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Alveolar-Capillary Region Exposure (ACRE) Model

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Method Article

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Abstract

This exposure model was designed as an *in vitro* representation of trans-alveolar effects of airway particulate matter exposures on microvascular endothelial cells in the alveolar capillary region of the lung. The alveolar epithelial cells are represented by the NCI-H441 cell line, interstitial fibroblasts are represented by the IMR90 cell line, and microvascular endothelial cells are represented by the HULEC cell line.

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

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

Introduction

Reagents

Reagents

1. Advanced RPMI 1640 Medium without L-glutamine & HEPES (ThermoFisher #12633-012; hereafter referred to as “basal medium”)
2. MCDB 131 Medium, no glutamine (ThermoFisher #10372-019)
3. EGF Recombinant Human Protein (ThermoFisher #PHG0311)
4. Hydrocortisone (Sigma #H0888)
5. Dexamethasone (Sigma #D4902)
6. Fetal bovine serum (ThermoFisher #16000-044)
7. GlutaMAX™ supplement (ThermoFisher #35050-061)
8. 100X penicillin/streptomycin solution (ThermoFisher #15140-122)
9. Dulbecco’s phosphate buffered saline (DPBS, 1X, ThermoFisher #14190-144)
10. 0.25% Trypsin-EDTA (ThermoFisher #25200-056)

11. Trypan Blue solution (0.4%, Sigma #T8154)

12. Dimethylsulfoxide (DMSO, Sigma #D8418)

13. Isopropanol (Sigma #I9516)

14. Ethanol (Sigma #E7023)

Equipment

Equipment/Supplies

1. Biosafety cabinet

2. Humidified tissue culture incubator with 5% CO_2

3. Water bath

2. IMR90 cells according to Vitucci and McCullough (2022a)

3. H441 cells according to Vitucci and McCullough (2022b)

4. HULEC cells according to Vitucci and McCullough (2022c)

1. Collagen coated Costar clear TC-treated multiple well plates prepared using 50 µg/mL collagen concentration according to McNabb and McCullough (2019)

a. 6 well plates (Corning #3506)

b. 12 well plates (Corning #3512)

c. 24 well plates (Corning #3524)

2. Collagen coated Transwell® 0.4 µm pore polyester membrane inserts prepared using 50 µg/mL collagen concentration according to McNabb and McCullough (2019)

a. 24mm inserts (Corning #3450)

b. 12mm inserts (Corning #3460)

c. 6.5mm inserts (Corning #3470)

3. Pipet aid

4. Serological pipettes

5. 50 mL conical tubes

6. Sterile 150 mL and 250 mL bottles

7. Tabletop centrifuge

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

9. Pipettes

10. Filter tips (low retention)

11. Hemocytometer

12. Cell scrapers (Corning #3008)

13. Sartorius Quintix 124-1S Analytical Balance

Optional Reagents

1. *Quick*-RNA Miniprep Kit performed according to Vitucci and McCullough (2022d) (Zymo Research #1055)

2. Diesel exhaust particulate (DEP) suspension, 1 mg/mL, prepared according to Faber et al. (2020)

Procedure

H441, IMR90, and HULEC cells are not known to be hazardous; however, they were derived from human samples and should be handled under BSL-2 precautions. H441, IMR90, and HULEC cells should be cultured on collagen coated tissue culture plates. Researchers should wear appropriate personal protective equipment during the completion of this protocol.

Quality Control:

Observe epithelial and endothelial cells prior to exposure to confirm desired confluency and an intact H441 monolayer. Observe cells prior to each harvest to identify potential cell death. To minimize variability, prepare PM suspensions in large batches, prepare single-use aliquots, snap freeze in liquid nitrogen, and store at -80 °C.

