Generation of human airway organoids from primary cells

PromoCell®

Application note

This application note describes the generation of functional 3D airway organoids with apical-out or inward-oriented cilia formation based on human bronchial epithelial cells (HBEpC) as an *in vitro* model. These organoids can be used for high-throughput drug screening or large-scale virus-host interaction studies.

Background

The cultivation of primary human airway epithelial cells has been an integral part of respiratory research since its establishment by Lechner et al. in 1982.[1] As cellular function is intrinsically linked to the structural architecture of native tissue, three-dimensional culture systems offer several advantages over traditional monolayer cultures for studying airway biology.[2–5] Human airway epithelial cells can be successfully maintained in various culture formats, including submerged 2D cultures, air-lifted monolayers on porous membranes (Air-Liquid Interface culture), or as self-organized 3D organoids embedded in basement membrane extract (BME).

Self-organized 3D structures from functional primary bronchial epithelial cells were first developed by Rock et al. in 2009.[6] This research showed that human basal bronchial epithelial cells, when sorted by progenitor markers Integrin α6 (ITGA2) and nerve growth factor receptor (NGFR), could form viable self-assembled spheroids embedded in extracellular matrix gel. These structures were termed bronchospheres and exhibited organization with basal cell lining on the exterior and differentiated ciliated and mucus-producing cells oriented toward the inner lumen, establishing them as powerful functional 3D cell culture models.[7–10]

Modern culture techniques enable bronchospheres to polarize in an apical-out configuration, featuring outward-oriented cilia formation. This apical-out orientation is valuable for experimental applications since cells lack restricted access to the luminal surface, allowing improved nutrient and agent accessibility while presenting cell surface receptors directly to the culture medium. This configuration facilitates virus infection studies without requiring microinjection techniques, as viral particles can be simply added to the culture medium. For instance, apical-out airway organoids have been used to study SARS-CoV-2 infection by presenting ACE2 receptors on the exposed external surface.[11]

Fully differentiated organoids are morphologically characterized by a cell-free central lumen surrounded by a polarized epithelial cell layer. Ciliated cells can be found either directed toward the inner lumen or in an outward-oriented configuration.[12] The synchronous beating of outward-oriented cilia may initiate swirling motion of the organoid within the BME matrix. Addition of the cell survival factor ROCKi (Rho-associated protein kinase inhibitor) Y-27632 to airway organoids has been shown to increase colony forming efficiency by approximately 20%.[13,14]

Long-term airway organoid cultures are widely used in research, including disease modeling, drug toxicity and efficacy studies, lung cancer research, and investigation of genetic variations.[15] These models are also useful for high-throughput drug screening and provide robust platforms for studying complex cellular interactions. Recent advances have expanded organoid applications to include lung regeneration, cancer research, drug screening, and studies on cilia motility and viral infection, demonstrating the broad utility of these 3D culture systems across diverse research domains.[16–18]

Our Air-Liquid Interface Medium (ALI-Airway) was developed for culturing human bronchial epithelial cells (HBEpC) on porous membranes to form tight functional epithelial barriers in a serum- and BPE-free environment.

Although porous membrane systems may limit high-throughput applications, ALI-Airway medium can be used for generating 3D bronchospheres by embedding HBEpC in BME, where they form organoid-like structures suitable for high-throughput drug screening. The first signs of cilia beating in our ALI-Airway medium can be observed microscopically after 2 weeks. This application note describes a protocol for cultivating airway organoids with high self-renewal potential for over 4 weeks.

Even more robust cell differentiation in 3D can be observed using our pre-screened bronchial epithelial cells in ALI-cultures or organoids when using our serum- and BPE-free ALI-Airway medium. For cultivation of 2D culture, we have two variants of Airway Epithelial Cell Growth Medium (see protocol). For a more regulated research environment, we recommend our safe xeno-free medium variant, which is adjustable for your EXCiPACT™-GMP needs.

Our medium supports reproducible research while maintaining the highest standards of scientific rigor. For more information, contact our GMP expert team at gmp@promocell.com.

