AAV Dosing and Solution Treatment Protocol

Goals	Key Steps	Associated Emulate Documentation
● Prepare AAV treatment solution at different MOIs. ● Treat cells in Emulate Organ-Chips with AAV through epithelial (top) and/or endothelial (bottom) dosing strategies. ● Assess the transduction levels using an appropriate reporter.	● Prepare AAV control stock solutions. ● Prepare treatment solution at varying MOIs. ● Treat chips with AAV at test MOIs in the epithelial channel.	● EP-008: Liver-Chip Co-Culture Protocol ● EP-123: Bright-Field & Phase-Contrast Imaging ● EP-124: Effluent Sampling ● EP-126: Fluorescence Imaging ● EP-137: Fixation and Immunofluorescence (IF) Staining ● EP-152: Compound Treatment Solution: Preparation and Treatment ● EP-177: Basic Research Kit Protocol

Key Words and Acronyms	Meaning
AAV	Adeno-Associated Virus
Null	Empty vector with no transgene to be used as a control vector
GFP	Green Fluorescent Protein that serves as the transgene reporter
MOI	Multiplicity of Infection for the number of virus particles per cell
Transduction	Transfer of genetic material into host cell via viral vector to express a transgene
GC	Genome Copies, or number of virus particles per mL

Part I. Introduction

Overview

Introduction

This protocol guides users through preparing AAV dosing media to use with Emulate Liver-Chips.

this protocol

This protocol contains the parts listed below.

Overview

Introduction

This protocol describes the steps for:

- Preparing Adeno-Associated Virus (AAV) stock solutions at different Multiplicity of Infection (MOI) levels.
- Treating Emulate Organ-Chips with AAV using dosing strategies for both the epithelial (top) and/or endothelial (bottom) channel.
- Assessing the transduction levels using an appropriate reporter.

Required Materials

- AAV6-CMV-NULL (Vector BioLabs)
- AAV6-CMV-GFP (Vector BioLabs)
- Liver Bio-Kit, Co-Culture, 12-Pack (Emulate BIO-LH-CO12)
- NucBlue™ (Thermo R37605)

For directions on co-culturing the Liver-Chip, please click here.

Part II. Experimental Timeline

Overview

Introduction

This chapter discusses the experimental timeline.

Contents

Timeline

Timeline

The total timeline for this protocol applies to the day after connecting the Liver-Chip (Day -1) for the remainder of the experiment.

Day	Description
-1	● To do a quality check, scan the chips through a phase contrast microscope, and remove poorly attached chips. ● Take representative morphology images (EP-123). ● Assign chips to groups, prepare AAV stocks, and administer them to the chips according to the design (Liver AAV Sample Dosing Calculator and EP-152).
0	● Collect/Aspirate effluent as needed (EP-124). ● Aspirate inlet media and replace it with blank media. ● Flush at 1000 µL / h for 5 minutes to remove any remaining dosing media from the channel. ● Image all chips (EP-123 and EP-126). ● Switch flow back to 30 µL / h.
1	● Image all chips. ● Collect effluent from channels. Store at -80°C for assays.
2	● Aspirate all channels. ● Refresh blank Media.
3	● Image all chips. ● Collect effluent from all channels and store at -80°C for assays.
4	● Repeat the steps from Day 2.
5	● Maintain.
6	● Repeat the steps from Day 2.
7	● Image all chips. ● Collect effluent from channels. Store at -80°C for assays. ● Live image chips for full chip tiles and GFP quantification. ● Fix chips in 4% PFA for 15 min, Wash with PBS and store them in 4°C untill future use (EP-137).

