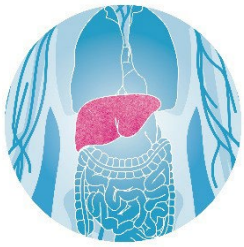

Liver-Chip Quad-Culture Protocol

August 4, 2023

EP-226 Rev. B

Liver-Chip Quad-Culture Protocol

Overview

Introduction

This protocol described the general steps for using the Liver-Chip Quad-Culture BioKit.

Contents

Part I. Liver Quad-Culture BioKit

Overview

Introduction

This part provides an overview of the Liver-Chip Quad-Culture BioKit as well as its key components, shipping information, and storage specifications.

Components

The Liver-Chip Quad-Culture BioKit includes the pre-qualified primary human liver cells listed in the table below.

Category	Channel Location	Type of Cells
Parenchymal epithelial cells	Top	• Hepatocytes
Non-parenchymal cells (NPC)	Bottom	• Liver sinusoidal endothelial cells (LSECs) • Kupffer cells • Stellate cells

Cell Shipping

Cells are shipped in cryogenic storage vacuum flasks.

Cell Storage

Cells must be stored in liquid nitrogen until use.

Part II. Experimental Overview

Overview

Introduction

This section gives an overview of the experimental workflow.

Day X: Reagent Preparation

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

Day -2: Thaw LSECs

- Prepare LSEC culture flask
- Thaw and plate LSECs

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: Hepatocytes to Chips

- Prepare hepatocyte seeding medium
- Prepare chips
- Thaw hepatocytes
- Adjust cell density
- Seed hepatocytes to epithelial channel
- Seed a well plate
- Gravity wash chips (3–4 hours post-seeding)

Day 1: Hepatocyte Overlay

- Prepare overlay medium
- Overlay hepatocytes

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Overview, Continued

Day 2: Non-Parenchymal Cells (NPCs) to Chips

- Prepare NPC seeding medium
- Wash chips
- Harvest LSECs
- Thaw stellate cells
- Thaw Kupffer cells
- Combine NPC mixture
- Seed non-parenchymal cells (LSECs, stellate cells, and Kupffer cells) to bottom channel
- Gravity wash with tips (4 hours post-seeding)

Day 3: Chips to Pods, and Pods to Zoë

- Gas equilibration of media
- Prime Pods
- Wash chips
- Chips to Pods
- Pods to Zoë

Day 4+: Maintaining and Sampling

- 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 materials needed.

Note

Exact catalog numbers are not provided for some required materials, as several brands and models are accepted.

Required Equipment

A list of equipment needed for this protocol in addition to the Emulate Liver-Chip Quad-Culture BioKit is provided below:

Equipment	Subscription	Supplier	Catalog Number
Human Quad-Culture Liver BioKit	Quad-Culture 12- or 24-pack	Emulate	BIO-LH-QUAD12 BIO-LH-QUAD24
Zoë-CM2® Culture Module	1 per 12 chips	Emulate	ZOE-CM2
Orb-HM1® Hub Module	1 per 4 Zoës	Emulate	ORB-HM1
Chip-S1® Stretchable Chip	12 per Zoë	Emulate	S1-3
Pod® Portable Module	1 per Chip-S1	Emulate	POD-3
UV Light Box	1 per Zoë	Emulate	UVLamp
Chip Cradle	Autoclaved, 1 per 6 chips	Emulate	CHIP-CRD
Steriflip®-HV Filters	Sterile, 0.45 um 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, 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	-	-	-
T25 Flask	-	-	-
T75 Flask	-	-	-
Ice bucket	-	-	-
70% ethanol wipes	For surface sterilization		

Note Low-binding tubes are not necessary but can be used to improve Kupffer cell yield.

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Required Materials

A list of reagents, media, and supplements needed for this protocol in addition to the Emulate Liver-Chip Quad-Culture BioKit is provided below:

Reagent	Description	Supplier	Catalog Number
ER-1	5-mg powder	Emulate	ER105
ER-2	25-mL bottle	Emulate	ER225
Dulbecco's PBS (DPBS -/-) (without Ca^{2+}, Mg^{2+})	1X	Corning	21-031-CV
10X DPBS (-/-) (without Ca++, Mg++)	10X	Corning	20-031-CV
Trypan blue	0.4% solution	Sigma	93595
Percoll® Solution	100% stock solution	Sigma	P4937
Trypsin-EDTA solution	0.05% trypsin	Sigma	T3924
WEM Medium (+)	Williams' medium E with phenol red (+)	Sigma	W4128
WEM Medium (-)	Williams' medium E no phenol red (-)	Sigma	W1878
CSC medium (Kit)	LSEC medium & supplements	Cell Systems	4Z3-500
Culture boost™	50X supplement	Cell Systems	4CB-500
Attachment Factor™	1X	Cell Systems	4Z0-210
Cell freezing medium	1X	Cell Systems	4Z0-705
Matrigel®	LDEV-free	Corning	354234
Fibronectin	Bovine protein, plasma	ThermoFisher	33010-018
Collagen type I	Rat tail; HC	Corning	354249
Penicillin-streptomycin	10,000 U / mL; 10 mg / mL	Sigma	P4333
L-GlutaMax™	200 mM	ThermoFisher	35050-061
L-Ascorbic Acid	100-mg powder	Sigma	5960

Dexamethasone	100-mg powder	Sigma	D4902
Fetal bovine serum (FBS)	Sterile, heat-inactivated	Sigma	F4135 or F8317
ITS+	Premix supplement	Corning	354352
Fungin™	10 mg / mL	InvivoGen	ANT-FN-1

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 above table.

Part IV. Workstation Preparation and Chip Handling Techniques

Workstation Preparation

Aseptic Techniques

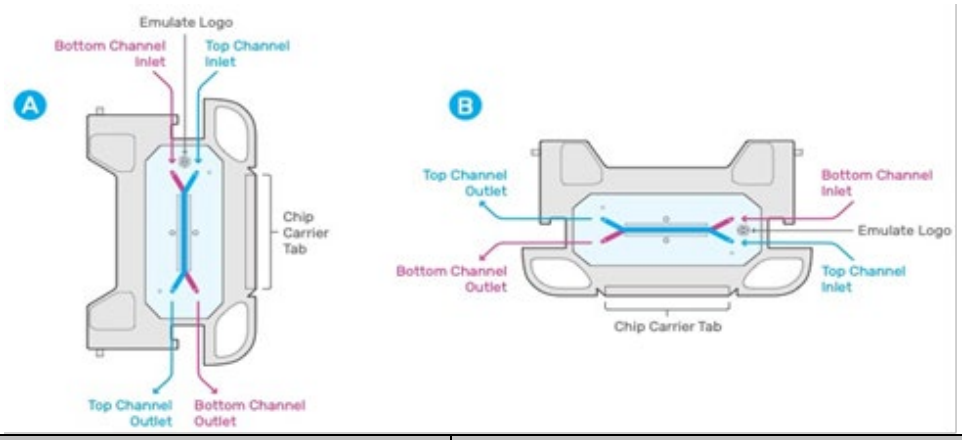
- Always work with chips in a sterile environment, such as the biosafety cabinet (BSC).
- Before beginning the experiment, prepare, sanitize, and organize the work surface of the BSC. Arrange tips, media, and other necessary materials in the sterile field so they are easily within reach but not blocking 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.

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.

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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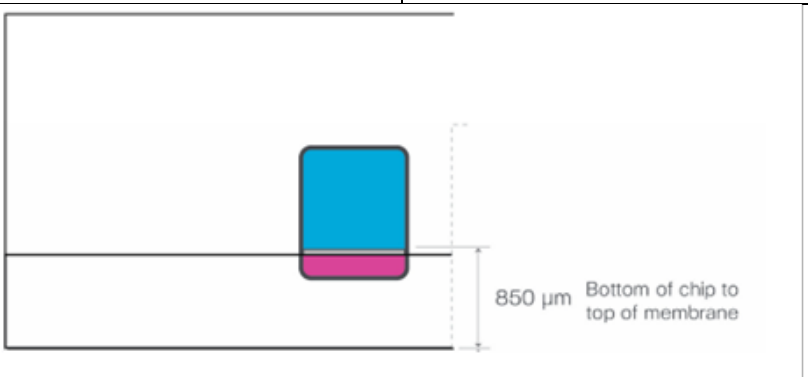
Channel and Membrane Dimensions

The specific channel and membrane dimensions are outlines 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

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. Liver-Chip Quad-Culture Protocol

Protocol Overview

Introduction

This section lists the basic steps for using Liver-Chip Quad-Culture BioKits 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.

Fibronectin (ECM)

Reagent	Conc. [Stock]	Amount	Volume	Solvent
Fibronectin	1 mg / mL	1 mg	1 mL	Cell culture grade water

- Resuspend 1 mg fibronectin in 1 mL of cell culture grade water according to manufacturer's instructions.
- Create single-use volume aliquots and store them 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, create 5-mg aliquots based on the specific stock concentration. For example, if the stock concentration is 10 mg / mL, 500 µL of solution will yield 5 mg of Matrigel.
- Store aliquots at -20°C.

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Aliquot Reagents, Continued

Culture Supplements

Reagent	Conc. [Working]	Volume	Solvent
L-Ascorbic acid	50 mg / mL	Calculate based on amount measured	Cell culture grade water
Dexamethasone	10 mM	Calculate based on amount measured	Cell culture grade DMSO
Dexamethasone	1 mM	Calculate based on amount measured	Cell culture grade DMSO

- Resuspend each supplement to the working concentration in the table above.
- Aliquot each supplement to single-use volumes.
- Store aliquots at -20°C.

