TECHNICAL MANUAL

Generation of Human Alveolar Organoids Using PneumaCult™ Alveolar Organoid Media

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1.0 Introduction

Alveoli are the breathing units of the human lung, located in the most distal portion of the airway. Alveolar epithelium comprises a mixture of alveolar epithelial cells, stromal cells, and extracellular matrix molecules. Alveolar type 1 (ATI) cells are large, flat, terminally differentiated cells responsible for gas exchange, and cuboidal alveolar type 2 (ATII) cells are the stem cells of the alveoli capable of self-renewal and differentiation to replenish ATII and ATI cells, respectively, during homeostatic turnover or after an injury. This homeostasis *in vivo* is achieved via soluble factors and cell-to-cell interactions within the alveolar niche, which regulate the biological functions of ATII cells. Traditional two-dimensional (2D) culture techniques have proven insufficient for the long-term culture of ATII cells.

PneumaCult™ Alveolar Organoid Media, which include PneumaCult™ Alveolar Organoid Expansion (AvOE) Medium and PneumaCult™ Alveolar Organoid Differentiation (AvOD) Medium, can be used in a two-part workflow for the long-term culture of ATII cells and their differentiation into ATI cells in a Matrigel®-embedded organoid culture. Using PneumaCult™ AvOE Medium, primary isolated human ATII cells can be seeded as single cells into organoid cultures and passaged for multiple passages. PneumaCult™ AvOE Medium is fully compatible with fresh and cryopreserved single cells, isolated from tissues or organoids dissociated during culture/passage in PneumaCult™ AvOE Medium. Mature organoid cultures in PneumaCult™ AvOE Medium can be differentiated into ATI organoids by switching the medium to PneumaCult™ AvOD Medium in the differentiation protocol.

2.0 Materials, Reagents, and Equipment Required

2.1 PneumaCult™ Alveolar Organoid Media

The components listed below are sold as complete kits and are not available for individual sale.

PRODUCT NAME	CATALOG #	COMPONENT NAME	COMPONENT #	QUANTITY
PneumaCult™ Alveolar Organoid Expansion Medium	100-0847	PneumaCult™ Alveolar Organoid Expansion Basal Medium	100-0848	450 mL
		PneumaCult™ Alveolar Organoid Expansion 10X Supplement	100-0849	50 mL
		PneumaCult™ Alveolar Organoid Expansion 100X Passage Supplement	100-0860	1.5 mL
PneumaCult™ Alveolar Organoid Differentiation Medium	100-0861	PneumaCult™ Alveolar Organoid Differentiation Basal Medium	100-0862	90 mL
		PneumaCult™ Alveolar Organoid Differentiation 10X Supplement*	100-0863	10 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for HIV-1 and -2, hepatitis B, and hepatitis C prior to donation. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
Heparin Solution	07980
Animal Component-Free Cell Dissociation Kit	05426
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free	Corning 356231
24-well non-tissue culture-treated plates	e.g. 100-0097
CryoStor® CS10	07930

For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor
- Hemocytometer or Nucleocounter®
- Pipettor with appropriate tips (e.g. Catalog #38058)
- Pipette-Aid with appropriate serological pipettes (e.g. Catalog #38002)
- Inverted microscope
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Preparation of Media

3.1 PneumaCult™ Alveolar Organoid Expansion (AvOE) Medium

Use sterile technique to prepare complete PneumaCult™ AvOE Medium (PneumaCult™ Alveolar Organoid Expansion Basal Medium + PneumaCult™ Alveolar Organoid Expansion 10X Supplement + Heparin Solution). The following example is for preparing 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw PneumaCult™ Alveolar Organoid Expansion 10X Supplement at room temperature (15 - 25°C) (~1 hour). Mix thoroughly, then store on ice.
2. Aliquot 89.75 mL of PneumaCult™ Alveolar Organoid Expansion Basal Medium into a sterile container.