Figure 1 Timeline visualization

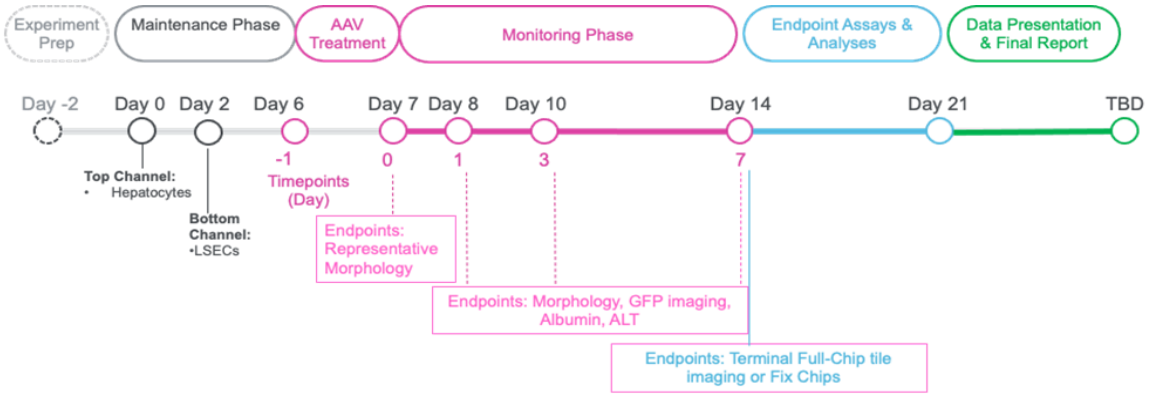


Figure 2 Dosing table

Model	Group Num.	Treatment Condition		MOI	Dosing Channel	Num. of Chips	Endpoints (0h)	Endpoints (24h, 72h, 168h)	Terminal Endpoints (168h)
Human Co-Culture Liver Chip	1	-	Vehicle Control	-	-	3	Morphology (n=1): • Brightfield & GFP (ECHO)	Morphology (n=3): • Brightfield & GFP (ECHO) Effluent (n=3): • Albumin • ALT	Live IF Imaging (n=3) • Full-Chip Tiles • Nuclear: NucBlue • Transduction: GFP • Quantifying average GFP intensity over total cell population Fix Chips (n=3)
	2	Customer Asset – Null	Negative Control	High	Epithelial/ Endothelial	3			
	3	AAV6 – GFP	Positive Control (Original Vector)	500,000	Epithelial	3			
	4	Customer Asset 1	-	TBD	Epithelial/ Endothelial	3			
	5	Customer Asset 2	-	TBD	Epithelial/ Endothelial	3			
	6	Customer Asset 3	-	TBD	Epithelial/ Endothelial	3			
					24				

Part III. Liver-Chip Co-Culture Protocol

Overview

Introduction

Timeline for establishing the Liver-Chip. To establish the Liver-Chip, please follow the instructions from EP008 Liver-Chip Co-Culture Protocol.

Contents

Timeline

Required Materials

To establish the Liver-Chip Co-Culture, you will need an Emulate Liver Bio-Kit. For complete information, please follow the instructions in EP008: Liver-Chip Co-Culture Protocol.

Days

Timeline for establishing the Liver-Chip Co-Culture.

Day	Description
-1	Chips are prepared.
0	Hepatocytes are seeded onto the chip.
1	Hepatocyte Overlay.
2	LSECs on chip.
3	Chips to Pods and Pods to Zoë.

Part IV. AAV Stock Solution Preparation

Overview

Introduction

This section provides guidance on preparing all stock solutions needed for AAV treatment.

Contents

Preparation of Hepatocyte Maintenance Medium

Base Hepatocyte Maintenance Medium

The materials required to prepare the Base Hepatocyte Maintenance Medium (500 mL) are as follows:

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
WEM (-)	490 mL	-	-	Sigma	W4128
Pen / Strep	5 mL	100X	1%	Sigma	P4333
L-GlutaMAX	5 mL	100X	1%	Gibco	35050-61

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

Complete Hepatocyte Seeding Medium

The materials required to prepare the Complete Hepatocyte Seeding Medium (200 mL) are as follows:

Reagent	Volume	Conc. [Stock]	Conc. [Final]	Source	Cat. No.
Base Hepatocyte Maintenance Medium	49.445 mL	-	-	Recipe above	-
ITS+Premix	500 µL	-	1%	Corning	354352
Ascorbic acid	200 µL	50 mg / mL	0.05 mg / mL	Sigma	5960
Dexamethasone	5 µL	1 mM	100 nM	Sigma	D4902

