Cutting Lab
CFTR2 Cell Center
Protocol: CFBE Cell Maintenance

I. STATEMENT OF PURPOSE

This protocol describes the techniques needed for maintaining CFBE 41o- cells, including plastic-ware coating, thawing, feeding, trypsinizing, and freezing.

II. SAFETY REQUIREMENTS

Gloves and lab coat are required.

III. EQUIPMENT/SUPPLIES

- Sterile cell culture set-up (See *Sterile Cell Culture Set-Up and Techniques Protocol (No.1)* for details).
- Beaker of sterile DI H2O warmed in bead bath.
- Sterile plastic bags
- Mr. Frosty
- Cryovials
- -80C Freezer
- Liquid Nitrogen Storage Dewar

IV. REAGENTS

- **Coating Solution** (per 50 ml)
  - 46 mL MEM (no FBS/antibiotics added) (Life, Cat. No. 11095098) (see *Procedure Notes*)
  - 5mL BSA (0.1% filtered PBS solution) (Albumin bovine serum, Sigma, Cat. No. A9056)
    - Instructions: add 0.1 g Albumin Bovine Serum to 100 mL PBS, swirl to dissolve, then filter using a sterile vacuum filter (ex. Millipore Express PLUS 0.22 um vacuum filter), aliquot and store at -20C
  - 500uL collagen I, rat tail (3mg/mL) (Life, Cat. No. A1048301)
  - 500uL human fibronectin (1mg/mL) (Sigma, Cat. No. F2006-1MG)
- **Cell culture media depends on stage/selection process:**

	CFBE parentals **"Complete Media"**	CF8Flp 100 ug/ml Zeo	Stbl. Trans. Step 1 50 ug/ml Hygro	Stbl. Trans. Step 2 100 ug/ml Hygro	Stbl. Trans. 200 ug/ml Hygro
MEM (Life, Cat.No. 11095098)	450 ml	450 ml	450 ml	450 ml	450 ml
FBS (Corning Cellgro, Cat. No. 35-010-CV)	50 ml	50 ml	50 ml	50 ml	50 ml
PS (Quality Biological, Cat. No. 120-095-721)	5 ml	5 ml	5 ml	5 ml	5 ml
Zeocin (Life, Cat.No. R25001)		500 ul			
Hygromycin B (Corning Cellgro, Cat.No. 30-240-CR)			500 ul	1 ml	2 ml

- **Sterile 1X PBS**, stored in cell culture hood
  - Instructions: Spray bottle of autoclaved DI H2O for 1X PBS (red label) and bottle of 10X PBS with 70% ethanol, and place in hood. Using sterile 50 ml tube, add 50 mls 10X PBS to bottle of sterile DI H2O. Mix by inverting and store in culture hood. Remove both the empty bottle of 1X PBS and 10X PBS, and place in the "Refill Box" and back on shelf, respectively.
- **0.25% Trypsin-EDTA** (Corning, Cat. No. 25-053-Cl)
- **Recovery Cell Culture Freezing Medium** (Life, Cat. No. 12648010)

V. PROCEDURE

A. Coating Plastic-Ware: Protocol as described by Gruenert Lab (http://www.uvm.edu/~uvmhmg/faq/07-coating.html)

1. Make 50 mls of the Coating Solution (See *Reagents*).
2. Label all plastic-ware to be coated with the letter "C" and the date.
3. Add an excess of the Coating Solution to the surfaces that are to be coated, using up all 50 mls.
4. After letting the coated plastic-ware sit for about 20 mins in the sterile hood, remove the Coating Solution using the Pipet Man and transfer to additional dishes to be coated.
   **Once you have coated all the dishes that are needed, discard the Coating Solution by aspirating (after incubation period).
5. After removing the Coating Solution, allow the coated plastic-ware to dry in the hood for ~1hr (T25 and T75 flasks will take longer)
6. After drying, items can be used directly for cell culture. Otherwise, the coated dishes are to be stored in sterile plastic bags at room temperature.
7. Always wash coated plastic ware with 1x PBS before use for cell culture.
8. Any plastic-ware not used within two weeks after coating is to be thrown away.

