Description

The below protocol outlines the isolation and initial expansion of nasal brush samples(s). To begin, obtain a single cell suspension through brief enzymatic digestion of nasal brush sample(s) by trypsin, and expand the cell population using the EpiX™ medium. In general, primary P0 cells should be plated at an appropriate density to ensure successful initiation of culture, as a majority of the cells are differentiated cells and may not divide *in vitro*. The examples provided below are for guidance only. Variations due to donors and isolation procedures may have significant impact on the establishment of nasal cell culture from brush samples, and appropriate adjustments need to be established by the end user.

To prevent bacterial and fungi contamination, extra antibiotics (Gentamicin and Amphotericin B) are used throughout P0 and P1 cultures in the EpiX medium and PBS solutions. From P2 culture, Gentamicin and Amphotericin B can be omitted from the medium and solutions and EpiX Epithelial Cell Expansion Protocol may be followed.

Reagents required

1. Nasal brush sample(s).
2. Collagen I coated culture vessels. e.g., Corning® BioCoat™ Cellware, Collagen Type I:
    a. 96-well, Multiwell Plates (Cat # 356407)
    b. 24-well, Multiwell Plates (Cat # 356408).
    c. T-25 flask (Cat # 356484) or
    d. T-75 flask (Cat # 356485)
3. 0.05% Trypsin-EDTA, e.g., ThermoFisher Scientific, Cat# 25300-054.
4. Ca^{2+}, Mg^{2+} free PBS, e.g., ThermoFisher Scientific, Cat# 10010-023.
5. 10% (v/v) FBS in PBS. Dilute FBS (e.g., ThermoFisher Scientific Cat# 26140-079) in PBS.
6. EpiX medium kit, e.g., Propagenix Cat # 276-201.
7. Antibiotics, such as:
    a. Gentamicin, e.g., ThermoFisher Scientific, Cat # 1570-058
    b. Amphotericin B, e.g., ThermoFisher Scientific, Cat # 15290-018
    c. Pen/Strep, e.g., ThermoFisher Scientific, Cat # 15140-122

Extra antibiotics used for P0 and P1 cultures

1. Gentamicin at 20 µg/mL.
2. Amphotericin B at 2.5 µg/mL.
3. Pen/Strep at 2X.

Preparation of EpiX medium

1. Thaw the EpiX medium supplement kit at room temperature. Add the following to each bottle (500 mL) of EpiX base medium to make complete medium:
    a. 100 µL supplement A
    b. Whole contents of supplement B vial
    c. 100 µL supplement C
2. Mix thoroughly by gently swirling. Store at 2 – 8°C until needed.

Nasal brushing sample collection overview

1. No topical anesthesia is used. Ask the subject to blow their nose thoroughly after 5 – 6 sprays of a commercial normal saline nasal spray to loosen and remove as much mucus as possible before brushing.
2. The inferior surface of the middle turbinate of one nare is brushed with a standard cytology brush. The brush is inserted into the nose and 2 back-and-forth motions are made, 2 – 3 cm in length. The brush is turned a quarter-turn and the 2 motions are repeated. Two more quarter-turns and brushes are performed. The total maneuver takes about 5 seconds. The brush is extracted and the brush tip is placed in a labeled container with sterile physiologic transport media until processing. The process is repeated for the other nare.
3. Once the nasal sample is collected, put the brush in a 15 mL tube with 2~3 ml medium (DMEM or RPMI, FBS is not needed) and shake the tube vigorously for 30~40 times to release the cells from the brush. Then transfer the brush into a new tube and add extra medium to fill up both tubes. Have both tubes shipped to your lab on the same day of collections. Upon receipt, you can examine the brush and recover any cells left on it.

Dissociate nasal brush sample and initiate P0 culture:

1. Upon receiving the sample in a 15mL falcon tube, centrifuge the tube at 200 – 300 x g for 5 – 10 minutes to pellet the cells/tissues to the bottom of the tube.
2. Gently aspirate the supernatant.

