Chapter 8

Characterization of Human Coronaviruses on Well-Differentiated Human Airway Epithelial Cell Cultures

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Abstract

The human airway serves as the entry point of human respiratory viruses, including human coronaviruses. In this chapter we outline the methods by which we establish fully differentiated airway epithelium and its use for human coronavirus propagation. Additionally, we outline methods for immunofluorescence staining of these cultures for virus detection, characterization of cell tropism, and how to perform antiviral assays and quantify viral replication.

Key words Human coronavirus, Antivirals, Cell tropism, Human airway epithelial cells, Virus detection

1 Introduction

The human airway serves as the entry point of human respiratory viruses, including human coronaviruses (HCoVs). In order to properly recapitulate the complex anatomy of the human lung specialized cell culture models have been developed to resemble both the upper and lower airways [1–3]. Primary human bronchial epithelial cells cultured in an air–liquid interface (ALI) system serve as a universal platform to study human respiratory viruses [4–6]. These human airway epithelial (HAE) cultures morphologically and functionally resemble the upper conducting airways in vivo. In these cultures, the epithelial layer is pseudostratified and after differentiation they contain many different cell types such as basal, ciliated, and goblet cells and furthermore, generate protective mucus equivalent to that of in vivo epithelium [7].

Establishment of HAE cultures requires time and patience but the differentiated cultures allow for a number of advantageous analyses in respiratory virus research. We have adapted and optimized our methods based on previously published work [8–10]. Moreover, we have standardized methods for the propagation of human coronaviruses and evaluation of the effects of antiviral

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compounds on both viral replication and cell viability. We are able to propagate all known human coronaviruses in this system and can easily evaluate their tropism by immunohistochemistry [5, 11]. In this chapter we outline the methods by which we establish fully differentiated airway epithelium and use it for human coronavirus propagation. Additionally, we outline methods for immunofluorescence staining of these cultures for virus detection, characterization of cell tropism and how to perform antiviral assays and quantify viral replication.

2 Materials

2.1 Human Airway Epithelial Cell Cultures

1. Primary human tracheobronchial epithelial cells can be obtained in accordance with local ethical guidelines from patients willing to give informed consent, who are undergoing bronchoscopy and/or surgical lung resections. Alternatively isolated primary human airway epithelial cells can be obtained commercially from a number of distributors.
2. 10× digestion solution: Minimum Essential Medium (MEM), 1 % m/v Protease from Streptomyces griseus Type XIV, 0.01 % m/v Deoxyribonuclease I from bovine pancreas.
3. Isolation/washing solution: MEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Amphotericin B Solution, 50 μg/ml gentamicin, 100 U/ml nystatin.
4. Bronchial epithelial cell serum-free growth medium (BEGM): LHC basal medium, supplemented with the required additives (Table 1).
5. Air–liquid interface (ALI) medium: LHC basal medium and Dulbecco's Modified MEM (DMEM) mixed in a 1:1 ratio, supplemented with the required additives (Table 1).
6. 12-Well inserts, pore size 0.4 μM and 12-well cluster plates or 12-well deep well cluster plates.
7. 24-Well inserts pore size 0.4 μM and 24-well cluster plates.
8. Human collagen Type I + III, Vitrocol 100.
9. Collagen Type IV from human placenta reconstituted in 5 ml filter-sterilized water with 0.25 % acetic acid. Dissolve for a few hours at 37 °C, occasionally swirling. Once dissolved, increase volume to 20 ml and maintain acetic acid concentration at 0.25 %, mix gently by pipetting. Filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 800 μl per eppendorf tube. The stock solution is stable for at least 1 year at −20 °C.