Coated plastic-ware is only to be used when thawing cells, plating CF8Flp cells for transfection, and growing up clones after hygromycin-selection

B. Thawing

1. Place a small plastic beaker, filled with autoclaved DI water, in the 37C bead bath for at least 30 minutes prior to thawing.
2. Remove cells from -80C/liquid nitrogen dewar.
3. Quickly thaw the cryovial of cells in the previously warmed water. Swirl/shake the cryovial in the water until the cell suspension has fully thawed.
   i. Cryovials can also be hand thawed by continuously rolling the vial between hands – thaw as quickly as possible to wash Cell Recover Freezing Media out of the cells ASAP (toxic)
4. Add 0.5 ml Complete Media (see *Reagents* for recipe) to the cryovial VERY CAREFULLY and gently mix by pipetting (1.5 ml cell suspension + 0.5 ml complete)
5. Add the contents of the cryovial, about 2 ml of cell suspension, to a 15 mL conical with 5 mls of Complete Media
6. Spin the cells down at 1 rcf x 5 min. Aspirate the media.
7. Add 5 ml of 1x PBS to the conical to further wash cells before plating (do not resuspend pellet, this is to further dilute the freezing media which is highly toxic to the cells)
8. Aspirate the PBS and resuspend the cells in 5 mL Complete Media and transfer into a T25 flask.
9. Store cells at 37C with 5% CO2.
10. The following day, change the culture media to media containing the appropriate antibiotic. The antibiotic depends on the cell stage (See the Cell Culture Media table in *Reagents*).

C. Feeding: Feed cells every 2 to 3 days

1. Aspirate the culture medium.
2. Briefly rinse the cell layer with sterile 1X PBS; the amount of 1X PBS should be enough to cover the dish surface.
3. After removing 1X PBS, add the appropriate amount (dependent on size of culture dish, see below) of culture media (dependent on the cell stage, see Cell Culture Media table in *Reagents*) to culture dish
   - 24 well: 500ul to 1 ml, cover bottom
   - 12 well: 1 ml

- 6 well: 2 ml
- T25: 5 ml (6 ml over weekend, if applicable)
- T75: 12 ml (15 ml over weekend, if applicable)
- Snapwell: 2 ml in bottom, 500 ul in top (aspirate bottom, then membrane + feed membrane, then bottom)

D. Hygromycin Kill Curve for CF8-Flp (before transfection)

1. CF8-Flp cells may have different tolerances to hygromycin after thawing and between aliquots
2. Plate 6 or more wells of CF8-Flp cells then allow to grow to 80-90% confluency
3. Expose each well to a concentration above or below 100 Hygromycin and observe (example: 50, 100, 150, 200, 250, 300)
4. Dying can take up to two weeks – death from over-confluency will appear as hole and dead patches, while hygromycin susceptibility will appear as sickly cells detaching until there are few sick cells then non left
5. Feed each well complete media for a week to look for any regrowth of cells that were not completely killed by hygromycin
6. Choose the lowest concentration of hygromycin that killed the well (300-500 will likely be too strong)

E. Trypsinizing

Volumes are given for a T75 flask. Increase or decrease the amounts needed proportionally for culture vessels of other sizes.

1. Aspirate culture medium.
2. Rinse cells two times with sterile 1X PBS; amount of 1X PBS should be enough to cover the dish surface, ~10 mls.
3. Add 1.5 ml of 0.25% Trypsin-EDTA solution to the T75 flask.
4. Place the flask back in the incubator while waiting for cells to detach, usually 5 to 10 minutes – cells will be white layer without patches – Fully detach cells by tapping flask, cells should not detach in fibrous strings but as single cells (may be over trypsinized if they are fibrous)
5. Once the cells are detached, add 8-10 mls of corresponding media (Hygromycin-containing media for CF8-stable cells and Zeocin-containing media for CF8Flps) and mix cells by gently pipetting, until any clumps are broken up and the solution looks homogeneous, rinse bottom while pipetting
6. Split the cell suspension between new flasks prefilled with a volume to add up to 12-15 ml (will depend on reseeding ratio)
    i. **PLEASE NOTE:** CF8Flps and CF8 Stable cells do not recover well when seeded at low densities. We suggest reseeding at a ratio no greater than 1:4 from a confluent T75 (for example, 1:10 is too dilute.)
7. Gently swirl cell suspension in new flask to ensure even distribution