Day -2: Thawing Liver Sinusoidal Endothelial Cells (LSECs)

Overview

Goals

- Expand LSEC culture media and flask

Required Materials

- Complete LSEC 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 LSEC Culture Media and Flask

LSEC Culture Media

Base LSEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
CSC basal medium	485 mL	-	-	Cell Systems	4Z3-500
Culture-boost	10 mL	-	2%	Cell Systems	4CB-500
Pen / strep	5 mL	-	1%	Sigma	P4333

- Store the LSEC Culture Medium at 4°C.
- Use the LSEC Culture Medium within 30 days of preparation.

Complete LSEC Culture Medium (50 mL)

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

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

Prepare Flask

Step	Action
1	Warm a sufficient amount of Complete LSEC Culture Medium and Attachment Factor™ to 37°C. 15 mL of medium is needed for thawing, and an additional 15 mL is needed for each flask.
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 to coat the surface. Maintain this temperature until the cells are plated (about 5 minutes).

Thaw and Plate LSECs

Note on LSECs

LSECs are the only cells in this protocol that must be plated and expanded before being seeded in the chip. LSECs arrive at Passage 3, are expanded once in a flask, and then seeded at Passage 4.

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 LSEC Culture Medium.
4	Rinse the vial(s) with 1 mL of Complete LSEC Culture Medium and collect the run-off in the 15-mL tube.
5	Bring the volume to 15 mL with Complete LSEC 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 pellet by gently flicking the tube.
9	Re-suspend cells in 15 mL of Complete LSEC Culture Medium.
10	Aspirate and discard excess Attachment Factor from the T-75 flask that was prepared. **Note:** It is unnecessary to rinse or dry the flask prior to adding cells.
11	Add the LSEC suspension to the freshly coated T-75 flask.
12	Incubate overnight at 37°C and 5% CO_2.
13	Refresh the Complete LSEC Culture Medium every other day until the cells are seeded in the chip.

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 I and fibronectin ECM proteins for cell attachment

Required Materials

- Chip-S1 Stretchable Chips (12 Chips per Zoë)
- ER-1 powder
- ER-2 solution
- 15-mL conical tubes
- DPBS (- / -) at room temperature
- DPBS (- / -) aliquot at 4°C
- Collagen I
- Fibronectin
- 70% ethanol
- Square Cell Culture Dish (120 x 120 mm)
- 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 with 70% ethanol and bring it 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. Organ-Chips being placed into a 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 BSC light and allow 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 working concentration of 0.5 mg / mL. Gently pipette the solution to mix it without creating bubbles. The ER-1 should be fully 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-uL 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).

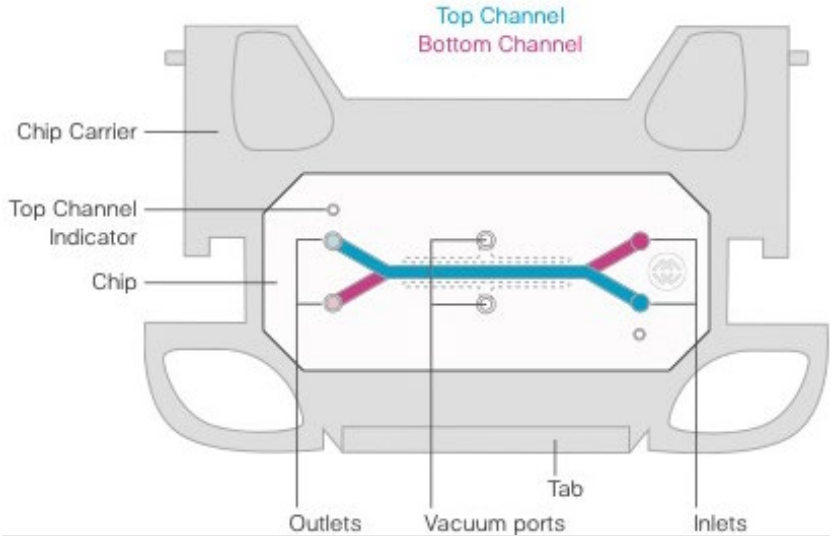

Figure 2. Top view of chip, with labelled ports, in the chip carrier.

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 exist 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).

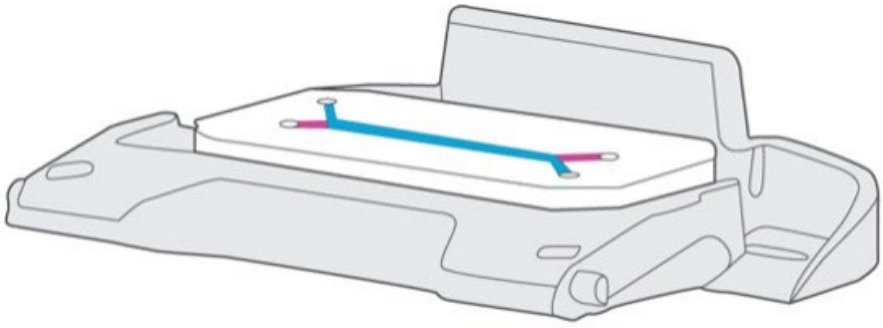

Figure 3. View of chip with no excess ER-1 solution on its surface.

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, it may be helpful to 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 in 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 sterile ER-2.
9	Fully aspirate the ER-2 from the channels.
10	Wash each channel with 200 µL of sterile cold DPBS.
11	Leave cold DPBS inside the channels.

Prepare ECM Solution

Before You Begin

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 Liver-Chips, the ECM working concentrations are:

Reagent	Concentration
Collagen	100 µg / mL
Fibronectin	25 µg / mL

Steps

Step	Action
1	Bring a full ice bucket to the BSC.
2	Thaw one aliquot of fibronectin (1 mg / mL) on 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 I stock concentration	8.41 mg / mL (C1)
Collagen I final concentration	0.1 mg / mL (C2)
Fibronectin stock concentration	1 mg / mL (C1)
Fibronectin final concentration	0.025 mg / mL (C2)
Stock Volume	Collagen I (X) or fibronectin (Y) (V1)
Final volume of ECM solution	1.5 mL (V2)

Collagen Calculation:

(8.41 mg / mL) x (X mL) = (0.1 mg / mL) x (1.5 mL)

X = 17.83 µL of collagen I stock solution

Fibronectin Calculation:

(1 mg / mL) x (Y mL) = (0.025 mg / mL) x (1.5 mL)

Y = 37.5 µL of fibronectin

DPBS Calculation

Volume DPBS =

(total volume of ECM needed) – (volume of collagen I) – (volume of fibronectin)

= 1500 µL – 17.83 µL – 37.5 µL

= 1444.67 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).

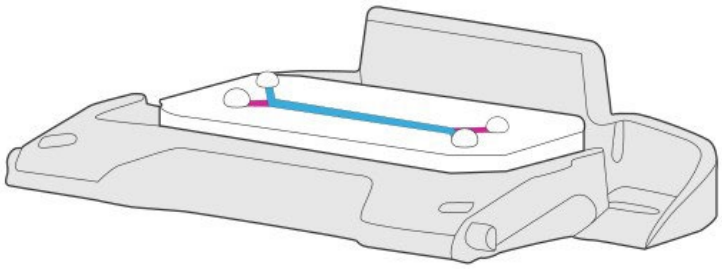

Figure 4. Chip in chip carrier with small ECM droplets at ports.

6	Wash any bubbles 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.

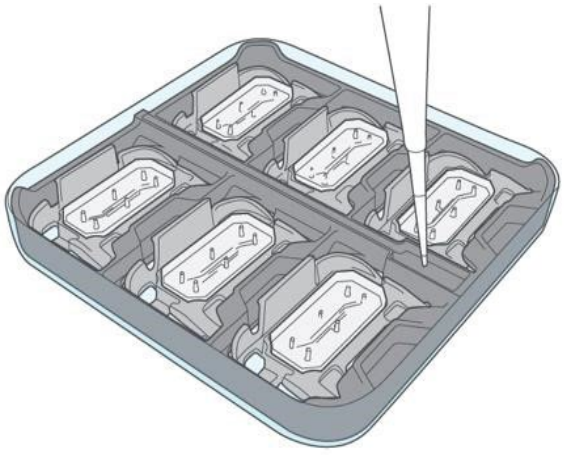

Figure 5. Pipette filling central reservoir of Chip Cradle with 0.75 mL DPBS.

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 seeding. **Note:** Chips can be stored at 4°C for up to 2 days if kept moist.

Day 0: Hepatocytes to Chip

Overview

Goals

- Seed hepatocytes in chip.

Required Materials

- Complete hepatocyte seeding medium (at 37°C)
- Percoll solution (at room temperature)
- 10X DPBS (at room temperature)
- 1X DPBS (at room temperature)
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- 50-mL conical tubes
- Diluted trypan blue counting solution
- Hemocytometer
- 24-well collagen I-coated plate
- 70% ethanol
- Microscope

Key Steps

Prepare Hepatocyte Seeding Media

Hepatocyte Seeding Media

Base Hepatocyte Seeding Medium (500-mL bottle)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM + (with phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	-	1%	Sigma	P4333
L-GlutaMAX	5 mL	-	1%	Gibco	35050-61

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

Complete Hepatocyte Seeding Medium (200 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	187.78 mL	-	-	Recipe Above	-
ITS+ premix	2 mL	-	1%	Corning	354352
Ascorbic Acid	200 µL	50 mg / mL	0.05 mg / mL	Sigma	5960
Dexamethasone	20 µL	10 mM	1 µM	Sigma	D4092
FBS	10 mL	-	5%	Sigma	F4135

- Store the Complete Hepatocyte Seeding Medium at 4°C.
- Use the Complete Hepatocyte Seeding Medium within 3 days of preparation.

