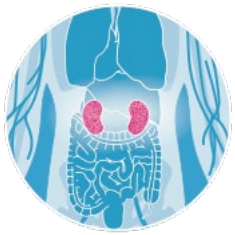
emulate



Proximal Tubule Kidney-Chip Co-Culture Protocol

May 09, 2024

EP-228 Rev. D

Proximal Tubule Kidney-Chip Co-Culture Protocol

Overview

Introduction

This protocol described the general steps for using the Emulate Kidney-Chip S1 BioKit.

Contents

Part I. Emulate Kidney-Chip S1 BioKit

Overview

Introduction

This part provides an overview of the Emulate Kidney-Chip S1 BioKit as well as its key components, shipping information, and storage specifications.

Components

The Emulate Kidney-Chip S1 BioKit includes the pre-qualified primary human kidney cells listed in the table below.

Category	Channel Location	Type of Cells
Human Renal Proximal Tubule Epithelial Cells (hRPTECs)	Top	• Epithelial Cells
Human Glomerular Microvascular Endothelial Cells (hGMVECs)	Bottom	• Endothelial Cells

Cell Shipping

Cells are shipped in cryogenic storage vacuum flasks.

Cell Storage

Always store cryopreserved cells in liquid nitrogen. Never store them in dry ice or an -80°C freezer. Chronic temperature fluctuations can cause severe damage to cell membranes and the cytoskeletal components.

Part II. Experimental Overview

Overview

Introduction

This section gives an overview of the experimental workflow.

Day X: Reagent Preparation

- Aliquot reagents (ECM, Matrigel, etc.)

Day -5: Thaw Cells

- Thaw human Glomerular Microvascular Endothelial Cells (hGMVECs)
- Thaw human Renal Proximal Tubule Epithelial Cells (hRPTECs)

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: Seeding hGMVECs and hRTPECs into Chips

- Prepare the necessary cell culture media
- Prepare chips
- Prepare hGMVECs for seeding
- Seed hGMVECs to the bottom channel
- Flip chips upside-down using Chip Cradle
- Allows cells to attach (2.5 h post-seeding)
- Gravity wash bottom channels
- Seed hRPTECs to top channel
- Allow cells to attach (2.5 h post-seeding)
- Gravity wash chips

Continued on next page

Overview, Continued

Day 1: Chips to Pods, and Pods to Zoë

- Gas equilibrate media
- Prime Pods
- Wash Chips
- Chips to Pods
- Pods to Zoë

Day 2+:

- Maintenance and the Regulate Cycle
- Sampling and Media Replenishment

Part III. Equipment and Materials Required

Overview

Introduction

Ensure all equipment, materials, and reagents are accessible prior to each experiment. Below are the catalog numbers for the specific equipment, consumables, and materials needed.

Note on Catalog Numbers

Exact catalog numbers are provided for specific materials required for successful experiments.

Required Equipment and Consumables

A list of equipment and consumables needed for this protocol in addition to the Emulate Kidney-Chip S1 BioKit is provided below:

Equipment	Description	Supplier	Catalog Number
Emulate Kidney-Chip S1 BioKit	12- or 24-pack	Emulate	BIO-KH-CO12 BIO-KH-CO24
Zoë-CM2® Culture Module	1 per 12 chips	Emulate	ZOE-CM2
Orb-HM1® Hub Module	1 per 4 Zoës	Emulate	ORB-HM1
UV Light Box	1 per Zoë	Emulate	UVLamp
Chip Cradle	Autoclaved, 1 per 6 chips	Emulate	CHIP-CRD
Steriflip®-HV Filters	Sterile, 0.45 µm PVDF filter	EMD Millipore	SE1M003M00
Square Cell Culture Dish (120 x 120 mm)	Sterile, 1 per 6 chips	VWR	82051-068
Collagen type-1 coated plates	24-well, flat-bottom, TC-treated	Corning	356408
Handheld vacuum aspirator	-	Corning	4930
Aspirating pipettes	2-mL, polystyrene, individually wrapped	Corning / Falcon	357558
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, filter, low-adhesion	-	-
Conical tubes	15-mL and 50-mL polypropylene, sterile	-	-
Eppendorf Tubes®	15-mL, sterile	-	-
Aluminum foil	-	-	-
Parafilm®	-	-	-
Microscope (with camera)	For bright-field imaging	-	-
Hemocytometer	-	-	-
Manual Counter	-	-	-
Water bath (or beads)	-	-	-
Vacuum set-up	Minimum pressure: -70 kPa	-	-
T75 flasks	-	-	-
Ice bucket	-	-	-
70% ethanol and wipes	For surface sterilization		

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Overview, Continued

Required Materials

A list of reagents, media, and supplements needed for this protocol in addition to the Emulate Kidney-Chip S1 BioKit provided below:

Reagent	Description	Supplier	Catalog Number
ER-1™ Reagent	5-mg powder	Emulate	ER105
ER-2™ Reagent	25-mL bottle	Emulate	ER225
Dulbecco's PBS (DPBS -/-) (without Ca^{2+}, Mg^{2+})	1X	Corning	21-031-CV
Trypan blue	0.4% solution	Sigma	93595
Trypsin-EDTA Solution	0.05% Trypsin	Sigma	T3924
REGM™ Renal Epithelial Cell Growth Medium BulletKit™	Epithelial Growth Medium & Supplements	Lonza	CC-3190
REBM™ Basal Medium	Base Epithelial Grown Medium	Lonza	CC-3191 (part of CC-3190)
REGM™ SingleQuots™ (Kit)	Supplements and Growth Factors	Lonza	CC-4127 (part of CC-3190)
Normal Blood Glucose Level Without Serum (Kit)	Endothelial Medium & Supplements	Cell Systems	4N3-500-R
Culture boost™	50X supplement	Cell Systems	4CB-500-R (part of 4N3-500-R)
Attachment Factor™	1X	Cell Systems	4Z0-210 (part of 4N3-500-R)
Matrigel®	LDEV-free	Corning	354234
Collagen type IV	5 mg powder	Sigma	C5533
Penicillin-streptomycin	10,000 U / mL; 10 mg / mL	Sigma	P4333
Fetal bovine serum (FBS)	Sterile, heat-inactivated	Sigma	F4135 or F8317

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Overview, Continued

Notes for ER-1 and ER-2

- Upon arrival, store the ER-1 powder unopened in the metalized pouch at -20°C, protected from light and humidity.
- Upon arrival, store the ER-2 solution at 4°C.
- Both ER-1 and ER-2 reagents should be discarded if stored at room temperature for over 3 weeks, as this can compromise the performance of the reagents.
- If additional ER-1 and ER-2 are needed, they can be purchased separately from Emulate using the product information in the table above.

Part IV. Workstation Preparation and Chip Handling Techniques

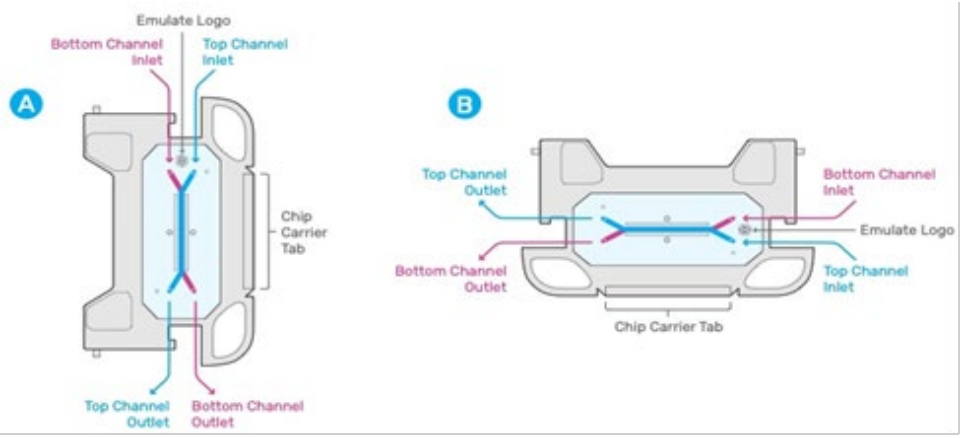
Workstation Preparation

Aseptic Techniques

- Always work with chips in a sterile environment, such as the biosafety cabinet (BSC).
- Before beginning the experiment, prepare, sanitize, and organize the work surface of the BSC. Arrange tips, media, and other necessary materials in the sterile field so they are easily within reach but not blocking the path of airflow.
- Use 70% ethanol to thoroughly sanitize all reagents and materials before placing them into the BSC.
- Always avoid touching the chip directly. Handle the chip carrier only by the sides, or by the tab, with gloves.
- Do not remove chips from the chip carrier until after the experiment.

Chip Handling Techniques

Possible Chip Orientations



Orientation A	Orientation B
The bottom channel inlet will be on the top left of the chip, while the top channel inlet will be on the top right of the chip. Conversely, the bottom channel outlet will be on the bottom right of the chip, while the top channel outlet will be on the bottom left of the chip.	The bottom channel inlet will be on the top right of the chip, while the top channel inlet will be on the bottom right of the chip. Conversely, the bottom channel outlet will be on the bottom left of the chip, while the top channel outlet will be on the top left of the chip.

Pipetting

- While 50 µL (top channel) and 20 µL (bottom channel) are the standard volumes used throughout the protocol, there can be some flexibility in the actual volumes used:

Channel	Volume Range
Top Channel	35–50 µL
Bottom Channel	15–20 µL

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 µL of the specific wash solution.

