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Rodent Liver 2-Step Collagenase Perfusion and Digestion using VitaCyte

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ABSTRACT

To obtain a viable single cell suspensions of total liver homogenate (TLH) that can be used for downstream experiments, such as *in vitro* experiments and more biologically representative transcriptomics with greater success, a slow *in situ* 2-step collagenase rodent liver perfusion and digestion is developed in both mice and rat. The process of dissociation can accelerate cell death if done harshly. Thus, an optimized gentle method will yield more viable cells, which this protocol has been inspired by the human caudate 2-step perfusion and digestion within the lab. VitaCyte neutral protease and collagenase is used because of its high purity of the enzymes.

IMAGE ATTRIBUTION

Created by BioRender.com

GUIDELINES

All biological samples and animals should be handled and processed according to the institutes guidelines, eg. University Health Network (UHN).

Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 92855

Keywords: Mouse, Rat, Liver, Perfusion, Digestion, Dissociation, Collagenase, Protease

MATERIALS

Equipment

- Biological Safety Cabinet
- Water Bath (42°C)
- Peristaltic Pump
- Bubble Trap
- Surgery Microscope
- Lighting
- Surgical Platform
- Anesthesia apparatus with chamber and tubing along with oxygen tank
- Shaving razor (BravMini+ Cordless Trimmer, WAHL (BrainTree Scientific Inc. [CLP-41590](#))

Material and Reagents

- 1 x ice bucket (replace ice when needed)
- 1 x ice pack
- Paper towel
- 4 x disposable sterile scalpels (Almedic [M92-23](#))
- 4 x petri dishes
- 2 x sterile forceps
- Pipette Gun
- 25 mL sterile serological pipettes
- P1000 micropipette & suitable sterile tips
- P200 micropipette & suitable sterile tips
- P10 micropipette & suitable sterile tips
- 4 x 50 mL conical tubes
- 1 x 500 µm cell strainers (pluriStrainer® [43-50500-03](#), El Cajon, CA, USA)
- 1 x 100 µm cell strainers (Falcon® [352360](#), Durham, DC, USA)
- 1 x 70 µm cell strainers (Falcon® [352350](#), Durham, DC, USA)
- 1 x 0.22 µm syringe filter (MilliporeSigma™ Millex™ [SLGP033RS](#))
- 1 x 50 mL Luer-Lok™ Syringe (Becton, Dickinson and Company [309653](#))
- 2 x Disposable Hemacytometers (SKC, Inc. C-Chip™ [DHC-N01](#))
- 1 x sterile scissor
- 1 x sterile titanium spring scissors (Fine Science Tools [15651-11](#))
- 3 x sterile surgical suture forceps (to hold mice open)
- 1 x sterile cannulation forceps (Fine Science Tools [00574-11](#))
- 2 x sterile microvasculature forceps
- 3 x sterile cannulation clamps (Fine Science Tools [00325-00](#))
- 1 x locked blunt clamp

- Isoflurane, USP (**Double Check DIN**)
- Milli-Q water or deionized water(dH₂O)
- 70% ethanol
- Kim wipes (Kimtech Science™ Kimwipes™ [05511](#))
- Virox wipes (PREempt™ [11221](#), Oakville, ON, Canada)
- Tape
- Sterile surgical cotton tips
- 20G IV Catheters (Becton, Dickinson and Company [381137](#))
- 24G IV Catheters (Becton, Dickinson and Company [381112](#))
- 1mL injection syringes (Becton, Dickinson and Company [309659](#))
- 27G precision glide needles (Becton, Dickinson and Company [305109](#))
- Heparin, 1000U/mL (Heparin LEO® LEO Pharma 006174-09) **Double Check**
- PBS 1X (Gibco 10010-023, Paisley, UK) (without Ca²⁺ and Mg²⁺)
- HBSS, Ca²⁺Mg²⁺ Free (Gibco [14170161](#), Grand Island, NY, USA)
- DMEM (high glucose, HEPES, no phenol red) (Gibco [21063029](#), Grand Island, NY, USA)
- Penicillin/streptomycin (Sigma-Aldrich [P4333](#), Oakville, ON, Canada)
- Trypan Blue Solution (Sigma-Aldrich [T8154](#), Oakville, ON, Canada)
- FBS (Heat Inactivated, Gibco 12484-028, Grand Island, NY, USA)
- HEPES, 1M (Gibco [15630080](#), Paisley, Scotland, UK)
- Calcium chloride solution, 1M (Sigma-Aldrich [21115](#), Oakville, ON, Canada)
- Sodium hydroxide solution, 10N (Fisher Chemical [SS255-1](#), Fair Lawn, NJ, USA)
- EGTA, 1M (BioShop, [EGT101](#), Burlington, ON, Canada)
- Collagenase MA (VitaCyte [001-2030](#), Indianapolis, IN)
- AOF BP Protease (VitaCyte [003-1000](#), Indianapolis, IN)

SAFETY WARNINGS

 Wear the proper protective personal equipment (PPE) when handling with rodents in a biological sterile environment in the manner of the institutions guidelines of safety.

ETHICS STATEMENT

All biological samples should be handled and processed according to UHN Biosafety Guidelines. Animal handling should be done according to UHN Animal Resource Centre (ARC) Guidelines. This procedure should be performed in accordance with all applicable safety procedures and using aseptic techniques.

BEFORE START INSTRUCTIONS

Please read the protocol first to work efficiently and prepare reagents as well materials to reduce the time required. Also, account for the number of materials and reagents required, to the number of animals being perfused and digested. Furthermore, work efficiently after the liver is extracted, to decrease the time before downstream experiments/analyzes to prevent any further cell death.

Buffers and Solutions

1 Solution 1 – EGTA, Ca^{2+} Mg^{2+} Free

MOUSE: Add 100 μL of 50mM EGTA into 50mL of Ca^{2+} Mg^{2+} free HBSS.

