Air-Liquid Interface Culture of Nasal and Tracheal Airway Epithelial Cells

PromoCell®

Application Note

Generation of a 3D human airway model with primary human nasal and tracheal epithelial cells using our Air-Liquid Interface Medium (ALI-Airway).

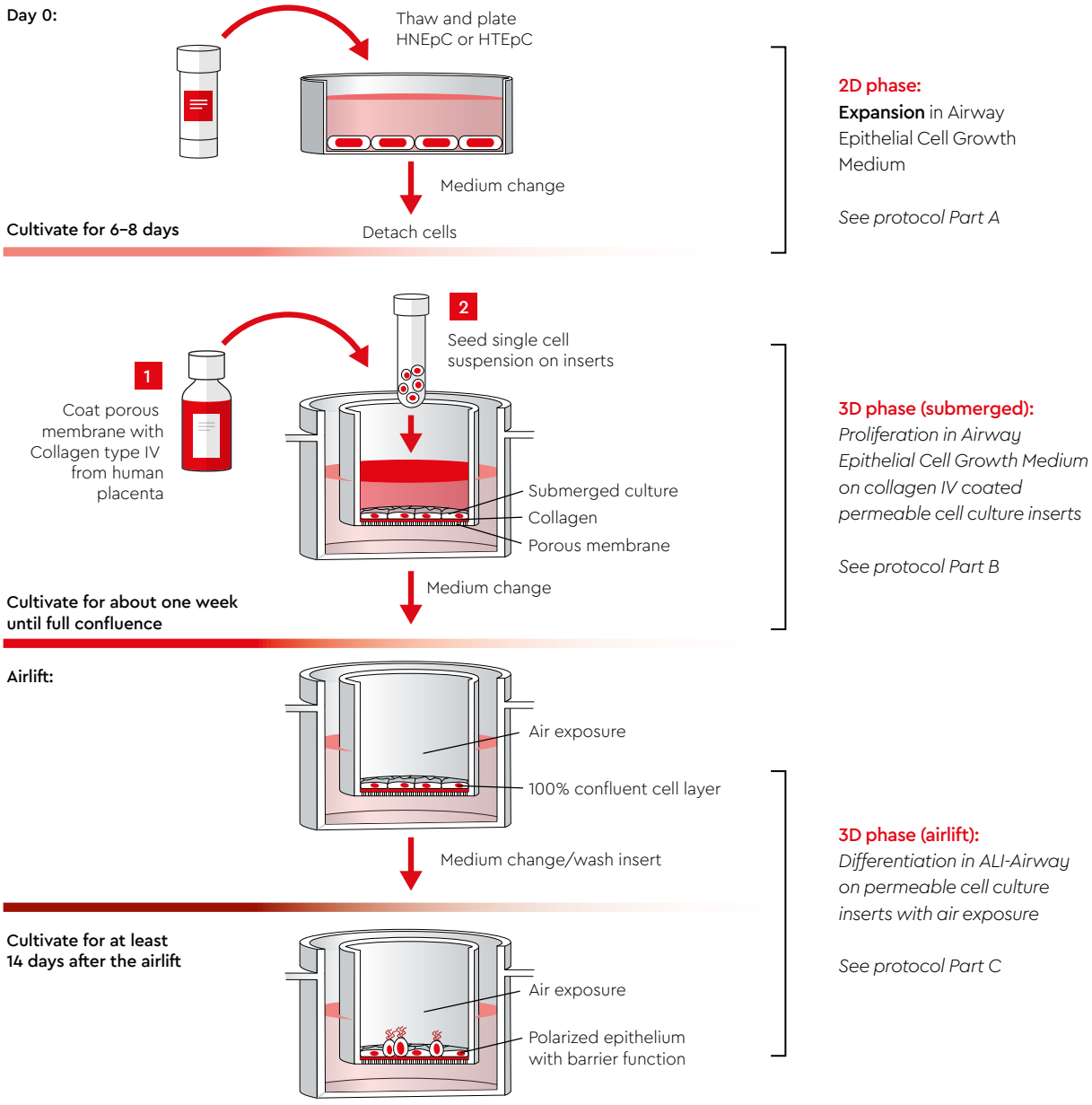


Fig. 1: Schematic overview of culture procedure for HNEpC or HTEpC differentiation in the Air-Liquid Interface Medium (ALI-Airway). *The workflow can be divided into various phases:* **A,** *Human primary cell expansion in 2D culture on plastic.* **B,** *Cell re-seeding and expansion in 3D culture on submerged collagen type IV coated porous membranes.* **C,** *Induction of differentiation by airlift in ALI-Airway. In the differentiation phase, the formation of a tight epithelial barrier occurs after at least 14 days post-airlift. For detailed steps, see the following protocol.*

Background

***In vitro* experiments on nasal (HNEpC), bronchial (HBEpC), and tracheal (HTEpC) epithelial cells** constitute an interesting translational step between experimental and clinical research. HNEpCs are primarily used to study respiratory infections – like the coronavirus – or allergies, and to test nasally applied drugs. In contrast, HTEpCs and HBEpCs are more often used to study COPD or investigate the effects of inhaled drugs. However, since HNEpC and HBEpC cilia are comparable both in structure and function, HNEpCs can be used as surrogates for HBEpCs in airway inflammation studies [1].

The airway epithelium is a key protective barrier whose integrity is preserved by the self-renewal and differentiation of basal progenitor cells. This is one of the reasons why **primary epithelial cells have many advantages** over immortalized cell lines [2]. In principle, HNEpCs are easier to obtain than HBEpCs or HTEpCs, as they are isolated from the normal human nasal mucosa. Conversely, the other two cell types are isolated from the surface epithelium of the human bronchi or trachea, respectively [3].

Air-liquid interface (ALI) *in vitro* lung cell models are instrumental for studying basic and applied aspects of respiratory tract biology, disease, and therapy. When HNEpCs, HBEpCs, or HTEpCs are grown on porous supports at an ALI, they undergo mucociliary differentiation, reproducing both *in vivo* morphology and key physiological processes [4,5].