90% Percoll Solution (20 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Percoll Solution	18 mL	100%	90%	Sigma	P4937
10X DPBS (- / -)	2 mL	100%	10%	-	-

- Maintain the 90% Percoll Solution at room temperature prior to use.
- Prepare the Percoll Solution fresh for each use.

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Prepare Hepatocyte Seeding Media, Continued

Hepatocyte Seeding Media, continued

Trypan Blue Cell Counting Solution (45 µL)

Reagent	Volume	Source	Cat. No.
Complete Hepatocyte Seeding Medium	40 µL	Recipe Above	-
Trypan Blue	5 µL	Sigma	93595

- Maintain the Trypan Blue Cell Counting Solution at room temperature prior to use.
- Prepare the Trypan Blue Cell Counting Solution fresh for each use.

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	Gently wash each channel with 200 µL of Complete Hepatocyte Seeding Medium. Aspirate the medium outflow on each chip's surface, leaving medium in both channels.
4	Repeat the wash with an additional 200 µL of Complete Hepatocyte Seeding Medium per channel, leaving the excess medium outflow that covers the inlet and outlet ports.
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.

Thaw Hepatocytes

Before You Begin

Before thawing the cryopreserved hepatocytes, make sure all equipment is organized and ready for use. Also, be sure that all required reagents are prepared and have reached the appropriate temperature.

Tips for Thawing Cells

- Up to 3 vials of cryopreserved hepatocytes can be thawed at the same time. Once they're thawed, the contents of each should be combined into one 50-mL conical tube (See Step 6 in "Steps" below") and processed as one sample.
- As the cells are thawing, it is critical to work as quickly but gently as possible. This will help maximize cell recovery and minimize damage to the hepatocytes.
- Do not allow the cells to thaw at room temperature or on ice.
- Once the hepatocytes are thawed, dilute them in the cell culture medium as soon as possible to prevent DMSO toxicity within the cryoprotectant.

Steps

Step	Action
1	Place 3 mL of warm Complete Hepatocyte Seeding Medium into a sterile 50-mL conical tube.
2	Remove the required number of cryovials.
3	Spray each cryovial with 70% ethanol and wipe it dry. Twist the cap a quarter of a full turn to relieve any internal pressure, then re-tighten it.
4	Immediately place the frozen vial in a 37°C water bath without submerging the cap. Rapidly thaw hepatocytes by gently swirling the vials in the water bath until only a small ice pellet remains. This process should take only 60–90 seconds. Thawing any longer will decrease viability and cell yield.
5	When one small ice pellet remains, immediately remove the vial from the water bath, wipe it dry, spray it with 70% alcohol, and wipe it dry again before placing it into the BSC.
6	Quickly transfer the contents of the vial into the 3 mL of Complete Hepatocyte Seeding Medium in the sterile 50-mL conical tube prepared in Step 1.
7	Rinse the cryovial with 1 mL of Complete Hepatocyte Seeding Medium and transfer it to the 50-mL conical tube.

8	With gentle agitation and swirling, slowly add enough of the Complete Hepatocyte Seeding Medium to bring the total volume to 35 mL.
9	Add 15 mL of 90% Percoll solution, bringing the total volume to 50 mL.
10	Cap the tube tightly and slowly invert it three times to mix the cell solution.
11	Centrifuge the cells at 96 x g for 6 minutes at room temperature.
12	Return the tube to the BSC. Carefully aspirate the supernatant, leaving 3–5 mL. Ensure the pellet remains undisturbed.
13	Tilt and rotate the tube to gently re-suspend the cell pellet in the remaining medium.
14	Gently add enough Complete Hepatocyte Seeding Medium to bring the total volume to 50 mL.
15	Centrifuge the cells at 72 x g for 4 minutes at room temperature.
16	Return the tube to the BSC. Carefully aspirate the supernatant, leaving 1–2 mL. Ensure the pellet remains undisturbed.

Adjust Cell Density

Overview

Human hepatocytes must be seeded in the Liver-Chip at a density of 3.5×10^6 cells / mL. It is essential to ensure the seeding density is accurate for viable, functional cells and long-term culture.

Steps

Step	Action
1	Tilt and rotate the tube to gently resuspend the cell pellet.
2	Measure the total suspension volume using a 5-mL pipette.
3	Confirm the cell pellet has disappeared, sufficiently rotate to homogenize the cell suspension, and transfer 5 µL of the cell suspension to the Trypan Blue Cell Counting Solution, generating a 1:10 dilution.
4	Mix the trypan blue solution thoroughly and count the cells using a hemocytometer.

Cell Counting and Viability Assessment

Cell Counting and Viability Assessment

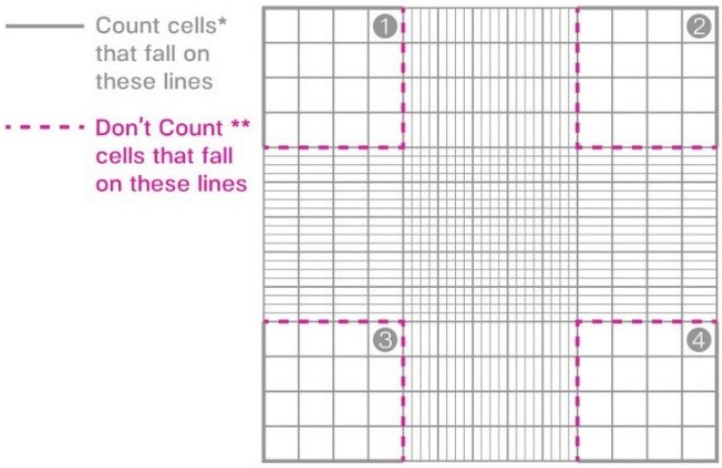


Figure 6. Example hemocytometer

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 solution above.

   Live Cell Count x (1 x 10 x 10^4) ÷ 4 = Viable Cell Concentration (cells / mL)

4. Calculate the viable cell yield.

5. **Viable Cell Concentration x Cell Suspension Volume = Viable Cell Yield (cells)**

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

Diluting Hepatocytes

After calculating the Viable Cell Yield, dilute the hepatocytes with warm Complete Hepatocyte Seeding Medium to the required final cell density: 3.5 x 10^6 x cells / mL.

Continued on next page

Cell Counting and Viability Assessment, Continued

Additional Steps

If the Viable Cell Concentration is less than 3.5 x 10^6 cells / mL:

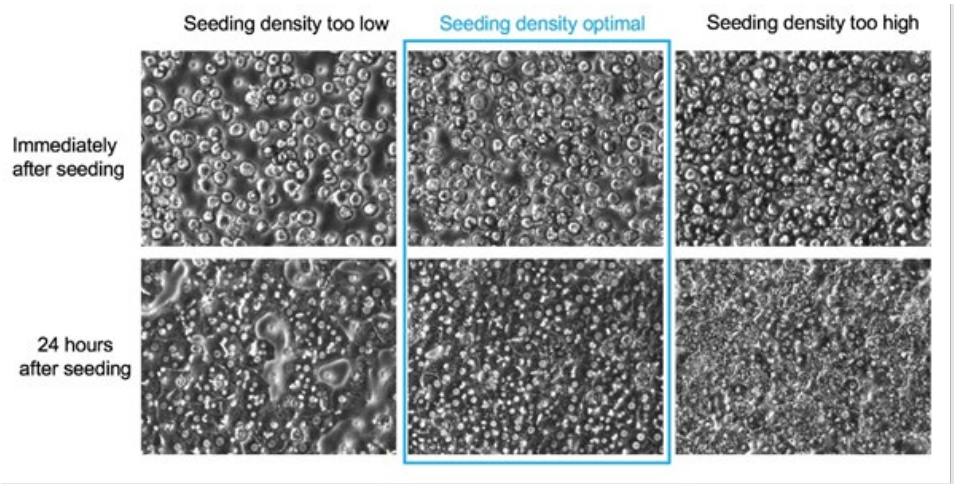
Step	Action
1	Leave the hepatocyte cell suspension undisturbed at room temperature for at least 5 minutes. This will allow the cells to settle at the bottom of the tube.
2	Gently remove enough from the top of the supernatant to decrease total cell suspension volume.
3	Re-count the cell suspension and recalculate the appropriate seeding density accordingly (**Steps 5 and 6 on the next page**). This will help to avoid subjecting hepatocytes to mechanical stress in centrifugation.

Seed Hepatocytes to the Top Channel

Overview

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 before seeding each chip to ensure a homogeneous cell suspension.
4	Quickly and steadily pipette 40 µL of the cell suspension (at 3.5 x 10^6 cells / mL) into the top channel inlet port while aspirating the outflow fluid from the surface. Do not directly touch the outlet port.
5	Cover the dish and transfer it to the microscope to check the seeding density within the chip (see Figure 7). **Note:** At this stage, cells with optimal seeding density will form an even cell layer with ~half a cell radius between individual cells.
	 Figure 7. Hepatocyte seeding density reference chart.
6	If the seeding density is not optimal or cell distribution is not even, return the chips to the BSC, and wash the channel with 200 µL of fresh medium twice. **Do not aspirate the medium from the**

	channels. Adjust the cell density accordingly and repeat steps 3–5 until the density in the channel is correct.
7	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 batches of no more than 12 chips at a time and by immediately placing the batches into the incubator at 37°C.
8	Place the chips (with the DPBS-filled reservoir) in the incubator at 37°C for 4 hours (see Figure 8 for examples of attachment). **Note:** Achieving the correct seeding density is essential for the success of the chips.

