



emulate



## Alveolus Lung-Chip Co-Culture Protocol

June 3, 2020

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## Please Read

Note that primary human alveolar epithelial cells are particularly difficult to isolate. Sourcing of high-quality cells is limited because of a lack of standardized isolation protocols, which results in highly inconsistent cell quality. Success of the chips is highly dependent on the quality of the cells.

## Introduction to the Human Emulation System<sup>®</sup>

This section provides an overview of key components of the Human Emulation System<sup>®</sup>, including the Chip-S1<sup>®</sup> Stretchable Chip, the Pod<sup>™</sup> Portable Module, the Zoë<sup>®</sup> Culture Module, and the Orb<sup>™</sup> Hub Module.

An Organ-Chip is a living, micro-engineered environment that recreates the natural physiology and mechanical forces that cells experience within the human body. The Pod provides the media to the Organ-Chip that is needed to promote cell growth; the Pod also acts as the interface between the Organ-Chip and the Zoë Culture Module. Zoë provides the flow of nutrient-rich media at a rate determined by the user and provides the mechanical forces that emulate the physical forces experienced *in vivo* by tissue. The Orb delivers a mix of 5% CO<sub>2</sub>, vacuum stretch, and power for up to four Zoë Culture Modules.

## Organ-Chip (Chip-S1<sup>™</sup>)

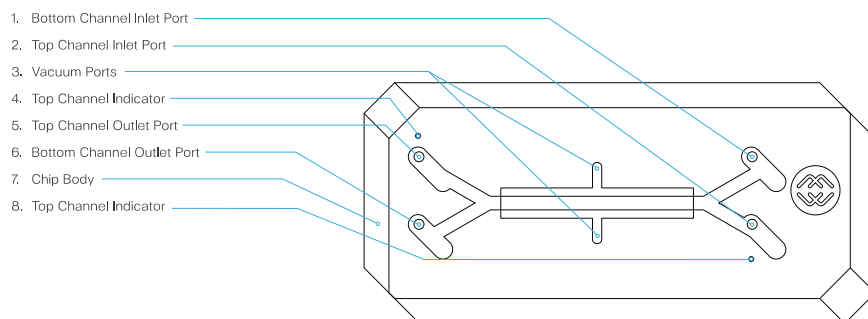
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Our Chip-S1 can be configured to emulate multiple different organ tissues including lung, liver, intestine, kidney, and brain. The chip, when connected to the Pod and Zoë, recreates the body's dynamic cellular microenvironment, including tissue-to-tissue interfaces, blood flow, and mechanical forces. This guideline describes the methodology for the Alveolus Lung-Chip.

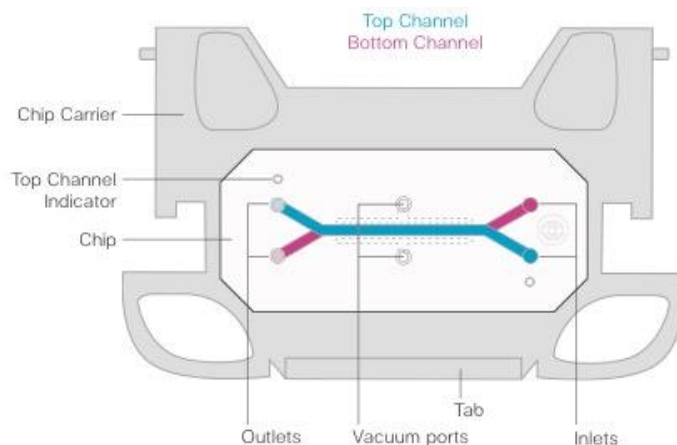
The microenvironment created within each Chip-S1 includes epithelial cells in the top channel and endothelial cells in the bottom channel. Top and bottom channels are separated by a porous membrane that allows for cell-cell interaction like those that are seen *in vivo*. These two channels are fluidically independent. Cells in each channel are seeded with organ- and cell-specific extracellular matrix proteins (ECM), and can be maintained in static culture for up to four days, depending on cell type, before being connected to Zoë, which provides continuous flow of cell culture media.

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## Organ-Chip Configuration



Chips are supplied pre-packaged in the Chip Carrier and should be maintained in the Chip Carrier at all times. The Chip Carrier is designed to hold chips in place, eliminating the need to disturb or distort the chip during preparation. In addition, the Chip Carrier connects securely to the Pod, which acts as the interface between Zoë and the chip. Use sterile tweezers when handling chips in chip carriers. Removal of the chip from the Chip Carrier prior to or during an experiment could result in chip failure. (The only appropriate time to remove the chip from the carrier is at end of the life of the chip in the experiment, when it is no longer used for live culture of the cells.)



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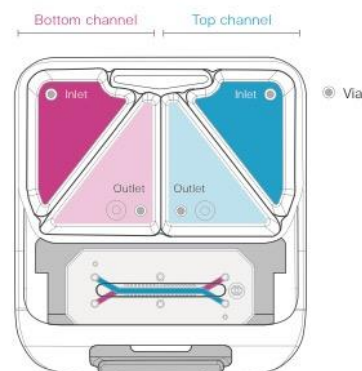
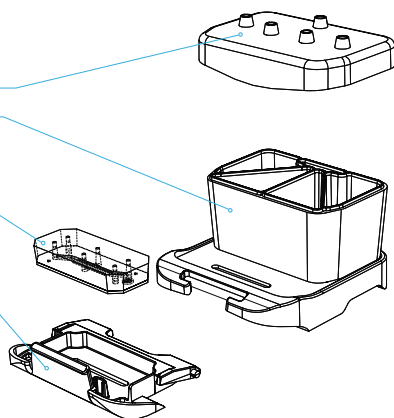
## Pod™ Portable Module

The Pod houses the chip, supplies media, and enables compatibility with microscopes and other analysis equipment. The Pod, which has a height of 41.5 mm, is designed to allow Organ-Chips to be easily transported and placed on standard microscopes for imaging. It also allows simple portability of Organ-Chips between our hardware and other analysis equipment.

The Pod's reservoirs allow users to introduce nutrient media, precisely control dosing to test drugs or other inputs, and sample chip effluent. Each Pod reservoir holds up to 4 mL.

### Pod™ Components

1. Pod Reservoir Lid
2. Pod Reservoir
3. Organ-Chip
4. Chip Carrier



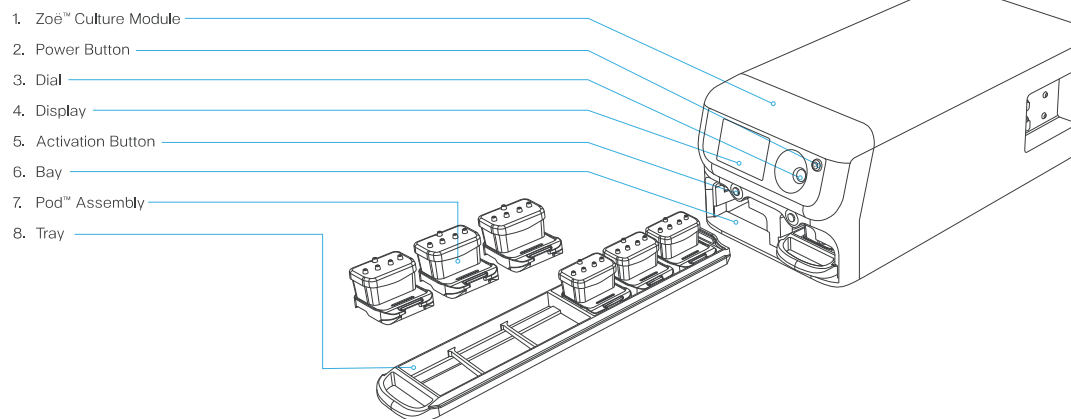
## Zoë® Culture Module

The Zoë Culture Module is designed to sustain the life of cells within Emulate's Organ-Chips. It provides the dynamic flow of media and the mechanical forces that help recreate the microenvironment cells experience *in vivo*. The instrument automates the precise conditions needed for simultaneous cell culture of up to 12 Organ-Chips.

Zoë enables independent control of the flow rate of media through the top and bottom channels of the Organ-Chips, as well as stretch parameters — including frequency and amplitude — of the Organ-Chip. Additionally, Zoë has automated algorithms to prime the fluidic channels of Pods with media, and programming to maintain the culture microenvironment for optimal cell performance.

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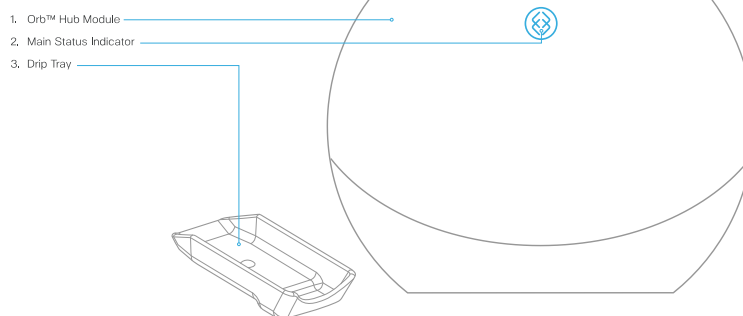
## Zoë-CM1™ Components



## Orb™ Hub Module

The Orb is a central hub that connects to up to four Zoë Culture Modules. It provides a simple solution for installing and operating the Human Emulation System within the lab environment. The Orb delivers a mix of 5% CO<sub>2</sub>, vacuum stretch, and power from standard lab connections. It generates a 5% CO<sub>2</sub> supply of gas to Zoë by combining air with an external 100% carbon dioxide supply (or from a portable CO<sub>2</sub> canister for increased flexibility) in a controlled mixture. The Orb also generates the vacuum required to apply Organ-Chip stretching (-70 kPa), and contains four individual power ports.

## Orb-HM1™ Components

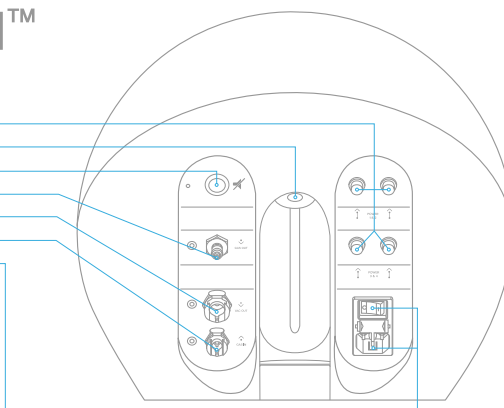


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## Orb-HM1™

### Control Panel

1. Power Ports
2. CO<sub>2</sub> Canister Connection
3. Alarm Silence Button
4. Mixed CO<sub>2</sub> Out
5. Vacuum Out
6. 100% CO<sub>2</sub> In
7. Power Input & On / Off Switch



## Chip Cradle

The Chip Cradle is an accessory that holds and organizes up to six chips. It offers several benefits, including:

- It allows inversion of the chips during seeding.
- It has a DPBS reservoir that keeps the chips humidified.
- It is made from an autoclavable plastic.
- It contains slots with numbered labels to help keep chips organized.

