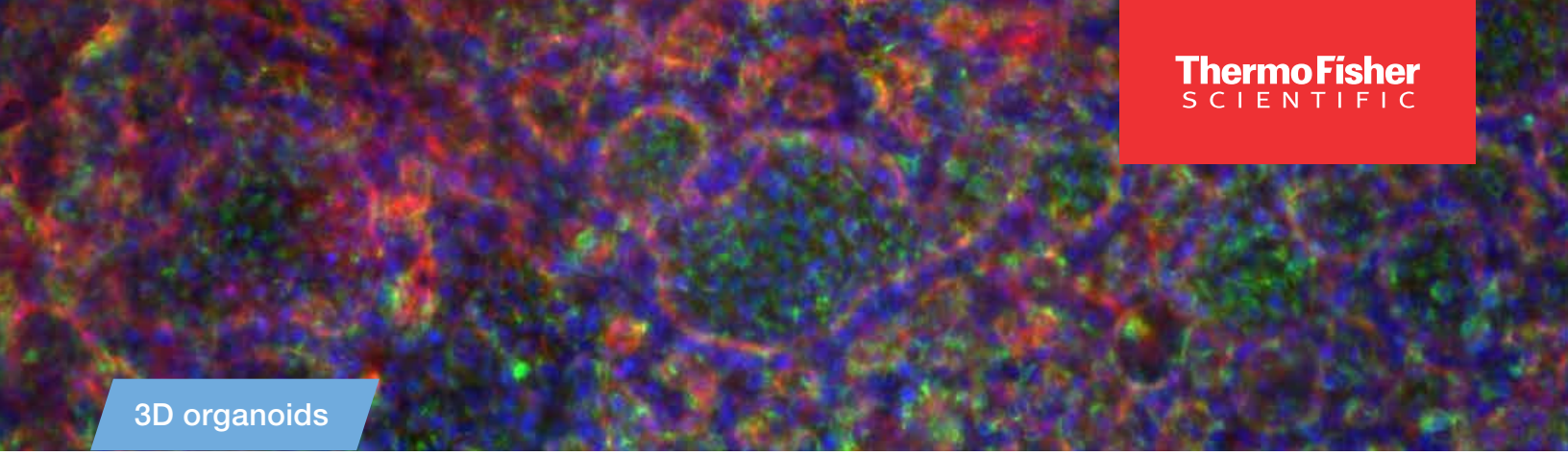

3D organoids

Five steps to creating a lung organoid disease model

For researchers interested in leveraging pluripotent stem cell (PSC)-derived organoid models to study respiratory diseases, this guide provides an overview of what is involved across the workflow, including tips and tricks from our scientists.

Whether you are just beginning your research with 3D cell culture models and need to learn more about organoids or spheroids, or you need appropriate tools to validate your *in vitro* models, we have outlined five easy steps to support your progress. Generating 3D cell culture models requires a commitment of time and resources, and you need assurance that your investment is going to provide you with a physiologically relevant model. Gibco™ media and reagents from Thermo Fisher Scientific are widely used for the growth, differentiation, and maturation of 3D lung cell culture models. The appropriate supplements and media are critical for growing and, in some cases, differentiating the cells within the *in vitro* model.

PSC-derived 3D lung organoids have significant potential for use in drug discovery and regenerative medicine. These organoids can be used to screen compounds for therapeutic efficacy and toxicity, as well as to model lung diseases, such as cystic fibrosis, pulmonary fibrosis, and lung cancer. Thermo Fisher Scientific's products and services can help provide researchers with the necessary tools to advance our understanding of lung development and disease, and to develop new therapies for patients in need.