RAT: Add 500 μL of 50mM EGTA into 250mL of Ca^{2+} Mg^{2+} free HBSS.

Note

Solution 1 composition is enough per one animal.

1.1 *MOUSE:*

Add 80U of heparin (80 μL of stock heparin) into solution 1 and invert the tube before running it through the pump.

RAT:

Add 400U of heparin (4mL of stock heparin) into solution 1 and invert the tube before running it through the pump.

RAT:

Add 400U of heparin (400 μL of stock heparin) into solution 1 and invert the tube before running it through the pump.

2 Dehydrate

Add 68.6 μL of 1M CaCl solution into 50mL of Ca^{2+} Mg^{2+} free HBSS.

3 Solution 2 – 2,000 NPU/mL collagenase and 500 NPU/mL protease with Ca^{2+} Mg^{2+}

MOUSE: Add 68.6 μL of 1M CaCl solution and 500 μL of HEPES into 49.43mL of Ca^{2+} Mg^{2+} free HBSS.

RAT: Add 343 μL of 1M CaCl solution and 2.5mL of HEPES into 247.16mL of Ca^{2+} Mg^{2+} free HBSS.

Note

Solution 2 composition is enough per one animal.

3.1 MOUSE:

Add 200 μ L of collagenase and 50 μ L of protease during the time dehydrate solution is being pumped. The enzymes will be thawed in their respective aliquots before dehydration and kept in the fridge to preserve enzymatic activity until added.

RAT:

Add 1250 μ L of collagenase and 275 μ L of protease during the time dehydrate solution is being pumped. The enzymes will be thawed in their respective aliquots before dehydration and kept in the fridge to preserve enzymatic activity until added.

4 0.2M NaOH

Add 1mL of 10N of NaOH into 49mL of MiliQ water. Always add base to water slowly in a fume hood.

5 Mincing Media (keep cold)

When opening a new 500mL DMEM, extract 5mL of DMEM and add 5mL of penicillin/streptomycin to a final concentration of 1%.

5.1 MOUSE:

Add 5mL of FBS into 45mL of DMEM with 1% penicillin/streptomycin.

RAT:

Add 15mL of FBS into 135mL of DMEM with 1% penicillin/streptomycin. Or make three 50mL tubes of mincing media if a container that can hold a large volume is not available.

Note

Suggest using a media that is supplemented for your cell of interest, (for example, supplement with 1x GlutaMAX™ and 1x MEM Non-Essential Amino Acids Solution) but 10% FBS is absolutely necessary to be in the media.

5.2 *Filter the media using 50mL syringe and 0.22 μ m syringe filter before use.*

Note

Can prepare buffers and solutions the day prior.

Before Perfusion

45m

- 6 Organize the surgical platform and BSC with all the materials and pre-labeled tubes prior to liver perfusion to obtain total liver homogenate (TLH) to be processed quickly.
- 7 Place prepared solution 1, dehydrate and solution 2 (enzyme free) into an incubator at  37 °C with loosened caps for 40 minutes or directly into  42 °C water bath with caps on firmly.
- 8 Prepare 2 tubes of 50mL each of dH₂O, 70% EtOH and 1x PBS.
- 9 Clean surgical platform.
- 10 Ensure anesthesia machine has enough oxygen and isofluorane for your procedure.
- 11 Place a paper towel into anesthesia chamber.

12 Fill a 1mL syringe with a 27G with heparin (stock, 1U/ μ L).

13 Right before perfusion, clean the perfusion machine.

14 Quickly, run 50mL of NaOH solution, followed by 50mL of milli-Q water or sterile dH₂O then 50mL of ethar 20m and then finally 50mL of PBS.

Anesthetizing the rodent

15m

15 Re-tighten the caps of the buffers and transfer from the incubator to the water bath if not done so in previous section.

16 Fill a 1mL syringe with warm Solution 1. Use the p200 to fill the dead space of the catheter.

MOUSE: 24G catheter

RAT: 20G catheter

17 Turn on the oxygen flow rate at 3.5 L/min and isofluorane at 4%.

18 Place the rodent into the anesthesia chamber.

19 Observe breathing rate.

- 20 Once breathing has slowed, switch valves to direct flow to the tube and waste valves.
- 21 Remove the rodent and tape it to the surgical platform with its snout in the anesthesia tube/hose.
- Note**
- Autoclave tape works very well.
- 22 Decrease oxygen to 2 L/min and isofluorane to 2-2.5%.
- 23 Observe the rodent's behavior. If its heartbeat/breathing is erratic (a strong intermittent pulsation is observed on its chest cavity), reduce the levels of isofluorane. Observe until slow steady breathing is achieved.
- 23.1 Pinch its hind paws for a pain withdrawal reflex. If no paw withdrawal reflex is observed, proceed to operation.

