

Culture of Primary Human Tracheobronchial Epithelial Cells

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Method Article

Keywords: primary, bronchial, tracheobronchial, epithelial, culture

Posted Date: March 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.pex-1379/v1>

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Abstract

This protocol is intended for culture of primary human tracheobronchial epithelial cells (pHBEC) obtained by brush biopsy during clinical bronchoscopy, or purchased commercially, using Lonza-based medium.

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 At the time of publication, this protocol has been superseded by a different version in the McCullough lab; however, it is being published to support the transparency and reproducibility of other studies by which it is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

Disclaimer: The information presented here has been reviewed and approved for publication by the US Environmental Protection Agency do not necessarily represent Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendations for use.

Introduction

Reagents

Primary bronchial epithelial cells obtained by brush biopsy

pHBEC, cryopreserved

BEBM media (Lonza #CC-3171)

BEGM bullet kit (Lonza #CC-3170)

Growth Supplements (Lonza #CC-4175)

Trypsin, 0.25% with EDTA (any brand)

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

Soybean trypsin inhibitor, SBTI (Sigma #T9128) 1mg/ml in DPBS, sterile filtered.

Cell Freezing Medium: 10% fetal bovine serum and 10% DMSO in growth medium.

Tissue culture treated plastic ware: 75 cm² tissue culture flasks (T75) (any brand), multi-well tissue culture plates (any brand); transwell inserts with 0.4 micron pores.

Sterile Pasteur pipets and plastic serological pipets; filtered pipet tips, polypropylene centrifuge tubes.

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

Equipment

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

Tissue culture incubator capable of maintaining 37 °C and 5% CO₂ (any manufacturer)

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

Light microscope for cell counting (any brand)

Hemocytometer for cell counts

Procedure

Thawing Cells

DAY 1:

1. If not already prepared, make BEGM by thawing growth supplements and adding to the basal medium and sterile filter (0.22 um pore).
2. Generally, one vial of cryopreserved cells is thawed into one T75 flask.
3. Add 18 mL of growth medium to the flask and pre-equilibrate in the incubator for approximately 30 minutes.
4. To thaw cells, place the vial(s) in a 37 °C water bath. Swirl frequently to check if the contents have thawed. It usually takes less than three minutes. Upon thawing, spray the vial with 70% ethanol to reduce surface contaminants.
5. Using aseptic technique, pipet the vial contents up and down to resuspend the cells. Add cells to the pre-equilibrated T75 flask.
6. Alternatively, instead of adding thawed cells directly to the flask, you can add the cells to a tube of pre-equilibrated medium, centrifuge cells at 1000 RPM for five minutes, resuspend the pellet in fresh medium then add to the flask. This removes the DMSO in which the cells were cryopreserved. Place flasks in a CO₂ incubator (5% CO₂, 37 °C).

OPTIONAL : Check cell viability by adding trypan blue to some cells on a microscope slide. About 50% or more of the cells should exclude the dye.

DAY 2:

1. Check cultures under the microscope to determine if cells have attached to the plastic.
 - a. Any floating cells did not survive the freeze/thaw process.
2. Aspirate the media and replace with 15 mL of fresh BEGM.
 - a. This will remove the dead cells and any left-over DMSO if cells were added directly to the flask without a prior rinse.
3. Place flasks back into the incubator.

DAY 4 and LATER:

1. Aspirate media and replace with 18 mL of BEGM.
2. Replace media every two days.
3. When cells reach approximately 80-100% confluence, passage cells into more T75 flasks.

Passage Protocol:

1. Aspirate off media.
2. Add 4 mL of trypsin-EDTA solution (room temperature) to each T75 flask. Incubate at 37 °C for 2-3 minutes.
3. 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 flask bottom.
 - a. This aids mixing of the trypsin and SBTI, and helps remove some of the still adherent cells.
4. Transfer the cells to a sterile centrifuge tube. Cells from individual flasks can be combined.
5. Centrifuge cells at 1000 RPM for five minutes at room temperature.
6. Aspirate off the supernatant. Agitate the pellet by tapping to break up the pellet. Add 10 mL BEGM, and vigorously and quickly pipet the cells to create a homogeneous suspension.
7. Transfer a 10 μ L aliquot to a hemocytometer and count the cells. A confluent T75 flask typically has 2-4 million cells.
8. Add 5×10^5 cells/T75 flask. Final volume of BEGM should be 18ml/flask.

