Mouse Hepatocyte Isolation

General Surgery Lab SOP

Last Updated 3/23/15 by Alicia Frank

Solutions and Supplies

Per I Stock Solution (10X)- 1 Liter

- diH_2O 800mL
- 1.42 M Sodium Chloride (NaCl) 83.0g Sigma Cat# S-7653
- 67mM Potassium Chloride (KCl) 5.0g Simga Cat# P-5405
- 100mM HEPES (pKa 7.5) 24.0g Sigma Cat# H-3375
- Adjust pH to 7.5 with 10M Sodium Hydroxide (NaOH)
- Bring to volume
- Filter into sterile bottle
- Store at 4°C

Per I Ready Solution- 1 Liter

- diH_2O 800mL
- 100mL Per I Stock Solution
- 2.23uM Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) 0.85g Sigma Cat# E-4378
- Adjust pH to 7.5 using 10M NaOH
- Bring to volume
- Filter into sterile bottle
- Store at 4°C

Per II Stock Solution - 4 Liters

- 19 mM Calcium Chloride ($CaCl_2$) 2.8g Sigma Cat# C7902
  - Dissolve in ~100mls of diH_2O before adding to remaining solution
- diH_2O 3500mL
- 151.52 mM Albumin 40.0g Sigma Cat# A7030
- 67mM NaCl 15.6g
- 6.7mM KCl 2.0g
- 100mM HEPES (pKa 7.5) 96.0g
- Stir to dissolve
- Adjust solution to pH 7.6 using 10M NaOH
- Bring to volume
- Use Micro-culture capsule to filter into sterile bottles VWR Cat#28145-134
- Store at 4°C

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Per II Ready Solution- 150mL per mouse

- 150mL Per II Stock
- 0.13mg/ml Collagenase H 20mg Roche Cat #11087789001
- Allow to dissolve for at least 30 mins at room temp
- Filter into sterile bottle
    - This solution should only be made on the day of the harvest. May be kept for up to 1 week at 4°C if necessary
    - Entire bottle of collagenase should be aliquotted at the same time

Liver media

- Williams E Media 500mL bottle Invitrogen Cat# 12551-032
- 100 U/mL Penicillin-Streptomycin 5mL Invitrogen Cat# 15140-122
- 2mM L-glutamine 5mL FisherSci Cat# BW17-605E
- .032 U/mL Insulin (Humulin R) 0.16mL Lilly USA, LLC #HI-213
- 15mM HEPES buffer 7.5mL FisherSci Cat# BW17-737E
- Store at 4°C

10% CS media

- Williams E Media 500mL bottle
- 100 U/mL Penicillin-Streptomycin 5mL
- 2mM L-glutamine 5mL
- .032 U/mL Insulin (Humulin R) 0.16mL
- 10mM HEPES buffer 7.5mL
- 10% Bovine Calf Serum 50mL FisherSci Cat# SH3007203
- Store at 4°C

Gel Coat Stock

- Liver Media 100mL
- 1ug/mL Gelatin 0.1g Sigma Cat#G-1890
- Dissolve in 37°C water bath
    - Will take a couple of hours
- Filter into sterile bottle
- Store at 4°C

Gel Coat Media

- Liver Media 100mL
- 1% Gel Coat Stock 1mL
- Store at 4°C

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Percoll Plus Solution

- 1.02g/mL Percoll Plus — 180mL — GE Healthcare Cat#17-544-01
- 1% HBSS — 20mL — Invitrogen Cat#14065-056
- Store in 75cm^2 cell culture flask at 4°C — FisherSci Cat# 13-680-65

Equipment

- Surgical instrument pack:
  - Large scissors — FST Cat#91401-12
  - Small scissors — FST Cat#14060-11
  - Forceps — FST Cat#11002-12
  - Small curved forceps — FST Cat#11009-13
- Sterile gauze
- Sterile 1cc syringe
- 22 gauge x 1 ½' catheter
- Sterile drape
- Sterile gloves
- Sterile tygon tubing
  - Thick tubing, cut to ~3.5-4 feet — ColeParmer Cat#HV-96440-16
  - Thin tubing, cut to ~6-9 inches — FisherSci Cat#02-587-1D
- Betadine solution
- Sterile 6cm petri dish of liver media on ice
- Cell scraper
- Sterile funnel with 2 ply layer of gauze

Procedure

Set up

- Place Per I Ready and Per II Ready in 37°C waterbath
- Prepare 6cm dishes with Liver Media on ice to collect livers
- Place blue pads on bench and sterile drape on top of blue pads
- Set up tubing in pump, place the tubing in the Per I and bleed through, place end of tubing back in bottle
  - Speed should be around 8mls/min
- Anesthetize mouse with 70mg/kg Nembutal (0.30 mL of 7mg/mL dilution for a 30g mouse)
- Shave mouse abdomen
- Tape down limbs
- Scrub abdomen with Betadine
- Cover the mice with the sterile drape

- Remove instruments from packs, being careful to maintain sterility
- Open catheter