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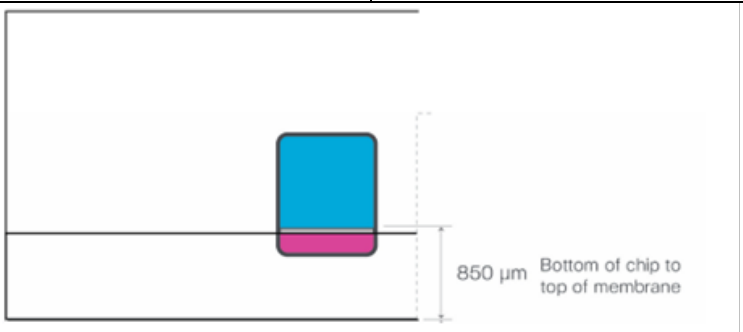
Chip Handling Techniques, Continued

Channel and Membrane Dimensions

The specific channel and membrane dimensions are outlined below:

Top Channel	
Width x Height Dimensions	1000 µm x 1000 µm
Area	28.0 mm^2
Volume	28.041 µL
Imaging distance from the bottom of the chip to the top of the membrane	850 µm
	
Bottom Channel	
Width x Height Dimensions	1000 µm x 200 µm
Area	24.5 mm^2
Volume	5.6 µL
Membrane	
Pore diameter	7.0 µm
Pore spacing	40 µm (hexagonally packed)
Thickness	50 µm
Co-Culture Region	
Area	17.1 mm^2

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Chip Handling Techniques, Continued

Pipetting Solution into Channels

Follow the steps below for pipetting solution into the Organ-Chip when coating, washing, and seeding cells prior to attaching the chip to Zoë.

Note: Always introduce liquid to the endothelial channel before the epithelial channel.

Step	Action
1	Take a P200 pipette with a sterile pipette tip and collect the solution to be added to the Organ-Chip.
2	Place the pipette tip perpendicular to the chip channel inlet, ensuring that the tip is securely in the port.
3	Steadily dispense the liquid through the channel.

Part V. Proximal Tubule Kidney Co-Culture Protocol

Protocol Overview

Introduction

This section lists the basic steps for using Proximal Tubule Kidney-Chips in experiments.

Timeline

Day X: Reagent Preparation

Aliquot Reagents

Introduction

Aliquot reagents prior to use so the stock solutions do not undergo multiple freeze-thaw cycles.

Collagen-IV (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Collagen-IV	1 mg / mL	5 mg	5 mL	DPBS

- Resuspend 5 mg collagen-IV in 5 mL of DPBS according to manufacturer's instructions.

- Create single-use volume aliquots and store them at -20°C.

Matrigel

Aliquot reagents including media supplements and ECM prior to use and store appropriately to avoid multiple freeze-thaw cycles.

Reagent	Conc. [Stock]	Conc. [Final]
Matrigel®	Varies per lot	100 µg / mL

The Matrigel bottle must be thawed overnight on ice either in the back of the 2–6°C refrigerator or in a cold room. Add water to ensure the ice is slushy, as the solution gels rapidly at temperatures above 10°C. Before aliquoting, use pipettes, tips, and tubes prechilled to -20°C.

- After the Matrigel is thawed, aliquot Matrigel to 100 µL aliquots (the specific concentration of the Matrigel solution (varies per lot) on each vial for reference when coating chips. The Matrigel concentration can be found on the product quality certificate).
- Aliquoting volumes of Matrigel smaller than 100 µL is not recommended due to the low accuracy associated within pipetting viscous solutions.

- Store aliquots at -20°C.

Day -5: Thaw hGMVECs and hRPTECs

Overview

Goals

- Thaw and expand hGMVECs and hRPTECs in flasks prior to seeding in chips.

Required Materials

- Complete hGMVEC culture medium (at 37°C)
- Complete hRPTEC culture medium (at 37°C)
- 15 mL conical tube
- Attachment Factor™
- T-75 flask
- Serological pipettes
- Pipettes and tips
- Aspirator
- Centrifuge
- 70% ethanol

Key Steps

Prepare hGMVEC Culture Media and Flask

hGMVEC Culture Media

Base hGMVEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Normal Blood Glucose Level Without Serum	485 mL	-	-	Cell Systems	4N3-500-R
Culture-boost-R	10 mL	-	2%	Cell Systems	4CB-500-R
Pen / strep	5 mL	-	1%	Sigma	P4333

- Store the Base hGMVEC Culture Media at 4°C.
- Use the Base hGMVEC Culture Media within 30 days of preparation.

Complete hGMVEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hGMVEC Culture Medium	45 mL	-	-	Recipe Above	-
FBS	5 mL	-	10%	Sigma	F4135

- Store the Complete hGMVEC Culture Medium at 4°C.
- Use the Complete hGMVEC Culture Medium within 7 days of preparation.

Prepare Flask

Step	Action
1	Warm a sufficient amount of Complete hGMVEC Culture Medium and Attachment Factor™ to 37°C. 15 mL of media and 5 mL of attachment factor are needed (see Step 3).
2	Label the culture flask with the relevant information (e.g., cell type, passage number, date, initials).
3	Pipette Attachment Factor onto the growth surface of the flask until it is fully covered. 5 mL of Attachment Factor is used for each T75 flask.
4	Place the prepared flask into the 37°C incubator for at least 10 seconds to coat the surface. Maintain this temperature until the cells are plated.

Thaw and Plate hGMVECs

Thawing and Maintaining Cells

Step	Action
1	Immerse the vial(s) of cells in a 37°C water bath without submerging the cap. Closely observe and gently agitate the vials. Remove them from the water bath just before the last of the ice disappears.
2	Spray the vial(s) with 70% ethanol and wipe them dry before placing them into the BSC.
3	Immediately transfer the contents of the vial(s) into a sterile 15-mL conical tube containing 3 mL of warm Complete hGMVEC Culture Medium.
4	Rinse the vial(s) with 1 mL of Complete hGMVEC Culture Medium and collect the run-off in the 15-mL tube.
5	Bring the volume to 15 mL with Complete hGMVEC Culture Medium.
6	Centrifuge 200 x g for 5 minutes at room temperature.
7	Aspirate and discard the supernatant, leaving approximately 100 µL of medium covering the cell pellet.
8	Loosen the cell pellet by gently flicking the tube.
9	Re-suspend cells in 15 mL of Complete hGMVEC Culture Medium.
10	Aspirate and discard excess Attachment Factor from the T75 flask that was prepared. **Note:** It is unnecessary to rinse or dry the flask prior to adding cells.
11	Add the hGMVEC suspension to the freshly coated T75 flask.
12	Incubate overnight at 37°C and 5% CO_2.
13	Exchange the Complete hGMVEC Culture Medium the following day (Day -4) and again on Day -2. If seeding takes place on Day 0, no further changes are needed.

Prepare hRPTEC Culture Media and Flask

hRPTEC Culture Media

Base hRPTEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No
REBM™ Renal Epithelial Cell Growth Basal Medium	492 mL	-	-	Lonza	CC-3191
REGM™ SingleQuots™ Kit containing:				Lonza	CC-4127
• Human Epidermal Growth Factor (hEGF)	0.5 mL	-	-	-	-
• Insulin	0.5 mL	-	-	-	-
• Hydrocortisone	0.5 mL	-	-	-	-
• Transferin	0.5 mL	-	-	-	-
• Triiodothyronine	0.5 mL	-	-	-	-
• Epinephrine	0.5 mL	-	-	-	-
Pen / Strep	5 mL	-	1%	Sigma	P4333

- Store Base hRPTEC Culture Media at 4°C.
- Use Base hRPTEC Culture Media within 30 days of preparation.

Note: Due to its nephrotoxic nature, gentamicin sulfate from the REGM™ SingleQuots™ Supplement Pack should not be used. If only one bottle of REBM™ Renal Epithelial Cell Growth Basal Medium will be used, omit the hEGF from the base hRPTEC Culture Medium and add it accordingly to prepare the Complete hRPTEC Culture Media. This is because, on day 1, the hEGF will need to be diluted to one-tenth of its original concentration in order to prepare the Complete hRPTEC Maintenance Medium.

Complete hRPTEC Culture Media (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hRPTEC Culture Medium	49.75 mL	-	-	Recipe Above	-
FBS	0.25 mL	-	0.5%	Lonza (from kit above)	CC-4217

- Store Complete hRPTEC Culture Media at 4°C

- Use Complete hRPTEC Culture Media within 7 days of preparation.

Prepare hRPTEC Culture Media and Flask, Continued

Prepare Flask

Step	Action
1	Warm 15 mL of Complete hRPTEC Culture Medium to 37°C.
2	Label the culture flask with the relevant information and place it into the 37°C incubator to pre-warm the T75 flask.

Thaw and Plate hRPTECs

Thawing and Maintaining Cells

Step	Action
1	Thaw the frozen vial(s) of cells by immersing in a 37°C water bath, without submerging the cap. Closely observe while gently agitating and remove from the water bath just before the last of the ice disappears.
2	Once only a small ice pellet remains, immediately remove the vial(s) from the water bath, wipe them dry, spray the vial(s) with 70% ethanol, wipe them dry again, and place them into the BSC.
3	Immediately transfer the contents of the vial(s) into a sterile 15-mL conical tube containing 3 mL of warm Complete hRPTEC Culture Medium.
4	Rise the vial with 1 mL of warm Complete hRPTEC Culture Medium and collect the run-off in a 15-mL tube.
5	Bring the volume to 15 mL with warm Complete hRPTEC Culture Medium—**Do Not Centrifuge.**
6	Add the hRPTEC suspension to the pre-warmed T75 flask.
7	Incubate overnight at 37°C and 5% CO_2.
8	Exchange the Complete hRPTEC Culture Medium the following day (Day -4) and again on Day -2. If seeding takes place on Day 0, no further changes are needed.

Day -1: Chip Preparation

Overview

Goals

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

Required Materials

- Chip-S1 Stretchable Chips (12 per Zoë)
- ER-1 reagent
- ER-2 buffer
- 15 mL conical tubes
- DPBS (- / -) at room temperature
- DPBS (- / -) aliquot at 4°C
- Collagen IV (aliquot at 4°C on ice)
- Matrigel (aliquot at 4°C on slushy ice)
- 70% ethanol
- 120 x 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

Key Steps

Prepare Chips

Steps

Step	Action
1	Spray the chip packaging, Chip Cradle, and 120 x 120-mm square dish with 70% ethanol before bringing them into the BSC.
2	Open the packaging, place the Chip Cradle in the dish, and then carefully insert 6 chips into the Chip Cradle (see Figure 1). **Note:** For ease of workflow, ensure the carrier's tab is pointing to the right and that all chips are facing the same direction within the dish.
	 Figure 1. Proper insertion of chip and chip carrier into Chip Cradle
3	Label each chip carrier tab with the corresponding chip's ID number.