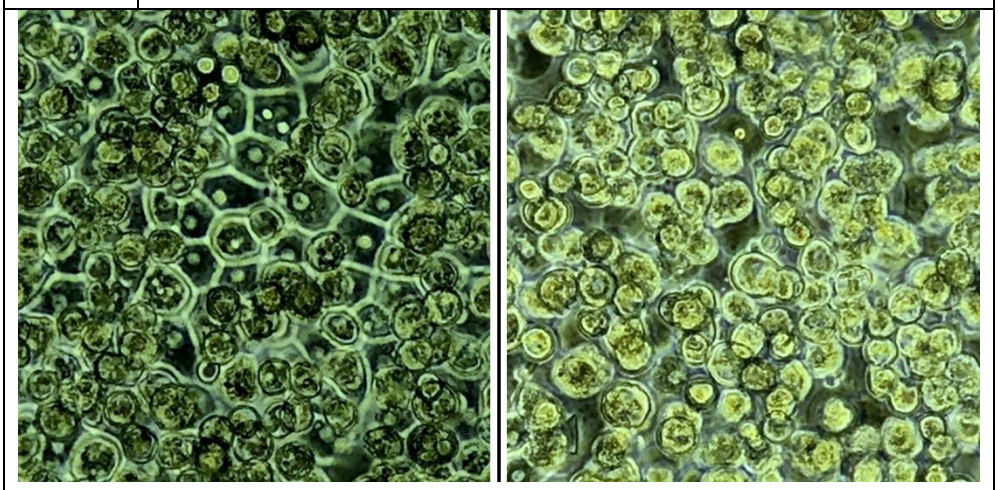


Figure 8. The left image shows appropriate attachment. The right image shows poor attachment.

Seed a Well Plate

Overview

It is recommended to always seed any remaining hepatocytes into a 24-well plate pre-coated with collagen I as control for cell quality.

Steps

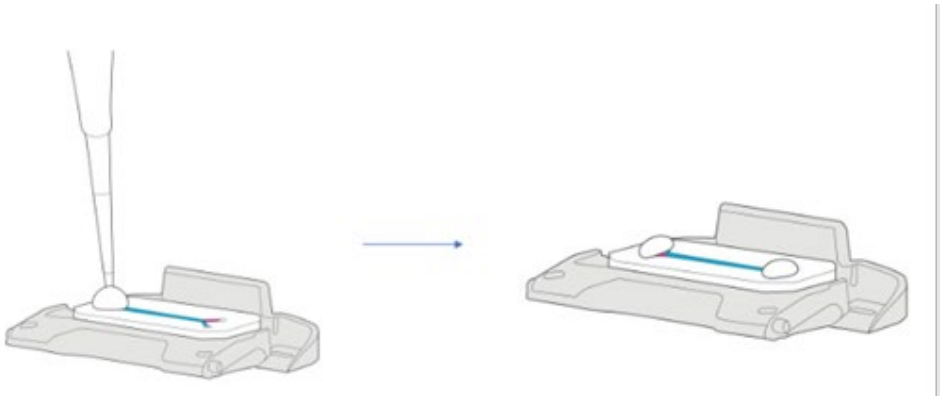
Step	Action
1	Dilute the hepatocyte suspension with warm Complete Hepatocyte Seeding Medium to a final density of **0.8 x 10^6 cells / mL.**
2	Add 400, 500, and 600 µL of the cell suspension to three separate wells of the 24-well plate.
3	Mix each well to ensure an even suspension and allow the cells to settle for 5 minutes on the microscope stage. After this, inspect the densities under a microscope.
4	Determine which of the three wells depict the optimal seeding density. Then, using that well's volume, plate the remaining cells into individual wells until no cells remain.
5	In the incubator, disperse the cells evenly across the bottom of the culture wells by moving the plate in a figure-eight motion flat 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 at least 3 times to evenly disperse the cells. Afterward, do not disturb the plate until the next day so the cells can fully attach.

Gravity Wash

Overview

Once the hepatocytes in the chip have attached (4 hours after seeding), perform a gentle gravity wash to ensure that nutrients are replenished, and the channels do not dry out. During the gravity wash, ensure the medium flows through the channel and exits through the outlets.

Steps

Step	Action
1	With a P200 pipette, gently drop 200 µL on top of both channel inlet ports. This should cause media to gently flow through the channel and exit from the outlets.
2	If the media does not flow through the channel, very gently pipette a small amount of media into the inlets until a small droplet appears on the outlet, or until a bubble is ejected from the outlet.
3	Pipette droplets of media until all inlet and outlet ports are fully covered. Doing so will help to prevent media evaporation from the ports (see Figure 9).
	 Figure 9. Chip with drops of media covering the inlet and outlet ports.
4	Incubate the chips overnight at 37°C.

Day 1: Hepatocyte Overlay

Overview

Required Materials

- Complete Hepatocyte Seeding Medium (at 4°C)
- Complete Hepatocyte Maintenance Medium (at 37°C)
- Hepatocyte Overlay Medium (at 4°C)
- Matrigel aliquot (at 4°C in slushy ice)
- 50-mL conical tube
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- Ice bucket and ice
- 70% ethanol
- Microscope

Key Steps

Prepare Hepatocyte Maintenance Medium

Hepatocyte Maintenance Media

Base Hepatocyte Maintenance Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM (without phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	100X	1%	Sigma	P4333
L-GlutaMAX	5 mL	100X	1%	Gibco	35050-61

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

Complete Hepatocyte Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	49.445 mL	-	-	Recipe above	-
ITS + premix	500 µL	-	1%	Sigma	354352
Ascorbic acid	50 µL	50 mg / mL	500 µg / mL	Sigma	5960
Dexamethasone	5 µL	1 mM	100 nM	Sigma	D4902

- Store the Complete Hepatocyte Maintenance Medium at 4°C in well-labeled tubes.
- Use the Complete Hepatocyte Maintenance Medium within 3 days of preparation.

Prepare Overlay Medium

Before You Begin

Slowly thaw the Matrigel aliquot on ice (which should be made slushy with water) for 30 minutes or until thawed. Keep the Matrigel aliquot in slushy ice at all times, as this solution gels rapidly at temperatures above 10°C. To maintain an even coating, use pre-chilled pipettes, tips, and tubes stored at -20°C prior to use as well as cold medium during preparation and overlay.

Hepatocyte Overlay Medium

Hepatocyte Overlay Medium (20 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium	19.5 mL	-	-	Recipe above	-
Matrigel	0.5 mL	10 mg / mL	0.25 mg / mL	Corning	354234

- Keep both the Matrigel aliquot and the Hepatocyte Overlay Medium on slushy ice.

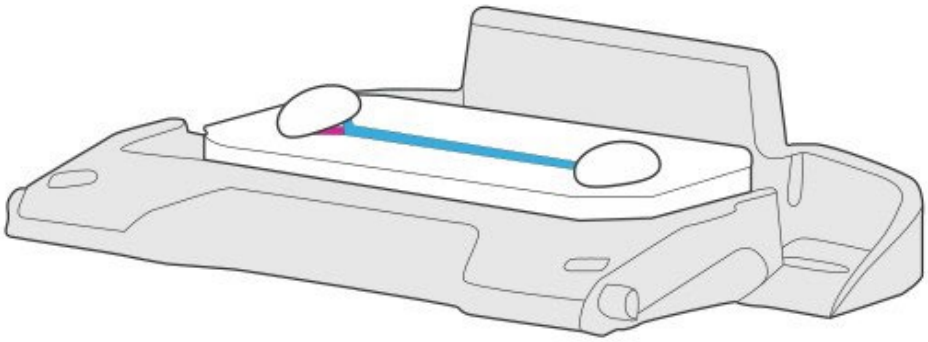
Steps for preparation:

Step	Action
1	Prepare the Hepatocyte Overlay Medium by diluting ice-cold, thawed Matrigel into ice-cold Complete Hepatocyte Maintenance Medium using prechilled tips to a final concentration of 0.25 mg / mL, as directed above.
2	Gently mix the overlay medium well, and keep the Hepatocyte Overlay Medium on ice at all times.

Note: Introducing polymerized Matrigel to chip channels can cause uneven distribution and may impact cell viability and chip performance. If any traces of solidified Matrigel are observed in the Hepatocyte Overlay Medium, discard the entire medium and prepare a fresh batch following the steps above, ensuring all reagents and equipment are kept at 4°C during this step.

Overlay Hepatocytes

Steps for Overlaying Hepatocytes in Chips

Step	Action
1	In the BSC, quickly pipette 200 µL of warm Complete Hepatocyte Maintenance Medium through the top channel of each chip to remove cell debris from the hepatocyte monolayer.
2	Aspirate the media outflow at the outlets, leaving the media within the channel.
3	Using cold tips, gently pipette 200 µL of the cold Hepatocyte Overlay Medium to the top channel of each chip, leaving droplets covering both the inlets and outlet ports (See Figure 10).
	 Figure 10. Chip with Hepatocyte Overlay Medium covering ports.
4	Incubate chips overnight at 37°C.

Steps for Overlaying Hepatocytes in Well Plates

Step	Action
1	In the BSC, vigorously swirl the 24-well plate of hepatocytes to release any cell debris and unattached cells from the monolayer.
2	Aspirate the medium from each well.
3	Add 500 µL of cold Hepatocyte Overlay Medium to each well and return the plate to the incubator to sit overnight.

Day 2: Non-Parenchymal Cells (NPC) to Chip

Overview

Goals

Seed mixture of non-parenchymal cells (LSECs, Kupffer cells, and stellate cells) in the chip.

