Preparation of a Single Cell Suspension from Bronchoalevolar Lavage

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

This protocol describes a method for the isolation of the immune cells, structural and epithelial cells, and progenitors from lavage fluid collected from human lung. By providing defined media formulations, volumes at each step, and a defined dilution factor for density centrifugation, it yields consistent single-cell suspensions across samples.

ATTACHMENTS

dzhjbk587.pdf

MATERIALS

Materials:

- Syringes Fitted with Luer Lock Valve (509353) **Millipore Sigma Catalog #509639**
  25mL Syringe
- BD Syringes without Needle 50 mL **Fisher Scientific Catalog #13-689-8**
- BD Angiocath Peripheral IV Catheter 12G x 76mm (10) **BD Biosciences Catalog #382277**
- Benzonase nuclease **Sigma Aldrich Catalog #E1014-5KU**
- 3-Way Stopcocks **Bio-rad Laboratories Catalog #7328103**

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Keywords: Lung, BAL, Airway, CD45, Lymphocytes, Myeloid, Isolation, Density gradient, Ficoll, Immune, 10x, scRNAseq, Flow cytometry, Leukocyte, Single cell suspension, T cell

- DPBS no calcium no magnesium **Thermo Fisher Scientific Catalog #14190144**
- Penicillin-Streptomycin-Glutamine (100X) **Thermo Fisher Catalog #10378016**
- Thermo Scientific™ Nunc™ 50mL Conical Sterile Polypropylene Centrifuge Tubes **Fisher Scientific Catalog #12-565-271**
- Gibco™ IMDM (Iscoves Modified Dulbeccos Medium) **Fisher Scientific Catalog #12-440-053**
- Gibco™ Fetal Bovine Serum qualified Australia **Fisher Scientific Catalog #10-099-141**
- UltraPure™ 0.5 M EDTA pH 8.0 **Thermo Fisher Scientific Catalog #15575020**

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- Thomas ScientificSupplier Diversity Partner Cell Strainer 100um Yellow Sterile Individually Wrap **Fisher Scientific Catalog #50-146-1428**
- Ficoll-Paque™ PLUS Media **Fisher Scientific Catalog #45-001-749**
- Mr. Frosty™ Freezing Container **Fisher Scientific Catalog #5100-0001**
- CryoStor CS10 100ML **Fisher Scientific Catalog #NC9930384**
- Corning™ Externally Threaded Cryogenic Vials **Fisher Scientific Catalog #09-761-71**
- 5mL Falcon™ Round-Bottom Polypropylene Test Tubes **Fisher Scientific Catalog #14-959-11A**
- Solution 13 AO – DAPI **Chemometec Catalog #910-3013**
- NC-Slide A8™ box with 25 Slides **Chemometec Catalog #942-0003**
- Falcon™ Plastic Disposable Transfer Pipets **Fisher Scientific Catalog #1368050**

Equipment

- Multi-Axle-Rotating Mixer/Shaker with Temperature Control
- Centrifuge
- Cell Counter - NC-3000
- Surgical scissors
- Scale

Preparing Mediums and Buffers

1 Create the following **IMDM-FBS-PSQ Media** in a 500 mL bottle of IMDM by using the table below:

	A	B	C	D
	Component	Volume (mL)	Starting Conc.	Final Conc.*
	IMDM	500	-	-
	Penicillin-Streptomycin-Glutamine	5	100X	1X
	FBS	50	100%	10%

Table 1.
*Final Concentration is approximate.

2 Create the following **DPBS-FBS-EDTA Solution** in a bottle of DPBS without calcium and magnesium by using the table below:

	A	B	C	D
	Component	Volume (mL)	Starting Conc.	Final Conc.*
	DPBS	500	-	-

	A	B	C	D
	FBS	25	100%	5%
	EDTA	50	0.5M	1mM

Table 2.
*Final Concentration is approximate.

Performing the Lavage

3 Identify, and using a scissors, make an incision in one of the secondary bronchi that connects to the lower lobes of the left lung.

4 Insert the catheter about 5 to 10 centimeters into the incision, remove the needle and attach a 3- way Stopper to the catheter.

5 Fill a 50 mL syringe with PBS and connect to the 3-way Stopper.

6 Slowly inject 25 mL of cold PBS into the lungs. Watch the lungs inflate, and do not overinflate.

7 Attach an empty 25 mL syringe to the final spot of the 3-way Stopper.

8 Collect about 10 mL of BAL fluid (BALF) from lungs.

9 Repeat the previous steps until 50 mL of PBS is injected into the lungs and at least 25 mL to 50 mL of BALF is collected.

Processing the BALF

5m

10 Spin for 00:05:00 at 400 x g at 4 °C, remove and save the supernatant in 2 mL cyrovials – record supernatant volume saved below: 5m

_____ mL

11 Resuspend the cell pellet in 10 mL of IMDM, add 10 µL of benzonase to the BALF and at 37 °C for 00:30:00. 30m

12 Add 40 mL of IMDM (NO ADDITIVES) to the cell suspension, spike in 0.500 mL of EDTA [M] 0.5 Molarity (M) pH 8.0.

Ficoll-Paque

40m

13 Filter the cell suspension through a [M] 100 micromolar (µM) cell strainer.

14 In two 50 mL tubes, layer 25 mL of cell suspension on top of 15 mL of Ficoll-Paque Media PLUS.

15 Spin for 00:20:00, 1200 x g at 20 °C with 4 acceleration and 0 brake, evenly distribute 20m tubes across the entire rotor to prevent wobbling (use all four buckets if possible as opposed to just two).

16 Remove the mononuclear cell layer from both tubes with a transfer pipet and combine in one 50 10m

tube. Add cold DPBS-FBS-EDTA Solution to a final volume of 50 mL and centrifuge the cell suspension for 00:10:00 at 400 x g, 4 °C.

17 Remove the supernatant and re-suspend the cell pellet in 50 mL cold DPBS-FBS-EDTA Solution 10m

centrifuge the cell suspension for 00:10:00 at 120 x g, 4 °C.

18 Remove the supernatant and re-suspend the cell pellet in cold 10 mL IMDM-FBS-PSQ Media.

Cell Count

19 Count cells, and viability by using the NC-3000 cell counter. Calculate total viable cells and record below:
cell number: _____ cells/mL, _____ %viable
final volume: _____ mL
$cell\ number\ (cells/mL) * viability(\%) * final\ volume(mL) = total\ viable\ cells$
Total Viable Cells: _____

Freeze-down and QC

20 (Optional QC) Aliquot 2 x 10^6 cells to a 5mL Falcon tube and place on ice for subsequent flow cytometric analysis.

21 Aliquot cells for analysis or experimentation, and then freeze down cells in up to 2 x 10^7 aliquots using Cryostor CS10 Medium, a Mr. Frosty, and a -80 °C freezer (1 mL - 1.5 mL aliquots, round down to the nearest 30 million cells and discard/freeze/use any left over cells). Record the number of vials frozen: _____.