Culture and Differentiation of Primary Human Tracheobronchial Epithelial Cells Using STEMCELL Technologies Pneumacult Media

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Method Article

Keywords: pHBEC, primary bronchial epithelial cells, penumacult, stemcell, air-liquid interface, ALI, differenitation

Posted Date: November 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.pex-1674/v1

Abstract

NOTE: A PDF methods document is attached in the supplementary materials.

SCOPE OF APPLICATION (LIMITATIONS)

This method describes the culture and differentiation of primary human tracheobronchial epithelial cells (pHBEC). Cells used for this method can be obtained by brush biopsy during clinical bronchoscopy or purchased commercially. This method replaces the previous version described in (Dailey and McCullough, 2021a; b).

Introduction

Reagents

Supplies

· Primary bronchial epithelial cells obtained by brush biopsy or commercially.

NOTE: Companies will supply vessels already plated with cells, or cryopreserved cells. Follow supplier's instructions on how to handle plated cells, which will then go into an incubator. Upon receiving frozen cells, make sure contents are still frozen. Store cells immediately in liquid nitrogen.

· Pneumacult Expansion-Plus (STEM CELL #05040) which is comprised of basal media (#05041) with 50X Supplements (#05042) and hydrocortisone (#07925). Follow manufacturer's instructions for storage and preparation.

· Pneumacult ALI medium (STEM CELL #05001) which is comprised of ALI basal media (#05002) with 10X ALI Supplements (#05003), ALI maintenance supplement (#05006). Hydrocortisone (#07925) and heparin (cat# 07980) are also required and are sold separately from the medium kit. Follow manufacturer's instructions for preparation and storage of individual components

· CryoStor CS10 (STEMCELL #07930): 10% DMSO in defined cryopreservation medium.

· Trypsin, 0.05% with EDTA (any brand) and Soybean trypsin inhibitor, SBTI (Sigma T-6522) 1mg/ml in PBS, sterile filtered

· ACF enzymatic dissociation and inhibition kit (STEMCELL #05426)

· Disposable vacuum filtration bottles (0.22 mm pore; Corning #430796)

· Tissue culture treated plasticware:

o 75 cm^2 tissue culture flasks (T75) (any brand)

o Multi-well tissue culture plates (any brand)

o Transwell inserts w/0.4 mm pores

§ 24 mm (Corning #3450)

§ 12 mm (Corning #3460)

§ 6.5 mm (Corning #3470)

· Sterile glass Pasteur pipets

· Sterile individually wrapped serological pipets

· Sterile filtered pipet tips

· Sterile polypropylene centrifuge tubes

· Portable pipet-men, single and multi-channel pipettors, repeat pipettors and appropriate tips

Equipment

Equipment

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

· Humidified tissue culture incubator at 37 °C and 5% CO_2 (any manufacturer)

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

· Light microscope for cell counting (any brand)

· Hemocytometer

Procedure

PROCEDURE

This protocol describes how to expand and cryopreserve pHBEC, as well as seed and differentiate pHBEC at air-liquid interface on Corning Transwell inserts.

Prepare Pneumacult Ex-Plus Growth Medium

1. Thaw the 50X supplement at room temperature. Mix by gently inverting the bottle.

2. Add the contents of the 50X supplement to a bottle of Ex-Plus Basal medium.

3. Add 0.5 mL of hydrocortisone stock solution to the above supplement-containing medium.

4. Sterile filter with a 0.22 mm vacuum filtration bottle and store at 4 °C for up to one month.

Prepare Pneumacult ALI Differentiation Medium

1. Prepare complete base medium by adding 1 bottle of 10X Supplement (thawed overnight at 4 °C) to a bottle of ALI base medium.

2. Prepare maintenance medium as follows: starting with 100 mL of complete base medium, add 1 vial of 100X Maintenance supplement (1 mL), 0.2 mL of heparin solution, and 0.5 mL of hydrocortisone stock solution. Sterile filter with a 0.22 mm vacuum filtration bottle and store at 4 °C for up to 2 weeks.

3. Prepare the required volume of ALI maintenance medium by adjusting the above volumes accordingly.

NOTE: Prepare aliquots of ALI media to avoid repeated warming and cooling.

Thawing Cryopreserved Cells

1. Warm Pneumacult Ex-Plus growth medium to 37 °C and prepare materials prior to obtaining a vial of cells from cryostorage.

