



3dGRO™ Human Lung Organoid Culture System

Product Manual for Catalog Nos.

SCC301
SCM302
SCM305
SCM306
SCM307
SCM308

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Not for use in diagnostic procedures.

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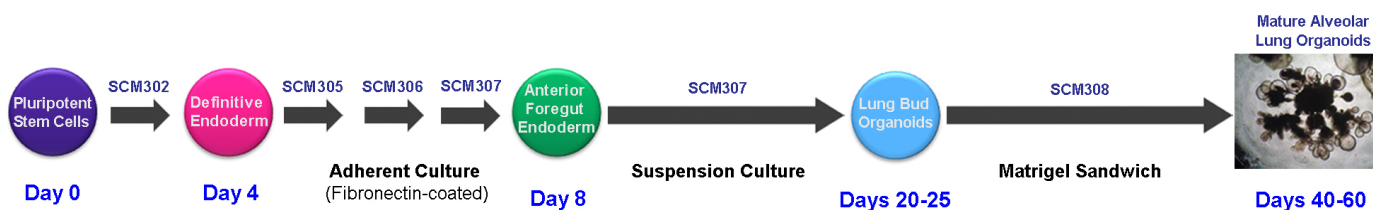
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Introduction

Lung organoids are useful tools to study human lung development and to model infectious respiratory diseases. The 3dGRO™ Human Lung Organoid Culture System is a chemically defined, serum-free, multi-stage culture system for efficient differentiation of human pluripotent stem cells to mature lung organoids that structurally resemble the *in vivo* branching airway and early alveolar structures. Using the 3dGRO™ Human Lung Organoid Culture System, large numbers of mature lung organoids may be generated that express appropriate markers indicative of multiple cell types found in the mature lung and airways including: SFTPB and SFTPC (surfactant-producing type II alveolar epithelial (ATII) cells), MUC5AC (airway goblet cells), EpCAM, Sox9 and Nkx2.1 (pulmonary endoderm), acetyl- α -tubulin (ciliated cells) and the mesenchymal marker Vimentin. Lung organoids also express ACE2, the receptor for the novel SARS-CoV2 virus that causes COVID-19 and TMPRSS2, the serine protease that enhances SARS-CoV2 viral entry.

In this multi-stage protocol, human pluripotent stem cells are first differentiated into definitive endoderm cells using a 3-day induction medium. From days 4-8, human definitive endoderm cells are directed towards anterior foregut endoderm. Anterior foregut endoderm (AFE) cells may be cryopreserved (SCC301) or further differentiated into branching lung bud organoids using 3dGRO™ Lung Organoid Branching Medium (SCM307) and further matured into branching and alveolar lung organoids using 3dGRO™ Lung Organoid Maturation Medium (SCM308). The culture system has been validated on two human iPS cell lines that were generated from reprogrammed human foreskin fibroblasts and PBMCs.

3dGRO™ Human Lung Organoid Culture System



3dGRO™ Human Lung Organoid Culture System			
Description	Catalog Number	Volume Provided	Storage Temp.
Definitive Endoderm Induction Medium	SCM302	50 mL	-20C
Anterior Foregut Endoderm Induction Medium I	SCM305	10 mL	-20C
Anterior Foregut Endoderm Induction Medium II	SCM306	10 mL	-20C
3dGRO™ Lung Organoid Branching Medium	SCM307	50 mL	-20C
3dGRO™ Lung Organoid Maturation Medium	SCM308	250 mL	-20C
Human iPSC Derived AFE Progenitors	SCC301	3x10 ⁶ cells	Liquid Nitrogen

Figure 1. Multi-stage protocol for efficient differentiation of pluripotent stem cells to mature branching and alveolar lung organoids. The 3dGRO™ Human Lung Organoid Culture System is comprised of 5 media that are sold separately and **NOT** as a kit. Cryopreserved Human iPSC Derived AFE Progenitors (SCC301) are available separately.

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Materials Required but Not Supplied

1. mTeSR™ 1 Medium (Stem Cell Technologies Cat. No. 85850) or PluriSTEM™ Human ES/iPS Cell Medium (SCM130)
2. Matrigel hESC-qualified Matrix, 5 mL (Corning Cat. No. 354277)
3. Growth Factor Reduced (GFR) Matrigel, 5 mL (Corning Cat. No. 354230)
4. DMEM/F12, with HEPES, L-Glutamine, 500 mL (Cat. No. DF-041-B)
5. AccuMax™ Cell Detachment Solution (Cat. No. SCR006)
6. EmbryoMax® 1X Dulbecco's Phosphate-Buffered Saline w/o Ca⁺⁺ or Mg⁺⁺, 500 mL (Cat. No. BSS-1006-B).
7. 24-well transparent cell culture inserts, 0.4uM (Corning Cat No. 353095)
8. Costar® Ultra-low attachment 24-well plate (Cat. No. CLS3473)
9. Non-tissue culture treated 24 well plate (Corning Cat. No. 351147)
10. 6-well tissue culture plates (any vendor)
11. 96-well, Cell Culture-Treated, U-Shaped Bottom Microplate (Fisher Cat. No. FB012932)
12. Cell Scraper (Sarstedt Cat. No. 83.1832)
13. Fibronectin (Cat. No. F0895)
14. ROCK Inhibitor, Y-27632 (Cat. No. SCM075)
15. 1 mL disposable glass serological pipets (Fisher Cat. No. 13-678-36A)
16. Sterile tweezers
17. Characterization antibodies

Storage and Stability

All media are provided frozen and can be stored at -20°C for up to 1 year. When ready to use, thaw medium overnight at 2 – 8°C. **Do not thaw or warm medium at 37°C.** Depending upon the amount required, mix thoroughly and aliquot into smaller volumes. Store unused aliquots at -20°C. Thawed aliquots may be stored at 2 – 8°C for up to 2 weeks. Before use, warm working aliquots at room temperature for 10 minutes.