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

Preparation of LSEC Media

Basic LSEC Culture Medium

The materials required to prepare the Basic LSEC Culture Medium (500 mL) are as follows:

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 4°C
- Use within 30 days of preparation

Complete LSEC Culture Medium

The materials required to prepare the Complete LSEC Culture Medium (50 mL) are as follows:

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

- Store at 4 °C
- Use within 7 days of preparation

AAV Stock Solution

AAV Stock Solution

The steps for preparing the AAV stock solution are as follows:

Step	Action
1	Ensure that all materials and reagents required to prepare the stock solution are sterile and ready.
2	Do not reconstitute the stock, as it comes ready for use.
3	Thaw AAV stock on ice.

Part V. AAV Treatment Solution Preparation

Overview

Introduction

This section provides guidance on preparing all AAV treatment solutions needed for this application.

Contents

AAV Treatment Solution

Goals

To prepare the AAV treatment solution using the Liver AAV Sample Dosing Calculator.

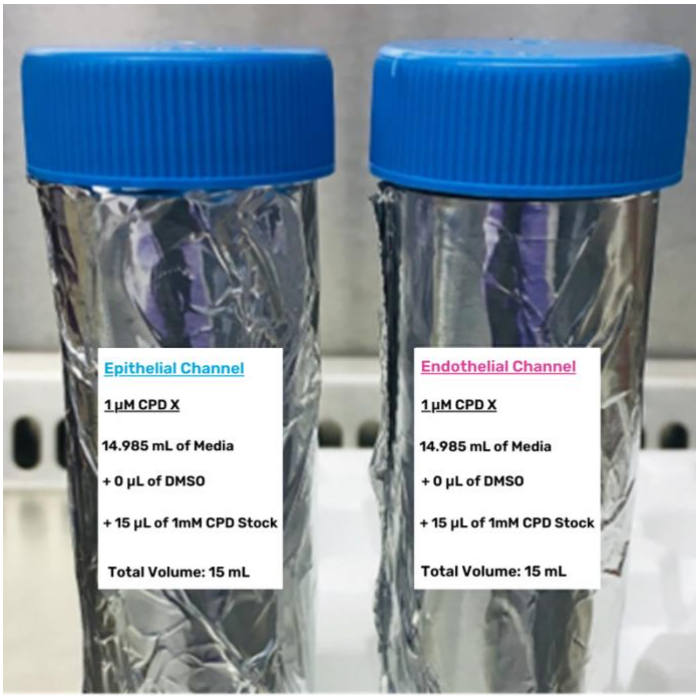
Note: Prepare all dosing solutions within an hour before use. This will help to minimize variability or instability.

Steps

The steps for AAV treatment are as follows:

Step	Action
1	Calculate the volume of media needed for each Organ-Chip using the Liver AAV Sample Dosing Calculator. Additionally, calculate volumes of media, stock solution, and solvent required for each concentration of the test compound based on the number of chips, flow rate, and duration of each concentration (see the calculation example below).
2	Pre-warm complete hepatocyte and/or LSEC media for chip treatment at 37 °C for 1 h. Ensure that you are preparing the solutions in the correct medium for each channel.
3	While the media is warming, gather and label the 50 mL conical tubes for each dosing group. (See Figure 3).
4	Once the media is warmed, collect the aliquot(s) of stock solution needed to prepare the test AAV treatment solution.
5	Following the dosing calculations below, add the appropriate stock volume to produce the treatment solution(s) in medium at the required concentrations / MOIs.
6	Mix medium by vortex to ensure the stock solution is completely dissolved in the medium.
7	Keep the medium warm until adding it to the Pod™ Portable Module to begin treatment.

Figure 3 Labelling best practice



Example Calculation

Below is an example calculation:

Calculate GC (vector genomes) by multiplying desired MOI by expected number of cells.

GC = MOI x Number of Cells

For the Liver-Chip with 750,000 MOI:

GC = 750,000 x 42,000 = 3.15×10^{10}

For 24 h flow at 30 µL / h, the total expected flow volume is 720 µL. So, for 750,000 MOI, the cells are exposed to 720 µL in 24 h. Thus, 3.15×10^{10} GC must be present in 720 µL.