Table 1
Preparation of stock additives for BEGM and ALI medium

Component	Stock concentration	Comment
Bovine Serum Albumin (BSA)	300× 150 mg/ml	*See* **Note 1**
Bovine pituitary extract (BPE)	1,000× ±14 mg/ml	
Insulin	2,000×, 10 mg/ml	Store at +4 °C
Transferrin (TF)	1,000×, 10 mg/ml	
Hydrocortisone (H)	1,000×, 0.072 mg/ml	
Triiodothyronine (T3)	1,000×, 0,067 mg/ml	
Epinephrine (EP)	1,000×, 0.6 mg/ml	
Epidermal Growth Factor (EGF)	1,000× or 50,000×, 25 μg/ml	1,000× for BEGM, 50,000× for ALI medium.
Retinoic acid (RA)	1,000×, 5×10^{-5} M	Light sensitive. *See* **Note 2**
Phosphorylethanolamine (PE)	1,000×, 70 mg/ml	
Ethanolamine (EA)	1,000×, 30 μl/ml	
Stock 11 (S11)	1,000×, 0,863 mg/ml	
Stock 4 (S4)	1,000×	*See* **Note 3**
Trace Elements (TR)	1,000×	*See* **Note 4**
Penicillin/Streptomycin (P/S)	100× 10,000 U/ml of penicillin and 10,000 μg/ml of streptomycin	Store at +4 °C
Gentamicin	1,000×, 50 mg/ml	Store at +4 °C. *See* **Note 5**
Amphotericin B	1,000×, 50 mg/ml	*See* **Note 5**

All additives should be aliquoted and stored at −20 °C unless stated otherwise

2.2 Human Coronavirus Propagation

1. Apical wash solution: Hank's Balanced Salt Solution (HBSS), without calcium and magnesium.
2. Virus transport medium (VTM): MEM, 25 mM HEPES-buffered, 0.5 % gelatin, 100 U/ml penicillin, 100 μg/ml streptomycin.
3. Aerosol barrier pipette tips and 1.5 ml Eppendorf Safe-Lock Tubes™.

2.3 Immuno-fluorescence Analysis

1. Fixation solution: 4 % formalin solution, neutral buffered (Formafix).
2. Confocal staining buffer (CB): 50 mM ammonium chloride (NH_4Cl), 0.1 % saponin, and 2 % IgG and protease-free BSA dissolved in 500 ml of phosphate buffered saline (PBS,

Table 2
Primary antibodies

Antibody	Target	Dilution	Host	Comment
Anti-β-Tubulin IV	Cilia	1:400	Mouse, IgG1	Clone ONS.1A6
anti-ZO1	Tight junctions	1:200–400	Goat	Directed against C-terminal domain
anti-dsRNA	dsRNA	1:500–1,000	Mouse, IgG2a	Clone J2
Anti-CD13	CD13/APN	1:200	Sheep	Receptor 229E
Anti-CD26	CD26/DPPIV	1:200	Goat	Receptor MERS
Anti-ACE2	ACE2	1:200	Goat	Receptor SARS and NL63
intravenous immunoglobulin (IVIG)	Viral proteins	1:1,000	Human	
Anti-β-Tubulin	Cilia	1:400	Mouse, IgG1	Clone Tub2.1, Cy3 conjugate

pH 7.4). Filter-sterilize (0.2 μm filter) solution and prepare aliquots of 40 ml and store at −20 °C.

3. Primary antibodies: *see* Table 2.
4. Fluorescent DNA dyes: DAPI or Hoechst 33528.
5. Wash solution: Phosphate buffered saline, pH 7.4, without calcium and magnesium.
6. Scalpel (No.10).
7. Rat-tooth forceps.
8. Fluorescence Mounting Medium.
9. Gyro-rocker.

2.4 Antiviral Assays

1. Inhibitors: e.g. K22 [12], recombinant Interferon Alpha and Lambda proteins [13].
2. CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

2.5 Virus Detection

2.5.1 Renilla Luciferase Assay

1. Renilla Luciferase Assay System (Promega).
2. White, non-transparent 96-well plates.
3. Gyro-rocker.
4. Luminometer.

2.5.2 Plaque Assay

1. Huh-7 cells.
2. Medium: DMEM, high glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM Sodium Pyruvate, 5 % heat-inactivated FBS.