SUB-CULTURE & EXPOSURE MEDIA FORMULATIONS

Prepare Complete Advanced RPMI Growth Medium:

1. Prepare complete advanced RPMI growth medium as described in Vitucci and McCullough (2022a, 2022b)

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Prepare Complete MCDB-131 Growth Medium:

1. Prepare complete MCDB-131 growth medium as described in Vitucci and McCullough (2022c)

Prepare a 51 μM Dexamethasone Growth Medium:

1. Weigh out 1 mg of Dexamethasone using Sartorius Quintix 124-1S Analytical Balance.

2. Transfer Dexamethasone to a 15 mL conical tube.

3. Resuspend Dexamethasone in 1 mL of 100% Ethanol using a P1000 pipette.

4. Mix by vortexing for 2-5 seconds.

5. Resuspend 1 mg/mL Dexamethasone in 49 mL of basal Advanced RPMI.

6. Mix by vortexing for 2-5 seconds.

7. Store in the 4°C until ready to use.

a. **NOTE**: 51 μM Dexamethasone solution expires 30 days from resuspension date.

Prepare Basolateral Exposure Medium:

1. To prepare basolateral exposure medium, combine the following constituents with the desired volume of basal MCDB-131 medium:

- Fetal bovine serum (FBS) to a final FBS concentration of 1%.
- 100X penicillin/streptomycin (P/S) to a final P/S concentration of 0.5%.
- Glutamax supplement to a final Glutamax concentration of 10 mM.

2. Mix thoroughly and label with the date and additives. Store in the 4°C until ready to use.

Prepare Apical Exposure Medium:

1. To prepare apical exposure medium, combine the following constituents with the desired volume of basal Advanced RPMI medium:

a. 100X penicillin/streptomycin (P/S) to a final P/S concentration of 0.5%.

b. Glutamax supplement to a final Glutamax concentration of 4 mM.

c. 51 µM Dexamethasone to a final Dexamethasone concentration of 0.5 µM.

2. Mix thoroughly and label with the date and additives. Store in the 4°C until ready to use.

PROCEDURE

Seed IMR90 Cells on Inverted Transwell® Inserts (Day 1)

Seed IMR90 cells on the underside of Transwell® insert membranes.

1. Pre-warm Complete Advanced RPMI growth medium and DPBS in a 37°C water bath and a trypsin aliquot at room temperature 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. Trypsinize, collect, and pellet IMR90 cells at 1,000 x *g* at room temperature according to Vitucci and McCullough (2022a).

a. **NOTE:** IMR90 cells are maintained according to Vitucci and McCullough (2022a). Cells should be split on the third day after being plated at 4.2 x 10^4 cells/mL.

4. Aspirate the supernatant and resuspend the cell pellet in 12 mL of complete growth medium using a 10 mL pipette. Triturate at least four times to thoroughly break up the majority of clumped cells. Add additional complete growth medium to dilute the cell suspension if desired (typically, a total volume of 12 mL per 2-3, 15 cm plates collected).

5. Prepare a 1:2 dilution of the cell suspension in Trypan Blue. Transfer 50 µL of Trypan Blue to a 1.5 mL Eppendorf tube using a P200 pipette and add 50 µL of the cell suspension using a P200 pipette. Mix by vortexing for 1-2 seconds.

6. Transfer 10 µL of the Trypan Blue-treated diluted 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.

7. Mix the cell suspension from Step #4 thoroughly by 5-10 inversions. Using this suspension, prepare a plating suspension at the desired density in complete growth medium in a 50 mL tube or larger sterile bottle to the total volume that will be used for plating.

a. **NOTE:** Make 2-5 mL extra cell suspension.

b. **NOTE**: See appendix for cell densities and plating volumes for desired plating format.

8. Invert a Transwell® dish and temporarily remove the bottom of the dish, leaving the inverted lid and inverted inserts. Remove the tops from sterile 1.5mL Eppendorf tubes and place one top in each corner of the inverted lid.

a. **NOTE**: Usage of the Eppendorf tops will elevate the bottom of the multi-well plate and prevent it from touching the plated inserts in Step #9.