## Chip Cradle

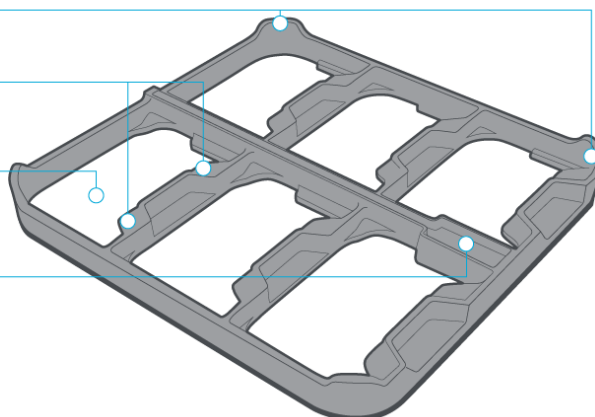
### Configuration

Chip Cradle Top Corners

Carrier Retention Wings  
2 Per Bay

Chip Carrier Bay  
6 per Cradle

Fluid Reservoir



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## Cells

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The commercial availability of human primary alveolar epithelial cells (HPAEC) is limited, and these cells are known to be difficult to culture *in vitro*. We recommend testing various sources and donors of cells for viability, morphology, and expression of key markers associated with type I and type II alveolar cells.

Type I cells are typically flat and large, while type II cells should retain a more cuboidal-like morphology. Type I markers include Podoplanin and Aquaporin 5, while type II markers include Surfactant Protein C and ABCA3 (ATP-binding cassette sub-family A member 3). HTI-56 (human alveolar type I cell 56kDa protein) and HTII-280 (human alveolar type II cell 280 kDa protein) are type I and type II markers respectively that will also aid in identifying cell populations. E-cadherin junctional protein should also be present to confirm healthy monolayer formation. Depending on the desired application and goals, it might be necessary to optimize conditions for a dominant type I population or type II population.

For endothelial cells, we recommend the use of Human Lung Microvascular Endothelial Cells (HMVEC-L) purchased from Lonza (#CC-2527).

## Media and Gas Equilibration

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Proper gas equilibration of media is essential for successful Organ-Chip culture. As the system is sensitive to bubbles, all media must be equilibrated to 37°C and excess gas must be removed prior to use in the chip and in Zoë. To do this, 37°C medium is placed under vacuum using the 0.45 µm PVDF filter of a Steriflip® conical filter unit. Failure to carry out this step could lead to failure of the chips in the experiment.

It is also important to look for bubbles throughout the experiment. Observed bubbles can often be flushed out of the system to avoid a disruption in medium flow and / or improper surface coverage. However, instances of chips with visible bubbles or blocked flow should be recorded and considered when analyzing experimental data.



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## Experimental Overview

### Workflow Overview

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#### Day X: Reagent Preparation

- Aliquot reagents (media supplements, ECM, etc.)

#### Day -3 & -1: Thaw and HPAEC & HMVEC-L

- Prepare HPAEC and HMVEC-L culture media and flasks
- Thaw and plate HPAEC and HMVEC-L

#### Day -1: Chip Preparation

- Prepare chips
- Prepare ER-1™ solution
- Introduce ER-1™ solution to channels
- Activate chips
- Prepare ECM solution
- Coat chips with ECM

#### Day 0: HPAEC to Chips

- Prepare HPAEC seeding medium
- Gently wash HPAECs on flask with PBS
- Harvest HPAEC from flask
- Re-suspend HPAECs in appropriate volume to obtain  $1 \times 10^6$  cells/mL
- Seed the top channel
- Incubate chips at 37°C for 2 hours
- Wash chips
- Incubate chips at 37°C

#### Day 1 and 2: Media Replenishment

- Rinse the top channel with culture medium
- Rinse the bottom channel with culture medium

#### Day 3: HMVEC-L to Chips

- Prepare HMVEC-L culture medium
- Wash chips
- Harvest HMVEC-L
- Re-suspend in appropriate volume to obtain  $5 \times 10^6$  cells/mL
- Seed HMVEC-L to bottom channel
- Incubate chips at 37°C for 1 hour
- Wash chips and insert tips

#### Day 4: Chips to Pods and Pods to Zoë

- Gas equilibration of media
- Prime Pods

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- Wash chips
- Chips to Pods
- Pods to Zoë

**Day 5: Introducing Air Liquid Interface (ALI)**

- Prepare ALI medium
- Establishing ALI & Maintenance

**Day 7: Initiating mechanical Stretch****Day 8+: Sampling**

- Sampling and media replenishment

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## Required Materials

Ensure all equipment, materials, and reagents are accessible prior to beginning each experiment. Exact catalog numbers are provided for specific materials required for successful experiments.

## Equipment & Materials

Equipment	Description	Supplier	Catalog Number
Zoë-CM1 Culture Module	1 per 12 chips	Emulate	-
Orb-HM1 Hub Module	1 per 4 Zoës	Emulate	-
Chip-S1 Stretchable Chip	12 per Zoë	Emulate	-
Pod Portable Modules	1 per Chip-S1	Emulate	-
UV Light Box	1 per Zoë	Emulate	-
Chip Cradle	Autoclaved, 1 per 6 chips	Emulate	-
Steriflip®-HV Filters	0.45 µm PVDF filter Sterile	EMD Millipore	<a href="#">SE1M003M00</a>
120 mm cell square dish	Sterile, 1 per 6 chips	Corning / Falcon	
Handheld vacuum aspirator	-	Corning	<a href="#">4930</a>
Aspirating pipettes	2 mL, polystyrene, individually wrapped	Corning / Falcon	<a href="#">357558</a>
Aspirating tips	Sterile (autoclaved)	-	-
Serological pipettes	2 mL, 5 mL, 10 mL, and 25 mL low endotoxin, sterile	-	-
Pipette	P20, P200, and P1000	-	-
Pipette tips	P20, P200, and P1000 sterile, low-adhesion	-	-
Conical tubes	15 mL and 50 mL polypropylene, sterile	-	-
Eppendorf Tubes®	1.5 mL, sterile	-	-
Aluminum foil	-	-	-
Parafilm®	-	-	-
Microscope (with camera)	For bright-field imaging	-	-
Hemocytometer	-	-	-
Manual counter	-	-	-

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Water bath (or beads)	Set to 37°C	-	-
Vacuum set-up	Minimum pressure: -70 kPa	-	-
T25 flasks	-	-	-
T75 flasks	-	-	-
Ice bucket	-	-	-
70% ethanol and wipes	For surface sterilization	-	-

## Reagents, Media & Supplements

Reagent	Description	Supplier	Catalog Number
ER-1™	5 mg powder	Emulate	-
ER-2™	25 mL bottle	Emulate	-
Dulbecco's PBS (DPBS -/-) (without Ca <sup>2+</sup> , Mg <sup>2+</sup> )	1X	Corning	<a href="#">21-031-CV</a>
Trypan blue	0.4% solution	Sigma	<a href="#">93595</a>
TrypLE™ Express	Enzyme (1X), no phenol red, 100 mL	Gibco	<a href="#">12604013</a>
Small Airway Epithelial Cell Growth Medium (SAGM)	Includes basal medium and SingleQuots™ Kit	Lonza	<a href="#">CC-3118</a>
EGM™ - 2 MV Microvascular Endothelial Cell Growth Medium-2	Includes basal medium and SingleQuots™ Kit	Lonza	<a href="#">CC-3202</a>
Fibronectin	Human plasma	Corning	<a href="#">356008</a>
Collagen type IV	Human placenta	Sigma	<a href="#">C5533</a>
Laminin	Human placenta	Sigma	<a href="#">L6274</a>
Penicillin-streptomycin	10,000 U/mL; 10 mg/mL	Sigma	<a href="#">P4333</a>
L-GlutaMax™	200 mM	Thermo Fisher	<a href="#">35050-061</a>
Dexamethasone	100 mg Powder	Sigma	<a href="#">D4902</a>
Fetal bovine serum (FBS)	Sterile heat-inactivated	Sigma	<a href="#">F4135</a> or <a href="#">F8317</a>
Keratinocyte growth factor (KGF)	Powder	Thermo	PHG0094
8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (cAMP)	Powder	Sigma	B7880

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Medium 199, no phenol red	500mL bottle	Thermo Fisher	11043023
Epidermal Growth Factor, human, recombinant	Powder	PromoCell	C-60170
Vascular Endothelial Growth Factor (human)	Powder	PromoCell	C-64420
Hydrocortisone	Powder	Sigma	H0135
Basic Fibroblast Growth Factor (FGF-2 or FGF-b)	Powder	PromoCell	C-60243
Heparin	Powder	Sigma	H3149
Isobutyl methylxanthine (IBMX)	Powder	Sigma	I7018

**Notes for ER-1™ and ER-2™**

- Upon arrival, store ER-1 (powder) unopened in the metalized pouch at -20°C, protected from light and humidity.
- Upon arrival, store ER-2 solution at 4°C.

## Aseptic Technique

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Prepare and sanitize the work surface of the biosafety cabinet (BSC) prior to beginning. Ensure that the work space within the BSC is organized and free from clutter. Arrange tips, media, and other necessary materials in the sterile field, easily within reach, without blocking the path of airflow.

Sanitize all reagents and materials entering the BSC with 70% ethanol. Spray and wipe thoroughly. Always avoid touching the chip directly. Handle the chip carrier only by the sides or by the tab with gloves. Never remove the chip from the chip carrier prior to during an experiment.

## Cell Storage

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**Cryopreserved cells must be stored in liquid nitrogen.** Severe damage to cell membrane and cytoskeletal components can result from chronic temperature fluctuations. Never store in dry ice or -80°C freezer.

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## Chip Handling Techniques

Always work with chips within the Chip Carrier in a sterile environment, such as the BSC.

When pipetting to fill each channel, 50  $\mu\text{L}$  volume is generally used for the top channel, and 20  $\mu\text{L}$  is used for the bottom channel. These volumes allow for simple pipetting and a slight overfill to avoid bubbles or dry channels. All wash steps, unless otherwise stated, are performed using 200  $\mu\text{L}$  of the specific wash solution.

