Culture of Primary Human Lung Microvascular Endothelial Cells

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Method Article

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Abstract

This protocol describes the thawing, culturing, and cryopreservation of primary human lung microvascular endothelial cells (pMVECs).

NOTE: There is a consolidated PDF version of this protocol in the supplementary files section below.

Introduction

Reagents

1. EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit (Lonza Cat#: CC-3202)
2. Accutase (Innovative Cell Technologies, Inc. #AT104)
3. Recovery Cell Culture Freeze Solution (ThermoFisher #12648010)
4. Ham's F-12 Nutrient Mix, GlutaMAX™ Supplement (ThermoFisher # 31765035)
5. Dulbecco's phosphate buffered saline (DPBS, 1X, ThermoFisher #14190-144)
6. Isopropanol (Sigma #I9516)
7. Trypan Blue solution (0.4%, Sigma #T8154)

Equipment

1. Biosafety cabinet
2. Humidified tissue culture incubator with 5% CO_2
3. Water bath
4. Collagen coated tissue culture dishes prepared using 50 µg/mL collagen concentration according to McNabb and McCullough (2019)

a. 100mm dishes (TPP #93100)

b. 150mm dishes (TPP #93150)

5. Pipet aid

6. Serological pipettes

7. 50 mL conical tubes

8. Sterile 150 mL and 250 mL bottles

9. Tabletop centrifuge

10. Eppendorf tubes (autoclaved; USA Scientific #1615-5500)

11. Pipettes

12. Filter tips (low retention)

13. Hemocytometer

14. PVDF membrane syringe filter with 0.22µm pore (Millipore #SLGVR33RS)

15. “Mr. Frosty” cell freezing apparatus (Nalgene; Fisher #5100-0001)

16. Long-term cell storage container with liquid nitrogen

Procedure

pMVECs are not known to be hazardous; however, they were derived from human samples and should be handled under BSL-2 precautions. Researchers should wear appropriate personal protective equipment during the completion of this protocol. A face shield should be worn while handling liquid nitrogen and cell vials that have been recently removed from liquid nitrogen storage as they may explode unexpectedly.

pMVECs should be thawed and sub-cultured on collagen coated tissue culture plastics (McNabb *et al.,* 2019). HULEC cells used within an APD range of 3-12 have high levels of reproducibility within sub-cultured experiments.

Quality Control:

Cells will be visually observed for culture quality prior to every passage. Cultures should be tested for the presence of the intracellular bacterial pathogen *Mycoplasma* spp.

Prepare Growth Medium

1. Thaw 1 x EGM-2 MV SingleQuots Supplement Pack in a 37°C water bath.

2. Remove 25 mL of volume from the bottle of EBM-2 basal medium using a 25 mL pipet and discard.

3. Add the following SingleQuots from the Supplement Pack to the EBM-2 basal medium using either a 25 mL pipet or P1000 pipette:

- 1 x bottle of fetal bovine serum (FBS), 25 mL
- 1 x natural cap vial with hydrocortisone, 0.20 mL
- 1 x gray cap vial with hFGF-B, 2.00 mL
- 1 x white cap vial with VEGF, 0.50 mL
- 1 x yellow cap vial with R3-IGF-1, 0.50 mL
- 1 x blue cap vial with ascorbic acid, 0.50 mL
- 1 x green cap vial with hEGF, 0.50 mL
- 1 x red cap vial with GA-1000, 0.50 mL

4. Mix thoroughly and label with the date and additives.

Thawing Cryopreserved Cells

1. Pre-warm Ham's F-12 medium in a 37°C water bath for at least 30 minutes and prepare materials prior to obtaining a vial of cells from cryostorage.

a. This reduces the amount of time cells are in the presence of DMSO during thawing.

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

a. It is important that cells are thawed quickly to prevent damage and reduced viability by minimizing exposure to DMSO.

b. Submerge to the level of the medium within the cryovial. Submerging past the junction of the tube and cap can result in contamination.

c. Once the vial has thawed, dry it with a paper towel and sanitize it by spraying with 70% ethanol.

3. Add 9 mL of pre-warmed growth medium to a 50 mL conical tube with a 10 mL pipet. Then, transfer the thawed cells from the cryovial into the growth medium with a P1000 pipette and gently mix by inversion.

a. **NOTE:** A 50 mL conical tube is used instead of a 15 mL conical tube here to reduce chances for contamination by contacting the inner wall of the tube with the pipette shaft and/or aspirator. It also reduces the possibility of overflow during trituration.