   Note: If antibiotics are required, reduce basal medium volume to accommodate antibiotic volume.
3. Add 10 mL of PneumaCult™ Alveolar Organoid Expansion 10X Supplement.

   Note: If not used immediately, aliquot supplement and store at -20°C. After thawing aliquots, use immediately; do not re-freeze. Do not exceed the expiry date as indicated on the label. Alternatively, store supplement at 2 - 8°C for up to 1 week.
4. Add 250 µL Heparin Solution. Mix thoroughly, avoiding generating bubbles.
5. Optional: Add antibiotic(s)/antimycotic(s) as desired.

   Note: If not used immediately, store complete medium at 2 - 8°C for up to 6 weeks. Do not exceed the expiry date of the individual components.

3.2 PneumaCult™ AvOE Seeding Medium

Use sterile technique to prepare PneumaCult™ AvOE Seeding Medium (complete PneumaCult™ AvOE Medium + PneumaCult™ Alveolar Organoid Expansion 100X Passage Supplement). The following example is for preparing 10 mL of seeding medium. If preparing other volumes, adjust accordingly.

1. Thaw PneumaCult™ Alveolar Organoid Expansion 100X Passage Supplement at room temperature (~30 minutes). Mix thoroughly, then store on ice.

   Note: If not used immediately, aliquot and store supplement at -20°C. Do not exceed the expiry date as indicated on the label. After thawing aliquots, use immediately; do not re-freeze. Alternatively, store supplement at 2 - 8°C for up to 1 week.
2. Add 9.9 mL of complete PneumaCult™ AvOE Medium (prepared in section 3.1) to a new sterile container.
3. Add 100 µL of PneumaCult™ Alveolar Organoid Expansion 100X Passage Supplement to the aliquoted medium. Mix thoroughly. Use immediately.

3.3 PneumaCult™ Alveolar Organoid Differentiation (AvOD) Medium

Use sterile technique to prepare complete PneumaCult™ AvOD Medium (PneumaCult™ Alveolar Organoid Differentiation Basal Medium + PneumaCult™ Alveolar Organoid Differentiation 10X Supplement). The following example is for preparing 10 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw PneumaCult™ Alveolar Organoid Differentiation 10X Supplement at room temperature (~1 hour). Mix thoroughly, then keep on ice.

   Note: If not used immediately, aliquot and store supplement at -20°C. After thawing aliquots, do not re-freeze. Alternatively, store supplement at 2 - 8°C for up to 1 week.

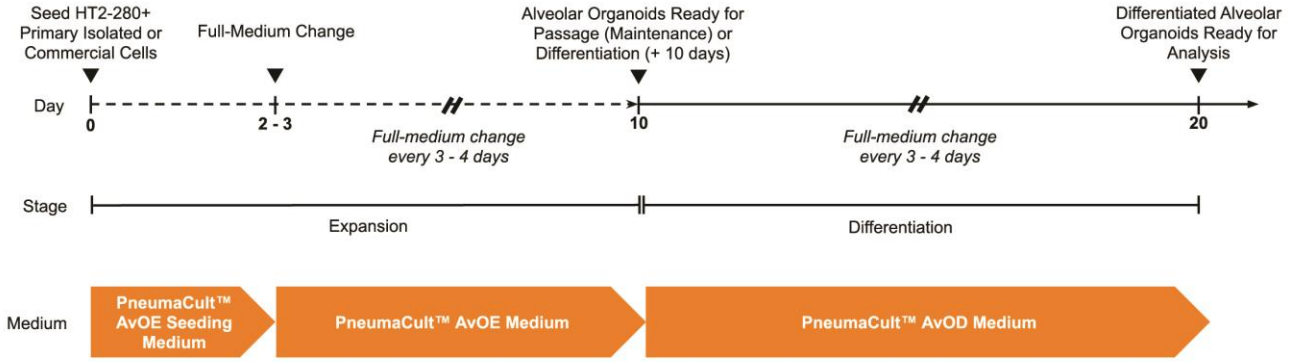
2. Aliquot 9 mL of PneumaCult™ Alveolar Organoid Differentiation Basal Medium into a sterile container.

