NOVABIOSIS

Primary Human Lung Epithelial Cell Culture Protocol (NhTE-P0, NhBE-P0, NhSAE-P0, NhTE-P1, NhBE-P1, NhSAE-P1)

Preparation of culture ware and media

1. Freshly isolated P0 airway epithelia thrive best on tissue culture-treated plastic that has been coated with Human Type IV Placental Collagen (also known as Bornstein and Traub Type IV Placental Collagen). This collagen provides an excellent substrate for the cells to interact with upon the initial attachment and cell propagation.

2. **Collagen Type IV stock solution (10x):** Add 50mg collagen to 83.1mL deionized water and 0.208mL glacial acetic acid together into a small glass flask with a sterile Teflon-coated stir bar. Place flask into a larger beaker with enough water to create a double boiler. Warm to 37°C. Stir slowly until collagen is dissolved. Aliquot into 10mL tubes, label, freeze at -80°C. This is the 10x stock.

3. **Collagen working solution:** Thaw 10x stock in room temperature water. In a glass bottle, dilute 1 part 10x stock with 9 parts deionized water to make 1x working collagen solution. Store 1x solution at 4°C for several weeks until used. Coat all tissue-culture plastic with a thin layer of collagen solution. Let air dry in a sterile tissue culture biosafety cabinet. Turn UV on for 10 minutes to finish drying and sterilizing the collagen layer. Cover, seal with tape, store at room temperature until used.

4. **Usage of collagen-coated tissue cultureware:** Wash stored collagen-coated tissue cultureware with PBS with Mg/Ca at least twice prior to plating any cells. *Optional:* Use any media with phenol red pH indicator to ensure all acetic acid has been washed from the cultureware. Any sudden change in pH will harm the airway epithelium and will prevent them from propagating. Avoid pH shock of the culture.

5. **Bronchial Epithelial Growth Medium:** Currently, Novabiosis does not offer a BEGM, so please select your preferred BEGM vendor. If you are adding the retinoic acid as a supplement, please keep in mind that the combined media formulation must be shielded from light and high temperatures at all times. Store at 4°C and use cold without warming to 37°C. Feeding cells with room temperature, or less, media does not harm them and is recommended as media freshness affects the cells more than the media temperature.

6. **Air/Liquid Interface (ALI) Differentiation Medium:** This medium contains retinoic acid and many recombinant growth factors, and therefore must be shielded from light and high temperatures at all times. The product must be stored at -20°C at all times when frozen. After initial thaw, the product must be stored at 4°C and use cold, without warming to 37°C. Feeding cells with room temperature, or less, media does not harm them and is recommended as media freshness affects the cells more than the media temperature. *ALI medium is good for 12 months from date of manufacturer. After thaw, the ALI media must be stored at 4°C and is good for 30 days from*

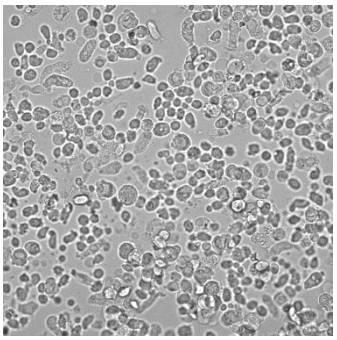
the date of thaw and provided that the product is continuously stored in a 4°C location shielded from light.

7. **Note:** Some lots of antimicrobials have negatively affected cell growth and differentiation. It is recommended to use USP grade antimicrobials when possible.

Thawing and initial plating procedure

1. Thaw cells according to standard practice. DO NOT CENTRIFUGE FRESHLY THAWED PRIMARY CELLS. Removal of the DMSO in the Freezing Media by centrifugation is not necessary. Cell loss can occur from the centrifugation step. Note: Freezing Medium contains FBS and DMSO.

2. Add to the freezing vial, 1 mL of warm, fresh -BEGM with desired antimicrobials. Add the cell suspension to a 35-mm^2 dish (or a single well of a 6-well plate) and add 2 mL additional fresh BEGM. Freshly isolated P0 epithelia contain ciliated and non-ciliated cells and can present as either single cells or small clumps as pictured below (30x magnification).