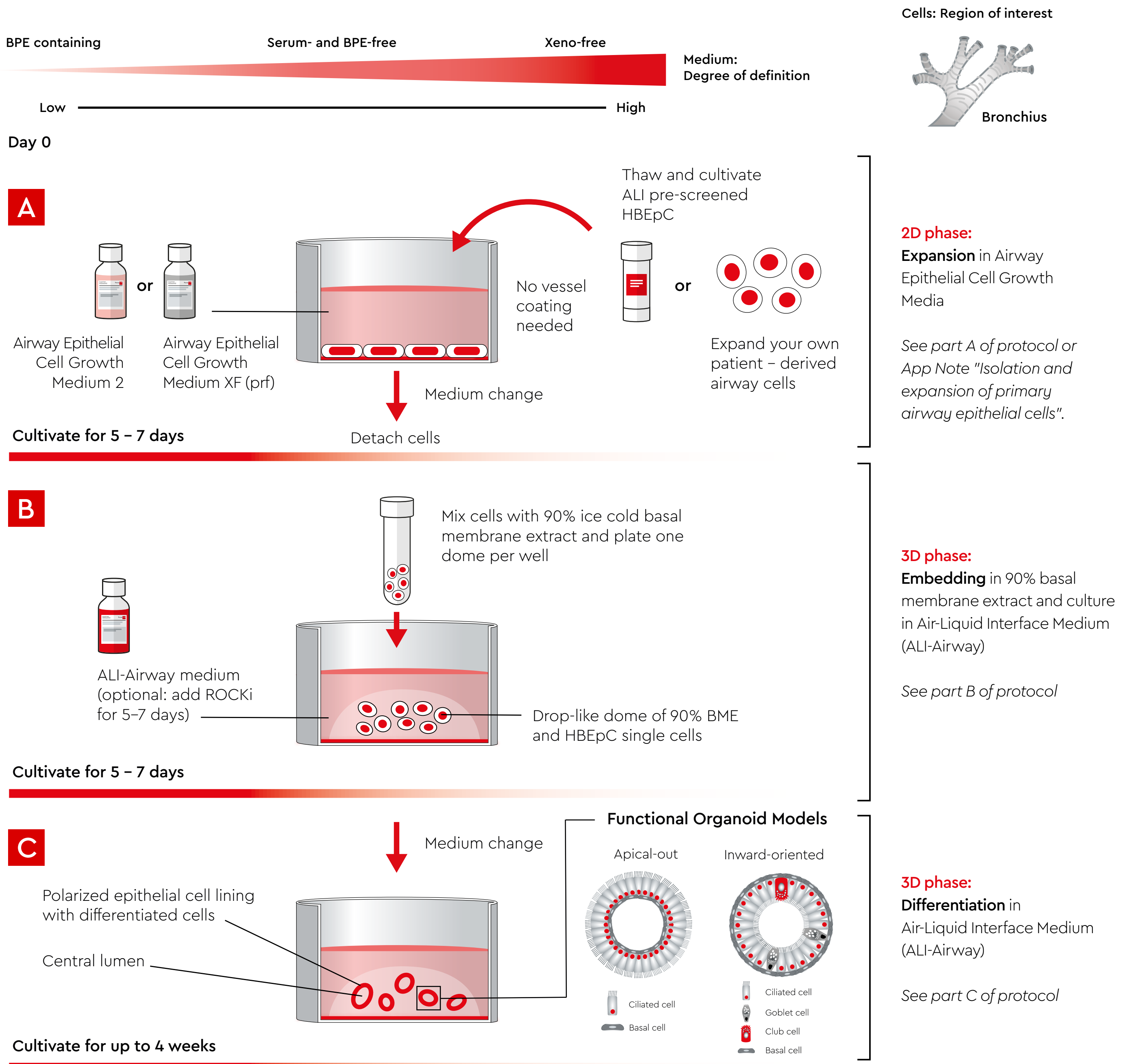


__Fig. 1: Workflow for generating functional 3D airway organoids from primary human bronchial epithelial cells.__ The workflow encompasses three phases: __A)__ Thawing and cultivation/expansion of isolated HBEpC in Airway Epithelial Cell Growth Medium (choose between Airway Epithelial Cell Growth Medium 2 or Airway Epithelial Cell Growth Medium XF (prf) for 5–7 days in 2D culture. __B)__ Cell detachment and embedding of a drop-like cell-containing dome of single-cell HBEpC in 90% ice-cold basement membrane extract (BME), followed by cultivation in Air-Liquid Interface Medium with optional ROCKi supplementation for enhanced colony formation efficiency. __C)__ Daily medium changes to promote differentiation into functional organoids with a polarized epithelial cell lining containing ciliated cells. Approximately 2 weeks after re-seeding, a central lumen of the 3D cell constructs will become visible, and ciliated cells can appear at the outside of the polarized epithelial cell lining with differentiated cells of the organoid (apical-out) or inward oriented towards the lumen (inward-oriented). Both Airway Epithelial Cell Growth Medium 2 (C-21040) and Airway Epithelial Cell Growth Medium XF (prf) (C-21050) can be used for the 2D phase. For isolation protocol, see application note "Isolation and expansion of primary airway epithelial cells".

Protocol for generation of airway organoid culture system - Part A

2D expansion of primary Human Bronchial Epithelial Cells

The protocol in this section describes the procedure for thawing and expanding the required amount of HBEpC in 2D culture.

For best results, we recommend our ALI pre-screened HBEpC. Alternatively you can use your own isolated cells derived from patient-tissue. For own isolated cells, we recommend to use the cells in early passage (P3) and to precultivate in Airway Epithelial Cell Growth Medium 2.

I. Materials

- ALI pre-screened Human Bronchial Epithelial Cells (ALI pre-screened HBEpC; C-12640) or Human Bronchial Epithelial Cells (HBEpC; C-12640)
- Airway Epithelial Cell Growth Medium 2 (C-21040) or Airway Epithelial Cell Growth Medium XF (prf) (C-21050)
- Cell culture vessel (e.g., Falcon®)