Culture

- StemScale PSC Suspension Medium
- RevitaCell Supplement (100X)
- TrypLE Express Enzyme (1X), no phenol red
- GlutaMAX Supplement
- Nunc Non-Treated Multidishes
- Countess 3 FL Automated Cell Counter
- Nunc non-treated T-flasks for suspension culture
- Nalgene shake flasks for suspension culture

Differentiation

- GlutaMAX Supplement
- Gibco N-2 Supplement (100X)
- Gibco B-27 Supplement (50X)
- Gibco PSC Definitive Endoderm Induction Kit
- StemPro Accutase Cell Dissociation Reagent
- Gibco Bovine Albumin Fraction V
- Gibco Human BMP-4 Recombinant Protein
- Iscove's Modified Dulbecco's Medium
- Ham's F-12 Nutrient Mix

Maturation

- Geltrex matrix
- Gibco Human FGF-7 Recombinant Protein
- PeproTech Recombinant Human KGF (FGF-7)
- Gibco Trypsin-EDTA (0.05%)
- Gibco DPBS, no calcium, no magnesium

Characterization

- Human Lung Total RNA
- QuantStudio Real-Time PCR Systems
- TaqMan Assays
- Fluorescence-based reagents
- CellInsight HCA systems
- Varioskan LUX microplate readers

Scale-up and cryopreservation

- Mr. Frosty Freezing Container
- Gibco FBS
- Nunc non-treated T-flasks for suspension culture
- Nalgene shake flasks for suspension culture

gibco

Culture

With Gibco™ advanced cell culture media and reagents, researchers can efficiently generate lung organoids from human pluripotent stem cells (hPSCs). Gibco™ StemScale™ PSC Suspension Medium promotes the expansion of hPSCs as self-nucleating spheroids in suspension culture. The medium enables efficient nucleation, allowing for high cell yields and control of spheroid size, with uniform size facilitating successful differentiation. The spheroids can be differentiated in suspension to definitive endoderm (DE) cells using the Gibco™ PSC Definitive Endoderm Induction Kit. The DE spheroids can subsequently be induced to lung progenitor cells. The cells from these spheroids can be embedded in an extracellular matrix (ECM), such as Gibco™ Geltrex™ matrix, for further differentiation into lung organoids. Geltrex matrix provides a supportive extracellular scaffold for lung organoids, mimicking the natural environment of lung tissue and enhancing cell attachment, proliferation, and differentiation.

Together, these products enable researchers to create robust and functional 3D lung organoids for various applications, including drug screening and disease modeling. These products provide researchers with the necessary tools to culture 3D lung organoids that closely mimic the cellular and architectural complexity of the human lung. In addition, Thermo Fisher offers the Invitrogen™ Countess™ 3 Automated Cell Counter, which allows researchers to visualize and quantitate organoid growth and development.

Note: Prior to culturing the induced PSCs (iPSCs), genomic editing and manipulation of iPSCs was completed to allow for the building of the lung organoids model. From precision genome editing and gene modification technologies to high-efficiency delivery systems and reliable validation methods, we have developed a complete set of trusted solutions to help create the modified genes and stable cell lines that researchers need to study the impact of specific genetic mutations on lung development, disease progression, and drug responses.

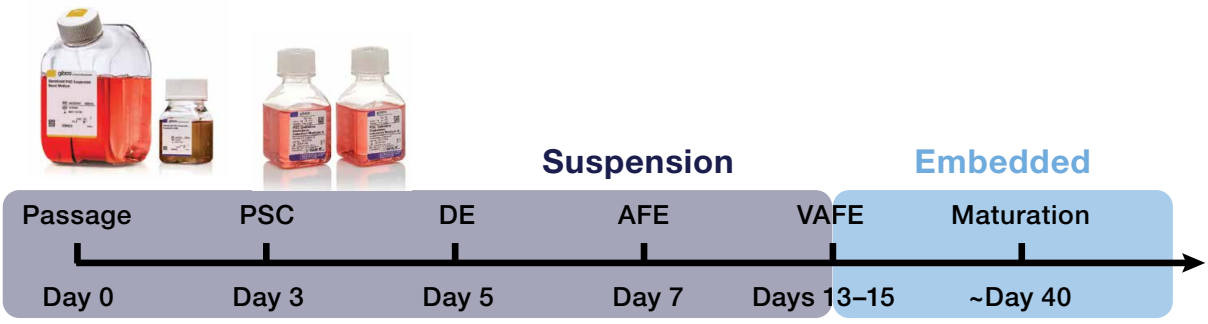

Figure 1. Timeline for the generation of lung organoids. The steps include passaging PSCs in suspension cultures; expanding PSC spheroids to sufficient size; differentiating to definitive endoderm (DE), anterior foregut endoderm (AFE), and ventralized anterior foregut endoderm (VAFE, also known as lung progenitor cells); and maturation to lung epithelial cells.