While 50  $\mu\text{L}$  (top channel) and 20  $\mu\text{L}$  (bottom channel) are standard volumes used throughout the protocol, there can be some flexibility in the actual volumes used.

Top Channel: 35–50  $\mu\text{L}$

Bottom Channel: 15–20  $\mu\text{L}$

The specific channel and membrane dimensions are outlined below:

### Top Channel

Width x height dimensions	1,000 $\mu\text{m}$ x 1,000 $\mu\text{m}$
Area	28.0 $\text{mm}^2$
Volume	28.041 $\mu\text{L}$
Imaging distance from bottom of chip to top of membrane	850 $\mu\text{m}$

### Bottom Channel

Width x height dimensions	1,000 $\mu\text{m}$ x 200 $\mu\text{m}$
Area	24.5 $\text{mm}^2$
Volume	5.6 $\mu\text{L}$

### Membrane

Pore diameter	7.0 $\mu\text{m}$
Pore spacing	40 $\mu\text{m}$ (hexagonally-packed)
Thickness	50 $\mu\text{m}$

### Co-Culture Region

Area	17.1 $\text{mm}^2$
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A P200 pipette with a sterile pipette tip is used to add solution directly to the channels of the chip, as when coating, washing, and seeding cells prior to attaching the chip to Zoë. To introduce solution to the channels, place the pipette tip perpendicular to chip channel inlet, ensuring that the tip is securely in the port, and steadily dispense liquid through the channel. Always introduce liquid to the bottom channel before pipetting into the top channel.

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## Alveolus Lung-Chip Culture Protocol

### Reagent Preparation

#### Aliquot Reagents

Aliquot reagents prior to use to avoid multiple freeze-thaw cycles of the stock solutions.

#### Extracellular Matrix (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
<b>Fibronectin</b>	1 mg / mL	5 mg	5 mL	Cell culture grade water
<b>Laminin</b>	1 mg / mL	5 mg	5 mL	Cell culture grade water
<b>Collagen IV</b>	1 mg / mL	5 mg	5 mL	Cell culture grade water

- Resuspend ECM according to manufacturer's instructions.
- Aliquot to single-use volumes and store at -20°C (Collagen IV, Fibronectin) and -80°C (Laminin).

#### Culture Supplements

Reagent	Conc. [Working]	Volume	Solvent
<b>Dexamethasone</b>	1mM	Reconstitute 25mg in 63.7008mL DMSO	Cell culture grade DMSO
<b>KGF</b>	10 ug / mL	Reconstitute 10ug in 1mL PBS	PBS + 0.1% BSA
<b>cAMP</b>	100 mM	Reconstitute 100mg in 2.3251mL water; sterile filter	Cell culture grade water
<b>Epidermal Growth Factor (human)</b>	1 mg / mL	Reconstitute 500µg in 500µL water	Cell culture grade water
<b>Basic Fibroblast Growth Factor (human)</b>	100 µg / mL	Reconstitute 50µg in 500µL water	Cell culture grade water
<b>Vascular Endothelial Growth Factor (human)</b>	50 µg / mL	Reconstitute in 50mM acetic acid + 0.1% BSA	50mM Acetic acid + 0.1% BSA
<b>Hydrocortisone</b>	50 µg / mL	Reconstitute 1mg in 1 mL absolute ethanol and 19 mL sterile medium	Absolute Ethanol + Sterile medium
<b>Heparin</b>	50 mg / mL	Reconstitute 50mg in 1mL water	Cell culture grade water
<b>Isobutyl methylxanthine (IBMX)</b>	1M	Reconstitute 100mg in 450uL DMSO	DMSO

- Resuspend each supplement to the working concentration in the table above or manufacturer's instructions.
- Aliquot each supplement to single-use volumes.
- Store aliquots at -20°C; replace Dexamethasone and KGF aliquots every 3 months, replace cAMP aliquots every year.

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## Day -3 & -1: Thawing HPAEC and HMVEC-L

### Goals:

- Expand HPAEC and HMVEC-L in flask prior to chip seeding

### Key Steps:

- Prepare HPAEC culture media and flask
- Prepare HMVEC-L culture media and flask
- Thaw and plate HPAEC and HMVEC-L

### Required Materials:

- Complete SAGM Culture Medium (at 37°C)
- Complete EGM-2MV Culture Medium (at 37°C)
- 15 mL conical tube
- T-75 flask
- T-25 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol



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### Day -3: Prepare HPAEC Culture Medium and Flask (Alveolus-Lung Epithelium Cells Expansion)

#### HPAEC Culture Medium

##### Complete SAGM Culture Medium

Reagent	Conc. [Final]	Source	Cat. No.
SABM™ Basal Medium	-	Lonza	CC-3119
SAGM™-SingleQuots™ Supplement Pack	-	Lonza	CC-4124
Fetal bovine serum (FBS)	5%	Sigma	F4135 or F8317

- Gentamicin (GA-1000) from the SAGM kit can be replaced by 1% Penicillin-Streptomycin
- Store at 4°C.
- Use within 30 days of preparation.

#### Prepare Flask

1. Warm sufficient amount of Complete SAGM Culture Medium to 37°C.
2. Label the culture flask with relevant information (e.g., cell type, passage number, date, initials).

### Thaw and Plate HPAEC

#### Thawing and Maintaining Cells

1. Coat a T-25 flask with gelatin solution and incubate at 37°C and 5% CO<sub>2</sub> for 5–10 minutes.
2. Thaw the vial(s) of cells by immersing in a 37°C water bath. Closely observe while gently agitating and remove from the water bath just before the last of the ice pellet disappears.
3. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC.
4. Immediately transfer the contents of the vial into 3 mL of warm Complete SAGM (5% FBS) in a sterile 15 mL conical tube.
5. Rinse the vial with 1 mL of Complete SAGM (5% FBS) and collect in the 15 mL tube.
6. Bring the volume to 15 mL with Complete SAGM (5% FBS) Culture Medium.
7. Centrifuge at 200 x g for 5 minutes at room temperature.
8. Aspirate and discard supernatant, leaving approximately 100 µL of medium covering cell pellet.
9. Loosen the cell pellet by gently flicking the tube.
10. Re-suspend cells in 7 mL of Complete SAGM (5% FBS) Culture Medium.
11. Add the HPAEC suspension to the T-25 flask.
12. Incubate overnight at 37°C and 5% CO<sub>2</sub>.
13. Exchange with fresh Complete SAGM (5% FBS) once a day until use for seeding in the chip.

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## Day -1: Prepare HMVEC-L Culture Medium and Flask

### HMVEC-L Culture Medium

#### Complete EGM-2MV Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
EBM™-2 Basal Medium	500 mL	-	-	Lonza	CC-3156
EGM™-2MV SingleQuots™ Supplement Pack		-	-	Lonza	CC-4147

- Gentamicin (GA-1000) from the EGM-2MV kit can be replaced by 1% Penicillin-Streptomycin
- Store at 4°C.
- Use within 30 days of preparation.

### Prepare Flask

1. Warm sufficient amount of Complete EGM-2MV Culture Medium to 37°C.
2. Label the culture flask with relevant information (e.g., cell type, passage number, date, initials).

## Thaw and Plate HMVEC-L

### Thawing and Maintaining Cells

1. Thaw the vial(s) of cells by immersing in a 37°C water bath. Closely observe while gently agitating and remove from the water bath just before the ice pellet disappears.
2. Spray vial(s) with 70% ethanol and wipe dry prior to placing them in the BSC.
3. Immediately transfer the contents of the vial into 3 mL of warm Complete EGM2-MV Culture Medium in a sterile 15 mL conical tube.
4. Rinse the vial with 1 mL of Complete EGM-2MV Culture Medium and collect in the 15 mL tube.
5. Bring the volume to 15 mL with Complete EGM2-MV Culture Medium.
6. Add the HMVEC-L suspension to the T75 flask.
7. Incubate for 6 hours (until 60 – 80% cells have adhered) at 37°C and 5% CO<sub>2</sub>
8. Aspirate the culture medium and add Complete EGM-2MV Culture Medium to remove traces of DMSO
9. Incubate overnight at 37°C and 5% CO<sub>2</sub>.
10. Exchange with fresh Complete EGM-2MV Culture Medium every other day until use for seeding in the chip.

**Note:** That is a guideline and primary cells confluency and viability can vary between different lot and suppliers. We recommend that cells confluency should be no larger than 90% on the day of seeding.

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## Day -1: Chip Preparation

### Goals:

- Activate the inner surface of the chip channels for proper ECM attachment
- Coat inner channels with a mixture of ECM proteins for cell attachment

### Key Steps:

- Prepare chips
- Prepare ER-1 solution
- Introduce ER-1 solution to channels
- Activate and wash chips
- Prepare ECM solution
- Coat chips with ECM

### Required Materials:

- Chip-S1 (12 chips per Zoë)
- ER-1 powder
- ER-2 solution
- Chip cradle
- 15 mL conical tubes
- DPBS (- / -) at room temperature
- DPBS (- / -) aliquot at 4°C
- Collagen IV
- Fibronectin
- Laminin
- 70% ethanol
- 120 mm square cell culture dish
- Ice and ice bucket
- Pipettes and filtered tips
- Aspirator and sterile tips
- Aluminum foil
- UV light box
- UV safety glasses

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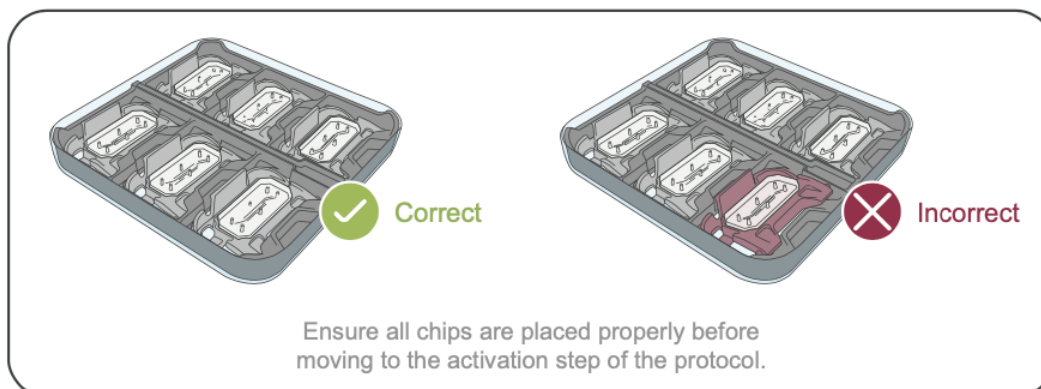
### Prepare Chips

1. Spray chip packaging, 120 mm square cell culture dish, and Chip Cradle with 70% ethanol and bring into the BSC.
2. Open the Chip Cradle sterile packaging and place the cradle into the 120 mm square dish, making sure the Chip Cradle is oriented properly with the corners facing up.
3. Open the chip packaging carefully and place the first chip into the cradle by sliding the back of the carrier under the tabs on the cradle (Figure 1).