- DetachKit (C-41210; 125 ml), which contains the following products: HEPES Buffered Saline Solution (HEPES BSS), 0.04% Trypsin/0.03% EDTA, and Trypsin Neutralization Solution (0.05% Trypsin Inhibitor in 0.1% BSA)
- Optional: Physiological buffer (Dulbecco's PBS w/o Ca^{++}/Mg^{++} C-40232, HEPES BSS C-40000 or similar)

II. Protocol

1

Prepare airway growth medium

Depending on your regulatory field, choose between serum and BPE-free or xenofree formulation. Thaw the SupplementMix at 15–25°C. Aseptically mix the supplement solution by carefully pipetting up and down. Then transfer the entire contents of the supplement to 500 ml of basal medium. Protect the medium from light.

2

Adjust the medium to room temperature

Calculate the amount of airway growth medium needed to start the culture. Prewarm only an aliquot of the complete medium protected from light, and store the remaining medium refrigerated at 4–8°C.

3

Thaw the HBEpC

Remove the cryovial from liquid nitrogen and transport it on dry ice. Under a laminar flow bench, release the pressure of the vial by briefly twisting the cap counterclockwise by a quarter turn and then retightening it. Allow the cell suspension to thaw in a water bath at 37°C for 2 minutes. Rinse the vial with 70% EtOH and place it under a laminar flow bench. Aspirate the ethanol from the threads of the screw cap. Carefully open the cryovial. Transfer the cell suspension to a centrifuge tube containing 9 ml of growth medium.

Note: Our cryopreserved cells are frozen in Cryo-SFM Plus (C-29920), which contains DMSO. Work quickly to prevent a longer incubation of the cell suspension in Cryo-SFM Plus, because the cells are very sensitive after thawing.

4

Collect the cells and determine the cell number and viability

Gently mix the 10 ml cell suspension and take an aliquot for cell counting. Centrifuge the 10 ml cell suspension (300 x g for 3 minutes) and aspirate the supernatant. Resuspend the pellet in airway growth medium. Count the cells from the aliquot using your standard method.

5

Incubate the cells

Use a seeding density of 5,000 cells per cm² in growth medium (180 µl per cm²). Gently swirl the vessel containing the cell suspension and place it in an incubator (37°C, 5% CO_2). The next day, check the cell adherence under a microscope.

6

Let the cells grow

Change the medium every 2–3 days.

