SOP MPS-001
CHIPSHOP DEVICE FOUR CELL LIVER MODEL (LAMPS) PROTOCOL

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A. PURPOSE AND APPLICABILITY

This protocol details the steps needed to produce the Liver Acinus Microphysiology system (LAMPS) model in ChipShop devices up to the point that they are put under flow, at which time they are ready for use. Disease models can be generated by manipulating the media used in the devices to generate various metabolic states.

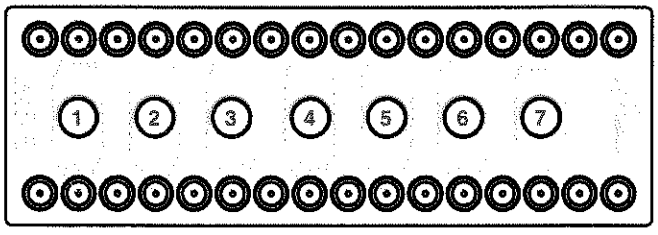
The proportions of the four cell types used in the model:

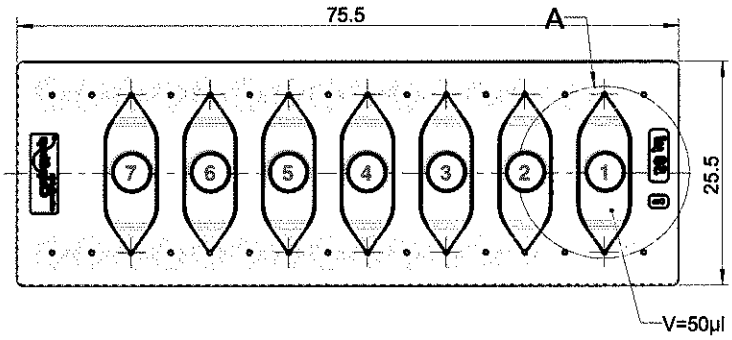
Cell Type	*Seeding Density [cells/mL]*	*% Total Cells*
Hepatocyte	2,500,000	51
Endothelial	1,500,000	30
Kupffer	800,000	16
Stellate	140,000	3

B. SUMMARY OF METHOD

Timeline Overview	
Day -3	Coat devices with ECM (fibronectin [100µg/mL] + collagen I [150µg/mL] in PBS) Treat THP-1 cells with PMA
Day -2	Seed hepatocytes Add Liver ECM [400µg/mL] over hepatocytes
Day -1	Seed THP-1 and LSEC Add collagen I overlay [1.5mg/mL] with LX-2
Day 0	Establish flow at 15 µL/hour (Zone 1) or 5 µL/hour (Zone 3)

B1. REFERENCE IMAGES OF CHIPSHOP DEVICE INDICATING CHAMBER NUMBERING SYSTEM





C. DEFINITIONS

DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix (col I + fibronectin)
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution
HPM	Hepatocyte Plating Medium
NFM	Normal Fasting Medium
EMS	Early Metabolic Syndrome medium
LMS	Late Metabolic Syndrome medium
O/N	Overnight
PBS	Phosphate Buffered Saline
PMA	Phorbol-12-myristate-13-acetate
RPMI	Roswell Park Memorial Institute 1640 medium
RT	Room Temperature
LAMPS	Liver Acinus Microphysiology System
Conditioned Medium	medium incubated at 37°C, 5% CO_2 for several hours
LSEC	Liver Sinusoidal derived Endothelial Cells
PFA	Paraformaldehyde

D. QUALIFICATIONS

1. Laboratory personnel will be trained on this SOP and essential instrumentation by qualified, experienced personnel.
2. Successful completion of training will be indicated by initialing and dating of the training record by the Trainer and Trainee.

E. HEALTH AND SAFETY WARNINGS

1. All chemicals must be handled in compliance with the University of Pittsburgh's Chemical Hygiene Plan Safety Manual.
2. All biological material must be handled in compliance with the University of Pittsburgh's Biosafety Guidelines.