Cannulation

25m

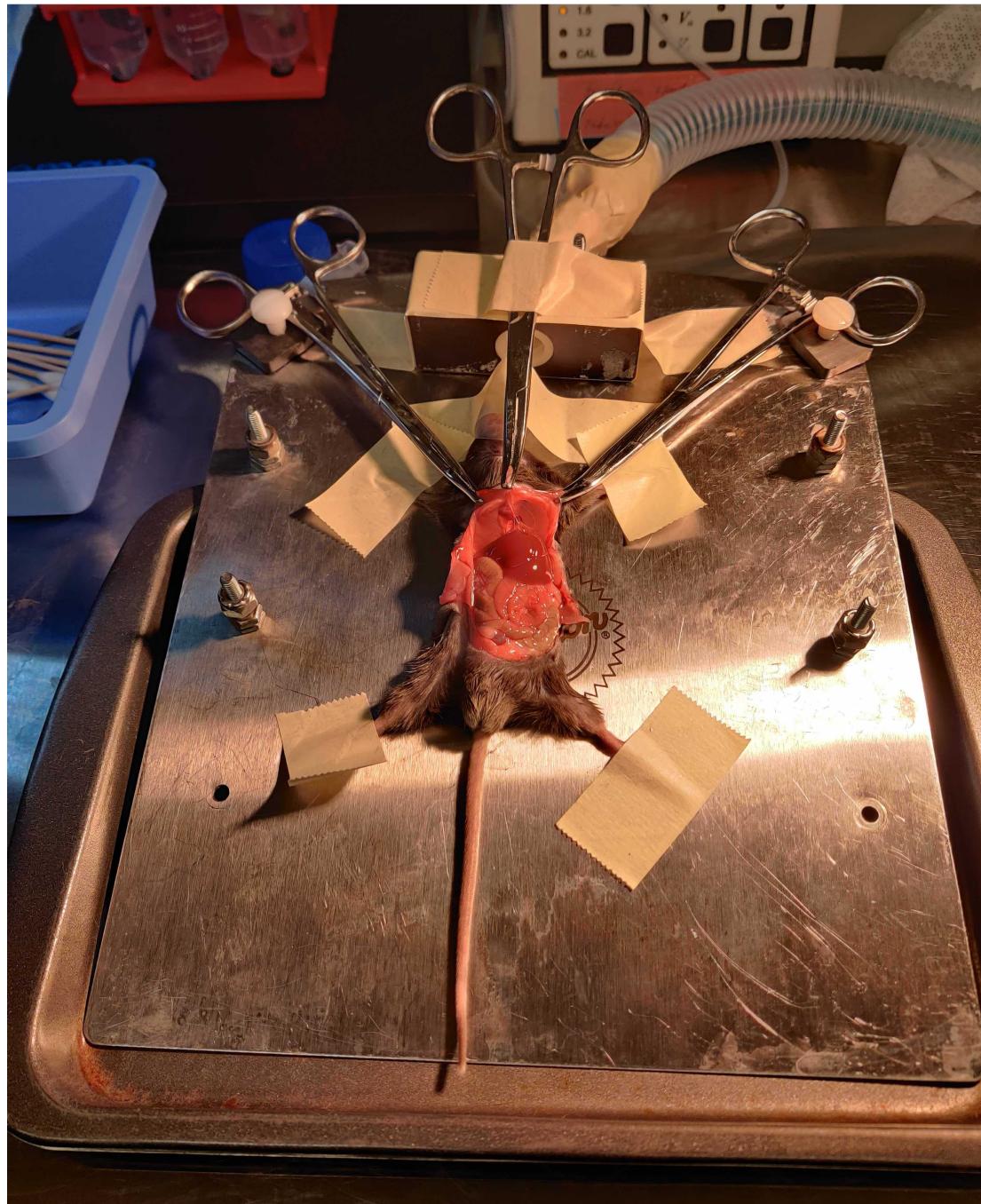
- 24 Shave the abdominal region of the mouse.
- 24.1 Remove of excess hairs with Kim wipes and any residual with tape.

- 25 Wipe the surgical region with ethanol-soaked wipes.
- 26 Inject the rodent peritoneally with:
MOUSE: 100U of heparin (100µL of stock heparin)
RAT: 400U of heparin (400µL of stock heparin diluted with 600µL PBS)
- 27 Wait 1 minute. Ensure breathing is still occurring normally.
- 27.1 During this time, wipe the surgical platform and surrounding area with Virox wipes for any residual fur.
- 28 Using the medium sharp forceps, pinch the skin in the middle of the lowest abdomen. Create a small incision using the sharp scissors.
- 29 Push the scissor through the separate the abdominal skin from the abdominal muscle. Create an incision running through the anterior-posterior axis of the rodent's abdomen.
- 30 Make lateral incisions/cuts of the abdomen/skin beneath the thoracic cage.

Note

At this step, perfusion tubing should be connected to solution 1 to fill the dead space.

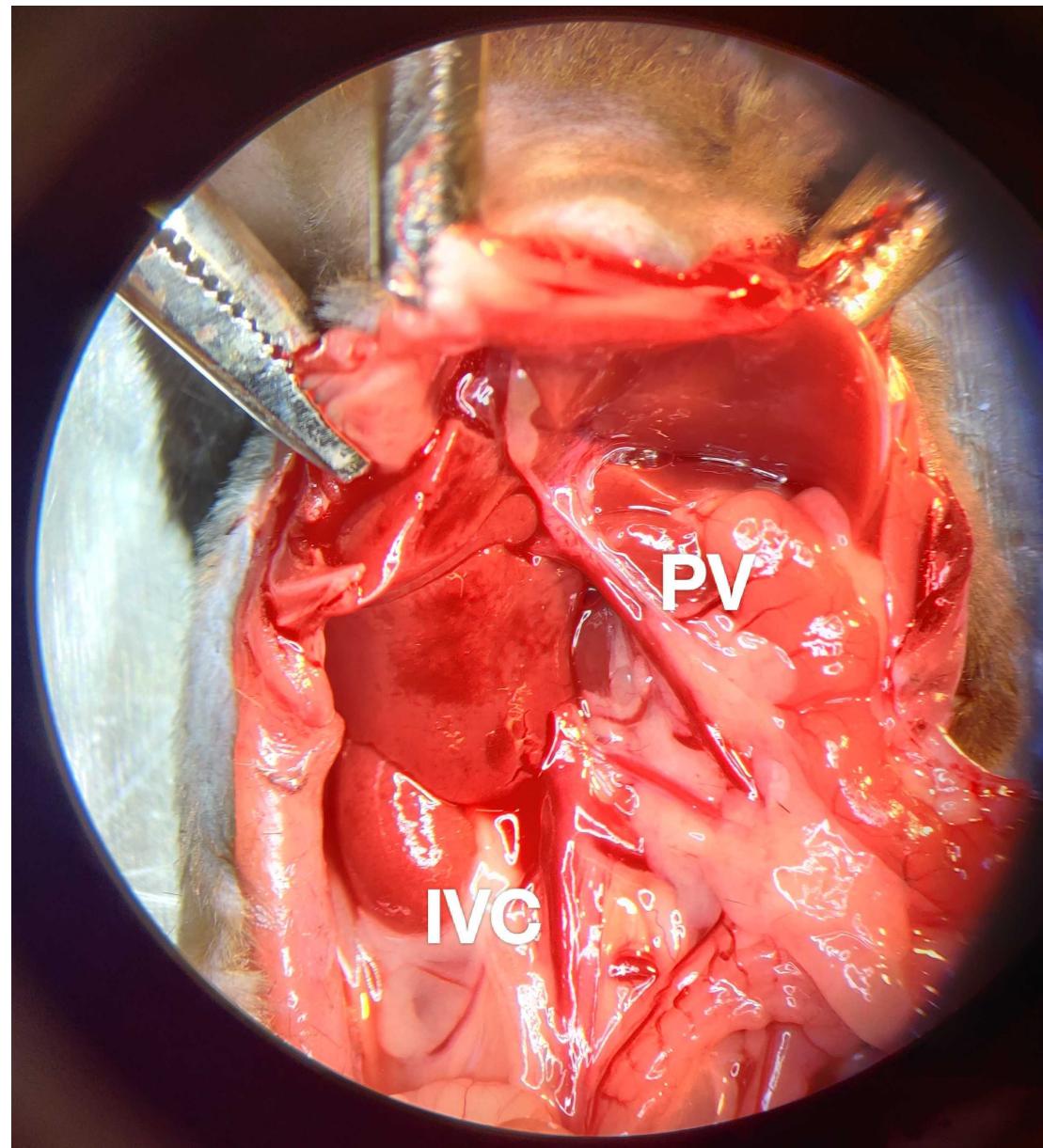
-
- 31 Repeat the steps 29 and 30 for the abdominal muscles of the rodent.
- 32 Hold the flap away from the surgery area by clamping it with the large forceps and securing it by inserting the finger holes/rings through the plastic screws on the upper right and left of the operation platform.