   Note: If antibiotics are required, reduce basal medium volume to accommodate antibiotic volume.

3. Add 1 mL of PneumaCult™ Alveolar Organoid Differentiation 10X Supplement. Mix thoroughly.
4. Optional: Add antibiotic(s)/antimycotic(s) as desired.

   Note: If not used immediately, store complete medium at 2 - 8°C for up to 6 weeks.

4.0 Protocol Diagram



Seed human ATII (HT2-280+) single cells in PneumaCult™ AvOE Seeding Medium (section 5.2). On day 2 - 3, switch to complete PneumaCult™ AvOE Medium, with full-medium changes every 3 - 4 days to obtain mature ATII organoids (section 5.3). On day 10, alveolar organoids are ready for passage (section 5.4) or differentiation (section 6.0). To differentiate organoids, switch to complete PneumaCult™ AvOD Medium, with full-medium changes every 3 - 4 days. At the end of the 10-day differentiation protocol (total of 20 days including the expansion phase), differentiated ATI organoids are ready for analysis.

5.0 Expansion of Human Alveolar Organoids

5.1 Thawing and Preparing Alveolar Epithelial Type 2 Cells

Cultures can be initiated using cryopreserved or fresh human ATII cells. The following protocol is for thawing and preparing cryopreserved cells. If using freshly isolated cells already in suspension, proceed to step 5.

1. Transfer all vials of cryopreserved cells (0.5 - 1 mL) for thawing from ultra-low temperature storage (e.g. liquid nitrogen) onto dry ice.
2. Process one tube at a time as follows:
    a. Take one vial off the dry ice and transfer it to a 37°C water bath, and swirl until ice has melted and only a very small amount of ice remains.
    b. Pipette cells carefully into a 15 mL conical tube.
    c. Add 1 mL of cold complete PneumaCult™ AvOE Medium into the empty vial to wash, pipetting up and down 3 times.
    d. Combine wash medium, dropwise, with the aliquot of cells in the conical tube, swirling to ensure mixing with each drop.
    e. Continue to add cold medium, dropwise, into the conical tube until cell suspension is 2.5 - 3X the original volume (e.g. for 1 mL of frozen cells, add medium dropwise until 2.5 - 3 mL total volume is reached).
    f. Slowly bring the volume up to 5 - 10X the original volume using cold complete PneumaCult™ AvOE Medium, and mix by gently pipetting up and down 1 - 2 times.
    g. Store on ice.
3. Centrifuge tubes at 400 x *g* for 5 minutes at 2 - 8°C.
4. Aspirate supernatant and gently resuspend pellet in 1 mL of cold complete PneumaCult™ AvOE Medium using a 1 mL pipettor. Add an additional 4 mL of cold medium.
5. Centrifuge tubes at 400 x *g* for 5 minutes at 2 - 8°C.
6. Aspirate supernatant and gently resuspend pellet in 1 mL of cold complete PneumaCult™ AvOE Medium.
7. Perform a live cell count and dilute cell suspension to a concentration of 4000 cells per 25 µL using complete PneumaCult™ AvOE Medium. If needed, concentrate cells by centrifuging and resuspending at 4000 live cells per 25 µL using complete PneumaCult™ AvOE Medium.
8. Store on ice. Proceed to section 5.2 for culture initiation.

5.2 Initiation of Human Alveolar Organoid Expansion Cultures

1. For each dome to be seeded, aliquot 25 µL of the suspension prepared in section 5.1 into a sterile microcentrifuge tube, up to 24 domes per tube (i.e. 600 µL).

   Note: Aliquot an additional 25 µL of the suspension to account for expected pipetting loss (e.g. prepare 25 domes if plating 24 domes).

