

Non-Cystic Fibrosis Cell Lines	Cystic Fibrosis Cell Lines
UNCN1T	UNCCF1T
UNCN2T	UNCCF2T
UNCN3T	UNCCF3T

Introduction

The limited availability of primary human bronchial epithelial (hBE) cells has driven the creation of cell lines. Bmi-1 is a proto-oncogene that maintains stem cells by suppressing cyclin dependent kinase inhibitors, and its expression creates cell lines that recapitulate normal cell structure and function. Using lentiviral vectors, we introduced Bmi-1 and the catalytic subunit of telomerase (hTERT) into three different non-Cystic Fibrosis (CF) and 3 Delta-F508 homozygous CF primary bronchial cell preparations. Bmi-1/hTERT expression extended cell lifespan while maintaining a diploid karyotype. When seeded at high density on porous supports, passage 15 cells exhibited a pseudostratified morphology with few ciliated cells but abundant mucus secretory cells. Ussing chamber studies demonstrated electrophysiologic properties mostly comparable to early passage primary cells. All non-CF and CF cell lines produced similar levels of IL-8 at baseline and equally increased IL-8 secretion in response to IL-1beta, TNFalpha and the Toll-like receptor 2 agonist, Pam3Cys. Bmi-1/hTERT cell lines are similar to primary hBE cells in their fastidious requirements for growth factors, substratum, adequate seeding density and good husbandry practices. We strongly recommend careful reading of Fulcher et al (1) in preparation for receiving and growing these cell lines. Furthermore, due to the limited growth capacity compared to traditional cell lines, there is a limited world supply of these cells.

The cell lines are shipped frozen. The overall strategy is to grow 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 and to freeze down a sufficient quantity of the cells for future studies. Once the cells have been expanded and aliquots are securely stored in liquid nitrogen, they can be seeded on human type IV collagen coated porous supports in air liquid interface (ALI) media 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.

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



Marsico Lung Institute/CF Center

PRODUCTS	VENDOR (Cat. #)	Substitute Products
DMEM-H	Corning (MT10-013-CV)	Gibco (11995-065)
LHC Basal	Invitrogen (12677-019)	
Insulin	Sigma (I6634)	Sigma (I1882)
Hydrocortisone	Sigma (H0396)	
Epidermal Growth Factor	Invitrogen (PHG0313)	Sigma (E4127)
Triiodothyronine	Sigma (T6397)	
Transferrin	Sigma (T0665)	
Epinephrine	Sigma (E4250)	
Phosphorylethanolamine	Sigma (P0503)	
Ethanolamine	Sigma (E0135)	
Bovine Pituitary Extract	Sigma (P1476)	Gibco (13028014) or MilliporeSigma (02-103)
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	Sigma (F8048)	
Magnesium chloride	J.T. Baker (2444)	
Calcium chloride	Sigma (C3381)	
Pen/Strep	Sigma (P3032) (S9137)	
Amphotericin B	Sigma (A2942)	Corning™ (MT30003-CF)
Gentamicin	Sigma (G1397)	Corning™ (MT30005-CR)
Retinoic Acid	Sigma (R2625)	
Stock 11	Sigma (Z0251)	

Performance

- ❖ Recommended seeding density for subculture: 1 x 10⁶ cells/100 mm dish
- ❖ Typical time from subculture to confluent monolayer: 5-9 days
- ❖ Recommended seeding density for cells on supports: 1.5 x 10⁵ cells/cm²
- ❖ Additional population doublings: 30

Quality Control

All cell lines have been tested for mycoplasma and were DNA fingerprinted to check for cross contamination. CFTR genotype was confirmed to be deltaF508 homozygous in the UNCCF1-3T lines. Cell viability, morphology and proliferative capacity are measured after recovery from cryopreservation.

UNPACK and STORE CELLS

Prepare Medias:
BEGM and ALI

Thaw cells: count,
record viability,
Expand on plastic

Plate on collagen type
I/III coated plastic dishes
for expansion (BEGM)