Figure 1. Place chips by sliding under the tab of the Chip Cradle.

4. Repeat as necessary for all of the chips included in the experiment.
5. Label each chip with ID numbers on the chip carrier tab.



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### Prepare ER-1 Solution

ER-1 is light-sensitive. Prepare ER-1 solution immediately before use and discard any remaining ER-1 solution 1 hour after reconstitution. Failure to protect from light or use of ER-1 solution that has not been freshly prepared will lead to failure of chips. Note: ER-1 is an eye irritant and must be handled in the BSC with proper gloves and eye protection.

1. Turn off the light in BSC and allow the ER-1 and ER-2 reagents to equilibrate to room temperature before use (Approximately 10 to 15 minutes).
2. Use a 15 mL amber conical tube or wrap an empty sterile 15 mL conical tube with aluminum foil to protect it from light.
3. In the BSC, remove the small vial of ER-1 powder from the packet, and briefly tap the vial to concentrate the powder at the bottom.
4. Add 8 mL of ER-2 buffer to the covered 15 mL conical tube.
5. Add 1 mL of ER-2 buffer to the ER-1 vial and transfer contents directly to the 15 mL conical tube. Note: The color of the solution transferred to the conical tube will be deep red.
6. Add an additional 1 mL of ER-2 buffer to the ER-1 vial, cap the bottle and invert to collect any remaining ER-1 powder from the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 10 mL ER-1 solution. Note: The color of the transferred ER-1 solution will become lighter with each additional wash of the ER-1 bottle.
7. The final working concentration of ER-1 should be 0.5 mg / mL. Pipette gently to mix without creating bubbles. ER-1 should be fully dissolved within the ER-2 solution prior to use.

### Introduce ER-1 Solution to Channels

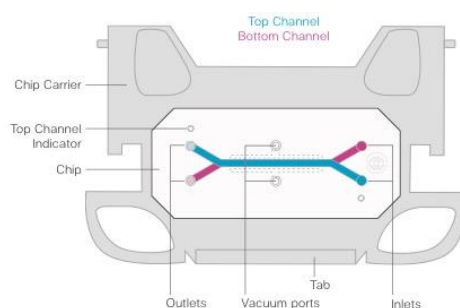


Figure 1 Top view of chip, with labelled ports, in chip carrier

1. Using a P200 pipette and a sterile 200  $\mu$ L filtered pipette tip, take up 200  $\mu$ L of ER-1 solution. Note: 200  $\mu$ L of ER-1 solution will fill approximately 3 chips.
2. Carefully introduce approximately 20  $\mu$ L of ER-1™ solution through the bottom channel inlet, pipetting until the solution begins to exit the bottom channel outlet.
3. Without releasing the pipetting plunger, take the pipette out from the bottom channel inlet, and move the pipette containing remaining ER-1 solution to the top channel inlet.

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4. Introduce approximately 50  $\mu$ L of ER-1 solution to the top channel inlet, pipetting until the solution begins to exit the top channel outlet.
5. Remove all excess ER-1 solution from the surface of the chip by gentle aspiration. Be sure only to remove ER-1 solution from the chip surface — do not aspirate ER-1 from the channels.
6. Repeat Steps 1 through 5 for each chip.
7. Inspect the channels for bubbles prior to UV activation. If bubbles are present, dislodge by washing the channel with ER-1 solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly reintroduce ER-1 solution.

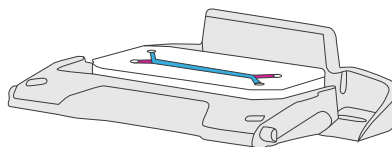


Figure 2 View of chip with no excess ER-1™ solution on surface

### Activate and Wash Chips

1. Bring the 120 mm square dish containing the ER-1-coated chips to the UV light box.
2. Before placing the square dish into the UV light box, make sure to remove the cover from the 120 mm square dish. Note: If the lid is not removed prior to placing the dish in the UV light box, the chips will not activate properly and could result in poor cell attachment.
3. Set the switch at the back of the UV light box to the “Constant” setting. Turn on the “Power,” and press the “On” button to begin UV activation.
4. Allow the chips to activate under UV light for 10 minutes.
5. After UV treatment, bring the chips back to the BSC.
6. Aspirate activated ER-1 from all channels and refill channels with fresh ER-1 as before.
7. Bring the chips back to the UV light box and activate under UV light for an additional 5 minutes.
8. While the chips are being treated, prepare ECM Solution. (For more information, see the next section: Prepare ECM Solution).
9. After UV treatment, bring chips back to the BSC. Note: The light may be on in the BSC from this point forward.
10. Fully aspirate the ER-1 solution from both channels.
11. Wash each channel with 200  $\mu$ L of ER-2 solution.
12. Fully aspirate the ER-2 from the channels.
13. Wash each channel with 200  $\mu$ L of sterile cold DPBS.
14. Leave cold DPBS inside the channels.

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### Prepare ECM Solution

The ECM solution is prepared fresh each time by combining the individual ECM components with cold DPBS to the final working concentrations. The ECM solution will be used to coat both the top and bottom channels.

For the top channel of the Alveolus Lung-Chip, the ECM working concentration is:

- Collagen IV: 200 µg / mL
- Fibronectin: 30 µg / mL
- Laminin : 5 µg / mL

For the bottom channel of the Alveolus Lung-Chip, the ECM working concentration is:

- Collagen IV: 200 µg / mL
- Fibronectin: 30 µg / mL

1. Bring an ice bucket and ice to the BSC.
2. Thaw one aliquot of fibronectin, one of collagen IV, and one of laminin on ice. Maintain all ECM components and mixture on ice at all times.
3. Calculate total volume of ECM solution needed to coat all chips.

Volume required per chip = approximately 100 µL. For every 12 chips to coat, prepare 1.5 mL of ECM solution (12 chips x 100 µL / chip + extra 300 µL = 1.5 mL of ECM solution).

#### ECM Calculation Example ( $C_1V_1 = C_2V_2$ )

Fibronectin stock concentration: 1 mg / mL ( $C_1$ )

Fibronectin final concentration: 0.030 mg / mL ( $C_2$ )

Stock volume: collagen I or fibronectin ( $V_1$ )

Final volume of ECM solution: 1.5 mL ( $V_2$ )

#### Fibronectin

$$(1 \text{ mg / mL}) \times (Y \text{ mL}) = (0.030 \text{ mg / mL}) \times (1.5 \text{ mL})$$

$$Y = 45 \text{ µL of fibronectin}$$

#### DPBS

Volume DPBS

$$= (\text{total volume of ECM needed}) - (\text{volume of collagen I}) - (\text{volume of fibronectin})$$

$$= 1500 \text{ µL} - 45 \text{ µL}$$

$$= 1,455 \text{ µL of DPBS}$$

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4. Combine components to prepare ECM working solution.
5. Keep the ECM solution on ice until ready to use.

### Coat Chips with ECM

1. Fully aspirate cold DPBS from both channels.
2. Using a P200 pipette and sterile 200  $\mu$ L filtered pipette tip, take up 200  $\mu$ L ECM solution.
3. Carefully introduce ECM solution through the bottom channel inlet until a small ECM droplet forms on the outlet.
4. Without releasing the pipetting plunger, take the pipette out from the bottom channel inlet, and move the pipette containing remaining ECM solution to the top channel inlet.
5. Introduce ECM solution through the top channel inlet, leaving small droplets of excess ECM solution on both ports in both channels (Figure 3).

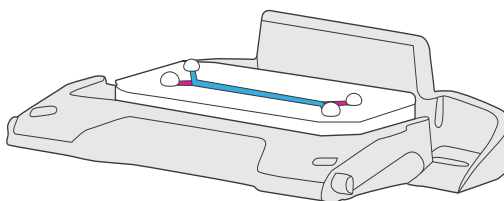


Figure 3. Chip in chip carrier with small droplets of ECM solution at ports

6. Inspect channels to ensure that no bubbles are present. If bubbles are present, dislodge by washing the channel with ECM solution until all bubbles have been removed.
7. Repeat steps 1 through 6 for each chip.
8. To prevent evaporation during incubation, fill central reservoir with 0.75 mL of DPBS (Figure 4) and place lid onto 120 mm square dish and incubate overnight at 37°C and 5% CO<sub>2</sub>.

**Note:** If desired, HPAEC can be seeded the same day as chip activation and ECM coating, though incubation overnight is preferred for best result. Chips can be ready for same day seeding of HPAEC 4 hours after adding the ECM and incubating chips at 37°C. If chips will be stored longer than overnight before seeding, store the chips at 4°C for up to 2 days.

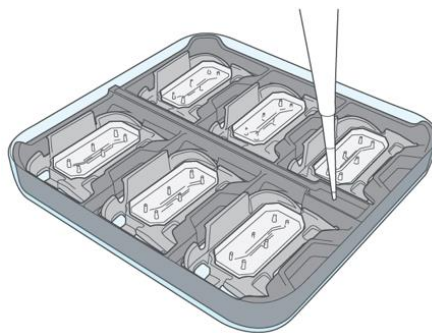


Figure 4. Fill central reservoir of Chip Cradle with 0.75 mL of DPBS



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## Day 0: HPAEC to Chip

### Goals:

- Seed HPAEC in chip

### Key Steps:

- Prepare HPAEC seeding medium
- Prepare chips
- Harvest HPAEC
- Adjust cell density
- Seed HPAEC to top channel
- Seed a well plate
- Wash chips (2 hours post-seeding)

### Required Materials:

- HPAEC seeding medium (at 37°C)
- 1X DPBS (at room temperature)
- TrypLE Express
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 15 mL conical tubes
- Diluted trypan blue counting solution
- Hemocytometer
- 24-well plate
- 70% ethanol
- Microscope

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## Prepare HPAEC Seeding Medium

### HPAEC Seeding Medium

#### Complete SAGM Culture Medium

Reagent	Conc. [Final]	Source	Cat. No.
SABM™ Basal Medium	-	Lonza	CC-3119
SAGM™ SingleQuots™ Supplement Pack	-	Lonza	CC-4124
Fetal bovine serum (FBS)	5%	Sigma	F4135 or F8317

- Store at 4°C.
- Use within 30 days of preparation.