Tips and tricks

- Check your media requirements; half or full media changes may be required for long-term culture or differentiation protocols.
- When preparing to aspirate media from a well, tilting the microplate at an angle will prevent the pipette tip from touching the bottom of the well where the spheroids are settled.
- A few ways to control the size of spheroids include adjusting the initial cell seeding density, avoiding culture vessels with large surface areas, such as a T-flask or large petri dish, and using a more confined physical space to promote spheroid formation, such as a low-attachment microplate (e.g., Thermo Scientific™ Nunclon™ Sphera™ 96-well U-bottom plate).

Product highlights

- **StemScale PSC Suspension Medium**—StemScale medium is a scalable, easy-to-use medium that supports large-scale generation of hPSCs in suspension culture. It is formulated to address the current technical challenges of aggregating PSC spheroid culture systems.
- **Gibco™ RevitaCell™ Supplement (100X)**—This supplement has been optimized for use with PSCs, either as a post-thaw recovery solution to improve cell viability or in combination with StemScale PSC Suspension Medium to enhance single-cell passaging applications.
- **Invitrogen™ Countess™ 3 FL Automated Cell Counter**—This cell counter is capable of bright-field or fluorescence illumination with three-channel flexibility (bright-field and two optional fluorescence channels), enabling researchers to count cells, monitor fluorescent protein expression, gain insights to apoptotic processes, and measure cell viability.

Differentiation

Stem cell differentiation is a process by which stem cells develop into specialized cells with specific functions (Figure 2). Thermo Fisher offers a range of products to support stem cell differentiation for 3D lung organoids, including a comprehensive catalog of Gibco™ PeproTech™ recombinant proteins. Recombinant proteins and small molecules play an important role in guiding stem cell differentiation. Lung organoids differentiated from PSCs are 3D structures that mimic the architecture and function of human lung tissue, making them valuable tools for studying lung development, disease, and drug discovery.

