UNC SCHOOL OF MEDICINE
Marsico Lung Institute/CF Center

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 homemeade ALI and BEGM media you will need the following products.

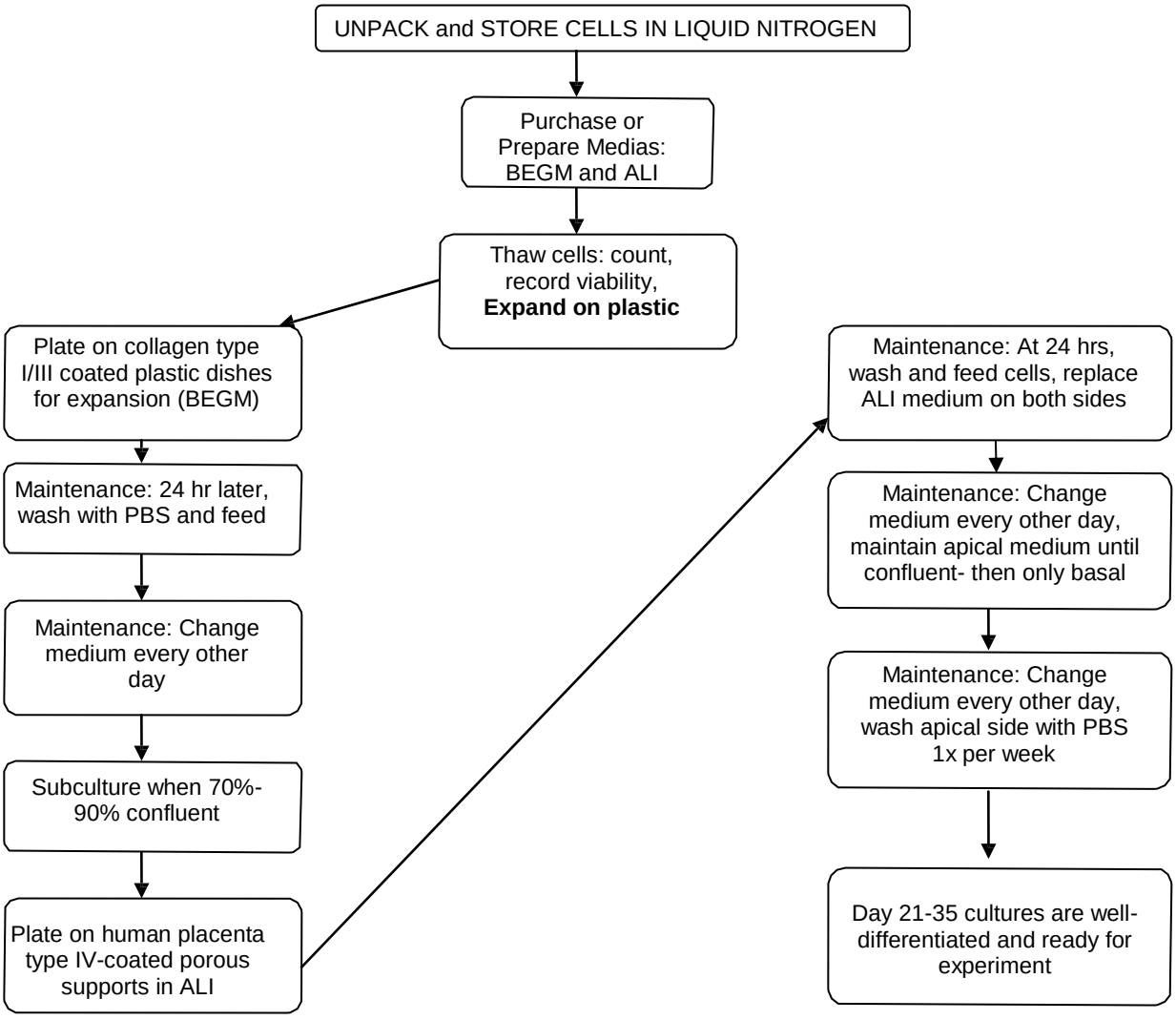
☐	**PRODUCTS**	☐	**VENDOR (Cat. #)**
☐	DMEM-H	☐	Corning (MT10-013-CV)
☐	LHC Basal	☐	Invitrogen (12677-019)
☐	Insulin	☐	Sigma (I6634)
☐	Hydrocortisone	☐	Sigma (H0396)
☐	Epidermal Growth Factor	☐	Invitrogen (PHG0313)
☐	Triiodothyronine	☐	Sigma (T6397)
☐	Transferrin	☐	Sigma (T0665)
☐	Epinephrine	☐	Sigma (E4250)
☐	Phosphorylethanolamine	☐	Sigma (P0503)
☐	Ethanolamine	☐	Sigma (E0135)
☐	Bovine Pituitary Extract	☐	Sigma (P1476)
☐	Bovine Serum Albumin	☐	Sigma (A7638)
☐	Trace Elements-Silicone	☐	Sigma (S5904)
☐	Selenium	☐	Sigma (S5261)
☐	Manganese	☐	Sigma (M5005)
☐	Molybdenum	☐	Sigma (M1019)
☐	Vanadium	☐	Sigma (398128)
☐	Nickle	☐	Sigma (N4882)
☐	Tin	☐	Sigma (243523)
☐	Stock 4-Ferrous Sulfate	☐	Fisherbrand (I146)
☐	Magnesium chloride	☐	Fisherbrand (M33)
☐	Calcium chloride	☐	Sigma (C3381)
☐	Pen/Strep	☐	Sigma (P3032) (S9137)
☐	Amphotericin B	☐	Sigma (A2942)
☐	Gentamicin	☐	Sigma (G1397)
☐	Retinoic Acid	☐	Sigma (R2625)
☐	Stock 11	☐	Sigma (Z0251)

Performance

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

Quality Control

Cell viability, morphology and proliferative capacity are measured after recovery from cryopreservation.



Storage Requirements

- **Cells**
  Remove cryoperserved 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

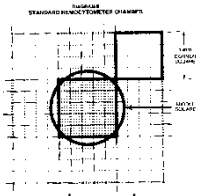
All human sourced material should be handled using universal precautions.

- 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 (>$1x10^6$cells per collagen coated 100 mm diameter dish).
- Place culture dishes in 37°C, 5% CO_2 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 mm^2 corner squares. Nonviable cells stain blue. Keep separate counts of viable and nonviable cells. Count cells falling on the perimeter on only two of the four sides.
- Each square of the hemocytometer, represents a total volume of 0.1 mm^3 or $10^{-4}cm^3$. 1 cm^3 is equivalent to 1mL, the subsequent cell concentration per mL (and the total number of cells) can be determined thusly:

CELLS PER mL = the average count per square x dilution factor (in this case 2) x 10^4

TOTAL CELLS = cells per mL x the original volume of fluid from which cell sample was removed.

% CELL VIABILITY = total viable cells (unstained) / by total cells (stained and

unstained)
x 100.

Collagen Coating Plates & Supports

- Coat 100 mm tissue culture dish with 1:75 dilution of Purecol in sterile dH_2O.
- 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 dH_2O 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 dH_2O.
- 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 10^6 cells per cm^2. This equals 1.25 - 2.50 x 10^5cells on 10-12mm supports and 1-3 x 10^6 cells per 24-30 mm support.
- Place culture dishes in 37°C, 5% CO_2 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. 2013;945:109-21.
2) ML Fulcher, SE Gabriel, JC Olsen, E Livanos, JR Tatreau, 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.