## Prepare Chips

1. Transfer ECM-coated chips from incubator into the BSC.
2. Gently wash each channel of the chip with 200 µL of warm Complete SAGM Culture Medium. Aspirate the medium outflow at the surface of the chips, leaving the medium in the channels.
3. Repeat the wash with an additional 200 µL of Complete SAGM Culture Medium per channel, leaving the excess medium outflow covering the inlet and outlet ports.
4. Cover the 150 mm dish and place the chips in the incubator until the cells are ready for seeding.

## Harvest HPAECs

HPAECs in culture must be harvested and counted for top channel seeding. HPAECs are adjusted to a density of  $1 \times 10^6$  cells / mL prior to seeding in the top channel.

1. Bring the culture flask containing HPAEC from the incubator into the BSC.
2. Aspirate culture media and add 5 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
3. Add 3 mL of TrypLE Express to the flask. Incubate for 10 to 15 minutes at 37°C.
4. Tap the side of the flask gently and inspect the culture under the microscope to assess complete detachment of cells from the culture surface.
5. Add 7 mL of warm Complete SAGM Culture Medium to the flask and pipette gently to mix, while collecting all cells from the culture surface.
6. Transfer the contents of the flask (10 mL) into a sterile 15 mL conical tube.
7. Centrifuge HPAEC at 200 x g for 5 minutes at room temperature.
8. Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. Note: The cell pellet will be very small. Aspirate carefully.
9. Loosen the cell pellet by flicking the tube gently.
10. Using a P1000 pipette, gently resuspend the cells by adding 500 µL of warm Complete SAGM Culture Medium.

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11. Pipette gently to create a homogeneous mixture, and transfer 10  $\mu\text{L}$  of the cell suspension to the trypan blue cell counting solution (This will make a 1:10 dilution).
12. Mix the counting solution thoroughly and count cells using a manual hemocytometer.

### Trypan Blue Cell Counting Solution

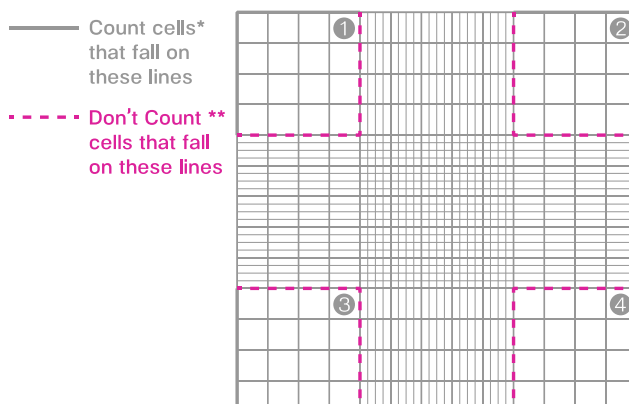
Reagent	Volume	Source	Cat. No.
Complete SAGM Culture Medium	80 $\mu\text{L}$	Recipe Above	-
Trypan blue	10 $\mu\text{L}$	Sigma	<u>93595</u>

- Maintain at room temperature prior to use.
- Prepare fresh for each use.

### Adjust Cell Density

Human primary alveolar epithelial cells seeded in the Alveolus Lung-Chip must be seeded at a density of  $1 \times 10^6$  cells / mL. Accurate seeding density is important to ensure viable and functional cells within the Alveolus Lung-Chip for long-term culture.

1. After resuspending the pellet to homogenize the cell suspension, transfer 10  $\mu\text{L}$  of the cell suspension to the trypan blue cell counting solution, generating a 1:10 dilution.
2. Mix the trypan blue solution thoroughly and count cells using a manual hemocytometer.



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### Cell Counting and Viability Assessment

- Count both viable and non-viable cells in each quadrant of the hemocytometer.  
Live Cell Count; Dead Cell Count; Total Cell Count
- Calculate percent viability of the cell solution.  
 $(\text{Live Cells}) \div (\text{Total Cells}) \times 100 = \% \text{ Viability}$
- Calculate viable cell concentration. The dilution factor is 10 when prepared in the trypan blue solution above  
 $(\text{Live Cell Count} \times 10 \times 10^4) \div 4 = \text{Viable Cell Concentration (cells / mL)}$
- Calculate viable cell yield.  
 $(\text{Viable Cell Concentration}) \div (\text{Cell Suspension Volume}) = \text{Viable Cell Yield (cells)}$

Dilute HPAEC with warm Complete SAGM Culture Medium to the required final cell density of  $1 \times 10^6$  cells / mL.

### Seed HPAEC to Top Channel

---

Work with one chip at a time. After seeding the first chip, assess cell density within the channel through the microscope, adjusting the density of the cell suspension accordingly for the subsequent chips if necessary.

- Bring the 120 mm dish containing the prepared chips to the BSC.
- Avoiding contact with the inlet and outlet ports, carefully aspirate excess medium droplets from the surface of one chip.
- Very gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension.
- Quickly and steadily pipette 35 to 50  $\mu\text{L}$  of the cell suspension (at  $1 \times 10^6$  cells / mL) into the top channel inlet port, while aspirating the outflow fluid from the chip surface. Avoid direct contact with the outlet port.
- Cover the dish and transfer to the microscope to check the seeding density within the chip. Note: at this stage, optimal seeding density should form an even layer with cell to cell spacing of about half the radius of a single cell.
- If seeding density is not optimal, return the chips to the BSC, and wash the channel with 200  $\mu\text{L}$  of fresh medium 2 times. Do not aspirate the medium from the channel. Adjust cell density accordingly, and repeat steps 3 through 5 until the correct density is achieved within the channel.
- After confirming the correct cell density, seed cells in the remaining chips. Note: minimize the amount of time the cells are outside the incubator by seeding no more than 12 chips at a time, immediately placing the chips in the incubator at  $37^\circ\text{C}$  after seeding each batch of 12.
- Place the chips (with DPBS in the central reservoir of the chip cradle) at  $37^\circ\text{C}$  for at least 2 hours or until cells have attached. Note: correct seeding density is essential for success of the chips

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### Seed a Well Plate

It is recommended to seed any remaining HPAEC into a plate as control for cell quality. HPAEC are seeded on a 24-well plate.

1. Further dilute HPAEC suspension with warm Complete SAGM Culture Medium to a final cell density of  $0.5 \times 10^6$  cells / mL.
2. Add 400, 500, and 600  $\mu$ L of the cell suspension to 3 separate wells of the 24-well plate.
3. Mix each well to ensure an even suspension and allow the cells to settle for 5 minutes on the microscope stage. Inspect densities under the microscope.
4. Determine which well depicts the optimal seeding density — either the well containing 400, 500, or 600  $\mu$ L of the cell suspension — and plate the remaining cells using that volume into individual wells of the 24-well plate.
5. In the incubator, disperse the cells evenly across the bottom of the culture wells by moving the plate in a figure eight motion across the shelf at least 3 times, while keeping the plate flat on the surface of the incubator. Finally, move the plate in a crisscross pattern to evenly disperse the cells at least 3 times. Once the cells are dispersed, do not disturb the plate until the next day to allow for cells to fully attach.

### Wash Chips

A gentle wash is performed 2 hours post-seeding, once the cells have attached to ensure that nutrients are replenished and the channels do not dry out. If cells are not attached, incubate overnight and then wash.

1. Gently pipette 200  $\mu$ L of warm Complete SAGM Culture Medium to the top and bottom channel of each chip to wash. Aspirate the outflow, leaving media in the channel.
2. Place additional droplets of media to fully cover all inlet and outlet ports to prevent evaporation from the ports (See Figure 3).
3. Incubate chips overnight at 37°C.

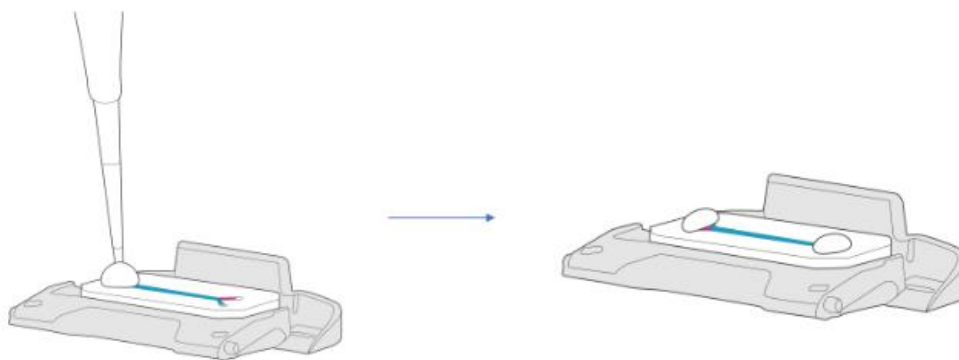


Figure 3 Chip with medium drops covering the inlet and outlet ports

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## Days 1 and 2: Media Replenishment

### Key Steps:

- Prepare HPAEC Maintenance Medium
- Replenish the medium for both the top channel and the bottom channel

### Required Materials:

- Complete HPAEC Maintenance Medium (at 37°C)
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 70% ethanol
- Microscope

## Prepare HPAEC Maintenance Medium

### HPAEC Maintenance Medium

#### Complete Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
SABM™ Basal Medium	500 mL	-	-	Lonza	CC-3119
SAGM SingleQuots™ Supplement Pack		-	-	Lonza	CC-4124
Fetal bovine serum (FBS)	Sterile heat-inactivated	-	2%	Sigma	F4135 or F8317
Dexamethasone		1mM	100nM	Sigma	D4902
Keratinocyte growth factor (KGF)		10µg/mL	5ng/mL	Thermo Fisher	PHG0094
8-br-cAMP		100mM	50µM	Sigma	B7880
Isobutyl methylxanthine (IBMX)		1M	25µM	Sigma	I7018

- Filter using 0.4µM filters
- Store at 4°C.
- Use within 15 days of preparation.

### Wash Chips

1. Gently pipette 200 µL of warm Complete Maintenance Medium to the top and bottom channel of each chip to wash. Aspirate the outflow, leaving media in the channel.
2. Place additional droplets of Complete Maintenance Medium to fully cover all inlet and outlet ports to prevent evaporation from the ports (See Figure 3).
3. Incubate chips overnight at 37°C.