3. Overlay medium: 2.4 g of Avicel RC-581 (FMC biopolymer) dissolved in 100 ml of distilled water and autoclaved for 20 min at 121 °C. 2.7 g of DMEM powder (high glucose) dissolved in 90 ml of distilled water and the pH adjusted to 7.4 with 1 M NaOH. Fill volume up to 100 ml and filter-sterilize (0.2 μm filter). Freshly prepare a 1:1 mixture of Avicel (2.4 %) and 2× DMEM solution, supplemented with 10 % FBS and 100 U/ml penicillin, 100 μg/ml streptomycin.
4. Crystal-violet solution: 25 g of Crystal Violet, 40 g NaCl dissolved in 2,500 ml of 99 % Ethanol. Add 2,250 ml of distilled water and 250 ml of 37 % formaldehyde. Mix solution overnight at room temperature (*see* **Note 6**).

2.5.3 Quantitative Reverse Transcriptase PCR

1. Nucleospin RNA isolation kit (Machery Nagel).
2. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT).
3. Random primers.
4. RNAse-free water.
5. FastStart Universal SYBR Green Master reaction mixture (Roche).
6. Positive control; in vitro transcribed RNA of target gene or plasmid DNA containing target gene.

3 Methods

Carry out all procedures in a biosafety cabinet according to local biosafety regulations.

3.1 Human Airway Epithelial Cell Cultures

Cell culture flasks are coated for 2 h with a mixture of Type I and III collagen that is necessary to efficiently expand the number of primary airway epithelial cells.

3.1.1 Collagen Type I and III Coating of Cell Culture Flasks

1. Use filter-sterilized dH_2O (0.22 μm) to prepare a 1:75 dilution of Vitrocol 100.
2. Use 4 ml per 75 cm^2, make sure the entire surface is covered with the collagen solution.
3. Incubate for 2 h at 37 °C.
4. Aspirate remaining liquid and wash twice with 10 ml of PBS to remove traces of acetic acid.
5. Culture flasks can be directly used. *Optional*: Store coated flasks at +4 °C for a maximum of 6 weeks.

3.1.2 Collagen Type IV Coating of Inserts

The inserts need to be coated overnight with collagen type IV, necessary for development and long-term maintenance of differentiated primary airway epithelial cell cultures.

1. Mix 7.2 ml of filter-sterile dH_2O with 800 μl of Collagen Type IV solution (0.5 mg/ml).
2. Apply 150 μl per 12-well inserts, or 50 μl per 24-well inserts. After completing one plate, make sure that the entire surface of each well is covered with the 1:10 collagen solution.
3. Air-dry the inserts overnight in a laminar flowhood, and afterwards expose them to UV-light (type C) for 30 min.
4. To remove traces of acetic acid wash inserts twice with at least 500 μl of PBS.
5. After these steps, coated inserts can be used directly. *Optional*: Store at +4 °C (wrapped in foil) for a maximum of 6 weeks. Repeat UV-exposure and washing steps before use.

3.1.3 Isolation of Primary Human Tracheal and/or Bronchial Cells

Primary epithelial cells can be isolated from whole lung tissue resections of tracheal and/or bronchial origin according to the following protocol. Smaller lung tissue resections can be processed with the same protocol. All procedures are performed at room temperature unless stated otherwise.

1. Trim the bronchial tissue free of connective tissue and fat using forceps and scissors or a scalpel. If needed, cut the bronchial tissue into 2 cm segments.
2. Wash the cleaned tissue three times in washing solution.
3. Fill the desired number of 50 ml tubes with 30 ml of wash solution and transfer as many tissue segments as possible into a single tube, until the volume reaches 36 ml. Then add 4 ml of 10× digestion solution to each tube, to end volume 40 ml (40 mg Protease/0.4 mg DNase).
4. Place tubes on a rocking platform/tube roller at +4 °C and incubate for 48 h.
5. Place the 50 ml tube containing the digested tissue on ice and add 4 ml of heat-inactivated FBS to each tube (to a final concentration of 10 % ($^v/_v$)), to neutralize protease activity. Invert tubes three times.
6. Pour solution along with the tissue onto a large petri dish, and gently scrape off the epithelium from the collagen-cartilage surface, using a scalpel in the reverted angle. Pool solutions containing dissociated cells into a 50 ml conical tube and wash the petri dish once with PBS.
7. Centrifuge for 5 min at $500 \times g$. Wash cells once with HBSS and resuspend cells in BEGM to a concentration of, approximately, 5×10^6 cells/ml.