For trypsinizing well plates, pipette enough trypsin to cover the surface and incubate the plate at 37C, add media after cell detachment, then tilt plate to wash cell surface using P200 (24 well) or P1000 (larger well sizes) by pipetting media over cells until they are completely resuspended

F. Freezing

1. Grow T25 or T75 to confluency.
2. If the Mr. Frosty is not thawed, place it in the 37C bead bath at least 30 minutes prior to starting protocol. Recovery Cell Culture Freezing Media should not be left at room temperature for too long, thaw and use immediately.
3. Follow the *Trypsinizing Protocol* (*Part D*).

4. At step 5 of the *Trypsinizing Protocol*, instead add 5 mls of Complete Media to a T25 or 10 mls of Complete Media to a T75. Mix cells by gently pipetting.
5. Remove cell suspension and place in a 15 ml conical tube.
6. Spin tube for 5 mins at RT at 1000 rcf.
7. At this step you can begin thawing aliquots of Recovery Cell Culture Freezing Media in 37C bead bath and labeling cryovials (1 for T25, 3 for T75).
8. Aspirate supernatant. **Be careful not to disturb the cell pellet.
9. Add 1.5 ml of Recovery Cell Culture Freezing Medium to tube with cells from a T25 and 4.5 mls to tube with cells from a T75, and resuspend by pipetting up and down.
10. Aliquot 1.5 ml of cell suspension to properly labeled cryovials

    Label should have the following:
    Cell Type
    Variant (if needed)
    Clone (if needed)
    Passage #
    Date
    Your initials
    Hygromycin concentration if different than 100 Hygromycin standard

    **Do this very quickly as the Recovery Cell Culture Freezing Media contains DMSO and is toxic. If possible, use multiple Mr. Frosties when freezing many flasks at once to reduce time cells spend in thawed media. Also be sure not to pipette any bubbles into the cryovial.
11. Place cryovials in Mr.Frosty and then place in -80C.
12. The following day, retrieve the cryovials and store in the proper location in liquid nitrogen.

G. Plating for Short Circuit Current Testing

1. Add 2 ml warmed DMEM Complete Media (Use DMEM instead of MEM and follow protocol for CFBE Parental Complete Media under *Reagents*) and 1 mL DMEM Complete Media to uncoated 6 well plate for RNA extraction
2. Follow *Trypsinizing protocol (Part D)* using fully confluent T75
3. At Step 5, resuspend the cells in 8.5 mL warmed DMEM Complete Media
4. Move the entire suspension to a 15 ml conical tube and centrifuge at 1 rcf for 5 min
5. Aspirate the media (be careful not to disturb cell pellet) and resuspend evenly in DMEM Complete Media
6. Plate the membranes of the snapwells with 0.5 ml suspension and add 1 ml cell suspension to RNA 6 well (add drop wise circling the well in order to evenly disperse the cells)
7. Add remaining cell suspension to new T75 flask filled with MEM 100 Hygromycin Media
8. Swirl the RNA plate to further disperse cells evenly
9. Feeding:
    i. Feed the T75 (100 Hygromycin Media), RNA Plate and Snapwell (DMEM Complete Media) next day
    ii. Snapwell: Aspirate from the membrane being very careful not to touch the membrane, then aspirate media from the bottom of the well – add DMEM Complete Media to the bottom of the well (2 ml) then to the snapwell membrane (0.5 ml) VERY carefully
    iii. Feed daily for 5 days (check confluency of RNA plate before ISC testing)
    iv. Testing should be done on the 6^{th} day

VI. PROCEDURE NOTES

- Cells should be stored at 37C and 5% CO2 at all times. Make sure to minimize time outside incubator.
- When making Complete Media or any of the other culture medias, reserve the 50 mls removed before FBS/antibiotic and store in a 50 ml tube at 4C. This media is used for coating and other protocols.

- When aspirating media/washes make sure to quickly replace media/wash so that cells do not dry out.
- Remember to always replace/spray gloves with 70% ethanol after touching anything outside the cell culture hood.
- Long hair should be pulled back.
- Questions about this protocol should be emailed to cftr2cellcenter@jhmi.edu.