1.0 Differentiation of Human ES/iPS cells to Definitive Endoderm (DE) (Cat. No. SCM302)

Important Notes Before Starting

- Read through the entire manual before starting.
- Use high quality undifferentiated human ES/iPS cells that are cultured on Matrigel. Remove any areas of spontaneous differentiation before starting. Failure to remove differentiated areas may result in decreased efficiency of definitive endoderm (DE) induction. For complete instructions on culture and maintenance of high quality undifferentiated human ES/iPS cells, refer to manufacturer's protocols.
- The definitive endoderm induction protocol is based on culture of human ES/iPS cells in feeder-free conditions and require the use of ROCKi, Y-27632 (Cat. No. SCM075; 10 μ m final) to enhance cell survival during the single cell dissociation step.
- Aliquot the Definitive Endoderm Induction Medium (SCM302) in 10 mL aliquots and store at -20°C until ready to use. After thaw, store aliquots at 2-8°C during the duration of the induction period.

MATRIGEL COATING:

Below are general guidelines for the coating of 6 well plates with hESC-qualified Matrigel (Corning Cat. No. 354277). For other culture wares, adjust volumes accordingly.

1. Thaw hESC-qualified Matrigel on ice. Keep on ice and use pre-cooled medium and pipettes to avoid gelling of the ECM gel. **IMPORTANT: Do not thaw Matrigel at temperatures higher than 15°C to avoid gelling.**
2. Dilute the hESC-qualified Matrigel 1:20 with cold DMEM/F12 medium. For example, to every 0.5 mL Matrigel, add 9.5 mL cold DMEM/F12 medium for a total volume of 10 mL. Scale according to the volumes required.
3. Aliquot 1.5 mL of the diluted hESC-qualified Matrigel to each well of a 6-well plate. Swirl the culture plates to spread the Matrigel evenly across the surface of the plate. Incubate at 2-8°C for at least 2 hour or 2 – 8°C overnight. If not used immediately, store coated cultureware at 2-8°C until ready to use.

Note: *If not used immediately, Matrigel coated cultureware should be sealed with parafilm to prevent evaporation and can be stored at 2 – 8°C for up to 3-4 days.*

4. Prior to seeding the cells, bring the plate back to room temperature for at least 30 minutes, remove the coating solution and add an appropriate volume of expansion medium. **IMPORTANT: Do not allow the plate to dry out.**

Day 0: Start with high quality undifferentiated human ES/iPS cells that are ~80-90% confluent and contain <5% differentiated cells. Reserve at least 1 well for QC flow analyses. Indicated volumes are for a single well of a 6-well plate. Scale according to the number of wells being differentiated. Adjust volumes as necessary.

1. Prepare Single Cell Passaging Media: Add ROCK Inhibitor, Y-27632 (SCM075) to the mTeSR™-1 or PluriSTEM™ Medium to a final concentration of 10 μ M.
2. Aspirate the medium from the 6-well plate containing high quality undifferentiated human ES/iPS cells. Wash each well with 2 mL of DMEM/F12 or 1X PBS. Aspirate and add 1 mL of AccuMax (Cat. No. SCR006) to each well. Incubate for 6-8 minutes at 37°C. Tap the plate firmly against the palm of your hand to help dislodge the cells.
3. Add 1 mL of the Single Cell Passaging Media (from step 1) to each well. Pipet up and down 1-3 times with a 5 mL pipettor to dislodge the cells. Be careful to not introduce any bubbles.
4. Collect the dissociated cells (~2 mL volume/well) to a 15 mL conical tube. Rinse each well with 1 mL Single Cell Passaging Media to collect any remaining cells and add to the conical tube containing the cell suspension. Centrifuge at 800 rpm for 5 minutes. Aspirate.
5. Resuspend the cell pellet in 1 mL of Single Cell Passaging Media. Count the total number of live cells using Trypan blue and a hemocytometer.
6. Add 1×10^6 cells per well to a Matrigel coated 6-well plate. The media used should be the Stem Cell Passaging Media (from step 1); Total volume = 3 mL per well. Incubate at 37°C overnight.

Day 1 to Day 3: Definitive endoderm differentiation

7. Aspirate the medium from each well. Add 2 mL of Definitive Endoderm (DE) Induction Medium (SCM302) to each well and incubate at 37°C overnight.
8. Repeat step 7 for days 2 and days 3.
9. On Day 3, prepare fibronectin coated 6-well plates in preparation for differentiation of definitive endoderm cells to anterior foregut endoderm. Dilute fibronectin (Sigma F0895-1MG; provided as 1 mg/mL) to 4 μ g/mL in PBS. Add 1.5 mL of diluted fibronectin per well. Store coated dishes overnight at 2-8°C.

Day 4: QC flow analysis

10. Sacrifice one well to perform a QC flow analysis using definitive endoderm markers (CXCR4 and c-kit). Flow analysis should indicate >80% c-kit+CXCR4+ double positive population with a compact cell distribution.