To make enough media to fill the pod, adjust the calculation to 1 mL

by multiplying GC by the new desired volume and then dividing by the original volume.

For 750,000 MOI in 1 mL:

C1V1 = C2V2 (C1 and C2 are GCs needed and V1 and V2 are volumes in mL)

3.15×10^{10} X 1 mL = GC needed X 0.72 mL

1 mL of media containing the vector will be needed for every chip, so multiply the chip count by 1 mL of media to get the final volume of media needed.

For 3 chips, 4 mL would be needed to allow for excess.

To get the volume of vector needed, multiply the total volume of media by the GC needed per mL and divide that value by the stock concentration.

If the vector stock is 2.8×10^{12} GC / mL

C1V1 = C2V2 (C1 and C2 are GCs needed and V1 and V2 are volumes in mL)

4.17×10^{10} X 4 mL = 2.8×10^{12} X Volume needed

Thus, volume needed is 0.06 mL

4 mL of the highest stock is needed, since 0.5 mL will be used to make the remaining stocks.

To make the 75,000 MOI stock, add 0.5 mL 750,000 MOI stock to 4.5 mL media.

To make a 7,500 MOI stock, add 0.5 mL 75,000 MOI stock to 4.5 mL of media.

From there, add 1 mL to appropriate chips.

Part VI. Cell Treatment in Organ-Chips with AAV

Overview

Introduction

Description of how to treat cells within the Liver-Chip successfully with prepared solutions.

Contents

Cell Treatment

Goal

To successfully treat the cells within the Liver-Chip with the AAV solution prepared in the previous section.

Before Starting

Ensure all solutions prepared beforehand are warmed to 37°C. Label all chips and Pods with their corresponding treatment conditions prior to adding the compound. For more information on working with Organ-Chips, please review the Basic Research Kit Protocol (EP-177).

Note for Multiple Collection Time Points

If there are multiple collection time points in the experiment, it is advised to organize the conditions so that there is 1 time point per tray. This will allow flow to be paused only for the tray from which effluent is being collected, resulting in a more accurate assessment of elapsed time.

Steps

Step	Action
1	Carefully remove the tray with the Pods from Zoë™ Culture Module and then transfer it to the biosafety cabinet. It is recommended to remove one tray at a time to minimize stress experienced by cells in the Organ-Chips while they are outside of the incubator.
2	Fully aspirate both the inlet and outlet reservoirs of each Pod while avoiding direct contact with the Pod reservoir Vias.
3	Add the calculated volume of warm, freshly prepared treatment medium to the appropriate channel.
4	Add warm, freshly prepared media to the other channels.
5	Once all Pods have been refreshed, ensure that all trays are returned to the appropriate Zoë. Flush the chips at 1000 µL / h for 5 minutes to flush the dosing solution through the Pod and to prime the channels.
6	After flushing the Pods and chips, carefully remove the trays from Zoë, one at a time. Once again, transfer them to the biosafety cabinet, and aspirate the accumulated media from the outlet reservoirs.

7	Return all trays to Zoë, reset Zoë's settings to the correct experimental conditions (e.g., flow rate, stretch), and note the time as the experimental start time (T = -1).
8	Sample each outlet reservoir independently at each timepoint following EP-124.
9.	After 24 h, replenish the Pods with freshly prepared culture medium without viral vector (T = 0). Replenish them again at least every other day, regardless of the collection timepoints, until the end of treatment period or experiment.

