell suspension is incubating add 0.200mL of BioMag Plus Streptavidin Beads to a 5mL Falcon Round-Bottom tube.

4.7.5. Add 2mL of DPBS Solution-EDTA to the BioMag Plus Streptavidin Beads and place on a magnet for 5 minutes.

4.7.6. Remove all the supernatant from the BioMag Plus Streptavidin Beads, remove from the magnet and resuspend the beads in 0.100mL of DPBS Solution-EDTA.

4.7.7. Once step 4.73 is complete, add 3mL of DPBS Solution-EDTA to the single cell suspension and centrifuge for 5 minutes at 400 × g, 4°C.

4.7.8. Resuspend the single cell suspension in the BioMag Plus Streptavidin Beads from step 4.7.6, and incubate at room temperature for 5 minutes.

4.7.9. Add 3mL of DPBS to the tube and place on a magnet for 5 minutes.

4.7.10. Remove supernatant from tube and transfer to a separate 5mL Falcon Round Bottom tube.

4.8. Sample Clean Up for scRNAseq – Dead Cell Removal

4.8.1. Centrifuge the single cell suspension for 5 minutes at 400 × g, 4°C, and discard supernatant.

4.8.2. Resuspend cell pellet in 0.100mL of Dead Cell Removal Microbeads, mix well, incubate, room temperature, 15 minutes.

4.8.3. While the cell suspension is incubating, place an MS Column onto the MACS Multistand and rinse with 0.500mL 1x Binding Buffer Solution.

4.8.4. Post incubation, apply cell suspension to the MS Column and capture the flow through in a 5mL Falcon Round Bottom tube. Rinse with 1.5mL of 1x Binding Buffer and capture in the same tube.

4.8.5. Centrifuge the single cell suspension for 5 minutes at 400 × g, 4°C, and discard supernatant.

4.8.6. Resuspend cell pellet in 0.500mL DPBS, and count cells.

4.9. Cell Counting of COVID Samples (10x)

4.9.1. Add 0.050mL of sample, 0.050mL of DPBS, and 0.005mL of Solution 13 to a 1.5mL centrifuge tube, incubate for 2 minutes at room temperature.

4.9.2. Add 0.100mL of BD Cytofix Fixation Buffer to the samples and incubate 30 minutes, room temperature, and protect from light.

4.9.3. Aliquot 0.010mL of sample to the well of a NC-Slide A8 and count on the NC-3000.

4.9.4. Record number and viability below, calculate total cells:
cell number: _____cells/mL,_____% viable

4.10. 10X Encapsulation

4.10.1. Follow the appropriate 10X protocol (Chromium Next GEM Single Cell 3' Reagent Kits v3.1 User Guide – Rev D) for encapsulation of cells from the airway sample.