33 Put surgical microscope over the surgical area to view the image.

34 Push the liver up and the guts away from the inferior vena cava (IVC) and portal vein (PV).

34.1 Locate the PV and IVC.



PV: Portal vein; IVC: Inferior vena cava

34.2 Use the locking clamp to secure the guts away from the surgical area if in the way, not absolutely necessary.

35 Observe the status of the IVC. If the IVC is deflated, then proceed the latter steps using the PV first.



If both IVC and PV are deflated, then the success of the perfusion is low regardless. If this is true, suggest not to proceed and start again with a new rodent if concerned about cell yield.

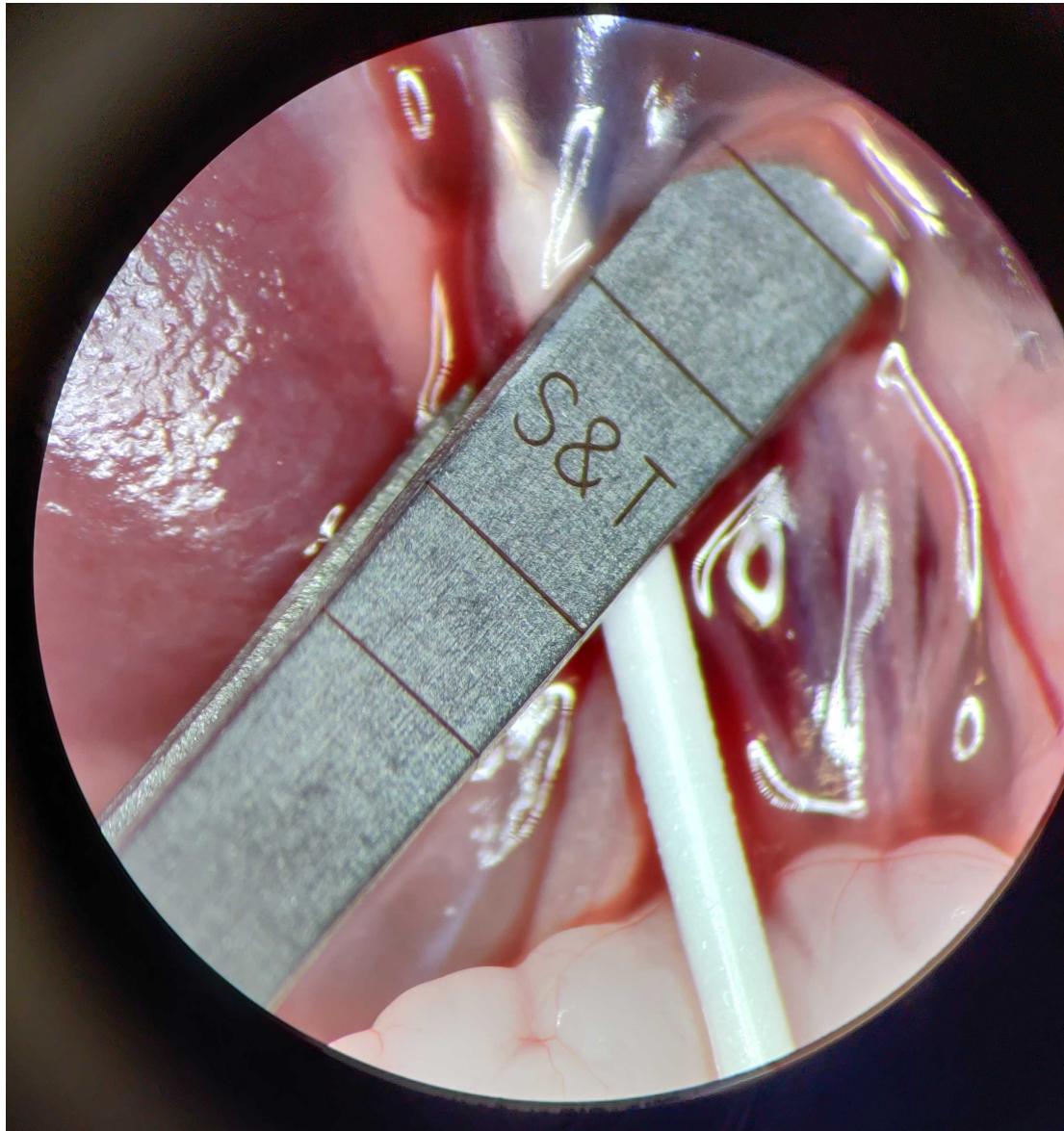
36 Observe the tissue surrounding the IVC. If the IVC is not clear and there are many layers of connective peritoneal tissue, then peel off these layers carefully with 2 forceps to not tear the vessel until you reveal the IVC. An exposed IVC should be a deep clear burgundy red with no opaqueness.