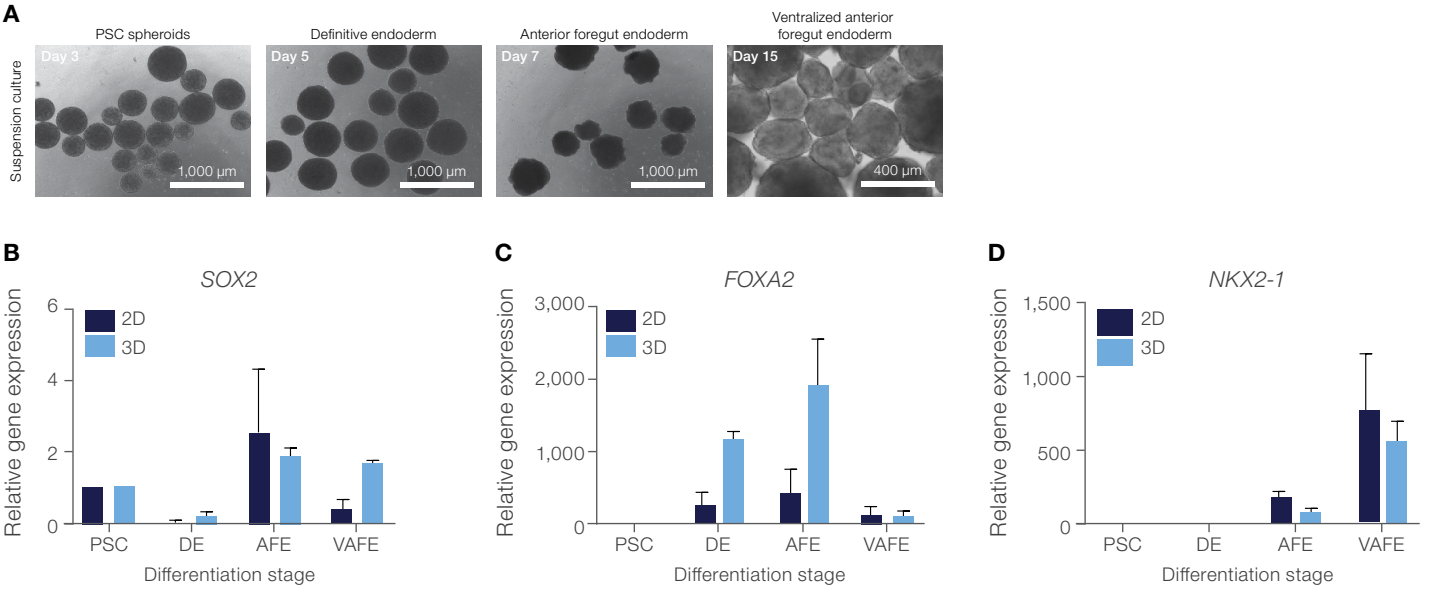

Figure 2. Analysis of spheroid differentiation. (A) Representative phase-contrast images of spheroids undergoing differentiation in suspension. **(B)** Gene expression measured by qPCR, normalized to *GAPDH,* and plotted relative to PSC expression of *SOX2*, **(C)** *FOXA2*, and **(D)** *NKX2-1* in cells undergoing differentiation in suspension (3D) or 2D adherent cultures.

Tips and tricks

- Geltrex matrix is a soluble form of basement membrane extracted from murine Engelbreth-Holm-Swarm (EHS) tumors and can be used as a replacement for products like Corning™ Matrigel™ matrix. **Learn more** about Geltrex basement membrane extract.
- It is critical to place the Geltrex matrix solution on ice to avoid premature gelling.
- When transitioning to dome culture, inverting the plate or dish can facilitate dome formation.

Product highlights

- **Gibco™ B-27™ Supplement**—This supplement is a defined yet complex mixture of antioxidant enzymes, proteins, vitamins, and fatty acids that are combined in optimized ratios to support neuronal survival in culture.
- **Gibco™ N-2 Supplement**—This is a chemically defined, serum-free supplement based on Bottenstein's N-1 formulation.
- **Gibco™ PSC Definitive Endoderm Induction Kit**—It consists of two xeno-free media that enable efficient induction of hPSCs to definitive endoderm.
- **Gibco™ GlutaMAX™ Supplement**—It is an alternative to L-glutamine, with increased stability that improves cell health. This supplement is suitable for both adherent and suspension culture of mammalian cells, with no adaptation required.

Services

- Our scientists can differentiate patient-derived iPSCs into a wide variety of terminal cell lineages.
- Our team can differentiate customer-provided PSCs or reprogram patients' somatic cells into iPSCs.
- Reduce complexity and stress with our dedicated project managers as your advocate.

Maturation

Confirming that 3D cell structures are developing and maintaining the appropriate morphology is paramount to establishing a 3D *in vitro* model with the correct cellular and biochemical makeup. Methods to monitor 3D cell cultures include measurement of cell count, cluster size, and growth patterns over time in multi-well plate formats. These measurements can be achieved with technologies such as microscopic imaging and high-content analysis.

As they mature, lung organoid cultures should adopt a more homogeneous cystic morphology (Figure 3). This typically happens over the course of a few passages between days 35 and 50, depending on cell line and experiment. The observed morphology is driven by differentiation to epithelial lineages that form the more organized cystic organoids.

Bright-field imaging can easily establish the diameter of spheroids and organoids, and these data can be used to calculate volume and estimate the number of cells in the 3D clusters. Quantification by fluorescence imaging of cells in a 3D cell culture model can be more difficult because of the dense cellular organization of a cluster of cells. Antifade mounting agents can help to visualize 3D cultures while maintaining the morphology of the cells, making them amenable to techniques like immunofluorescence (IF), immunocytochemistry (ICC), and immunohistochemistry (IHC). 3D staining can be improved by using Invitrogen™ Alexa Fluor™ Nano secondary antibodies, which have high affinities and reduced molecular weights.