In addition, the mechanical integrity of cells and cell-to-cell junctions plays a vital role in the formation of organized monolayers of epithelial cells in the lungs, whereas their instability is related to pathological processes. Thus, ***in vitro* models** using three-dimensional tissue scaffolds and microfluidic devices to **reproduce cell barriers** are useful for studying the system's basic biology, disease pathogenesis, gene therapy, and drug administration [6]. For instance, self-isolated HNEpCs can reach high transepithelial electrical resistance (TEER)-values between 500–900 Ohm*cm^2 [7].

Culture conditions are an important factor determining the suitability of ALI cultures for functional and biologically relevant studies. Not every porous membrane is equally successful at driving cell differentiation and achieving functionality. The material, roughness, stiffness, pore density, or surface treatment impacts cell attachment, spreading, proliferation, migration, and differentiation [6,8]. On the other hand, the ALI culture medium greatly influences a cell's capacity for achieving a mucociliated phenotype and associated physiological functionality, as demonstrated by Luengen et al. [9].

In our Air-Liquid Interface Culture System, differentiation of ALI pre-screened HNEpC/HTEpC is stimulated both by the Air-Liquid Interface Medium (ALI-Airway, C-21080) and air exposure. Due to variations in the differentiation capacity between donors, we recommend testing an ALI-culture before performing an assay. The ALI-Airway medium lacks attachment factors, therefore collagen type IV is a prerequisite for optimal attachment of HNEpC and HTEpC. The ALI-Airway medium consists of a Basal Medium and a BPE- and serum-free SupplementMix and was developed to have a high barrier-forming capacity over several weeks.

The following **application note** details the ALI culture procedure and recommended TEER measurement technology.

Air-Liquid Interface Culture Protocol Part A

2D expansion of nasal (HNEpC) or tracheal (HTEpC) epithelial cells

The protocol in Part A describes how to thaw and expand an ALI-suitable HNEpC or HTEpC donor for 2D culture on plastic.

