HUMAN PRIMARY AIRWAY EPITHELIAL CELL CULTURING PROTOCOL

Introduction
The hBE passage 1 (P1) cells are shipped frozen. The overall strategy is to grow passage 1 and expand the cells in either BEGM (bronchial epithelial growth medium) or a suitable commercially available medium on collagen type I/III coated plastic tissue culture dishes. Once the P1 cells reach 70-90% confluence, then they can be passaged and seeded on human type IV collagen coated porous supports in air liquid interface (ALI) medium for differentiated cultures.

Materials
- Homemade BEGM (see reference 1) or commercial BEGM™ Bronchial Epithelial Cell Medium Bulletkit™ (www.Lonza.com).
- Tissue culture dishes-usually 100 mm circular.
- Type I/III Collagen – PureCol® (www.advancedbiomatrix.com).
- Collagen type IV (Sigma, C7521).
- For ALI cultures- one or more of the following porous supports: Corning® Transwell®-Clear (Cat. # 3460, 3450); Corning® Snapwells (Cat. # 3801); Millipore Millicel® CM (Cat. #: PICM01250 and 03050).
- ALI media as per reference 1.
- Accutase- (Cat #: AT104) www.Innovativecelltech.com (Used in place of trypsin)
- Ham’s F12 medium

To make homemade ALI and BEGM media you will need the following products.
Performance

- Recommended seeding density for subculture: \(1 \times 10^6\) cells/100 mm dish
- Typical time from subculture to confluent monolayer: 5-8 days
- Recommended seeding density for cells on supports: \(1.5 \times 10^5\) cells/cm\(^2\)

Quality Control

Cell viability, morphology and proliferative capacity are measured after recovery from cryopreservation.
Storage Requirements
- **Cells**
  Remove cryopreserved cells from dry ice and place immediately into vapor phase liquid nitrogen storage.
- **Medium**
  Purchase medium and store at 4°C or make homemade BEGM and ALI media and store at 4°C.
- **Reagents & Solutions**
  Ham’s F-12 medium and Accutase, both stored at 4°C.

Safety Precautions
All human sourced material should be handled using universal precautions.
- Always wear gloves when handling human material.
- Exercise caution when removing and thawing cells out of liquid nitrogen, wear eye protection.
- Wash hands after handling human material.
- Never mouth pipet.

Cryopreserved Cells
- Warm Ham’s F-12 media to 37°C.
- Rapidly thaw cryovial in 37°C water. Transfer as soon as ice melts.
- Decontaminate cryovial by wiping the exterior down with 70% ETOH.
- Resuspend cell suspension slowly and transfer cells to 15 mL conical tube.
- Dilute the cell suspension by slowly filling tube with warm F-12; centrifuge at 600g for 5 min at 4°C.
- Aspirate supernatant and gently resuspend cells in appropriate plating medium, count cells and assess viability.
- Plate cells according to recommended cell seeding density and the surface area of vessel being used (>1x10⁶ cells per collagen coated 100 mm diameter dish).
- Place culture dishes in 37°C, 5% CO₂ incubator. The cells will adhere to the bottom of the dish.
- Remove medium 24 hr after plating and wash away any dead cells with PBS. Feed every other day thereafter with fresh media. Examine cells daily.