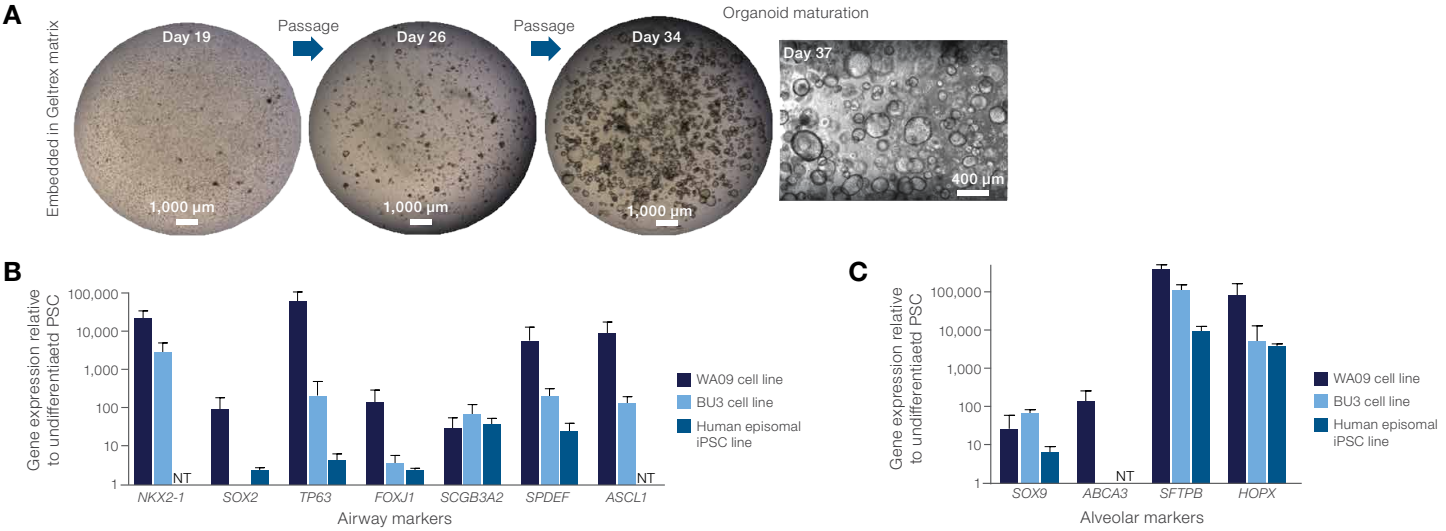

Figure 3. Dissociation and maturation of stem cells into organoids and their gene expression analyses. (A) Representative photos of dissociated cells embedded in Geltrex matrix as they form spheroids and differentiate into lung organoids with a cystic, ring-like morphology. Embedded cultures are passaged when the average organoid diameter reaches ~250 µm. **(B)** Gene expression analysis of differentiated lung organoids indicates enrichment for airway epithelial cell lineages, including basal (*TP63*), ciliated (*FOXJ1*), secretory (*SCGB3A2*), goblet (*SPDEF*), and pulmonary neuroendocrine cells (*ASCL1*). NT: not tested. **(C)** Gene expression analysis of differentiated lung organoids indicates enrichment for alveolar epithelial cell lineages (*SOX9*), including alveolar type II (*ABCA3*, *SFTPB*) and alveolar type I (*HOPX*) cells.

Tips and tricks

- Passage lung organoid cultures when they become overcrowded or reach an average diameter of ~250 µm.
- Replace the maturation medium every 48–72 hours.
- When passaging early spheroids and mature organoids, enzymatic and mechanical dissociation should be performed in parallel, as one method may work well for one cell line but not another.

Product highlights

- **PeproTech Recombinant Human KGF**—KGF (FGF-7) is one of 23 known members of the FGF family. KGF is a mitogen factor specific for epithelial cells and keratinocytes, which signals through FGFR2b. It also plays a role in kidney and lung development, as well as in angiogenesis and wound healing.
- **Geltrex matrix**—It is a basement membrane extract that contains laminin, collagen IV, entactin, and heparan sulfate proteoglycans, and supports growth of a variety of cells in 2D and 3D cell culture.
- **Gibco™ Trypsin-EDTA (0.05%)**—This solution is made from trypsin powder—an irradiated mixture of proteases derived from porcine pancreas. Because of its digestive strength, Trypsin-EDTA solution is widely used for cell dissociation, routine cell culture passaging, and primary tissue dissociation.