F. CAUTIONS

1. Do not exceed a flow rate of 50 µL/hr.
2. Aseptic technique is required throughout the experimental process.

G. INTERFERENCES

None known

H. EQUIPMENT AND SUPPLIES

- Device – ChipShop Reaction Chip 50µL (Fluidic 557)
- Cryopreserved Human hepatocytes – (Variable lots from ThermoFisher, AnaBios)
- Lots are tested prior to use. Endothelial cells — Cryopreserved liver derived endothelial cells (LifeNet.Health, cat no HL160019)
  - Expanded to Passage 2 (P2) in house and cryopreserved according to the manufacturer's protocol.
- Kupffer cells – THP-1 (ATCC, cat no TIB-202)
  - Differentiated with 200ng/ml PMA for 48 hours before harvesting attached macrophages and seeding into device
- Stellate cells – LX-2 (Sigma, cat no SCC 064)
- Fibronectin Bovine – (Sigma, cat no F1141)
  - 1 mg/mL
- Collagen – type I Rat tail 8-10 mg/ml (Corning, cat no 354249)
- Porcine Liver ECM (10 mg/ml) from Dr. Badylak research group
- Hepatocyte Recovery medium (Life Technologies, cat no CM7000)
- Hepatocyte plating medium (HPM)
  - 93% Williams E medium (Thermo Fisher Cat no A1217601) with 2 mM glutamine (Gibco cat no 25-030-081)
  - 5 % FBS (Corning cat # MT35010CV)
  - 1 % pen/strep (Cytiva HyClone Cat no SV30010)
- Normal Fasting Media (NFM): refer to NFM preparation protocol (SOP-003).
- DMEM (Life Tech, cat no 12100-061), 10% or 2% FBS, 1% pen/strep
- RPMI (hyclone, cat no SH30096.01) with 10 % FBS, 1% pen/strep, 1% glutamine
- EGM-2-MV BulletKit (Lonza, order from Fisher NC9902887)
- Tryple (Life Tech, cat no 12605-010)
- PBS pH 7.4, sterile filtered (Gibco, cat no 21600-069)
- 10X HBSS, sterile filtered (Sigma, cat no H1641)
- NaOH, 1 M (Fisher, cat no SS277)
- PEEK tubing, 0.8 mm OD, 0.2 mm ID (IDEX, cat no1569XL) and 2-4 cm c-flex strips used as sleeves to make connections, (c-flex tubing, 0.5 mm ID, 2.1 mm OD (Cole-Parmer, cat no 06422-00)
- Male Mini Luer tube PEEK connector (ChipShop cat no 10001765)
- Mini Luer to Pipette Adapter (ChipShop cat no. 10000057)
- Green Cap (ChipShop cat no 10001686)
- Syringe pump (KD Scientific, cat nos KDS220; KDS250, New Era Pump Systems cat no NE1800) or Harvard pump (Harvard Apparatus Pump cat no 703007)
- 20 or 22 g ½ inch blunt needles (Instech laboratories, cat no LS22,)
- 100 or 150 mm Petri dishes (Fisher, cat no FB0875711, cat no FB0875714)
- Efflux tubes (plastic/glass) depending on experiment - Glass (Fisher, cat no 03-376-462), Plastic (Fisher, cat no 02-681-272)
- PMA (phorbol-12-myristate-13-acetate (Calbio, cat no 524400)

- Williams E medium (life technologies, cat no A12176-01)
- Fetal bovine Serum (Corning, cat no 35010CV)
- Pen/Step (Corning, cat no SV300010)
- L-Glutamine (Corning, cat no SH30034-01)
- Media filters, 0.22 μm pore size (Fisher, cat no 565 0020)
- Experiment preparation: Autoclave following items:
  - blunt needles, tubing, metal tube "blunts", glass syringes, ChipShop end caps, end plugs and ferrule port.