3. Place cells into the incubator (37°C, 5% CO_2). This is Growth Day 0.

4. On Growth Day 1, draw the remaining floating cells and media into a 22 gauge needle/syringe and aspirate back onto the same dish. This breaks up clumps of cells. Supplement the 35-mm^2 dish (or a single well of a 6-well plate) with 1 mL fresh BEGM with antibiotics.

5. On Growth Day 2, aspirate all remaining cells and replace the media with 2-3 mL fresh BEGM with antibiotics.

6. Replace the media every other day until obvious cell propagation. With freshly isolated P0 epithelia, this process can typically take 7-14 days and is dependent on initial cell attachment.

7. When cells are 75% confluent, detach all cells with Accutase (as below) and expand the culture into a larger but appropriately sized flask (e.g., a T25-flask). Primary cells do not thrive well with low seeding densities.

8. Continue propagation until enough cells are produced for differentiation. Propagation and differentiation conditions are subject to empirical testing by each investigator.

Propagation and plating

1. Wash T75-flask containing cells with 5 mL PBS without calcium and magnesium (PBS -/-). Aspirate.

2. Incubate T75 with 10 mL PBS -/- supplemented with 1 mM EDTA for 3-7 minutes at 37°C. Aspirate.

3. Add 5 mL fresh, room temperature Accutase dissociative to each T75. Let sit at room temp for 3-10 minutes. Check every 3 minutes by microscope for rounding of cells. Do not tap flask until cells are well-rounded.

4. Place a sterile 100 micron Cell Strainer (filter) into a new sterile 50 mL conical tube.

5. Detach primary epithelia by mechanical dislodgement. Place cells solution into a sterile 50 mL conical tube without the filter. Place all flasks of the same primary cell line into the same tube.

6. Pipette cell solution a few times to help break up the cell clumps.

7. Apply the cell solution to the filter to strain out the clumps. If done correctly, few clumps should remain. If the flask was over-confluent or if the EDTA and/or Accutase was left on too long, cells form large clumps and will not pass through the filter. Obtaining a near single-cell suspension is helpful for achieving a monolayer of cells on permeable support filters.

8. Wash used flask with 5 mL media (DMEM/F-12 Media is preferred). Pass wash through the same Cell Strainer as the primary cells in the same 50 mL conical. Discard the T75-flask.

9. Wash filter with 5 mL fresh media.

10. Centrifuge cell suspension at 300xg for 10 minutes.

11. Resuspend in 3-5 mL BEGM for propagation on collagen-coated tissue culture plastic, including permeable support filters. Cells plated onto permeable filters should be submerged and have media placed on both sides of the permeable filter to enable a liquid-liquid interface culture.

12. Suggested plating densities for cells at liquid-liquid interface (LLI) as follows, for all uses:
    a. 500,000 cells per T75-flask (75 cm_2)
    b. 100,000 cells per 24-well Transwell (0.33 cm_2)
    c. 250,000 cells per 12-well Transwell (1.12 cm_2)
    d. 250,000 cells per 6-well Snapwell (1.12 cm_2)
    e. 1,000,00 cells per 6 well Transwell (2.4 cm_2)

Differentiation into a mucociliary culture

1. The day of plating is Differentiation Day 0. The day after plating is Differentiation Day 1. The day of air-liquid interface (ALI) culture until ALI Day 6 is Week 0. Seven days after ALI is the beginning of Week 1 (1wk ALI).

2. Cells plated in BEGM on Day 0 at LLI are to be left alone until Day 2.

3. On Day 2, the media is changed to bilateral ALI. For some cultures, the BEGM on both sides (bilaterally) should be renewed on Day 2 due to slow growth. This requires empirical testing by the investigator.

4. On Day 4, the media is aspirated bilaterally. Only replace the basolateral media compartment with ALI. This is the initiation of ALI culture. This is ALI Day 0.

5. Cultures are to be fed basolaterally in the same manner until they are used. The ALI-D Media can support cultures for no longer than 72 hours and should be changed M/W/F of each week. Differentiated cultures should be used between ALI Days 14-28 for best practice and consistent results. Cells begin to ciliate at ALI Day 7 and continue to ciliate until ALI Day 28.