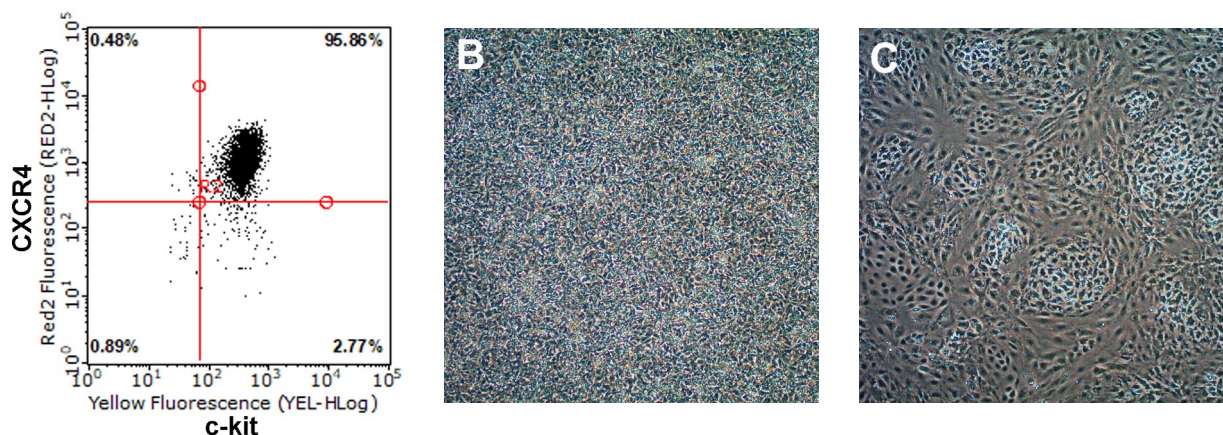


Figure 2. QC flow analysis of definitive endoderm cells (**A**). Cells should be ≥ 80 CXCR4⁺ c-kit⁺ double positive. Morphology of definitive endoderm cells before differentiation to anterior foregut endoderm (AFE) (**B**). Morphology of AFE cells at Day 8 (**C**).

2.0 Differentiation of Definitive Endoderm (DE) Cells to Anterior Foregut Endoderm (AFE) (Cat. Nos. SCM305, SCM306, and SCM307)

Important Notes Before Starting:

- The anterior foregut endoderm induction media protocol starts from day 4 of definitive endoderm cells.
- High cell density should be observed at both day 4 of definitive endoderm cells and also at day 8 after differentiation to anterior foregut endoderm (AFE) cells. See figure 2. The protocol below provides a reference seeding density in a 6-well plate format. Adjust the seeding density for other plate formats.

Protocol (Anterior Foregut Endoderm Differentiation from DE cells)

From Section 1, step 10 (Day 4): Start with a 6-well plate of cells that have been determined through flow analysis to be $\geq 80\%$ positive for definitive endoderm markers (CXCR4, c-Kit) (Figure 2A).

1. Remove the 6-well plate containing day 4 definitive endoderm cultures from the incubator.
2. Aspirate the medium from each well. Wash each well with 2 mL 1X PBS (Sigma BSS-1006-B). Aspirate.
3. Add 1 mL AccuMax (Sigma SCR006) per well to dissociate definitive endoderm cells. Incubate at 37°C for 6-8 minutes.
4. After 5 minutes, visually inspect the plate. Gently tap the edge of the plate to further detach the cells. Most of the cells should come off as a suspension. If not, wait 2 minutes more.
5. Add 2 mL per well of 1X PBS (Sigma BSS-1006-B) to dilute the AccuMax. Pool the cell suspension into 50 mL conical tube(s). Rinse each well with 1 mL 1X PBS to collect any remaining cells and add to the 50 mL conical tube. Pipette up and down several times to get a single cell suspension.

6. Centrifuge the cell suspension at 800 rpm for 5 minutes at room temperature.
7. Aspirate the supernatant. Resuspend the cell pellet in 2 mL of AFE Induction Medium I (SCM305). Count the cell number using a cell counter/hemocytometer.
8. Take the fibronectin coated plates prepared in Section 1, step 9 and aspirate the coating solution. Air dry in the hood with the lid off for 5 minutes. After 5 minutes when the plates are dried, close the lid and set aside until cells are ready.
9. Plate 1 million cells per well onto fibronectin coated 6-well plates. Add the appropriate volume of AFE Induction Medium I (SCM305) to each well to obtain a total volume of 2 mL per well.
10. Place the plate in a 37°C incubator. Agitate the plate gently from side to side and forward and backwards to ensure that the cells are evenly distributed across the surface of the well. Incubate in a 37°C incubator overnight.
11. **Day 5:** Replace with 2 mL per well of AFE Induction Medium II (SCM306, sold separately). Incubate in 37°C incubator for 24 hours.
12. **Day 6:** Replace medium with 2 mL per well of 3dGRO™ Lung Organoid Branching Medium (SCM307, sold separately). Incubate in 37°C incubator for 24 hours.
13. **Day 7:** Replace medium with 2 mL per well of 3dGRO™ Lung Organoid Branching Medium (SCM307, sold separately). Incubate at 37°C for 24 hours.
14. **Day 8:** AFE cells should exhibit morphological changes comprised of confluent areas interspersed with cell clusters/aggregates (see Figure 2C). Follow the protocol to further differentiate into mature lung organoids, the extra remaining AFE cells could be cryopreserved.

Troubleshooting:

Observation	Possible Cause(s)	Recommended Action
AFE cells at day 8 appear too sparse and are not confluent.	Seeding 1×10^6 definitive endoderm cells/ well of a 6-well plate at day 4 should be sufficient to obtain a confluent layer of AFE cells by day 8 (refer to Figure 2C). There may be some areas that appear sparse, but the overall plate confluency should be >80%. Different hiPS cell lines may have different growth kinetics, if so, adjust the seeding density at day 4 accordingly.	Adjust seeding density for day 4 definitive endoderm cells to obtain a confluent layer of AFE cells by day 8.
CXCR4+ c-kit+ population at day 4 definitive endoderm cells is <80%.	The starting human ES/iPS culture may be of lower quality and contain a higher degree of spontaneous differentiation which may affect the quality and efficiency of the differentiation.	Restart with high quality undifferentiated human ES/iPS cell that are 80-90% confluent and contain <5% differentiated cells. Remove any spontaneously differentiated areas.