9. Mix the cell plating suspension from Step #7 thoroughly by 10-20 inversions. Slowly dispense the diluted cell suspension atop each collagen-coated, inverted insert in the appropriate volume indicated in Table 1 of the appendix.

a. **NOTE**: Both the top and bottom of the Transwell® insert membrane need to be collagen coated in two separate steps. See Figure 1 in the appendix for reference.

10. Carefully replace the bottom of the multi-well plate from Step #8 atop the seeded, inverted inserts.

a. **NOTE**: The Eppendorf tops will lift the lid and prevent it from touching the plated inserts.

b. **NOTE**: When returning the lid, be careful not to disrupt the seeded inserts.

11. Slowly transfer the inverted Transwell® inserts, housed in their inverted multi-well plate, to a humidified incubator.

a. **NOTE**: The raised lid serves as a barrier to protect the seeded inserts from contamination when transferring inserts from the biosafety cabinet to the incubator.

12. Incubate the seeded, inverted inserts in a humidified 37 °C incubator with 5% CO_2 and ambient O_2 concentration for 2 hours before combining with H441 cells (next step).

Seed H441 Cells in Transwell® Inserts (Day 1)

Seed H441 cells on the upper face of the IMR90-seeded Transwell® insert membranes.

1. Pre-warm Complete Advanced RPMI growth medium and DPBS in a 37°C water bath and a trypsin aliquot at room temperature for at least 30 minutes.

a. **NOTE:** Begin this procedure at an appropriate time so that Step #7 of this procedure is completed when the 2 hour incubation period for seeding the IMR90 from above is complete.

2. Trypsinize, collect and pellet H441 cells at 1,000 x *g* at room temperature according to Vitucci and McCullough (2022b).

a. **NOTE:** H441 cells are maintained according to Vitucci and McCullough (2022b). Cells should be used on the third day after being plated at 1.4 x 10^5 cells/mL.

3. Aspirate the supernatant and resuspend the cell pellet in 12 mL of complete growth medium using a 10 mL pipette. Triturate at least four times to thoroughly break up the majority of clumped cells. Add additional complete growth medium to dilute the cell suspension if desired (typically, a total volume of 12 mL per 1-2 15 cm plates collected).

4. Prepare a 1:5 dilution of the cell suspension in DPBS. Transfer 800 µL of DPBS to a 1.5 mL Eppendorf tube using a P1000 pipette and add 200 µL cell suspension using a P200 pipette. Mix by vortexing for 1-2 seconds.

5. Prepare a 1:2 dilution of the cell suspension from Step #8 in Trypan Blue. Add 50 µL of Trypan Blue to a new 1.5 mL Eppendorf tube using a P200 pipette then add 50 µL of 1:5 cell suspension from Step #13 using a P200 pipette. Mix by vortexing for 1-2 seconds.

6. Transfer 10 µL of the Trypan Blue-treated diluted 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.

7. Mix the cell suspension from Step #3 thoroughly by 5-10 inversions. Using this suspension, prepare a cell plating suspension at the desired density in complete growth medium in a 50 mL tube or larger sterile bottle to the total volume that will be used for plating.

a. **NOTE:** Make 2-5 mL extra cell suspension.

b. **NOTE**: See Table 1 in the appendix for plating volumes and cell densities for desired plating format.

c. **NOTE**: This step should coincide with the completion of the 2 hour IMR90 attachment step from above.

8. After the IMR90s have attached to the Transwell^{Ò} insert for 2 hours, carefully remove the inverted multi-well plate and inserts from the incubator.

9. Gently aspirate the media and unattached cells from the inverted inserts. Remove the tops of the 1.5 mL Eppendorf tubes and replace the lid of the Transwell^{Ò} plate. Revert the closed plate to its upright position, with the inserts being in their correct, “hanging” position within their multi-well plate.