2. Add 25 µL Matrigel® for every 25 µL of cells (i.e. one volume of total cell suspension) into the suspension above to create a 50% Matrigel® suspension. Store tubes on ice.
3. Process one tube/plate at a time as follows, using a 24-well non-tissue culture-treated plate:
   a. Working quickly and minimizing contact between fingertips and the tube, take one tube of 50% Matrigel® suspension off the ice. Using a 1 mL pipettor, pipette up and down 8 - 10 times quickly but gently, avoiding bubbles by stopping at the first stop of the pipette, to create a uniform suspension.
   b. Set a 200 µL pipettor to 50 µL. Add 50 µL of suspension to the center of the well, with a slight swirling motion to enlarge the 'foot' of the dome, gradually moving the tip upwards as the volume is dispensed to create a dome/droplet shape. Stop at the first stop of the pipettor to avoid bubbles.

      (optional): To seed domes to determine organoid forming efficiency (OFE), refer to section 7.3

   c. Repeat step b in the next well of the plate until all cell suspension is plated.
   d. Place the lid on the culture plate and leave in the biosafety cabinet undisturbed for 5 minutes. Carefully transfer the plate to the incubator without disturbing the domes.
   e. Incubate plate at 37°C for 35 - 50 minutes.
   f. Prepare PneumaCult™ AvOE Seeding Medium (section 3.2).
   g. Gently add 500 µL of room temperature PneumaCult™ AvOE Seeding Medium to each well against the wall to avoid disturbing the dome.
   h. Carefully transfer plate to the 37°C incubator. Incubate for 2 - 3 days, then proceed to section 5.3.
4. Repeat step 3 for all remaining tubes.

   Note: Step 3a can be started for the next tube/plate while the current plate incubates in step 3e.

5.3 Maintenance of Human Alveolar Organoid Expansion Cultures

1. On day 2 or 3, perform a full-medium change by carefully aspirating all of the medium from each well, then adding 500 µL of fresh, room temperature complete PneumaCult™ AvOE Medium.
2. Perform a full-medium change every 3 - 4 days.
3. On day 10 (up to day 14 if more growth is desired), proceed as follows:
   - For maintenance of expansion cultures, passage organoids as described in section 5.4
   - For differentiation of human alveolar organoids, proceed to section 6.0
   - For immunocytochemical staining, proceed to section 7.1
   - For flow cytometric analysis, proceed to section 7.2

5.4 Passaging and Cryopreservation of Human Alveolar Organoids

1. Without contacting the dome(s), aspirate all medium from each well to be passaged.
2. Add 500 µL ACF Enzymatic Dissociation Solution per 50 µL Matrigel® in the well.
3. Triturate by pipetting up and down 10 times forcefully and quickly (but avoid bubble formation by stopping at the first stop of the pipette) to shear large Matrigel® aggregates.
4. Incubate at 37°C for 10 minutes.
5. Using a 1 mL pipettor, triturate each well again, 5 - 10 times. Incubate at 37°C for 5 minutes.
6. Triturate 5 times, then add an equal volume of ACF Enzyme Inhibition Solution. Mix by pipetting 3 - 5 times. Immediately transfer to sterile 15 mL conical or microcentrifuge tubes as volumes permit.
7. Centrifuge at 400 x *g* for 5 minutes at 2 - 8°C.
8. Aspirate supernatant, then resuspend in a minimum volume of 250 - 500 µL of room temperature complete PneumaCult™ AvOE Medium. Mix by pipetting 5 - 8 times.
9. Perform a live cell count.
10. **Optional**: To cryopreserve cells, centrifuge suspension at 400 x *g* for 5 minutes at 2 - 8°C. Resuspend pellet in CryoStor® CS10 at 0.5 - 1 x 10^6 live cells/mL, with a minimum volume of 200 µL, and aliquot into cryopreservation tubes.
    a. Transfer tubes into an insulated freezing container and store overnight at -80°C.
    b. Transfer tubes to ultra-low temperature (e.g. -150°C) or liquid nitrogen for long-term storage.
11. Dilute cell suspension to a concentration of 4000 cells per 25 µL using complete PneumaCult™ AvOE Medium. If needed, concentrate cells by centrifuging (400 x *g* for 5 minutes at 2 - 8°C) and resuspending at 4000 cells per 25 µL with complete PneumaCult™ AvOE Medium.
12. To seed into the next passage, refer to section 5.2.