Required Materials

- NPC Seeding Medium (at 37°C)
- NPC Seeding Medium (at 4°C)
- Complete Hepatocyte Maintenance Medium (at 37°C)
- Trypan blue counting solution
- Hemocytometer
- 15-mL conical tube
- Serological pipettes
- Pipettes and filtered tips
- Aspirator and sterile tips
- Ice bucket and ice
- 70% ethanol
- Microscope

Key Steps

Prepare NPC Seeding Medium

Before You Begin

The LSECs, Kupffer cells, and stellate cells are seeded within the bottom channel, in the NPC Seeding Medium.

NPC Seeding Media

NPC Seeding Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium (omitting dexamethasone)	22.5 mL	-	-	Prepared above, but omit dexamethasone	-
Base LSEC Culture Medium	22.5 mL	-	-	Prepared above	-
FBS	5 mL	-	10%	Sigma	F4135

- Store the NPC Seeding Medium at 4°C.
- Use the NPC Seeding Medium within 3 days of preparation.

Trypan Blue Cell Counting Solution (45 µL)

Reagent	Volume	Source	Cat. No.
NPS Seeding Medium	40 µL	Recipe Above	-
Trypan Blue	5 µL	Sigma	93595

- Maintain the Trypan Blue Cell Counting Solution at room temperature prior to use.
- Prepare three tubes of the Trypan Blue Cell Counting Solution.
- Always prepare the solution fresh before each use.

Wash Chips

Steps

Step	Action
1	Gently pipette 200 µL of warm Complete Hepatocyte Maintenance Medium into the top channel of each chip. Aspirate the outflow, leaving the media in the channel.
2	Gently pipette 200 µL of warm NPC Seeding Medium to the bottom channel of each chip. Aspirate the outflow, leaving the media in the channel.
3	Return the chips to incubator until the NPCs are ready for seeding.

Harvest LSECs

Before You Begin

- To seed the bottom channel, LSECs in culture must be harvested and counted. LSECs are adjusted to a density of 9 x 10^6 cells / mL (3 times the final seeding concentration) before being combined with stellate and Kupffer cells.
- If the LSECs are not as proliferative as expected, the concentration can be increased up to 12 x 10^6 cells / mL (3 times the final seeding concentration) to form a confluent monolayer within the channel.

Steps

Step	Action
1	Bring the culture flask containing LSECs from the incubator 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 2–3 minutes at 37°C.
4	Tap the side of the flask gently. Inspect the culture under the microscope to verify complete cell detachment from the culture surface.
5	Add 9 mL of warm NPC Seeding 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 NPC Seeding Medium the tube, bringing the total volume to 15 mL.
8	Centrifuge LSECs at 200 x g for 5 minutes at room temperature.
9	Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. **Note:** The cell pellet will be very small, so be sure to aspirate gently.
10	Loosen the cell pellet by flicking the tube gently.
11	Using a P1000 pipette, gently resuspend the cells by adding 200 µL of cold NPC Seeding Medium.
12	Pipette gently to create a homogenous mixture, and transfer 5 µL of the cell suspension to the Trypan Blue Counting Solution. This will create a 1:10 dilution.

13	Mix the counting solution thoroughly. Count the cells using a hemocytometer (See “Cell Counting and Viability Assessment”).
14	Dilute the LSECs to 9 x 10^6 cells / mL (3 times the final seeding concentration) in cold NPC Seeding Medium.
15	Keep the LSEC cell suspension on ice until the stellate and Kupffer cells are ready.

Thaw Stellate Cells

Before You Begin

Stellate cells must be thawed and counted before bottom cell seeding. Stellate cells are adjusted to a density of 0.3 x 10^6 cells / mL (3 times the final seeding concentration) prior to combining with LSECs and Kupffer cells.

Steps

Step	Action
1	Place 3 mL of warm NPC Seeding Medium into a sterile 15-mL conical tube.
2	Remove the required number of cryopreserved stellate cell vials from the liquid nitrogen.
3	Spray the cryovial(s) with 70% ethanol and wipe it dry. Untwist the cap a quarter of a full turn to relieve any internal pressure, then retighten it. **Note:** Adjusting the cap this way will prevent the cryovial from popping due to rapid expansion of any liquid nitrogen that may have been trapped inside the vial.
4	Immediately place the frozen vial in a 37ºC water bath without submerging the cap. Rapidly thaw the stellate cells by gently swirling the vial in the water bath until only one small ice pellet remains. **Note:** This should take 60–90 seconds. Thawing for longer will result in decreased cell viability and yields.
5	Immediately remove the vial from the water bath, wipe it dry, spray it with 70% alcohol, and dry it once more before placing it into the BSC.
6	Quickly transfer the vial's contents into the 15-mL conical tube prepared in Step 1.
7	Rinse the cryovial with 1 mL of warm NPC Seeding Medium and transfer it to the 15-mL conical tube.
8	Bring the volume within the conical tube to 15 mL using cold NPC Seeding Medium.
9	Centrifuge stellate cells at 250 x g for 5 minutes at room temperature. Once done, cool centrifuge to 4°C to prepare for Kupffer cell seeding.
10	Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. **Note:** The cell pellet will be very small. Aspirate carefully.

11	Gently flick the tube to loosen the cell pellet.
12	Using a P1000 pipette, carefully resuspend the cells by adding 200 µL of cold NPC Seeding Medium.
13	Pipette gently to recreate a homogenous mixture, and transfer 5 µL of the cell suspension to the trypan blue cell counting solution (1:10 dilution).
14	Mix the counting solution thoroughly. Count the cells using a manual hemocytometer (See “Cell Counting and Viability Assessment”).
15	Dilute the stellate cells to 0.3 x 10^6 cells / mL (3 times the final seeding concentration) in cold NPC Seeding Medium and keep them on ice until the rest of the cells (Kupffer) are ready.

Thaw Kupffer Cells

Before You Begin

- Kupffer cells must be thawed and counted for bottom channel seeding. Kupffer cells are adjusted to a density of 6 x 10^6 cells / mL (3 times the final seeding concentration) prior to combining with LSECs and stellate cells.
- Kupffer cells are very sticky at the physiological temperature of 37°C. If the medium is warmed to 37°C, the Kupffer cells will attach to any substrate, including the walls of the conical tube and the pipette tip. Therefore, use cold NPC Seeding Medium and pre-chilled tips throughout the thawing process.

Steps

Step	Action
1	Place 3 mL of cold NPC Seeding Medium into a sterile 15-mL conical tube.
2	Remove the required number of cryopreserved Kupffer cell vials from the liquid nitrogen.
3	Spray the cryovial(s) with 70% ethanol and wipe it dry. Untwist the cap a quarter of a full turn to relieve any internal pressure, then retighten it. **Note:** Adjusting the cap this way will prevent the cryovial from popping due to rapid expansion of any liquid nitrogen that may have been trapped inside the vial.
4	Immediately place the frozen vial in a 37ºC water bath without submerging the cap. Rapidly thaw the Kupffer cells by gently swirling the vials in the water bath until only one small ice pellet remains. **Note:** This should take 60–90 seconds. Thawing for longer will result in decreased cell viability and yields.
5	Immediately remove the vial from the water bath, wipe it dry, spray it with 70% alcohol, and dry it once more before placing it into the BSC.
6	Quickly transfer the vial's contents into the 15-mL conical tube containing 3 mL of NPC Seeding prepared in Step 1.
7	Rinse the cryovial with 1 mL of warm NPC Seeding Medium and transfer it to the 15-mL conical tube.
8	Bring the volume within the conical tube to 15 mL using cold NPC Seeding Medium.
9	Centrifuge the Kupffer cells at 500 x g for 5 minutes at 4°C.

Steps, continued

Step	Action
10	Carefully aspirate the supernatant, leaving approximately 100 µL of medium above the cell pellet. **Note:** The cell pellet will be very small. Aspirate carefully.
11	Gently flick the tube to loosen the cell pellet.
12	Using a P1000 pipette, carefully resuspend the cells by adding 200 µL of cold NPC Seeding Medium.
13	Pipette gently to recreate a homogenous mixture, and transfer 5 µL of the cell suspension to the Trypan Blue Cell Counting Solution (1:10 dilution).
14	Mix the counting solution thoroughly. Count the cells using a hemocytometer (See "Cell Counting and Viability Assessment").
15	Dilute the Kupffer cells to 6.0 x 10^6 cells / mL (3 times the final seeding concentration) in cold NPC Seeding Medium and keep them on ice until use.

Combine NPC Mixture

Before You Begin

With all three cell types prepared and at the proper density, prepare the cell suspension mixture that will be seeded into the bottom channel.

Combining to Final Densities

The final density of each cell type in the bottom channel will be:

Cell Type	Densities
LSECs	3 x 10^6 cells / mL
Stellate cells	0.1 x 10^6 cells / mL
Kupffer cells	2 x 10^6 cells / mL

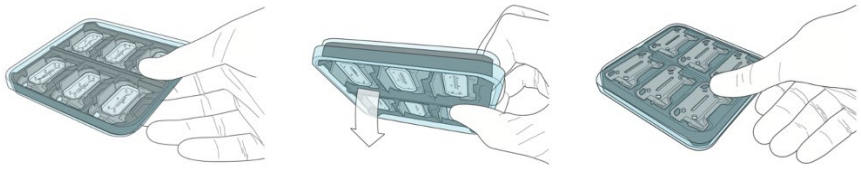
- Mix the three NPC cell suspensions in a 1:1:1 ratio (v/v/v) inside a sterile, 15-mL conical tube on ice. Ensure there is enough seeding solution for all chips—calculate up to 15 µL per chip, leaving a small amount of extra volume.

Seed NPC Mixture to Bottom Channel

Before You Begin

Work with one chip at a time. After seeding the first chip, assess the cell density within the channel using a microscope, and then immediately invert the chips. Adjust the density of the cell suspension accordingly for the next chips if necessary.

