Establishing Differentiated Air-Liquid Interface Primary Human Bronchial Epithelial Cell Cultures

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Method Article

Keywords: air-liquid interface, ALI, pHBEC, primary, human, bronchial, epithelial

Posted Date: March 16th, 2021

DOI: https://doi.org/10.21203/rs.3.pex-1380/v1

Abstract

This protocol describes the establishment and maintenance of differentiated primary human bronchial epithelial cell (pHBEC) cultures from primary bronchial epithelial cells using Lonza and Gibco media.

Note: This is a historical protocol. At the time of publication, this protocol has been superseded by a different version in the McCullough lab; however, it is being published to support the transparency and reproducibility of other studies by which it is referenced.

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Introduction

Reagents

Primary bronchial epithelial cells obtained by brush biopsy or purchased from various commercial companies: Lonza, Cell Applications, etc.

Differentiated cell culture medium (recipe below)

Trypsin, 0.25% with EDTA (any brand)

Dulbecco's phosphate buffered saline ("DPBS", Gibco #14190-144)

Soybean trypsin inhibitor, SBTI (Type II crude powder, Sigma #T9128) 1 mg/mL in DPBS, sterile filtered.

All-trans Retinol (Sigma #R7632) (recipe below)

Equipment

Laminar flow hood (any manufacturer) Biosafety Level II equipped with vacuum connection.

Tissue culture incubator capable of maintaining 37 °C and 5% CO_2 (any manufacturer)

Olympus CK2 (or comparable) inverted microscope with 4x, 10x and 20x objectives

Transwell-Clear filters (Corning): Membranes must have the 0.4 mm pore size. Larger pore sizes have proven unsuccessful.

· 24 mm insert (6-well format), Corning #3450

· 12 mm insert (12-well format), Corning #3460

· 6.5 mm insert (24-well format), Corning #3470

Sterile glass and plastic pipets and pipet tips, tissue culture cluster plates and flasks.

Procedure

Human bronchial epithelial cells are grown in culture as per the method “Culture of Primary Human Tracheobronchial Epithelial Cells.”

Prepare reagents prior to starting.

Air Liquid Interface (ALI) Medium:

· 250 mL DMEM-H (Gibco #11995-065)

· 250 mL BEBM (Lonza #CC-3171)

· 1 complete BEGM bullet kit (Lonza #CC-4175)

· 1 additional Bovine Pituitary Extract (BPE) singlequot, approximately 25 mg. (Gibco #13028-014)

· 1.5 mg/mL BSA (Make a 10 mg/mL solution in sterile water: store at -20 °C) Add 75 µL of the stock BSA (Sigma #A9418) solution to 500 mL medium.

· 1mL aliquot of nystatin (Sigma #N1638, 20 U/mL)

· Sterile filter the above medium, over 0.2 mm pore (Nalgene #156-4020 or equivalent).

BEGM medium (Lonza #CC-3170)

All-trans Retinol (Sigma #R7632)

· All-trans retinol (RE) has a coefficient of extinction of 52480 in ethanol at 325 nm. Dissolve 25 mg in 0.75 mL absolute ethanol.

· Dilute a small amount 1:10,000 in absolute ethanol. Read on the UV spectrophotometer in a quartz cuvette. Divide the absorbance by the extinction coefficient then multiply by the dilution factor. This will

give you the molarity of the stock solution.

· While growing ALI cultures, prepare the retinol stock solution monthly.

· Make a 10 mM working solution in ethanol from the above stock solution. Store both solutions at -80 °C.

As needed: Dilute 5 µL of 10 mM retinol (RE) solution in 1 mL of ALI medium. This yields a 50 µM solution. Use this to make the appropriate RE concentration in ALI medium to feed the cells.

Setting up and maintaining the cell cultures.

Day 1:

1. Plate bronchial epithelial cells on Transwell-clear filters.

2. Determine the number of wells you are going to plate, based on a seeding density suggested below. These suggestions are for passage 3 cells. Passage 2 cells may be plated less densely.