I. PROCEDURAL STEPS

Prior to adding hepatocytes, it is permissible to empty and refill the chamber. Once hepatocytes are in the chamber, only new media solutions are used to reduces the chance for bubbles in the device and increases the viability of cells. A detailed guide on bubble avoidance in setting up flow-thru media and bubble removal after flow is initiated is in the appendix.

I1. DAY -3 – STERILIZATION AND MIXED MATRIX COATING OF CHIPSHOP DEVICES

1. Sterilize devices with mercury UV lamp following MPS020 protocol.
2. Create fibronectin [100μg/mL] and collagen I [150μg/mL] ECM solution in PBS
   a. First, add fibronectin to PBS
   b. Then, add collagen
   c. Mix gently without creating bubbles
   d. Example calculation in Appendix
3. Evenly fill empty chambers of device with mixed ECM solution
   a. If large bubbles are present, fill other chambers on device, then return to the chamber with the bubble. Empty that chamber and refill with ECM solution
4. Once chambers are filled and bubble free, fill inlet and outlet reservoirs, then cap using either a pre-sterilized end cap.
5. Place one device into a 100mm sterile petri dish with cover.
6. Store devices in petri dishes O/N at 4°C.

I2. DAY -3 – PMA TREATMENT OF THP-1 CELLS

1. Place 5 million viable THP-1 cells into a T75 flask
2. Add PMA to a final concentration of 200 ng/ml in RPMI medium
3. Incubate 48 hours before use; cells will become adherent (macrophage like)

I3. DAY -2 – DRYING DEVICES

1. Remove ECM solution from devices with vacuum aspiration
2. Fill chambers hot (~60°C) H_2O
3. Remove H_2O with vacuum
4. Allow to air dry in biosafety cabinet for at least 15 minutes

I4. Day -2 – Seeding Hepatocytes

1. Thaw hepatocytes, check viability with trypan blue, and count cells.
    a. Warm recovery medium and plating medium to 37 °C.
    b. Thaw 1 vial of cryopreserved hepatocytes
    c. Place 1 ml of cryopreserved hepatocytes into 50 ml of pre-warm recovery medium.
    d. Invert the tube 1X to mix.
    e. Centrifuge 100g for 11 min.
    f. Remove and discard supernatant.
    g. Resuspend pellet and in 4 mL of HPM.
    h. Perform cell count
        i. Viability must be greater than or equal to 80%
        ii. Use the number of viable cells to determine the cell density
2. Re-suspend hepatocytes in hepatocyte plating media (HPM) to **density of $2.5x10^6$ viable cells/mL**
3. Mix hepatocyte solution and add to device chambers
    a. Remix hepatocyte solution between seeding each chamber
    b. Budget ~100µL per chamber.
    c. Avoid bubbles as much as possible. Empty and reseed if necessary
4. Fill inlet/outlet reservoirs with remaining cell solution, or HPM if no solution remains
5. No need to cap reservoirs if ECM coating same day
6. Return device to 100mm petri dish, and place in 37°C, 5% CO_2 incubator

I5. Day -2 – LECM (Liver ECM layering)

Typically, this step is performed four hours after hepatocyte seeding. If the hepatocytes have poor attachment, this can be performed the following day.

1. Prepare a 200 µg/mL solution of LECM in NFM
    a. Thaw 50µL of stock 10mg/mL LECM
    b. Sonicate the thawed LECM for 2 minutes
    c. Add 3µL of 1N HCl
    d. Vortex
    e. Add 600µL of NFM
    f. Vortex until no large chunks of LECM are present
    g. Add 600µL of NFM and vortex to create final solution
2. Flow thru LECM solution to replace HPM in chambers of device
3. Fill reservoirs and cap
4. Return device to 100mm petri dish, and place in 37°C, 5% CO_2 incubator O/N

I6. Day -1 – LSEC and THP-1 Seeding

Begin by preparing solutions of LSEC and THP-1 cells at twice (2-times) the final density. Then, combine the two solutions 1-to-1 and add to devices

1. LSEC (endothelial cells) at 3.0x10^6 cells/mL

1. Remove vials of LSECs from liquid nitrogen cryo-bank.
    a. Each vial contains 1.0x10^6 viable cells
2. Thaw in water bath and transfer LSEC to a centrifuge tube
3. 9mL NFM per 1mL cyro-solution
4. Spin at 200g for 5 minutes
5. Re-suspend the pellet **in NFM at 3.0x106 cells/mL**
6. Set aside.