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## Day 3: HMVEC-L to Chip

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### Goals:

- Seed HMVEC-L in chip

### Key Steps:

- Prepare HMVEC-L Culture Medium
- Wash chips
- Harvest HMVEC-Ls
- Seed HMVEC-Ls to bottom channel
- Wash chips

### Required Materials:

- Complete HMVEC-L Culture Medium (at 37°C)
- Trypan blue counting solution
- Hemocytometer
- 15 mL conical tubes
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- Ice bucket and ice
- 70% ethanol
- Microscope

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## Prepare HMVEC-L Culture Medium

### HMVEC-L Culture Medium

#### Complete EGM-2MV Culture Medium

Reagent	Volume	Source	Cat. No.
EBM™-2 Basal Medium	500 mL	Lonza	CC-3156
EGM™-2MV SingleQuots™ Supplement Pack		Lonza	CC-4147

- Store at 4°C.
- Use within 30 days of preparation.

#### Trypan Blue Cell Counting Solution

Reagent	Volume	Source	Cat. No.
Complete EGM-2MV Culture Medium	80 µL	Recipe Above	-
Trypan blue	10 µL	Sigma	93595

- Maintain at room temperature prior to use.
- Prepare fresh for each use.

## Wash Chips

1. Gently pipette 200 µL of warm Complete EGM-2MV Culture Medium to the top channel of each chip to wash. Aspirate the outflow, leaving medium in the channel.
2. Pipette 200 µL of warm Complete EGM-2MV Culture Medium to the bottom channel of each chip. Aspirate the outflow, leaving medium in the channel.
3. Return chips to the incubator until HMVEC-Ls are ready for seeding.

## Harvest HMVEC-Ls

HMVEC-Ls in culture must be harvested and counted for bottom channel seeding. HMVEC-Ls are adjusted to a density of  $5 \times 10^6$  cells / mL prior to seeding in the bottom channel. Bring the culture flask containing HMVEC-Ls from the incubator into the BSC.

1. Aspirate culture medium and add 15 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
2. Add 5 mL of TrypLE to the flask. Incubate for 5-10 minutes at 37°C.
3. Tap the side of the flask gently, and inspect the culture under the microscope to assess complete detachment of cells from the culture surface.
4. Add 7 mL of warm Complete EGM-2MV Culture Medium to the flask and pipette gently to mix, while collecting all cells from the culture surface.



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5. Transfer the contents of the flask (12 mL) into a sterile 15 mL conical tube.
6. Centrifuge HMVEC-Ls at 200 x g for 5 minutes at room temperature.
7. Carefully aspirate the supernatant, leaving approximately 50-100  $\mu$ L of medium above the cell pellet. Note: The cell pellet will be small. Aspirate carefully.
8. Loosen the cell pellet by flicking the tube gently.
9. Using a P1000 pipette, gently resuspend the cells by adding 500  $\mu$ L of Complete EGM-2MV Culture Medium.
10. Pipette gently to create a homogeneous mixture, and transfer 10  $\mu$ L of the cell suspension to the trypan blue cell counting solution (this will make a 1:10 dilution).
11. Mix the counting solution thoroughly and count cells using a manual hemocytometer (as described above)
12. Dilute the HMVEC-Ls to  $5 \times 10^6$  cells / mL in Complete EGM-2MV Culture Medium.

### Seed HMVEC-L to Bottom Channel

Work with one chip at a time. After seeding the first chip assess cell density within the channel through the microscope, adjusting the density of the cell suspension accordingly for the next chips if necessary.

1. Bring the 120 mm square dish containing the prepared chips to the BSC.
2. Avoiding contact with the ports, aspirate DPBS from cradle reservoir and carefully aspirate excess medium droplets from the surface of one chip.
3. Gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension.
4. Seed 15 to 20  $\mu$ L of the endothelial cell suspension into the bottom channel of one chip first, while aspirating the outflow.
5. Cover the dish and transfer to the microscope to check the seeding density within the chip.
6. If seeding density is not optimal, return the chip to the BSC and wash the channel 2 times with 200  $\mu$ L of fresh endothelial cell culture medium. Do not aspirate the medium from the channel prior to washing. Adjust the volume of the cell suspension as needed to obtain correct seeding density, and repeat steps 3 through 5 until the correct density is achieved within the channel.
7. After confirming the correct cell density, seed the remaining chips in the chip cradle. Note: Minimize the amount of time the cells are outside the incubator by seeding no more than 12 chips at a time, immediately placing the chips in the incubator at 37°C after seeding.
8. Once all 6 chips have been seeded in the cradle, cover the dish and carefully invert the dish.
9. To prevent evaporation during incubation, fill central reservoir with 0.75 mL of DPBS and place cover onto square dish.
10. Proceed with remaining chips until all have been seeded.
11. Incubate at 37°C for 2 hours, or until cells in the bottom channel have attached.
12. Once endothelial cells have attached (approximately 2 hours post-seeding), aspirate DPBS from the central reservoir, and flip the dish back so the chips are in an upright position in the chip cradle. Note: It is recommended to always seed any remaining cells into a plate as control for cell quality.

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### Wash with Tips

1. Once HMVEC-Ls have attached (approximately 1 hour post-seeding), flip the chips back to an upright position. Note: Remove the chip cradle, wipe with 70% ethanol to clean, and autoclave for use in next experiment.
2. A wash with 200  $\mu$ L of Complete Maintenance Medium for the top channel and Complete EGM-2MV Culture Medium for the bottom channel per chip will provide nutrients to cells. Since there are two different media being used, these two media must be separated by keeping them in filtered tips instead of drops. (See Figure 9.)
3. Return chips with pipette tips inserted in each inlet and outlet port to the incubator overnight.
4. Maintain cells in static culture in chips until connecting to Pods and Zoë the next day. Note: If desired, chips can be connected at least 2 hours post-attachment.

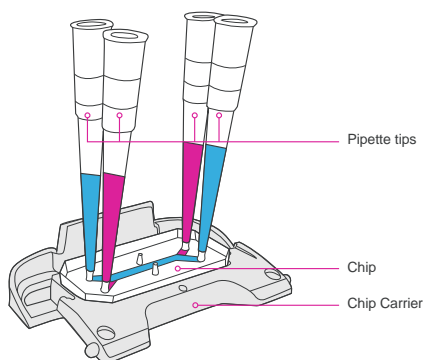


Figure 9 Chip with filtered tips inserted into ports with respective media

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## Day 4: Chips to Pods and Pods to Zoë

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### Goals:

- De-gas and equilibrate media
- Connect chips to Pods
- Connect Pods to Zoë

### Key Steps:

- Gas equilibration of media
- Prime Pods
- Wash chips
- Connect chips to Pods
- Connect Pods to Zoë
- Run Regulate cycle
- Begin experimental flow

### Required Materials:

- Installed and qualified Zoë
- Prepared chips
- Pods (sterile) — 1 per chip
- Tray — 1 per 6 chips
- Steriflip® filtration unit: PVDF filter 0.45 µm (sterile)
- Vacuum source (minimum -70 kPa)
- Serological pipettes
- Pipettors and filtered tips
- 37°C water or bead bath
- 70% ethanol

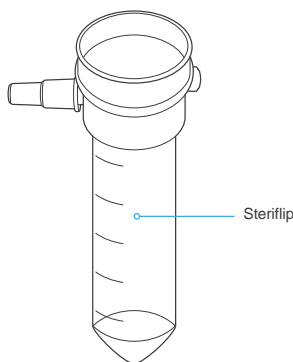
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### Gas Equilibration of Media

The media equilibration step is important for the success of Organ-Chip culture. Omission of this step will cause the eventual formation of bubbles in the chip, the Pod, or both, which will in turn cause irregular flow and negatively impact cell viability. Minimize the time media is outside of a warmed environment (such as an incubator or bath) to no more than 10 minutes, as gas equilibrium can become compromised when media is allowed to cool.

1. Place at least 3 mL of Complete Maintenance Medium for each chip in a 50 mL conical tube.
2. Place at least 3 mL of Complete EGM-2MV Culture Medium for each chip in a separate 50 mL conical tube.
3. Warm both 50 mL conical tubes of media at 37°C in a water or bead bath for at least 1 hour.
4. Immediately connect the 50 mL tube containing each warmed medium to a Steriflip® unit.
  - Attach each conical tube containing warmed medium to a Steriflip® unit.
  - With the unit "right-side-up" (medium in the bottom conical tube), apply vacuum for 10 seconds.
  - Invert the Steriflip®-connected tubes, and check that the medium begins to pass from the top conical tube to the lower tube. Note: The vacuum source must operate at least -70 kPa. At this correct pressure, it should take about 2 seconds for every 10 mL of medium to flow through the filter. If it takes longer, stop and see the troubleshooting protocol, as this indicates the medium not equilibrated properly.
  - Leave the filtered medium under vacuum for 5 minutes.
5. Remove the vacuum tubing from the Steriflip® units.
6. Separate the conical tubes containing media from the Steriflip® unit and immediately place the conical tubes containing media in the incubator with the caps loose.

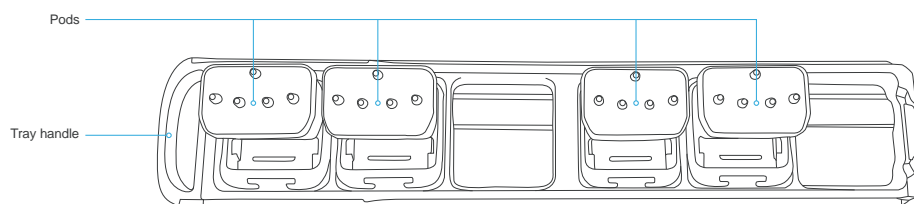
As noted above, minimize the time media is outside of the incubator during Pod preparation to maintain temperature. This is an important step to ensure success of the chips.