I. Materials

- Human Nasal Epithelial Cells (HNEpC, C-12620) / Human Tracheal Epithelial Cells
- (HTEpC, C-12644)
- Airway Epithelial Cell Growth Medium, containing the Basal Medium and either SupplementMix (Ready-to-use; C-21060) or SupplementPack (Kit; C-21160).
- Cell culture vessel (e.g., Falcon®; Corning® Inc.)
- Water bath at 37°C
- Timer
- Cell counting equipment

Use aseptic techniques and a laminar flow bench.

II. Protocol

To prepare the medium, thaw the SupplementMix or SupplementPack at 15–25°C. Aseptically mix the supplement by carefully pipetting it up and down. Transfer all components to the 500 ml bottle of Basal Medium. Close the bottle and swirl gently until a homogenous mixture is formed. After adding the supplement(s) to the Basal Medium, its shelf life (complete growth medium) is 6 weeks. Store the complete growth medium at 2–8°C.

1

Thaw the HNEpC or HTEpC

Remove the cryovial of the respective cell type from liquid nitrogen and transport it on dry ice. Under a laminar flow bench, release the vial pressure 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 EtOH from the threads of the screw cap. Carefully open the cryovial. Transfer the cell suspension to the cell culture vessels containing the prewarmed medium from step 1.

Note: Our cryopreserved cells are frozen in Cryo-SFM (C-29910), which contains DMSO. Work quickly to prevent long incubation of the cell suspension in Cryo-SFM, as cells are very sensitive after thawing.

2

Incubate the cells

Gently swirl the vessel containing the cell suspension and place it in an incubator (37°C, 5% CO_2). After 16–24 hours, check cell adherence under a microscope and replace the growth medium. There should be only a few floating cells.

3

Cultivate cells while regularly changing the medium

Change the medium every 2–3 days (e.g., Mon-Wed-Fri). Use prewarmed complete Airway Epithelial Cell Growth Medium (180 µl medium/cm²). Regularly check cell confluence. Once they have reached 70–80% confluence, passage the cells.

Note: Avoid confluence >90%. Cells can become contact-inhibited, resulting in slower proliferation after passaging.

4

Subcultivate the expanded cells

Once cells have reached 70–80% confluence, subcultivate them as described in Part B. The required confluence can be typically reached 7 days after thawing. Cell morphology should correspond to the typical cobblestone pattern of epithelial cells (see Figure 2).

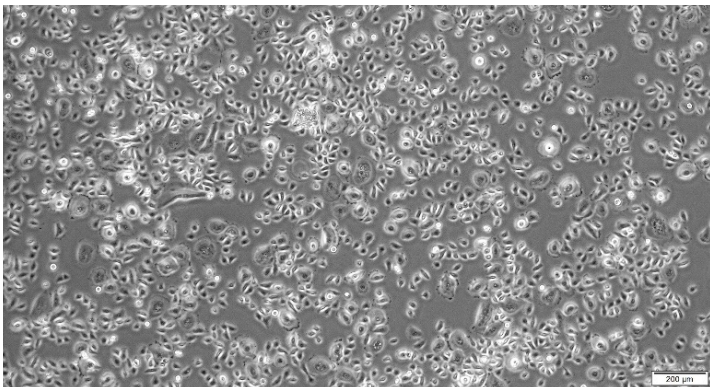


Fig. 2A: Typical HNEpC morphology in 2D culture. *Cells were thawed and seeded in our Airway Epithelial Cell Growth Medium at 5,000 cells/cm². Cells were expanded until reaching 70–80% confluence. Image acquired 7 days after seeding.*

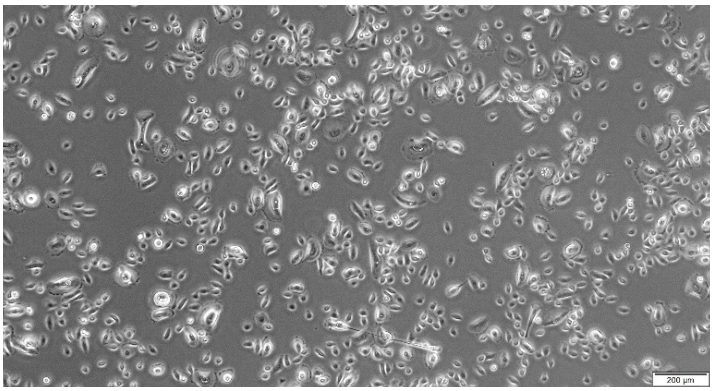


Fig. 2B: Typical HTEpC morphology in 2D culture. Cells were thawed and seeded in our Airway Epithelial Cell Growth Medium at 5,000 cells/cm². Cells were expanded until reaching 70–80% confluence. Image acquired 7 days after seeding.