3. Plating procedures:

Plating densities and medium volumes are listed in Table 1.

a. Warm media, trypsin-EDTA and SBTI.

b. Trypsinize cells from stock culture vessels as described in the protocol for culturing primary/NHBE cells.

c. Cells are resuspended in ALI medium without retinol and added to the apical compartment of the Transwell-clear insert. BEGM is used to feed the cells from the basolateral compartment.

Day 2:

· Apical ALI medium is replaced to remove any cells that have not adhered to the filter. The basolateral medium is also replaced with fresh BEGM.

Day 4:

· If cells are not confluent:

o Replace both apical and basolateral medium as indicated in Day 2 above.

· If the cells are confluent (cover the entire filter membrane):

o Add medium with 500 nM retinoic acid to both compartments (use ALI medium in the apical compartment and BEGM in the basolateral compartment) and incubate cells for 48 hours.

o A working solution of RE is prepared fresh from the 10mm frozen stock as described earlier.

o Take as much medium as you will need to feed the cells on that day, and add the appropriate amount of RE.

Day 5 or 7:

· The air-liquid interface is established 48 hours after the addition of medium with RE. ALI is established by removing the apical medium and replacing only the basolateral medium, supplemented with 100 nM RE. At this point, the medium used to feed the cultures and to prepare the 100 nM RE is the ALI medium. This is day 0 at ALI.

Basolateral medium, supplemented with 100 nM RE, is replaced every 48 hours until a Monday/Wednesday/Friday feeding pattern can be established. Periodically examine the cultures with the microscope to assess the health of the culture and to follow progression of cilia formation.

Before replacing the medium, 1 mL of DPBS is added to the apical compartment of each Transwell insert. Carefully rinse the surface of the filter by gently swirling the plate or using a 1ml pipet tip to wash the surface. Remove both the apical rinse medium and the basolateral medium, then replace the basolateral medium. Periodically examine the cultures with the microscope to assess the health of the culture. Apical mucin secretions may appear and can be removed by this rinse. It also makes it easier to see the ciliary motion if the cells have just been rinsed.

Day 8/10/12/*et cetera* post start of ALI:

Examine the cultures for the first signs of ciliation. Apical secretions (mucin) may also be observed. Cells have usually achieved uniform differentiation by day 24; this is the standard point to begin exposing cells to desired pollutants or treatments.

The lifetime of the culture will vary with the cell donors. Other factors will also influence the length of time you are able to maintain the differentiated culture. These include the age of the ALI medium, the freshness of the RE solution, the gentleness with which you have handled the inserts during rinsing, etc.

Quality control rationale

The health of the cells can be determined visually using an inverted microscope. Cultures showing signs of bacterial or fungal contamination should be immediately removed from use and disposed of properly. Cells are maintained in a water-jacketed CO_2 incubator; the concentration of CO_2 should be checked periodically (at least once per week) using a Fyrite test kit. The temperature within the chambers should also be monitored with a thermometer. Incubators are periodically (at least every two weeks) cleaned with anti-bacterial/antifungal agents. An antifungal agent (benzalkonium chloride) is added to the internal water trays to deter growth of contaminants.

References

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Figures

Filter Size	Surface Area	Seeding Density	Apical Volume	Basolateral Volume
6.5 mm	0.33 cm^2	$\geq 0.5 \times 10^5$ cells/well	0.2 mL	0.5 mL
12 mm	1.00 cm^2	$\geq 1.0 \times 10^5$ cells/well	0.5 mL	1.0 mL
24 mm	4.79 cm^2	$\geq 2.0\text{-}5.0 \times 10^5$ cells/well	0.75 mL	2.0 mL

Figure 1

Cell seeding densities and medium volumes.

Supplementary Files

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- McCulloughMethodsALICultureofpHBEC.docx