Part VII. Endpoint Readouts

Readout Protocols

Readout Protocols

For steps on conducting the following readout analyses, please refer to the associated protocol:

Readout	Protocol
Morphology	Please refer to the protocol https://emulatebio.com/wp-content/uploads/2021/06/EP123_v1.0_Bright_Field_Phase_Contrast_Imaging.pdf
Albumin	Please refer to the Emulate protocol https://emulatebio.com/wp-content/uploads/2021/06/EP139_v2.0_Albumin_Quantification_Assay.pdf
ALT	Please refer to the Emulate protocol https://emulatebio.com/wp-content/uploads/2022/05/EM143-rev-A-Alanine-Transaminase-Protein-Quantification-Assay.pdf

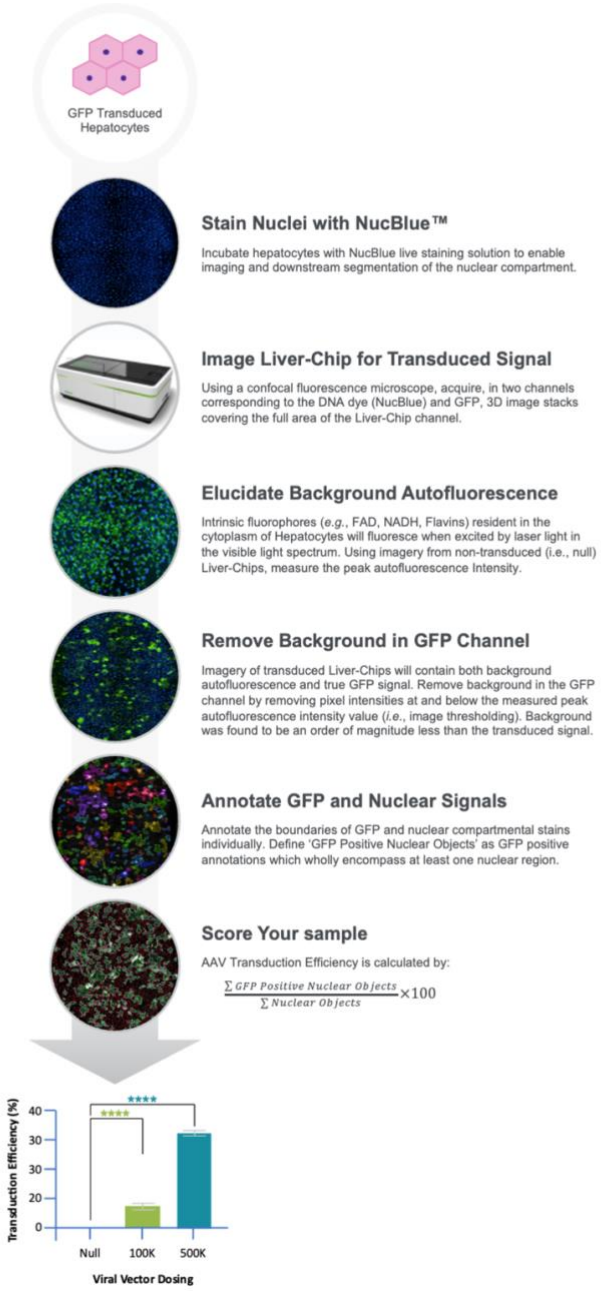
AAV Image Analysis

Overview

Overview

This section describes the steps for carrying out AAV Image Analysis

Steps



Part VIII. Takedown

Overview

Introduction

Protocol for takedown.

Takedown

The steps for takedown are as follows:

Step	Action
1	On Day 7, disconnect hips from pods.
2	Wash once with channel respective media
3	Prepare NucBlue™ live staining solution, 2 drops / mL serum free media (-FBS).
4	Add 100 µL of staining solution in each channel according to Live Staining of cells Protocol (EP155).
5	Incubate at room temperature for 15 minutes.
6	Wash twice with 200 µL serum free media (-FBS).
7	Image for Liver-Chip immunofluorescence.
8	After imaging has concluded, fix chips with 4% PFA for 15 min at room temperature.
9	Wash twice with 1X PBS and store at 4˚C for any further investigation desired.

Part IX. Troubleshooting

Troubleshooting

If you experience any issues, try the following:

If ...	Then ...
Transduction of the AAV GFP positive control is not observed 24 h post treatment.	● Review dosing calculations. ● Ensure vector stock concentration is updated as this varies from batch to batch. ● Ensure GFP and Null vector were not mixed up.

Revision History

Version	CR#	Date	By	Description
A	CR-219	14Sept2022	J. Wells	Review/CR
B	CR-248	04Oct2022	J. Wells	Content Update/CR