Prepare ER-1 Reagent

CAUTION

ER-1 is light sensitive. Prepare ER-1 solution immediately before use and discard any remaining ER-1 solution 1 hour after reconstitution. Using ER-1 solution that has been exposed to light or that has not been freshly prepared will lead to chip failure.

Before You Begin

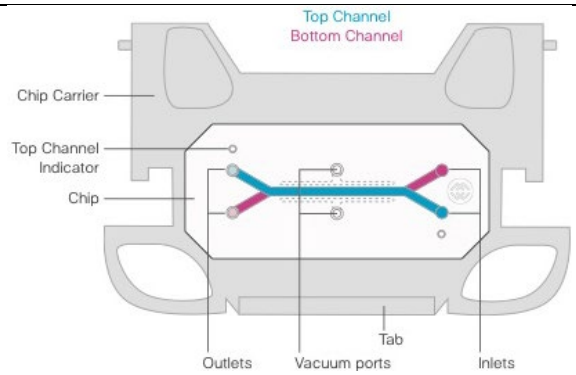
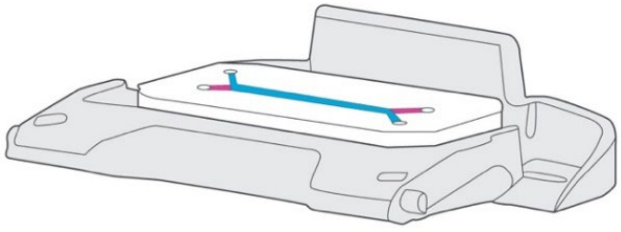
- For complete activation, prepare ER-1 immediately before use, and discard any remaining solution 1 hour after reconstitution.
- **Note:** ER-1 is an eye irritant and must always be handled in the BSC with proper gloves and eye protection.

Steps

Step	Action
1	Turn off the light in the BSC and allow the ER-1 and ER-2 to reach room temperature before use (approximately 10–15 minutes).
2	Wrap an empty, sterile, 15-mL conical tube with foil to protect it from light.
3	In the BSC, remove the small vial of ER-1 powder from the packet. Tap the vial to concentrate the powder at the bottom.
4	Add 1 mL of ER-2 to the vial and transfer the contents directly to the bottom of the 15-mL conical tube. Do not pipette to mix.
5	Add 1 mL of ER-2 to the ER-1 vial to collect any remaining material and transfer the solution directly to the 15-mL conical tube. **Note:** The color of the transferred ER-1 solution will become lighter each time the bottle is washed.
6	Repeat Step 5 two more times, adding another 1 mL of ER-2 each time.
7	On the last ER-2 addition, cap and invert the bottle to collect any remaining ER-1 powder in the lid. Transfer the collected solution to the conical tube, bringing the total volume in the tube to 4 mL of ER-1 solution.
8	Add 6 mL of ER-2 solution to the 4 mL of ER-1 solution in the 15 mL conical tube for a final volume of 10 mL (working concentration of 0.5 mg / mL). Gently pipette the solution to mix it without creating bubbles. The ER-1 should be fully dissolved within the ER-2 solution prior to use.

Introduce ER-1 Solution to Channels

Steps

Step	Action
1	Using a P200 pipette and a sterile 200-µL filtered tip, draw 200 µL of ER-1 solution. **Note:** 200 µL of ER-1 solution will fill approximately 3 chips.
2	Carefully introduce approximately 20 µL of ER-1 solution to the bottom channel inlet until it begins to exit the outlet (see Figure 2).
	 Figure 2. Top view of chip, with labelled ports, in the chip carrier.
3	Without releasing the plunger, take the pipette out from the bottom channel inlet, and move it to the top channel inlet.
4	Introduce approximately 50 µL of ER-1 solution to the inlet until it begins to exit the outlet.
5	Gently aspirate all excess ER-1 solution from the surface. Be sure to only remove ER-1 solution from the chip surface—do not aspirate any solution from the channels (see Figure 3).
	 Figure 3. Chip in chip carrier with no excess ER-1 on the surface.
6	Repeat Steps 1–5 for each chip.

7	Inspect the channels for bubbles prior to UV activation. Dislodge any bubbles by washing the channel with ER-1 solution. If bubbles persist, aspirate the channel dry and slowly re-introduce the ER-1 solution.

Activate and Wash Chips

Steps

Step	Action
1	Bring the Square Cell Culture Dish (120 x 120 mm) containing the ER-1-coated chips to the UV light box.
2	Remove the cover from the dish. Place the open dish into the UV light box.
3	Set the switch at the back of the UV light box to “Constant.” Turn on the power and press the “On” button to begin UV activation.
4	Allow the chips to activate under UV light for 15 minutes.
5	While the chips are being treated, prepare the ECM solution. (For more information, refer to the next section, “Prepare ECM Solution.”)
6	After UV treatment, bring chips back to the BSC. **Note:** The BSC light may be on from this point forward.
7	Fully aspirate the ER-1 solution from both channels.
8	Wash each channel with 200 µL of ER-2.
9	Fully aspirate the ER-2 from the channels.
10	Wash each channel with 200 µL of sterile cold DPBS. Aspirate excess DPBS from the surface.
11	Leave cold DPBS inside the channels.

Prepare ECM Solution

Before Beginning

Prepare fresh ECM before each use by combining the individual ECM components with cold DPBS to reach the final working concentrations. The ECM solution will coat both channels.

Needed Volumes

For human Kidney-Chips, the ECM working concentrations are:

Reagent	Concentration
Collagen-IV	50 µg / mL
Matrigel	100 µg / mL

Steps

Step	Action
1	Bring a full ice bucket to the BSC.
2	Thaw one aliquot of Collagen IV (1 mg / mL) on slushy ice. Always maintain each ECM component and mixture on ice.
3	Calculate the volume of ECM solution needed to coat all chips. 1. Volume required per chip: ~100 µL 2. For every batch of 12 chips, prepare 1.5 mL of ECM solution: 12 chips x 100 µL / chip = 1.2 mL of ECM solution. 1.2 mL + extra 300 µL = **1.5 mL of ECM solution**.
4	Combine the components to prepare the ECM working solution.
5	Keep the ECM solution on ice until it is used.

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Prepare ECM Solution, Continued

Example ECM Calculation

ECM Calculation Example:

Solution	Concentration
Collagen IV stock concentration	1 mg / mL (C_1)
Collagen IV final concentration	0.05 mg / mL (C_2)
Matrigel stock concentration	10 mg / mL (C_1)
Matrigel final concentration	0.1 mg / mL (C_2)
Stock Volume	Collagen IV (X) or Matrigel (Y) (V_1)
Final volume of ECM solution	1.5 mL (V_2)

Collagen IV Calculation:

(1 mg / mL) x (X mL) = (0.05 mg / mL) x (1.5 mL)

X = 75 µL of collagen IV stock solution

Matrigel Calculation:

(10 mg / mL) x (Y mL) = (0.1 mg / mL) x (1.5 mL)

Y = 15 µL of Matrigel stock solution

DPBS Calculation

Volume DPBS =

(total volume of ECM needed) – (volume of collagen IV) – (volume of Matrigel)

= 1500 µL – 75 µL – 15 µL

= 1410 µL of DPBS

Coat Chips with ECM

Steps

Step	Action
1	Fully aspirate the cold DPBS from both channels.
2	Using a P200 pipette, draw 100 µL of ECM solution. (Each chip will use 100 µL)
3	Carefully introduce ECM solution through the bottom channel inlet until a small droplet forms on the outlet.
4	Without releasing the plunger, move the pipette containing the remaining ECM solution to the top channel inlet.
5	Introduce ECM solution through the inlet, leaving small droplets of excess ECM solution on both ports in both channels (see Figure 4).

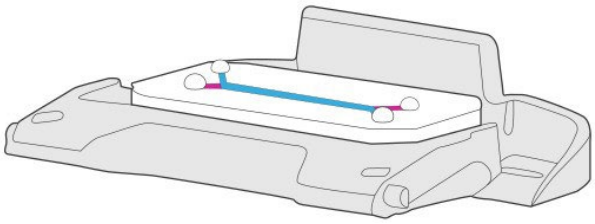


Figure 4. Chip in chip carrier with small ECM droplets at ports.

6	If bubbles are present, wash them from the channel with the ECM solution.
7	Repeat steps 1–6 for each chip.
8	To prevent evaporation during incubation, fill the central reservoir with 0.75-1 mL of DPBS (see Figure 5). Place the lid onto the dish and incubate overnight at 37°C.

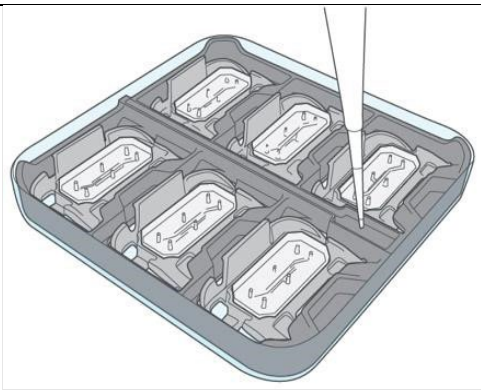


Figure 5. Pipette filling central reservoir of Chip Cradle with DPBS.

9	For best results, incubate the chips at 4˚C overnight, then at 37˚C for at least 1 hour the following day prior to cell seeding. **Note:** Chips can be stored at 4°C for up to 2 days if kept moist.

Day 0: Seeding hGMVECs and hRPTECs into Chips

Overview

Goals

- Harvest hGMVECs and hRPTECs from flasks and seed them into the chips.