3.0 Optional: Cryopreserved Anterior Foregut Endoderm (AFE) Cells (Cat. No. SCC301)

Anterior Foregut Endoderm (AFE) cells may be cryopreserved at Day 8 in 3dGRO™ Organoid Freeze Medium (Cat. No. SCM301) at $3-6 \times 10^6$ cells per vial.

Thawing Cryopreserved AFE Cells

1. Cryopreserved AFE cells are thawed in 3dGRO™ Lung Organoid Branching Medium (SCM307) in fibronectin-coated 6-well plates. Refer to Section 1, step 9 for details on fibronectin coating. Prepare fibronectin coated plates 1 day prior to thawing AFE cells.
2. On day of thaw, take the fibronectin coated plates and aspirate the coating solution. Air dry in the hood with the lid off for 5 minutes. After 5 minutes when the plates are dried, close the lid and set aside until cells are ready.
3. Remove the vial of frozen AFE cells from liquid nitrogen and incubate in a 37°C water bath. Closely monitor until small ice pieces still remaining in the vial. Pull out the cryovial.
4. Disinfect the outside of the vial with 70% ethanol. Proceed immediately to the next step.
5. In a laminar flow hood, use a 1 or 2 mL pipette to transfer the cells to a sterile 15 mL conical tube. Be careful not to introduce any bubbles during the transfer process.
6. Using a 10 mL pipette, slowly add dropwise 9 mL of 3dGRO™ Lung Organoid Branching Medium to the 15 mL conical tube.

IMPORTANT: Do not add the entire volume of media all at once to the cells. This may result in decreased cell viability due to osmotic shock.

7. Gently mix the cell suspension by slowly pipetting up and down twice. Be careful not to introduce any bubbles.
8. Centrifuge the tube at 800 x rpm for 5 minutes to pellet the cells.
9. Decant as much of the supernatant as possible. Steps 5-8 are necessary to remove residual cryopreservative (DMSO).
10. Resuspend the cells in 1-2 mL of 3dGRO™ Lung Organoid Branching Medium. Count the cell number using a cell counter/hemocytometer.
11. Plate 1 million **viable** cells/well into a fibronectin-coated 6-well plate in 2-3 mL 3dGRO™ Lung Organoid Branching Medium (SCM307). Total volume = ~2-3 mL per well.
12. Replace with 3dGRO™ Lung Organoid Branching Medium (SCM307) every other day until cells are at confluent status. Once the cells are confluent, you can proceed to Section 4. This usually takes 3 days.

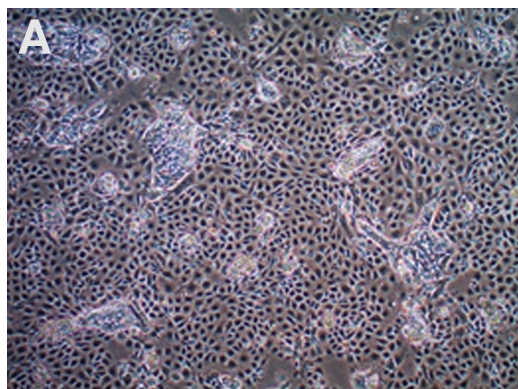


Figure 3. Brightfield images of anterior foregut endoderm (AFE) cells three days after thaw. Cells were thawed and plated at 1 million cells/well in a fibronectin coated 6-well plate. The overall confluency > 80-90%.