6.0 Differentiation of Organoids into Human Alveolar Epithelial Type 1 (ATI) Cells

On day 10 (or up to day 14) of expansion culture, expansion-phase organoids can be differentiated in an endpoint assay as follows:

1. Without contacting the dome(s), carefully aspirate the medium from each well to be differentiated.
2. Gently add 500 µL of room temperature complete PneumaCult™ AvOD Medium to each well, without disturbing the domes.
3. Change medium by repeating steps 1 - 2 every 3 - 4 days.
4. On day 10, cultures are considered fully differentiated into ATI cells and can be used in standard assays or harvested for immunostaining or flow cytometry analysis. For more information, refer to section 7.0.

   Note: Including 10 days in expansion culture, cells are in culture for a total of 20 days after a 10-day differentiation. Representative images of expansion and differentiation organoid cultures, displaying potential donor variation, are presented in Figure 1.

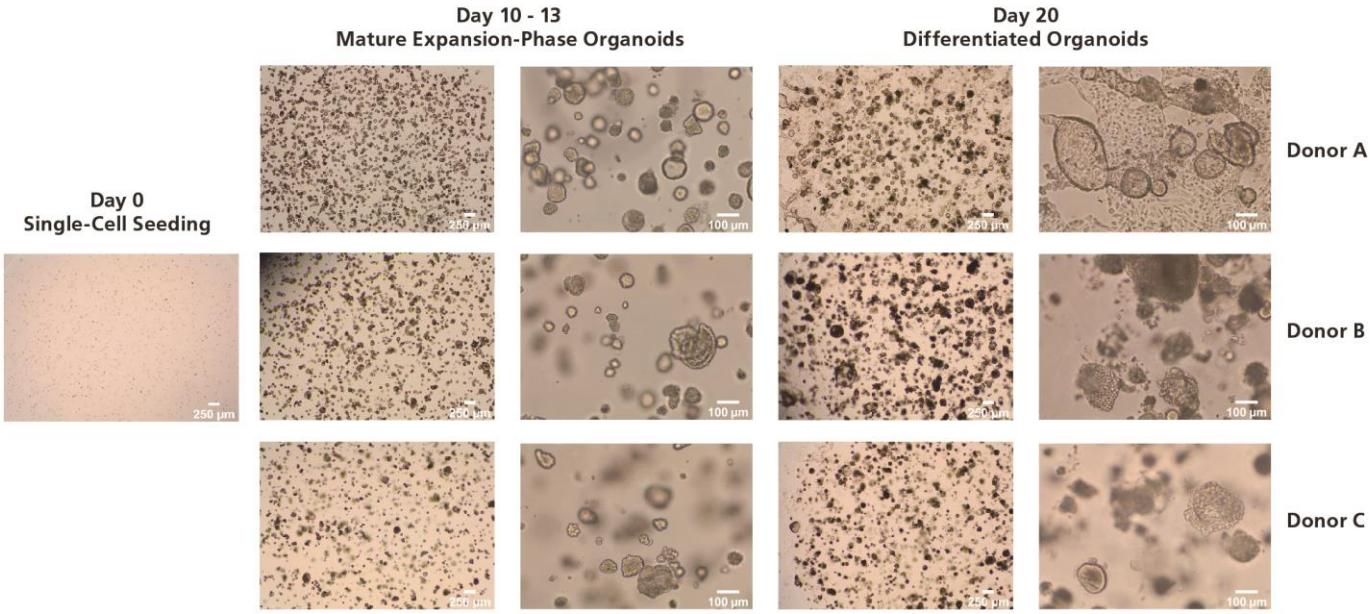


Figure 1. Representative Images of Cells After Seeding and Organoids at Different Stages of the Workflow

Brighfield images of organoids with varying morphologies generated from 3 different donors. Magnifications: 2X and 10X.