4. Pellet cells by centrifugation at 600 x *g* for 5 minutes at room temperature.

5. Carefully aspirate the supernatant.

6. Gently resuspend the cells in 3 mL of pre-warmed EGM-2 growth media using a 5 mL pipette.

a. **NOTE:** 3 mL resuspension volume will result in an appropriate cell dilution for a frozen vial of ~2 x 10^7 cells. Resuspension volume can be adjusted for frozen vials of cells with different cell counts.

7. Prepare a 1:2 dilution of the cell suspension in a 1.5 mL Eppendorf tube by mixing 50 µL of the cell suspension with 50 µL of Trypan Blue using a P200. Mix by vortexing for 1-2 seconds.

8. Transfer 10 µL of the Trypan Blue-treated cell suspension to each side of a hemocytometer using a P20 pipette. Count and record the number of cells that exclude Trypan Blue (*i.e.*, intact cells) in the four corner grids on each side of the hemacytometer

a. **NOTE**: Do not use an automated cell counter as these cells display variability in size and are not accurately quantified by automated methods).

b. **NOTE**: Blue staining of cells indicates a damaged membrane. Blue-stained cells are considered to be non-viable.

9. Dilute cells to 2 x 10^4 cells/mL in EGM-2 growth medium in a 50 mL tube or larger sterile bottle to the total volume that will be used for plating (10 mL for each 10 cm dish and 25 mL for each 15 cm dish, making 2-5 mL extra).

a. If larger volumes are required, then they can be prepared in sterile disposable bottles. Mix cell suspensions in bottles by swirling for 10 seconds instead of inversion.

10. Mix thoroughly by inversion and dispense the diluted cell suspension into each collagen coated dish using a 10 mL or 25 mL pipet for 10mL or 25 mL volume, respectively.

a. **NOTE**: Cells spread out more evenly when collagen coated dishes are pre-warmed to 37°C.

11. Distribute the cells evenly by gentle rocking and swirling for at least 10 seconds.

12. Incubate in a humidified 37 °C incubator with 5% CO_2 at ambient O_2 concentration overnight.

13. Check cells the following day with a microscope for attachment to the dish. Aspirate medium to remove dead cells (rounded, unattached cells) and add fresh pre-warmed EGM-2 growth medium (10 mL for each 10 cm dish and 25 mL for each 15 cm dish) using a 10 mL or 25 mL pipet, respectively. It is preferable to culture thawed cells for at least two passages before using in experiments.

a. **NOTE**: Perform every-other-day-media changes until cells have reached 75-90% confluency and are ready for sub-culture.

b. **NOTE**: Cells from different donors may grow at different rates. As a result, cells may be ready to begin sub-culture 4-7 days after plating.

c. **NOTE:** Plating cells at a determined cell density will result in more predictable sub-culture and more reproducible performance in subsequent experimental assays.

Sub-Culturing Cells

NOTE: Cells should be split when they have reached 75-90% confluency after being plated at 2 x 10^4 cells/mL. Cells from different donors may grow at different rates. As a result, cells may be ready to split 4-7 days after plating.

1. Pre-warm an aliquot of EGM-2 growth medium and DPBS in a 37°C water bath for at least 30 minutes.

2. Sanitize biosafety cabinet working surface by spraying a paper towel with Cavicide then wiping the working area. Then spray the working surface 70% ethanol and wipe until dry.

3. Transfer plates of cells being passaged from the incubator to the biosafety cabinet.

4. Aspirate the cell culture medium.

5. Gently add warmed DPBS to each plate and rinse by swirling carefully.

a. 20 mL for 15 cm dishes (25 mL pipet)

b. 10 mL for 10 cm dishes (10 mL pipet)

6. Aspirate the PBS wash.

7. Add Accutase and rotate/rock to spread evenly across the surface of the dish.

a. 6 mL for 15 cm dishes (10 mL pipet)

b. 3 mL for 10 cm dishes (10 mL pipet)

c. **NOTE:** Accutase should be added cold and returned to 2°-8°C immediately after use.