10. Mix the cell plating suspension from Step#7 thoroughly by 5-10 inversions and dispense the H441 cell suspension into the apical compartment of each collagen-coated insert in the appropriate volume indicated in Table 1 of the appendix.

11. Dispense complete Advanced RPMI growth medium to the basolateral compartment of each well in the appropriate volume indicated in Table 1 of the appendix.

a. **NOTE:** Check that there are no air bubbles trapped underneath insert membrane. If so, angle the plate slightly and tap gently to remove them.

12. Incubate in a humidified 37°C incubator with 5% CO_2 and ambient O_2 concentration for 3 days until combining with HULECs.

Change Media and Add Dexamethasone Growth Medium (Day 2)

Change apical media on the H441 cells into polarization medium. Refresh basolateral complete growth media.

1. Pre-warm Complete Advanced RPMI Growth Medium in a 37 °C water bath for approximately 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. Prepare polarization medium. Transfer complete growth medium to a 50 mL canonical tube or larger sterile bottle to the total volume that will be used for an apical media change, making 2-5 mL extra. Add the necessary volume of 51 µM Dexamethasone Growth Medium to a final concentration of 0.5 µM.

a. **NOTE**: Make this 0.5 µM solution fresh for each daily use.

NOTE: See Table 1 of the appendix for medium volumes used for desired format.

4. Aspirate the medium from the basolateral compartment of the wells containing Transwell^{Ò} inserts seeded with H441 and IMR90 cells.

5. Replace with Complete Advanced RPMI Growth Medium in each well using the appropriate volume indicated in Table 1 of the appendix:

a. **NOTE**: Check that there are no air bubbles trapped underneath insert membrane.

6. Aspirate the medium from the apical compartment of the Transwell^{Ò} inserts seeded with H441 and IMR90 cells.

a. **NOTE**: Be careful not to disrupt seeded H441 cells when aspirating.

7. Replace with polarization medium in the apical compartment of each Transwell® insert using the appropriate volume indicated in Table 1 of the appendix.

8. Return inserts to a humidified 37 °C incubator with 5% CO_2 and ambient O_2 concentration for 24 hours until next media change.

Refresh Apical Polarization Medium (Day 3)

Refresh apical medium only with polarization medium.

1. Pre-warm Complete Advanced RPMI Growth Medium in a 37°C water bath and 51uM Dexamethasone solution at room temperature for approximately 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. Prepare polarization medium as described above in Day 2, Step #2.

4. Aspirate the medium from the apical compartment of the Transwell® inserts seeded with H441 and IMR90 cells.

a. **NOTE**: Be careful not to disrupt seeded H441 cells when aspirating.

5. Replace with polarization medium in each insert using the appropriate volume indicated in Table 1 of the appendix.

NOTE: Check that there are no air bubbles trapped underneath insert membrane.

6. Return inserts to a humidified 37 °C incubator with 5% CO_2 and ambient O_2 concentration for 24 hours until next media change.

Seed HULECs in Transwell® wells (Day 4)

Seed HULECs in wells of multi-well plates.

1. Pre-warm Complete MCDB-131 growth medium and DPBS in a 37°C water bath and a trypsin aliquot at room temperature for at least 30 minutes.

a. **NOTE:** Begin this procedure in the morning of Day 4 so that Step #8 of this procedure is completed 8 h prior to combining with Transwell® inserts (next section).

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. Trypsinize, collect, and pellet HULEC at 1,000 x *g* at room temperature according to Vitucci and McCullough (2022c).

a. **NOTE:** HULEC are maintained according to Vitucci and McCullough (2022c). Cells should be split on the third day after being plated at 4.5 x 10^4 cells/mL.

4. Aspirate the supernatant and resuspend the cell pellet in 12 mL of complete growth medium using a 10 mL pipette. Triturate at least four times to thoroughly break up the majority of clumped cells. Add additional complete growth medium to dilute the cell suspension if desired (typically, a total volume of 12 mL per 2-3, 15 cm plates collected).