3. Rinse the pellet for 2 – 3 times with 10mL PBS containing extra antibiotics using a serological pipette.
4. Centrifuge again at 200 – 300 x g for 5 – 10 minutes.
5. Gently aspirate the supernatant.
6. Add 0.5 – 1 mL of 0.05% Trypsin-EDTA, depending on the size of the pellet, and gently disrupt the pellet.
7. Incubate in 37°C water-bath for 2 minutes.
8. Observe under the microscope, if single cell suspension is achieved, stop trypsinization. If aggregates are still observed, continue trypsinization for an additional 2 minutes at room temperature as needed.
9. Using a P200 pipette, pipette 3 – 4 times to dissociate the remaining aggregates.
10. Add 4 – 5 mL of 10% FBS in PBS containing extra antibiotics to the tube and pipette 1 – 2 times.
11. Centrifuge at 200 – 300 x g for 5 minutes.
12. Gently aspirate the supernatant and add 200 – 500 µL of EpiX medium containing extra antibiotics depending on the size of the pellet.
13. Using a P200 pipette, pipette 3 – 4 times to resuspend the cells in EpiX medium.
14. Perform manual cell counts using a hemocytometer and trypan blue.
    a. Generally, we obtain 20,000 – 50,000 viable cells in total from a nasal brush sample. Most cells are differentiated cells (such as multi-ciliated cells) and may not replicate further in vitro.
15. P0 primary cells are generally plated at 50,000 cells/cm^2 to 300,000 cells/cm^2, with a median density around 100,000 cells/cm^2. Culture the cells using EpiX medium with extra antibiotics at 37°C with 5% CO_2.
    a. Initiate the P0 primary culture in one collagen-coated 96-well or one collagen-coated 24-well, depending upon the number of viable cells
16. After 24 hours, transfer the spent medium and non-attached cells from the original 96-well or 24-well into a new empty well. Observe if there is good cell attachment in the original well. Add EpiX medium containing extra antibiotics into the original 96-well or 24-well.
    a. Monitor cells transferred to new well for attachment and growth. These cells may be combined with original 96-well or 24-well at initial sub-culture if desired.
17. Change the medium every 2 – 3 days until the cells reach 80 – 90% confluence.

Sub-culture and expand P0 and P1 nasal epithelial cell culture:

1. The nasal epithelial cell culture should be split when the cells reach 80 – 90% confluence. DO NOT allow the cells to reach complete confluence, as this will induce the cells to differentiate and significantly decrease their ability for continuous replication.
2. The growth of P0 and P1 cultures generally are slow and may not result in a net increase of cell number. Growth rate will increase from P2 and onwards.
3. P0 and P1 cultures should not be expanded too aggressively. For example, if P0 culture is established in one 96-well, P1 culture could be expanded into one 24-well, and the P2 culture should be expanded into one 6-well plate. When the culture in 6-well is ready for split, it can be expanded more aggressively (see below).

Volumes used here are for one 96-well; proportionally increase the amount of media for one 24-well

1. Remove spent medium using a P200.
2. Add 100 µl PBS to the well to rinse the cells and discard the rinse solution.
3. Add 100 µl warm 0.05% Trypsin-EDTA to the well. Incubate at 37°C for 5 min to detach the cells. Pipette gently for several times across the whole well to help loosen the cells.
4. Neutralize the trypsin by adding 100 µl warm 4% FBS in PBS, and pipette up and down for several times.
5. Transfer the cell suspension into a sterile 15 mL centrifuge tube. Rinse the 96-well with an additional 200 µl 4% FBS in PBS and pool into the centrifuge tube with cells. Confirm that all cells in the 96-well are harvested. If there are still many cells in the 96-well, repeat step 2 – 4 to collect the remaining cells.
6. Centrifuge cells at 200 x g for 5 min at room temperature.
7. Aspirate most of the supernatant from the centrifuge tube containing the cell pellet. Flick the tube briefly to loosen the pellet.
8. Dilute the cells in 500 µl of warm EpiX medium containing extra antibiotics. Determine cell count and viability using a hemocytometer with Trypan Blue.
9. Seed cells into one collagen-coated 24-well (this is approximately 1:6 split). Use 0.5 mL EpiX medium with extra antibiotics.
10. Incubate the cultures at 37°C in a 5% CO_2 humidified incubator.
11. Change the growth medium every 2 – 3 days.