7

Detach the cells when reaching ≥70% confluence

Once the cells have reached ≥ 70% confluence, they can be passaged. Carefully aspirate the culture medium. Add 0.15 ml HEPES BSS per cm² of vessel surface to wash the cells and agitate the vessel carefully for 15 seconds. Aspirate the HEPES BSS (optional Dulbecco's PBS w/o Ca^{++}/Mg^{++}) and cover the cells with 50 µl Trypsin/EDTA Solution per cm². Transfer the vessel to an incubator for 4 minutes.

8

Harvest the detached cells

Monitor the round-up process of the cells under a microscope. Tap the side of the culture vessel to enhance the cell dissolution from the plastic. When cells are detached, add 50 µl Trypsin Neutralization Solution per cm² and gently agitate. Resuspend the cells and pipette the cell suspension into a centrifuge tube containing an appropriate volume of airway growth medium. Place the culture vessel under a microscope and check if all the airway epithelial cells are harvested. Centrifuge the cell suspension at 300 x g for 3 minutes.

Note: Epithelial cells stick tightly to plastic because of the large number of adherens junctions. If the cells do not round up after 4 minutes of incubation at 37°C, you can place the vessel in the incubator for 1 additional minute. Do not over-trypsinize them. If they are still sticking after 1 minute of incubation, use a 1000 µl pipette to wash them down.

9

Resuspend the cell pellet

Discard the supernatant and resuspend the cell pellet carefully by pipetting up and down in an appropriate volume of airway growth medium.

10

Count the cells using your standard method

Note: For further cultivation, reseed the HBEpC at a density of 5,000 cells/cm² in a cell culture vessel containing 180µl per cm² of prewarmed airway growth medium. Incubate at 37°C with 5% CO_2. If you wish to proceed with organoid seeding, continue with Part B of this AppNote. Store the cell suspension in a precooled centrifuge tube until mixing it with BME within the next 15 minutes.

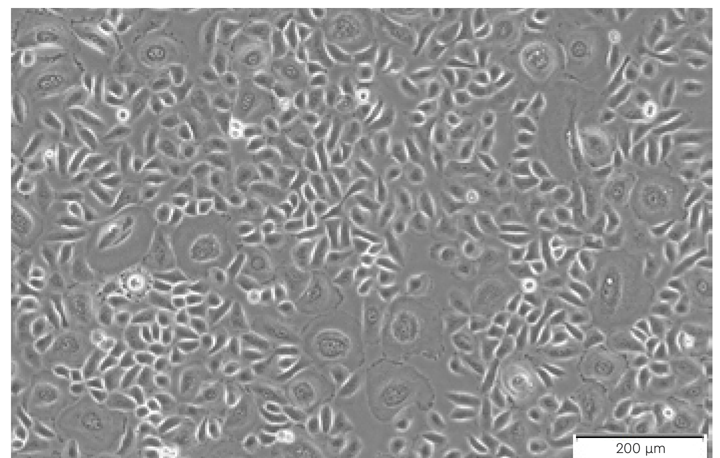


Fig. 2: Growth and morphology of HBEpC in 2D in serum- and BPE-free Airway Epithelial Cell Growth Medium 2. *Microscopy of HBEpC in 2D culture five days after thawing. Picture was taken before passaging (10x magnification, scale bar = 200 µm).*

Protocol for generation of airway organoid culture system - Part B

Embedding of HBEpC into basal membrane extract (BME)

This section describes the detachment from 2D culture and embedding of HBEpC into BME for 3D culture to initiate 3D airway organoid formation under defined culture conditions.