- i. OPTIONAL: Additionally, some cells can be set aside for cryopreservation at this point. (See below).
- a. Culture cells as above, changing the media every two days until cells reach confluence.
- b. When flasks reach confluence: 1) cryopreserve the cells, 2) transfer to Transwells or other plastic ware for studies, and/or 3) further passage cells.
- c. After cells have been removed by trypsin, the trypsin neutralized with SBTI, and cells are counted, adjust cell density to the desired one by either adding more media and/or centrifuging and re-suspending in less media. Usually we adjust the density to 10^6 cells/ml.

For Transwells:

Determine the number of inserts to be seeded and adjust cell density and volume as outlined in the table below:

Suggested Cell Seeding Densities/Volumes are given in Table 1

Example: seed twelve 24 mm inserts at 300,000 cells per insert. Include a fudge factor so you do not run out of cells. In this case, calculate as if you were seeding 14 inserts.

Fourteen x .75 mL = 10.5 mL final volume. Fourteen x 300,000 cells = 4.2 million cells.

If your cells were resuspended at a concentration of 1 million/ml, you would add 4.2 mL of the cell suspension to 6.3 mL of additional medium to get the desired plating density. (300,000 cells/0.75 mL added to each insert).

Let cells adhere for 16-24 hours. After the adhesion period, aspirate off nonadherent cells, and then add fresh medium apically. Once the cells reach confluence, apical medium is removed and cells are fed basolaterally every other 48 hours, until used.

For Plasticware: Lonza suggests seeding cells at $1 \times 10^5/\text{cm}^2$ in 1 mL BEGM/ 5cm^2 growth area. Cells should be used at 90-100% confluence for experiments. As cells near confluence, cells may need to be fed daily as they quickly deplete the nutrients in the medium.

Suggested Cell Seeding Densities/Volumes are given in Table 2

pHBEC CRYOPERSERVATION:

1. Cells can be frozen using standard techniques.
2. Trypsinize the cells as usual. Determine the number of cells present and repellet.
3. Resuspend the pellet in Cell Freezing Medium (10^6 cells/1.5-2.0 mL) and pipet up and down to mix.
4. Transfer to cryovials and let sit on ice for 20 minutes.
5. Add vial in upright position to a Styrofoam rack.
6. Place cooler in a -80 °C freezer for 16-24 hours.
7. Place vial in liquid N_2 .

Notes:

pHBEC cells generally can be passaged 3-4 times before they stop proliferating.

pHBEC cells received from Lonza have been passaged once previously.

Alternatively, if buying cells commercially, you can get cells already plated in a flask; this would bypass thawing of the cells.

Quality Control

Cells should be observed microscopically to insure there is no bacterial or fungal contamination. Cells that fail to thrive (do not reach confluence) should not be used for further passaging or experimental procedures. Also, cells that show unusual morphology should also be considered carefully before proceeding further. Generally poor growth of the cells is associated with aging of the cells. If all culture conditions have been checked (CO_2 concentration, humidity levels, incubator temperature, etc.), it is likely that the poor growth is characteristic of the cells themselves. 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.

Troubleshooting

Time Taken

Anticipated Results

References

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Figures

Transwell size	Growth Area, cm ²	Seeding Density	Apical Volume	Basolateral Volume
6.5 mm	0.33	30-50,000	0.1 ml	0.75ml
12 mm	1.12	75-100,000	0.25 ml	1.0 ml
24 mm	4.67	200-500,000	0.75 ml	2.0ml

Figure 1

pHBEC Seeding Densities for Transwell inserts

Cluster Plates	Effective Growth Area, cm ²	Seeding Density/well	Volume of Medium/well
96 well plates	0.32	5-10,000 cells	0.2 mL
48 well plates	0.75	30,000 cells	0.5 mL
24 well plates	2.0	50,000 cells	0.5-1.0 mL
12 well plates	3.8	100,000 cells	2.0 mL
6 well plates	9.6	200-500,000 cells	3.0-5.0 mL

Figure 2

pHBEC Seeding Densities for multi-well plates.

Supplementary Files

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

- [McCulloughMethodspHBECculture.docx](#)