8. Count cells using a hemocytometer and seed into collagen coated flasks with 20 ml of pre-warmed BEGM. An appropriate amount of cells for T75 flasks ranges between 0.5 and 1.0×10^6 cells.
9. Change medium the next day to remove red blood cells and any unattached epithelial cells.
10. To prevent acidification of the medium change it every 2–3 days, until 80–90 % confluence.

3.1.4 Establishment of Fully Differentiated HAE Cultures

When the primary cells have reached 80–90 % confluence in the expansion phase one can dissociate and seed the dedifferentiated primary cells on collagen type IV coated inserts, according to the following protocol. All procedures are performed at room temperature unless stated otherwise.

1. Remove BEGM and transfer it into a 50 ml tube and wash the cell monolayer twice with 12 ml of HBSS.
2. Dissociate the bronchial cells for 3 min at 37 °C in a humidified 5 % CO_2 incubator with the appropriate amount of trypsin (25 cm^2: 1 ml, 75 cm^2: 3 ml). If needed tap the flask to dissociate the cells (*see* **Note** 7).
3. Collect the cells in the previously collected BEGM and centrifuge for 5 min at $500 \times g$.
4. Carefully discard the supernatant and resuspend cells in HBSS and centrifuge the suspension for 5 min at $500 \times g$.
5. Discard the supernatant and resuspend cells in pre-warmed ALI medium and count using a hemocytometer.
6. For generation of differentiated HAE cultures the number of cells seeded should be $1.0–2.0 \times 10^5$ cells per 12-well insert in 500 μl, or $0.3–0.6 \times 10^5$ cells per 24-well insert in 200 μl of ALI medium. A single 75 cm^2 flask should provide enough cells for preparing 48 individual 12-well inserts or 96 individual 24-well inserts.
7. Fill the basolateral compartment of the plates with 1 ml of ALI medium (500 μl for 24-well inserts), and transfer 500 μl (200 μl for 24-well inserts) of diluted cell suspension to the upper chamber of the collagen coated inserts and incubate overnight at 37 °C in a humidified 5 % CO_2 incubator. Cells are now in liquid–liquid interface.
8. The next day, medium in the apical compartment must be changed to remove any unattached cells. Discard the old medium and wash the apical surface with 500 μl HBSS and apply 500 μl of pre-warmed ALI medium to the apical side. Adjust volume to 200 μl for 24-well inserts.

9. To prevent acidification of the medium it should be changed every 2–3 days until cells have reached complete confluence (*see* **Note 8**).
10. During media change in liquid–liquid interface change apical medium first (as described in **step 8**) followed by exchange of medium in the basolateral compartment.
11. To establish air–liquid interface, aspirate apical side medium, once cells have reached complete confluence, and wash twice with HBSS (500 μl for 12-well inserts and 200 μl for 24-well inserts).
12. Incubate cultures for a few hours at 37 °C in a humidified 5 % CO_2 incubator and monitor if seeping of basolateral medium into the apical compartment occurs. If no seepage occurs cultures can be maintained at air–liquid interface. Otherwise cultures have to be cultured at liquid–liquid interface for another day.
13. Incubate cultures for 4–6 weeks to allow differentiation. Appearance of active ciliated cells can be used as an indicator of differentiation. During the extended culture time medium must be changed regularly (every 2–3 days). If desired, inserts can be transferred to deep well plates that only require medium renewal every 7 days.
14. After differentiation HAE cultures are suitable for human coronavirus propagation.

3.2 Human Coronavirus Propagation

1. Wash the apical surface of the HAE culture twice with 500 μl of HBSS solution prior to inoculation with human coronavirus specimen to remove excess of mucus.
2. Dilute the clinical material or virus supernatant in HBSS and inoculate 200 μl dropwise to the apical surface and incubate for 2 h at either 33 °C or 37 °C (*see* **Note 9**), in a humidified 5 % CO_2-incubator. *Optional*: Centrifuge inoculum solution for 4 min at $1{,}500 \times g$ at room temperature to remove cell debris prior to inoculation.
3. Collect the inoculum and transfer it to a container and store at −80 °C for later analysis, and wash the apical surface three times with 500 μl HBSS. *Optional*: Transfer the collected inoculum into an equal volume of VTM.
4. Incubate the infected cultures for the desired amount of time at the appropriate temperature in a humidified 5 % CO_2-incubator, e.g. 48 h at 33 °C for HCoV-229E.
5. Apply 200 μl of HBSS dropwise to the apical surface 10 min prior to the desired collection time and incubate in the humidified 5 % CO_2-incubator. Then collect progeny virus and transfer it to a container and store at −80 °C for later analysis. *Optional*: Transfer the collected progeny virus into an equal volume of VTM.