Required Materials

- Complete hGMVECs Culture Medium (at 37°C)
- Complete hRPTECs Culture Medium (at 37°C)
- 1X DPBS (at room temperature)
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 50 mL conical tubes
- Trypan Blue Cell Counting Solution
- Hemocytometer
- 70% ethanol
- Microscope
- Trypsin-EDTA

Key Steps

Prepare Complete hGMVEC and hRPTEC Culture Medium

Complete hGMVEC and hRPTEC Culture Medium

Complete hGMVEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hGMVEC Culture Medium	45 mL	-	-	Recipe	-
FBS	5 mL	-	10%	Sigma	F4135

- Store the Complete hGMVEC Culture Medium at 4°C.
- Use the Complete hGMVEC Culture Medium within one week of preparation.

Complete hRPTEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hRPTEC Culture Medium	49.75 mL	-	-	Recipe	-
FBS	0.25 mL	-	0.5%	Sigma	F4135

- Store the Complete hRPTEC Culture Medium at 4°C.
- Use the Complete hRPTEC Culture Medium within one week of preparation.

Prepare Chips

Steps

Step	Action
1	Transfer the ECM-coated chips from the incubator into the BSC.
2	Fully aspirate the ECM from both channels.
3	Pipette 200 µL of warm Complete hGMVEC Culture Medium to the bottom and top channels of each chip. Wash the channel by aspirating the outflow, leaving media in the channel.
4	
5	Cover the Square Cell Culture Dish (120 x 120 mm) and place the chips into the incubator until the cells are ready for seeding.

Harvest hGMVECs

Before You Begin

- hGMVECs in culture must be harvested and counted for bottom channel seeding. hGMVECs are adjusted to a density of 3.0 x 10^6 cells / mL prior to seeding the bottom channel.
- If the hGMVECs are not as proliferative as expected, the concentration can be increased up to 4 x 10^6 cells / mL to achieve a confluent monolayer within the channel.

Steps

Step	Action
1	Bring the culture flask containing hGMVECs from the incubator into the BSC.
2	Aspirate culture media and add 15 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.
3	Add 3 mL of trypsin-EDTA to the flask. Incubate for 2 to 3 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 9 mL of warm Complete hGMVEC 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 (12 mL) into a sterile 15-mL conical tube.
7	Add 3 mL of warm Complete hGMVEC Culture Medium to bring the total volume of the tube to 15 mL.
8	Centrifuge hGMVECs at 200 x g for 5 minutes at room temperature.
9	While the cells are in the centrifuge, prepare Trypan Blue Cell Counting Solution in a 1.5 mL tube: **Trypan Blue Cell Counting Solution (45 µL)** Reagent: Complete hGMVEC Culture Medium — Volume: 40 µL Reagent: Trypan Blue — Volume: 5 µL

10	Aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. **Note:** Aspirate carefully, as the cell pellet will be very small.
11	Gently flick the tube to loosen the cell pellet.
12	Using a P1000 pipette, gently resuspend the cells by adding 400 µL of warm Complete hGMVEC Culture Medium.
13	Pipette gently to create a homogeneous mixture and transfer 5 µL of the cell suspension to the Trypan Blue Cell Counting Solution. (This will make a 1:10 dilution).

Cell Counting and Viability Assessment

Cell Counting and Viability Assessment

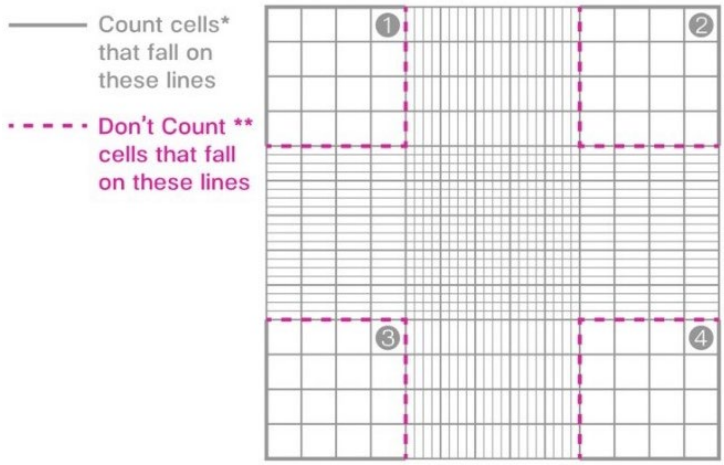


Figure 6. Example hemocytometer and cell counting.

1. Count both viable and non-viable cells in each quadrant of the hemocytometer (see Figure 6).

   Live Cell Count, Dead Cell Count, Total Cell Count.

2. Calculate the percentage viability of the cell solution.

   Live Cells ÷ Total Cells x 100 = % Viability

3. Calculate the viable cell concentration. The dilution factor is 10 when prepared in the Trypan Blue Cell Counting Solution above.

   (Live Cell Count x 10 x 10^4) ÷ 4 = Viable Cell Concentration (cells / mL)

4. Calculate the viable cell yield.

   Viable Cell Concentration ÷ Cell Suspension Volume = Viable Cell Yield (cells)

5. **Viable Cell Yield ÷ Desired Density = Reconstitution Volume**

Diluting hGMVECs

After calculating the Viable Cell Yield, dilute the hGMVECs with warm Complete hGMVEC Culture Medium to the required final cell density of 3.0 x 10^6 cells / mL.

Seed hGMVECs to the Bottom Channel

Before You Begin

Work with one chip at a time. After seeding the first chip, use a microscope to assess the cell density within the channel. Adjust the density of the cell suspension as necessary for the rest of the chips.

Steps

Step	Action
1	Bring the square dish containing the prepared chips to the BSC.
2	Without touching the ports, carefully aspirate excess medium droplets from the surface of one chip.
3	Very gently agitate the cell suspension to ensure homogeneity before seeding each chip.
4	Quickly and steadily pipette 15–20 µL of the cell suspension (at 3.0 x 10^6 cells / mL) into the bottom channel inlet port while aspirating the outflow fluid from the surface. Do not directly touch the outlet port. **Note:** The rapid injection technique will provide homogeneous cell distribution throughout the culture area in the channel.
5	Cover the dish and transfer it to the microscope to check the seeding density within the chip (see Figure 7).

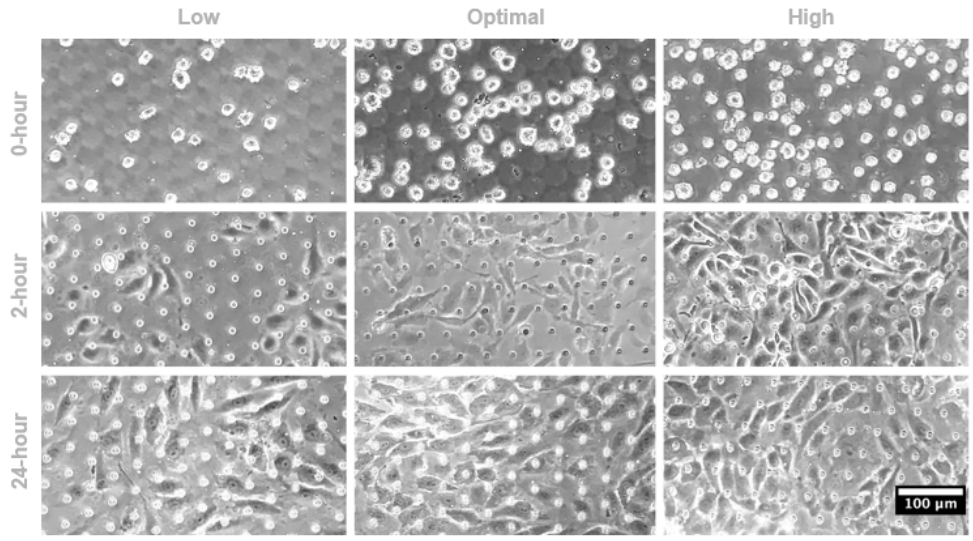
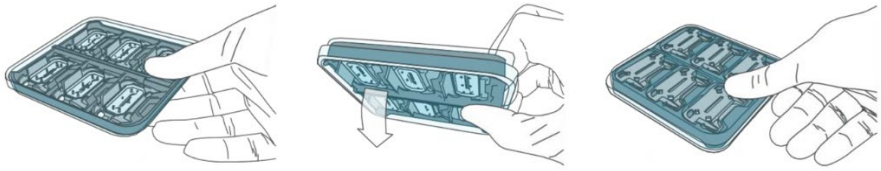


Figure 7. 10X brightfield images showing optimal hGMVEC density and attachment.

<table>
<tr><td>6</td><td>If the seeding density is not optimal, follow these steps:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Return the chips to the BSC</td></tr>
<tr><td>2</td><td>Using a P200 pipette, aspirate the hGMVECs through the outlet port. Then, dispose of the pipette.</td></tr>
<tr><td>3</td><td>Wash the channel with 200 µL of fresh medium twice. Aspirate the outflow.</td></tr>
<tr><td>4</td><td>Repeat steps 3–5 until the correct density is achieved within the channel.</td></tr>
</table></td></tr>
<tr><td>7</td><td>After confirming the correct cell density, seed cells in the remaining chips. Then, cover the 120 x 120-mm square dish and flip the dish (see Figure 8). Add 0.75 mL DPBS to the reservoir on the chip cradle (see Figure 5).
Note: Minimize the amount of time the cells are outside the incubator by seeding batches of no more than 12 chips at a time and by immediately placing the batches into the incubator at 37°C.</td></tr>
<tr><td colspan="2"> 
Figure 8. Inverting chips during endothelial seeding for cell attachment to the ECM-coated membrane.</td></tr>
<tr><td>8</td><td>Place the chips (with the DPBS reservoir) at 37°C for 2.5 hours.</td></tr>
<tr><td>9</td><td>Once hGMVECs have attached (approximately 2.5 hours post-seeding), aspirate DPBS from reservoir, and flip the dish back so that chips are in the upright position.</td></tr>
<tr><td>10</td><td>With a P200 pipette, gently wash the bottom channel with 200 µL of Complete hGMVEC Culture Medium and the top channel with 200 µL of Complete hRPTEC Culture Medium. Return the chips to the incubator until ready to seed the hRPTECs in the top channel.</td></tr>
</table>

Seed a Well Plate

Before You Begin

It is recommended to always seed any remaining hGMVECs into a conventional well-plate as a control for cell quality. If desired, transwells can be used as controls.