2. Remove vial from liquid nitrogen (wearing eye protection) and thaw in 37 °C water bath (1-2 min).

a. It is important that cells are thawed quickly to reduce the duration of exposure to the high concentration of DMSO in the freezing medium, which will negatively impact cell viability.

3. Add 24 mL of growth medium to a 50 mL conical tube then transfer the thawed cells from the cryovial into the growth medium using a P1000 pipette and gently mix by inverting 10 times.

NOTE: do not vortex

4. Pellet cells via centrifugation at 1,000 x *g* for 4 minutes at room temperature in centrifuge with a swinging bucket rotor.

5. Carefully aspirate the supernatant.

6. Gently resuspend the cells in pre-warmed growth medium and transfer to a flask. Distribute cell suspension on the dish through a combination of gentle rocking and swirling of the dish for approximately 15 seconds. Some investigators prefer to thaw cells into a collagen coated vessel, although this is not necessary. All subsequent passages use non-coated vessels.

NOTE: If a vial containing $1.0x10^6$ cells is thawed, then the cells should be plated in a 75 cm^2 flask.

7. Place in a tissue culture incubator overnight.

8. Check cells the following day for attachment to the dish. Aspirate and replace medium.

Passaging and Expansion of pHBEC

It is recommended to passage the cells when they are >50-70% confluent.

1. Pre-warm Pneumacult ALI growth medium and DPBS in a 37 °C water bath and a trypsin aliquot at room temperature for approximately 30 minutes.

2. Aspirate growth medium from flasks to be passaged. Cells may be rinsed once with DPBS but this is not required.

3. Add trypsin-EDTA solution and place in a tissue culture incubator for 2-3 minutes.

a. 500 mL/well of 12 well plate.

b. 4.0 mL/T75 flask.

4. When approximately 80-90% of the cells are detached (which can be facilitated by tapping sides and bottom of the flask), add a half volume of SBTI to the flask, pipet up the trypsin/SBTI mixture, and rinse the vessel bottom. This aids mixing of the trypsin and SBTI, and helps remove remaining adherent cells.

5. Transfer the cells to a sterile centrifuge tube.

NOTE: Cells from the same donor source plated over individual wells or flasks can be combined.

6. Pellet cells at 200-600 x *g* for 5 minutes at room temperature in a centrifuge with a swinging bucket rotor.

7. Aspirate the supernatant. Note the size of the cell pellet before tapping the tube to break up the pellet. Ideally, the pellet should be resuspended in a volume of medium that will yield a cell density of $1.0x10^6$ cells/mL. Triturate the cells with a serological pipet to create a homogeneous suspension.

NOTE: With experience, you will be able to judge this volume based on the pellet size. For a barely visible pellet, start with 1 mL medium; for a pellet of 1 cm, start with 10ml medium (generally, resuspension volumes will range from 5-10 mL).

8. Transfer a 10 mL aliquot of the cell suspension to a hemocytometer and count the cells. Viability can be determined, if desired, by diluting a sample of the cells with an equal volume of trypan blue.

9. Adjust the number of cells to approximately $1.0x10^6$/mL. If the cell density is less than $1.0x10^6$/mL, re-pellet the cells and suspend in the appropriate volume. If the cells density is greater than $1.0x10^6$/mL, dilute the cell suspension with the appropriate volume of medium.

10. Plating density will be based on how quickly you want the cells to be ready for subsequent passage/plating. Cells are typically seeded at 0.5 x 10^4 cells/cm^2 but can be seeded more heavily if desired.

11. Change the medium every other day until cells reach approximately 70% confluence. Denser cultures may require more frequent medium replacement.

12. Upon approaching confluence cells can be:

a. Passaged further for culture (seeding densities and volumes in table below)

b. Plated on Transwell inserts for ALI differentiation (seeding densities and volumes in table below)

c. Cryopreserved (procedure described below

Refer to Tables 1A and B for Suggested Cell Seeding Densities/Volumes in Flasks and Multi-Well Plates.

Cells should be used at 90-100% confluence for experiments.

Differentiation of pHBEC Cultures at ALI

1. Trypsinize and collect cells from expansion flasks as described in steps #1-9 of the "Passaging and Expansion of pHBEC" section above.

2. Starting with a cell density of 1.0x10^6 cells/mL, prepare a volume and dilution of cell suspension according to the size and number of Transwell inserts needed based on the seeding density and volume table below.