I. Materials

- ALI pre-screened Human Bronchial Epithelial Cells (ALI pre-screened HBEpC C-12640) or Human Bronchial Epithelial Cells (HBEpC; C-12640)
- Airway Epithelial Cell Growth Medium 2 (C-21040) or Airway Epithelial Cell Growth Medium XF (prf) (C-21050)
- Air-Liquid Interface Medium (ALI-Airway, C-21080)
- Phosphate Buffered Saline without Ca++/Mg++ (PBS, C-40232)
- 0.04% Trypsin/0.03% EDTA (Trypsin/EDTA, C-41010)
- 0.05% Trypsin Inhibitor, 0.1% BSA (TNS, C-41110)
- Gentamicin sulfate solution with a final concentration of 50 µg/ml in the medium
- 96-well plate for suspension cells (e.g., Cellstar® 96-Well Suspension U-bottom Plate, product number 650185 Greiner, Bio-One)
- Growth factor-reduced basal membrane extract of Engelbreth-Holm-Swarm mouse sarcoma (e.g., Cultrex™ UltiMatrix Reduced Growth Factor Basement Membrane Extract, product number BME001-05, R&D Systems)
- Optional: 10 mM Y-27632 stock solution (e.g., Y-27632, product number S6390, Sellekchem)

II. Protocol

1

Prepare a 10 mM Y-27632 stock solution (optional)

For optimal seeding results of airway organoids, the ALI-Airway medium can be optionally supplemented with 10 µM Y-27632, a ROCK inhibitor. The addition of ROCKi enhances colony forming efficiency and promotes the development of larger 3D cellular constructs. To prepare the working solution, create a 1,000x stock solution by dissolving 1 mg of Y-27632 powder in 312 µl of DMSO, creating a 10 mM stock solution. Vortex thoroughly until completely dissolved, then aliquot into small volumes and store at -20°C until use. Avoid repeated freeze-thaw cycles to maintain the stability of the compound.

2

Prepare ALI-Airway medium for use as airway organoid seeding medium

Remove an appropriate aliquot of fully supplemented ALI-Airway medium (e.g., 50 ml) from storage and allow it to reach room temperature. We strongly recommend supplementing the medium with gentamicin sulfate (final concentration of 50 µg/ml) to prevent contamination. If using the optional ROCKi for increased colony forming efficiency, add 10 µM Y-27632 (e.g., 50 µl of 10 mM Y-27632 stock per 50 ml of ALI-Airway medium). The prepared airway organoid seeding medium is stable for 6 weeks when stored at 4–8°C.

Note: The addition of Y-27632 to the airway organoid seeding medium is not essential for achieving cilia beating airway organoids, but it improves colony forming efficiency when added for 5–7 days following seeding in the BME.

3

Prepare aliquots of basal membrane extract

BME stock solution requires storage at -20°C and undergoes gelation at room temperature. To prevent premature gelling, minimize exposure to temperatures above 8°C. We recommend preparing BME aliquots in advance for convenient use. Thaw the BME stock solution overnight at 4–8°C, keeping the vial on ice during transfer to the laminar flow bench. Work quickly to prevent gelation, pipetting BME aliquots into fresh precooled vials. Maintain aliquots on ice and store at -20°C.

4

Calculate a cell suspension

We recommend preparing a cell suspension with a concentration ranging from 1×10^6 cells/ml to 2×10^6 cells/ml. If you use your own isolated cells, we recommend using them in early passage (passage 3 or earlier) and starting with a density of 2×10^6 cells/ml.

Take the cell suspension from step 10 of Part A under laminar flow bench. After completing cell counting, calculate the volume required to prepare a cell suspension at 2×10^6 cells/ml and spin them down (3 minutes at 300 x g). Resuspend the cell pellet in 500 µl ALI-Airway medium (optionally supplemented with 10 µm Y-27632) at the target concentration and maintain the cell suspension on ice under the laminar flow bench for optimal results.

Note: This protocol optimizes the seeding density at 10,000 cells per 50 µl cell-matrix mixture for one 96-well plate using HBEpC in passage three. Cells in higher passages may require adjusted seeding densities for optimal results.