Maintenance: 24 hr later,
wash and feed

Maintenance:
Change medium every
other day

Subculture when a 70%-
90% confluent

Split cells 1:4, Expand/
freeze aliquots/plate on
coated porous supports

Repeat Expansion and
freezing step until
adequate freezer stocks

Thaw vial from stock and
plate on coated plastic

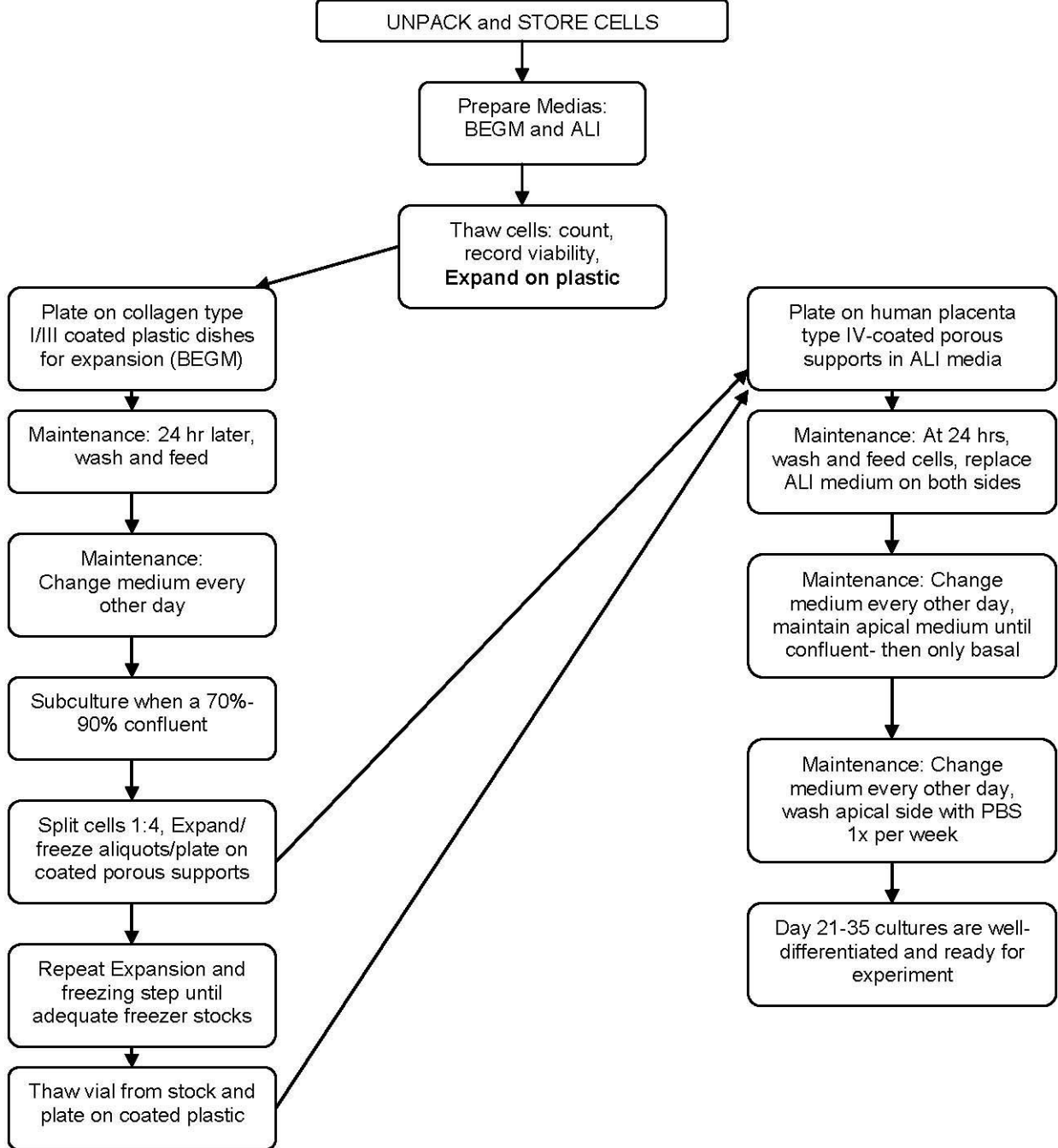
Plate on human placenta
type IV-coated porous
supports in ALI media

Maintenance: At 24 hrs,
wash and feed cells, replace
ALI medium on both sides

Maintenance: Change
medium every other day,
maintain apical medium until
confluent- then only basal

Maintenance: Change
medium every other day,
wash apical side with PBS
1x per week