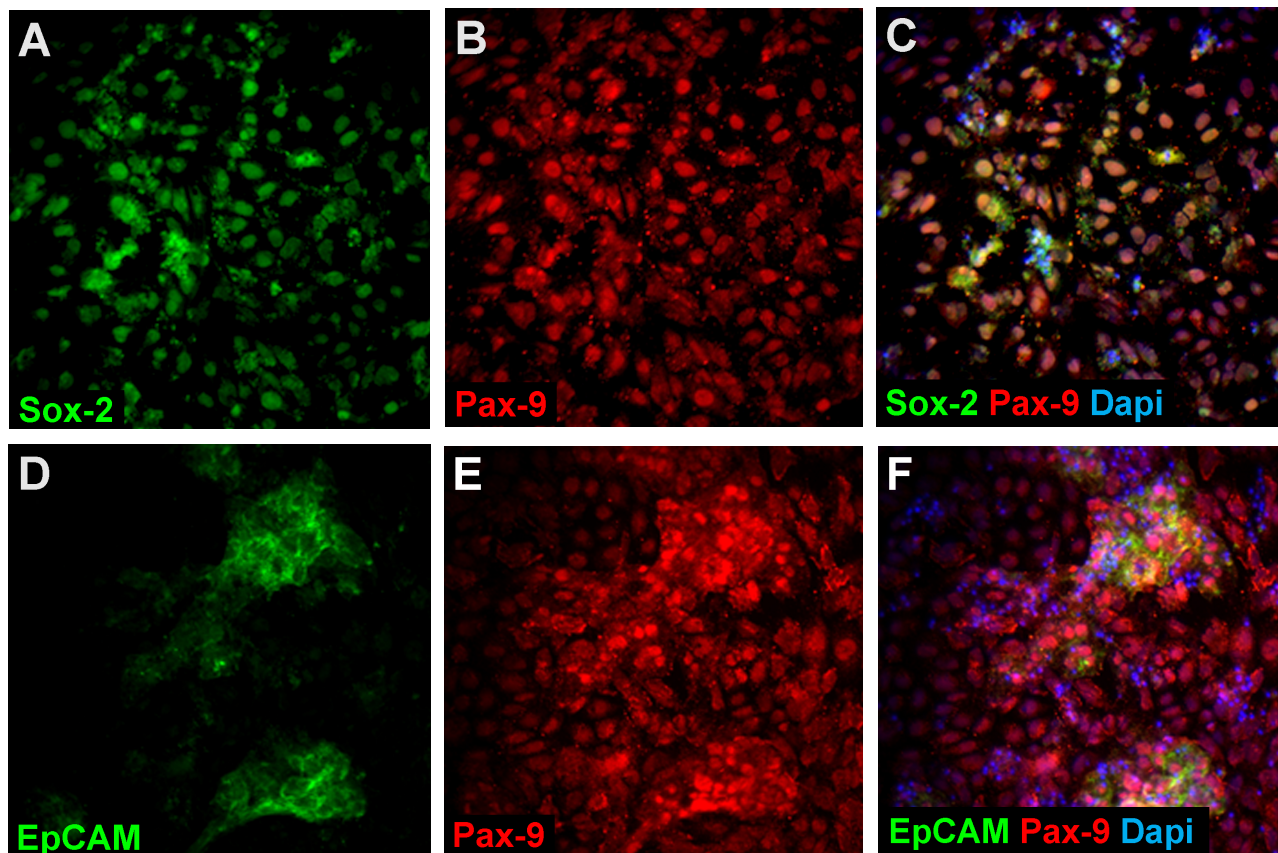


Figure 4. Approximately 95-100% AFE cells are double positive for Sox2 (A,C; Sigma AB5603A4; 1:50) and Pax9 (B, C; Abcam ab28538; 1:200). Approximately 20-30% AFE cells are double positive for EpCAM (D, F; Sigma MAB4444; 1:500) and Pax9 (E, F).

4.0 Transfer AFE cells to Suspension Culture (Cat. No. SCM307)

Continued from Section 2, Step 14 or Section 3, Step 12.

1. Aspirate and add 1 mL fresh 3dGRO™ Lung Organoid Branching Medium (SCM307) to each well of the 6-well plate containing the confluent layer of AFE cells.
2. Using a 5mL pipette, gently scrape along the surface of each well to dislodge the cell monolayer as aggregates. **Be careful to not triturate into single cells.** Transfer the cell aggregates from 1 well of a 6-well plate to a well of a Costar® Ultra-Low Attachment 24-well plate (Cat. No.

CLS3473). The transfer ratio is 1:1; one well of a 6-well plate of adherent AFE cells is scrapped and transferred to one well of a low attachment 24-well plate for suspension culture.

Note: Alternatively, a cell scraper (SARSTEDT Cat No. 83.1832) may be used to harvest the cells as aggregates. If a cell scraper is used, pipette up and down several times.

3. Add 0.5 mL of 3dGRO™ Lung Organoid Branching Medium (SCM307) to collect any remaining cells and add to the well of cells in the low attachment 24-well plate. Total volume ~ 1.5 mL cell suspension. Incubate at 37°C.
4. Exchange the media every other day until Days 20 -25. As the organoids are in floating culture, use the following protocol for the media exchanges:
 - a. Use a 1 mL glass serological pipet (Fisher Cat. No. 13-678-36A) to transfer the floating organoids to a sterile 15 mL conical tube. Wait 10 minutes for the organoids to settle down to the bottom of the tube.
 - b. Attach a 2 mL aspirating pipette to the vacuum pump. To the 2 mL aspirating pipette, attach a sterile, 20 µL **non-barrier** pipette tip. Carefully aspirate the medium. Be careful to leave a small volume of medium above the organoids as a buffer to avoid disturbing the organoids.
 - c. Add 0.5 mL 3dGRO™ Lung Organoid Branching Medium to the 15 mL tube containing the organoid pellet. Pipette up and down twice with a 1 mL glass pipette (Fisher Cat. No. 13-678-36A). Transfer the organoids back to the same well in the ultra-low attachment 24 well plate (Cat No. CLS3473).
 - d. Add 0.5 mL 3dGRO™ Lung Organoid Branching Medium to the 15 mL conical tube to rinse and collect any remaining organoids. Add the rinse to the same well of the ultra-low attachment 24 well plate. Total volume = 1 mL/well.

Day 23: Floating immature LBOs have further maturation potential

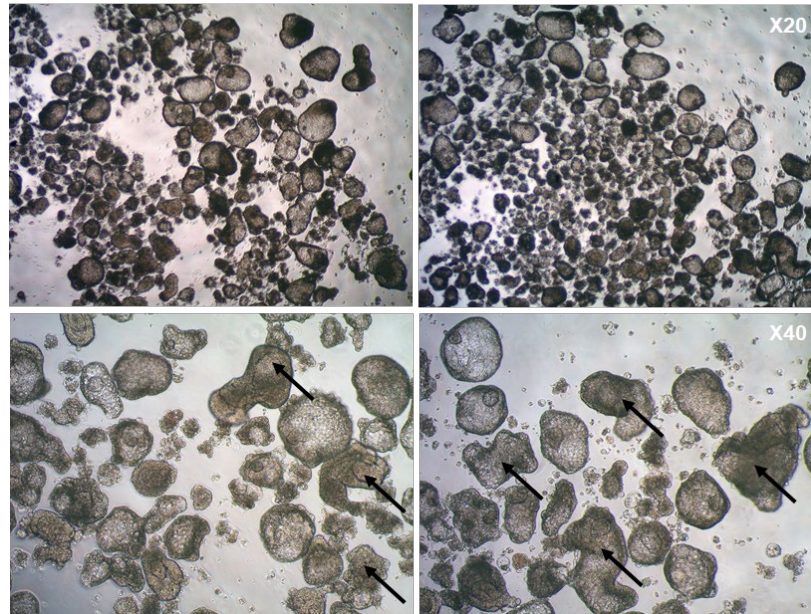


Figure 5. Representative morphologies of immature lung bud organoids (LBOs) on Day 23. Day 20 to Day 25 is the time period that LBOs may be selected and transferred to Matrigel sandwiches for further maturation. The lower panels show optimal organoids with folded structures that may be selected for maturation in Matrigel sandwiches (arrows).