Air-Liquid Interface Culture Protocol Part B

Subcultivation and re-plating of HNEpC or HTEpC on collagen type IV precoated permeable cell culture inserts

This section describes HNEpC and HTEpC detachment from 2D culture and seeding at high density on collagen type IV precoated Transwell® inserts as a submerged 3D culture.

I. Materials

- Airway Epithelial Cell Growth Medium, containing Basal Medium and either SupplementMix (Ready-to-use; C-21060) or SupplementPack (Kit; C-21160).
- Phosphate Buffered Saline without Ca^{2+}/Mg^{2+} (PBS, C-40232)
- 0.04 % Trypsin / 0.03% EDTA (Trypsin/ EDTA, C-41010)
- 0.05 % Trypsin Inhibitor, 0.1% BSA (TNS, C-41110)
- Collagen type IV solution from human placenta (Sigma-Aldrich®, product number C5533–5MG)
- 6.5 mm Transwell® inserts, 0.4 µm pore size, tissue culture treated polyester membrane polystyrene plates (we strongly recommend Costar®, product number 3470-Clear, Corning® Inc.), alternative products see Material List on page 9)

Use aseptic techniques and a laminar flow bench.

II. Protocol

On the day of use, coat the permeable cell culture inserts with collagen type IV from the human placenta and seed the HNEpC or HTEpC on the coated inserts. We strongly recommend using cells in early passages (P3), as this results in higher differentiation capacity and TEER-values. Passages >3 may result in a decrease in barrier formation indicated by lower TEER-values.

1

Coating of permeable cell culture inserts with collagen type IV and cell seeding

Coat the porous membranes of 24-well permeable cell culture inserts with collagen type IV from the human placenta at 10 µg/cm². For optimal collagen distribution, gently rock the plate from side to side and from front to back. Do not swirl the plate. Then, incubate the plate for 45 minutes **in an incubator (37°C, 5% CO_2). Carefully aspirate the collagen solution from the inserts. * Immediately wash the inserts with 150 µl of PBS without Ca^{2+}/Mg^{2+}. If a break is required, keep the PBS on the inserts and store the plate at 37°C.**

**Aspiration of culture medium on inserts: use a 1 ml pipette tip on top of the aspiration pipette. This gives better control and facilitates the handling of inserts. Be careful not to damage the membrane with the pipette tip.*

Note: We strongly recommend using collagen type IV solution from the human placenta. Collagen stock solution should be stored at 2–8°C. Acclimate stock solution to room temperature (20–25°C) before diluting the working stock solution in PBS without Ca^{2+}/Mg^{2+}. A cold collagen solution is much more viscous and therefore more difficult to pipette. Depending on the experimental design, remember to include a collagen coated permeable cell culture insert without cells as a "blank."

2

Wash the cells

Between 6–8 days after thawing, HNEpC or HTEpC should reach 70-90% confluence. Aspirate the medium and wash the cells by adding an equal volume of PBS without Ca^{2+}/Mg^{2+}.

Note: Acclimate the PBS without Ca^{2+}/Mg^{2+} to room temperature before adding it to the cells.

3

Detach the cells

Aspirate the PBS without Ca^{2+}/Mg^{2+} from the vessel and add prewarmed Trypsin/EDTA (100 µl/cm^2). Gently swirl the vessel to ensure that cells are completely covered with Trypsin/EDTA. Place the vessel in an incubator (37°C, 5% CO_2) for 4 minutes. Check detachment under a microscope. The cells should appear round. To encourage detachment, you can gently tap the vessel horizontally against the tabletop. Return the vessel to the laminar flow bench and add an equal amount of Trypsin Neutralization Solution (TNS) to the cells. Gently swirl the vessel.

