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APPLICATION NOTE

High-throughput in-vitro airway modelling with ThinCert® 96 Well HTS Insert

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1. INTRODUCTION

Growing cells in an air-liquid interface (ALI) is a popular method to generate a stable and functional model of the respiratory tract by recreating a pseudostratified epithelium *in vitro*. ALI cell cultures are frequently adopted to investigate different processes associated to lower airways including disease modelling [1,2], drug testing, cell-to-cell signalling and air pollutant-induced pulmonary toxicity studies [3]. To establish an ALI cell culture, normal human bronchial epithelial cells (NHBEs) are cultivated on a porous membrane insert (ThinCert® 96 well HTS insert). In the initial phase the cells are grown under submerged conditions to form a confluent cell layer. Once this is achieved, the media is removed from the membrane insert and the cells are transferred to the air-liquid interphase. Due to the two-component design of the ThinCert® HTS insert, the basal side of the cells are still in direct contact with the cell culture medium while the apical side is exposed to air. In this way the cells undergo a morphological and functional differentiation similar to that found in intact tissues. This includes the formation of tight junctions, the development of cilia and mucus and an established cellular polarity.

ALI cell cultures have attracted even more attention in course of COVID-19 disease modelling and are frequently applied for SARS-CoV-2 related research. Single permeable membrane inserts such as ThinCert® cell culture insert have proven to be an essential tool for modeling airway-epithelia *in vitro* [4, 5, 6, 7]. With the addition of an automation-friendly permeable cell culture support specifically optimised for high-throughput assays, the ThinCert® 96 well HTS insert expands and enhances the well-established Greiner Bio-One ThinCert® portfolio. In contrast to single inserts, the 96 well HTS insert can be handled as a single unit significantly reducing the manual effort.

2. MATERIALS AND METHODS

Item	Manufacturer	Item No.
Cells		
Normal Human Bronchial Epithelial Cells	Lonza	CC-2540S
Cell culture media kits		
PneumaCult-Ex Plus culture medium	Stemcell Technologies	05040
PneumaCult-ALI Medium for differentiation	Stemcell Technologies	05001
Cell culture chemicals		
Hydrocortisone, 2 x 10^{-4} M	Stemcell Technologies	07925
Heparin, 2 mg/ml / 0,2 %	Stemcell Technologies	07980
Accutase cell detachment solution	Stemcell Technologies	07920
Rat Tail Collagen Coating Solution, 50 µg/ml	Sigma Aldrich	122-20
Trypsin/EDTA solution (0.05 %/0.02 %)	PAN Biotech	P10-023100

Item	Manufacturer	Item No.
Immunofluorescent staining		
Mouse anti-ß-Tubulin IV	Sigma-Aldrich	T7941-.2ML
Alexa 488 goat anti-mouse	Life Technologies	A11001
Rabbit anti-ZO1	Life Technologies	40-2200
Alexa 546 goat anti-rabbit	Life Technologies	A11010
Alcian blue staining		
Alcian blue 8GX	Sigma Aldrich	A3157-10G
DAPI staining		
DAPI	Sigma-Aldrich	D8417-10MG

3. ASSAY PRINCIPLE

3.1 SEEDING PREPARATION (GENERAL CELL CULTURE)

1. NHBE cells are cultivated according to standard cell culture protocols (e.g., 60 % confluence)
2. Remove and discard the cell culture media and rinse with PBS
3. Add Accutase solution to initiate cell detachment
4. Transfer the cell suspension to a tube and centrifuge at 350 X g for 5 min.
5. Resuspend the cell pellet in pre-warmed cell culture media
6. Dilute the solution to a seeding density of approximately 150,000 cells/cm^2 (420,000 cells/ml)

3.2 COATING WELLS WITH COLLAGEN

1. Add 28 µl of rat tail collagen stock solution (10 µg/cm^2) to each well of the membrane plate
2. Incubate the collagen at room temperature for 1 h
3. Rinse with PBS

3.3 SEEDING CELLS IN THINCERT® 96 WELL HTS INSERT (SUBMERGED EXPANSION PHASE)

1. Add 50 µl of the above prepared cell suspension to each well (approx. 21,000 cells/well)
2. Add 200 µl cell culture media to each well of the receiver plate
3. Place the ThinCert® 96 well HTS insert into the receiver plate
4. Place the plate in the incubator (37 °C, 5 % CO_2)
5. Cultivation for 5 days until a confluent cell layer has formed
6. Perform media change after 48 h

3.4 MEDIA CHANGE

1. Remove media from the wells of the receiver plate
2. Remove media from the wells of the insert plate (upper chamber wells)
3. Add media to the wells of the receiver plate
4. Add media to the wells of the insert plate (upper chamber wells)

General remark: The media change can be performed through the access ports (see figure 1), e.g., with a multi-channel pipette. Alternatively, the insert plate can be lifted and the media change of the receiver plate can be performed accordingly.