Note

Connective peritoneal tissue is thin and translucent – the more layers, the whiter it will be on top of the IVC.

37 Cannulate the IVC below the kidneys with the (mouse: 24G, rat: 20G) catheter/syringe filled with solution 1 from earlier. Secure wth micro-vasculature clamp.





Note

Cannulate by approaching horizontally parallel to the vessels to prevent piercing through the vessel if approached at an angle above. Also, ensure the cannulae end is not poking at the vessel's wall, but be parallel to the vessel for non-disruptive flow.

- 37.1** Ensure there are no bubbles at the tip of the catheter or inside the syringe chamber.





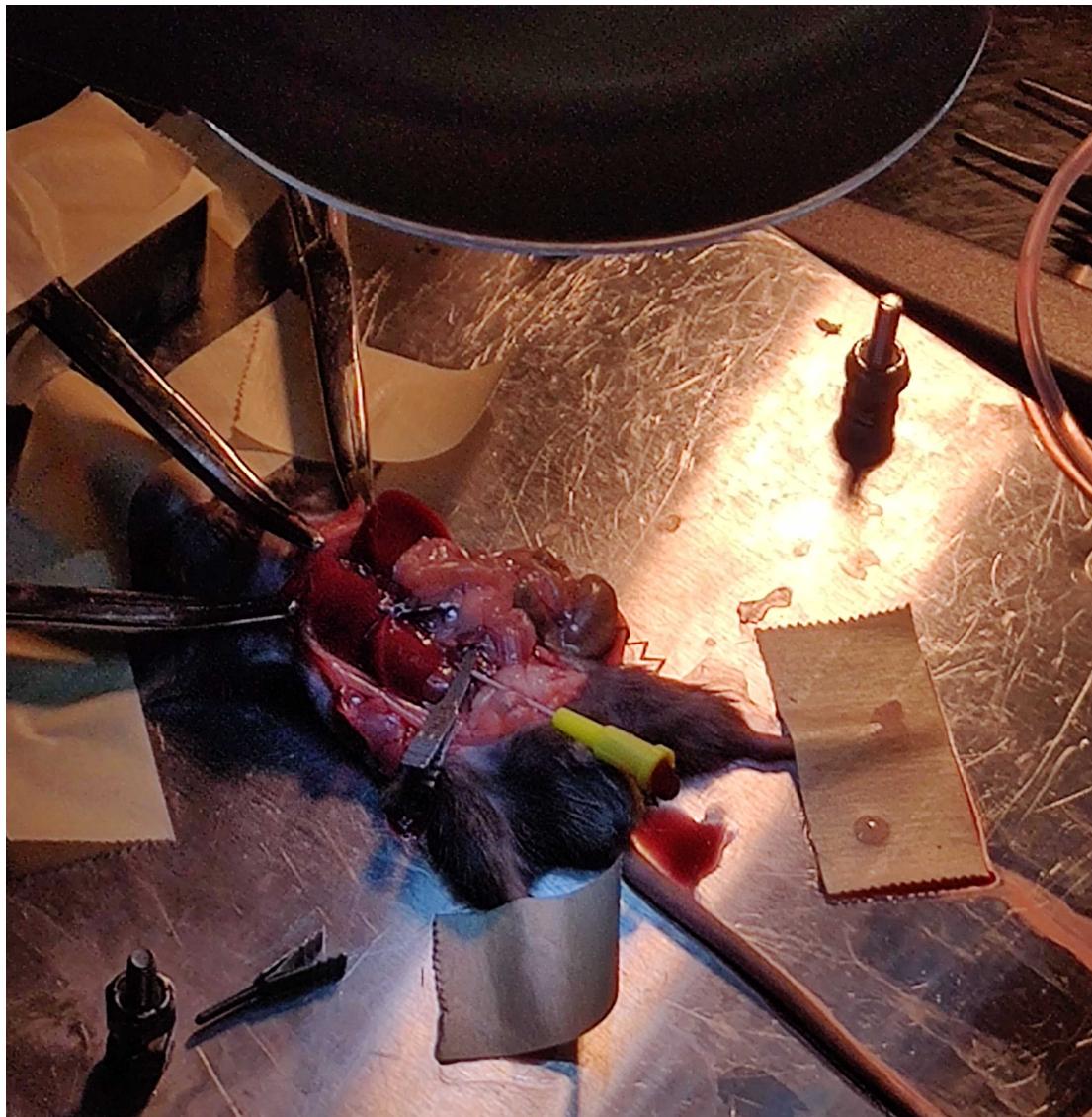
Note

To test that a proper connection was made to the IVC lumen, ensure the catheter moves freely within the IVC.

- 38 To increase the subsequent PV cannulation success, the slow removal of the cannulae needle (not the cannulae catheter), a small amount of solution 1 at 0.5-1mL/min is pumped.

Expected result

A successful flush should show backwards flow of blood and solution 1 after removal of the cannulae needle. Liquid should flow out of the catheter away from the liver from the IVC cannula.