5

Prepare the matrix and embed cells in the 90% BME gel

Remove a BME aliquot from -20°C storage and place it at 4–8°C to thaw overnight the day before use. Once thawed, place the vial on ice, maintaining cold conditions throughout the procedure. Preheat multiwell plates at 37°C in an incubator for at least two hours to accelerate matrix gelling upon plating. Keep the cell suspension on ice and use precooled tubes for all steps. For a 96-well plate format, combine 500 µl of prepared cell suspension (2×10^6 cells per ml) with 4,500 µl of matrix on ice to create a 90% BME-cell solution. Mix gently and dispense 50 µl of the cell-matrix mixture to each well of the preheated plate, resulting in 10,000 cells per well. We strongly recommend using U-bottom suspension plates to prevent cell adherence and proliferation on plastic surfaces. Avoid introducing air bubbles during pipetting and work efficiently within 60 seconds. Do not agitate the plate after plating. Incubate the plate at 37°C with 5% CO_2 for 30 minutes to solidify the cell-matrix without movement. After gelation, examine the cell-matrix under a microscope to confirm gelled cell-matrix and even cell distribution throughout each well. Return the plate to the laminar flow bench and add approximately 200 µl of ALI-Airway medium (optionally including 10 µM Y-27632) to each well. The medium volume should cover approximately 90% of the well. Place the plate in an incubator (37°C, 5% CO_2) for cell growth initiation.

Note I: For reduced well usage in 96-well suspension plates, mix 5 µl of precooled cell suspension (2×10^6 cells per ml) with 45 µl of ice-cold BME and pipette the entire 50 µl volume into one well of the preheated plate to achieve 10,000 cells per well.

Note II: If you wish to use a 24-well format, you can use 24-well suspension plates (see Related products table). It is important to preheat the 24-well plate in a 37°C incubator at least two hours to enhance a fast-gelling process when pipetting the 50 µl domes in the well. If the plate is not preheated, the domes will not form a proper 3D "drop-like" architecture and melt. Pipet the 50 µl cell-matrix mix in the center of one well by holding the pipet vertically. Do not touch the prewarmed plastic bottom of the well with the pipet tip to avoid gelling in the tip. Gently dispense the cell-matrix mix and avoid air bubbles. After pipetting the domes quickly incubate the plate for 30 minutes at 37°C in an incubator. Do not move the plate. After the specified time, check for proper gelling. Afterwards add 500 µl of ALI-Airway (optional: incl. 10 µM Y-27632) to each well and incubate at 37°C and 5% CO_2 in a humidified incubator.

Protocol for generation of airway organoid culture system - Part C

3D cultivation and differentiation of airway organoids

This section outlines the daily maintenance of airway organoids in ALI-Airway medium, leading to the formation of differentiated, ciliated 3D structures within two to four weeks.

I. Materials

- Air-Liquid Interface Medium (ALI-Airway, C-21080)
- Gentamicin sulfate solution with a final concentration of 50 µg/ml
- Optional: 10 mM Y-27632 stock solution (e.g., Y-27632, product number S6390, Sellekchem)

II. Protocol

1

Cultivate 3D cell culture in ALI-Airway medium

Bring ALI-Airway medium (optionally supplemented with 10 µM Y-27632) to room temperature before use. Implement daily medium changes for optimal organoid development. Carefully aspirate approximately 180 µl of the medium, taking care not to contact the cell-matrix mixture with the pipette tip. Gently dispense approximately 180 µl of fresh ALI-Airway medium (optionally including 10 µM Y-27632) to each well. Spheroid structures typically form during the first three days of culture. If using the optional 10 µM Y-27632 supplementation, transition to medium without ROCKi after 5–7 days of seeding.

Important considerations: Avoid using vacuum pumps for medium removal as aspiration can result in sample loss. Exercise caution during medium changes since the cell-matrix mixture has a soft consistency and can be easily aspirated with a 1,000 µl pipette tip. If uncertainty exists regarding the separation between the cell-matrix and culture medium phases, aspirate a smaller volume of medium. Due to the daily medium change requirement in 96-well plate format, the precise volume of fresh medium is less critical. For weekend coverage, perform medium changes on Friday afternoon and Monday morning. Daily medium changes are essential because of the high metabolic activity of the cells. If you use a 24-well plate format, a medium change every 2–3 days is required instead of daily changes.