Note: Epithelial cells stick tightly to plastic because of the large number of adherens junctions. If cells do not round after 4 minutes of incubation at 37°C, you can place the vessel in the incubator for an additional minute. Do not over-trypsinize them. If they are still sticking after this extra incubation, use a 1,000 µl pipette to wash them down.

4

Collect the cells and determine their number and viability

Transfer the cell suspension to a 15 ml conical tube. To collect all remaining cells, add complete Airway Epithelial Cell Growth Medium to the vessel and transfer it into the same 15 ml conical tube. Examine the vessel under a microscope to check if all cells have been collected. Use an appropriate volume of detached cell suspension to determine the cell number. Use standard methods for cell counting and viability assessments. Spin the cells (3 minutes at 300 x g) and aspirate the supernatant. Resuspend the cell pellet by pipetting up and down in Airway Epithelial Cell Growth Medium. Keep the cells under laminar flow until seeding.

5

Plate the cells on a collagen type IV coated permeable cell culture inserts (24-well plate format)

Make sure the Transwell® plate inserts have been collagen-coated for ≥45 minutes at 37°C in an incubator, according to step 1. Aspirate the collagen solution from the inserts and wash each insert with 150 µl of prewarmed PBS without Ca^{2+}/Mg^{2+}. To avoid evaporation, you can fill the outer walls of the plate with 200 µl PBS (optional). After removing the PBS without Ca^{2+}/Mg^{2+} to seed the cells, work quickly to avoid drying out the semipermeable membrane. After cell counting calculate the desired number of cells. For a 6.5 mm permeable cell culture insert (24-well plate) use a seeding density of 150,000 cells/cm^2 (the volume of the apical chamber is 100 µl [500,000 living cells/ml]). Mix with an appropriate volume of Airway Epithelial Cell Growth Medium for a final concentration of 500,000 cells/ml in a conical tube. For one 6.5 mm permeable cell culture insert plate, you need ≥600,000 living cells. If you plan to do TEER measurements, leave one Transwell® insert without cells as a blank. Transfer 500 µl of Airway Epithelial Cell Growth Medium to the insert in each basal chamber. Then, use a 1,000 µl pipette to transfer 100 µl cell suspension into each upper chamber. If you use a blank insert, put 500 µl of Airway Epithelial Cell Growth Medium in the lower chamber and 100 µl in the upper chamber (see Figure 3). For optimal cell distribution, gently rock the plate from side to side and from front to back, but do not swirl the plate.

6

Expansion of the cells in submerged culture

Change the medium 24 hours after seeding. Hold the plate at an angle and carefully collect the medium from the lower and upper chambers using an aspiration pipette or 1,000 µl pipette. Be careful to avoid contact of the pipette tip with the cell layer. Transfer 500 µl of Airway Epithelial Cell Growth Medium to the lower chamber and 100 µl to the upper chamber.