Characterization

Characterizing 3D lung organoids is an essential step in stem cell research, as it allows researchers to understand the differentiation and maturation of stem cells into specific cell types within the organoid. Thermo Fisher offers a range of products to support the characterization of 3D lung organoids, including specific antibodies, fluorophores, and imaging systems. Gene expression, which can be assessed by qPCR or sequencing using Ion Torrent™ instruments, provides information about cellular state, identity, and signaling pathways (Figures 2B–2D and 3B–3C). Protein expression is particularly useful for identifying relative abundance of cell populations within these cultures, and can be performed with immunostaining or flow cytometry. Key protein markers of lung development are observed in PSCs differentiated to lung organoids (Figure 4). To examine global transcriptome changes induced by this differentiation, bulk RNA sequencing was used to compare undifferentiated PSCs to the induced lung organoids. Principle component analysis demonstrated that the lung organoid transcriptomes were distinct from the undifferentiated PSCs. The experimental replicates from the same cell line cluster slightly closer together, possibly indicating cell line–dependent differences in the differentiation. Hierarchical clustering also grouped these samples by differentiation status and revealed clusters of activated or inactivated genes in the lung organoids. The 12 genes most enriched in the human lung were upregulated in lung organoid cells compared to undifferentiated PSCs; the expression levels of these genes tend to be lower than in primary lung cells (measured from Invitrogen™ Human Lung Total RNA, Cat. No. AM7968), suggesting that other niche factors may be required for complete maturation. Proximal airway and distal airway (alveoli) gene markers were also enriched compared to undifferentiated cells.

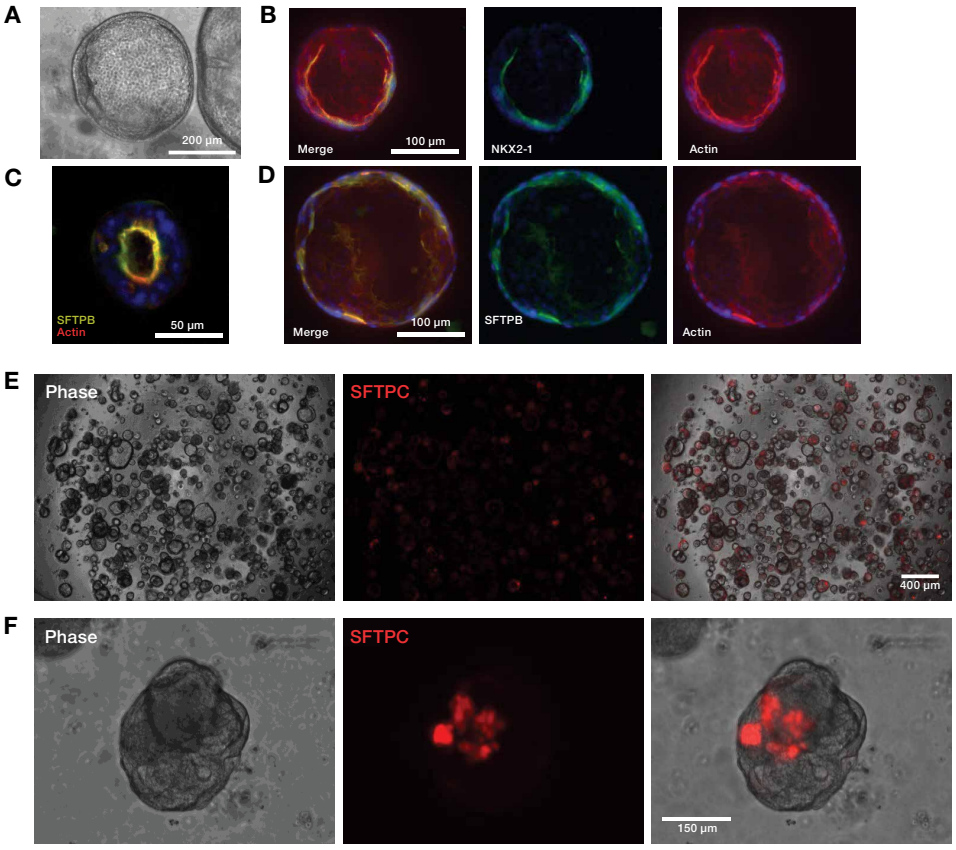

Figure 4. Protein detection of lung markers in organoids. (A) Representative phase-contrast image of cystic organoid morphology. **(B)** Immunofluorescent detection of NKX2-1 (green) in embedded lung organoids. Actin is stained with phalloidin (red). Nuclei are counterstained with Hoechst™ 33342 dye (blue). **(C,D)** Embedded lung organoids stained for SFTPB (green). Actin is stained with phalloidin (red). Nuclei are counterstained with Hoechst 33342 dye (blue). **(E,F)** iPSC cells, engineered with an endogenous fluorescent reporter for SFTPC (red), were differentiated to lung organoids and indicate the presence of alveolar type II cells expressing this marker, as well as other cell types.

