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 Millicell® 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.

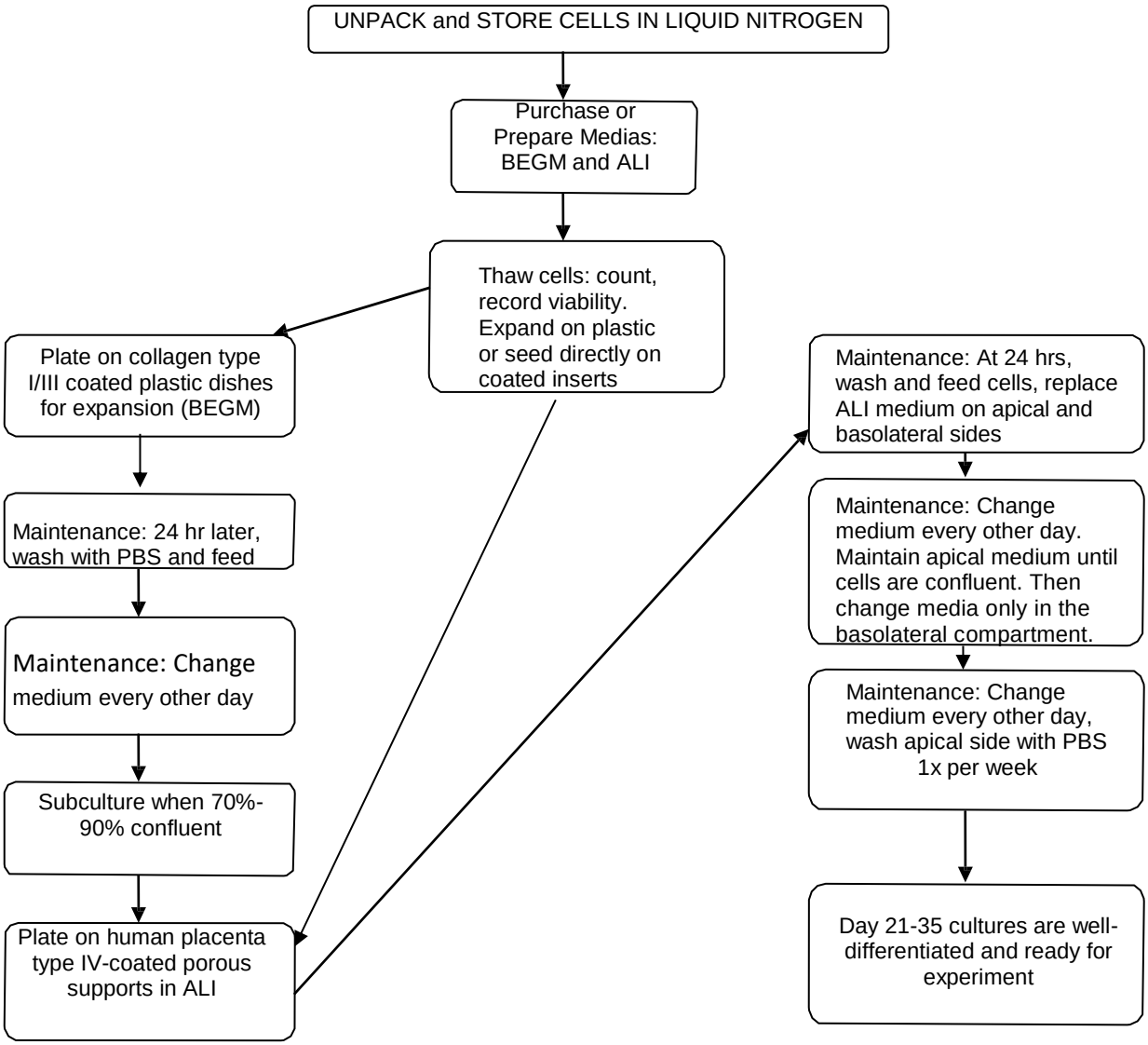
□	**PRODUCTS**	□	**VENDOR (Cat. #)**
□	DMEM-H	□	Corning (MT10-013-CV)
□	LHC Basal	□	Invitrogen (12677-019)
□	Insulin	□	Sigma (I6634)
□	Hydrocortisone	□	Sigma (H0396)
□	Epidermal Growth Factor	□	Invitrogen (PHG0313)
□	Triiodothyronine	□	Sigma (T6397)
□	Transferrin	□	Sigma (T0665)
□	Epinephrine	□	Sigma (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 x 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 x 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 commercial medium or make homemade BEGM and ALI media and store at 4°C. UNC BEGM and ALI can now be purchased through the MLI Tissue Procurement and Cell Culture Core (www.med.unc.edu/mlicellcore).
- **Reagents & Solutions**
  Ham's F-12 medium and Accutase, follow manufacture's storage recommendations.