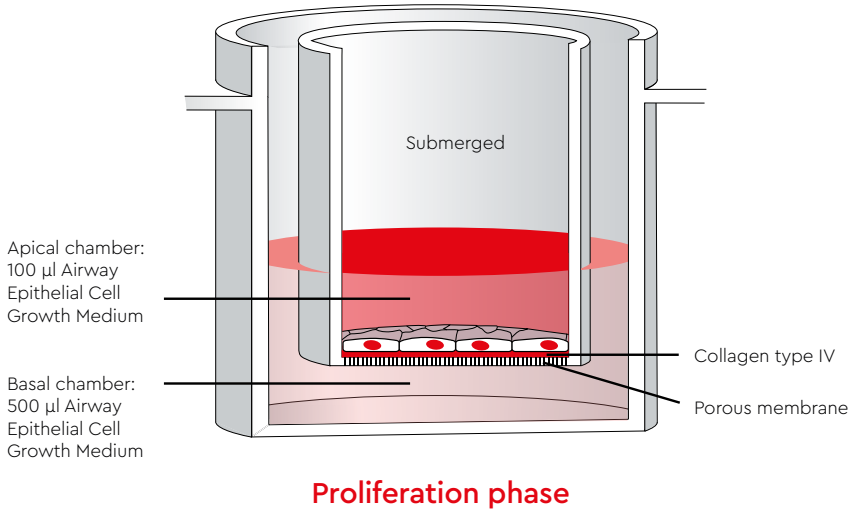


Fig. 3: Collagen type IV coated permeable cell culture insert with submerged cultivated HNEpC or HTEpC. *The porous membrane was coated with collagen type IV solution. The basal chamber is only filled with Airway Epithelial Cell Growth Medium, whereas the apical chamber is filled with a cell suspension. Cells attach to the collagen-coated*

Air-Liquid Interface Culture System Protocol Part C

HNEpC or HTEpC differentiation on the air-liquid interface

This section describes the 3D culture of HNEpC or HTEpC on permeable cell culture inserts cultivated in ALI-Airway medium to promote differentiation for at least 14 days while exposed to air.

I. Materials

- Air-Liquid Interface Medium (ALI-Airway; C-21080)
- Phosphate Buffered Saline without Ca^{2+}/Mg^{2+} (PBS, C-40232)
- Gentamicin-sulfate solution at 50 µg/ml in the medium

Use aseptic techniques and a laminar flow bench.

II. Preparation of ALI medium

The PromoCell Air-Liquid Interface Medium (ALI-Airway) is designed for differentiating plated airway epithelial cells on permeable cell culture inserts under airlift conditions. It does not contain adherence factors, making its use mandatory with collagen coated inserts. To prepare the medium, thaw the SupplementMix at 15–25°C. Aseptically mix the supplement by carefully pipetting up and down. Transfer all supplements to the 500 ml bottle of Basal Medium. Close the bottle and swirl gently until a homogenous mixture is formed. We recommend the addition of 50 µg/ml of gentamicin-sulfate solution for long-term cultivation, especially if you want to perform TEER measurements with an electrode pair. After adding the SupplementMix, the shelf life of the ALI-Airway medium is about 4 weeks. Store the complete growth medium at 2–8°C. Do not prewarm the bottle at 37°C. At the time of use, allow the medium to warm up to room temperature. ALI-Airway medium contains light-sensitive components, and we therefore recommend protecting it from light.

1

Initiate differentiation and kick start the airlift culture

The cells should be 100% confluent one week after seeding on the permeable cell culture inserts. Check confluence under a microscope. Carefully aspirate the Airway Epithelial Cell Growth Medium in lower and upper chambers. Transfer 500 µl of ALI-Airway medium to the lower chamber. Avoid pipetting any medium into the upper chamber. It should remain empty, as air exposure will stimulate differentiation (see Figure 4).

Note: Make a timetable for your ALI experiment to prevent issues over the weekend (e.g., thaw cells on Tuesday, passage cells on Monday the following week and seed on inserts, change medium on Tuesday, change to ALI-Airway medium on Monday, and airlift the cultures). If cells do not reach 100% confluence on inserts, change the medium, and let them grow for another day. The cell layer needs to be completely closed when airlifting.

2

Cultivate the cells under airlift conditions

Replace the ALI-Airway medium in the lower chamber every 2–3 days (e.g., Mon-Wed-Fri). On Mondays, change the medium in the morning, but on Fridays do it in the evening. The intact cell layer will prevent medium diffusion from the lower to the upper chamber. If some medium diffuses to the insert it should be removed. Wash the upper chamber once a week with 150 µl of prewarmed PBS without Ca^{2+}/Mg^{2+}. Carefully aspirate the PBS without damaging the cell layer, as doing so would disrupt the epithelial barrier.