Steps

Step	Action
1	Seed 15-20 µL of the combined NPC cell suspension into the bottom channel while aspirating the outflow.
2	After seeding, aspirate the DPBS from the reservoir and invert the Chip Cradle (see Figure 11). **Note:** Each Chip Cradle can support up to six chips inside a Square Cell Culture Dish (120 x 120 mm).
3	Place the small reservoir (15-mL conical tube cap containing sterile DPBS) inside the Square Cell Culture Dish (120 x 120 mm) to provide humidity for the cells. Replace the dish lid.
 Figure 11. Inverting chips during endothelial attachment	
4	Place the dish containing the chips into the 37°C incubator for 4 hours, or until the cells in the bottom channel have attached.

Prepare NPC Maintenance Medium

NPC Maintenance Medium

NPC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium omitting Dexamethasone	24.5 mL	-	-	-	-
Base LSEC Culture Medium	24.5 mL	-	-	-	-
FBS	1 mL	-	2%	Sigma	F4135

- Store at 4°C.
- Use the NPC Maintenance Medium within three days of preparation.

Note: You can use 10% FBS if your seeding density is low for 24-48 h and then switch to 2% FBS.

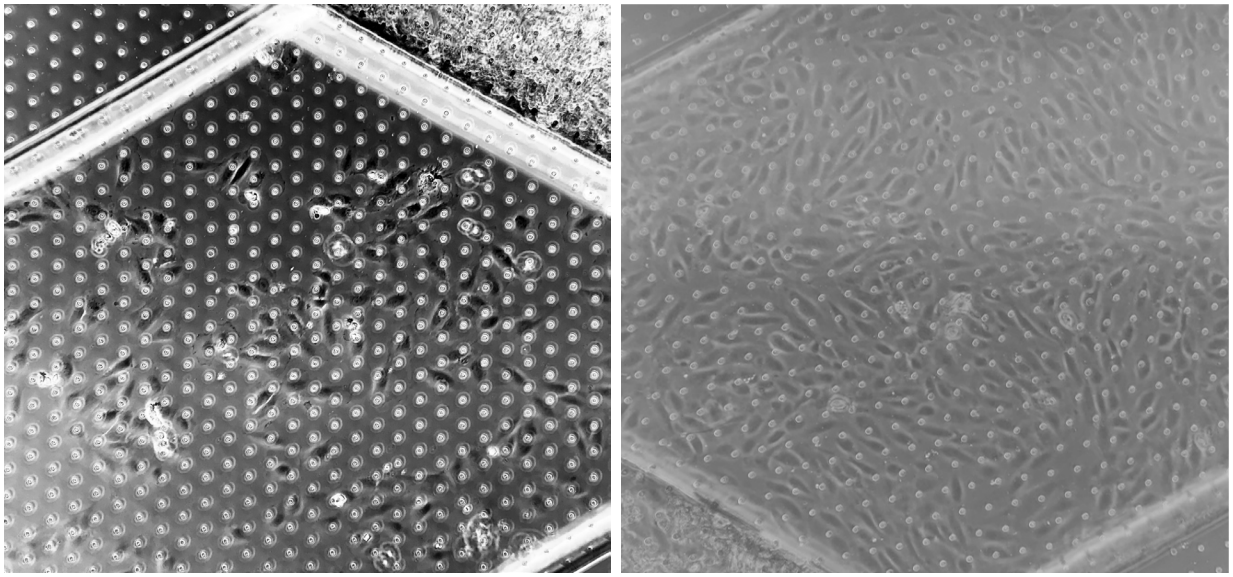
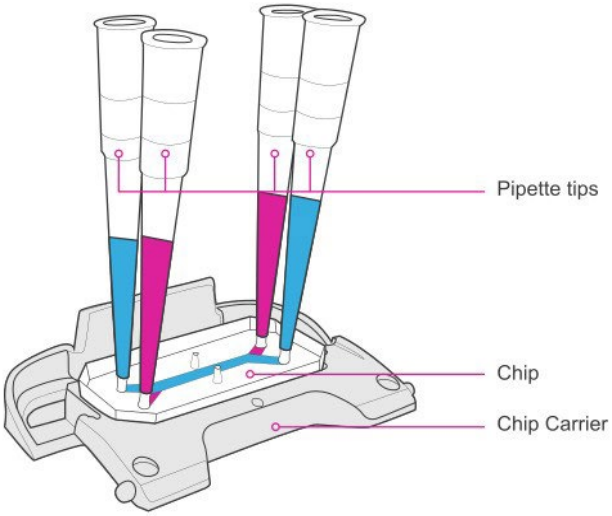


Figure 12: If seeding density is low (left), continued use of 10% FBS is recommended. If seeding density is optimal (right), culture cells with 2% FBS.

Gravity Wash with Tips

Steps

Step	Action
1	Once NPCs have attached (approximately 4 hours after seeding), orient the chips upright.
2	Gravity wash the top channel with 200 µL of Hepatocyte Maintenance Medium and the bottom channel with NPC Maintenance Medium for each chip to provide cells with nutrients. The two media in use must be maintained in filtered tips instead of drops (see Figure 13).
	 Figure 13. Chip with filtered tips inserted into ports with respective media.
3	Return the chips with pipette tips inserted into each outlet port to the incubator, where they will remain overnight.
4	Maintain cells in static culture within the chips until the next day, when Pods are connected to Zoë. **Note:** If desired, chips can be connected as early as 2 h post attachment (6 h post seeding).

Day 3: 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
- Prepared chips
- Pods (sterile), 1 per chip
- Tray, 1 per 6 chips
- Steriflip® filtration unit: PVDF filter 0.45 µm (sterile)

Key Steps

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

- Once the culture media for both chip channels have been warmed for at least 1 h, the gas equilibration step can be performed.
- 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	Place at least 3.3 mL of Complete Hepatocyte Maintenance Medium for each chip in a 50-mL conical tube.
2	Place at least 3.3 mL of NPC Maintenance 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 bath or bead bath for at least 1 h.
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 14).
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 tube. **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 “Media take too long to pass through Steriflip” in the troubleshooting section.
	4	Leave the filtered medium under vacuum for at least five minutes.

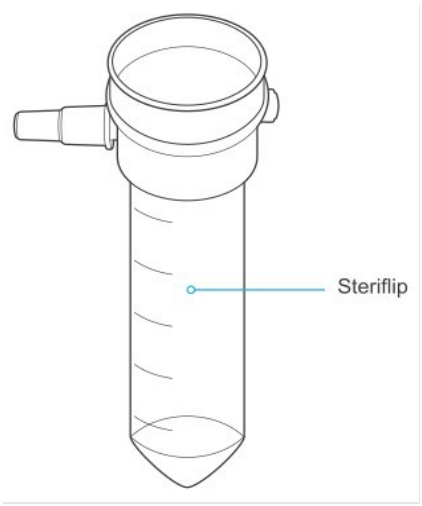


Figure 14. Steriflip unit

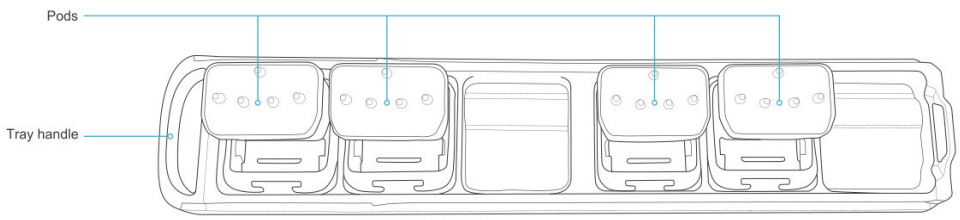
5	Remove the vacuum tubing from the Steriflip unit.
6	Separate the conical tubes containing media from the Steriflip unit, and immediately place them into the incubator with the caps loose to maintain the degassed state and allow bubbles to escape. **Note:** Minimize the time media is outside of the incubator when the Pods are 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 15).
	 Figure 15. 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 Hepatocyte Maintenance Medium; in the bottom channel inlet reservoir, add NPC Maintenance Medium.
4	Pipette 300 µL of pre-equilibrated, warm media to each outlet reservoir, directly over each outlet Via (see Figure 16).

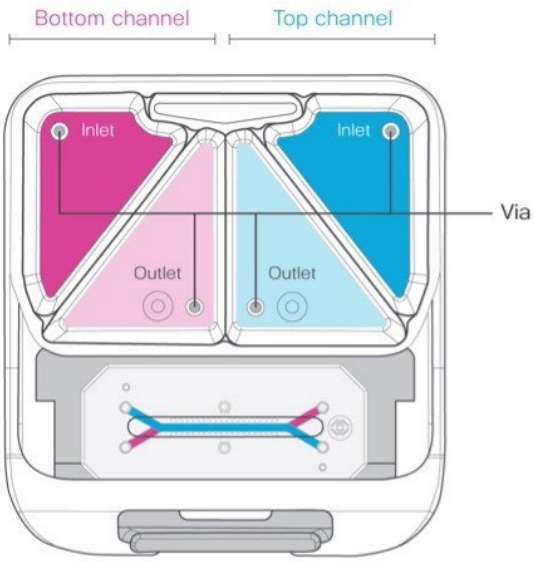


Figure 16: Top view of a Pod.

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 engages 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 them to the BSC.
9	Verify that the Pods were successfully primed. This is critical for success.