2. THP-1 (Kupffer cells) at 1.6x10^6 cells/mL

1. Aspirate medium from flask(s) of PMA-treated THP-1 cells
2. Add 2 ml of RT TryPLE to flask(s) for cell detachment
    a. ~10 to 15 minutes at 37°C
3. Add 8 ml of RPMI 10% FBS to flask(s)
4. Count the resulting cell suspension and perform trypan blue viability stain
5. Should have > 90% viable cells
6. Spin at **300g for 5 min.**
7. Re-suspend the pellet **in NFM at 1.6x10^6 cells/mL**

3. Pooling LSEC and THP-1

1. Pool equal volumes THP-1 into LSEC to prepare a mixed cell suspension in NFM
    a. 1.5x10^6 LSEC per mL
    b. 0.8x10^6 THP-1 per mL

4. Seeding LSEC and THP-1 into Device

1. Remove device from incubator
2. Flow thru LSEC+THP-1 mixture
3. Fill inlet/outlet reservoirs with remaining solution, or NFM is no solution remains
4. Return device to 100mm petri dish, and place in 37°C, 5% CO_2 incubator for 2 to 4 hours

I7. DAY -1 – LX-2 IN COLLAGEN

1. Detach LX-2 cells from flask with 2mL RT TryPLE
    a. ~10 to 15 minutes at 37°C
2. Suspend the detached cells in 8mL NFM
3. Count cells with trypan blue viability stain
    a. >= 80% viable
4. Spin down LX-2 cells at 100g for 5 minutes
5. While cells are spinning, collect stock collagen I, 10x HBSS, NFM and 1N NaOH
6. Store HBSS, stock collagen type 1 and NaOH on ice until used.
7. Create around 1mL of solution at 1.5mg/mL collagen type 1 with 140,000 LX-2 cells in NFM.
    a. This calculation will change depending on the stock collagen concentration
    b. This solution *cannot be kept on ice*, as the LX-2 cells do not respond well

   c. See appendix for example calculation and detailed solution preparation protocol
8. Using the 1mL solution, quickly seed up to 2 devices
9. Transfer device(s) back into 37°C, 5% CO2 incubator
   a. Place any remaining solution in incubator as well to check for collagen gelling
10. After 40 to 60 minutes, remove gelled collagen from inlet and outlet reservoirs
11. Refill inlet/out reservoirs with NFM
12. Cap devices
13. Return device(s) back into 37°C, 5% CO_2 incubator O/N

I8. Day -1 – Media Conditioning

Media (NFM, EMS, LMS) should be conditioned O/N in 37°C, 5% CO_2 incubator in at vented flask. If possible, condition drug solutions O/N as well, if the compounds are not absorbed by the plastic flask.

Devices require a minimum of 360µL/day/chamber (Zone 1) or 120µL/day/chamber (Zone 3). Typically, 500µL/chamber are necessary to prime the tubing before starting flow.

I9. Day 0 – Starting Flow in ChipShop Devices

1. Pump Setup

1. Adjust pump for syringe diameter by entering the diameter:
   a. 4.70 mm for 1mL BD syringe
   b. 12.36 mm for 5mL BD syringe
   c. 14.48 mm for 10mL BD syringe
   d. 14.57 for 10mL Hamilton Glass-syringe
2. Adjust the pump flow rate to 15 (Zone 1) or 5 (Zone 3) µL/hour
3. Set total volume in syringe if desired, but not necessary for flow

2. Collect materials into the hood

- Tray
- Pump
- Syringes
  - Glass if drugs being used, plastic otherwise
- Conditioned medium
- Sterile, autoclaved inlet and outlet PEEK tubing
- Efflux collection vials
  - 1.5 ml Eppendorf (plastic) or 4 mL glass with hole in top to accommodate tubing.