7.0 Alveolar Organoid Culture Evaluation

The following section details protocols used to evaluate the organoids and cells grown with the PneumaCult™ Alveolar Organoid Media.

7.1 Immunocytochemistry

For complete instructions, refer to the web protocol: Performing Immunocytochemical Staining of Epithelial Organoids, available at www.stemcell.com. Refer to Table 1 for recommended antibodies.

Table 1. Recommended Primary Antibodies for Immunocytochemistry

ANTIBODY	CATALOG #	ISOTYPE	DILUTION
Primary antibodies			
Anti-RAGE/AGER	R&D Systems AF1145	Goat IgG	1 in 400
Anti-GPRC5a	Sigma HPA007928	Rabbit IgG	1 in 500
Anti-human HT2-280	Terrace Biotech TB-27AHT2-280	Mouse IgM	1 in 200
Anti-Pro-SPC	Sigma AB3786	Rabbit IgG	1 in 2000

7.2 Flow Cytometry

The following protocol is for preparation of alveolar organoid cells for flow cytometry. The markers HT1-56 (ATI marker) and HT2-280 (ATII marker) are used to quantify the level of differentiation and expansion, respectively.

7.2.1 Materials Required

PRODUCT	CATALOG #
D-PBS (Without Ca++ and Mg++)	37350
Animal Component-Free Cell Dissociation Kit	05426
AO•DAPI Staining Reagent	Chemometec 910-3018
96-well tissue culture plate	e.g. 38044
Normal Donkey Serum	Sigma D9663-10ML
15 mL conical tube	e.g. 38009
EasySep™ Buffer	20144
DAPI (Hydrochloride)	75004

7.2.2 Alveolar Organoid Cell Preparation

The following protocol is for dissociating alveolar organoids embedded in Matrigel® in a 24-well plate (prepared in section 5.3 or 6.0). If using cells from organoid passaging (section 5.4), proceed to step 7.

1. Aspirate medium from each well.
2. Add 500 µL of ACF Enzymatic Dissociation Solution per well. Dislodge organoids in each well by triturating 5X with a pipette.
3. Incubate plate at 37°C for 5 minutes.
4. Further dislodge organoids by triturating 5 times with a pipette. Incubate plate at 37°C for 5 minutes.
5. Observe cells under a microscope; if cells are still clumpy, repeat step 4.
6. Add 500 µL of ACF Enzyme Inhibition Solution per well. Transfer all medium from the plate to a 15 mL conical tube.
7. Centrifuge at 300 x *g* (low brake) for 5 minutes.
8. Remove the supernatant and resuspend the pellet in 200 - 500 µL of PneumaCult™ AvOE Medium (if passaging expansion-phase organoids as well as performing flow cytometry) or D-PBS (if only performing flow cytometry).
9. Count cells using a hemocytometer or NucleoCounter® NC-250™ cytometer (20 µL cells + 1 µL AO•DAPI Staining Reagent).
10. Calculate and aliquot the volume required for 20,000 live cells/well into a 96-well plate.

    Note: The ideal amount of cells can range from 10,000 to 50,000, as long as all samples being tested have the same amount of cells aliquoted into each well of the 96-well plate.

7.2.3 Preparation of Reagents

Primary Antibody Mix

1. Prepare a 1 in 100 dilution of anti-HT1-56 and anti-HT2-280 antibodies in EasySep™ Buffer (ESB) with 5% Normal Donkey Serum (NDS). Refer to Table 2 for recommended antibodies.
2. For a secondary control, prepare 5% NDS in ESB.

Secondary Antibody Mix

Prepare a 1 in 500 dilution of each secondary antibody in ESB; refer to Table 2 for recommended antibodies.

DAPI Solution

Add DAPI to ESB to a final concentration of 2.5 µg/mL. Mix thoroughly.