5. Prepare a 1:2 dilution of the cell suspension in Trypan Blue. Transfer 50 µL of Trypan Blue to a 1.5 mL Eppendorf tube using a P200 pipette and add 50 µL of the cell suspension using a P200 pipette. Mix by vortexing for 1-2 seconds.

6. Transfer 10 µL of the Trypan Blue-treated diluted 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.

7. Mix the cell suspension from Step #4 thoroughly by 5-10 inversions. Using this suspension, prepare a plating suspension at the desired density in complete growth medium in a 50 mL tube or larger sterile bottle to the total volume that will be used for plating.

a. **NOTE:** Make 2-5 mL extra cell suspension.

b. **NOTE**: See Table 1 in the appendix for cell densities and plating volumes for desired plating format.

8. Dispense the diluted cell suspension into each collagen-coated well in the appropriate volume indicated in Table 1 of the appendix.

9. Incubate the seeded wells in a humidified 37 °C incubator with 5% CO_2 and ambient O_2 concentration for 8 hours before combining with Transwell® inserts (next section).

Combine HULECs with Transwell® Inserts (Day 4)

Transfer inserts seeded with IMR90s and H441s into wells seeded with HULECs to create the tri-culture, ACRE model.

1. 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.

2. Prepare appropriate volume of Basolateral Exposure Medium, for two basolateral media changes in a 50 mL tube or larger sterile bottle.

a. **NOTE**: See Table 1 of the appendix for appropriate plating volumes for 6, 12, and 24 well plates.

3. Prepare appropriate volume of polarization medium for one apical media change in a 50 mL tube or larger sterile bottle:

a. **NOTE**: See Table 1 of the appendix for appropriate plating volumes for 6, 12, and 24 well plates.

4. Prepare appropriate volume of Apical Exposure Medium for one apical media change in a 50 mL tube or larger sterile bottle:

a. **NOTE**: See Table 1 of the appendix for appropriate plating volumes for 6, 12, and 24 well plates.

b. **NOTE**: Save Apical Exposure Medium in 4 °C to be used for Day 5 exposure.

5. Pre-warm Basolateral Exposure Medium and 0.5 µM Dexamethasone Growth Medium in a 37 °C water bath for approximately 30 minutes.

6. Aspirate media from seeded HULECs and replace using the appropriate volume of Basolateral Exposure Medium indicated in Table 1 of the appendix.

a. **NOTE**: Save the remaining Basolateral Exposure Medium in 4 °C to be used for Day 5 exposure.

7. Aspirate apical then basolateral media from seeded Transwell® inserts. Transfer inserts atop seeded HULECs and replace apical media using the appropriate volume of polarization medium indicated in Table 1 of the appendix.

a. **NOTE**: Check that there are no air bubbles trapped underneath insert membrane after addition atop HULECs.

8. Return the seeded ACRE model to a humidified 37 °C incubator with 5% CO_2 and ambient O_2 concentration overnight (approximately 14 hours).

Alveolar Capillary Region Exposure and Harvest (Day 5)

Add PM suspension to H441 cells and begin exposure for desired length.

1. Pre-warm Apical Exposure Medium and Basolateral Exposure Medium in a 37°C water bath for approximately 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. Thaw frozen PM preparation, dilute to desired concentration in Apical Exposure Medium, and vortex thoroughly.

a. **NOTE:** Vortex PM suspension occasionally to mix the settling particles.

4. Aspirate the medium from the basolateral compartment of the Transwell^{Ò} inserts and replace with Basolateral Exposure Medium in the appropriate volume indicated in Table 1 of the appendix.

5. Aspirate the medium from the apical compartment of the Transwell^{Ò} inserts and replace with either vehicle (Apical Exposure Medium) or diluted PM suspension in the appropriate volume indicated in Table 1 of the appendix.

a. **NOTE:** Vortex PM suspension occasionally to mix the settling particles before adding the suspension to inserts.