Useful hint:
Please handle the plate carefully to avoid excessive movement of the medium in the receiver plate.

Figure 1: Access port is located next to the well and is accessible for (multi-channel) pipettes, automated liquid handling robots as well as for electrodes for Transepithelial Electric Resistance (TEER) measurements

3.5 AIR-LIFT CELL CULTURE (DIFFERENTIATION PHASE)

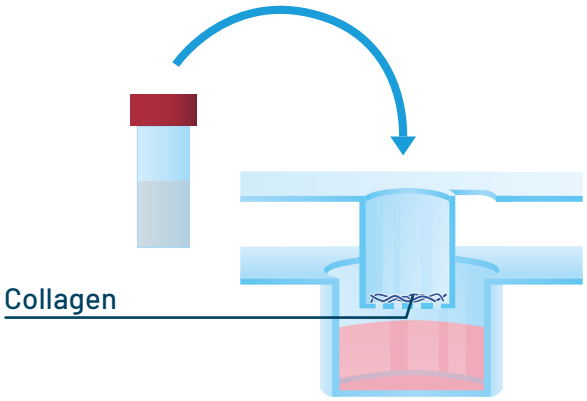
1. Remove media from the wells of the receiver plate
2. Remove media from the wells of the insert plate (upper chamber wells)
3. Add 150 µl differentiation media to the wells of the receiver plate
4. The wells of the insert plate remain empty to facilitate the ALI cell culture
5. Exchange the differentiation media of the receiver plate every second day
6. Wash the cells in the insert plate with 100 µl PBS buffer once a week to remove the mucus
7. Carefully aspirate the PBS

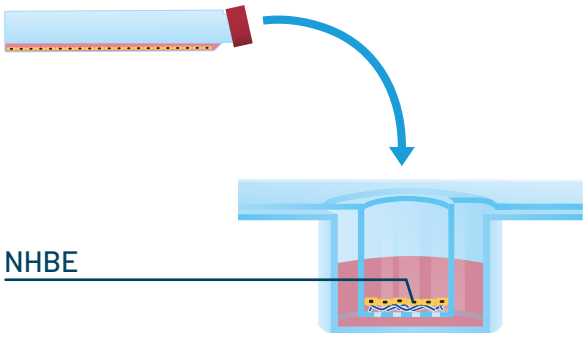
Note: Do not damage the cell layer to maintain the integrity of the epithelial barrier.

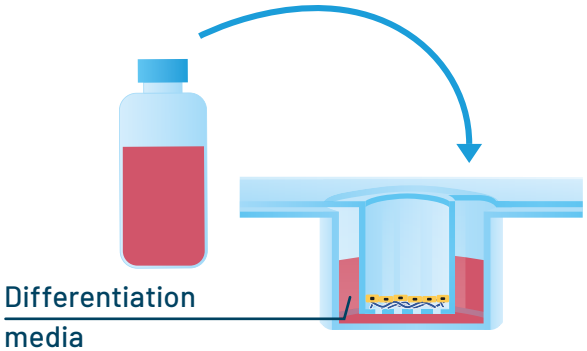
8. The cells are cultivated over a period of 20 to 25 days

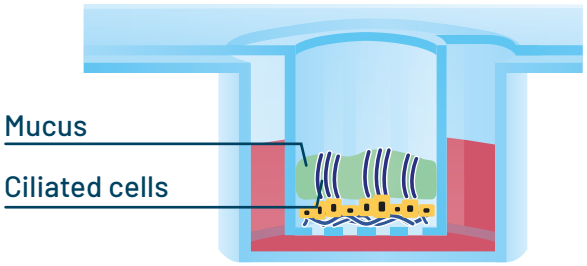
Note: If media diffuses from the receiver well to the upper chamber, remove it carefully.

4. AIR-LIFT CULTURE









1 **Insert plate preparation**
Coat membrane with collagen

2 **Submerged cell culture (expansion)**
Add cell suspension to each well of the insert plate

5 days (media change every 48 h)

3 **Air-lift culture (differentiation)**
Add differentiation media to the wells of the receiver plate and remove media from the well of the insert plate

20-25 days media change every 48 h
Wash with PBS buffer once a week

4 **Air-lift culture (differentiation)**
Cultivate the cells for 20-25 days

4.1 DIFFERENTIATION MARKER

To assess the differentiation performance, anti-ß-tubulin IV was used to detect ciliated cells that make up most of the cells found in the airway epithelium. The presence of mucus-producing goblet cells was proven by an alcian blue dye which stains sulfated and carboxylated polysaccharides found in the mucus layer of the respiratory tract. Both cells play a major role in mucociliary clearance, a self-clearing mechanism and first-line defence against particles and pathogens. To prove that tight junctions have formed as a selective barrier on the porous membrane the tight junction protein-1 (ZO-1) was marked with the respective rabbit antibody.