Sub-culture and expand P2 nasal epithelial cell culture and beyond:

Volumes used in this protocol are for one 6-well; proportionally increase the amount of media for culture vessels of other sizes.

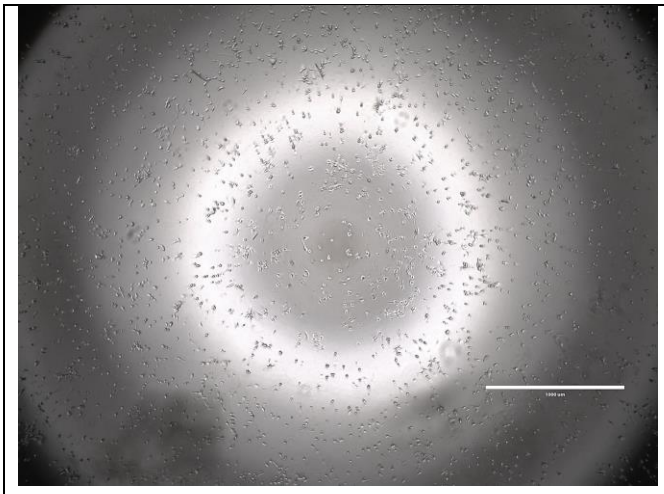
1. Remove spent medium.
2. Add 1 mL PBS to the well. Briefly rinse the cells and discard the rinse solution.

3. Add 0.5 mL warm 0.05% Trypsin-EDTA to the well. Incubate at 37°C for 5 min, or longer time if necessary, to detach the cells. Tap the plate several times against your palm to loosen the cells.
4. Neutralize the trypsin by adding 1 mL warm 4% FBS in PBS, and pipette up and down several times to break up any cell clumps into suspension.
5. Transfer the cell suspension into a sterile 15 mL centrifuge tube. Rinse the well with an additional 1 – 2 mL 4% FBS in PBS and pool into the centrifuge tube with cells.
6. Centrifuge the cells at 200 x g for 5 min at room temperature.
7. Aspirate most of the supernatant from the centrifuge tube containing the cell pellet. Flick the tube briefly to loosen the pellet.
8. Dilute the cells in 1 mL of warm EpiX medium. Determine cell count and viability using an automated cell counter such as Countess II, or a hemocytometer using Trypan Blue.
9. Use the following equation to determine the number of population doubling.

$$PD = 3.32 \quad x \quad \log\left(\frac{[total\ cell\ number\ at\ harvest]}{[total\ viable\ cell\ number\ seeded\ at\ the\ beginning]}\right)$$

10. Seed cells into a new subculture vessel(s) at a density of 2,000 – 4,000 cells/cm2. Mix desired number of nasal epithelial cells in the EpiX medium to ensure a uniform suspension, and dispense the calculated volume into the subculture flasks.
11. Incubate the cultures at 37°C in a 5% CO_2 humidified incubator.
12. Change the growth medium every 2 – 3 days. As the cells become more confluent, increase the volume of media as follows: under 50% confluence feed 1 mL per 5 cm^2; over 50% confluence feed 1.5 mL per 5 cm^2.

Example of nasal epithelial cell culture:



Day 2 culture of nasal epithelial cells in EpiX medium, passage 0 seeded at 86,000 cells/cm^2. Photo is taken at 4X.