6. Return the ACRE model to a humidified 37 °C incubator with 5% CO_2 and ambient O_2 concentration for the desired exposure duration.

References

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Figures

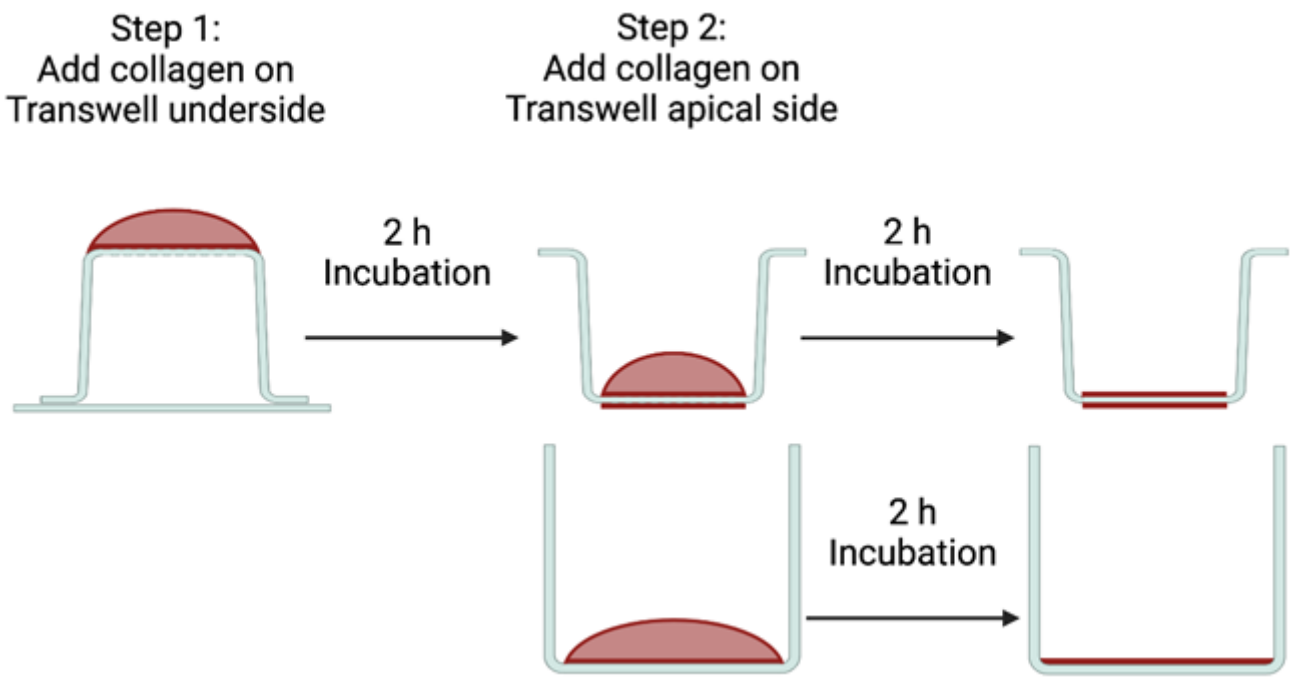

Figure 1

Figure 1: Collagen-coating format for Transwell^{Ò} inserts and well.