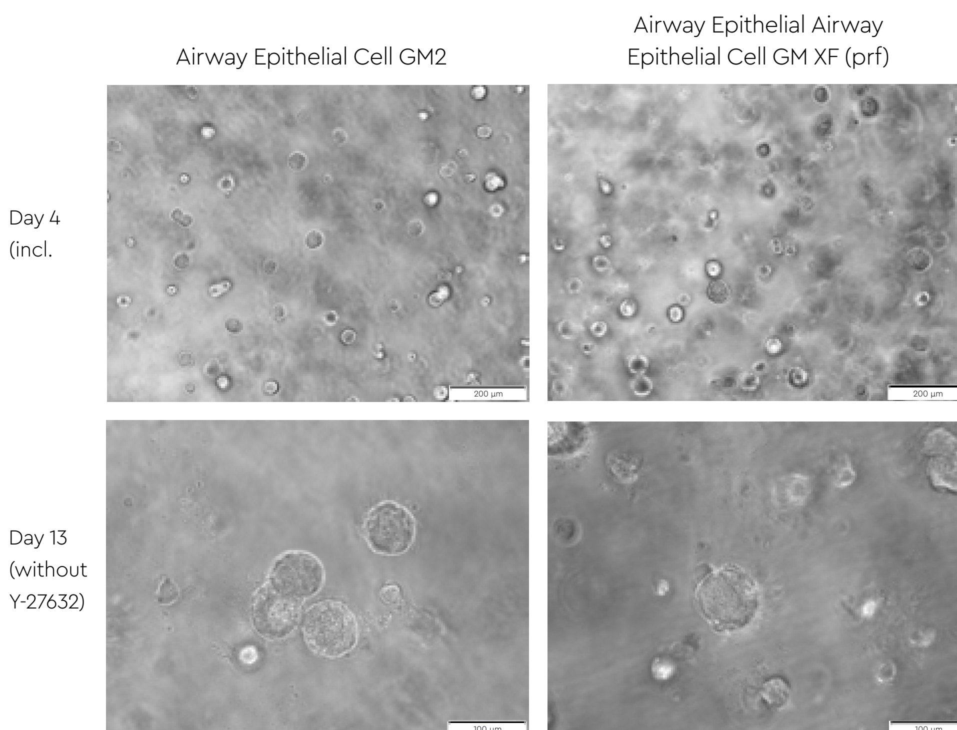


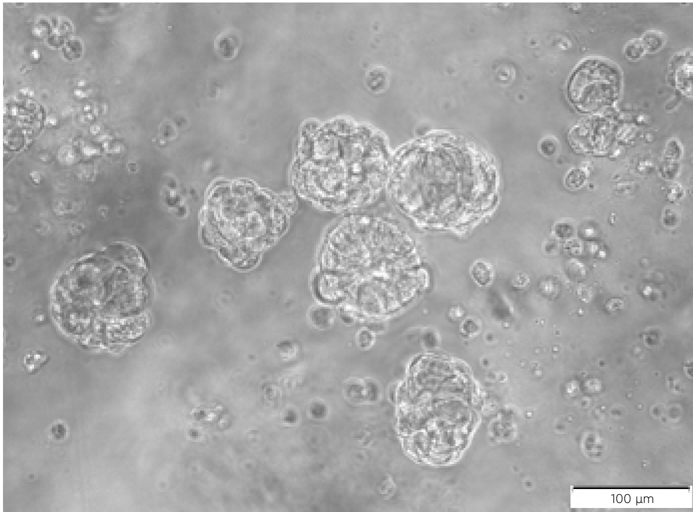
Fig. 3: Time-dependent formation of an organoid-like structure of HBEpC embedded in 90% BME. *Cryopreserved primary HBEpC were seeded and precultured in Airway Epithelial Cell Growth Medium 2 or Airway Epithelial Cell Growth Medium XF (prf) at 5,000 cells per cm². Following detachment with Trypsin/EDTA, cells were embedded in 90% growth factor-reduced basement membrane extract (BME) (Cultrex™ UltiMatrix, R&D Systems) in 96-well U-bottom plates (Cellstar® Greiner Bio-One) at a seeding density of 10,000 cells per well. ALI-Airway medium supplemented with 10 µM ROCKi (Y-27632) served as the organoid seeding medium. After gelation, the cell-matrix mixture was covered with ALI-Airway medium (including Y-27632) with daily medium changes. After six days of incubation, cells were switched to medium without ROCKi.* ***Upper panel:*** *Microscopy of HBEpC at day four in organoid culture shows cell aggregation and early colony formation with ROCKi supplementation to enhance colony forming efficiency (10x magnification, scale bar = 200 µm).* ***Lower panel:*** *Microscopy of HBEpC at day 13 of organoid culture demonstrates organoid-like structures with round, compact morphology and emerging central lumens (20x magnification, scale bar = 100 µm).*