Continued on next page

Pod Priming Verification

Take out the tray and inspect the top of the Pods (See Figure 17) 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 “Priming 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 17

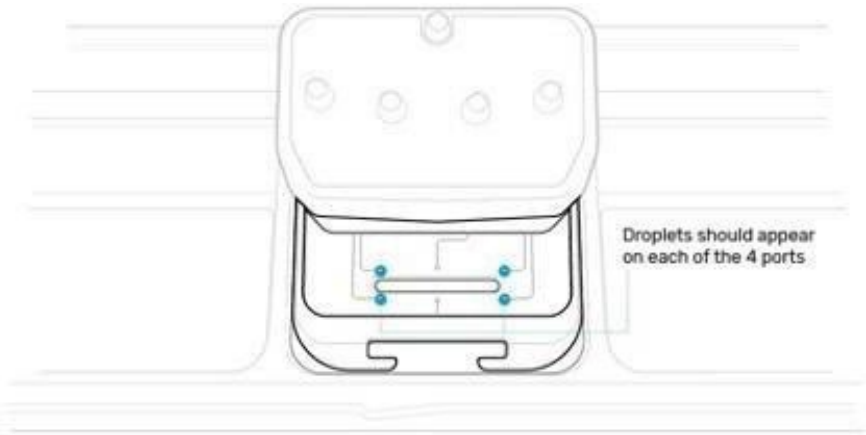


Figure 17. Top view of chip in Pod with fluidic posts covered with droplets.

Wash Chips

Steps

Step	Action
1	Transfer the seeded chips in the Square Cell Culture Dish (120 x 120 mm) 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 Hepatocyte Maintenance Medium to remove any bubbles.
4	Place small droplets of equilibrated Complete Hepatocyte Maintenance Medium on each chip's inlet and outlet port.
5	Gently wash each chip's bottom channel with warm, equilibrated NPC Maintenance Medium to remove any possible bubbles as well as to replace with de-gassed media.
6	Place small droplets of equilibrated NPC Maintenance 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 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. Additionally, clean all excess media from the trays and bottoms of pods using a wipe sprayed with 70% ethanol.
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.
7	Bring the tray to the incubator until all samples are connected to keep the equilibrated media warm.

Pods to Zoë

Steps

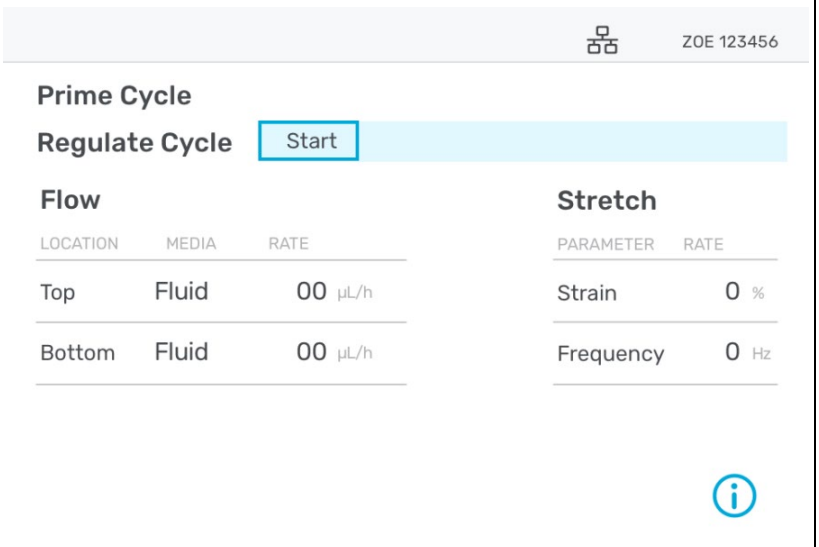
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Immediately place the trays holding Pods and chips into Zoë to prevent media from cooling and losing its gas equilibration.</td></tr>
<tr><td>2</td><td>Program the appropriate Organ-Chip culture conditions on Zoë.
These conditions will start as soon as the Regulate Cycle is complete.
For human Quad-Culture Liver-Chips, set the flow rate 30 µL / h for both top and bottom channels.</td></tr>
<tr><td>3</td><td>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 18).
Note: Once start is selected, there will be a sound as Zoë engages the Pods.</td></tr>
<tr><td colspan="2"> 

Figure 18. 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 — The Regulate Cycle takes 2 hours to finish. Once it is complete, Zoë will begin flow at the preset Organ-Chip culture conditions (see Figure 19).

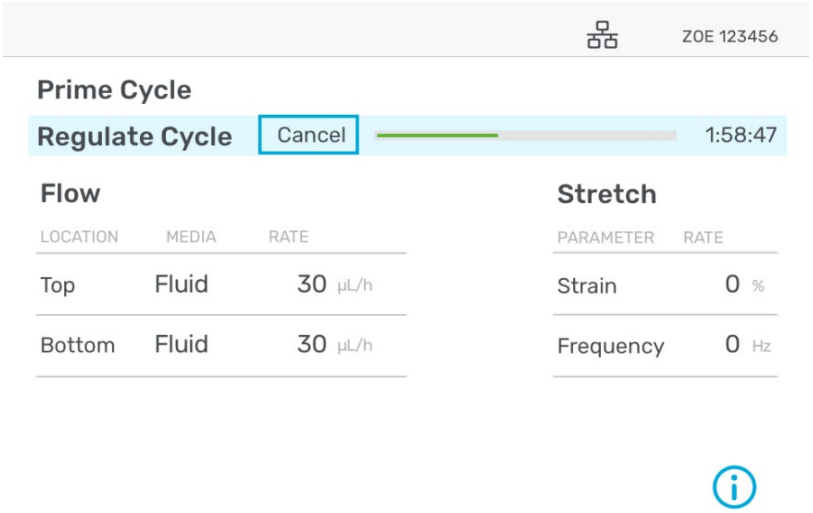


Figure 19. Zoë UI screen showing Regulate Cycle in progress.

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 4: Chip Maintenance and Sampling

Overview

Goals

- Maintain chips in Zoë.
- Cell culture inspection
- Collect samples for analysis

Required Materials

- Chips in Pods
- Cell culture media

Key Steps

Maintenance and the Regulate Cycle

Steps

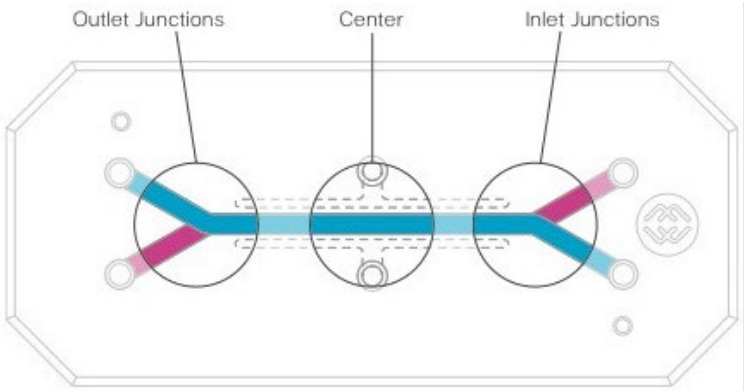
Step	Action
1	The day after connecting chips and Pods to Zoë (beginning the Organ-Chip culture process), pause Zoë by pressing the silver “Activation” button located above the tray bays. This stops flow and releases the Pods.
2	Slide the tray out of the bay, and transfer it to the BSC
3	Remove the Pod lids. Using a 200-µL pipette, perform a Via wash on each Pod inlet and outlet reservoir using the following steps: **Step** / **Action** 1 — Pipette 200 µL of media from the Pod reservoir directly on top of the Via to dislodge any bubbles that may be present. 2 — Repeat this wash step for each of the four Pod reservoirs.
4	Place Pod lids back on and return the trays to Zoë.
5	Run the Regulate Cycle again. **Step** / **Action** 1 — Using the Dial, highlight the “Regulate” field. 2 — Press the Dial Button to select “Regulate,” and rotate the Dial clockwise to “Start.” 3 — 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. 4 — Make sure the “Activation” button is glowing blue. 5 — The Regulate Cycle takes 2 hours to finish. Once it is complete, Zoë will begin flow at the preset Organ-Chip culture conditions.

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 3.5 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 20): • Inlet Junction • Center of Channel • Outlet Junction
	 Figure 20. Chip with marked locations for image capture.
5	Remove Pod lids and collect effluent from the Pod outlet reservoirs 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 cell culture 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	Place the Pod lids back on 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 take 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 2 hours 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 Pods 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 out 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 the Pod with media, and reconnect the chip to the Pod.