Day 21-35 cultures are well-
differentiated and ready for
experiment



Storage Requirements

- Cells**
 Remove cryopreserved cells from dry ice and place immediately into vapor phase liquid nitrogen storage.
- Medium**
 Purchase Lonza BEGM Bulletkit™ and store at 4°C or make homemade BEGM and ALI media and store at 4°C. Aliquot amount needed and return stock bottle to the refrigerator.
- Reagents & Solutions**
 Ham's F-12 medium and soybean trypsin inhibitor (STI), both stored at 4°C. Cell freezing solution and trypsin/EDTA solution, stored at -20°C (for preparation see reference 1).
- Resuspend cell suspension slowly and transfer cells to 15 mL conical tube.
- Dilute the cell suspension by slowly filling tube with warm F-12 media and centrifuge at 600g for 5 min at 4°C.
- Aspirate supernatant and gently resuspend cells in appropriate plating media, 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 10 cm diameter dish).
- Place culture dishes in 37°C, 5% CO₂ incubator. The cells will adhere to the bottom of the dish.
- Remove media 24 hr after plating and wash away dead cells with PBS. Feed every other day thereafter with fresh media. Examine cells daily.

Safety Precautions

All human sourced material should be handled using universal precautions.

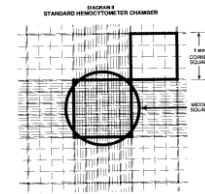
- Always wear gloves when handling human material.
- Exercise caution when removing and thawing cells out of liquid nitrogen. Wear eye protection.
- Wash hands after handling human material.
- Never mouth pipet.

Cryopreserved Cells

- Warm Ham's F-12 media and plating media to 37°C.
- Rapidly thaw cryovial in a beaker of 37°C water. Thaw one cryovial at a time and transfer as soon as ice melts.
- Decontaminate cryovial by wiping the exterior down with 70% ETOH.

Using a Hemocytometer

- Prepare a cell suspension in Ham's F-12 media.
- Clean hemocytometer and make sure all surface areas are dry. Center the coverslip on the hemocytometer.
- Quickly resuspend cell suspension. Pipette out 12 μL 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 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 on only two of the four sides.

• Each square of the hemocytometer, with the coverslip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3 . Since 1 cm^3 is equivalent to 1 mL, 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 dH₂O.
- Add 3 mL to each dish, swirl and incubate at 37°C for 2-24 hr.
- Transfer plates to the hood and aspirate any remaining liquid.
- Dry completely in hood. UV 30 min.
- Store coated plates 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 50 µL of concentrated acetic acid.

- Incubate solution at 37°C until dissolved (>4hrs) and syringe filter (0.2µm)
- Store aliquots at -20°C.
- To coat porous supports, 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 at room temperature 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 8 weeks.

Subculture Preparation

- Subculture the cells when they are 70-90% confluent.
- Thaw trypsin/EDTA.
- Rinse each 100 mm culture dish with PBS, add 3 mL trypsin/EDTA 100 mm dish. Incubate 5-10 min at 37°C.
- Periodically check to see if cells are detached. If detached (cells will round up and have smooth edges and appear shiny), harvest cell suspension by gently tapping plate and transfer cells to conical tube on ice. Add an equal volume of STI. Repeat trypsinization process with fresh reagents. Rinse dish with Ham's F-12 media or PBS to harvest remaining cells. Add cells to conical tube.
- Check plate for attached cells and repeat trypsinization process if necessary, pooling cells.



Marsico Lung Institute/CF Center

- Centrifuge tube 10 min at 600g, 4°C. Aspirate supernatant and resuspend pellet in Ham's F-12 media for counting (See above instructions).
- Plate cells according to recommended cell seeding density and the surface area of vessels being used (plate $> 1 \times 10^6$ /100 mm dish).
- If plating subcultured cells on collagen coated porous supports, plate at a density of $0.1-0.25 \times 10^6$ cells per cm^2 . This equals $1.25 - 2.50 \times 10^5$ cells on 10-12mm supports and $1-3 \times 10^6$ cells per 24-30 mm support (Note: these cell lines require plating at a higher density on the larger supports than primary HBE cells, otherwise they will retract into islands rather than gain confluence).
- Place culture dishes in 37°C, 5% CO₂ incubator. The cells will attach to the bottom of the dish.
- 24 hr after plating, wash away dead cells with PBS and change medium. Feed every other day thereafter. Examine cells daily.
- Most cell lines grown on plastic dishes will grow and become confluent between days 5-9.
- 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.

Maintenance after Subculturing

- 24 hr after plating, examine the cells under the microscope. 30% or more of the cells should have attached and show signs of spreading. Most cells will be single or in small islands.
- Change the culture medium to remove dead cells and debris. For cells on supports, add media to the apical side until a confluent monolayer is formed.