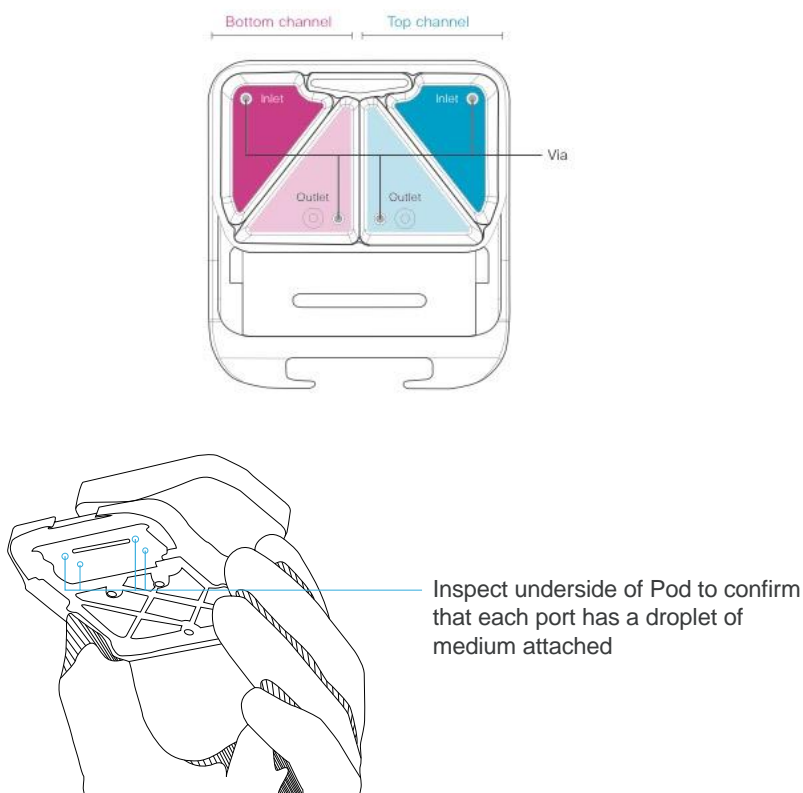
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### Prime Pods

1. Sanitize the exterior of the Pod packaging and trays with 70% ethanol, wipe, and transfer to the BSC.
2. Open the Pod package and place the Pods into the trays. Orient the Pods with the reservoirs toward the back of the tray.
3. Pipette 2 mL of pre-equilibrated, warm media to each inlet reservoir. In the top channel inlet reservoir, add Complete Maintenance Medium; in the bottom channel inlet reservoir, add Complete EGM-2MV Culture Medium .
4. Pipette 300  $\mu$ L of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet via.
5. Bring trays containing Pods to the incubator and slide completely into Zoë with the tray handle facing outward.
6. Run the Prime cycle on Zoë.
  - Use the rotary dial to highlight “Prime” on the display.
  - Press the rotary dial to select “Prime.”
  - Rotate the dial clockwise to highlight “Start.”
  - Press the dial again to select “Start” and begin. Note: Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.
7. Close the incubator door and allow Zoë to prime the Pods; this process takes approximately 1 minute. Note: When the status bar reads “Ready,” the “Prime” cycle is complete.
8. Remove the tray from Zoë, and bring to the BSC.
9. Verify that the Pods were successfully primed. This is important for success.
  - Inspect the underside of each Pod — look for the presence of small droplets at all four fluidic ports. Droplets will vary in size from a small meniscus to larger droplets; often, droplets on the outlet ports will be larger.
  - If any Pod does not show droplets, re-run the “Prime” cycle on those Pods.
  - If any media dripped onto the tray (this may occur more often by the outlet ports), clean tray with a wipe sprayed with 70% ethanol.
10. Once it is confirmed that all Pod ports are wet with droplets, put the tray of Pods to the side in the BSC.



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## Wash Chips

1. Transfer the seeded chips in a 150 mm dish from the incubator to the BSC.
2. Remove the pipette tips from the chip inlet and outlet ports.
3. Gently wash the top channel of each chip with warm, equilibrated Complete Maintenance Medium to remove any possible bubbles in the channel.
4. Place small droplets of equilibrated Complete Maintenance Medium on the top of each inlet and outlet port of each chip.
5. Gently wash the bottom channel of each chip with warm, equilibrated Complete EGM-2MV Culture Medium to remove any possible bubbles in channel.
6. Place small droplets of equilibrated Complete EGM-2MV Culture Medium on the bottom channel inlet and outlet ports of each chip.

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### Chips to Pods

1. Holding one chip (while it remains in the Chip Carrier) in the dominant hand and one Pod in the non-dominant hand, slide the chip carrier into the tracks on the underside of the Pod until the chip carrier has seated fully.
2. Place thumb on the Chip Carrier tab and gently, but firmly, depress the tab in and up to engage the tab of the Chip Carrier with the Pod.
3. Aspirate any excess medium on the chip surface from the Pod window.
4. Place the Pod with connected the chip onto the tray.
5. Repeat steps 1 through 4 for each Pod and Chip Carrier.
6. Confirm that there is sufficient media in each Pod inlet and outlet reservoirs and that the Pod lids are flat and secure.

### Pods to Zoë

1. Place trays that are holding Pods and chips immediately into Zoë to prevent media from cooling and losing its gas equilibration.
2. Program the appropriate Organ-Chip culture conditions on Zoë. These conditions will start as soon as the Regulate cycle is complete.
3. Set top channel flow rate to 0  $\mu$ L / hr and bottom channel flow rate to 30  $\mu$ L / hr.
4. Run Regulate cycle.
  - Using the rotary dial, highlight the “Regulate” field.
  - Press the dial to select “Regulate,” and rotate the dial clockwise to “Start.”
  - Press the dial again to select “Start” and begin the Regulate cycle. Note: Once “Start” is selected, there will be an audible sound as Zoë engages the Pods.
  - At this point the “Activation” button will glow blue.
5. The Regulate cycle lasts 2 hours. After the cycle has finished, Zoë will begin flow at the preset Organ-Chip culture conditions. To cancel the Regulate cycle (only if needed) on Zoë, select the “Regulate” field with the dial and press the button to select. Rotate the dial counter-clockwise to select “Cancel.” Press the dial once more and wait 1 minute for the cycle to end, after which the tray can be removed. If cancelled, always rerun a complete Regulate cycle before proceeding.

ZOë-CM1

Pod ID

001

Prime

Ready

Regulate

Start

CHANNEL

Top

Fluid

Flow

30 uL/hr

Bottom

Fluid

Flow

30 uL/hr

Stretch

0.0%

Freq.

0.0 Hz

Left - Paused

Right - Paused

ZOë-CM1

Pod ID

001

Prime

Ready

Regulate

In Progress

60%

CHANNEL

Top

Fluid

Flow

30 uL/hr

Bottom

Fluid

Flow

30 uL/hr

Stretch

0.0%

Freq.

0.0 Hz

Left - Active

Right - Active

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## Day 5: Introduction of Air-Liquid Interface (ALI)

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### Goals:

- Introduction of ALI using Zoë

### Key Steps:

- Via wash
- Establishing ALI & Maintenance

### Required Materials:

- Chips in Pods
- Cell culture media

## Via Wash and the Regulate Cycle

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1. The day after connecting chips and Pods to Zoë, which begins the process of Organ-Chip culture, pause the Zoë by pressing the silver “Activation” button located above the tray bays. This stops flow and releases the Pods.
2. Slide the tray out of the bay and transfer to the BSC.
3. Remove the Pod lids. Using a 200  $\mu$ L pipette, perform a via wash on each Pod inlet and outlet reservoir:
  - Using media within the Pod reservoir, pipette 200  $\mu$ L of media directly over the top of the via to dislodge any bubbles that may be present.
  - Repeat this wash step for each of the four Pod reservoirs.
4. Replace Pod lids and return the trays to Zoë.



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## Prepare Culture Medium for Maintenance of Air-Liquid Interface (ALI-Medium)

### ALI-Medium

#### Complete ALI-Medium

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Medium 199	500mL	-	-	Thermo Fisher	11043023
Epidermal Growth Factor (human)		1mg / mL	10ng / mL	PromoCell	C-60170
Basic Fibroblast Growth Factor (human)		100µg / mL	3ng / mL	PromoCell	C-60243
Vascular Endothelial Growth Factor (human)		50µg / mL	0.125ng / mL	PromoCell	C-64420
Hydrocortisone		50µg / mL	1µg / mL	Sigma	H0135
Heparin		50mg / mL	10µg / mL	Sigma	H3149
di-butyryl cAMP		100mM	80µM	Sigma	B7880
L-Glutamax		200mM	1mM	Thermo Fisher	35050-061
Dexamethasone		1mM	20nM	Sigma	D4902
Penicillin Streptomycin		10,000 U / mL; 10 mg / mL	1%	Sigma	P4333
Fetal Bovine Serum		-	2%	Sigma	F4135 or F8317

- Store at 4°C.
- Use within 15 days of preparation.

### Establishing ALI & Maintenance

Note: Only establish ALI 12 or more hours after running regulate. Do not re-run Regulate cycle once ALI is established.

1. Pause Zoë by pressing the silver "Activation" button and move Pods into the biosafety cabinet.
2. Using complete aspiration technique, aspirate all media from both inlet and outlet reservoirs along all four edges of the reservoir for the top channel for all Pods that require culture at ALI. Transfer all trays and Pods back into Zoë.
3. Set top channel flow rate to 1,000 µL / hr and bottom channel to 0 µL/h. Start flow and allow to run for 1 minute. This step gently pushes any remaining media from the top channel and collects it in the top channel outlet reservoir.
4. Pause Zoë as previously described and move Pods into the biosafety cabinet. Immediately aspirate all remaining media from top channel outlet reservoir to avoid media backflow from the outlet reservoir into the channel.
5. Using the microscope, check to confirm that the top channel does not contain medium.
  - If channels are not completely free of medium, place back in Zoë and run for another 1 minute at 1,000µl / hr
6. Aspirate all medium from bottom channel inlet and outlet reservoirs, leaving a small liquid layer over the bottom inlet reservoir via. This will prevent introducing unwanted bubbles during flow.
7. Add 2-4mL of warm Complete ALI-medium to the bottom channel inlet reservoirs.

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8. Pipet 1.0 mL of media in the air channel Pod inlet reservoir first, then immediately pipet 1.0 mL of media in the air channel Pod outlet reservoir. Note: This equal media distribution in the Pod reservoirs is required to maintain static pressure in the air channel.
9. Transfer all trays and Pods back to the Zoë.
  - Set top channel to “Air.”
  - Set bottom channel flow rate to 30  $\mu$ L / hr.
10. Press the activation button to resume Zoë operation.
11. Refresh medium in bottom channel inlet reservoir every other day or as needed.

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## Day 7: Initiating Mechanical Stretch

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### Goals:

- Initiating mechanical stretch (breathing motion)

### Key Steps:

- Set up Zoë for mechanical stretch

### Required Materials:

- Chips in Pods
- Cell culture media

### Initiating Mechanical Stretch

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1. Pause Zoë and move Pods into the biosafety cabinet.
2. Using the microscope, check to confirm that the top channel does not contain medium.
3. Replenish the cell culture medium in the bottom inlet with fresh ALI medium.
4. Transfer all trays and Pods back into Zoë.
  1. Check to confirm that the top channel is set to "Air."
  2. Check to confirm that the bottom channel flow rate is set to 30  $\mu$ L / hr.
5. Move the dial to the "Stretch Field" to set mechanical stretch on the chip.
6. Rotate the dial to set the stretch to 5%.
7. Set the Frequency field to 0.20 Hz.
8. Re-start Zoë.