8. Place in tissue culture incubator for 5 minutes (time varies by trypsin lot and will need to be determined empirically for each lot). To ensure uniform heating of the bottom surface of each plate, do not stack dishes on top of each other in the incubator during this step. Following incubation, use the palm of your hand to gently hit the side of the dish to facilitate detachment. Check detachment with microscope before proceeding.

a. **NOTE**: If cells are still attached, incubate for another 2 minutes. Incubate and tap dish until >95% of the cells are detached.

9. Transfer Accutase and cell suspension to a 50 mL conical tube.

10. Wash the plate by adding 6 mL of additional growth medium with a 10 mL pipet. Triturate once to break up any large cell clumps, swirl to mix, and transfer the cell suspension to the same 50 mL conical tube from Step #9.

a. **NOTE**: Growth medium should be added in a 1:1 dilution. Volume can be adjusted accordingly.

b. **NOTE**: 6 mL wash can be used to wash 1-2, 15 cm dishes.

11. Centrifuge at 1,000 x *g* for 4 minutes to pellet cells.

12. Carefully aspirate the supernatant and resuspend the cell pellet in 3 mL of growth medium using a 10 mL pipet. Using the same pipet, triturate two times to thoroughly break up the majority of clumped cells.

a. **NOTE**: Typically, medium is added such that there is a total volume of 3 mL per 1-2 15 cm plates collected.

13. Prepare a 1:7 dilution of the cell suspension in a 1.5 mL Eppendorf tube by mixing 200 µL of the cell suspension with 1200 µL of DPBS using a P1000. Mix by vortexing for 1-2 seconds.

14. Prepare a 1:2 dilution of the diluted cell suspension in a 1.5 mL Eppendorf tube by mixing 50 µL of the diluted cell suspension with 50 µL of DPBS using a P200. Mix by vortexing for 1-2 seconds.

15. Transfer 10 µL of the Trypan Blue-treated cell suspension to each side of a hemocytometer using a P20 pipette. Count and record the number of cells that exclude Trypan Blue (*i.e.*, intact cells) in the four corner grids on each side of the hemacytometer

a. **NOTE**: Do not use an automated cell counter as these cells display variability in size and are not accurately quantified by automated methods).

b. **NOTE**: Blue staining of cells indicates a damaged membrane. Blue-stained cells are considered to be non-viable.

16. Calculate the number of population doublings that occurred during the last passage using the following formula (include the indicated values in the associated worksheet):

([3.32(log(cells collected per plate)-log(cells plated per plate)) + previous APD])

17. Prepare a plating suspension at 2.0 x 10^4 cells/mL in growth medium in a 50 mL tube or larger sterile bottle to the total volume that will be used for plating (10 mL for each 10 cm dish and 25 mL for each 15 cm dish, making 2-5 mL extra).

a. If larger volumes are required, then they can be prepared in sterile disposable bottles. Mix cell suspensions in bottles by swirling for 10-20 seconds instead of inversion.

18. Mix thoroughly by inversion and dispense the diluted cell suspension into each collagen coated dish using a 10 mL or 25 mL pipet for 10mL or 25 mL volume, respectively.

a. **NOTE**: Cells spread out more evenly when collagen coated dishes are pre-warmed to 37°C.

19. Incubate in a humidified incubator at 37°C with 5% CO_2 and ambient O_2 concentration. Cells will be ready to split 4-7 days after plating, or when 75-90% confluency is reached.

a. **NOTE:** Plating cells at a determined cell density will result in more predictable sub-culture and more reproducible performance in subsequent experimental assays.

20. Discard any remaining aliquot of medium and return DPBS to 2°-8°C storage.

Preparing Cells for Cryopreservation

1. Prepare freezing medium.

a. Thaw Recovery Cell Culture Freezing Medium, mix well and keep at 2°-8°C until use.

2. Follow sub-culturing protocol through Step #15.

3. Calculate and record the total number of viable cells in the cell suspension.

4. Pellet cells by centrifugation at 1,000 x *g* for 4 minutes at room temperature.

5. Resuspend the cell pellet to a cell density of 1.2 x 10^6 cells/mL in freezing medium by gently triturating three times.

6. Transfer 1 mL of the cell suspension per cryovial using a P1000 pipette.

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

a. The chamber regulates cooling to about 1 °C per minute.

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

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

8. On the following morning, transfer the frozen vials to liquid nitrogen storage.

References

McNabb, N. and McCullough, S. (2019) Collagen Coating for Tissue Culture. *Protocol Exchange*.

Supplementary Files

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

- CultureofpMVECcells.docx