Using a Hemocytometer
- Prepare a cell suspension in Ham’s F-12 media.
- Clean hemocytometer. Center the coverslip on the hemocytometer.
- Quickly resuspend cell suspension. Pipette out 12µL of cells and mix with 12 µL of trypan blue. Mix and take 12 µL of cell mixture in pipette tip and slowly fill the chamber.
- Count the cells in four 1 mm² corner squares. Nonviable cells stain blue. Keep separate counts of viable and nonviable cells. Count cells falling on the perimeter only two of the four sides.
- Each square of the hemocytometer, represents a total volume of 0.1 mm³ or 10⁻⁴cm³. 1 cm³ is equivalent to 1mL, the subsequent cell concentration per mL (and the total number of cells) can be determined thusly:
  \[
  \text{CELLS PER mL} = \text{the average count per square x dilution factor} \times 10^4 \\
  \text{TOTAL CELLS} = \text{cells per mL x the original volume of fluid from which cell sample was removed.}
  \]
  \[
  \% \ \text{CELL VIABILITY} = \frac{\text{total viable cells (unstained)}}{\text{by total cells (stained and}}
  \]
n
ll
l
p
m
ice.
Transfer cells to conical tube on
detach.
Add 3 mL to each dish, swirl and
incubate at 37°C for 2-12 hr.
Transfer plates to the hood and
aspirate any remaining liquid.
Dry completely in hood. UV 30
min. Store coated dishes at 4°C
for up to 8 weeks.
Coating porous supports, prepare
stock solution by dissolving 10 mg
type IV collagen in 20 mL dH₂O
and add 50µL of concentrated
acetic acid.
Incubate solution at 37°C until
dissolved (~4 hrs), filter.
Store aliquots at -20°C.
Dilute frozen stock 1:10 with
sterile dH₂O.
Place supports in appropriate
vessels, add 150 µL diluted
solution per 10-12 mm
support, 400 µL per 24-30 mm
support on apical side.
Allow to dry in the hood overnight
with blower on.
UV sterilize for 30 minutes and use
or wrap vessels in parafilm and
store at 4°C for up to 6 weeks.

Collagen Coating Plates
& Supports
- Coat 100 mm tissue culture dish
  with 1:75 dilution of Purecol in
  sterile dH₂O.
- Add 3 mL to each dish, swirl and
  incubate at 37°C for 2-12 hr.
- Transfer plates to the hood and
  aspirate any remaining liquid.
- Dry completely in hood. UV 30
  min. Store coated dishes at 4°C
  for up to 8 weeks.
- Coating porous supports, prepare
  stock solution by dissolving 10 mg
type IV collagen in 20 mL dH₂O
  and add 50µL of concentrated
  acetic acid.
- Incubate solution at 37°C until
dissolved (~4 hrs), filter.
- Store aliquots at -20°C.
- Dilute frozen stock 1:10 with
  sterile dH₂O.
- Place supports in appropriate
  vessels, add 150 µL diluted
  solution per 10-12 mm
  support, 400 µL per 24-30 mm
  support on apical side.
- Allow to dry in the hood overnight
  with blower on.
- UV sterilize for 30 minutes and use
  or wrap vessels in parafilm and
  store at 4°C for up to 6 weeks.

Subculture Preparation
- Subculture the cells when they are
  70-90% confluent.
- Rinse dish with PBS, add 3 mL
  Accutase to 100 mm dish. Incubate at 37°C until cells
detach.
- Transfer cells to conical tube on
  ice. Centrifuge tube 5 min at
  600g, 4°C. Aspirate supernatant
  and resuspend pellet in plating
  medium for counting.
- Plating cells on collagen coated
  porous supports, plate at a
density of 0.15- 0.25 x 10⁶ cells
  per cm². This equals 1.25 - 2.50 x
  10⁵ cells on 10-12 mm supports
  and 1-3 x 10⁶ cells per 24-30
  mm support.
- Place culture dishes in 37°C,
  5% CO₂ incubator.
- Change the medium 24 hr after
  plating. Wash away debris
  with PBS. Feed every other day
  thereafter.

Maintenance after
Subculturing
- 24 hr after plating, examine
  the cells under microscope.
  ~30%+ of the cells should
  attach and show signs of
  spreading. Most cells will be
  single or in small islands.
- Change the medium to remove
dead cells and debris. For cells
  on supports, add one or two
drops of media to the apical
  side until confluent.
- Most hBE cells grown on
  plastic dishes will become
  confluent between days 5-8.
- Once cells on porous supports
  become confluent, the culture
  can be made into air-liquid
  interface (ALI) conditions.
  Most cultures are well-
differentiated after day 21.

References:
1) Fulcher ML, Randell SH.
   Human nasal and tracheo-
   bronchial respiratory epithelial
cell culture. Methods Mol Biol.
2) ML Fulcher, SE Gabriel, JC
   Olsen, E Livanos, JR Tateau,
   MT Saavedra, P Salmon and
   SH Randell. Novel Human
   Bronchial Epithelial Cell Lines
   For Cystic Fibrosis Research,
   AJP Lung Cell and Mol Physiol,
   2009 Jan;296(1):L82-91.