2

Differentiation can be observed after two weeks of organoid culture

Following approximately two weeks of organoid culture, ciliated cells become visible under a microscope, indicating successful differentiation. Ciliated cells may be positioned at the exterior of the polarized epithelial cell lining (apical-out configuration) or located at the inner surface of organoid lumens. With extended culture time, central lumens become clearly visible and can be analyzed using confocal microscopy techniques. Organoids featuring apical-out positioned ciliated cells characteristically rotate within the matrix due to powerful synchronous cilia beating. Complete differentiation is achieved after four weeks of 3D culture (see Fig. 4).

Airway Epithelial Cell GM2



Airway Epithelial Airway Epithelial Cell GM XF (prf)

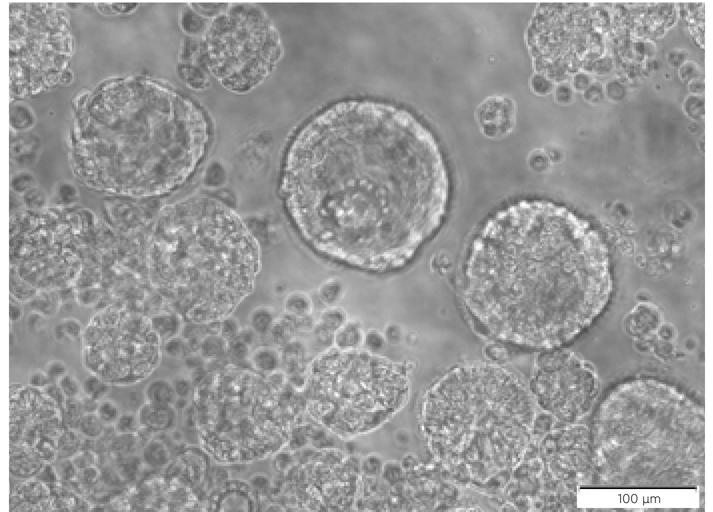


Fig. 4: Functional airway organoids can be used for long-term culture over 4 weeks in complete BPE-free culture system. *Cryopreserved HBEpC were seeded and precultured in Airway Epithelial Cell Growth Medium 2 or Airway Epithelial Cell Growth Medium XF (prf). Following passaging with Trypsin/EDTA, cells were embedded in 90% BME (Cultrex™ UltiMatrix, R&D Systems) and cultivated in 96-well U-bottom plates (Cellstar® Greiner Bio-One) using ALI-Airway medium for more than four weeks. Both apical outward and inward-oriented ciliated airway organoids were successfully formed, demonstrating the versatility and robustness of the culture system (20x magnification, scale bar = 100 µm).*

A video demonstrating rotating apical outward-oriented airway organoids in our airway media can be accessed here:
https://youtu.be/sJpD3W1nvU8

Trademark references

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Products

Product	Size	Catalog number
Airway Epithelial Cell Growth Medium 2 (ready to use)	500 ml	C-21040
Airway Epithelial Cell Growth Medium XF (prf) (ready to use)	500 ml	C-21050
Air-Liquid Interface Medium (ready to use)	500 ml	C-21080
HBEpC, ALI pre-screened	500,000 cryopreserved cells	C-12640
HBEpC	500,000 cryopreserved cells	C-12640
HEPES Buffered Saline Solution	250 ml	C-40020
Dulbecco's PBS, without Ca^{2+}/Mg^{2+}	500 ml	C-40232
DetachKit	125 ml	C-41210

Related products

Product	Size	Catalog number
Cultrex™ UltiMatrix Reduced Growth Factor Basement Membrane Extract (R&D Systems)	5 ml	BME-001–05
Y-27632 (Sellekchem)	5 mg	S6390
96-well Suspension U-bottom Plate (Cellstar® Greiner Bio-One)	–	650185
24-well plate Nunclon™ Sphera™ (Thermo Scientific)	–	174930
Falcon® Easy Grip TC-treated (Corning®)	–	353004

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