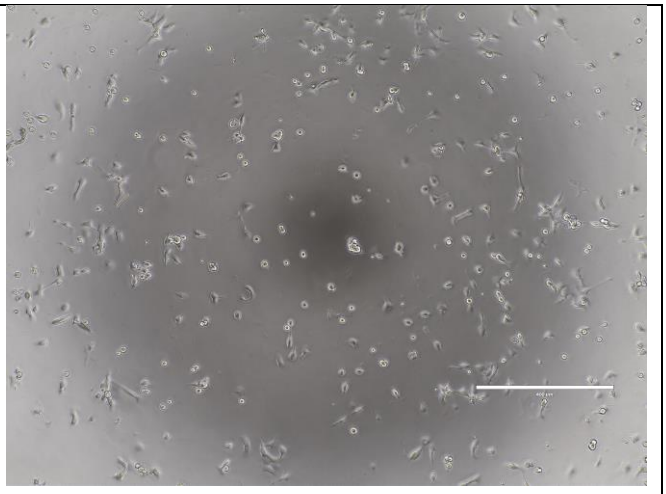
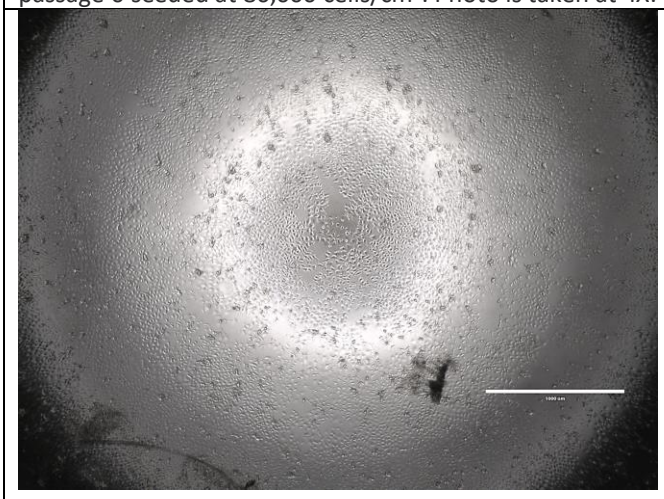


Image of the cell culture to the left at 10X.



Day 4 culture of nasal epithelial cells in EpiX medium, passage 2 seeded at 3,000 cells/cm^2. Photo is taken at 4X. The cells should be passaged.

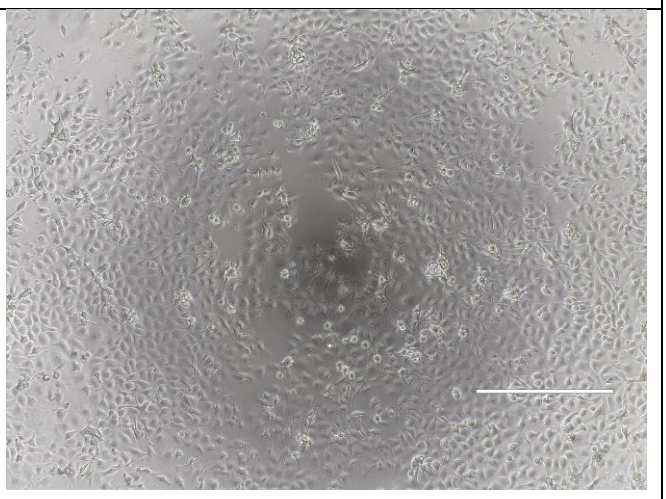
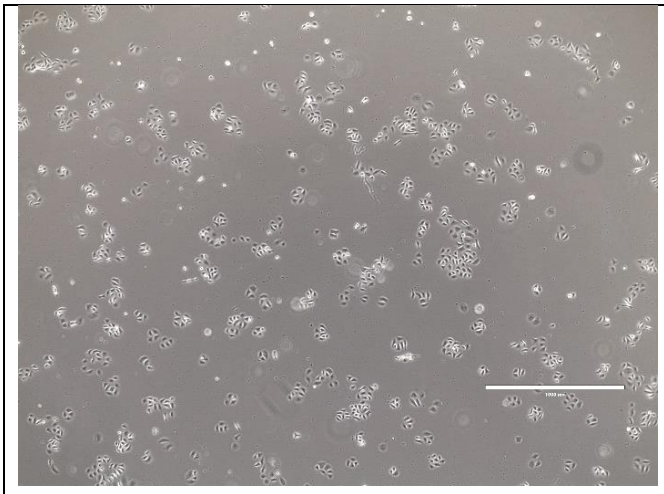


Image of the cell culture to the left at 10X.