5.0 Embed organoids in Matrigel Sandwiches at Days 20 to 25:

Note: At days 20 – 25, organoids with folded structures (Figure 5, arrows) are transferred, 4-6 organoids per insert, to make the Matrigel sandwiches (see below).

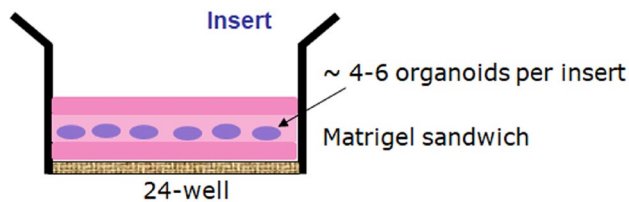


Figure 6. Schematic of Matrigel sandwich. Approximately 4-6 lung bud organoids are embedded between two layers of Matrigel to help induce further maturation.

1. Thaw Growth-Factor Reduced Matrigel (Corning Cat. No. 354230) on ice. **Note: The Matrigel tube should be kept on ice during the whole process to avoid gelling.**
2. Use sterile forceps to transfer sterile 24-well inserts (Corning Cat No. 353095) into non-tissue culture-treated 24 well plate (Corning Cat. No. 351147), one insert per well.
3. Aliquot 50 μL of cold 100% Growth Factor Reduced Matrigel to each insert. **Note: Be careful to not poke through the insert bottom.** Wait 5 minutes for the Matrigel to solidified.
4. During the 5 minutes wait time, transfer one well of floating LBOs to a 6 CM petri-dish. In a separate 96-well U-bottom plate (Fisher Cat. No. FB012932), add 60 μL of 3dGRO™ Lung Organoid Maturation Medium (SCM308) to each well.

Note: By days 20-25, there will be many floating lung organoids (see Figure 5). Each Matrigel sandwich should contain approximately 4-6 organoids. Calculate the number of Matrigel sandwiches you plan to make and that's the total number of wells in the 96-well plate that you will need to prepare the organoid/Matrigel suspension.

5. In a sterile dissection hood, use a sterile 20 μL pipette tip that is set at 10 μL volume to select and transfer 6 folded organoids (refer to Figure 5 for representative images of folded organoids) from 6 CM dish to 1 well of the 96-well U-bottom plate. **Note** It's easy to harvest all 6 LBOs in one suction due to high density of LBOs in 6 CM dish. Six total organoids transferred will roughly translate to $\sim 70 \mu\text{L}$ total volume per well of the 96-well plate.
6. Transfer the 96-well plate from the dissection hood to the tissue culture hood.

Note: To avoid premature gelling of Matrigel, we recommend preparing no more than 3 inserts at a time.

- a. Add 60 μL of cold 100% Growth Factor Reduced (GFR) Matrigel into each well of the 96-well plate from step 5 above. Do not do more than 3 inserts at a time. Mix gently by pipetting several times; avoid generating any bubbles. Total volume = 130 μL
- b. Immediately transfer the organoid-GFR Matrigel mixture ($\sim 130 \mu\text{L}$ volume) to the center of each insert in the 24-well plate prepared in step 3 above.
- c. Wait 5 minutes for the Matrigel to solidify.

- d. Repeat with the next set of 3 inserts until total 6 sandwiches are processed, continue to Step 7.
7. Add 75 μL of cold 100% Growth Factor Reduced (GFR) Matrigel to the top of the inserts to create the Matrigel sandwich. Put the 24 well plates in the incubator for at least 30 minutes to ensure Matrigel sandwiches have solidified. If you have more than 6 sandwiches to embed, repeat Step 4 to Step 7.
8. Add 500 μL of 3dGRO™ Lung Organoid Maturation Medium (SCM308) on top of the insert. Add 500 μL 3dGRO™ Lung Organoid Maturation medium into the wells underneath the inserts.
9. Exchange the medium every other day.
 - a. Attach a 2 mL aspirating pipette to the vacuum pump. To the 2 mL aspirating pipette, attach a sterile, 20 μL **non-barrier** pipette tip. Carefully aspirate the medium on top of the sandwiches and also underneath the inserts.
 - b. Add 500 μL of 3dGRO™ Lung Organoid Maturation Medium (SCM308) each, to the top of the insert and also to the wells underneath the inserts.
 - c. During the later stage of maturation, the embedded organoids may grow in size and some may extend complex processes (see Figure 7). During this time, the medium may turn yellow quickly. If this happens, add 750 μL of 3dGRO™ Lung Organoid Maturation Medium (SCM308) to the top of the inserts and still the same 500 μL to the well underneath the inserts.