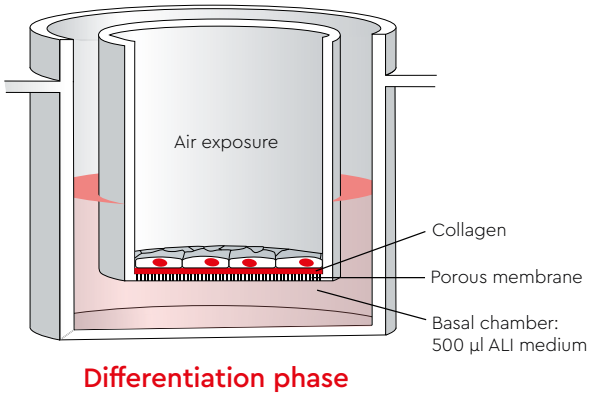


Fig. 4: Collagen type IV coated permeable cell culture insert with air exposure of the epithelial layer. *During the 3D culture differentiation phase, the ALI-Airway medium is only in the basal chamber. Here, the medium is changed every 2–3 days whereas the apical chamber is only washed with PBS once a week.*

3

Differentiation until week 3

Differentiation will be complete at least 14 days after airlifting. We guarantee TEER values >500 Ω*cm² with HNEpC or HTEpC (donors proven suitable for ALI-culture) if these instructions are followed.

Epithelial barrier integrity

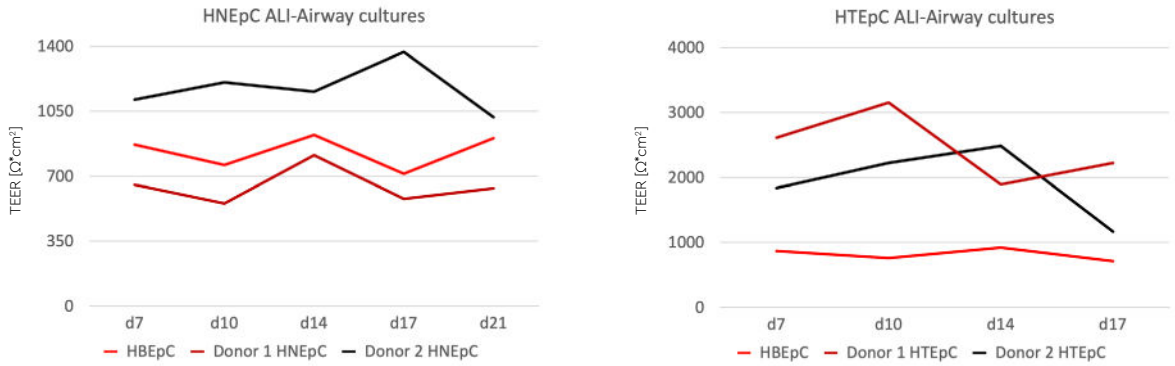
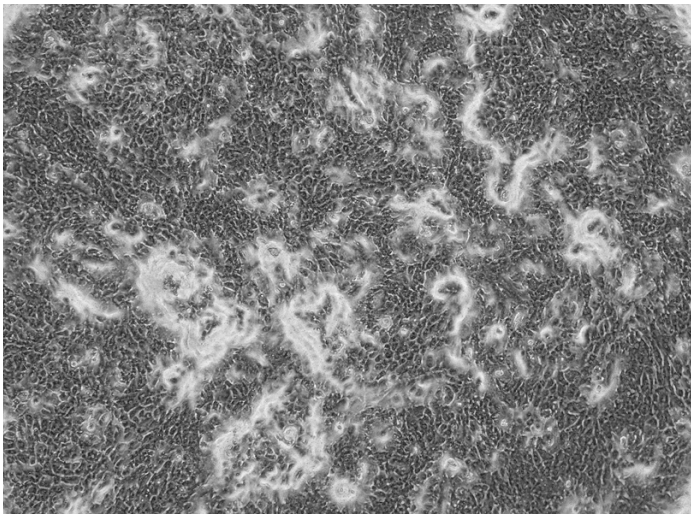