Example of nasal epithelial cell culture:



Day 2 culture of nasal epithelial cells in EpiX medium, passage 2 seeded at 3,000 cells/cm^2. Photo is taken at 4X.

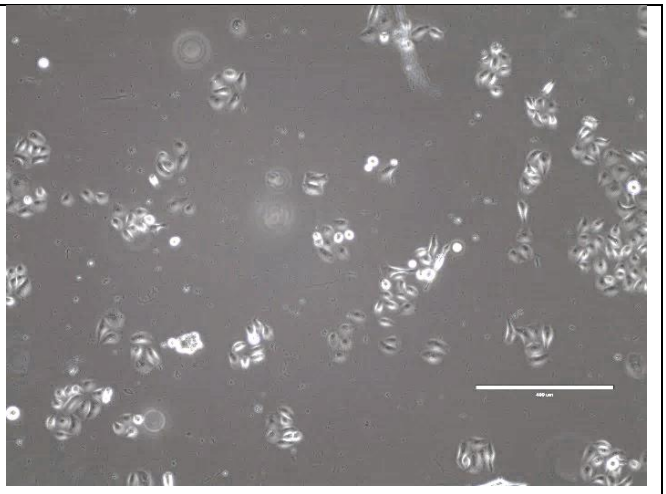
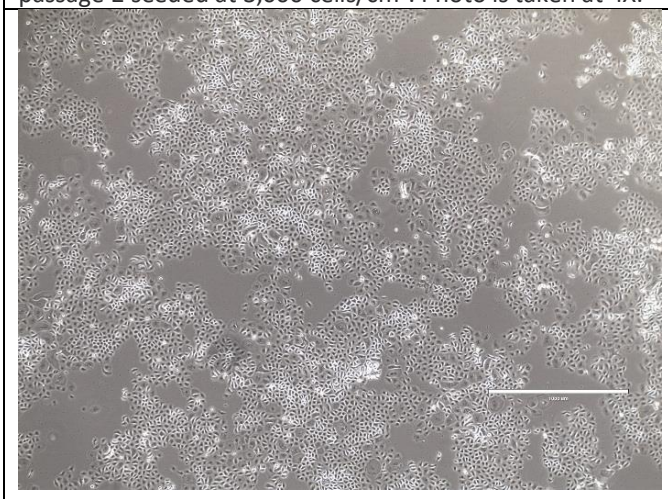


Image of the cell culture to the left at 10X.



Day 4 culture of nasal epithelial cells in EpiX medium, passage 2 seeded at 3,000 cells/cm^2. Photo is taken at 4X. The cells should be passaged.

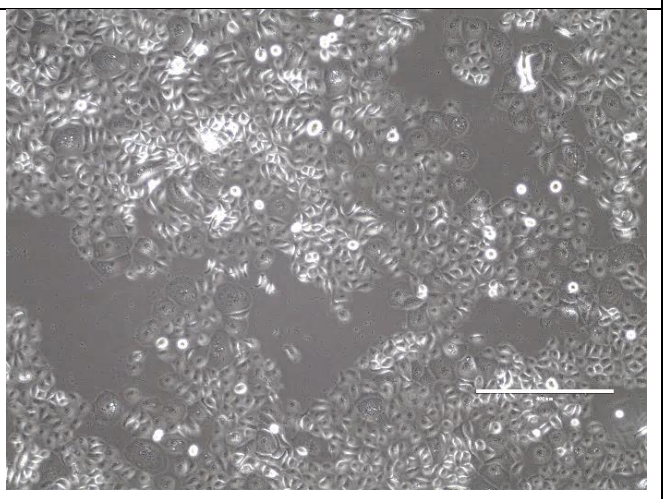


Image of the cell culture to the left at 10X.