Tips and tricks

- Select culture formats that support your desired imaging outputs. Embedded organoid cultures can be scaled down to 96-well plates for imaging and plate reader–based assays.
- Immunostaining 3D cultures tends to require longer incubation steps than traditional 2D cultures.
- Fixation of embedded cultures using 4% paraformaldehyde tends to dissolve the basement membrane extract domes, which is desirable for handling and imaging organoid structures in suspension. Alternatively, adding up to 1% glutaraldehyde to the fixation solution can preserve the domes for immunostaining or sectioning approaches. Note that glutaraldehyde can increase background fluorescence, so consider quenching this after fixation and include appropriate unstained controls.

Product highlights

- **Human Lung Total RNA**—This highest-quality RNA is DNase-treated, subjected to unsurpassed quality control standards, and certified to contain small RNAs (miRNA, siRNA, and snRNA).
- **Applied Biosystems™ QuantStudio™ Real-Time PCR Systems**—This family of systems detects changes in gene expression as low as 1.5-fold and supports a broad range of genomic applications, such as analyses of gene expression, microRNAs and noncoding RNAs, copy number variation, drug metabolism enzymes, and protein expression; SNP genotyping; and mutation detection.
- **Applied Biosystems™ TaqMan™ Assays**—These are the industry-leading choice for 5′ nuclease qPCR assays. They are designed using a highly sophisticated oligonucleotide probe/primer design pipeline that includes robust primer design algorithms and an extensive array of bioinformatics tools and processes to automate assay design.

Services

- Our team offers characterization services as a stand-alone option or in addition to other reprogramming, differentiation, or assay development projects.
- With access to a complete portfolio of characterization tools and cutting-edge instruments, our team can perform numerous experiments, including genomic analysis, pluripotency assays, and sterility testing.

Scale-up and cryopreservation

A previous protocol was longer and involved embedded culture (Figures 1A, 5A–B) , which limited its ease of use and scalability. To improve scalability, a protocol was developed to culture differentiated lung organoids in suspension, where diluted ECM protein (5% Geltrex matrix volume) promotes cell nucleation and reformation of organoids after seeding as single cells or small clusters (Figure 5C). Overall, the suspension protocol is more economical, saving time and cost. While expanding the lung organoids to larger cell numbers, reduced matrix consumption is beneficial for high-throughput experiments or for cryopreservation.

Lung organoids can be cryopreserved as small clusters of cells following mechanical passaging, which regrow into organoids upon thawing (Figure 5D). This enables an end-to-end workflow for biobanking where PSCs are differentiated to lung progenitors in suspension, induced to lung epithelial organoids in embedded culture, passaged into suspension culture for scale-up, and finally cryopreserved to create large working banks of frozen organoid cells.