3.3 Immunofluorescence Analysis

All incubation steps are performed at room temperature on a gyrorocker (20–30 rpm), unless stated otherwise

1. After the apical washing has been collected the apical surface is washed twice with 500 μl of PBS before cells are fixed with formalin-solution for later immunofluorescence analysis.
2. Apply 500 μl of 4 % formalin-solution to the apical compartment and 1 ml to the basolateral. Incubate for 15–30 min.
3. Remove the formalin-solution and wash both compartments three times with equal volumes of PBS.
4. Transfer the fixed HAE cultures to a new conventional 12-well plate.
5. Discard washing solution and apply 500 μl and 1 ml of confocal buffer (CB) solution to apical and basolateral compartments, respectively.
6. Incubate fixed cultures for 30–60 min to block non-specific binding of antibodies (*see* **Note 10**).
7. Remove the CB solution from the apical and basolateral compartments.
8. From this stage one should only apply CB solution to the apical compartment.
9. Wash the apical surface once with 500 μl of CB solution for 5 min.
10. Apply primary antibodies (*see* Table 2) diluted in 250 μl CB solution dropwise to the apical surface and incubate for 120 min.
11. Wash the apical surface three times with 500 μl of CB solution for 5 min (*see* **Note 11**).
12. Apply the appropriately diluted conjugated secondary antibodies in 250 μl CB solution dropwise to the apical surface and incubate for 60 min.
13. Wash the apical surface twice with 500 μl of CB solution for 5 min.
14. Incubate cells with nucleic acid counter stain solution diluted in 250 μl of CB solution for 5 min.
15. Wash the apical surface once with 500 μl of CB solution for 5 min.
16. Lastly, wash the apical surface twice with 500 μl of PBS for 5 min to remove residual saponin and restore cell membrane integrity.
17. Before removing the washing solution, apply mounting medium on a glass slide (use 1–2 drops). Remove any air bubbles.

18. Excise the membrane from the plastic holder and carefully place the basolateral side of the membrane on top of the mounting medium, without generating air bubbles.
19. Then slowly add one drop of mounting medium on top of each membrane.
20. Slowly place the coverslip, in a tilted fashion, on top of the membrane without generating air bubbles.
21. Allow the mounting medium to polymerize for 30 min, after which the slide can directly be analyzed.

3.4 Antiviral Assays

3.4.1 Treatment

1. Pre-warm ALI medium to 37 °C.
2. Mix antiviral compounds (e.g. K22, recombinant interferons) in various concentrations or by serial dilution in ALI medium. Include non-treated controls. Also, to exclude viral inhibition by solvents (e.g. DMSO) include solvent controls.
3. For evaluation of either prophylactic or therapeutic effects of antivirals, the HAE cultures can be incubated with the compounds diluted in the basolateral medium prior to, during or after infection.
4. Infect cultures apically with human coronaviruses as described in Subheading 3.2.
5. Collect apical washings in HBSS as described in Subheading 3.2 for viral quantification by plaque assay and cells for viral quantification by Renilla Luciferase Assay or qRT-PCR.

3.4.2 Cytotoxicity Assay

1. Thaw CellTiter-Glo buffer and equilibrate both buffer and CellTiter-Glo substrate to room temperature.
2. Transfer the buffer to the amber bottle containing the substrate to reconstitute the enzyme. Mix by gently swirling the bottle.
3. Wash the apical side of the HAE cultures three times with 500 μl HBSS to remove excess mucus.
4. Apply 50 μl of HBSS to the apical side and mix with equal volume of reconstituted CellTiter-Glo enzyme solution (optimized for 24-well inserts, for other insert sizes adjust buffer amount accordingly) and incubate for 5 min at room temperature on a gyro-rocker to induce cell lysis.
5. Next incubate the plate for 10 min at room temperature to allow for stabilization of the luminescence signal.
6. Transfer 20 μl of cell lysate to a white, non-transparent 96-well plate for analysis.
7. Record luminescence (*see* **Note 12**). To account for background signal include empty wells in your analysis.