NOTE: Prepare a volume of diluted cell suspension that is greater than the exact amount needed for the desired plating to account for volume loss and/or slight variations in pipetting.

Example: If seeding 12 24mm inserts at 3.0x10^5 cells per insert then prepare sufficient cell suspension for seeding 14 inserts to allow for volume lost during handling.

14 x 0.75 ml = 10.5 mL final volume.

14 inserts x $3.0x10^5$ cells/insert = $4.2x10^6$ cells needed

If your cells were resuspended at a concentration of $1.0x10^6$/mL, then you would add 4.2 mL of the cell suspension to 6.3 mL of additional medium to get the desired plating density ($3.0x10^5$/0.75 mL added to each insert).

3. Allow cells to adhere to Transwell inserts for 16-24 hours. After the adhesion period, aspirate medium to remove nonadherent cells and add fresh Pneumacult Ex-Plus growth medium in the apical compartment. Continue to grow in Pneumacult Ex-Plus growth medium until the cells are confluent.

4. Once cells are confluent, withdraw the apical medium and replace the basolateral medium with Pneumacult ALI differentiation medium.

5. Replace the Pneumacult ALI differentiation medium in the basolateral compartment every 48 hours.

NOTE: Cilia will become visible in as little as 7 days. Cells will continue to differentiate and the majority of culture surface will become ciliated. Washing the apical surface with DPBS at least once per week is recommended.

Determine the number of inserts to be seeded and adjust cell density and volume as outlined in the table below. STEMCELL recommends 0.5 x 10^5 cells/cm^2. We have seen the best results with cells seeded on the membranes at passage 2 and 3, but we have obtained highly ciliated cultures from differention of passage 5 cells.

Refer to Table 2 for Suggested Cell Seeding Densities/Volumes for Corning Transwell Inserts.

Cryopreservation of pHBEC

1. Trypsinize and collect cells from expansion flasks as described in steps #1-8 of the “Passaging and Expansion of pHBEC” section above.

2. Pellet cells again at 200-600 x *g* for 4 minutes at room temperature in centrifuge with a swinging bucket rotor.

3. Resuspend the pellet in CryStor10 Freezing Medium at a minimum of $1.0x10^6$ cells/mL.

4. Gently mix by pipetting. Transfer 1.0 mL of the cell suspension to each cryovial.

5. Place cryovials in a Mr. Frosty freezing container (filled to indicated line with isopropyl alcohol) and place in a -80 °C freezer overnight.

a. **NOTE:** Isopropanol should be changed after every three freezing cycles.

b. **NOTE:** Do not leave vials at -80 °C longer than overnight as it will impact viability after thawing.

Transfer the frozen vials in liquid nitrogen storage.

Figures

Cluster Plates	Effective Growth Area (cm^2)	Minimum Seeding Density/Well	Volume of Medium/ Well
96 well plates	0.32	$1.6x10^4$ cells	0.2 mL
48 well plates	0.75	$3.75x10^4$ cells	0.5 mL
24 well plates	2.0	$1.0x10^5$ cells	0.5-1.0 mL
12 well plates	3.8	$1.9x10^5$ cells	1.0-2.0 mL
6 well plates	9.6	$5.0x10^5$ cells	3.0-5.0 mL

Figure 1

Table 1A. Suggested Cell Seeding Densities/Volumes in Multi-Well Plates

Flasks	Growth Area (cm^2)	Minimum Seeding Density	Typical Seeding Density Used	Volume of Medium
T-25	25	$6.25x10^5$	$1.0\text{-}3.0x10^5$	5 mL
T-75	75	$1.86x10^5$	$3.0\text{-}5.0x10^6$	15 mL

Figure 2

Table 1B. Suggested Cell Seeding Densities/Volumes in Flasks

Transwell size	Growth Area (cm^2)	Seeding Density	Apical Volume	Basolateral Volume
6.5 mm	0.33	$3.0\text{-}5.0 \times 10^4$	0.1 mL	0.7 mL
12 mm	1.12	$0.75\text{-}1.0 \times 10^5$	0.25 mL	1.0 mL
24 mm	4.67	$2.0\text{-}5.0 \times 10^5$	0.75 mL	2.0 mL

Figure 3

Table 2. Suggested Cell Seeding Densities/Volumes for Corning Transwell Inserts

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 2021CultureandDifferentiationofpHBECinPneumacultMediaV1.pdf