Steps

Step	Action
1	Once the chips have been seeded, dilute the remaining hGMVECs to a final cell density of 1.6 X 10^5 cells / mL in Complete hGMVEC Culture Medium.
2	Add 500 µL of cell suspension to each well of a 24-well plate.
3	In the 37°C 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 three times while keeping it flat on the surface of the incubator.
4	Finally, move the plate in a crisscross pattern at least three times to evenly disperse the cells. Once the cells are dispersed, do not disturb the plate until the next day to allow for cells to fully attach.

Harvest hRPTECs

Before You Begin

Work with one chip at a time. After seeding the first chip, use a microscope to assess the cell density within the channel. Adjust the density of the cell suspension as necessary for the rest of the chips.

Steps

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Bring the culture flask containing hRPTECs from the incubator into the BSC.</td></tr>
<tr><td>2</td><td>Aspirate the culture media and add 15 mL of 1X DPBS to wash the culture surface. Aspirate the DPBS wash.</td></tr>
<tr><td>3</td><td>Add 3 mL of trypsin-EDTA to the flask. Incubate for 2 to 3 minutes at 37°C.</td></tr>
<tr><td>4</td><td>Tap the side of the flask gently and inspect the culture under the microscope to assess complete detachment of cells from the culture surface.</td></tr>
<tr><td>5</td><td>Add 9 mL of warm Complete hRPTEC Culture Medium to the flask and pipette gently to mix while collecting all cells from the culture surface.</td></tr>
<tr><td>6</td><td>Transfer the contents of the flask (12 mL) into a sterile 15-mL conical tube.</td></tr>
<tr><td>7</td><td>Add 3 mL of warm Complete hRPTEC Culture Medium, bringing the total volume of the tube to 15 mL.</td></tr>
<tr><td>8</td><td>Centrifuge hRPTECs at 200 x g for 5 minutes at room temperature.</td></tr>
<tr><td>9</td><td>While the cells are in the centrifuge, prepare Trypan Blue Cell Counting Solution in a 1.5 mL tube:
<table>
<tr><th>Reagent</th><th>Volume</th></tr>
<tr><td>Complete hRPTEC Culture Medium</td><td>40 µL</td></tr>
<tr><td>Trypan Blue</td><td>5 µL</td></tr>
</table></td></tr>
<tr><td>10</td><td>Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet.
Note: The cell pellet will be very small, so aspirate carefully.</td></tr>
<tr><td>11</td><td>Loosen the cell pellet by flicking the tube gently.</td></tr>
</table>

12	Using a P1000 pipette, gently resuspend the cells by adding 400 µL of warm Complete hRPTEC Culture Medium.
13	Pipette gently to create a homogenous cell mixture and transfer 5 µL of the cell suspension to the Trypan Blue Cell Counting Solution. This will make a 1:10 dilution.
14	Mix the counting solution thoroughly and count the cells using a hemocytometer (See “Cell Counting and Viability Assessment”).

Seed hRPTECs to the Top Channel

Before You Begin

Work with one chip at a time. After seeding the first chip, use a microscope to assess the cell density within the channel. Adjust the density of the cell suspension as necessary for the rest of the chips.

Steps

Step	Action
1	Bring the square dish containing the chips to the BSC.
2	Avoiding contact with the ports, carefully aspirate excess medium droplets from the surface of one chip.
3	Very gently agitate cell suspension before seeding each chip to ensure a homogeneous cell suspension.
4	Quickly and steadily pipette 35 to 50 µL of the cell suspension (at 1.0 x 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). **Note:** The rapid injection technique will provide homogeneous cell distribution throughout the culture area in the channel.
5	Cover the dish and transfer to the microscope to check the seeding density within the chip (see Figure 9).

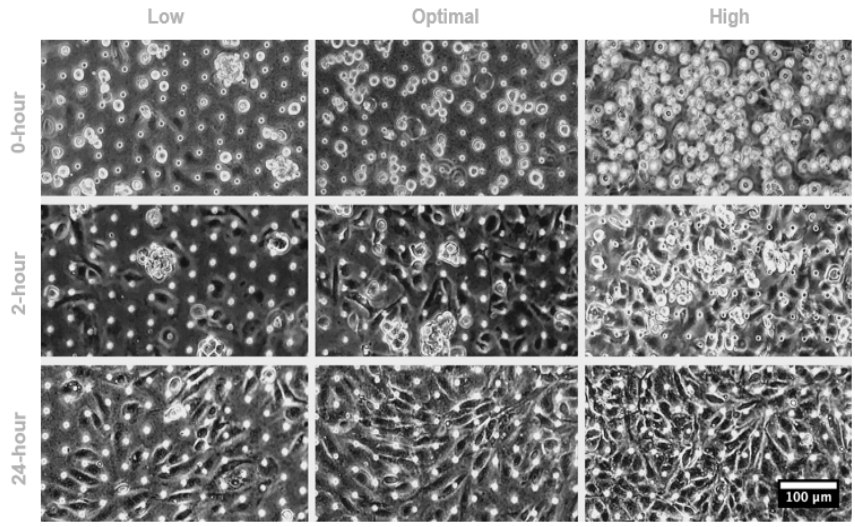


Figure 9. 10X brightfield images showing optimal hRPTEC density and attachment.

<table>
<tr><td>6</td><td>If seeding density is not optimal, follow these steps:<table><tr><th>Step</th><th>Action</th></tr><tr><td>1</td><td>Return the chips to the BSC</td></tr><tr><td>2</td><td>Using a P200 pipette, extract the hRPTECs through the outlet port. Then, dispose of it.</td></tr><tr><td>3</td><td>Wash the channel with 200 µL of fresh medium twice. Aspirate the outflow.</td></tr></table></td></tr>
<tr><td>7</td><td>After confirming the correct cell density, seed cells in the remaining chips.</td></tr>
<tr><td>8</td><td>Add 0.75 mL of DPBS into the chip cradle reservoir and replace the lid of the square dish.</td></tr>
<tr><td>9</td><td>Place the dish holding the chips at 37°C for 2.5 hours.</td></tr>
</table>

Seed a Well Plate

Before You Begin

It is recommended to always seed any remaining hRPTECs into a plate as a control for cell quality. If desired, transwells can be used as controls.

Steps

Step	Action
1	Once the chips have been seeded, dilute the remaining hRPTECs to a final cell density of 1.6 X 10^5 cells / mL in complete culture medium.
2	Add 500 µL of cell suspension to each well of a 24-well plate.
3	In the 37°C 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 three times while keeping it flat on the surface of the incubator.
4	Finally, move the plate in a crisscross pattern at least three times to evenly disperse the cells. Once the cells are dispersed, do not disturb the plate until the next day to allow for cells to fully attach.

Gravity Wash

Before You Begin

- A gentle gravity wash is performed after cells have fully attached (typically 2.5 hours) to ensure that nutrients are replenished, and the channels do not dry out. During a gravity wash, the medium should be observed to flow through the channel and outflow into the outlet.
- Because two different media are being used, they must be separated by keeping them in different filtered tips.
- Chips can be maintained overnight under static condition using pipette tips, as depicted below.

Steps

Step	Action
1	With a P200 pipette, gently insert 200 µL of Complete hGMVEC Culture Medium into the bottom channel inlet until a small droplet appears on the outlet, or until a bubble is ejected through the outlet.
2	While the inlet has a pipette tip with medium, carefully place another fresh, sterile, 200-µL pipette tip into the chip outlet port. Once you see the medium flow in the outlet tip, gently release the pipette tip in the inlet port. **Note:** Avoid pushing tips all the way down and release the tip gently using the pipettor's tip ejector—avoid forceful release so that the tip does not block the channel.
3	Repeat Step 3 for the top channel using warm Complete hRPTEC Culture Medium (see Figure 10).

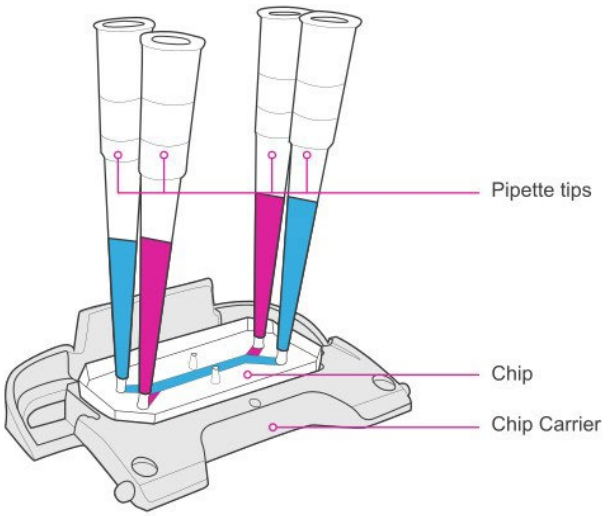


Figure 10. Chips with filtered tips inserted into ports with respective media

4	Return chips with pipette tips inserted in each inlet and outlet port to the 37°C incubator overnight.
5	Maintain cells in static culture in chips until connecting to Pods and Zoë the next day.

Day 1: Chips to Pods, and Pods to Zoë

Overview

Goals

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

Required Materials

- Installed and qualified Zoë-CM2 Culture Module
- 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

Key Steps

Prepare Complete hGMVEC Culture and Maintenance Media for Degassing

Before You Begin

The culture of hGMVECs will continue in Complete hGMVEC Culture Medium for Chip-to-Pod and Pod-to-Zoë connection (Day 1); hGMVECs culture medium will be switched to Complete hGMVEC Maintenance Medium after the second Regulate Cycle is completed or until a fully matured monolayer is formed (generally after 3 days).

Optional: Degassing media after chips are connected to Pods may help prevent bubble formation.