4.2 IMMUNOFLUORESCENT STAINING WITH ANTI-β-TUBULIN IV

1. Fix the cells on the apical side with cold 10 % formalin for 15 min at room temperature
2. Wash the wells 2 x with 100 µl PBS buffer
3. Permeabilise the cells with 50 µl 0.1 % Triton X-100 in PBS buffer for 5 min at room temperature
4. Wash the wells with 100 µl PBS buffer
5. Use blocking buffer (PBS + 5 % goat serum) and incubate for 1 h
6. Wash the wells with 100 µl PBS buffer
7. Incubate primary antibody (mouse anti-ß-Tubulin IV) in blocking buffer for 1 h at room temperature or overnight at 4 °C
8. Wash the wells 3 x with 100 µl PBS buffer
9. Incubate secondary antibody (Alexa 488 goat anti-mouse and DAPI if needed 10 µg/ml) in blocking buffer for 1 h at room temperature in the dark
10. Wash the wells 3 x with 100 µl PBS buffer
11. Cut out membranes with a scalpel
12. Place the membrane on a microscope slide and mount in properly

4.3 IMMUNOFLUORESCENT STAINING WITH ANTI-ZO-1

1. Fix the cells on the apical side with cold 10 % formalin for 15 min at room temperature
2. Wash the wells 2 x with 100 µl PBS
3. Permeabilise the cells with 50 µl 0.1 % Triton X-100 in PBS for 5 min at room temperature
4. Wash the wells with 100 µl PBS
5. Use blocking buffer (PBS + 5% goat serum) and incubate for 1 h
6. Wash the wells with 100 µl PBS
7. Incubate primary antibody (rabbit anti-ZO-1) in blocking buffer for 1 h at room temperature or overnight at 4 °C
8. Wash the wells 3 x with 100 µl PBS
9. Incubate secondary antibody (Alexa 546 goat anti-rabbit) in blocking buffer for 1 h at room temperature in the dark
10. Wash the wells 3 x with 100 µl PBS buffer
11. Cut out membranes with a scalpel
12. Place the membrane on a microscopy slide and mount in properly

4.4 ALCIAN BLUE STAINING

Preparation of alcian blue solution: 10 ml HCl + 90 ml H_2O demin. + 1 g alcian blue (powder)

1. Fix the cells on the apical side with cold 10 % formalin for 30 min at room temperature
2. Wash the wells 3 x with 100 µl PBS
3. Incubate 50 µl alcian blue solution for 30 min at room temperature
4. Wash the wells 3 x with 0.1 N HCl
5. Apply distilled water and microscope (no need to cut out the membrane here)

5. RESULTS

In this application note the ThinCert® 96 well HTS insert was used to generate an in-vitro model of the respiratory epithelium. Therefore, NHBEs were cultivated and subjected to 'air-lift'.

5.1 IMMUNOFLUORESCENT STAINING

To analyse the differentiation status of the pseudostratified epithelium, the ALI cultures were fixed and stained with anti-ß-tubulin IV and anti-ZO-1 to detect ciliated cells and the characteristic tight junctions. A DAPI staining was used to visualize the nuclei and to assess gross cell morphology.

The immunocytochemistry shows an intact monolayer of epithelial cells that have developed tight junctions (Fig. 4) as a primary boundary between the apical and basolateral region. As a major cilia protein, ß-tubulin IV is an excellent differentiation marker to prove the presence of a differentiated cell composition (Fig. 2).