Safety Precautions

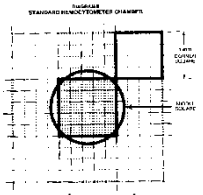
All human sourced material should be handled using universal precautions.

- 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 mix 12μL of homogenous cell suspension with 12 μL of trypan blue. Dispense 12 μL of cell suspension into one side of the counting 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 2-24 hr.
- Transfer plates to the biological cabinet and aspirate any remaining liquid.
- Dry completely in cabinet. UV 30 minutes to bind collagen to dish. Store coated dishes at 4°C for up to 8 weeks. (Recoat after expiration date).
- 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 (4-6 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 12 mm support, 400 μL per 24-30 mm support on apical side.
- Allow to dry in the biological cabinet overnight with blower on.
- UV for 30 minutes to crosslink collagen to membrane. Wrap vessels in parafilm and store at 4°C for up to 6 weeks. (Recoat after expiration date).

Subculture Preparation

- Subculture the cells when they are 70-90% confluent.
- hBE cells expanded on coated dishes will become confluent between days 5-8.
- Aspirate cells expansion medium and rinse dish with PBS.
- Add 3 mL Accutase to 100 mm dish. Incubate at 37°C until cells detach. Tap dish to release cells.
- 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 12mm supports and 1-3 x 10^6 cells per 24-30 mm support.
- Place culture dishes in 37°C, 5% CO_2 incubator.

Maintenance after Subculturing

- 24 hrs. after plating cells, examine the cells under microscope.
- Day one attachment can be difficult to see until the apical side is washed.
- Change the medium (apical and basolateral side) 24 hrs. after plating cells.
- Wash debris on the apical side with PBS. Add appropriate medium volume to basolateral compartment. (Refer to manufacture's recommendation).
- Add one or two drops of media to the apical side until cells are confluent. (Do not fill support as this will slow the differentiation process).
- 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.

Notes:

- We recommend using tissue culture treated dishes for cell expansion. These dishes must be coated with collagen and UV cross linked prior to use for any primary cells or passage 1 cells being thawed. Use of flasks is not recommended and we cannot guarantee cells will expand at the best quality using these vessels.
- Cell must be counted and seeded at the recommended seeding density on coated dishes and coated porous supports, otherwise cells may not proliferate at the appropriate growth rate.
- Our custom media is nonproprietary. While commercial medias are readily available, they contain proprietary additives.
- Other options for expanding HBE cells: Use of the Conditionally Reprogrammed Cell Method. Gentzsch M, Boyles SE, Cheluvaraju C, Chaudhry IG, Quinney NL, Cho C, Dang H, Liu X, Schlegel R, Randell SH**.** Pharmacological rescue of conditionally reprogrammed cystic fibrosis bronchial epithelial cells.Am J Respir Cell Mol Biol. 2017 May;56(5):568-574. See Supplement for more details.
- https://www.med.unc.edu/mlicellcore/