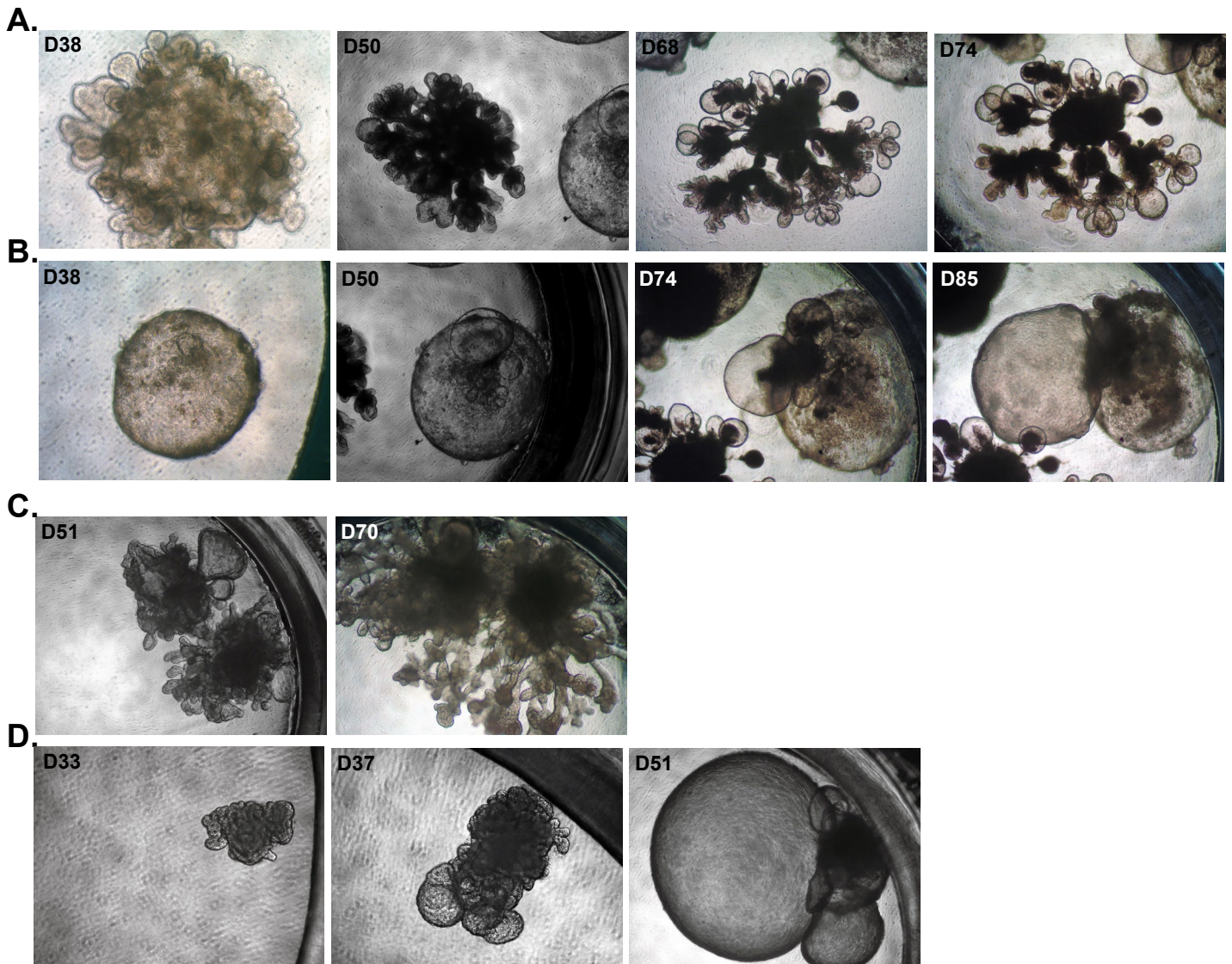


Figure 7. Maturation time course tracking of hiPSC-derived Lung Bud Organoids.

(A or B): Tracking LBOs in two individual wells derived from a human foreskin fibroblasts (HFF) derived iPSC line. **(C or D):** Tracking LBOs in two individual wells derived from a human PBMC-derived iPSC line. Both iPSC lines were seeded at 1×10^6 cells/well in a 6-well-plate at Day 1. At Day 20-25, around 4-6 immature LBOs were embedded in a Matrigel sandwich. Varied morphologies were observed which included branching structures and/or rounded expansion at the tips with dense materials in the center resembling alveolospheres. When the culture is maintained beyond Day 60-70, some large rounded structures may rupture, releasing a mucus-like material.