38.1 Stop the pump. Disconnect the tubing from the needle and connect it to the cannulae.

39 After step 39, the PV should be slightly under pressure and visibly prominent. Remove any obstructing fat layers away from the PV delicately without tearing the vessel.

39.1 Use the small tweezer to pinch the tissue around the PV, and lift/secure the PV.

40 Insert a (mouse: 24G, rat: 20G) cannulae into the PV, and secure with micro-vasculature clamp.

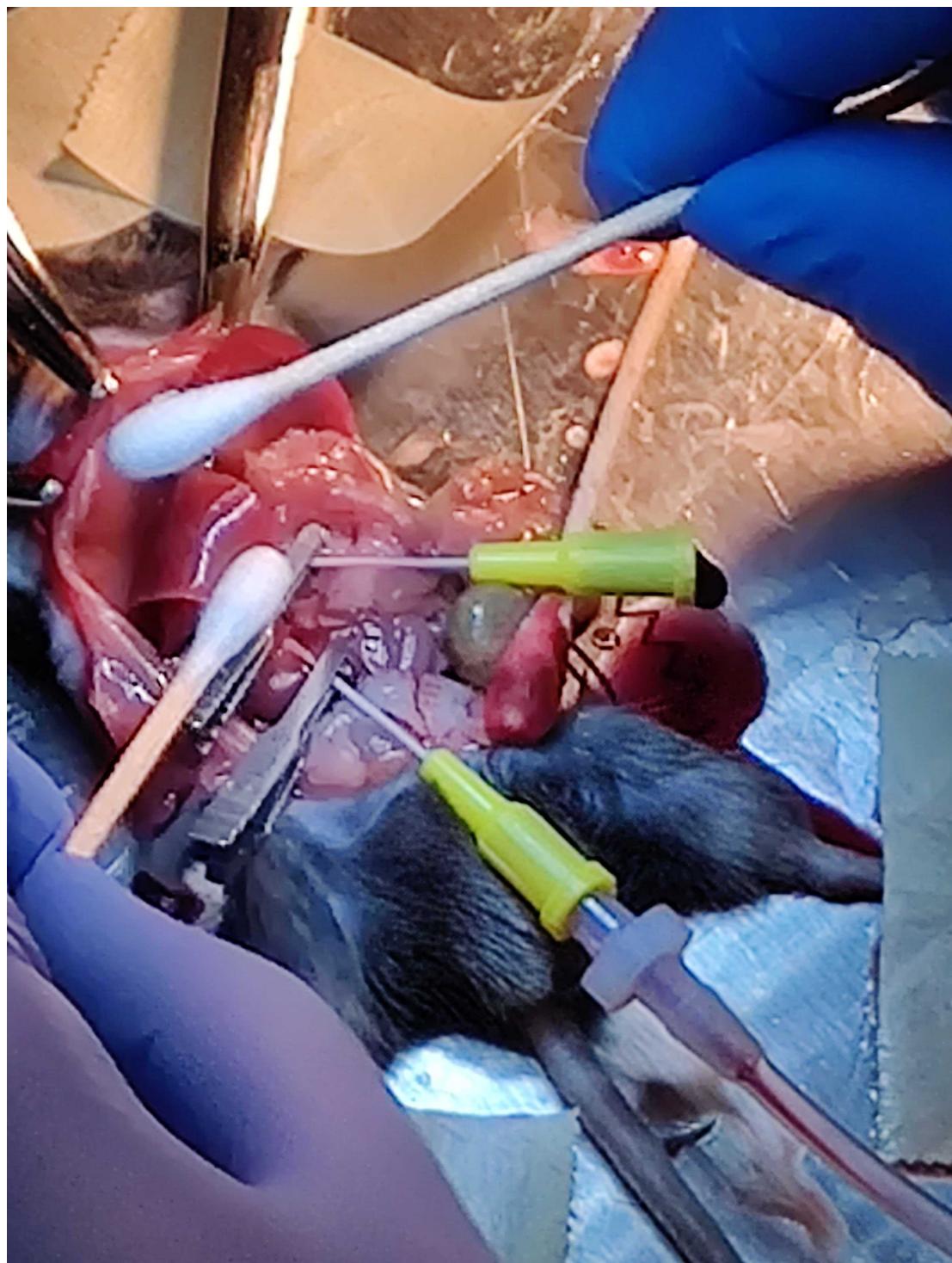
40.1 Remove the cannulae needle.



Expected result

Similarly to step 39, a backward flush of liquid should be observed upon removal of the cannulae needle.

41 Ensure angle of the catheter is fixed and secure parallel to the blood vessel to not disrupt flow of the solution running.