Table 2. Recommended Primary and Secondary Antibodies for Flow Cytometry

ANTIBODY	CATALOG #	ISOTYPE	DILUTION
Primary antibodies			
Anti-human HT1-56	Terrace Biotech TB-29AHT1-56	Mouse IgG1	1 in 100
Anti-human HT2-280	Terrace Biotech TB-27AHT2-280	Mouse IgM	1 in 100
Secondary antibodies*			
PE-conjugated anti-mouse IgG1	BioLegend 406607	Rat IgG	1 in 500
Alexa Fluor® 488-conjugated donkey anti-mouse IgM	Jackson ImmunoResearch 715-545-020	Donkey IgG	1 in 500

*Alexa Fluor® 647-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch 715-605-151) is an alternative to either secondary antibody; this antibody binds to both anti-HT1-56 and anti-HT2-280. Alternatively, other secondary antibodies can be used instead, e.g. highly cross-adsorbed donkey anti-mouse IgG, Alexa Fluor® 555 (Invitrogen A31570); this antibody binds to both anti-HT1-56 and anti-HT2-280.

Notes

- Anti-CD49f and anti-CD271 antibodies can be used to stain for contaminating basal cells, while anti-EpCAM antibody can be used to stain for epithelial cells and gate out contaminating non-epithelial cells.
- For samples with few cells (< 5000), it is recommended to include DRAQ5™ dye, which stains both live and dead cells; this will help to differentiate cells from debris.

7.2.4 Immunostaining Alveolar Organoid Cells for Flow Cytometry

The following protocol is for one well of a 96-well plate. If using other cultureware, adjust volumes accordingly.

1. Top up the 96-well plate (prepared in section 7.2.2) to 200 µL with ESB. Centrifuge the plate at 700 x *g* for 5 minutes.
2. Give the plate one quick flick to dispose of the supernatant into a sink/waste container (if done quickly, the pellets should remain intact at the bottom of their wells).
3. Add 100 µL/well of primary antibody mix. Incubate at room temperature (15 - 25°C) for 15 minutes. Repeat steps 1 and 2 three times.
4. Add 100 µL/well of secondary antibody mix. Incubate at room temperature for 15 minutes.
5. Repeat steps 1 and 2 two times.
6. Gently resuspend cells with 75 µL DAPI Solution per well.
7. Proceed to flow cytometer.

7.2.5 Flow Cytometry Gating

Refer to Figure 2 and Figure 3 for a gating strategy using FCS Express 5.

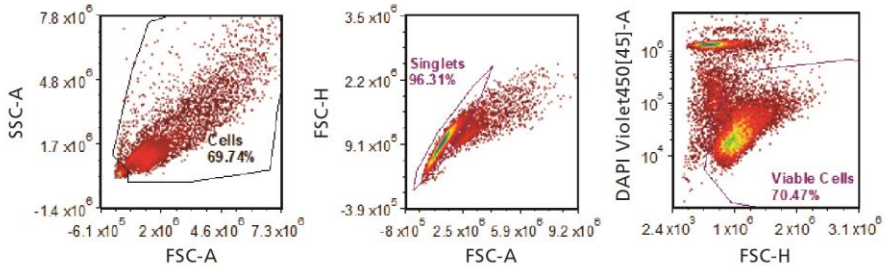


Figure 2. Gating Strategy

The first three gates shown here serve to gate out and eliminate debris, doublets, and dead cells, respectively, from the analysis.