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## Day 8+: Sampling and Media Replenishment

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### Goals:

- Sampling and media replenishment

### Key Steps:

- Organ-Chip maintenance

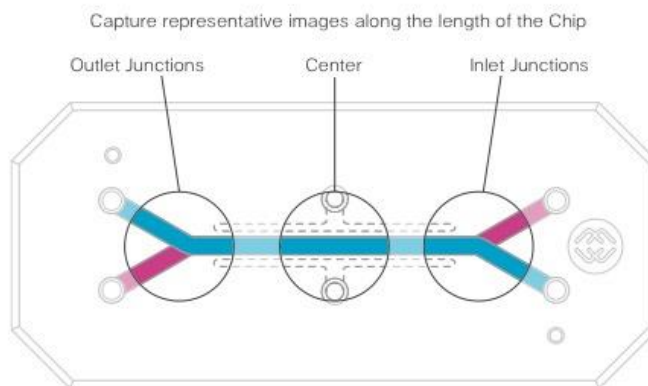
### Required Materials:

- Chips in Pods
- Cell culture media

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### Sampling and Media Replenishment

1. Pause Zoë by pressing the silver “Activation” button.
2. Remove the trays and place in the BSC.
3. Inspect each chip for bubbles by eye.
4. Using a microscope, inspect cells in the chips to assess morphology and viability. Capture representative images at 10X or 20X magnification at the following locations:
  - Inlet junction
  - Center of channel
  - Outlet junction
5. Remove the Pod lids and collect effluent medium from Pod outlet reservoirs for analysis. Collect effluent from the indicated regions, avoiding disturbing the Pod reservoir vias.
6. Gently aspirate any medium not collected for analysis, ensuring that a thin liquid film still covers the reservoir vias so that no air is introduced into the vias.
7. Refill the Pod media reservoirs with appropriate fresh cell culture medium and perform a via wash: using medium within the Pod reservoir, pipette 1 mL of medium directly over the top of the via to dislodge any bubbles that may be present.
8. Replace the Pod lids and return trays to Zoë.
9. Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions.
10. Zoë will engage when the “Activation” button glows blue.



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## Troubleshooting

Issue	Section	Step	Recommendation
<b>Bubbles are present in channel</b>	Chip Preparation	Introduce ER-1™ Solution to Channels; Activate Chips; Coat Chips with ECM	Dislodge by washing the channel with appropriate solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly reintroduce the solution.
<b>Bubbles in the ports upon introduction of media into the chip</b>	Chips to Pods and Pods to Zoë	Wash Chips	Since the chip material is hydrophobic, bubbles could get trapped at the ports. Dislodge bubbles using pipette tip or aspirate the channels and reintroduce equilibrated media.
<b>Media take too long to pass through Steriflip®</b>	Chips to Pods and Pods to Zoë	Equilibration of Media	Vacuum pressure is not reaching 70kPa. Need to find an alternate vacuum source.
<b>Pods do not prime</b>	Chips to Pods and Pods to Zoë	Prime Pods	Ensure that medium is directly over all Pod vias. If problem persists, record the Pod lot number and replace with new a Pod.
<b>Screen is frozen or unresponsive</b>	Chips to Pods and Pods to Zoë; Maintaining and Sampling	Pod to Zoë; Maintenance and Regulate	Power off Zoë and then turn Zoë on again; contact our support team if problem persists.
<b>Pods stuck in Zoë</b>	Maintaining and Sampling	Maintenance and Regulate	Pod lid not secured on Pod. Try wiggling tray to the right and left as you slide it out while keeping it level. If problem persists, contact our support team.
<b>Pods not flowing properly or evenly; Bubbles observed in chip</b>	Maintaining and Sampling	Maintenance and Regulate	There is inherent variability with Zoë, however large fluctuations and major flow issues primarily result from bubbles. Remove chip from Pod; flush chip with media; re-prime Pod with degassed media; connect chip to Pod; and run the Regulate cycle.

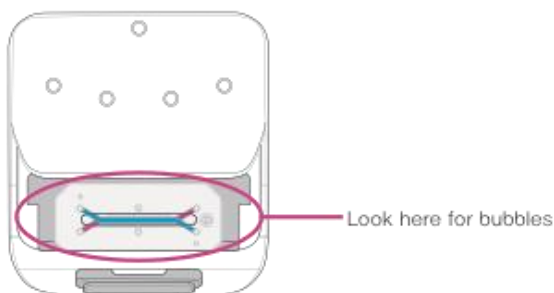
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### Potential Root Causes of Bubbles

Check the following if a high failure rate due to bubbles is observed or if bubbles are persistent, despite following the mitigation steps above.

- Medium not sufficiently equilibrated before adding to Pods: be sure to follow media preparations steps in section Gas Equilibration of Media.
- Vacuum for Steriflip® too weak: ensure that media is passing through the Steriflip® in about 10 seconds.
- Incorrect Steriflip® used: confirm correct Steriflip® unit (Millipore SE1M003M00).
- Medium not warmed correctly before Steriflip® step: Be sure to follow media preparations steps in section Gas Equilibration of Media.
- Insufficient priming: disconnect chip and reprime Pod.

Images of Bubbles in Chip



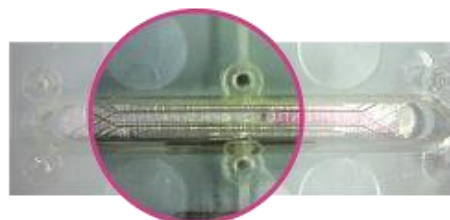
Channels filled with fluid



Small bubbles in Top Channel



Both Channels dry



Bubble plug in Top Channel



Bubble plug in Bottom Channel



Bubble plug in Top Seeding

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## Appendices

### Reagent Aliquots

Aliquot reagents prior to use to avoid multiple freeze-thaw cycles of the stock solutions.

#### Extracellular Matrix (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
<b>Fibronectin</b>	1 mg / mL	5 mg	5 mL	Cell culture grade water
<b>Laminin</b>	1 mg / mL	5 mg	5 mL	Cell culture grade water
<b>Collagen IV</b>	1 mg / mL	5 mg	5 mL	Cell culture grade water

- Resuspend ECM according to manufacturer's instructions.
- Aliquot to single-use volumes and store at -20°C (Collagen IV and Fibronectin) and -80°C (Laminin).

#### Culture Medium Supplements

Reagent	Conc. [Working]	Volume	Solvent
<b>Dexamethasone</b>	1mM	Reconstitute 25mg in 63.7008mL DMSO	Cell culture grade DMSO
<b>KGF</b>	10ug / mL	Reconstitute 10ug in 1mL PBS	PBS + 0.1% BSA
<b>cAMP</b>	100mM	Reconstitute 100mg in 2.3251mL water; sterile filter	Cell culture grade water
<b>Epidermal Growth Factor (human)</b>	1mg / mL	Reconstitute 500µg in 500µL water	Cell culture grade water
<b>Basic Fibroblast Growth Factor (human)</b>	100µg / mL	Reconstitute 50µg in 500µL water	Cell culture grade water
<b>Vascular Endothelial Growth Factor (human)</b>	50µg / mL	Reconstitute in 50mM acetic acid + 0.1% BSA	50mM Acetic acid + 0.1% BSA
<b>Hydrocortisone</b>	50µg / mL	Reconstitute 1mg in 1 mL absolute ethanol and 19 mL sterile medium	Absolute Ethanol + Sterile medium
<b>Heparin</b>	50mg / mL	Reconstitute 50mg in 1mL water	Cell culture grade water
<b>Isobutyl methylxanthine (IBMX)</b>	1M	Reconstitute 100mg in 450uL DMSO	DMSO

- Resuspend each supplement to the working concentration in the table above or manufacturer's instructions.
- Aliquot each supplement to single-use volumes.
- Store aliquots at -20°C; replace Dexamethasone and KGF aliquots every 3 months, replace cAMP aliquots every year.



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## Media Recipes

### HPAEC Seeding & Culture Medium

#### Complete SAGM Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
SABM™ Basal Medium	500 mL	-	-	Lonza	<u>CC-3119</u>
SAGM™-2 SingleQuots™ Supplement Pack		-	-	Lonza	<u>CC-4124</u>
Fetal bovine serum (FBS)	Sterile heat-inactivated	-	5%	Sigma	<u>F4135 or F8317</u>

- Store at 4°C.
- Use within 30 days of preparation.

### HMVEC-L Culture Medium

#### Complete EGM-2MV Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
EBM™-2 Basal Medium	500 mL	-	-	Lonza	<u>CC-3156</u>
EGM™-2MV SingleQuots™ Supplement Pack		-	-	Lonza	<u>CC-4147</u>

- Store at 4°C.
- Use within 30 days of preparation.

### HPAEC Maintenance Medium

#### Complete Maintenance Medium (500 mL)

Reagent	Conc. [Final]	Source	Cat. No.
SABM™ Basal Medium	-	Lonza	<u>CC-3119</u>
SAGM™- SingleQuots™ Supplement Pack	-	Lonza	<u>CC-4124</u>
Fetal bovine serum (FBS)	5%	Sigma	<u>F4135 or F8317</u>
Dexamethasone	100nM	Sigma	<u>D4902</u>
Keratinocyte Growth Factor	5 ng/mL	Thermo	<u>PHG0094</u>
Isobutyl methylxanthine (IBMX)	25µM	Sigma	<u>I7018</u>
8-Br-cAMP	50µM	Sigma	<u>B7880</u>

- Store at 4°C.
- Use within 15 days of preparation.

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## ALI-Medium

### Complete ALI-Medium (500 mL)

Reagent	Conc. [Final]	Source	Cat. No.
Medium 199	-	Thermo Fisher	11043023
Epidermal Growth Factor (human)	10ng / mL	PromoCell	C-60170
Basic Fibroblast Growth Factor (human)	3ng / mL	PromoCell	C-60243
Vascular Endothelial Growth Factor (human)	0.125ng / mL	PromoCell	C-64420
Hydrocortisone	1µg / mL	Sigma	H0135
Heparin	10µg / mL	Sigma	H3149
di-butyryl cAMP	80µM	Sigma	B7880
L-Glutamax	1mM	Thermo Fisher	35050-061
Dexamethasone	20nM	Sigma	D4902
Penicillin Streptomycin	1%	Sigma	P4333
Fetal Bovine Serum	2%	Sigma	F4135 or F8317

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