Surgery

- Put on sterile gloves
- Cut a hole in the sterile drape to expose each mouse's abdomen
- Make a U-shaped incision in mouse skin
- Make a similar U-shaped incision in the muscle wall, being careful not to cut liver or diaphragm
- Push viscera out of the way with sterile gauze to expose the vena cava and hepatic portal vein
- Insert catheter into vena cava (remove needle after insertion), connect tubing to allow Per I to flow in
- When liver begins to fill, cut portal vein
- Perfuse with Per I solution for 2-3 minutes until liver is clear of blood
- Pinch tubing and switch to Per II
- Perfuse with Per II for about 10 minutes (75-100mL)
- When perfusion is complete, dissect whole liver out and place in 6cm dish with liver media
- Remove gallbladder and any excess non-hepatic tissue
- Set aside small curved forceps for later use – be careful not to touch tips
- Move to cell culture hood

Hepatocyte isolation:

Note: Cells are very sensitive, so use care when pipetting and adding media (No air bubbles).
All work should be done in hood

- Using a cell scraper and forceps, scrape half of the liver into each side of the petri dish filled with media.
- Pour the suspension through sterile gauze funnel and rinse with media
- Spin at 400rpm for 2-4 min
- Remove supernatant containing non-parenchymal cells (see NPC protocol)
- Resuspend hepatocyte pellet in media and add Percoll Plus solution
    - Media to Percoll is 3:2 (Example 15mL media and 10mL Percoll)
- After adding Percoll gently invert tube to mix, do not pipette
- Spin at 400rpm for 10 min
- Remove supernatant (contains dead cells, typically there will be a layer at the top of the supernatant)
- Resuspend in liver media and spin at 400rpm for 2-4 min
- Resuspend in liver media and spin at 400rpm for 2-4 min (second time)
- Resuspend liver pellet in 10% CS liver media
- Count cells

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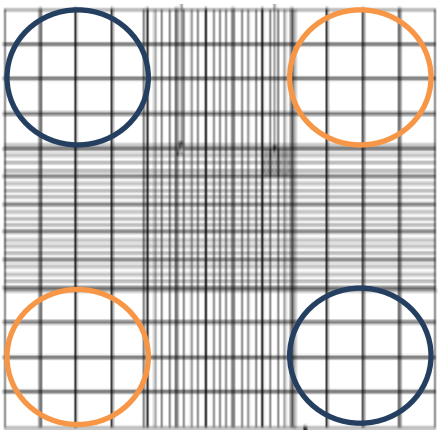
Determining Cell Count

- Add 190µL Trypan blue to 10µL of the above cell suspension into an 1.5mL eppendorf tube
- Mix by flicking tube – do not pipet up and down or vortex, you will kill the cells!
- Put 10µL of this suspension into each side of the hemocytometer.
- Count 2 opposite 'squares'of cells on each side of the hemocytometer – both alive and dead.
    - The white/yellow cells are live cells
        - These should be within 10 of each other. If not, take fresh samples and count again.
    - The blue cells are dead cells



- Average your counts together and multiply by your dilution factor to obtain total number of cells and viability percentage.
    - Ex: Cells are suspended in 15mL of media
      Side 1 – 30 alive, 4 dead; Side 2 – 34 alive, 1 dead
      Average 32 alive, 2 dead = 34 total
      Total # of Cells = 32 x $1.5x10^6$ = $48x\ 10^6$
      Viability Percent= 32/34 x 100 = 94%

Plating Cells

Gel coat media should be added to all dishes or plates in the same volume as the cell media will be (see below).

Dish Size	#Cells/Plate (Rat)	#Cells/Plate (Mouse)	Total Volume (mL)
15cm	$18X10^6$	--	15
10cm	$5x10^6$	$3x10^6$	5
6cm	$3x10^6$	$2x10^6$	3
35mm	--	$0.4x10^6$	1

When preparing dishes, add one extra dish onto total required to negate pipetting error. IE, 2 mouse 6 cm dishes are requested, prepare for 3: 3 x ($3x10^6$ cells) = 9.0 x 10^6 cells in 3 x 3ml = 9mls of media

Plate Size	#Cells/mL	#mL/well	Total #Cells/Plate	Total Volume/Plate (ml)
6 well	$2x10^5$	2	$2.4x10^6$	12
12 well	$2x10^5$	1	$2.4x10^6$	12
24 well	$1.5x10^5$	1	$3.6x10^6$	24
96 well	$2x10^4$	100ul	$1.92x10^6$	9.6

When preparing dishes, add 0.25 onto total required to negate pipetting error. IE, 2 6 well plates are requested, prepare for 2.25: 2.25 x ($2.4x10^6$ cells) = 5.4 x 10^6 cells in 2.25 x 12ml = 27mls of media

- Gel coat each well or plate, let sit in hood for 45 min
- Vacuum off excess fluid
- Add suspended cells (make sure to spread cells over the area of each well or plate)
    - To calculate volume of cells in media and volume of 10%CS media multiply the number of plates/dishes requested to get total number of cells. Divide this number by the average cell count. This number is the volume of cells in media needed. Subtract this from the total media volume to determine the volume of 10%CS media needed.
        - Ex: 2 x 6cm dishes mouse cells = $9.0x\ 10^6/3.2x\ 10^6$ = 2.8mL cells in media
          9mL of total media - 2.8mL cells in media = 6.2mL 10% CS media
        - Ex: 2 x 6 well = $5.4x10^6/3.2x\ 10^6$ = 1.7 mL cells in media
          27mL of total media – 1.7mL cells in media = 25.3 mL 10% CS media
- Incubate cells at 37°C and 5% CO2 overnight