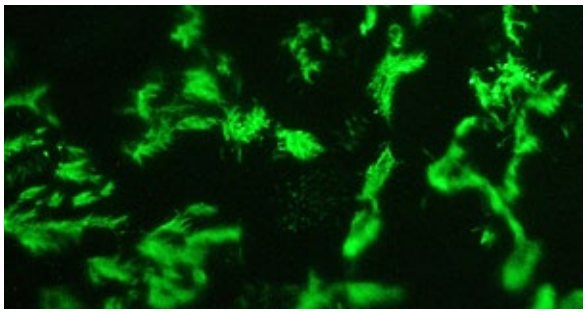


Figure 2: Anti-tubulin IV staining (green) of ciliated cells on the membrane of the ThinCert® HTS insert (63 x)

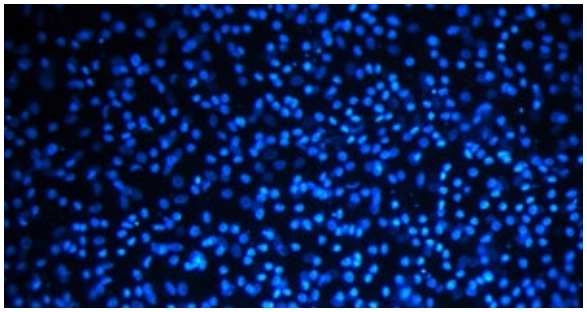


Figure 3: DAPI staining (blue) of cell layer on the membrane of the ThinCert® HTS insert (20 x)

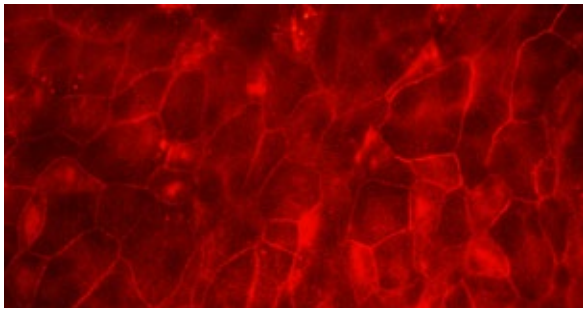


Figure 4: Anti-ZO-1 staining of tight junctions (red) on the membrane of the ThinCert® HTS insert (63 x)

5.2 HISTOCHEMICAL STAINING

After 25 days post air-lift, the fixed cells were stained with the alcian blue dye (Fig. 5) to mark the typical polysaccharides and glycoproteins found in the mucus of the pseudostratified epithelium. The staining acts as a further indicator for the differentiation under air-liquid conditions.

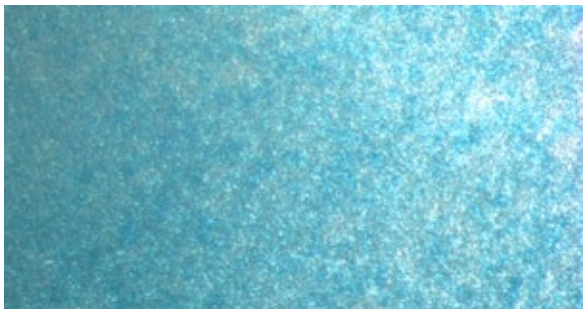


Figure 5: Staining of mucus polysaccharides with alcian blue (10x)

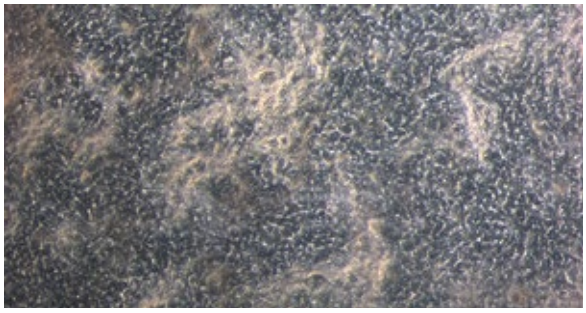


Figure 6: Unfixed NHBE cells after culturing in air-lift for 25 days (10x)

The ThinCert® 96 well HTS insert enables in vivo-like growth conditions *in vitro* and facilitate the reconstruction of epithelia from individual cells. The ALI cultures develop tissue-specific features, such as tight junctions, cellular polarity and barrier function, and may be used to study tissue-specific phenomena.

ThinCert® 96 Well HTS Insert (Membrane plates and receiver plates)

Growth area: 14 mm², Working volume (well of membrane plate): 15 - 160 µl, Working volume (well of receiver plate): 120 – 300 µl, Lid: yes, condensation rings

Item no.	Pore density	Ø Pores	Optical features of membrane	Surface treatment	Sterile	Qty. inner / outer
655640	1 x 10^8/cm²	0.4 µm	translucent	TC	+	1 / 5
655641	2 x 10^7/cm²	0.4 µm	optimised transparency	TC	+	1 / 5

Receiver plates for ThinCert® 96 Well HTS Insert

Working volume (well of receiver plate): 120 – 300 µl, Lid: yes, condensation rings

Item no.	Growth area	Surface treatment	Sterile	Qty. inner / outer
655169	-	non-treated	+	8 / 32
655167	53 mm²	TC	+	8 / 32

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