3. Connect Devices to Flow

1. Carefully fill syringes with conditioned media
2. Place and lock in filled syringes onto pump
3. Connect blunt needle on inlet tubing to the syringe
   a. Take care so that the mini-luer ferrules remain sterile. (e.g., store ends in a sterile conical tube)

4. Advanced syringes with fast-forward until fluid is coming out of all ferrules
5. Start flow to keep pressure in tubing
6. Take device out of incubator
7. Push ~50µL medium from the inlet to the outlet for each chamber of device
8. Attach outlet tubing to outlet reservoir of device
9. Attach inlet ferrule to inlet reservoir of device
10. Put device in a slide holder and affix device+holder to a solid service (e.g., Styrofoam)
11. Turn off pump
12. Transfer entire setup into 37°C, 5% CO_2 incubator
13. Turn on pump
14. Double check that flow rate (number and unit) and volume are accurate

I10. Day 1+ – Effluent Collection and Monitoring

Efflux volume determination

Determine the volume for day 2 efflux samples using a pipettor.

For later days calculate the volume of efflux by multiplying the flow rate by the time of efflux collection.

For later days check volume using a pipettor for two samples per device per day.

If the volumes differ significantly (how much is acceptable?) use a pipettor to determine the volume for all samples for that device for that day.

Efflux flow time

Record the time period over which efflux samples are collected for each day using either the time display on the pump or by recording the time of collection of each samples and calculating the duration using the time recordings.

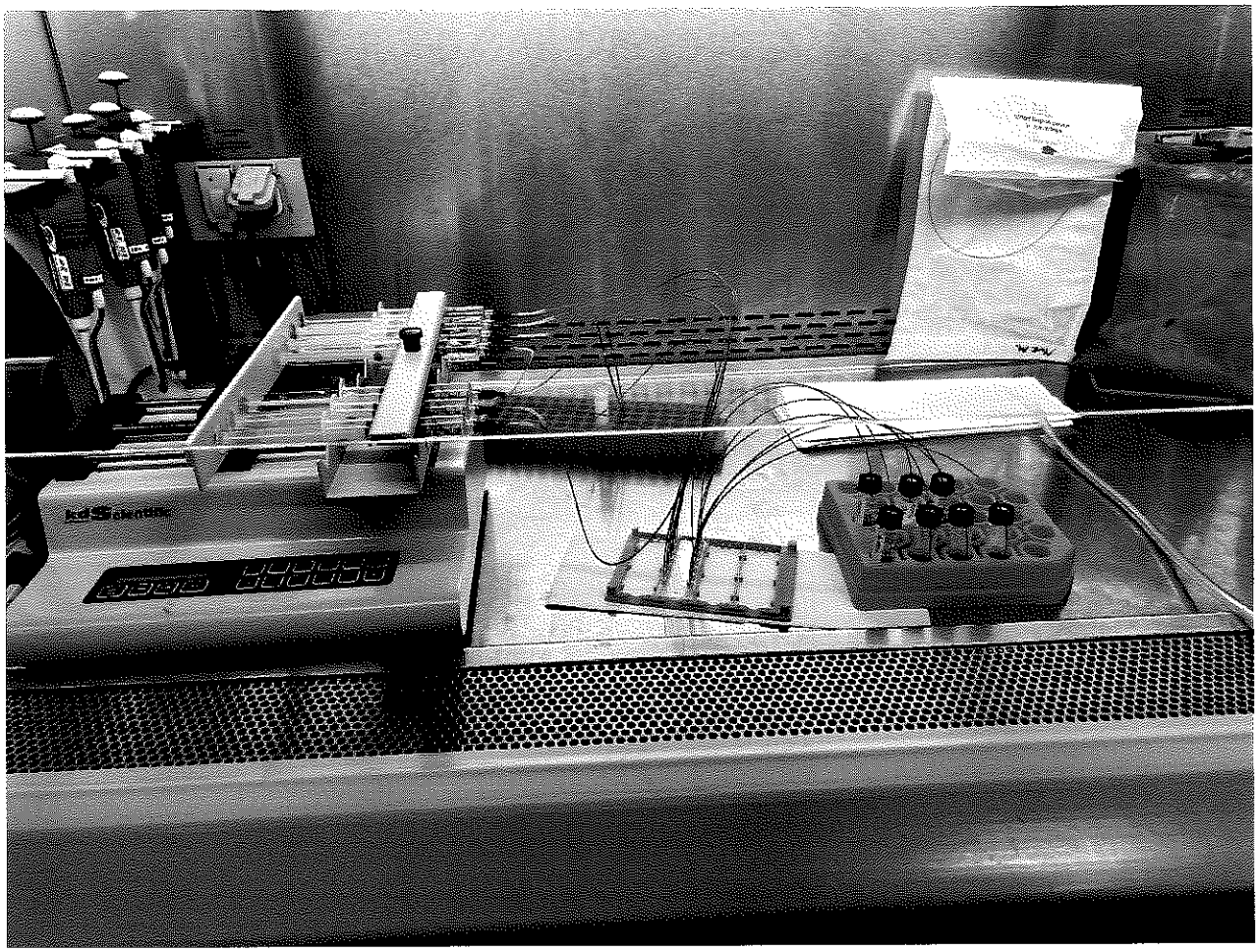
Store efflux samples at –80degC after collection and until testing.