3.5 Virus Detection

3.5.1 Renilla Luciferase Assay (See Note 13)

1. Thaw Renilla Luciferase Assay buffer and dilute 1:5 in water.
2. Wash HAE inserts with HBSS three times prior to cell lysis.
3. Incubate inserts with 80 µl of Renilla lysis buffer on a gyro-rocker for 30 min at room temperature (optimized for 24-well inserts, adjust lysis buffer amount accordingly for other insert sizes).
4. During incubation, thaw Renilla Assay buffer.
5. Transfer the cell lysate to a 96-well plate.
6. Transfer 20 µl of the lysate to a white, non-transparent 96-well plate for analysis.
7. Add Renilla substrate at 1:200 dilution to the required amount of Renilla Assay buffer (100 µl per sample). Protect from light (*see* **Note 14**).
8. Program your luminometer settings with 10 s measure time followed by a 2 s delay. 100 µl of assay buffer should be dispensed into each well. If the luminometer is not equipped with an injector the assay buffer can be added manually using a multichannel pipette.
9. To adjust samples for background include empty wells in your analysis.
10. Plot your values as Log_{10} RLU (Relative Light Units).

3.5.2 Plaque Assay

The current protocol is optimized for HCoV-229E, but can easily be adapted to any other cell line and coronavirus strain.

1. Seed 150,000 target cells in a 12-well cluster plates with 1 ml of complete medium per well and incubate overnight at 37 °C in a humidified 5 % CO_2-incubator.
2. Make 6 tenfold serial dilutions of the harvested virus supernatants in 1 ml and inoculate the cells.
3. Incubate inoculum for 2 h at 33 °C in a humidified 5 % CO_2-incubator before removing the serial diluted virus inoculums from the cells and replace with 1 ml of overlay medium.
4. Incubate titration plates for 3–4 days at 33 °C in a humidified 5 % CO_2-incubator.
5. Remove overlay and wash wells twice with water to remove residual Avicel.
6. Subsequently add approximately 0.5–1 ml of crystal violet solution to each well and incubate for 10 min.
7. Remove crystal violet solution and wash the cells once with water and allow the plates to air-dry before counting the number of plaques.

3.5.3 Quantitative Reverse Transcriptase PCR

1. Isolate viral RNA with NucleoSpin RNA kit according to the manufacturer's protocol and elute in the appropriate amount of RNase-free water.
2. For reverse transcription use M-MLV reverse transcriptase (100 U), M-MLV buffer, and random primers and 10 µl of extracted total RNA in a total volume of 20 µl, at 37 °C for 60 min. *Optional*: include serial dilutions of in vitro transcribed RNA of the target gene for virus yield quantification.
3. To quantify viral HCoV RNA yields from contemporary strains use the FastStart Universal SYBR Green Master reaction mixture. Amplify 2 µl of cDNA according to the manufacturer's protocol, using the previously published sense and antisense strain-specific primers (*see* **Note 15**). Measurements and analysis can, for instance, be done on a LightCycler 480 II instrument, using the LightCycler 480 software package (Roche). Use the following cycle profile of 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C followed by a melting curve step to confirm product specificity.