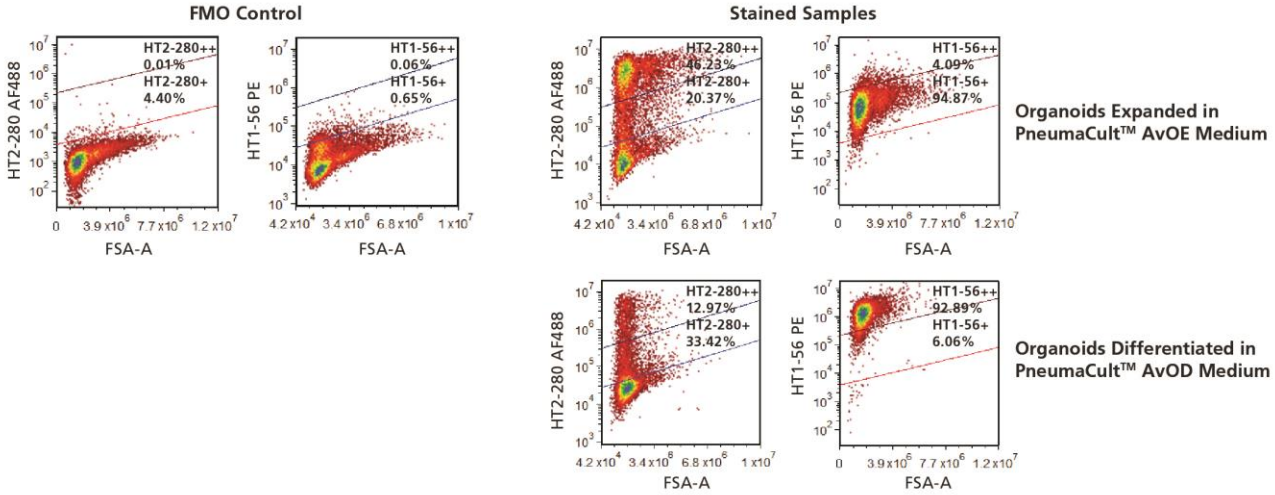


Figure 3. Secondary-Only Control and Stained Sample

The first two images show fluorescence minus one (FMO) controls for HT1-56+ and HT2-280+ populations. The higher HT1-56+/HT2-280+ gates were labeled "++". Diagonal gates were due to larger cells displaying higher background fluorescence intensity. ATII cells are typically HT2-280++ and HT1-56+, while ATI (differentiated) cells are HT2-280- and HT1-56++. Both ATI and ATII cells can be HT2-280+.

Notes

- Gating is done in order: cells > viable cells > viable singlets > HT2-280+/HT1-56+
- HT1-56 has two gates: HT1-56+ (gated above negative secondary control) and HT1-56++ (gated above expansion population).
- HT2-280 has two gates: HT2-280+ (gated above negative secondary control) and HT2-280++.
- When only using AF488, DAPI, and PE, compensation is not necessary.
- Optimally, FMO controls should be used. However, only the removal of anti-HT2-280 shows a difference from the secondary-only control. Primary FMO controls (removing primary antibodies) are recommended, but secondary FMO controls (removing secondary antibodies) are optional.

7.3 Organoid Forming Efficiency Assay

Organoid forming efficiency (OFE) is a measure of a culture's stemness, i.e. the percentage of cells in culture that form organoids. In a typical healthy expansion organoid culture, an OFE ≥ 5% is to be expected (tested across 5 independent donors), which diminishes with culture passage. OFE is also a measure of self-renewal and organogenic capacity of the seeded cells that can serve as a readout for applications such as drug or toxicity assays.

Refer to the following protocol to assess OFE.

1. Instead of seeding 1 x 50-µL dome (section 5.2 step 3b), seed 44-µL, 46-µL, or 48-µL domes into each well, followed by up to 3 x 2-µL domes into the same well, such that the total volume of suspension seeded is 50 µL (i.e. 4000 cells per well).

   Note: The small domes can be combined with the large dome during passaging and used in expansion after they are first quantified (on or after day 10) with the following steps before passage (section 5.4).
2. On an inverted microscope (4X objective), locate a seeded 2-µL dome.
3. Adjust focus so that all organoids are in focus or visible for accurate counting.
4. Count the number of organoids in the dome, or capture an image for counting at a later time.
5. Repeat steps 2 - 4 for each 2-µL dome seeded.
6. Calculate the OFE:

$$\textit{OFE (\%)} = \frac{\textit{Number of Organoids per Dome}}{\textit{Number of Cells Seeded per Dome}} \textit{ X 100}$$

TECHNICAL MANUAL

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