Complete hGMVEC Culture and Maintenance Medium

Complete hGMVEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hGMVEC Culture Medium	45 mL	-	-	Recipe	-
FBS	5 mL	-	10%	Sigma	F4135

Complete hGMVEC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hGMVEC Culture Medium	49.75 mL	-	-	Recipe	-
FBS	0.25 mL	-	0.5%	Sigma	F4135

- Store the Complete hGMVEC Culture and Maintenance Medium at 4°C.
- Use the Culture and Maintenance Medium within 7 days of preparation.

Prepare Complete hRPTEC Maintenance Media for Degassing

Before You Begin

The hRPTECs will now be kept in maintenance media for the duration of Organ-Chip culture.

hRPTEC Maintenance Media

Base hRPTEC Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
REBM™ Renal Epithelial Cell Growth Basal Medium	492.45 mL	-	-	Lonza	CC-3191
REGM™ SingleQuots™ Kit containing:				Lonza	CC-4127
• Human Epidermal Growth Factor (hEGF)	0.05 mL	-	-	-	-
• Insulin	0.5 mL	-	-	-	-
• Hydrocortisone	0.5 mL	-	-	-	-
• Transferin	0.5 mL	-	-	-	-
• Triiodothyronine	0.5 mL	-	-	-	-
• Epinephrine	0.5 mL	-	-	-	-
Pen / Strep	5 mL	-	1%	Sigma	P4333

- Store the Base hRPTEC Maintenance Medium at 4°C.
- Use the Base hRPTEC Maintenance Medium within 30 days of preparation.

Note: The hEGF concentration is now reduced 10-fold compared to the base culture media prepared on Day -5 that was used for flask culture and cell seeding.

Complete hRPTEC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hRPTEC Maintenance Medium	49.75 mL	-	-	Recipe Above	-
FBS	0.25 mL	-	0.5%	Lonza (kit from above)	

- Store the Complete hRPTEC Maintenance Medium at 4°C.
- Use the Complete hRPTEC Maintenance Medium within 7 days of preparation.

Gas Equilibration of Media

CAUTION

The media equilibration step is imperative to successfully culture Organ-Chips. Omitting this step will cause bubbles to form in the chip, in the Pod, or both, which will in turn negatively impact cell viability and result in irregular flow.

Before You Begin

- The media equilibration step is critical to the success of Organ-Chip culture. Omitting this step will eventually lead to bubble formation in the chip, the Pod, or both, which will in turn cause irregular flow and negatively impact cell viability.
- Ensure the medium is outside of a warmed environment (such as an incubator or bath) for no longer than 10 minutes, as gas equilibrium can become compromised when medium is allowed to cool.
- If the vacuum pump is not located close to the water bath (e.g., inside the BSC), it is recommended to place some clean water warmed at 37°C inside the BSC to minimize cooling during the media equilibration step.

Steps

Step	Action
1	Determine the total volume of media needed for chip connection: Multiply the total number of chips by the volume of media needed per chip (number of Chip x 4.3 mL)
2	Aliquot the total Complete Culture or Maintenance Medium needed in separate 50-mL conical tubes.
3	Warm the 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 using the following steps:

Step	Action
1	Attach each conical tube containing warmed media to a Steriflip unit (see Figure 11).
2	With the unit “right-side up” (medium in the bottom conical tube), apply vacuum for 10 seconds.

	3	Invert the Steriflip-connected tubes, and check that the medium begins to pass from the top conical tube to the bottom one. **Note:** The vacuum must operate at a minimum of -70 kPa. At this pressure, it should take about 2 seconds for 10 mL of media to flow through the filter. If it takes longer, stop and refer to the "Media takes too long to pass through Steriflip" in the troubleshooting section.
	4	Leave the filtered medium under vacuum for five minutes

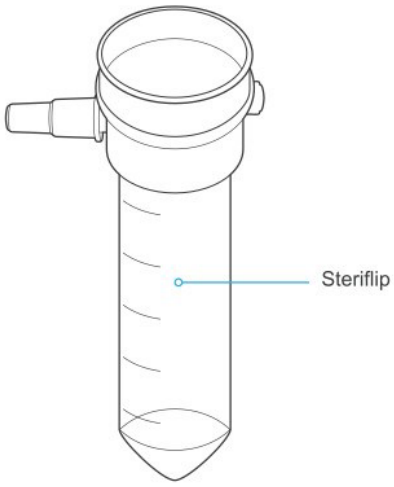


Figure 11. Steriflip unit

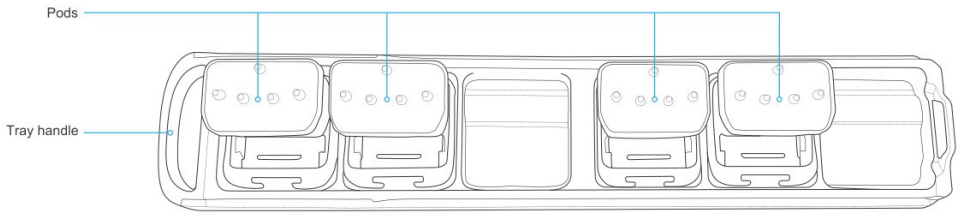
5	Remove the vacuum tubing from the Steriflip units.
6	Separate the conical tubes containing media from the Steriflip unit, and immediately place them into the incubator with the caps loose. **Note:** Minimize the time media is outside of the incubator when the Pod is being prepared to maintain the correct temperature. This is critical to ensure chip success.

Prime Pods

CAUTION

Ensuring successful Pod priming is best practice for successful liquid-liquid connection between the chip and Pod. Failure to do so can cause bubbles to form in the chip, in the Pod, or both, which will in turn negatively impact cell viability and result in irregular flow.

Steps

Step	Action
1	Sanitize the exterior of the Pod packaging and trays with 70% ethanol, wipe them, and transfer them to the BSC.
2	Open the Pod package and place the Pods into the trays. Orient the Pods with the reservoirs facing the back of the tray (see Figure 12).
	 Figure 12. Chips and Pods inserted into a tray.
3	Pipette 3 mL of pre-equilibrated, warm media to each inlet reservoir. In the top channel inlet reservoir, add Complete hRPTEC Maintenance Medium; in the bottom channel inlet reservoir, add Complete hGMVEC Culture Medium.
4	Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet Via (see Figure 13).

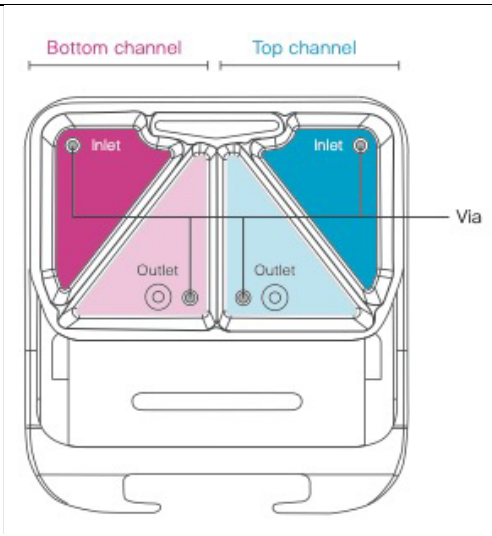


Figure 13. Top view of Pod and reservoirs.

5	Bring trays with Pods to the incubator and slide them completely into Zoë with the tray handle facing outward.
6	Run the Prime Cycle on Zoë. **Step** / **Action** 1 — Use the Dial to highlight “Prime” on the display. 2 — Press the Dial Button to select “Prime.” 3 — Rotate the Dial clockwise to highlight “Start.” 4 — Press the Dial again to select “Start” and begin. **Note:** Once “Start” is selected, there will be a sound as Zoë engaging the Pods.
7	Close the incubator door and allow Zoë to prime the Pods; this process takes approximately one minute. **Note:** The status bar will read “Ready,” if the Prime Cycle is complete.
8	Remove the tray from Zoë and bring it to the BSC.
9	Verify that the Pods were successfully primed. This is critical for success.

Continued on next page

Prime Pods, Continued

Pod Priming Verification

Take out the tray and inspect the top of the Pods (See Figure 14) to verify the presence of small media droplets through the Pod window at all four fluidic ports.

If…	Then…
Droplets are not visible through the top window	Re-run the Prime Cycle on those Pods. If the issue persists, contact Emulate Support.
Any outlet port does not show a droplet, but the inlet port does.	Ensure Step 4 of “Prime Steps” has been performed correctly.
Any media escaped onto the tray (this may occur more often by the outlet ports).	Clean the tray using a wipe sprayed with 70% ethanol.

Figure 14

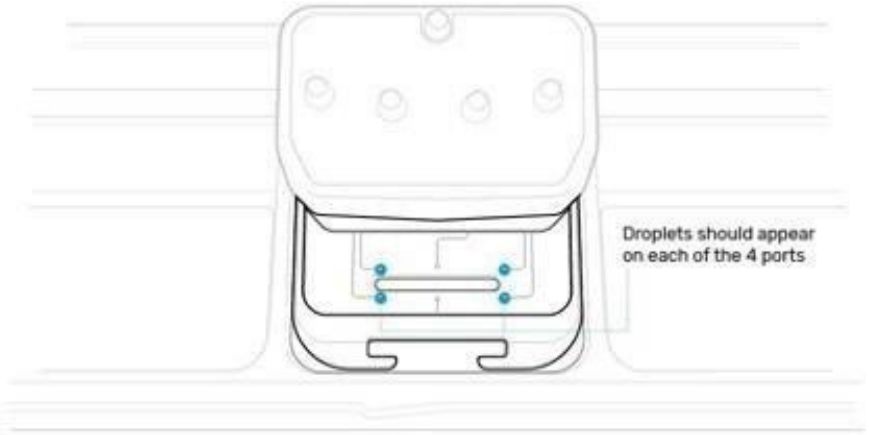


Figure 14. Top view of chip in Pod with fluidic posts covered with droplets.