Something like check for bubbles daily, change tubing as needed

I11. Endpoint – Device Removal for Imaging and/or Fixation

1. Remove the pump, ChipShop chamber slides and efflux collection system from the incubator.
2. Transfer devices and pump into cell culture hood.
3. Dismantle the connections saving the re-usable parts (mini-luer ferrules, caps)
4. Proceed to LAMPS Fixation and Staining Protocol MPS 011

A) Pump connect configuration



J. DATA AND RECORDS MANAGEMENT

1. All study parameters and data must be entered into EveAnalytics database
    a. Model Name
    b. Typical Conditions
2. Laboratory notebooks are used in addition to the database to record study parameters and data, some of which would be eventually entered into the database.

K. QUALITY ASSURANCE AND QUALITY CONTROL

1. Cell Quality control plate
    a. At each step in the seeding of the ChipShop devices aliquots of the media and cells that are added are also placed into a 96 well collagen coated plate.
    b. Plating medium
    c. Maintenance medium
    d. Hepatocytes

e. Endothelial/Kupffer
f. LX2 in collagen
g. The plate is examined the next day and evaluated using the following criteria:
    i. Presence of contamination
    ii. Appropriate cell morphology
    iii. The plate is held for up to 1 week and examined at the end of this time for signs of contamination and toxic or unhealthy cells.

2. Microscopic and visual inspection of devices
    a. Performed after seeding then periodically thereafter depending on the length of the experimental protocol.
    b. Devices are monitored for appropriate cell morphology and absence of contamination.
    c. Devices are examined for evidence of bubbles, leaking and for appropriate flow volume.
3. Lot control
    a. Lot numbers of ChipShop devices, cells, media, and media components are recorded in laboratory notebooks.
4. QC reporting
    a. Should evidence of contamination or other deterioration be noted the study monitor and project manager are notified and a decision made as to whether or not to continue monitoring/studying the devices.
    b. Should the cell morphology indicate unhealthy cells or toxicity (rounded, unattached, no spreading) the project manager is notified and a decision made as to whether or not to continue monitoring/studying the devices.
    c. Problems with ChipShop devices should be reported to the project manager who will notify the manufacturer.
5. Recordkeeping
    a. Results of the quality control examinations are kept in laboratory notebooks and/or the MPS database as appropriate.