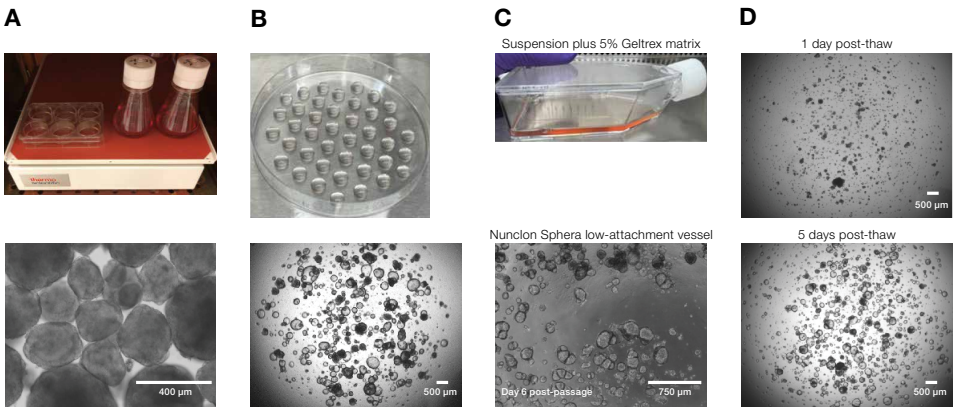

Figure 5. End-to-end workflow for differentiation and scale-up of large numbers of lung organoids for biobanking. (A) Top: Representative suspension cultures (6-well plate and 125 mL shake flask) on an orbital shaker placed in a cell culture incubator. Bottom: Phase-contrast image of ventralized anterior foregut endoderm spheroids grown in suspension. **(B)** Top: 50 µL Geltrex matrix domes containing organoid cells plated in a 100 mm petri dish. Bottom: Phase-contrast image of embedded lung organoids. **(C)** Top: T-75 flask containing suspension culture with dilute (5% by volume) Geltrex matrix. Bottom: Lung organoids grown in suspension in the 5% Geltrex matrix. **(D)** Lung organoids recovered from cryopreservation and embedded in Geltrex matrix 1 day (top) or 5 days (bottom) post-thaw.

Tips and tricks

- If cells are stuck to the bottom of the non–tissue culture treated plate, rinse with several mL of basal medium and collect in the same 15 mL conical tube.
- Cryopreservation based on a split ratio is recommended. For example, cryopreserve 3 domes in 1 cryovial and reseed those into ~9 domes upon thawing.

Product highlights

- **Gibco™ FBS**—It offers essential growth factors for the maintenance and growth of cultured cells.
- **Thermo Scientific™ Nunc™ EasYFlask™ flasks**—These non-treated T-flasks enhance cell attachment, growth, and differentiation.
- **Thermo Scientific™ Nalgene™ Shake Flasks for suspension culture**—These single-use Erlenmeyer cell culture flasks are ideal for suspension culture and media preparation.

Services

Obtain validated, high-throughput, screening-ready cells more efficiently with our cryopreservation service. Our standardized, high-quality process yields validated cell lines typically within 2 to 4 weeks.

- Provide your own cells or purchase cell lines.
- Our scientists scale up and produce cryopreserved cells.
- Cell lines are quality control–tested and validated.

Ordering information

Description	Cat. No.
Culture	
StemScale PSC Suspension Medium	A4965001
RevitaCell Supplement (100X)	A2644501
TrypLE Express Enzyme (1X), no phenol red	12563011
GlutaMAX Supplement	35050079
Nunc Non-Treated Multidishes	144530
Countess 3 FL Automated Cell Counter	AMQAF2000
Nunc EasYFlask Cell Culture Flasks—for suspension culture	156340
Nalgene Single-Use PETG Erlenmeyer Flasks with Plain Bottom—shake flasks for suspension culture	4115-0250
Differentiation	
GlutaMAX Supplement	35050079
N-2 Supplement (100X)	17502001
B-27 Supplement (50X), serum free	17504044
PSC Definitive Endoderm Induction Kit	A3062601
StemPro Accutase Cell Dissociation Reagent	A1110501
Bovine Albumin Fraction V	J64655.A1
Human BMP-4 Recombinant Protein	PHC9533
IMDM (Iscove's Modified Dulbecco's Medium)	12440053
Ham's F-12 Nutrient Mix	11765054
Maturation	
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A1413202
Human FGF-7 Recombinant Protein	PHG0094
PeproTech Recombinant Human KGF (FGF-7)	100-19
Trypsin-EDTA (0.05%), phenol red	25300062
DPBS (Dulbecco's Phosphate-Buffered Saline), no calcium, no magnesium	14190094
Characterization	
Human Lung Total RNA	AM7968
QuantStudio Real-Time PCR Systems	Request info
TaqMan Assays	Request info
CellInsight CX7 High-Content Analysis Platform	CX7B1112
Varioskan LUX Multimode Microplate Reader	VLB000D0
Scale-up and cryopreservation	
Mr. Frosty Freezing Container	5100-0001
FBS	16000069
Nunc EasYFlask Cell Culture Flasks—for suspension	156340
Nalgene Single-Use PETG Erlenmeyer Flasks with Plain Bottom—shake flasks for suspension	4115-0250

Learn more at **thermofisher.com/ipsc**

gibco