Wash Chips

Steps

Step	Action
1	Transfer the seeded chips in a 120 x 120-mm square dish from the incubator to the BSC.
2	Remove the pipette tips from the chip inlet and outlet ports.
3	Gently wash each top channel with warm, equilibrated Complete hRPTEC Maintenance Medium to remove any bubbles.
4	Place small droplets of equilibrated Complete hRPTEC Maintenance Medium on each chip's inlet and outlet port.
5	Gently wash each chip's bottom channel with warm, equilibrated Complete hGMVEC Culture Medium to remove any possible bubbles.
6	Place small droplets of equilibrated Complete hGMVEC Culture Medium on each chip's bottom channel inlet and outlet ports.

Chips to Pods

Steps

Step	Action
1	Hold one chip (in a chip carrier) in the dominant hand and one Pod in the other hand. Slide the chip carrier into the tracks on the underside of the Pod until the chip carrier has fully seated.
2	Place a thumb on the chip carrier tab. Gently, but firmly, press the tab in and up until it engages with the Pod.
3	Aspirate any excess media on the chip surface from the Pod window.
4	Place the Pod and connected chip onto the tray.
5	Repeat Steps 1–4 for each Pod and chip carrier.
6	Confirm that there is sufficient media in each Pod inlet and outlet reservoir. Also, ensure that the Pod lids are flat and secure.

Pods to Zoë

Steps

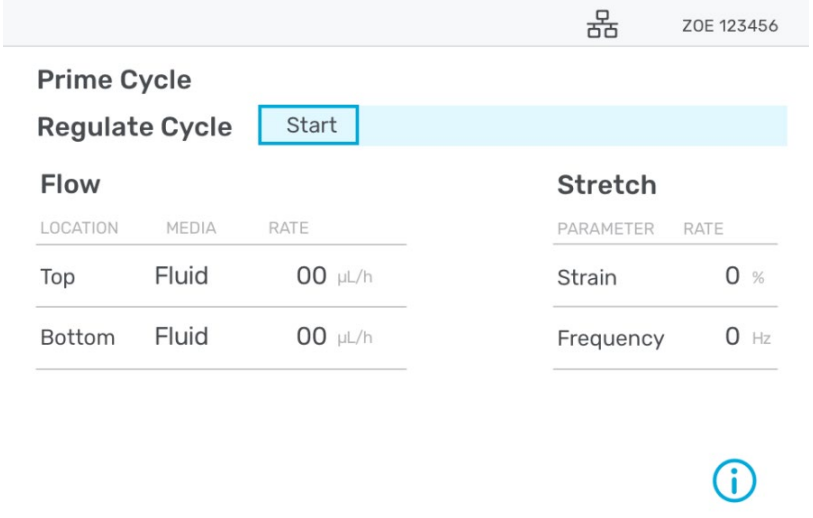
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Immediately place the trays holding Pods and chips into Zoë to prevent media from cooling and losing its gas equilibration.</td></tr>
<tr><td>2</td><td>Program the appropriate Organ-Chip culture conditions on Zoë. These conditions will start as soon as the Regulate Cycle is complete. For human co-culture Proximal Tubule Kidney-Chips, set the flow rate to 60 µL / h for both channels.</td></tr>
<tr><td>3</td><td>Once Organ-Chip culture conditions are set, run the Regulate Cycle.
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Using the Dial, highlight the “Regulate” field.</td></tr>
<tr><td>2</td><td>Press the Dial Button to select “Regulate,” and rotate the Dial clockwise to “Start.”</td></tr>
<tr><td>3</td><td>Press the Dial again to select “Start” and begin the Regulate Cycle (see Figure 15).
Note: Once start is selected, there will be a sound as Zoë engages the Pods.</td></tr>
<tr><td colspan="2"> 

Figure 15. Zoë UI showing Regulate Selection</td></tr>
<tr><td>4</td><td>Make sure the “Activation” button is glowing blue.</td></tr>
</table></td></tr>
</table>

	5	Wait for the Regulate Cycle to finish, which will take two hours. After the cycle has finished, Zoë will begin flow at the preset Organ-Chip culture conditions (see Figure 16).

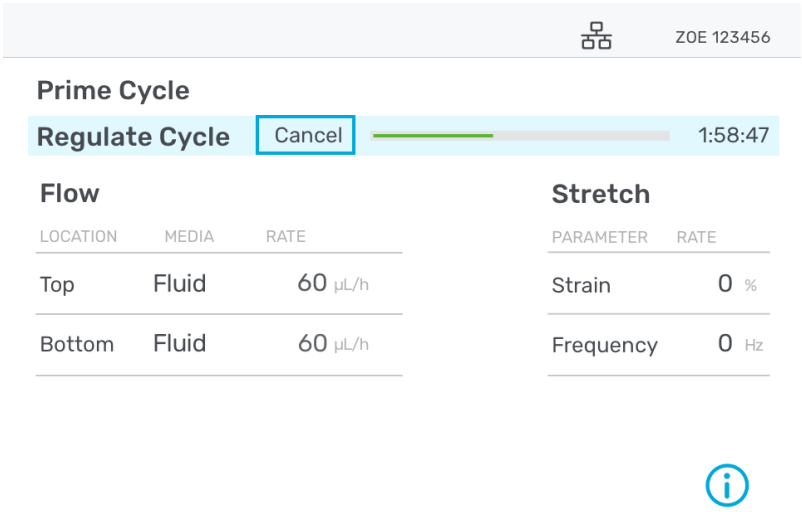


Figure 16. Zoë UI screen showing Regulate Cycle in progress.

Note

Avoid canceling the Regulate Cycle; however, if it is necessary, follow these steps:

Step	Action
1	Using the Dial, highlight the “Regulate” field.
2	Press the Dial Button to select “Regulate,” and rotate the Dial counterclockwise to “Cancel.”
3	Press the Dial again to select “Cancel,” 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.

Day 2+: Chip Maintenance and Sampling

Overview

Goals

- Maintain chips in Zoë
- Inspect cell culture
- Collect samples for analysis

Required Materials

- Chips in Pods
- Cell culture media

Key Steps

Maintenance and the Regulate Cycle

Steps

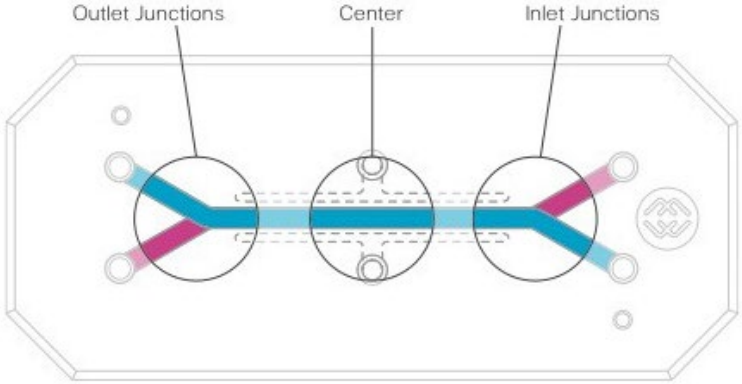
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>The day after connecting chips and Pods to Zoë (beginning the Organ-Chip culture process), pause Zoë by pressing the silver “Activation” button located above the tray bays. This stops flow and releases the Pods.</td></tr>
<tr><td>2</td><td>Slide the tray out of the bay and transfer it to the BSC.</td></tr>
<tr><td>3</td><td>Remove the Pod lids. Using a 200 µL pipette, perform a Via wash on each Pod inlet and outlet reservoir using the following steps:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Pipette 200 µL of media from the Pod reservoir directly on top of the Via to dislodge any bubbles that may be present</td></tr>
<tr><td>2</td><td>Repeat this wash step for each of the four Pod reservoirs.</td></tr>
</table></td></tr>
<tr><td>4</td><td>Replace Pod lids and return the trays to Zoë.</td></tr>
<tr><td>5</td><td>Run the Regulate Cycle again.
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Using the Dial, highlight the “Regulate” field.</td></tr>
<tr><td>2</td><td>Press the Dial Button to select “Regulate,” and rotate the Dial clockwise to “Start.”</td></tr>
<tr><td>3</td><td>Press the Dial Button again to select “Start” and begin the Regulate Cycle.
Note: Once “Start” is selected, there will be a sound as Zoë engages the Pods.</td></tr>
<tr><td>4</td><td>Make sure the “Activation” button is glowing blue.</td></tr>
<tr><td>5</td><td>Wait for the Regulate Cycle to finish, which will take two hours. After the cycle has finished, Zoë will begin flow at the preset Organ-Chip culture conditions.</td></tr>
<tr><td>6</td><td>The next day and upon formation of a complete monolayer, replace the hGMVECs bottom inlet medium with Complete hGMVEC Maintenance Medium.</td></tr>
</table></td></tr>
</table>

Sampling and Media Replenishment

CAUTION

Failure to replenish media on time will cause air to be introduced into the Pod and chip, which ultimately results in cell death and may cause damage to Zoë Culture Module. Do not fill reservoir past 4 mL of the total volume during replenishment.

Steps

Step	Action
1	Pause Zoë by pressing the silver “Activation” button.
2	Remove the trays and place them into the BSC.
3	Visually inspect each chip for bubbles.
4	Using a microscope, assess the morphology and viability of cells in the chips. Capture representative images at 10X or 20X magnification at the following locations (see Figure 17): • Inlet Junction • Center of Channel • Outlet Junction
	 Figure 17. Chip with marked locations for image capture.
5	Remove Pod lids and collect effluent from the Pod outlet reservoirs at the indicated regions while not 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 them.

7	Refill the Pod media reservoirs with the appropriate fresh complete culture or maintenance medium. Then, perform a Via wash by pipetting 1 mL of the medium in the reservoir directly over the top of the Via to dislodge any bubbles.
8	Replace the Pod lids and return the trays to Zoë.
9	Press the silver “Activation” button to resume pre-set Organ-Chip culture conditions. Zoë will engage when the “Activation” button glows blue.