4 Notes

1. Dissolve 5 g of BSA, globulin free, powder in 20 ml PBS in a 50 ml tube (do not vortex). Place the tube on a shaker/rollerbank for 2–4 (max 24) hours at +4 °C, until the BSA is completely dissolved. Add the volume up to 34 ml, mix gently by inverting the tube three times. Filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 3.5 ml in 15 ml tubes. Invert the tube three times before usage.
2. Dissolve 12 mg of Retinoic Acid (RA) in 40 ml absolute EtOH in a 50 ml tube wrapped in aluminum foil, the RA–EtOH stock (1×10^{-3} M) should be stored at −20 °C. To prepare the 1,000× stock, first confirm the RA concentration of the ethanol stock by diluting it 1:100 in absolute EtOH. Measure the absorbance at 350 nm using a spectrophotometer and a 1 cm light path quartz cuvette (or NanoDrop with 0.1 cm light path), blanked on 100 % EtOH. The absorbance of the diluted stock should equal 0.45 (0.045 on a NanoDrop). RA absorbance readings below 0.18 should be discarded. If the absorbance equals 0.45, add 3 ml of RA–EtOH stock solution to 53 ml PBS and add 4 ml of BSA 150 mg/ml stock. For absorbance values less than 0.45, calculate the needed volume of RA–EtOH stock as 1.35/absorbance and adjust the PBS volume appropriately. The 1,000× stock solution should be stored at −20 °C in aliquots of 1 ml per eppendorf tube.
3. Dissolve 42 mg ferrous sulfate, 12.2 g magnesium chloride, and 1.62 g calcium chloride-dihydrate in 80 ml H_2O, add

500 µl concentrated hydrochloric acid (HCl). Filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 1,100 µl per eppendorf tube.

4. Prepare seven separate 25 or 50 ml stock solutions (*see* Table 3a and b) in H_2O. Filter-sterilize (0.22 µm) each component after preparation. Afterwards, transfer an aliquot of 50 µl from each separate component into 49,600 µl filter-sterilized water (0.22 µm) and add a volume of 50 µl concentrated HCl solution. Mix the solution well through gentle vortexing and filter-sterilize the solution through a 0.22 µm filter, and store at −20 °C in aliquots of 1,100 µl per eppendorf tube.
5. Gentamicin and Amphotericin B should be omitted from ALI medium. These antibiotics are only required in BEGM medium right after cell isolation to prevent contamination.
6. For preparation of crystal violet solution safety glasses and protective clothing should be worn. Any spillage must be cleaned with 96 % ethanol.
7. Cells might take longer to dissociate from the bottom of the flask due to the collagen coating. If the cells are not dissociated after 3 min additional rounds of 1-min incubations can be performed until all cells have detached.
8. The seeded primary cells should reach confluence on the inserts within 1 week. If this takes longer the success rate of establishing well differentiated HAE cultures declines exponentially.

Table 3
Stock solutions for trace elements

(a)

Component	Formula	Amount/25 ml	Comment
Selenium	$NaSeO_3$	130.0 mg	Solution stable for 30 days at +4 °C
Silicone	$Na_2SiO_3 \cdot 9H_2O$	3.55 g	
Molybdenum	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	31.0 mg	
Vanadium	NH_4VO_3	14.75 mg	Heat >100 °C to dissolve

(b)

Component	Formula	Amount/50 ml	Comment
Nickel	$NiSO_4 \cdot 6H_2O$	13.0 mg	
Tin	$SnCl_2 \cdot 2H_2O$	5.5 mg	
Manganese	$MnCl_2 \cdot 4H_2O$	10.0 mg	

9. Human coronavirus NL63, 229E, HKU1, and OC43 are predominantly found in the upper respiratory tract and are therefore incubated at 33 °C. Both MERS-CoV and SARS-CoV are predominantly found in the lower respiratory tract and are therefore incubated at 37 °C.
10. The fixed HAE cultures can be kept for 1–3 months at 4 °C if the CB is filter-sterilized (0.2 μM) and all the procedure were performed under sterile conditions. After cold storage it is preferential to acclimatize the fixed cultures for 15 min to room temperature on a gyro-rocker (20–30 rpm) prior to continuation of the staining protocol.
11. To prevent bleaching of the fluorophores one should cover the inserts from daylight exposure during each incubation step.
12. Luminometer settings depend on the manufacturer. However, a measurement time of 1–2 s per well has proved effective.
13. For this assay cultures must be infected with coronaviruses expressing a Renilla Luciferase reporter gene.
14. If your luminometer is equipped with an injector you must remember to account for priming by increasing the volume of required Renilla Assay buffer by 2–3 ml.
15. Primers targeting HCoV-NL63, HCoV-HKU1, HCoV-229E, and HCoV-OC43 have been characterized and described [4, 14, 15].

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