Preparation of a Single Cell Suspension from Bronchoalevolar Lavage

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1. 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.

2. Materials

25mL Syringe (Millipore Sigma, Cat. No.: 509639)

50mL Syringe (Fisher Scientific, Cat. No.: 13-689-8)

BD Angiocath 10G (BD, Cat. No.: 382277)

Benzonase Nuclease (Millipore Sigma, Cat. No.: E1014-5KU)

3-Way Stopcock (Bio-Rad Laboratories, Cat. No.: 732-8103)

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

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

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

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

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

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

100µM cell strainer (Fisher Scientific, Cat. No.: 50-146-1428)

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

Mr. Frosty (Fisher Scientific, Cat. No.: 51000001)

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

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

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

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

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

Falcon™ Plastic Disposable Transfer Pipets (Fisher Scientific, Cat. No.: 13-680-50)

3. Equipment

Multi-Axle-Rotating Mixer/Shaker with Temperature Control

Centrifuge

Cell Counter - NC-3000

Surgical scissors

Scale

4. Protocol

4.1. Preparing Mediums and Buffers

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

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.*

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

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.**
DPBS	500	-	-
FBS	25	100%	5%
EDTA	1	0.5M	1mM

Table 2.
**Final Concentration is approximate.*

4.2. Performing the Lavage

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

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

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

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

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

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

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

4.3. Processing the BALF

4.3.1. Spin for 5 minutes at 400 × g at 4°C, remove and save the supernatant in 2mL cyrovials – record supernatant volume saved below:

_____ mL

4.3.2. Resuspend the cell pellet in 10mL of IMDM, add 10µL of benzonase to the BALF and at 37°C for 30 minutes.

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

4.4. Ficoll-Paque

4.4.1. Filter the cell suspension through a 100µM cell strainer.

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

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

4.4.4. Remove the mononuclear cell layer from both tubes with a transfer pipet and combine in one 50mL tube. Add cold DPBS-FBS-EDTA Solution to a final volume of 50mL and centrifuge the cell suspension for 10 minutes at 400 × g, 4°C.

4.4.5. Remove the supernatant and re-suspend the cell pellet in 50mL cold DPBS-FBS-EDTA Solution and centrifuge the cell suspension for 10 minutes at 120 x g, 4°C.

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

4.5. Cell Count

4.5.1. 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

$$cell\ number\left(\frac{cells}{mL}\right) * viability(\%) * final\ volume(mL) = total\ viable\ cells$$

Total Viable Cells: _____

4.6. Freeze-down and QC

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

4.6.2. 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 (1-1.5mL aliquots, round down to the nearest 30 million cells and discard/freeze/use any left over cells). Record the number of vials frozen: _____.