Perfusion

10m

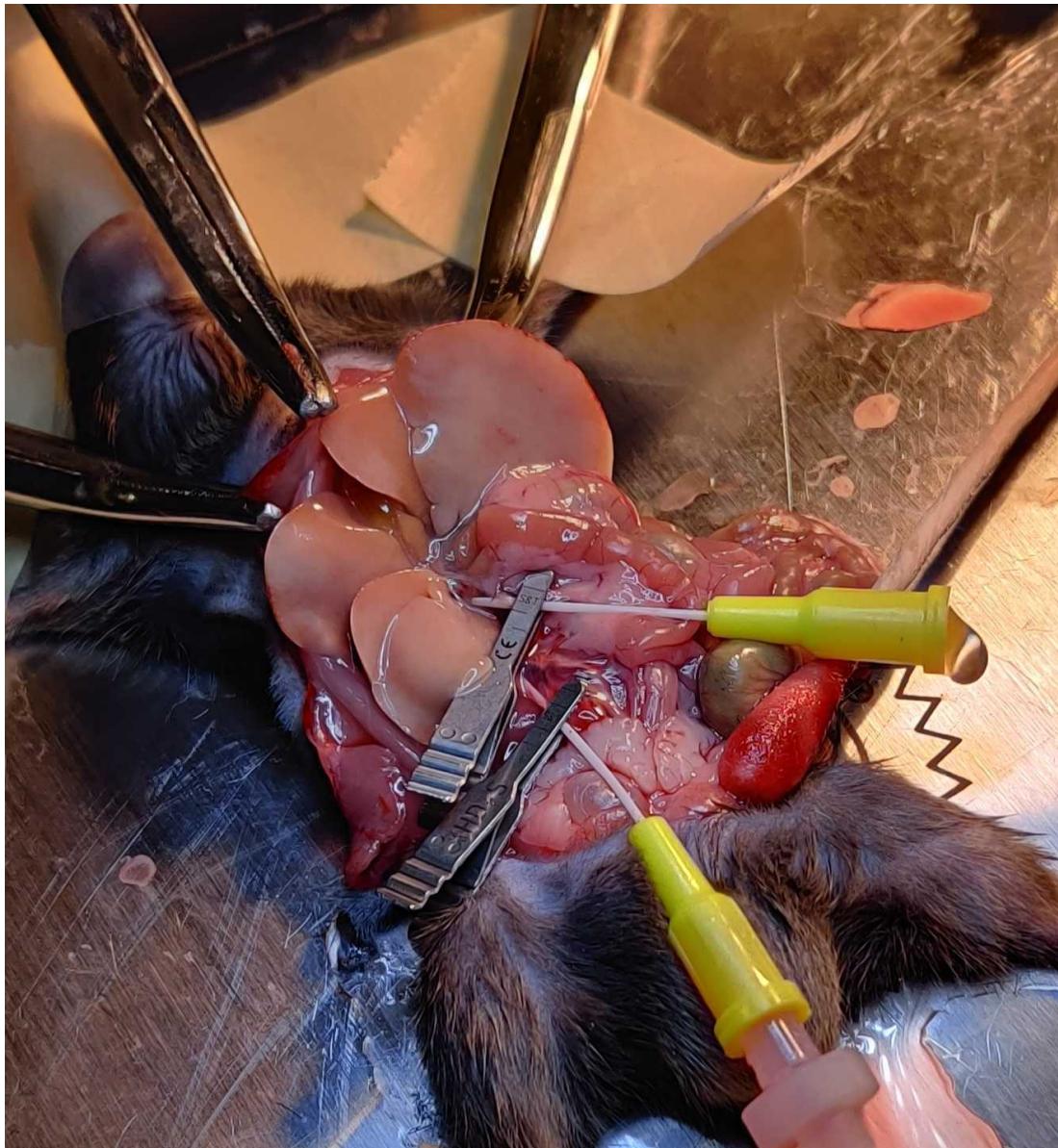
42

Start the perfusion pump flow of solution 1 from the IVC. Observe for bubbles, if it is clear - connect the perfusion tube to the PV cannula and let it run at 1-2mL/min for mouse (or 12mL/min for rat) for 1 minute whilst shaking the lobes of the liver to ensure proper perfusion.



**Note**

Flow rate is determined how inflated the lobes are to ensure every point of the liver is being flushed.

**43**

After 1 minute and liver looks pale* instead of burgundy red, disconnect from PV catheter and connect to the IVC catheter (same flow rate for mouse but 16mL/min for rat). The liquid running out of the top of the PV catheter should be clear.



Note

Solution 1 connection switching from the PV catheter to the IVC catheter is done to ensure every point of the liver is being perfused.

*The color will be of a pale yellow, white or brown depending on the lighting used. No red should remain.

- 44** Run 2-3 more minutes while shaking the liver until satisfactorily clear.

Expected result

Liver has a pale yellow/brown color with no more blood being flushed out of the IVC/PV catheter.

- 44.1** During this time, thaw 1 aliquot of VitaCyte collagenase and protease. Once thawed, place vials in the 4°C fridge or keep on ice.



- 45** Pause the pump and switch to dehydrate solution.



MOUSE: Run for 2 minutes.

RAT: Run for the entire 50mL dehydrate solution.

- 45.1** During this time, add collagenase and protease into solution 2 and invert the sealed tube several times.

**Digestion**

30m

- 46** Pause the perfusion run and switch to solution 2.



- 47** Run solution 2 while shaking the liver, then disconnect after 15-22 minutes (or 3/4 of the solution 2 running time) switch to PV perfusion for the remainder of the digestion.



Note

Shaking the liver should only be done within the first 2-3 minutes of solution 2 perfusion.

IVC catheter to PV cathether switch is typically after 20 minutes due to using a slow flow rate.

Expected result

At the end of the digestion, the liver should be:

MOUSE: Observably full of dissociate patches of cells within the Glisson capsule.

RAT: Easily dented by gentle touching of the forceps.

Liver Extraction

5m

- 48** Carefully remove by lifting the liver up via the PV and remove the liver by removing the tissue connecting to the stomach and small intestines, as well as the posterior abdominal wall, kidneys and the IVC.

- 49** Place the liver into a petri dish with (mouse: 10mL, rat: 50mL) of cold mincing media.



Mincing the liver for TLH collection

10m

- 50** For rats, place a 500µm cell strainer on top of a 250mL sterile collection vessel on ice for each rat being perfused.

Skip to the next step for mice.

- 51** Place a (mouse: 50mL tube, rat: 250mL collection vessel) with a 100µm cell strainer and a (mouse: 50mL tube, rat: 250mL collection vessel) with 70µm cell strainers in the ice box for each rodent being perfused.

Note

Low temperatures deactivates the collagenase and the FBS in the mincing media deactivates the natural protease. Thus, critical for the liver and the TLH to be kept cold in mincing media, which contains 10% FBS.

52

Place the perfused and digested rodent liver in a petri dish containing (mouse: 10mL, rat: 50mL) of cold mincing media on an ice pack.

**Note**

Don't forget to weigh the perfused and digested liver before mincing if a record is being kept.

53

Flip the liver and swirl it around and flip it back gently.

54

Make multiple vertical thin cuts. While doing this, occasionally swirl the liver.