L. APPENDIX

L1. TUBING STANDARDS

- PEEK tubing, 0.8 mm OD, 0.2 mm ID (IDEX, cat no1569XL) and 2-4 cm c-flex strips used as sleeves to make connections, (c-flex tubing, 0.5 mm ID, 2.1 mm OD (Cole-Parmer, cat no 06422-00)
    - Cut PEEK into 16-inch lengths (influx) and 12-inch lengths (efflux) and autoclaved prior to use, add 2-4 cm c-flex strips onto each end of both influx and efflux lines, bag both one influx line and one efflux line into a single autoclave bag [note needle blunts, port ferrules and glass vials with Teflon caps to collect efflux media can be added to peek tubing prior to autoclaving]

L2. Flow Through Procedure

Bubble prevention

a. Equilibrate medium to 37 deg for at least 2 hours before use
b. Remove bubbles from syringe
    i. Aspirate slowly without needle
c. Remove bubbles from tubing
    i. Watch for clear drop at tip of tubing before connecting it
    ii. Liquid to liquid, have a small drop of media on the male fitting that will insert into the influx port of the ChipShop device
    iii. If necessary carefully pipette a small liquid drop into the influx port
    iv. Connect the male fitting into influx port
d. Connect efflux tubing in the same manner using liquid to liquid connection

Bubble removal

a. Turn off pumps, manipulate the ChipShop device to position the bubble near an exit port. Tilt ChipShop device upright
b. Briefly pulse syringes or
c. Increase flow on pump – 50 to 200 ul/hour
d. Stubborn bubbles are easier to clear with lowered exit resistance by removing efflux male fitting and peek tubing
e. Reconnect efflux fitting, return ChipShop device to former position and turn pump back on

L3. Example Calculations

A. To make 1 ml of fibronectin (stock at 1 mg/ml) diluted 100 ul stock fibronectin into 900 ul PBS. Mix well. Calculate volume of collagen type 1 to the 1 ml of fibronectin as follows:

Volume of stock Collagen needed to prepare 1 ml ECM solution at 150 µg/ml =

$$\frac{0.150 \text{ mg/ml}}{\text{Stock Concentration in mg/ml}} \times 1 \text{ ml (fibronectin/PBS)} = \text{volume in ml}$$

Example: Stock concentration Collagen = 10.08 mg/ml protein

$$\frac{0.150 \text{ mg/ml}}{10.8 \text{ mg/ml}} \times 1 \text{ ml} = 0.014 \text{ ml } (14\mu l)$$

Mix gently without creating bubbles

B. Volume of stock Collagen needed to prepare 2 ml gelling solution at 1.5 mg/ml =

$$\frac{1.5 \text{ mg/ml}}{\text{Stock Concentration in mg/ml}} \times 2 \text{ ml} = \text{volume in ml}$$

Example: Stock concentration Collagen = 10.08 mg/ml protein

$$1.5 \frac{\text{mg/ml}}{[10.08 \text{ mg/ml}]} \times 2 \text{ ml} = 0.277 \text{ ml}$$

Volume of 10X HBSS = volume of Collagen/10

Example: 0.277 ml/10 = 0.027 ml

Volume of 1N NaOH = 6 µl

Volume of NFM to add to cell suspension
= 1 ml – volume collagen – volume of HBSS - volume 1 NaOH
Example: 1 ml – 0.277 ml – 0.027 ml – 0.006 ml = 0.690 ml

Using this example add 0.277 ml Collagen, 0.690 ml HMM, 0.0277 ml 10X HBSS and 0.006 mg/ml 1N NaOH to prepare 1 ml cell gelling solution

L4. LIVE IMAGING ON PHENIX SEE PROTOCOL MPS 013

M. REFERENCES

1. Vernetti L, Senutovitch, N, Boltz, R, DeBiasio R, Gough A, Shun, TY, Taylor DL. A human Liver Microphysiology Platform for Investigating Physiology, Drug Safety and Disease Models. Exp Biol Med, 2016, Jan: 241.1:101-114.
2. Lee-Montiel, FT et al. Control of Oxygen Tension Recapitulates Zone-Specific Functions in Human Liver Microphysiology Systems. Exp. Biol. Med. 2017 Oct, 242(16):1617-1632.