HBE Tissue Culture Protocol for Flasks and Filters

(slightly modified version of the Vertex Protocol, Neuberger et al, 2011)

Need:

- 3T3 conditioned media (see 3T3 protocol for procedure)
- Sterile PBS
- Accutase (cat # A11105-01 100 ml)
- Versene (cat # 15040-066 Gibco 100 ml)

Thawing cells:

1. Coat T25 cm^2 or T75 cm^2 flasks with 2 or 5 ml of 3T3 conditioned media respectively. for at least 4 hours in 37ºC CO_2 incubator.
2. Remove conditioned media from flasks, add 5 or 15 ml of hBE Growth media (BEGM or alternative), and place back in 37ºC CO_2 incubator.
3. Thaw 1 vial of cells from liquid nitrogen tank. Warm for two minutes in 37ºC water bath to thaw cell suspension. Carefully mix with a 2 ml pipette to resuspend cells.
4. Count cells using Hemocytometer:
    a. Pipette 10 µl of 0.4% trypan blue stain and 10 µl of cell suspension into a 2 ml microcentrifuge tube. Mix and add 10 µl to one slot of the hemocytometer. Count cells and record cell count and viability.
5. Add enough cell suspension to each flask to seed at 1.7 x 10^5 cells/T25 cm^2 flask [6.6 x10^3 cells/cm^2] or 4.95 x10^5 cells/T75cm^2 flask [6.6 x 10^3 cells/cm^2]. If there is any cell suspension left divide it evenly among all of the flasks.
6. The next day remove old media and feed with 5 ml of fresh hBE Growth media every other day for until flasks are 80-90% confluent.

Expanding cells into T75 cm^2 Flasks:

1. Coat T75 cm^2 flasks with 5 ml of 3T3 conditioned media [usually can make four T75 cm^2 flasks per T25 cm^2 flask] for at least 4 hours in 37ºC CO_2 incubator.
2. Remove conditioned media from the T75 cm^2 flasks, add 15 ml of HBE Growth media, and place back in 37ºC CO_2 incubator.
3. Washflasks containing cells with 5 or 10 ml of warm PBS (T25/T75) and then remove PBS from flask.
4. Add 2 or 5 ml of cold Versene to flask (T25/T75) and allow to sit in the tissue culture hood for 10-20 minutes.
5. Remove Versene from the flask and then add 3.3 or 10 ml of Accutase (T25/T75). Place flask in 37ºC CO_2 incubator for 10 minutes or until cells have detached.
6. Remove the cell suspension from the flask and place in a 15 ml conical tube. Wash the flask with 3 or 5 ml (T25/T75) of HBE Growth Media. Collect and add to the 15 ml conical tube with the cell suspension.
7. Spin the tube containing the cell suspension at 1,000 rpm for 5 minutes at 24ºC.
8. Carefully remove the supernatant. Add 2 ml of HBE Growth media per flask to the conical tube and resuspend pellet.
9. Count cells and add appropriate volume of cell suspension to the T75 cm^2 flasks to seed at 4.95 x10^5 cells/T75cm^2 flask [6.6 x 10^3 cells/cm^2]. If there is any cell suspension left divide it evenly among the four flasks.
10. Feed the flasks every other day until the flasks are 80-90% confluent.

Making Filters from T75cm^2 Flasks:

1. Coat filters with 3T3 conditioned media for at least 4 hours in 37ºC CO_2 incubator:
    - For HTS 24 well Transwell plates (Corning, cat# 3378) and Transwell filters (Corning, cat # 3470) add 100 µl conditioned media to top of the filter [and 10ml of conditioned media to the bottom reservoir – optional].
    - For Snapwell filters (Corning, cat# 3801) add 200 µl of conditioned media to the top of the filter [and 1 ml conditioned media to the bottom well – optional]

2. Remove conditioned media from the filters and place back in the 37ºC CO_2 incubator.
3. Wash the T75 cm^2 flask with 15 ml of warm PBS and then remove the PBS from the flask.
4. Add 5 ml of cold Versene to the T75 cm^2 flask (2 ml/T25) and allow to sit in the tissue culture hood for 10-20 minutes.
5. Remove the Versene from the flask and add 10 ml of Accutase. Place the flask in the 37ºC CO_2 incubator for 10 minutes or until cells have detached.
6. Add 5 ml of HBE Growth (BEGM or alternative) media to each T75 flask. Collect the cell suspension from flasks and place in a 50 ml conical tube.
7. Spin the tube containing the cell suspension at 1,000 rpm for 5 minutes at 24ºC.
8. Carefully remove the supernatant. Add 3 ml of HBE Growth media per flask to each conical tube and resuspend pellet. Collect all cells in a 50 ml Falcon tube.
9. Count cells and add appropriate volume of cell suspension to the top of the filters
    - For HTS 24 well Transwell plates and Transwell filters add enough to seed at 1.7 $x10^5$ cells/filter.
    - For Snapwell Filters add enough to seed at 5.0 $x10^5$ cells/filter.
10. Add HBE Growth media to bottom well of filter plate:
    - For HTS 24 well Transwell reservoir plates add 20 ml or 0.75 ml per well in 24 well plate.
    - For Snapwell filters add 2 ml.
12. Place back in 37ºC CO_2 incubator for 24 hours.
13. After 24 hours switch cells into HBE Differentiation media with Pen-Strep Amphotericin (PSA) (when aspirating media remove the media from bottom well first and then the top of the filter):
    - For HTS 24 well Transwell plates add 100 µl of media to top of filter first and then 20 ml of media to the bottom well.
    - For Snapwell filters add 200 µl of media to top of filter first and then 2 ml of media to the bottom well.
13. Feed every other day with HBE Differentiation media with PSA, after 96 hours (4 days) switch the filters to an air/liquid interface (remove media from the top of the filter and only replace media on the bottom well).
14. Feed every other day, after 3 weeks at air/liquid interface switch to HBE Differentiation media with Pen-Strep only.
15. Filters are ready to use after 4 weeks at air/liquid interface.