Part VII. Troubleshooting

Overview

Troubleshooting

Issue	Section	Step	Recommendation
Bubbles are present in channel	Any step related to chip handling, such as Chip Activation, ECM coating, and cell seeding.	Any step related to chip handling, such as Chip Activation, ECM coating, and cell seeding.	Wash the channel with the appropriate solution until all bubbles have been removed. If bubbles persist, it may be helpful to aspirate the channel dry and slowly re-introduce solution.
Bubbles in the ports upon introduction of media into the chip	Any step related to chip handling, such as Chip Activation, ECM coating, and cell seeding.	Any step related to chip handling, such as Chip Activation, ECM coating, and cell seeding.	Since the chip material is hydrophobic, bubbles could get trapped at the ports. Dislodge bubbles using pipette tip or aspirate the channels and reintroduce appropriate media.
Media takes too long to pass through Steriflip	Chips to Pods and Pods to Zoë	Equilibration of Media	Vacuum pressure is not reaching -70kPa. Find an alternate vacuum source with the appropriate pressure. If this solution is unavailable, leave the media in the incubator with the caps loose for at least 16 hours (preferably overnight) before adding to Pods.
Pods do not prime	Chips to Pods and Pods to Zoë	Prime Pods	If Pods do not prime on the first attempt, ensure that medium covers all Pod Vias, and run the Prime Cycle again. If the problems persist, record the Pod lot number, and replace it with a new Pod.

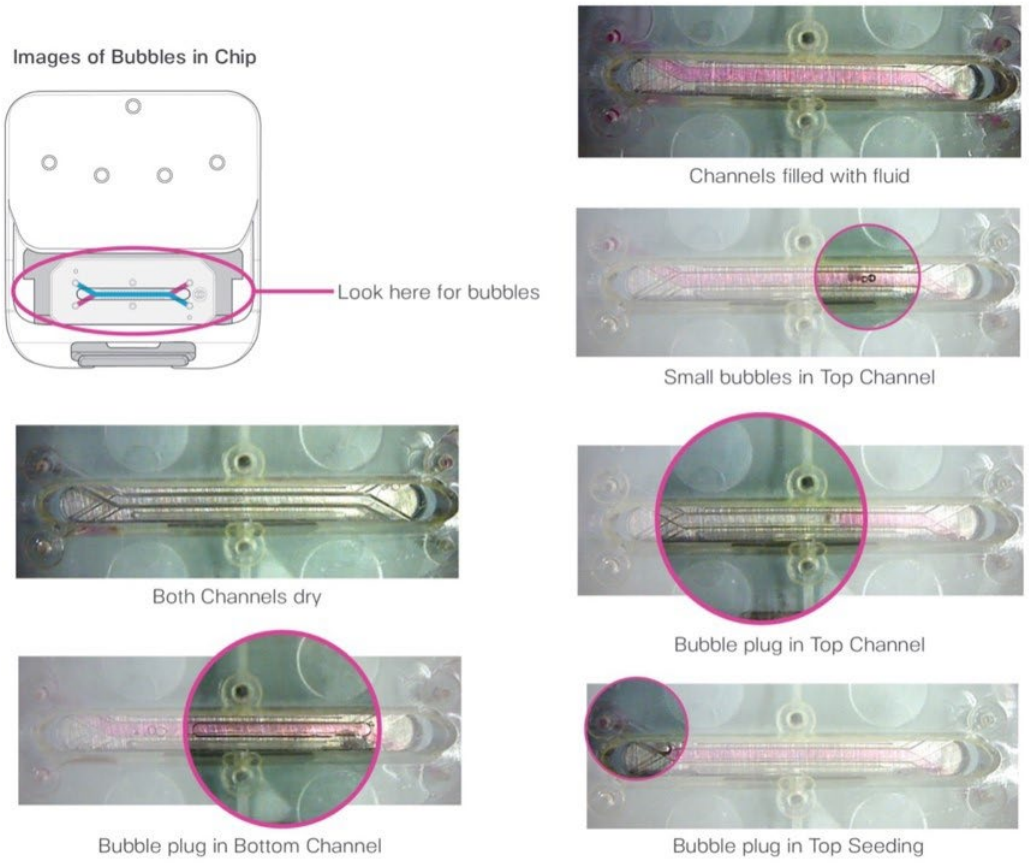
Screen is frozen or unresponsive	Chips to Pods and Pods to Zoë; Maintaining and Sampling	Any step related to Organ-Chip culture on Zoë	Power off Zoë and turn it on again. If the problem persists, contact our support team.
Pods stuck in Zoë	Maintaining and Sampling	Any step related to Organ-Chip culture on Zoë	The Pod lid is not secured. Try wiggling the tray to the right and left as you slide it out while keeping it level. If the problem persists, contact our support team.
Pods not flowing properly or evenly; Bubbles observed in chip	Maintaining and Sampling	Maintenance and Regulate	There is inherent variability with Zoë; however, large fluctuations and major flow issues primarily result from bubbles. To remove bubbles and allow for flow, remove the chip from the Pod, flush the chip with media, re-prime Pod with degassed media, reconnect the chip to the Pod, and run the Regulate Cycle.

Potential Root Causes of Bubbles

If there is a high failure rate due to bubbles, or if bubbles are persistent, despite performing the above mitigation step(s) (See Figure 18 on the next page), check for the following:

If ...	Then ...
Medium is not sufficiently equilibrated before adding to Pods	Be sure to follow media preparation steps in section “Gas Equilibration of Media”.
Vacuum for Steriflip too weak	Ensure that 10mL media passes through the Steriflip in ~10 seconds.
Incorrect Steriflip used	Confirm the correct Steriflip unit is being used (Millipore SE1M003M00).
Medium not warmed correctly before Steriflip step	Be sure to follow the media preparation steps in the section “Gas Equilibration of Media”.
Insufficient priming	Disconnect the chip and re-prime the Pod.

Figure 18 Images of Bubbles in an Organ-Chip



Part VIII: Appendices

Overview

Contents

Reagent Aliquots

Reagents

Collagen-IV (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Collagen-IV	1 mg / mL	5 mg	5 mL	DPBS

- Resuspend 5 mg Collagen-IV in 5 mL of DPBS according to the manufacturer's instructions.

- Aliquot to single-use volumes and store at -20°C.

Matrigel (Overlay)

Reagent	Amount	Volume
Matrigel	5 mg per aliquot	Varies per lot

The Matrigel bottle must be thawed overnight on ice either in the back of the 2–6°C refrigerator or in a cold room. Add water to ensure the ice is slushy, as the solution gels rapidly at temperatures above 10°C. Before aliquoting, use pipettes, tips, and tubes prechilled to -20°C.

- After the Matrigel is thawed, aliquot Matrigel to the desired volume (e.g., 100–200 µL) based on the specific stock concentration.

- Store aliquots at -20°C.

Media

hGMVEC Culture Media

Base hGMVEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Normal Blood Glucose Level Without Serum	485 mL	-	-	Cell Systems	4N3-500-R
Culture-boost-R	10 mL	-	2%	Cell Systems	4CB-500-R
Pen / strep	5 mL	-	1%	Sigma	P4333

- Store the Base hGMVEC Culture Media at 4°C.
- Use the Base hGMVEC Culture Media within 30 days of preparation.

Complete hGMVEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hGMVEC Culture Medium	45 mL	-	-	Recipe above	-
FBS	5 mL	-	10%	Sigma	F4135

- Store the Complete hGMVEC Culture Medium at 4°C.
- Use the Complete hGMVEC Culture Medium within 7 days of preparation.

hGMVEC Maintenance Medium

Complete hGMVEC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hGMVEC Culture Medium	49.75 mL	-	-	Recipe Above	-
FBS	0.25 mL	-	0.5%	Sigma	F4135

- Store Complete hGMVEC Maintenance Medium at 4°C.
- Use within 7 days of preparation.

Continued on next page

Media, Continued

hRPTEC Culture Media

Base hRPTEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
REMB™ Renal Epithelial Cell Growth Basal Medium	492 mL	-	-	Lonza	CC-3191
REGM™ SingleQuots™ Kit containing:				Lonza	CC-4127
• Human Epidermal Growth Factor (hEGF)	0.5 mL	-	-	-	-
• Insulin	0.5 mL	-	-	-	-
• Hydrocortisone	0.5 mL	-	-	-	-
• Transferin	0.5 mL	-	-	-	-
• Triiodothyronine	0.5 mL	-	-	-	-
• Epinephrine	0.5 mL	-	-	-	-
Pen / Strep	5 mL	-	1%	Sigma	P4333

- Store Base hRPTEC Culture Medium at 4°C.
- Use Base hRPTEC Culture Medium within 30 days of preparation.

Note: Do not use gentamicin sulfate from the REGM™ SingleQuots™ Supplement Pack.

Complete hRPTEC Culture Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hRPTEC Culture Medium	49.75 mL	-	-	Recipe above	-
FBS	0.25 mL	-	0.5%	Lonza (from kit above)	CC-4217

- Store Complete hRPTEC Culture Medium at 4°C.
- Use Complete hRPTEC Culture Maintenance within 7 days of preparation.

Continued on next page

Media, Continued

hRPTEC Maintenance Media

Base hRPTEC Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
REBM™ Renal Epithelial Cell Growth Basal Medium	492.45 mL	-	-	Lonza	CC-3191
REGM™ SingleQuots™ Kit containing:				Lonza	CC-4127
• Human Epidermal Growth Factor (hEGF)	0.05 mL	-	-	-	-
• Insulin	0.5 mL	-	-	-	-
• Hydrocortisone	0.5 mL	-	-	-	-
• Transferin	0.5 mL	-	-	-	-
• Triiodothyronine	0.5 mL	-	-	-	-
• Epinephrine	0.5 mL	-	-	-	-
Pen / Strep	5 mL	-	1%	Sigma	P4333

- Store the Base hRPTEC Maintenance Medium at 4°C.

- Use the Base hRPTEC Maintenance Medium within 30 days of preparation.

Note: the hEGF concentration is now reduced 10-fold compared to the culture media prepared on Day -5 that was used for flask culture and cell seeding.

Complete hRPTEC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base hRPTEC Maintenance Medium	49.75 mL	-	-	Recipe Above	-
FBS	0.25 mL	-	0.5%	Lonza (kit from above)	-

- Store the Complete hRPTEC Maintenance Medium at 4°C.

- Use the Complete hRPTEC Maintenance Medium within 7 days of preparation.