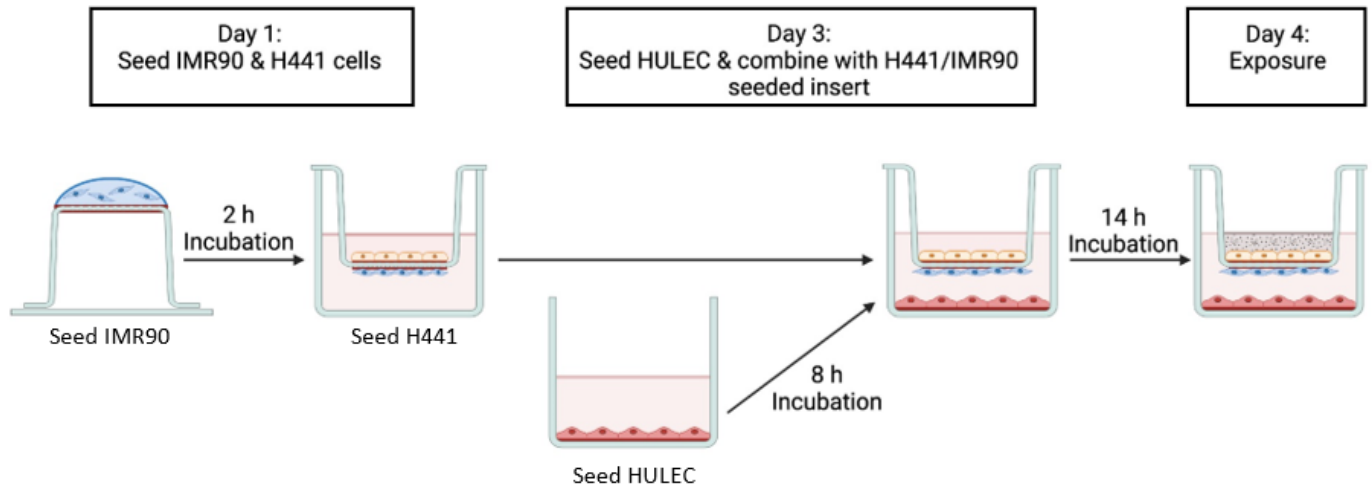

Figure 2

Figure 2: ACRE Model Set-Up Summary.

	ACRE Model		
COMPONENTS	6-well (24 mm insert)	12-well (12 mm insert)	24-well (6.5 mm insert)
Insert (Apical Compartment) Growth Area	4.67 cm^2	1.12 cm^2	0.33 cm^2
Well (Basolateral Compartment) Growth Area	9.5 cm^2	3.8 cm^2	1.9 cm^2
Particulate Matter (PM) Apical Suspension Volume	1000 µL	250 µL	100 µL
Apical Media Volume	2000 µL	500 µL	100 µL
Basolateral Media Volume	2000 µL	1000 µL	500 µL
Inverted Plating Volume	1000 µL	250 µL	74 µL
Diluted IMR90 Cell Suspension	2.9×10^4 cells / mL	2.8×10^4 cells / mL	2.78×10^4 cells / mL
IMR90 Cell Density	6.25×10^3 / cm^2	6.25×10^3 / cm^2	6.25×10^3 / cm^2
IMR90 Cell Count	29,000 cells / insert	7,000 cells / insert	2063 cells / insert
Diluted H441 Cell Suspension	1.75×10^5 cells / mL	16.8×10^4 cells / mL	24.8×10^4 cells / mL
H441 Cell Density	7.5×10^4 cells / cm^2	7.5×10^4 cells / cm^2	7.5×10^4 cells / cm^2
H441 Cell Count	3.5×10^5 cells / insert	8.4×10^4 cells / insert	2.48×10^4 cells / insert
Diluted HULEC Cell Suspension	1.38×10^5 cells / mL	1.12×10^5 cells / mL	1.1×10^5 cells / mL
HULEC Cell Density	2.9×10^4 cells / cm^2	2.9×10^4 cells / cm^2	2.9×10^4 cells / cm^2
HULEC Cell Count	275,500 cells / B.C.	112,000 cells / B.C.	55,100 cells / B.C.

Figure 3

Table 1: Cell seeding density and plating volumes for 6-well, 12-well, and 24-well inserts and plates. For plating volumes of < 200 µL use a P200 pipette. For plating volumes > 200 µL use a P1000 pipette.

Supplementary Files

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

- ACREModelSetup.docx