Potential Root Causes of Bubbles

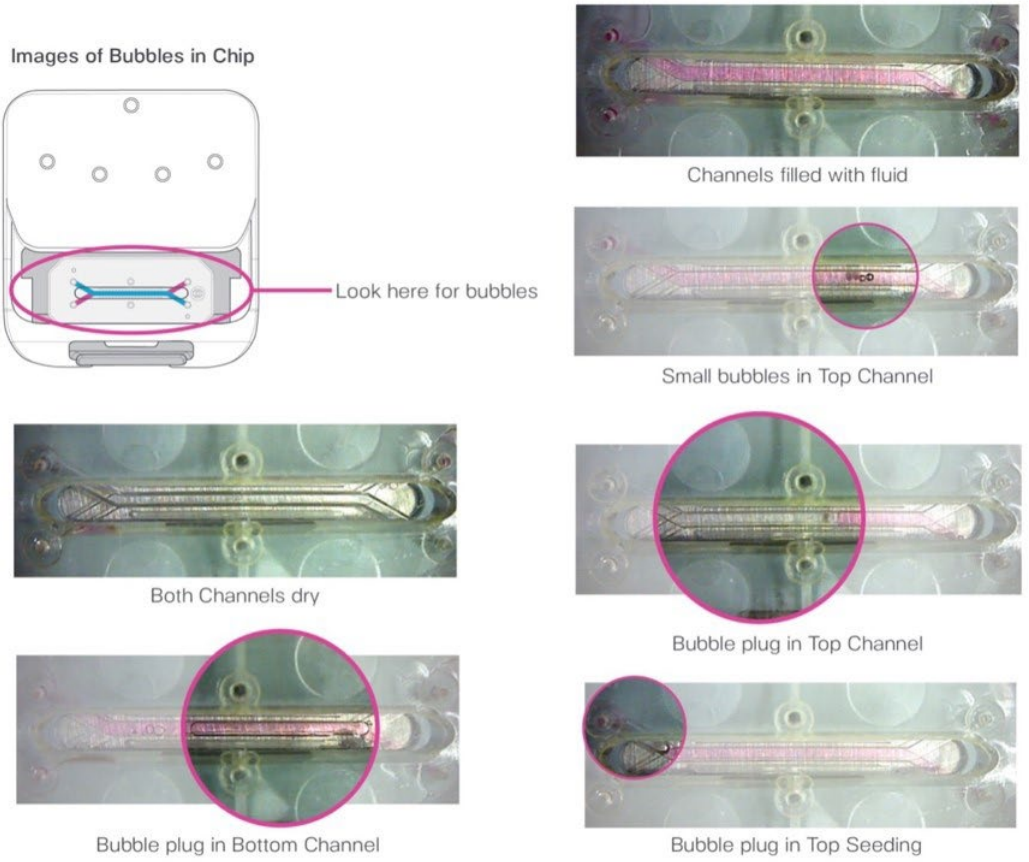
If there is a high failure rate due to bubbles, or if bubbles are persistent, despite performing the above mitigation step (See Figure 21 on the next page), check for the following:

If ...	Then ...
Medium is 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 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.

Continued on next page

Overview, Continued

Figure 21 Images of Bubbles in an Organ-Chip



Part VIII: Appendices

Overview

Introduction

This section contains all the reagent and media preparation steps found in this protocol.

Contents

Reagent Aliquots

Reagents

Fibronectin (ECM)

Reagent	Conc. [Stock]	Volume	Solvent
Fibronectin	1 mg / mL	1 mg	Cell culture grade water

- Resuspend 1 mg fibronectin in 1 mL of cell culture grade water according to manufacturer's instructions.

- Create single-use volume aliquots and store them 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, create 5-mg aliquots based on the specific stock concentration. For example, if the stock concentration is 10 mg / mL, 500 µL of solution will yield 5 mg of Matrigel.

- Store aliquots at -20°C.

Culture Supplements

Reagent	Conc. [Working]	Volume	Solvent
L-Ascorbic Acid	50 mg / mL	Calculate based on amount measured	Cell culture grade water
Dexamethasone	10 mM	Calculate based on amount measured	Cell culture grade DMSO
Dexamethasone	1 mM	Calculate based on amount measured	Cell culture grade water

- Resuspend each supplement to the working concentration in the table above.

- Aliquot each supplement to single-use volumes.

- Store aliquots at -20°C.

Media Recipes

LSEC Culture Media

Base LSEC Culture Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
CSC Basal Medium	485 mL	-	-	Cell Systems	4Z3-500
Culture-boost	10 mL	-	2%	Cell Systems	4CB-500
Pen / strep	5 mL	-	1%	Sigma	P4333

- Store the LSEC Culture Medium at 4°C.
- Use the LSEC Culture Medium within 30 days of preparation.

Complete LSEC Culture Medium (50 mL)

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

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

Continued on next page

Media Recipes, Continued

Hepatocyte Seeding Media

Base Hepatocyte Seeding Medium (500 mL bottle)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM + (with phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	-	1%	Sigma	P4333
L-GlutaMAX	5 mL	-	1%	Gibco	35050-61

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

Complete Hepatocyte Seeding Medium (200 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	187.78 mL	-	-	Recipe Above	-
ITS+ premix	2 mL	-	1%	Corning	354352
Ascorbic Acid	200 µL	50 mg / mL	0.05 mg / mL	Sigma	5960
Dexamethasone	20 µL	10 mM	1 µM	Sigma	D4092
FBS	10 mL	-	5%	Sigma	F4135

- Store the Complete Hepatocyte Seeding Medium at 4°C.
- Use the Complete Hepatocyte Seeding Medium within 3 days of preparation.

90% Percoll Solution (20 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Percoll Solution	18 mL	100%	90%	Sigma	P4937
10X DPBS (- / -)	2 mL	100%	10%	-	-

- Maintain the 90% Percoll Solution at room temperature prior to use.
- Prepare the Percoll Solution fresh for each use.

Continued on next page

Media Recipes, Continued

Hepatocyte Seeding Media, cont.

Trypan Blue Cell Counting Solution (45 µL)

Reagent	Volume	Source	Cat. No.
Complete Hepatocyte Seeding Medium	40 µL	Recipe Above	-
Trypan Blue	5 µL	Sigma	93595

- Maintain the Trypan Blue Cell Counting Solution at room temperature prior to use.
- Prepare the Trypan Blue Cell Counting Solution fresh for each use.

Hepatocyte Maintenance Media

Base Hepatocyte Seeding Medium (500 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM (without phenol red)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	100X	1%	Sigma	P4333
L-GlutaMAX	5 mL	100X	1%	Gibco	35050-61

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

Complete Hepatocyte Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Seeding Medium	49.445 mL	-	-	Recipe above	-
ITS+ premix	500 µL	-	1%	Sigma	354352
Ascorbic acid	50 µL	50 mg / mL	500 µg / mL	Sigma	5960
Dexamethasone	5 µL	1 mM	100 nM	Sigma	D4902

- Store the Complete Hepatocyte Maintenance Medium at 4°C in well-labeled tubes.
- Use the Complete Hepatocyte Maintenance Medium within 3 days of preparation.

Continued on next page

Media Recipes, Continued

Hepatocyte Overlay Medium

Hepatocyte Over Medium (20 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium	19.5 mL	-	-	Recipe Above	-
Matrigel	0.5 mL	10 mg / mL	0.25 mg / mL	Corning	354234

- Keep both the Matrigel aliquot and the Hepatocyte Overlay Medium on slushy ice.

NPC Seeding Media

NPC Seeding Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium (omitting dexamethasone)	22.5 mL	-	-	Prepared above, but omit dexamethasone	-
Base LSEC Culture Medium	22.5 mL	-	-	Prepared above	-
FBS	5 mL	-	10%	Sigma	F4135

- Store the NPC Seeding Medium at 4°C.
- Use within 3 days of preparation.

Trypan Blue Cell Counting Solution (45 µL)

Reagent	Volume	Source	Cat. No.
NPS Seeding Medium	40 µL	Recipe Above	-
Trypan Blue	5 µL	Sigma	93595

- Maintain the Trypan Blue Cell Counting Solution at room temperature prior to use.
- Prepare three tubes of the solution.
- Always prepare the solution fresh before each use.

Continued on next page

Media Recipes, Continued

NPC Maintenance Medium

NPC Maintenance Medium (50 mL)

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Complete Hepatocyte Maintenance Medium (omitting dexamethasone)	24.5 mL	-	-	Prepared above, but omit dexamethasone	-
Base LSEC Culture Medium	24.5 mL	-	-	Prepared above	-
FBS	1 mL	-	2%	Sigma	F4135

- Store at 4°C.
- Use within 3 days of preparation.

Note: You can use 10% FBS if your seeding density is low for 24-48 h and then switch to 2% FBS.

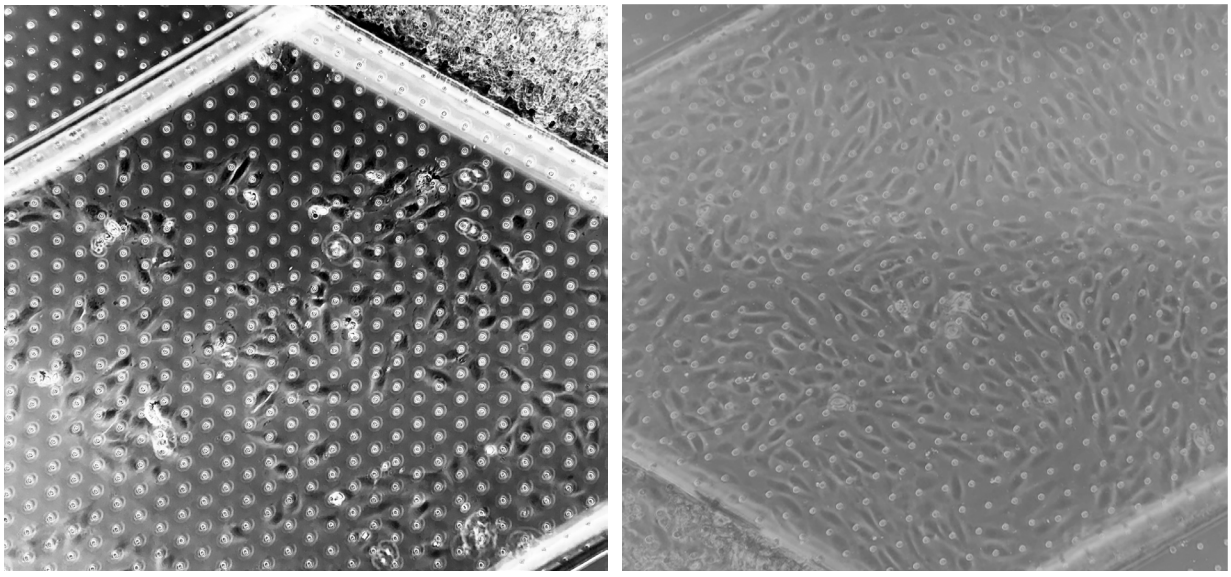


Figure 12: If seeding density is low (left), continued use of 10% FBS is recommended. If seeding density is optimal (right), culture cells with 2% FBS.