55

Carefully, place the top of the petri dish under the dish on one end to cause the liver contents to be on the other side the dish (tilted down towards the operator). Chop the liver contents in this soup multiple times.

56

Pipette up and down multiple times to dissociate any remaining tissue with a 25mL serological pipette.

57

Skip this step for mice.

For rats, transfer the TLH into the 250mL vessel with 500 μ m cell strainer (1 vessel for each rat). Proceed to next step 59.

Note

Due to a larger amount of undigestible connective tissues and matrices, the rat liver must first strained by a large pore strainer.

- 58 Transfer the TLH into the (mouse: 50mL tubes, rat: 250mL collection vessels) with 100 μ m cell strainer (1 vessel for each animal).

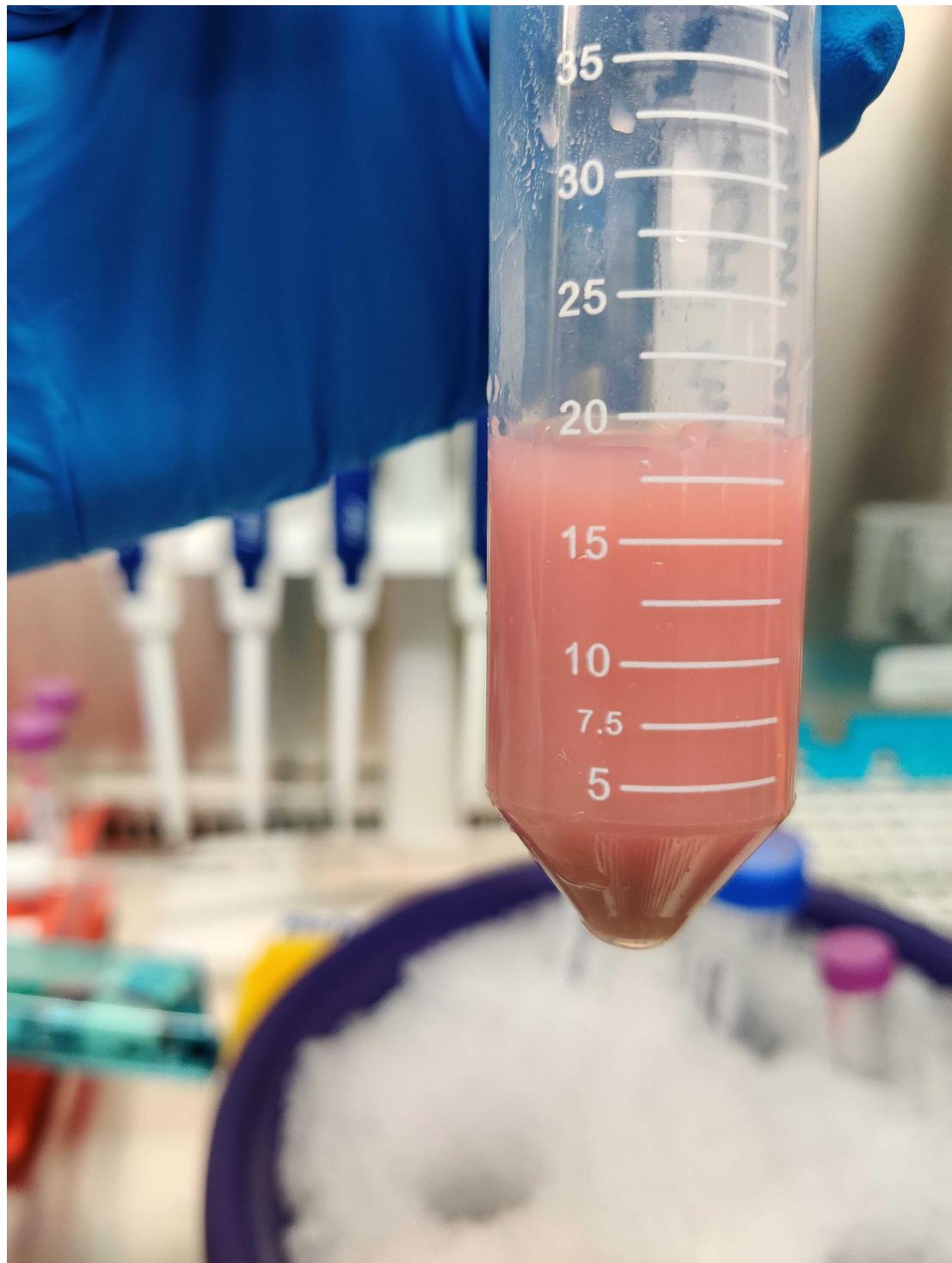
Note

Wash the dish with additional 2-5mL of macrophage media if needed.

- 59 Transfer the TLH into the (mouse: 50mL tubes, rat: 250mL collection vessels) with 100 μ m cell strainer into the appropriate vessels with 70 μ m cell strainer (1 vessel for each animal).

Note

The TLH is first strained by a 100 μ m cell strainer to remove lipids that may block the 70 μ m cell strainer.



Mouse total liver homogenate after mincing

60



Perform trypan blue on TLH by adding 20 μ L of cells into 180 μ L of PBS and take 10 μ L of 1:10 diluted cells

with 10 μ L of trypan blue. Load 10 μ L of the trypan blue with cells (1:20) into hemocytometer to count cells of interest (ex. hepatocytes or non-parenchymal cells).

Expected result

Viability of cells are \geq 80%, but mostly \geq 90%.

Note

This is done for each animal to assess liver perfusion and digestion.

Troubleshooting

- 61** If the IVC and/or PV cannot be located or is teared, then clamp the superior vena cava (SVC) or the side vasculatures.
*
- 62** If the lobes are not inflated with solution 1 running, then increase the flow rate to ensure the edges are being flushed and perfused. But if the lobes are too inflated during the perfusion, then decrease the flow rate, thus decreasing the pressure and preventing cell damage.