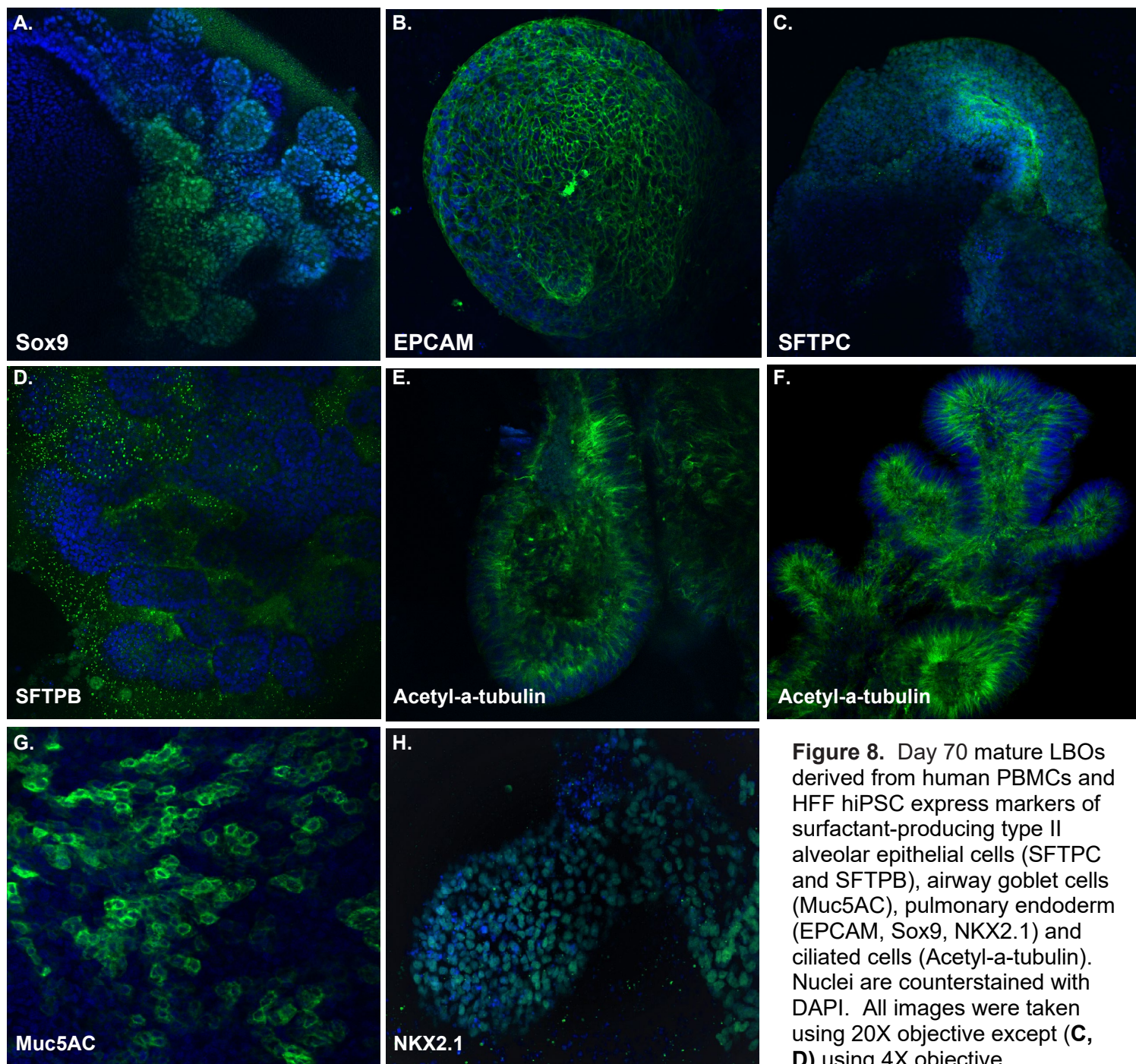


Figure 8. Day 70 mature LBOs derived from human PBMCs and HFF hiPSC express markers of surfactant-producing type II alveolar epithelial cells (SFTPC and SFTPB), airway goblet cells (Muc5AC), pulmonary endoderm (EPCAM, Sox9, NKX2.1) and ciliated cells (Acetyl-a-tubulin). Nuclei are counterstained with DAPI. All images were taken using 20X objective except (C, D) using 4X objective.

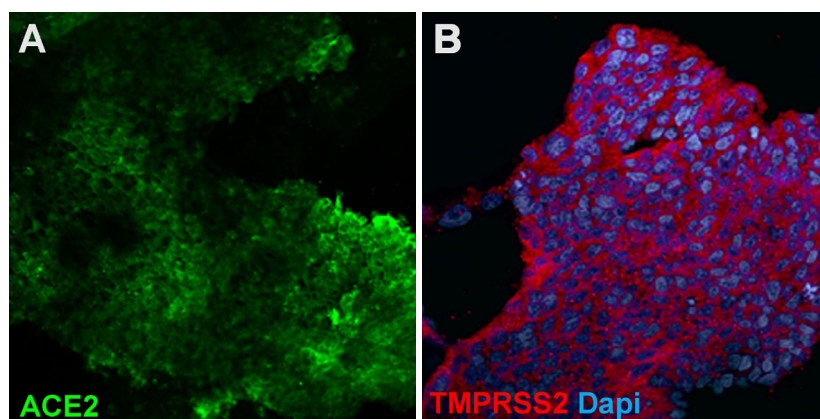


Figure 9. Lung bud organoids express ACE2, the SARS-CoV2 binding receptor (A) and the serine protease, TMPRSS2 (B), that enhanced SARS-CoV2 viral entry.

Frequently Asked Questions (FAQs):

1. After transferring LBOs at D20-25 to Matrigel Sandwiches, I do not see any branching or alveolar structures after two weeks?
 - a. The 3dGRO™ Lung Organoid Branching Medium (SCM307) and 3dGRO™ Lung Organoid Maturation Medium (SCM308) contain components that are temperature and light sensitive. Aliquot based on the experiment planned. Avoid repeated freeze/thaw. It is important to follow the instructions in “Storage and stability” section in the manual. Thaw and use a new aliquot of 3dGRO™ Lung Organoid Branching Medium (SCM307) and/or 3dGRO™ Lung Organoid Maturation Medium (SCM308).
 - b. Select lung bud organoids (LBOs) that have folded structures to transfer to make Matrigel sandwiches. Refer to Fig 4 for examples of LBOs with folded structures.
 - c. High cell density should be observed at both Day 4 and Day 8 before starting the floating suspension culture; adjust seeding density based on your cell types.
2. Floating LBOs attach to the bottom of plate during suspension culture? Use Ultra-low attachment 24-well plate. DO NOT just use tissue culture treated 24 well plate or Petri dishes.
3. When I change the medium for the Matrigel sandwiches, the top layer appears soft or even collapses? Add 75 µL instead of 50 µL 100% Growth Factor Reduced (GFR) Matrigel to the top layer of the sandwich. Put the 24 well plates in the incubator for ≥30 minutes to ensure that the Matrigel sandwiches have solidified.
4. Can I continue the differentiation even though the c-kit+CXCR4+ positive cells are below 80% at day 4? A compact c-kit+CXCR4+ should be observed at Day 4 with the percentage of 80-90% or higher. We do not recommend continuing the differentiation without successful definitive endoderm induction at Day 4. Restart using high quality, undifferentiated human iPSC cultures.

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