Fig. 5: Different HNEpC and HTEpC donors can be used for successful ALI-Airway cultures. *Two of our HNEpC and HTEpC donors were expanded in Airway Epithelial Cell Growth Medium. As a positive control, ALI pre-screened HBEpC were used. For ALI culture, the porous membranes of 24-well Transwell® inserts (Corning # 3470) were coated with collagen type IV from the human placenta (Sigma # C5533–5MG) at 10 µg/cm². Airway Epithelial Cells in passage 3 were seeded on Transwell® inserts at 150.000 cells/cm². Submerged cultures were airlifted approximately one week after seeding. The Airway Epithelial Cell Growth Medium in the basal chamber was replaced by 500 µl of ALI-Airway medium and the apical chamber remained empty (= airlift). The medium was changed every 2–3 days. The barrier-forming function of Airway Epithelial Cells was analyzed by TEER using an EVOM® voltameter and an STX® Electrode Set (World Precision Instruments). Our ALI-Airway Medium can be used with different HBEpC, HNEpC, and HTEpC donors; it can result in TEER values ≥ 500 Ω*cm² for over 2 weeks after airlifting.*

A



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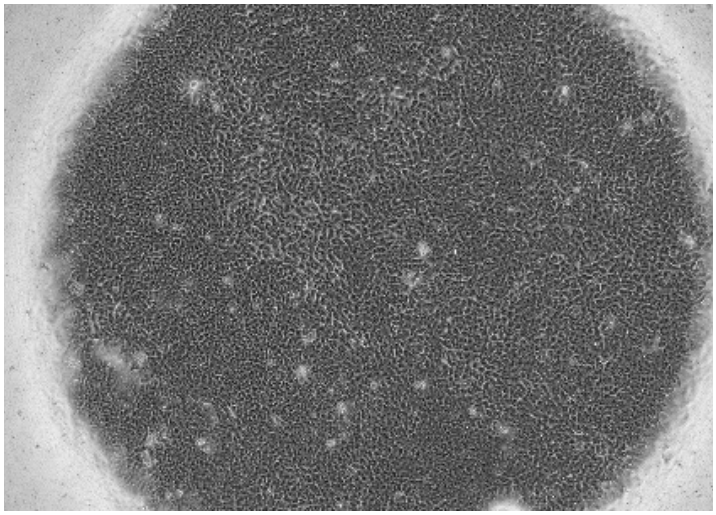

Fig. 6: After three weeks of airlift culture in ALI-Airway medium, HNEpC and HTEpC show the typical morphology and barrier-forming function. *HNEpC and HTEpC were seeded on collagen type IV coated 24-well Transwell® at 150.000 cells/cm² in Airway Epithelial Cell Growth Medium. After 5 days, the medium changed to ALI-Airway. ALI-Airway (500 µl) remained in the basal chamber while the upper chamber was airlifted without medium. Microscopic image showing a porous membrane with confluent HNEpC or HTEpC cell layers at day 22 post-airlift. TEER-values were ≥ 500 Ω*cm² for ≥17 days post-airlift.*

Trademark References

Material

Product	Size	Catalog Number
Human Nasal Epithelial Cells (HNEpC)	500,000 cryopreserved cells	C-12620
Human Tracheal Epithelial Cells (HTEpC)	500,000 cryopreserved cells	C-12644
Airway Epithelial Cell Growth Medium (Ready-to-use)	500 ml	C-21060
Airway Epithelial Cell Growth Medium Kit	500 ml	C-21160
Air-Liquid Interface Medium (ALI-Airway)	500 ml	C-21080
Phosphate Buffered Saline without Ca^{2+}/Mg^{2+}	500 ml	C-40232
Trypsin/EDTA (0.04% (w/v) Trypsin / 0.03% (w/v) EDTA)	125 ml	C-41010
Trypsin Neutralizing Solution (0.05% (w/v) Trypsin Inhibitor, 0.1% (w/v) BSA)	125 ml	C-41110

Additional products strongly recommended for ALI culture

Product	Size	Catalog Number
Collagen Type IV from human placenta (Sigma-Aldrich®)	1 mg/ml	C5533–5MG
Costar® Transwell® (24 well plate) (Corning®)	-	3470
CELLTREAT® Permeable Cell Culture Inserts Packed in 24 Well Plate, 0.4 µm PET (CELLTREAT® Scientific Products)	-	230635
EVOM® (World Precision Instruments®)	-	-
STX® Electrode Set (World Precision Instruments®)	-	-
1.000 Ω test resistor (World Precision Instruments®)	-	91750

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